

Skin test to assess virus-specific cytotoxic T-cell activity

(delayed-type hypersensitivity/diagnosis/peptide antigens/activated T cells)

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ABSTRACT A way to assess specific CD8⁺ T-cell activity in a skin test analogous to the conventional delayed-type hypersensitivity (DTH) reaction for CD4⁺ T cells is presented. Local injection of viral class I binding peptides caused a specific CD8⁺ T-cell-mediated DTH in footpads of virally infected mice. The DTH was inducible only during the acute phase of the infection. Apparently because of the short half-life of locally available peptide, only activated CD8⁺ effector T cells could mediate the reaction. This skin test may prove to be particularly interesting for use in humans to evaluate the activation status of CD8⁺ T cells during acute viral infections and of memory CD8⁺ T cells, for example, in chronically active immunopathological disease or infection.

The standard *in vivo* assay for cell-mediated immunity is injection of antigen into the skin and assessment of the subsequent reaction. When the antigen is given in the form of a protein it is taken up by skin resident antigen-presenting cells (1), which will virtually exclusively present it in association with class II major histocompatibility complex (MHC) molecules (2–4). If memory CD4⁺ T cells are present, they will mediate an infiltration dominated by mononuclear cells that is clinically detected within 24–72 hr as a so-called delayed-type hypersensitivity (DTH) reaction (5–11). In humans, CD4⁺ T-cell-mediated immunity against bacterial and fungal infections such as tuberculosis, leprosy, brucellosis, lymphogranuloma inguinale, and histoplasmosis, as well as parasitic infections, can be tested by such a skin reaction (12). Interestingly, these infections are all chronic and granulomatous in nature.

In contrast, CD8⁺ T-cell-mediated immunity can only be detected if the antigen is presented on class I MHC molecules. Therefore the antigen usually has to be introduced into the cytoplasm of a cell (2–4). Consequently, only local injection of live virus (13) readily induces a CD8⁺ T-cell-mediated DTH in influenza- (14) and lymphocytic choriomeningitis virus (LCMV)-immune mice (15). In order to observe a clinically detectable swelling, high challenge virus doses have to be inoculated (13), because memory CD8⁺ T cells efficiently inhibit viral replication. Since an inoculation with a high dose of live virus is too dangerous, particularly in a nonimmune person, no skin test for CD8⁺ T-cell memory exists in humans.

To overcome these difficulties, the present study aimed at investigating CD8⁺ T-cell-mediated DTH reactions after subcutaneous injection of class I-binding viral peptides, to circumvent infection and the need for intracellular processing. Vesicular stomatitis virus (VSV) and LCMV were used as model antigens. After intravenous infection, CD8⁺ T-cell responses were monitored by DTH induced with the relevant immunodominant class I-binding nucleoprotein peptides, VSV-NP peptide (amino acids 49–62) in H-2^b (16) mice and LCMV-NP peptide (amino acids 118–132) in H-2^d (17) mice.

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MATERIALS AND METHODS

Mice. Inbred C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained from the breeding colony of the Institut für Zuchthygiene, Tierspital Zürich, Zurich. Generation of H-2^b mice transgenic for the T-cell receptor against LCMV glycoprotein (-GP) has been described in detail (18); these animals were bred in the Biologisches Zentrallabor, Universitätsspital Zürich, Zurich. Mice were usually between 8 and 16 weeks of age at the beginning of the experiments.

Virus. VSV serotype Indiana (Mudd–Summer isolate) seed stocks had originally been obtained from D. Kolakofski (University of Geneva, Geneva) and were grown with low multiplicity of infection on BHK 21 cells and plaqued on Vero cells. LCMV strain WE was obtained as a triple-plaque-purified stock from F. Lehmann-Grube (Heinrich-Pette Institut, Hamburg, F.R.G.).

Peptides. Synthetic peptides were purchased from Neosystem, Strasbourg, France. The purity was around 85% for the VSV-NP peptide, 65% for the LCMV-NP peptide, and 85% for the LCMV-GP peptide (amino acids 32–42) as assessed by high-performance liquid chromatography. They were solubilized in balanced salts solution by sonification.

Assessment of DTH. Peptide solubilized in balanced salts solution (30 μ l) was injected into both hind footpads. Before injection and at the indicated times thereafter, thickness of footpads was measured with a spring-loaded caliper (Kroepelin, Schluchtern, Hessen, F.R.G.). Average swelling of four to six individual footpads is given as percent increase of footpads thickness. Interindividual variation was <20%.

***In Vivo* Restimulation.** For *in vivo* restimulation of VSV-NP-specific cytotoxic T lymphocytes (CTLs), C57BL/6 mice were injected intraperitoneally every 4 days with 2×10^6 EL-4_{NP} cells that had been γ -irradiated (4000 rads; 1 rad = 0.01 Gy) by a ⁶⁰Co source. EL-4_{NP} is an H-2^b thymoma cell line originating from C57BL/6 and has been transfected with the nucleoprotein of VSV serotype Indiana (19). EL-4_{neo} is a control cell line without expression of the nucleoprotein (19). Both cell lines were grown in Iscove's modified Dulbecco's medium containing 5% fetal bovine serum. For *in vivo* restimulation of LCMV-specific CTL, BALB/c mice were injected intraperitoneally with 5×10^6 syngeneic peritoneal macrophages from BALB/c mice that had been infected with LCMV strain WE. The BALB/c donor mice were injected intraperitoneally with 2 ml of thioglycolic acid (3%, wt/vol, in distilled water, BBL Microbiology Systems, Becton Dickinson) on day -6 and then infected intraperitoneally with 10^3 plaque-forming units (pfu) of LCMV strain WE on day -4; macrophages were washed out of the peritoneum with ice-cold balanced salts solution on day 0.

Abbreviations: VSV, vesicular stomatitis virus; VSV-NP, VSV nucleoprotein; LCMV, lymphocytic choriomeningitis virus; LCMV-NP, LCMV nucleoprotein; LCMV-GP, LCMV glycoprotein; DTH, delayed-type hypersensitivity; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; pfu, plaque-forming unit(s).

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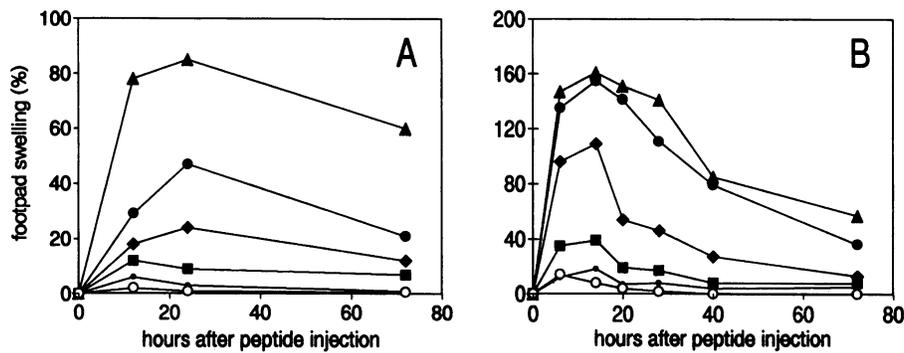


FIG. 1. Induction of DTH by injection of MHC class I-binding peptides into the footpads of virus-infected mice. (A) C57BL/6 mice were infected with VSV (2×10^6 pfu); 6 days later VSV-NP peptide was injected into the hind footpads subcutaneously. The subsequent swelling depended critically on the concentration of the injected peptide: \blacktriangle , 9000 $\mu\text{g}/\text{ml}$; \bullet , 900 $\mu\text{g}/\text{ml}$; \blacklozenge , 90 $\mu\text{g}/\text{ml}$; \blacksquare , 9 $\mu\text{g}/\text{ml}$; \circ , 0.9 $\mu\text{g}/\text{ml}$. Uninfected control mice were given 9000 $\mu\text{g}/\text{ml}$ (\circ). (B) BALB/c mice were infected with LCMV (200 pfu); 9 days later LCMV-NP peptide was injected into the hind footpads: \blacktriangle , 30 $\mu\text{g}/\text{ml}$; \bullet , 3 $\mu\text{g}/\text{ml}$; \blacklozenge , 0.3 $\mu\text{g}/\text{ml}$; \blacksquare , 0.03 $\mu\text{g}/\text{ml}$; \circ , 0.003 $\mu\text{g}/\text{ml}$. Unprimed mice were given 3000 $\mu\text{g}/\text{ml}$ (\circ).

Depletion of T-Cell Subsets. CD8^+ and CD4^+ T-cell subsets were depleted *in vivo* by intravenous injection of 1 mg of monoclonal antibody YTS 169.4 (anti- CD8) or YTS 191.1 (anti- CD4) (20). Depleted cells were below the detection level of microfluorometric analysis. Lack of functional CD8^+ T cells was confirmed by complete abrogation of vaccinia-specific CTL responses (21). The absence of functional CD4^+ T cells was confirmed by the loss of the IgM to IgG class switch of neutralizing antibodies against VSV serotype Indiana (21).

RESULTS

Specific Induction of DTH by Viral Class I-Binding Peptides. C57BL/6 (H-2^b) mice were intravenously infected with VSV (2×10^6 pfu) and tested 6 days later, and BALB/c mice (H-2^d) were immunized with LCMV (200 pfu) and tested 9 days later, at the respective times when CD8^+ T-cell activities are known to be maximal (22, 23). Four hours after subcutaneous injection of the relevant immunodominant class I-binding nucleoprotein peptide (VSV-NP peptide or LCMV-NP peptide, respectively), a swelling reaction developed that peaked after 12–24 hr. The swelling produced up to 100% increase in footpad thickness in VSV-immune animals (Fig. 1A) and up to 160% in LCMV-immune mice (Fig. 1B). The feet slowly regained their normal thickness within 5–6 days. Injection with the highest peptide concentrations (9 mg/ml for VSV-NP and 3 mg/ml for LCMV-NP) did not induce a measurable swelling in uninfected mice. The swelling reaction in virally infected mice depended on the concentrations of the injected peptides: below concentrations of 3 mg/ml for the VSV-NP peptide and 3 $\mu\text{g}/\text{ml}$ for the LCMV-NP peptide

the swelling reaction was limited by the amount of peptide injected. When higher concentrations were injected, the swelling reaction was not further enhanced. Thus, when the peptides were injected at these apparently saturating concentrations, the CD8^+ T-cell response became the limiting factor for the swelling. Therefore, in all further experiments, the peptides were used at the saturating concentrations, 3 mg/ml for VSV-NP peptide and 30 $\mu\text{g}/\text{ml}$ for LCMV-NP peptide. Antigen specificity of the swelling reaction was also tested in VSV-infected BALB/c mice, where no swelling was induced after injection of LCMV-NP peptide, and in LCMV-infected C57BL/6 mice, where no swelling was induced after injection of VSV-NP peptide (data not shown).

Peptide-Induced DTH Depends on CD8^+ T Cells and Not on CD4^+ T Cells. Similarly, DTH was assessed in day 6 VSV-immune C57BL/6 and day 9 LCMV-immune BALB/c mice that were depleted *in vivo* of either CD4^+ or CD8^+ T cells by a single injection of the respective monoclonal antibody 1 day before the DTH was elicited. The swelling reaction was not altered in mice depleted of CD4^+ T cells but was completely abrogated in mice depleted of CD8^+ T cells (Fig. 2).

Peptide-Induced DTH Is Short-Lived in VSV- and LCMV-Immune Mice. C57BL/6 mice were immunized with VSV (2×10^6 pfu) 4, 6, 8, 12, 20, and 45 days before injection of peptides and BALB/c mice were infected with LCMV (200 pfu) 7, 13, 56, 87, 216, and 280 days before injection of peptides. All mice, including uninfected control groups, were injected in the hind footpads at the same time with an optimal peptide concentration. In VSV-infected mice DTH was inducible only early after infection, with the highest response on day 6, and was undetectable after 45 days (Fig. 3A). For LCMV the response peaked on days 8–13, was drastically

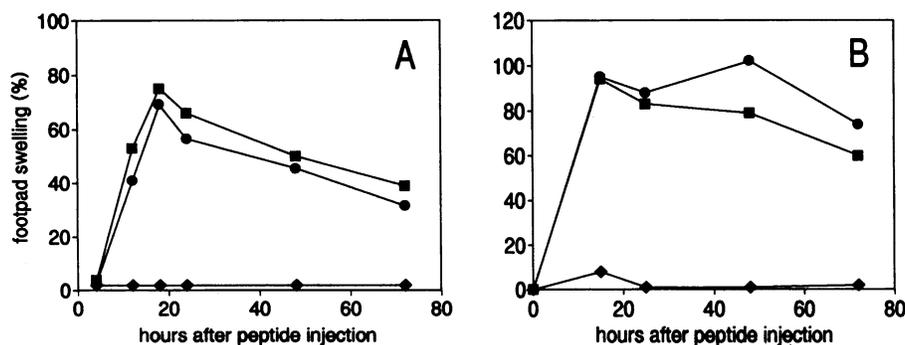


FIG. 2. Abrogation of peptide-induced DTH after *in vivo* depletion of CD8^+ T cells but not after depletion of CD4^+ T cells. (A) C57BL/6 mice were infected with VSV (2×10^6 pfu); 6 days later VSV-NP peptide was injected into their hind footpads (\blacksquare). Some mice received anti- CD8^+ (\blacklozenge) or anti- CD4^+ (\bullet) treatment 1 day before the peptide was injected. (B) BALB/c mice were infected with LCMV (200 pfu) 13 days before peptide injection (\blacksquare). Some mice received anti- CD8^+ (\blacklozenge) or anti- CD4^+ (\bullet) treatment 1 day before peptide injection.

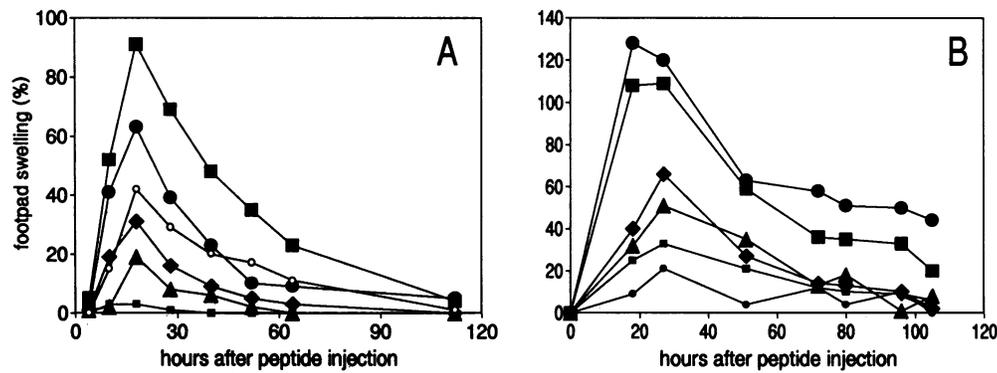


FIG. 3. Dependence of DTH upon kinetics of CD8⁺ T-cell immunity to virus. (A) C57BL/6 mice were infected with VSV (2×10^6 pfu) 4 (○), 6 (■), 8 (●), 12 (◆), 20 (▲), or 45 (■) days before VSV-NP peptide was injected into footpads. (B) BALB/c mice were infected with LCMV (200 pfu) 7 (■), 13 (●), 56 (◆), 87 (▲), 216 (■), or 280 (○) days before LCMV-NP peptide was injected.

reduced after 56 days, and was only questionably significant 216 and 280 days after infection (Fig. 3B).

Need for Highly Activated Specific CD8⁺ T Cells for DTH After Injection of Relevant Peptides. Although CD8⁺ T-cell memory after infection with VSV and LCMV is known to be long-lived when analyzed by secondary restimulation *in vitro* or by protection assays *in vivo* (24, 25), peptide-induced DTH was evanescent. The following experiments show that DTH can be restored in long-term memory mice after periodic or short-term activation of virus-specific CD8⁺ T cells. C57BL/6 mice infected with VSV (2×10^6 pfu) were injected intraperitoneally every 4 days with a transfected EL-4 cell (a T-cell lymphoma originating from C57BL/6) expressing the nucleoprotein of VSV (EL-4_{NP}) (19) or with a control EL-4 cell without nucleoprotein expression, EL-4_{neo}. After 45 days both groups were injected with the VSV-NP peptide. Only in mice infected with VSV and given EL-4_{NP} cells every 4 days, but not in those given the control EL-4_{neo} cells, was the peptide-induced DTH maintained (Fig. 4A), demonstrating the need for periodic restimulation. EL-4_{NP} given to uninfected animals with the same protocol did not prime them for a DTH.

Similarly, DTH was restored in BALB/c mice infected with LCMV (200 pfu) 70 days before, which without additional restimulation show only a weak DTH upon peptide challenge (Figs. 3B and 4B); after short-term restimulation of specific CTLs by intraperitoneal injection of LCMV-infected macrophages 2 days before the peptide challenge, the DTH reaction was fully restored (Fig. 4B). In uninfected control animals intraperitoneal injection of LCMV-infected macrophages did not immunize for a DTH within 2 days.

That DTH was fully restored within 2 days, a period that is too short for massive proliferation, suggested that the level of activation of CD8⁺ T cells was more important in mediating the DTH reaction than the frequency of specific CD8⁺ T cells. Further support for this notion was obtained in transgenic mice in which >70% of all CD8⁺ T cells are specific for the LCMV-GP in association with the D^b MHC class I molecule (18). Challenge with the specific class I-binding glycoprotein peptide recognized by the transgenic T-cell receptor (amino acids 32–42; 30 μg/ml) produced only a weak footpad swelling response after 14 hr (Fig. 5). Only if the specific CD8⁺ T cells were activated during 4 days of infection with high doses of LCMV (10^6 pfu intravenously) did peptide injection into the footpads induce a swelling with the usual rapid onset. The weaker swelling with the delayed onset that was observed in uninfected transgenic mice did not develop in nontransgenic control animals.

DISCUSSION

According to the classification of hypersensitivity reactions by Coombs and Gell (26), the observed swelling in response to peptide injection has to be classified as a type IV hypersensitivity, or DTH, reaction because of the following characteristics. (i) It is antigen-specific as confirmed in VSV-infected BALB/c mice, where no swelling was induced after injection of LCMV-NP peptide and vice versa. (ii) It requires induced effector T cells or established immunological memory, since peptide injected into nonimmune mice did not elicit a swelling. (iii) It is cell-mediated and antibodies are not involved, as demonstrated in mice depleted of CD8⁺ T cells,

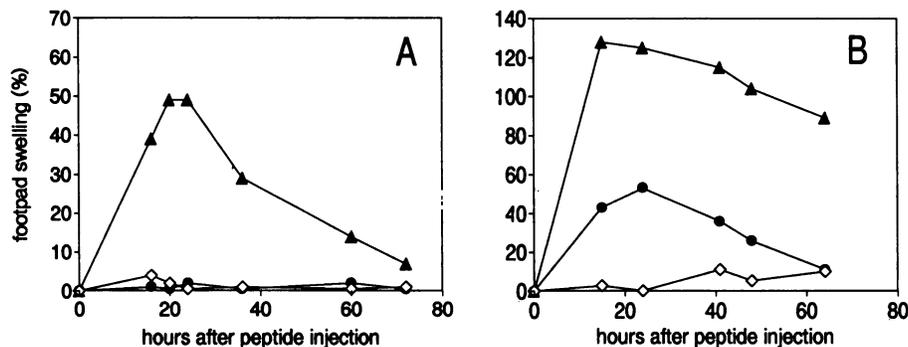


FIG. 4. Restoration of DTH reaction in VSV and LCMV long-term memory mice by periodic or short-term *in vivo* restimulation of virus-specific CD8⁺ T cells. (A) C57BL/6 mice were infected with VSV (2×10^6 pfu) 48 days before VSV-NP peptide was injected subcutaneously. Mice were injected intraperitoneally every 4 days with irradiated EL-4_{NP} cells (▲) or the EL-4_{neo} control cells (●). Uninfected control group was given EL-4_{NP} (◇). (B) BALB/c mice were infected with LCMV (200 pfu) 70 days before LCMV-NP peptide was injected subcutaneously (●). Some of these memory mice were given LCMV-infected syngeneic macrophages intraperitoneally 2 days before peptide injection (▲). Uninfected control mice were given LCMV-infected macrophages 2 days before peptide injection (◇).

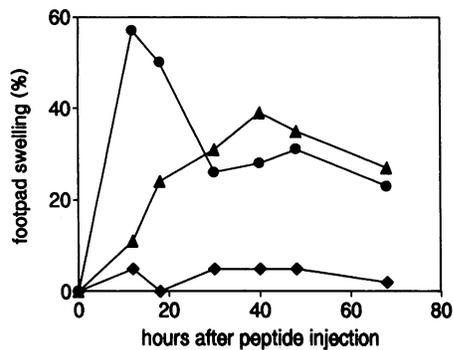


FIG. 5. Dependence of DTH on activation of specific CD8⁺ T cells. Transgenic H-2^b mice in which >70% of CD8⁺ T cells express a T-cell receptor specific for the LCMV-GP presented on the D^b MHC molecule were injected with the relevant glycoprotein peptide (amino acids 32–42). ▲, Uninfected transgenic mice; ●, transgenic mice intravenously infected with LCMV 4 days before peptide challenge; ◆, nontransgenic H-2^b control mice challenged with the same glycoprotein peptide at the same concentration (30 μ l, 30 μ g/ml).

where the swelling was completely abrogated. The peptide-induced DTH developed slightly faster than the classical CD4⁺ T-cell mediated DTH, probably because every cell is class I-positive and thus a potential target and because no recruitment of class II-positive macrophages to the site of protein injection and no antigen processing is required.

The peptide-induced virus-specific DTH reaction was only transiently inducible despite the fact that antiviral CD8⁺ T-cell memory against VSV and LCMV is known to be long-lived when analyzed by secondary stimulation *in vitro* or by protection assays *in vivo* (24, 25). This apparent contradiction probably indicates that only highly activated memory CD8⁺ T cells mediated DTH before the eliciting peptide had waned due to its relatively short local half-life *in vivo* (27). That the effective half-life of the injected peptide was short was supported by the following finding. In mice injected with VSV-NP peptide 4 days after a VSV infection, the DTH was weak (around 40%) and declined rapidly after 20 hr. If the peptide were still present locally >24 hr after challenge on day 5 or 6 after VSV infection, the swelling should have further increased after 20 hr according to the kinetics of the anti-VSV CTL response, which becomes maximal on day 6 (22). Similarly to the evanescence of the peptide-induced swelling reaction, DTH to epidermal reinfection of humans with live vaccinia virus was found some 90 years ago to last only for a few weeks after vaccination (28). This classical example also suggests that for certain routes of reinfection, only highly activated memory CD8⁺ T cells are fast enough to inhibit massive viral replication.

The peptide-induced LCMV-specific DTH was restored in long-term memory mice by antigenic restimulation for only 2 days. This is too short a time to allow massive replication of specific memory CD8⁺ T cells. This result suggests that the level of activation is more important than relative frequency of virus-specific memory CD8⁺ T cells for mediating the DTH. The requirement for priming and activation of specific CD8⁺ T cells was also demonstrated in transgenic mice. Despite the fact that >70% of all CD8⁺ T cells expressed the transgenic T-cell receptor specific for the subcutaneously injected LCMV-GP peptide in the tested H-2^b mice, the induced swelling was weak and started only after a delay. Only when the CTLs were primed and activated during 4 days of systemic infection with LCMV did a DTH with the usual rapid onset develop. The weak footpad swelling that was observed in uninfected transgenic animals is most probably explained by local priming and stimulation of specific CD8⁺ T cells by the injected peptide; these cells then exert their

function with a corresponding delay necessary for priming and activation to occur. Apparently, only when high numbers of specific T cells are present at the site of peptide injection, and probably in the draining lymph node, as in the transgenic mice, is this mechanism effective enough to induce a delayed swelling; no swelling developed in uninfected nontransgenic control animals.

The lack of a DTH with the usual rapid onset in transgenic mice demonstrates that a high frequency of unprimed specific CD8⁺ T cells is not sufficient to mediate a DTH. That the DTH was absent in long-term memory mice and could be fully restored only after 2 days of restimulation suggests that the activation level of the specific memory CD8⁺ T cells has to be increased by the antigen presented in an immunogenic form before they can mediate a DTH.

Because proteins injected into the skin can be locally detected for only about 1 day (29), it may be assumed [and has also been illustrated (10)] that only highly activated CD4⁺ T cells can mediate a DTH before the antigen has disappeared. This offers a reasonable explanation why the classical skin tests based on DTH are used successfully and therefore routinely only for granulomatous infectious diseases (12) and contact hypersensitivity (26, 30). Granulomas function basically as strongly immunogenic antigen depots, and the antigen-presenting macrophages and epithelioid cells keep the CD4⁺ T cells on a high level of activation. In various experimental systems it has been demonstrated that DTH is long-lived only after immunization with antigens in granuloma-forming complete Freund's adjuvant (31). In contrast, after immunization without adjuvant, DTH is short-lived (11, 29, 32, 33) as first described by Jones-Mote (33). This DTH is evanescent not because of increasing antibody responses (29, 32, 34) but, in analogy to the results shown here, probably because of decreasing activation of CD4⁺ T cells and the short half-life of local class II-presented peptides (27). Long-term demonstration by skin tests of contact hypersensitivity might be explained by persistence of the sensitizing substances or by frequent reexposures. Because most sensitizers are also primary irritants (30, 35, 36), these antigens might keep the T cells highly activated without granuloma formation.

The demonstrated peptide-induced DTH has characteristics analogous to those of the well-described CD4⁺ T-cell-mediated DTH. It is evanescent if the CD8⁺ T cells are not kept on a high level of activation by antigen persisting in a highly immunogenic form. The DTH described here is an alternative to the injection of live virus, which is impossible in humans. Obviously, because of MHC (HLA)-restricted antigen presentation, either the relevant HLA type has to be known or a cocktail of peptides will have to be used to elicit the CD8⁺-dependent DTH in humans (37).

This skin test might provide a quick and easy diagnostic tool, especially for studying CD8⁺ T cells in humans. It could be very useful for vaccine development [e.g., against malaria (38)] or for detecting acute viral infections. Of particular interest is the possibility to evaluate T-cell immunoreactivity in chronic persistent viral infections (e.g., chronic aggressive hepatitis or human immunodeficiency virus infection) or in chronic immunopathological disease where CD8⁺ T cells may be constantly activated.

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