Cytotoxicity Is Mandatory for CD8+ T Cell-mediated Contact Hypersensitivity

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Summary

Contact hypersensitivity (CHS) is a T cell–mediated skin inflammation induced by epicutaneous exposure to haptens in sensitized individuals. We have previously reported that CHS to dinitrofluorobenzene in mice is mediated by major histocompatibility complex (MHC) class I–restricted CD8+ T cells. In this study, we show that CD8+ T cells mediate the skin inflammation through their cytotoxic activity. The contribution of specific cytotoxic T lymphocytes (CTLs) to the CHS reaction was examined both in vivo and in vitro, using mice deficient in perforin and/or Fas/Fas ligand (FasL) pathways involved in cytotoxicity. Mice double deficient in perforin and FaasL were able to develop hapten-specific CD8+ T cells in the lymphoid organs but did not show CHS reaction. However, they did not generate hapten-specific CTLs, demonstrating that the CHS reaction is dependent on cytotoxic activity. In contrast, Fas-deficient lpr mice, FaasL-deficient gld mice, and perforin-deficient mice developed a normal CHS reaction and were able to generate hapten-specific CTLs, suggesting that CHS requires either the Fas FaasL or the perforin pathway. This was confirmed by in vitro studies showing that the hapten-specific CTL activity was exclusively mediated by MHC class I–restricted CD8+ T cells which could use either the perforin or the FaasL pathway for their lytic activity. Thus, cytotoxic CD8+ T cells, commonly implicated in the host defense against tumors and viral infections, could also mediate harmful delayed-type hypersensitivity reactions.

Key words: cytotoxic T lymphocyte • contact hypersensitivity • contact dermatitis • hapten • dinitrofluorobenzene • CD8+ T cells

Contact hypersensitivity (CHS) is a T cell–mediated cutaneous inflammatory reaction occurring after epicutaneous exposure to haptens in sensitized individuals (1–3). In humans, it frequently manifests as an inflammatory dermatosis referred to as contact dermatitis. Haptens are low molecular weight chemicals that covalently bind to discrete amino acid residues on self or exogenous proteins (4). The sensitization phase, also referred to as the afferent phase, occurs after the first contact of the skin with the hapten. Hapten-modified proteins are loaded onto dendritic epidermal Langerhans cells (LCs) which migrate from the epidermis to the regional draining LNs, where priming of hapten-specific CD4+ T cells occurs (5, 6). The elicitation phase, also known as the efferent phase, develops within a few hours after subsequent contact with the hapten, and is mediated by the activation of hapten-specific T cells in the skin.

For many years, CHS was considered, like classical delayed-type hypersensitivity (DTH), to be mediated by CD4+ T cells (7). Recent studies have demonstrated that CHS to dinitrofluorobenzene (DNFB) was mediated by IFN-γ–producing CD8+ T cells only, whereas CD4+ T cells downregulated this response (8–10). Thus, CHS can be
considered as an antigen-specific inflammation mediated by hapten-specific CD8\(^+\) T cells, which differs from classical DTH to protein antigens (1, 2). Although haptens are potent inducers of CD8\(^+\) CTLs (11, 12), it is not known whether CD8\(^+\) cells mediate the skin inflammation through such cytotoxic activity or through the secretion of type 1 cytokines.

CD8\(^+\) CTLs are major effector cells of the immune defense system against viruses and tumors (13) and exert their lytic functions through two main independent mechanisms (14–16). The secretory pathway involves the release of perforin and granzymes from cytolytic granules. The nonsecretory pathway involves interaction of the FasL upregulated during T cell activation, with the apoptosis-inducing Fas molecule on the target cell.

In this study, we investigated the contribution of CD8\(^+\) T cell-mediated cytolysis to the pathophysiology of CHS, using mice deficient in the Fas/FasL pathway (Ipr and gld mice), the perforin pathway (perforin-deficient [P\(^0/0\)] mice), and in both cytolytic pathways (P\(^0/0\) gld mice). The results provide evidence that CD8\(^+\) T cells mediate CHS through their cytolytic activity.

**Materials and Methods**

Mice. C57BL/6 mice were purchased from IFFA Credo. Mice homozygous for Ipr mutation (Ipr) and lacking the Fas (CD95) molecule were obtained from Harlan. Mice homozygous for perforin gene disruption (P\(^0/0\)) completely lack perforin-dependent cytolysis, while the Fas/FasL pathway remains fully functional (17, 18). Mice homozygous for the gld mutation (gld), homozygous for the gld mutation and heterozygous for the perforin deletion (P\(^+/0\) gld), and mice double deficient for perforin and carrying the gld mutation (P\(^0/0\) gld, unable to generate antigen-specific CTLs (19)) were provided by M. Michael Hahn (Institute of Biochemistry, Lausanne, Switzerland). P\(^0/0\) gld mice were obtained by mating P\(^+/0\) gld mice, and the offspring were tested for perforin deletion as described by Lowin et al. (18). Mice with a mutation in the β2 microglobulin gene (MHC class I–deficient [I\(^0/0\)] or in the I-A\(^\beta\) gene (MHC class II–deficient [II\(^0/0\)]) were provided by Christophe Benoist and Diane Mathis (20, 21).

All mutant mice were on a C57BL/6 (H-2\(b\)) background (backcrossed more than eight times with C57BL/6 mice) and were used between 8 and 12 wk of age. Lpr, gld, and P\(^0/0\) gld mice, which develop a diffuse lymphoproliferation by the age of 2 mo, which could interfere with the development of the CHS reaction, were used at the age of 6 wk, at a time when they show no clinical sign of disease and have normal sized lymphoid organs. P\(^0/0\), I\(^0/0\), and II\(^0/0\) mice were bred at the IFFA Credo/Transgenic Alliance specific pathogen-free facility (L’Arbresle, France).

Chemicals. DNFB and its water-soluble form, dinitrobenzene sulfonic acid (DNBS), were obtained from Sigma and used for in vivo and in vitro experiments, respectively.

Antibody. Ascites from the anti-M HC class I (heavy chain) hybridoma 20.8.4.S was obtained from Jean-Pierre Abastado (Institut Pasteur, Paris, France).

A assay for CHS to DNFB. DNFB was diluted in acetone/olive oil (4:1) immediately before use. The procedure used for the CHS, i.e., the mouse ear swelling test (MEST), has been described elsewhere (22). In brief, 25 μl of 0.5% DNFB solution was applied to a 2-cm\(^2\) area of shaved dorsal skin. After 5 d, test and control animals received 10 μl of 0.15% (nonirritant concentration) DNFB applied on both sides of the left ear, and the solvent (acetone/olive oil) alone on the right ear. Ear thickness was measured using a micrometer (J15; Blet SA, France), before challenge and every day after challenge. The ear swelling was calculated as [(T\(_2\) - T\(_1\)) left ear] - [(T\(_2\) - T\(_1\)) right ear], where T\(_1\) and T\(_2\) represent values of ear thickness after and before challenge, respectively.

In each experimental group, some mice were killed at different time intervals after DNFB challenge for histological and PCR analysis.

R NA Extradition and Reverse Transcription PCR Analysis of CD8 and IFN-γ mRNA. At different intervals after challenge, ear samples were collected from sensitized or unsensitized mice and frozen in liquid nitrogen. The detection of R NA was conducted as described in detail elsewhere (23). In brief, total R NA was extracted using an R NAX EL kit (Eurobio). After DNase I treatment, 1 μg of total R NA was reverse transcribed using poly dT15 primers and Superscript II RT (90 min, 37°C; Gibco BRL). The amount of R NA to be used for detection was normalized using the housekeeping gene hypoxanthine phosphoribosyltransferase (HPR T) as reference. The cDNA was amplified using different sets of primers: for HPR T (5' primer, 5'-GTA ATG ATC AGT CAA CGG GGG AC-3'; 3' primer, 5'-CCA GCA AGC TTG CAA CCT TAACCA-3'), for CD8 (5' primer, 5'-AGG ATG CTC TTG GCT CT TCC-3'; 3' primer, 5'-TCA CAG GCG AAG TCT AAC-3'), and for IFN-γ (5' primer, 5'-GCT CTC AGA CAA TGA AGC CT-3'; 3' primer, 5'-AAA GAG ATA ATC TGG CTC TGC-3'). The amplifications were carried out with 29 cycles for HPR T and 33 cycles for IFN-γ and CD8 (1 min at 94°C, 1 min 30 s at 60°C, 2 min at 72°C). The PCR products were analyzed on 1.5% agarose gel.

In Vivo Secondary T Cell Proliferation. Spleen cells from DNFB-sensitized C57BL/6 and P\(^0/0\) gld mice were collected 5 d after sensitization. T lymphocytes were purified through negative selection using anti-Ig columns (Biotech) as described elsewhere (6). The resulting cell suspensions contained >90% CD3\(^+\) viable cells. CD8\(^+\) T cells were isolated from the spleen T cells by elimination of CD4\(^+\) T cells using columns coated with goat anti-mouse and goat anti-rat IgG and a rat anti-mouse CD4\(^+\) mAb (YTS191.1; Biotech). FACS\(^+\) analysis of cells eluted from the column showed <0.5% contaminating CD4\(^+\) T cells. In vivo DNFB-primed unfractionated or CD8\(^+\) T cells (2.5 × 10\(^6\) cells) (6) obtained on day 5 after DNFB sensitization were cocultured for 3 d at 37°C in 96-well plates with 10\(^6\) mitomycin C–treated syngenic spleen cells from naive mice, that were either DNBS-derivatized as described (6) or left untreated. In brief, 10\(^6\) cells were incubated for 30 min with 25 μg/ml of mitomycin C (Sigma), washed, and subjected to 30-min incubation at 37°C with 4 mM DNBS, pH 8.0, in serum-free RPMI medium. The proliferative responses were assessed on day 3 of culture by [\(^3\)H]thymidine incorporation (1 μCi/well) for the last 6 h of culture. The results are expressed as proliferation indices (cpm in cultured T cells + DNBS-treated spleen cells)/(cpm in cultured T cells + untreated spleen cells).

IFN-γ Enzyme-linked Immunospot Assay. Inguinal and axillary LN s were harvested 5 d after DNFB sensitization. Cell suspensions were restimulated in vitro by overnight culture in complete RPMI medium supplemented with 10% FCS and containing a final concentration of 0.4 mM DNBS. Control cultures included cells cultured overnight in medium supplemented with 0.2 mM of the irrelevant hapten TNP, or in medium alone.
The number of IFN-γ-producing cells was determined using an enzyme-linked immunospot (ELISPOT) assay. In brief, 96-well nitrocellulose plates (M AHA 45; M lllipore) were coated overnight at 4°C with anti–IFN-γ antibody (R 46A2) and blocked with PBS/2% BSA for 2 h at 37°C. The plates were washed three times with PBS/Tween 0.1% before use. The cell suspensions were washed, and incubated at different concentrations in duplicate wells for 4 h at 37°C, 5% CO₂. Plates were washed three times with PBS/0.1% Tween and incubated with a biotinylated anti–IFN-γ antibody (AN 18). IFN-γ spot-forming cells (SFCs) were developed using streptavidin–alkaline phosphatase (Boehringer Mannheim), incubated for 2 h, and extensively washed before adding the substrate (5-bromo-4-chloro-3-indolyl-phosphate; Sigma). The number of IFN-γ SFCs present in each well was counted using a microscope, and the results were expressed as IFN-γ SFCs/10⁶ cells.

Production of CTLs. DNBS-specific CTLs were recovered from splenocytes of various H-2bk mice. Spleens were recovered 5 d after cutaneous sensitization with DNFB, perfused with RPMI 1 to eliminate red blood cells. 10⁷ splenocytes were used either fresh or after restimulation for 5 d in culture with 10⁷ syngeneic mitomycin C–treated, DNBS-derivatized spleen cells from either normal C57BL/6, 100. or 100. mice.

Target Cells. Target cells included MBL2 and MBL2-Fas cell lines, provided by Maries van den Broeck (Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland [24]), and EL-4 cells, all maintained in RPMI 1 plus 10% FBS. Target cells were simultaneously haptenated and labeled by incubating 2 × 10⁶ cells in 10 μl of RPMI 1 with or without 40 mM DNBS and 100 μC of Na₂⁵₁CrO₄ (sodium chromate solution, 1 Ci/mM) for 1 h at 37°C with periodic mixing. Labeled targets were washed thoroughly before use.

Cytotoxicity Assays. Various numbers of effector cells were incubated at 37°C for 4 h with 10⁴ labeled target cells. Supernatants were then collected, and ³¹Cr release was counted in a γ counter. The percentage of cytotoxicity was calculated using the formula: 100 × (experimental cpm – spontaneous cpm)/(maximal cpm – spontaneous cpm), where the maximal cpm and spontaneous cpm are the radioactivity released from targets exposed to 0.5 M HCl or medium, respectively. The results presented are representative of at least three independent experiments.

Statistical Analysis. Data were examined for normality and equal variance, and groups were compared by a two-tailed Student’s t test.

Results

CHS Reaction to DNFB Is Impaired in Perforin and FasL Double Knockout (P0/0 Gld) Mice. The contribution of CD8+ T cell–mediated cytotoxicity to the CHS reaction to DNFB was examined in mice double deficient for Fas/FasL and perforin (P0/0 gld), which are devoid of CTL activity (15). The CHS reaction was dramatically reduced in P0/0 gld mice compared with that observed in C57BL/6 mice (Fig. 1 a). No difference in ear swelling could be observed between the sensitized and unsensitized groups of P0/0 gld mice and the sensitized C57BL/6 mice. Similarly, none of the characteristic pathological changes occurring during a normal CHS reaction, namely edema, vascular enlargement, and mononuclear cell infiltration, was observed in P0/0 gld mice (Fig. 1 b).

Spleen cells from sensitized animals were tested for their ability to lyse haptenated targets either directly or after in vitro restimulation. As shown in Fig. 1 c, T cells from DNFB-sensitized C57BL/6 mice exhibited a potent hapten-specific CTL activity after restimulation in vitro but which was already detectable directly ex vivo (data not shown). In contrast, DNFB-specific CTLs could never be

![Figure 1](image_url)

**Figure 1.** CHS to DNFB is impaired in P0/0 gld mice. (a) CHS to DNFB was analyzed in groups of four wild-type C57BL/6 (squares) or P0/0 gld (circles) mice either sensitized with DNFB (filled symbols) or unsensitized (open symbols) and challenged 5 d later on the left ear. Results are expressed as the mean ear swelling (in μm) at different time points after challenge. Results are representative of three independent experiments. (b) Histological analysis of the CHS reaction 24 h after challenge in C57BL/6 and P0/0 gld mice. Major changes were observed in C57BL/6 mice, including edema of the dermis, mononuclear cell infiltration, and enlargement of blood vessels. No main histological modification was noted in the skin of P0/0 gld mice. Original magnification: ×100. (c) Cytotoxic activity of spleen cells from DNFB-sensitized C57BL/6 mice (●), P0/0 gld mice (■), or naïve C57BL/6 mice (□) was assessed after in vitro restimulation for 5 d with haptenated C57BL/6 mice spleen cells. Specific cytotoxic activity was determined by hapten-pulsed MBL2-Fas target lysis in a 4-h ³¹Cr-release assay. Results are representative of three independent experiments.
demonstrated in double-deficient P0/0 gld mice ex vivo, nor after in vitro restimulation with DNBS-derivatized cells. Thus, P0/0 gld mice cannot produce a CHS reaction to DNFB and are unable to develop any of the pathological changes associated with CHS, suggesting that hapten-specific CTL activity is mandatory for expression of the CHS reaction.

DNFB Can Prime for Specific CD8+ T Cells in the Lymphoid Organs of Perforin and FasL Double-deficient Mice. To determine whether the lack of CHS reaction in P0/0 gld mice is secondary to an impairment in the priming of specific CD8+ T cells in the lymphoid organs, P0/0 gld and C57BL/6 mice were sensitized with DNFB, and lymphoid cells were recovered 5 d later and tested for hapten-specific proliferative responses and IFN-γ production.

Lympocytes from P0/0 gld mice responded vigorously to hapten-treated syngenic cells in secondary proliferative responses, with stimulation indices identical to those observed with T cells recovered from sensitized C57BL/6 mice (Fig. 2 a). Similarly, CD8+ T cells from P0/0 gld mice exhibited hapten-specific responses indistinguishable from those of control C57BL/6 cells. Thus, CD8+ T cell priming has occurred in the lymphoid organs of P0/0 gld mice.

We next used an ELISPOT assay to determine the frequency of DNFB-specific, IFN-γ-producing LN cells in primed C57BL/6 and P0/0 gld mice. In C57BL/6 mice, the IFN-γ-producing cells were entirely contained in the CD8+ T cell subset, with a mean frequency of 3 IFN-γ SFCs/10^5 LN cells (Fig. 2 b). Interestingly, P0/0 gld mice exhibited comparable levels of hapten-specific, IFN-γ-producing cells (Fig. 2 c).

These data indicate that the lack of CHS in double-deficient P0/0 gld mice is not due to impaired priming of hapten-specific CD8+ T cells nor to an altered production of IFN-γ, but rather to the deficient CTL activity.
FasL-deficient (gld), and P0/0 mice to exhibit specific CHS and CTL responses after DNFB sensitization.

In marked contrast to double-deficient P0/0 gld mice, lpr, gld, or P0/0 mice developed a normal CHS reaction to DNFB (Fig. 4 a), with intensity, kinetics, and histological characteristics comparable to those observed in C57BL/6 mice. Mice homozygous for the gld mutation and heterozygous for the perforin deletion (P0/0 gld) also had a similar reaction to DNFB (data not shown). Interestingly, lpr, gld, and P0/0 mice exhibited a strong hapten-specific CTL response (Fig. 4 b) as well as normal quantities of DNFB-specific, IFN-γ-producing LN cells (data not shown).

Thus, exclusion of only one cytolytic pathway did not prevent the induction of hapten-specific CTLs nor the development of a CHS reaction to DNFB, indicating that CTLs can use either the Fas/FasL or the perforin pathway to mediate CHS.

Hapten-specific CTL activity was restricted to the MHC class I-restricted CD8+ T cell subset. Indeed, depletion of CD8+ cells, but not of CD4+ cells, totally abolished the CTL activity of primed C57BL/6 spleen cells (data not shown). MHC class I molecules were mandatory for the induction, expansion, and effector function of specific CD8+ CTLs, since: (a) primed spleen cells from C57BL/6 and II0/0, but not from I0/0, mice could lyse haptenated targets (Fig. 5 a); (b) hapten-specific lysis was not observed when effector cells were restimulated in vitro with haptenated APCs from I0/0 mice (Fig. 5 b); and (c) CTL activity of C57BL/6 spleen cells was suppressed by incubation with mAbs to the Kb MHC class I molecules (Fig. 5 c). These results indicate that the hapten-specific CTLs are "classical" MHC class I-restricted CD8+ T cells.

The contribution of perforin or Fas/FasL to the CTL activity was tested using perforin (+) from C57BL/6 mice and perforin-deficient (from P0/0 mice) effectors and targets comprising Fas+ (MBL2-Fas) and Fas-deficient (MBL2)
Discussion

This study demonstrates that CD8+ T cells require cytotoxic activity to mediate CHS. The invalidation of the two main cytolytic pathways, as observed in mice deficient in perforin and FasL (P0/0, gld), is responsible for the lack of generation of specific CTLs and for the abolition of the CHS reaction. Interestingly, the presence of a single cytolytic pathway, in lpr mice, gld mice, and P0/0 mice, is sufficient for the development of a CHS reaction and for the priming of specific CTLs, indicating that the Fas/FasL and the perforin pathways could be used independently and with similar efficiency for cytolytic activity. These results are in line with recent data on influenza virus infection showing that virus clearance by CTLs could be achieved only if one of the two main lytic pathways remained functional (25).

Until our study, cytokines, and especially IFN-γ and TNF-α, were thought to mediate skin inflammation through their ability to activate keratinocytes and endothelial cells (2, 26–29). Recent studies excluded a pivotal role for IFN-γ in the elicitation phase of CHS, since IFN-γ receptor-deficient mice developed a normal CHS reaction (30). In addition, studies in TNF-α-deficient mice indicated that the cytokine was not involved in the elicitation phase but played an important role in the sensitization phase by inducing the emigration of LCs to the draining LN (29).

CHS develops in two phases, the sensitization (i.e., afferent) phase leading to the priming of hapten-specific CD8+ T cells and the elicitation (i.e., effferent) phase occurring after challenge and leading to the development of skin inflammation. MHC class I molecules expressed by LCs are mandatory for the priming of hapten-specific CD8+ T cells in the LN during the afferent phase (6, 31). These MHC class I–restricted CD8+ T cells exert CHS through CTL activity using classical cytolytic pathways that cytotoxicity is the effector mechanism responsible for the effenter phase of CHS is supported by the observation that hapten-primed, IFN-γ–producing CD8+ T cells are present in lymphoid organs of P0/0 gld mice and respond vigorously in secondary proliferative responses, demonstrating that the lack of CHS reaction is not due to an impairment of the sensitization phase of CHS, but rather reflects an alteration during the elicitation phase.

The mechanisms involved in the development of skin inflammation upon challenge in sensitized mice, i.e., during the elicitation phase, associate hapten-specific and nonspecific steps (2). First, lymphocytes have to emigrate from the blood to the skin. Infiltration of the skin by mononuclear cells has been reported to occur within a few hours after challenge (32). Haptens are able to directly induce expression of E- and P-selectins on endothelial cells 2 h after skin painting (33, 34). It is thought that circulating antigen-specific memory T cells which carry homing receptors (the cutaneous leukocyte-associated antigen [CLA] molecule) are able to enter the skin (35) through interaction with P- and E-selectins expressed on endothelial cells (36). Second, specific T cells are activated on hapten presentation by skin-resident cells, as revealed by detection of IFN-γ mRNA in situ between 4 and 8 h after challenge (37, 38). Third, activation of skin-resident cells by T cell cytokines results in the amplification of the inflammatory reaction leading to the cellular inflammatory infiltrate. Upon IFN-γ activation, keratinocytes upregulate intercellular adhesion molecule 1 (ICAM-1) and Ia molecules and produce a wide array of inflammatory cytokines and chemokines such as IL-8 (2, 26, 39, 40). Likewise, endo-
epithelial cell activation is followed by leukocyte migration from the blood vessels to the dermis, leading to the formation of inflammatory cellular infiltrate (2, 26). In this scheme, the hapten-specific limb of the CHS reaction corresponds to the activation of hapten-specific T cells and occurs ~6 h after challenge, whereas the nonspecific phase corresponds to the infiltration of the skin by the polymorphic cellular infiltrate, which peaks 24 h after challenge.

It appears from our data that the failure of P0/0 gd mice to exhibit a CHS reaction is not due to the inability of CD8+ T cells to infiltrate the skin or to be activated, since IFN-γ–producing CD8+ T cells could be detected in the skin 6 h after challenge, at levels comparable to those found in C57BL/6 mice. However, these early parameters of the CHS reaction were not followed by an increase in cellular infiltration in situ, as observed in C57BL/6 mice at 24 and 48 h. These observations suggest that cytolytic function of hapten-specific CD8+ T cells is required for the recruitment of inflammatory cells and full development of the CHS response. Both epidermal dendritic LCs and keratinocytes express MHC class I molecules and may produce upon activation (or lethal hit) a wide array of inflammatory cytokines and chemokines. Destruction of haptenated LCs by CTLs may account for the recent observation that LCs undergo apoptotic cell death in CHS (41). Alternatively, keratinocytes, which represent >90% of epidermal cells, could be the targets of antihapten CTLs, the more so since IFN-γ, produced in situ during the course of CHS, upregulates Fas expression by keratinocytes (42).

In conclusion, our data demonstrate that CHS to DNFB is mediated by hapten-specific CTLs which may use either the Fas/FasL or the perforin pathway for the induction of cutaneous inflammation. The precise nature of the MHC class I–expressing skin cells able to present the hapten to CTLs in vivo during the elicitation phase is currently under investigation.

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