Afferent and Efferent Phases of Allergic Contact Dermatitis (ACD) Can Be Induced After a Single Skin Contact with Haptens: Evidence Using a Mouse Model of Primary ACD

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Allergic contact dermatitis is a T cell-mediated delayed type hypersensitivity reaction that occurs upon hapten challenge in sensitized individuals. The inflammatory response in classical allergic contact dermatitis requires both a sensitization phase and an elicitation phase responsible for the recruitment and activation of specific T cells at the site of hapten skin challenge. Conversely, previously unsensitized patients may develop a "primary allergic contact dermatitis" after the first skin contact with potent contact sensitizers leading to a skin inflammation with all the features of classical allergic contact dermatitis. In this study we used an experimental murine model, referred to as contact hypersensitivity, to study the pathophysiology of primary allergic contact dermatitis and its relationship to classical allergic contact dermatitis. We show that one epicutaneous application of a nonirritant dose of hapten (2,4-dinitrofluorobenzene, fluorescein isothiocyanate) was sufficient to induce an optimal allergic contact dermatitis reaction at the site of primary contact with the hapten without subsequent challenge. As in classical allergic contact dermatitis, the skin inflammation in primary allergic contact dermatitis was mediated by interferon-γ producing, $CD8^+$ effector T cells that were induced in the draining lymph nodes at day 5 postsensitization and downregulated by $CD4^+$ T cells. Reverse transcription–polymerase chain reaction analysis revealed that the primary allergic contact dermatitis reaction was mediated by a recruitment of $CD8^+$ T cells at the sensitization skin site at day 6 postsensitization. Analysis of the fate of the hapten fluorescein isothiocyanate applied once on the skin revealed its persistence in the epidermis for up to 14 d after skin painting. These results suggest that the development of primary allergic contact dermatitis (i.e., without secondary challenge) is associated with persistence of the hapten in the skin, which allows the recruitment and activation of $CD8^+$ T cells at the site of the single hapten application. ***Key words: contact hypersensitivity/hapten persistence/leukocyte trafficking/skin. J Invest Dermatol 120:641–647, 2003***

Allergic contact dermatitis (ACD), one of the most common skin diseases with a great socio-economic impact, is a T cell-mediated inflammatory reaction occurring at the site of challenge with a contact allergen (hapten) in sensitized individuals (Krasteva *et al*, 1999a). Knowledge of the pathophysiology of ACD is derived chiefly from animal models in which the skin inflammation induced by hapten painting of the skin is referred to as contact hypersensitivity (CHS) (Garrigue *et al*, 1994; Van Der Valk, 2002). ACD and CHS are thus considered as synonymous and define a hapten-specific T cell-mediated skin inflammation. They represent a form of delayed type hypersensitivity reactions. As in all delayed type hypersensitivity reactions, the pathophysiology of ACD consists classically of two distinct phases, i.e., the sensitization and the elicitation phases, which are considered to be temporally and spatially dissociated (Enk and Katz, 1995; Grabbe and Schwarz, 1998; Krasteva *et al*, 1999b; Cavani *et al*, 2001). The sensitization phase (also referred to as the afferent phase of ACD) occurs at the first contact of skin with the hapten. Hapten is taken up by skin dendritic cells (DC) that migrate to the draining lymph nodes (LN), where they present haptenated peptides on major histocompatibility complex class I and II molecules resulting in the induction of hapten-specific $CD8^+$ and $CD4^+$ T cells, respectively (Bour *et al*, 1995; Krasteva *et al*, 1997). The sensitization step lasts 8–15 d in humans, 5–7 d in the mouse, and is thought to have no clinical consequence. The elicitation phase, also known as efferent phase or challenge phase of CHS. Challenge with the same hapten in sensitized individuals leads in a few hours to the appearance of ACD. Upon subsequent contacts of the skin with the hapten, effector T lymphocytes are recruited and activated in the dermis, and trigger the inflammatory process responsible for the cutaneous lesions. This efferent phase of CHS takes 72 h in humans, and 24–48 h in the mouse. The inflammatory reaction persists during several days and progressively decreases upon physiologic downregulating mechanisms. Although in some experimental systems $CD4^+$

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Abbreviations: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; DC, dendritic cell; DNBS, 2,4-dinitrobenzenesulfonic acid.

T cells have been shown to mediate the CHS responses (Gocinski and Tigelaar, 1990; Wang B *et al*, 2000), recent data from our laboratory and others pointed to the major role of $CD8^+$ T cells as effector cells in CHS to different haptens (Bour *et al*, 1995; Xu *et al*, 1996; Bouloc *et al*, 1998; Cavani *et al*, 2001). Indeed, CHS to 2,4-dinitro-fluorobenezene (DNFB) is mediated by $CD8^+$ cytotoxic T lymphocytes (CTL), generated in the lymphoid organs during the sensitization phase and rapidly recruited into the skin after challenge where they induce ACD through cytotoxicity (Kehren *et al*, 1999; Akiba *et al*, 2002). Infiltration of challenged skin by $CD8^+$ T cells was associated with keratinocyte apoptosis, suggesting that epidermal cells were the cell target of specific $CD8^+$ CTL. Conversely, $CD4^+$ T cells, in this model, were downregulating the inflammatory reaction as, in their absence, the ACD reaction was increased and prolonged (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Bouloc *et al*, 1998).

Although it has been postulated that ACD is elicited by hapten exposure in sensitized individuals, clinical evidence has demonstrated that "primary ACD" could develop in previously unsensitized patients. These reactions, known as "primary allergic reactions" or "active sensitization" develop 7–15 d after skin contact with potent contact sensitizers such as DNFB/dinitrochlorobenzene, urushiol, and primine (Adams and Fischer, 1990; Kanerva *et al*, 1994; Vigan *et al*, 1997). The eczematous lesions are morphologically indistinguishable from conventional ACD and have the same kinetics in the amplification and the spontaneous regression of skin inflammation, suggesting that similar effector and downregulatory mechanisms are involved in classical and primary ACD; however, the relationship between primary and classical ACD has never been firmly established. In order to get a better insight into the mechanisms of induction of skin inflammation in primary ACD, we have developed a mouse model in which a single topical application of the strong haptens DNFB and fluorescein isothiocyanate (FITC) elicit an ACD response. We show that the pathophysiology of one-step primary ACD is identical to that of two-step classical ACD and involves $CD8^+$ effector T cells primed in the draining LN and recruited at the site of primary sensitization. Use of FITC to follow the hapten fate shows its persistence in epidermal cells for as long as 14 d after a single skin application. Our mouse model provides evidence that primary allergic reactions are elicited and downregulated by immune mechanisms similar to those of ACD in sensitized subjects, allowing them to be called "primary ACD".

MATERIALS AND METHODS

Mice Female BALB/C mice, 6 wk old, were purchased from Iffa-Credo (L'Arbresle, France). Animals were left to acclimate for 1 wk before entering the study. Five mice were used per group. Mice were housed individually and were provided food and water ad libidum.

Reagents DNFB (Sigma, St Louis, MO) and 4-ethoxy-methylene-2-phenyloxazol-5-one (oxazolone; Aldrich, Milwaukee, WI) were diluted in acetone/olive oil (4 : 1). FITC (Sigma) was diluted in acetone/dibutylphthalate (1 : 1). Haptens were freshly prepared before application.

Assay for murine ACD: the mouse ear swelling test

Assay for classical ACD The original procedure of the mouse ear swelling test has been described in detail elsewhere (Garrigue *et al*, 1994; Bour *et al*, 1995) and is summarized **Fig 1**(***A***). Briefly, mice were sensitized by epicutaneous application of haptens on to 2 cm^2 of fur-shaved dorsal skin at an irritant concentration of either 0.5% of DNFB or 2% of oxazolone diluted in acetone/olive oil (4 : 1; 25 μl). Five days later, animals were challenged on the ear by a nonirritant concentration of 0.2% of DNFB or 0.3% of oxazolone diluted in acetone/olive oil (4 : 1; 5 μl) applied on to each side of one ear, whereas the same volume of vehicle was applied on the contralateral ear. FITC was applied at 0.5% concentration both for sensitization (40 μl) and for challenge (2 × 5 μl) on each side of one ear. Ear thickness measurements were taken for each ear prior to hapten application with a spring-loaded micrometer (J15, Blet SA, Lyon, France). Measurements were repeated once daily after challenge. Ear swelling was calculated by subtracting the initial value from the value recorded on the corresponding day, and further subtracting any swelling recorded for the vehicle-control ear from the swelling recorded for the hapten-challenged ear.

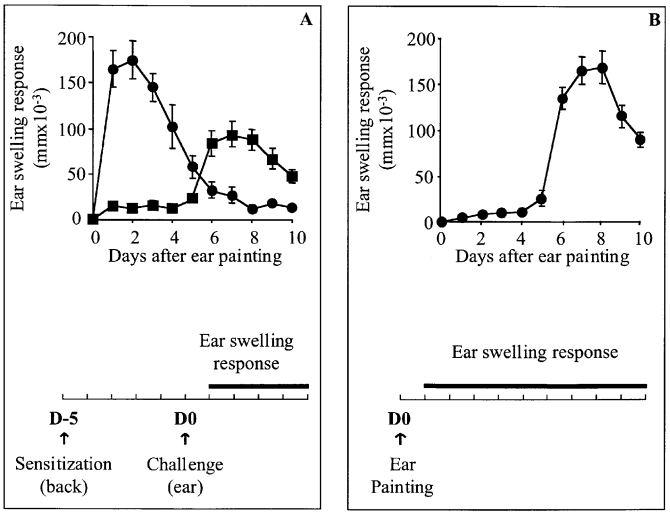

Figure 1. Kinetics of the inflammatory response to DNFB during classical ACD and primary ACD. (*A*) Inflammatory response in sensitized and challenged mice (classical ACD). Mice were sensitized by skin painting with DNFB 0.5% on the shaved back skin. Five days later they were ear challenged with DNFB 0.2% (●) or oxazolone 0.3% (■). (*B*) Inflammatory response induced by a single hapten application (primary ACD). Unsensitized mice were ear painted with DNFB 0.2% (●). Results are expressed as the mean ear swelling at different time points after challenge and are representative of three independent experiments.

Assay for primary ACD Naive mice were sensitized by a single application of a nonirritant concentration of haptens (DNFB 0.2%, oxazolone 0.3%, and FITC 0.5%) on to the left ear, whereas the right ear was painted with an equal volume of vehicle. The protocol is summarized in **Fig 1**(***B***). At various times after ear sensitization, ear thickness was measured and ear swelling calculated as described for the classical murine ACD reaction.

RNA extraction and reverse transcription-polymerase chain reaction (reverse transcription-PCR) analysis of CD8 and interferon (IFN)-γ mRNA At different intervals after challenge (classical ACD) or sensitization (primary ACD), ear samples were collected from sensitized or unsensitized mice and frozen in liquid nitrogen. The detection of RNA was conducted as described in detail elsewhere (Delassus *et al*, 1994). In brief, total RNA was extracted using the RNAXEL kit (Eurobio, F-91953, Lesulis, France). After DNase I treatment, 1 μg of total RNA was reverse transcribed using poly dT15 primers and Superscript II reverse transcriptase (90 min, 37°C; Gibco BRL) (Invitrogen, F95613, Cergy Pontoise). The amount of RNA to be used for detection was normalized using the housekeeping gene hypoxanthine phosphoribosyltransferase as reference. The cDNA obtained was amplified using different sets of primers:

For hypoxanthine phosphoribosyltransferase: 5′ primer, 5′-GTA ATG ATC AGT CAA CGG GGG AC-3′; 3′ primer, 5′-CCA GCA AGC TTG CAA CCT TAA CCA-3′

For CD8: 5′ primer, 5′-AGG ATG CTC TTG GCT CTT CC-3′; 3′ primer, 5′-TCA CAG GCG AAG TCC AAT CC-3′

For IFN-γ: 5′ primer, 5′-GCT CTG AGA CAA TGA ACG CT-3′; 3′ primer, 5′-AAA GAG ATA ATC TGG CTC TGC-3′.

The amplifications were carried out with 29 cycles for hypoxanthine phosphoribosyltransferase 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.

IFN-γ Enzyme-linked Immunospot Assay (ELISPOT) Inguinal plus axillary (classical ACD) or auricular (primary ACD) draining LN were harvested 5 d after DNFB sensitization. The number of IFN-γ-producing cells was determined using an ELISPOT assay. In brief, 96-well nitrocellulose plates (MAHA 45; Millipore) (Millipore, F-78054, ST. Quentin en Yuelines) were coated overnight at 4°C with anti-IFN-γ-antibody (R46A2) and blocked with phosphate-buffered saline (PBS)/1% bovine serum albumin for 2 h at 37°C. The plate were washed three times with PBS/Tween 0.1% before use. The LN cell suspensions were incubated

with 0.4 mM of DNBS directly on the plates overnight at 37°C, 5% CO_2. Plates were washed three times with PBS/Tween 0.1% and incubated 2 h at room temperature with a biotinylated anti-IFN-γ-antibody (XMG1.2) and extensively washed. IFN-γ spot-forming cells were developed using streptavidin–alkaline phosphatase (Boehringer Mannheim), incubated for 2 h at room temperature, and extensively washed before adding the substrate (5-bromo-4-chloro-3-indolyl-phosphate; Sigma). The number of IFN-γ spot-forming cells present in each well was counted using the Carl Zeiss vision ELISPOT (Carl Zeiss Vision, D-85399, Hallberghoos, Germany) and the results were expressed as IFN-γ spot-forming cells/10^5 LN cells.

***In vivo* depletion of CD4$^+$ and CD8$^+$ T cells** The rat anti-mouse CD4 monoclonal antibody (MoAb) GK 1.5 (Kehren *et al*, 1999) was obtained from the American Type Culture Collection (Rockville, MD) and the rat anti-mouse CD8 MoAb H35.17.2 (Akiba *et al*, 2002) was kindly provided by G. Milon (Institut Pasteur, Paris). Mice were given i.p. injections of 200 μl of anti-CD4 or anti-CD8 MoAb as 1 : 20 dilution of ascites on days −1, 0, +1, and + 4 of skin sensitization. Cell depletion was assessed in each mouse by staining peripheral blood mononuclear cells drawn from the tail vein, using FITC- or phycoerythrin-labeled anti-CD4 or anti-CD8 MoAb (Tebu, Le Perray-en-Yvelines, France).

Immunohistochemical analysis of epidermal sheets Epidermis from the challenged ears was peeled off the connective tissue by incubation for 1 h at 37°C in PBS supplemented with ethylenediamine tetraacetic acid 20 mM (Sigma). The sheets were cut into small pieces, rinsed, and incubated overnight at 4°C with rat IgG2b anti-mouse Ia (clone CD311) or isotype-matched irrelevant antibody. After three washes in PBS/1% bovine serum albumin, the sheets were incubated for 30 min at room temperature with biotinylated F(ab′)2 fragment of goat antibodies specific for rat IgG (H + L) (Pierce, Interchim, Montluçon, France), washed, and then further incubated with streptavidin conjugated to peroxidase (ABC kit, Dako, Carpinteria, CA). The reaction was developed using 3-amino-9-ethylcarbazole (AEC) substrate and H_2O_2 (Dako).

RESULTS

Primary ACD can develop after a single hapten exposure in unsensitized mice The classical murine model of the ACD

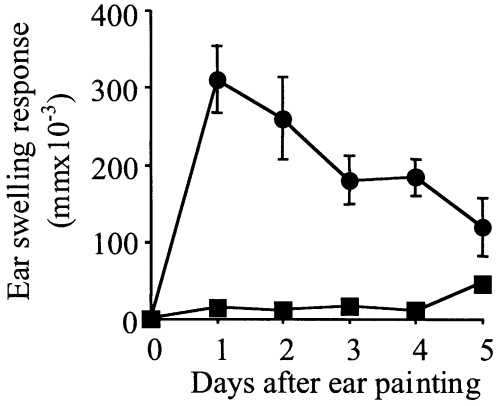

Figure 2. Hapten specificity of the inflammatory reaction induced in primary ACD. DNFB 0.2% (●) or oxazolone 0.3% (■) were applied on the right ear of mice that, 30 d previously, have developed a primary ACD following skin application of DNFB 0.2% on the left ear.

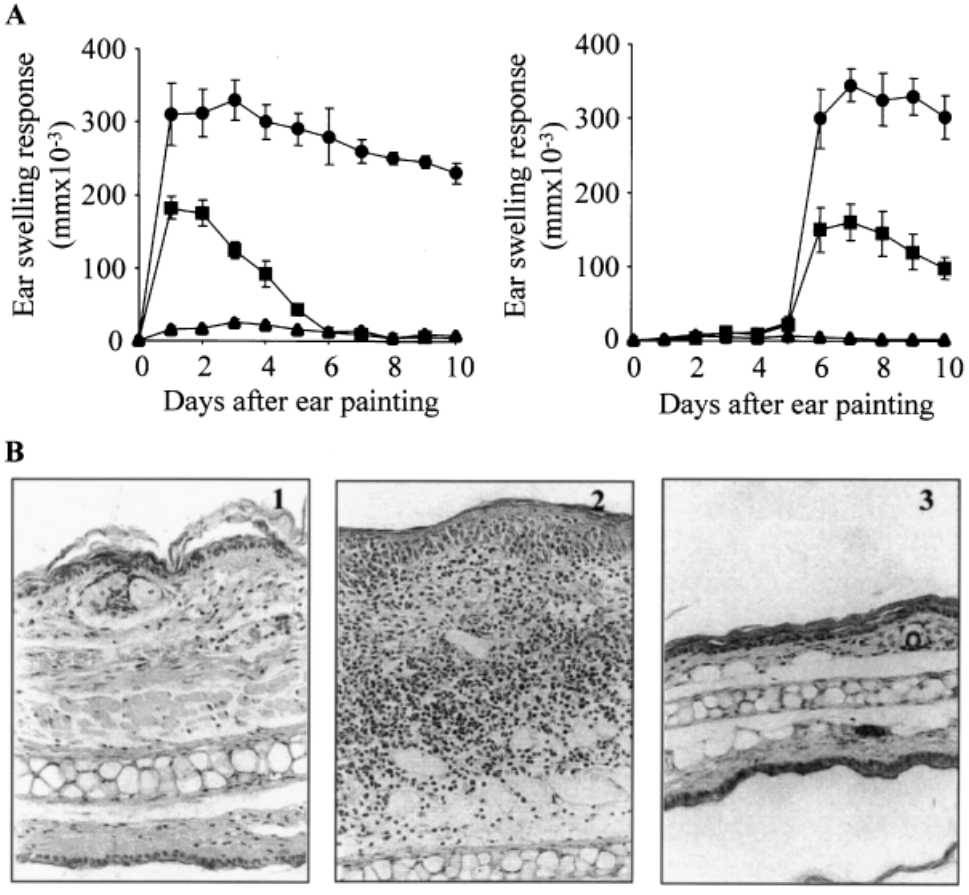

Figure 3. Effect of CD4$^+$ and CD8$^+$ T cell depletion on the primary ACD reaction. (*A*) Mice were subjected either to the classical ACD (*left panel*) assay or to the primary ACD assay (*right panel*). CD4$^+$ T cell-depleted (●), CD8$^+$ T cell-depleted (▲) and untreated mice (■) from both groups were ear painted simultaneously with DNFB 0.2% at day 0. Results are expressed as the mean ear swelling at different time points after challenge. (*B*) Histologic analysis of the skin inflammatory reaction in primary ACD. Mice were ear painted with DNFB 0.2% at day 0 (as in *A*). Biopsies were taken at day 6. (*B1*) Typical histology of ACD reaction in DNFB sensitized, untreated mice. (*B2*) Exaggerated inflammatory skin reaction in CD4$^+$ T cell-depleted mice, including enhanced edema, vasodilatation and prominent mononuclear cell infiltration. (*B3*) Lack of skin inflammation in CD8$^+$ T cell-depleted mice. HES staining; original magnification × 40.

reaction requires two applications of the hapten. Application of DNFB on the ears of previously sensitized mice induces an ear swelling that peaks 24–48 h postchallenge and then rapidly decreases (**Fig 1*A***). An irrelevant hapten (oxazolone) applied on the ear of DNFB-sensitized mice fails to produce a classical ear swelling response at 24–48 h, thus demonstrating the hapten specificity of the inflammatory reaction (**Fig 1*A***). Interestingly, these DNFB-sensitized, oxazolone-challenged mice developed a delayed skin inflammatory reaction at the site of challenge with kinetics comparable with that observed in ACD. This reaction, which has been previously described and referred to as "primary immune response to cutaneous antigens" (Williams *et al*, 1994), corresponds to a primary ACD that is studied in detail below.

Primary ACD is obtained by application of DNFB on the ear of unsensitized mice, which induced an inflammatory reaction starting at day 6, peaking at days 7–8 and quickly subsiding thereafter (**Fig 1*B***). The magnitude of primary ACD is similar to that of classical ACD but has a different kinetic since the onset of the skin inflammation is delayed by 5 d. This period might correspond to the time required to achieve T cell priming (Macatonia *et al*, 1986; Kripke *et al*, 1990). Challenge with DNFB on the contralateral ear 30 d later induced a classical ACD with ear swelling peaking at 24 h, confirming that the initial inflammatory reaction was responsible for the generation of immunologic memory (**Fig 2**). Alternatively, challenge with an irrelevant hapten (i.e., oxazolone 0.3%) did not induce any inflammatory response in the DNFB-sensitized mice, demonstrating that primary ACD is a hapten-specific inflammatory reaction (**Fig 2**).

Primary ACD is mediated by $CD8^+$ effector T cells and downregulated by $CD4^+$ T cells We have previously shown that $CD8^+$ CTL were effector cells in CHS reactions to DNFB in BALB/C mice (Bour *et al*, 1995). In order to analyze the contribution of $CD8^+$ and $CD4^+$ T cells in the primary ACD reaction, mice were depleted in $CD4^+$ or $CD8^+$ T cells by treatment with specific MoAb. In both classical ACD (**Fig 3*A***, left) and primary ACD (**Fig 3*A***, *right*), $CD8^+$ T cell depletion totally abolished the inflammatory response, whereas $CD4^+$ T cell depletion yielded to enhanced and sustained skin inflammation. Histologic examination of the ears of DNFB-sensitized, but untreated mice, at the peak of the skin inflammation (i.e., at day 6 after skin painting) showed that the ear swelling in primary ACD was associated with dermal edema, vascular enlargement, and mononuclear cell infiltration (**Fig 3*B1***). These changes were similar to those observed in classical murine ACD (not shown). Primary ACD induced in $CD4^+$ T cell-depleted mice resulted in a dramatic increase in dermal thickness, which was due to edema, vascular enlargement, and massive infiltration of mononuclear cells (**Fig 3*B2***). In contrast, $CD8^+$ T cell-depleted mice exhibited a normal skin histology, undistinguishable from that of naive untreated mice (**Fig 3*B3***). Thus, T cell involvement in primary ACD to DNFB is similar to that observed in classical ACD and comprises $CD8^+$ effector T cells and $CD4^+$ downregulatory T cells.

Studies of the afferent and efferent phase of primary ACD The pathophysiology of classical ACD is well-known and requires two temporally dissociated steps: the afferent phase (sensitization) and the efferent phase (elicitation). In order to get insight into the pathophysiology of primary ACD we next analyzed the afferent and efferent phases involved in the generation of hapten-specific inflammation following a single skin contact with DNFB.

We first studied the mechanisms of T cell priming occurring during the afferent phase in primary ACD. BALB/C mice were sensitized with DNFB in the classical or primary ACD model and lymphoid cells of the draining LN (inguinal plus axillary LN and auricular LN, respectively) were recovered 5 d later and tested in an ELISPOT assay to determine the frequency of DNFB-specific, IFN-γ-producing LN cells. As previously shown, in this system, IFN-γ production is restricted to $CD8^+$ effector T cells (Xu *et al*, 1996; Kehren *et al*, 1999). **Figure 4*A*** shows that the frequency of DNFB-specific, IFN-γ-producing T cells was equivalent in the primary and classical ACD reactions, demonstrating that application of hapten on the ear led to efficient T cell priming in auricular draining LN.

Study of the efferent phase of CHS was done by analyzing the recruitment of IFN-γ-producing $CD8^+$ T cells in the ear skin at days 4, 5, and 6 after ear skin sensitization. We have previously shown that recruitment of $CD8^+$ T cells could be followed by reverse transcription-PCR for CD8 and IFN-γ mRNA (Kehren *et al*, 1999; Akiba *et al*, 2002). In classical ACD, $CD8^+$ T cells infiltrate the skin early after hapten challenge and are maximal 24/48 h postchallenge (Akiba *et al*, 2002; **Fig 4*B***). In primary ACD, PCR analysis showed that CD8 and IFN-γ mRNA were neither found in the skin of nave mice nor in the ears at days 3 or 4 after hapten painting. In contrast, upregulation of both CD8 and IFN-γ mRNA occurred at day 5 and were maximum at day 6 (**Fig 4*B***).

These data indicate that the mechanisms involved in the generation of primary ACD are identical to those involved in classical ACD and comprise temporally dissociated afferent and efferent phases.

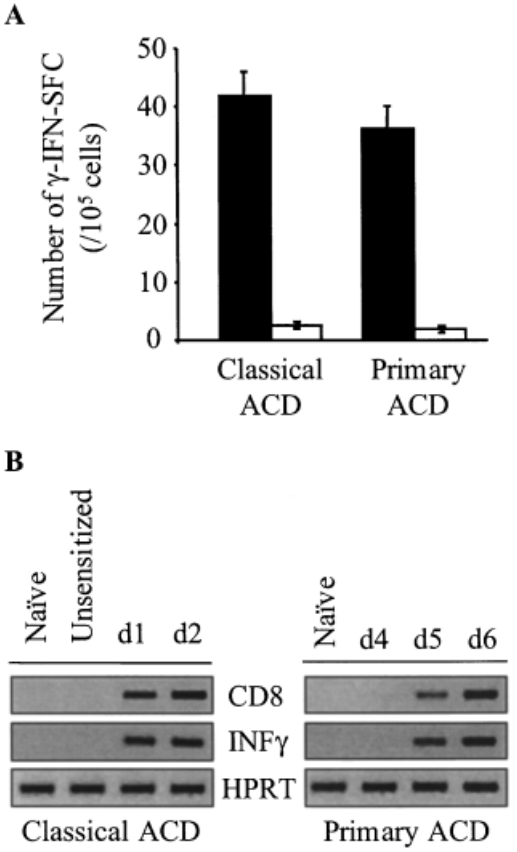

Figure 4. Priming of specific T cells in draining LN and their subsequent recruitment in haptenized skin in primary and classical ACD. (*A*) DNBS-specific IFN-γ spot-forming cells in LN from DNFB-sensitized BALB/C mice in the classical or primary ACD model after overnight restimulation with (*black bars*) or without (*white bars*) DNBS. (*B*) Detection of CD8 and IFN-γ mRNA in the ear skin during classical and primary ACD. CD8 and IFN-γ mRNA expression *in situ* was analyzed using semiquantitative reverse transcription-PCR; mRNA was obtained from the ear of mice at days 1 and 2 postchallenge in classical ACD and at days 4, 5, and 6 post ear sensitization. Controls included ears of untreated (nave) and ears of unsensitized but challenged mice.

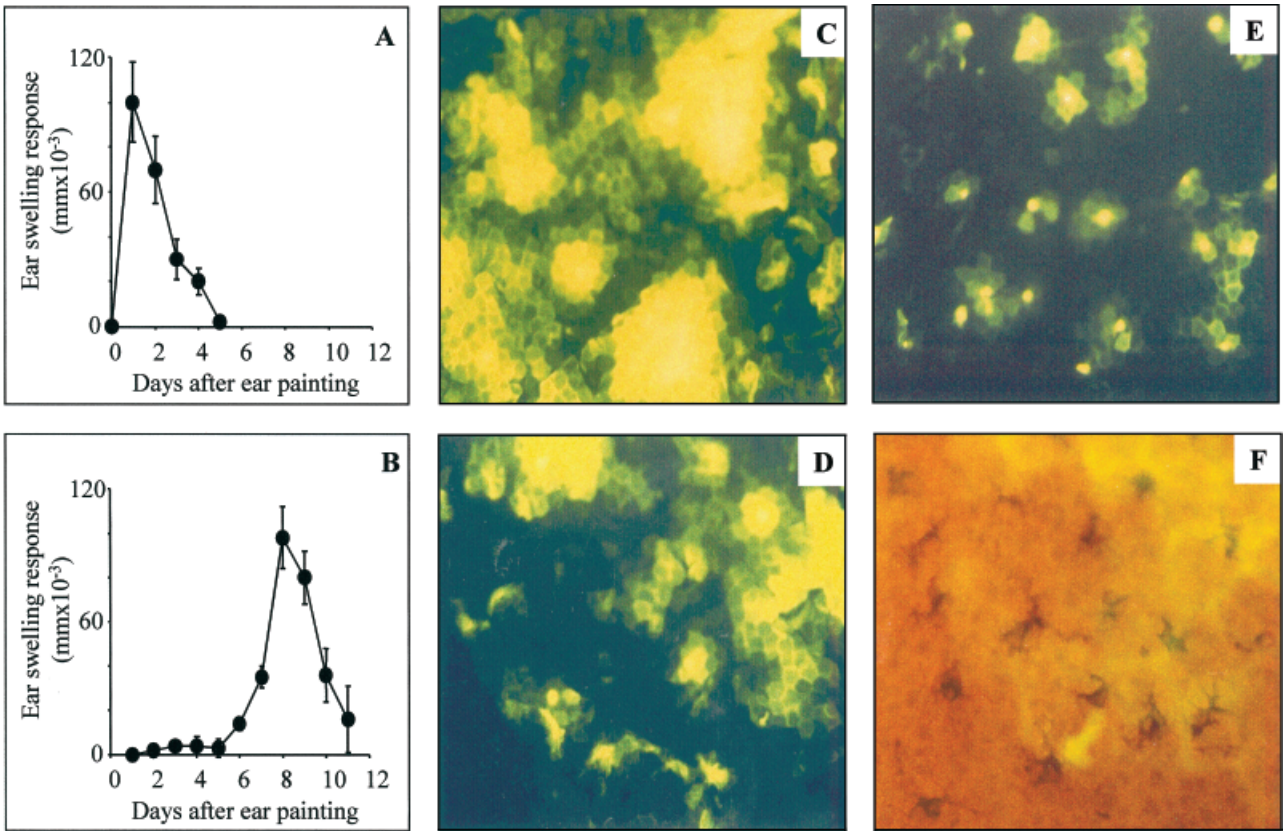

Figure 5. Primary ACD reaction to FITC is associated with persistence of the hapten in the skin. Kinetics of the classical (*A*) and primary (*B*) ACD to FITC. Mice were either sensitized by applying FITC 0.5% on the back skin at day – 5 (*A*) or unsensitized prior to ear painting (*B*). Both experimental groups were ear painted simultaneously at day 0, by application of FITC 0.5% on the left ear. Results are expressed as the mean ear swelling at different time points after challenge and are representative of three independent experiments. Immunohistochemical analysis of epidermal sheets showing FITC staining of epidermal cells at 24 h (*C*), day 6 (*D*), and day 14 (*E*) after ear painting. Staining of epidermal sheets with CD311 (anti-Ia) monoclonal antibody at day 9 after ear painting (*F*) showed that FITC staining was mostly found on Ia^- epidermal cells. Double-stained epidermal cells with dendritic cell morphology, presumably Langerhans cells, comprised only a minority of FITC-positive epidermal cells (original magnification × 100 for *C–E*; × 250 for *F*).

Primary ACD is associated with hapten persistence in the skin The observation that primary ACD developed 6–7 d after a single skin painting with DNFB and resolved after days 9–12 suggested that the hapten persisted in the skin for at least 7 d. In order to test this hypothesis, the fluorescent hapten FITC was used to examine both the fate of hapten in the skin and the induction of FITC-specific ACD.

We first observed that the hapten FITC induced a classical ACD **(Fig 5*A*)** (sensitization on the back skin at day 0 and challenge on the ear skin at day 5) as well as a primary ACD (single application of FITC at day 0 on the ear) (**Fig 5*B***). We next followed the distribution of FITC in the epidermis at various time points after the single hapten application on the ear skin. Epifluorescence examination of epidermal sheets revealed FITC persistence for up to 14 d. Large areas of confluent brightly fluorescent epidermal cells, with morphologic features of keratinocytes were observed at 24 h after FITC painting (**Fig 5*C***) and were still present at day 6 (**Fig 5*D***). From day 6, the number of $FITC^+$ cells and the fluorescence intensity of individual cells declined, leading to a pattern of perifollicular staining, which was observed up to day 14 (**Fig 5*E***). Double staining of epidermal sheets using anti-Ia MoAb showed that, at day 9 post-FITC painting, $FITC^+$ cells comprised mostly keratinocytes and that only a limited number of major histocompatibility complex class II^+ Langerhans cells had taken up the hapten (**Fig 5*F***).

Thus, our results show that primary ACD, which is associated with the recruitment of effector T cells at the site of the initial contact with hapten, is due to the persistence of the hapten in the skin.

DISCUSSION

It has been postulated that ACD in humans is elicited by hapten exposure in a previously sensitized individual; however, "primary allergic reactions" characterized by lesions appearing 10–14 d after either skin contact with dinitrochlorobenzene or patch testing with other strong contact allergens have been reported in patients who have never been exposed before (Adams and Fischer, 1990; Kanerva *et al*, 1994; Vigan *et al*, 1997). In this study we have developed a murine model of primary ACD, which demonstrates that a hapten-specific skin inflammation can develop in naive mice within 7 d after a single epicutaneous exposure to strong contact sensitizers, including DNFB and FITC. This immune response was hapten-specific, induced immunologic memory and exhibited immunologic features similar to those observed in classical ACD, which develops in previously sensitized mice upon hapten challenge. The kinetics of the skin inflammation (bell-curve shape) as well the intensity of the ear swelling was similar to that of classical ACD.

The afferent phase of primary ACD was, as expected, similar to that of classical ACD, despite differences in the protocol of sensitization concerning the site of sensitization and dose of hapten used. The sensitization site was the back or abdominal skin in classical ACD and the ear skin in primary ACD. The dose of hapten was lower in primary ACD and did not induce a clinical irritant inflammatory reaction as assessed by the lack of ear swelling 24 h after skin painting. Primary ACD is mediated by hapten-specific $CD8^+$ T cells, which are primed in the draining auricular LN by skin migrating DC. In primary ACD, hapten

(FITC)-loaded skin DC were found in the draining auricular LN 24 h after ear skin painting and expressed markers of mature DC, similar to what is observed in classical ACD (data not shown) (Macatonia *et al*, 1987). It is noteworthy that ear priming gave rise 5 d after skin painting to the same number of hapten-specific $CD8^+$ T cells/LN as regular sensitization on the abdominal or back skin.

Studies of the development of the primary ACD reaction demonstrated that the efferent phase of ACD could develop in the absence of challenge. In classical ACD, the recruitment in the skin and the subsequent activation of specific effector T cells is measured at the site of the challenge, which is distant from the site of sensitization. Application of the hapten on the ear skin in sensitized mice induces activation of innate skin immune cells, especially keratinocytes and endothelial cells, leading to synthesis of proinflammatory cytokines, chemokines, and expression of adhesion molecules by endothelial cells (Enk and Katz, 1992). The latter allow the recruitment at the challenged site of T cells bearing appropriate homing receptors, including $CD8^+$ T cells, which have emigrated from the LN and traffic through the blood (Robert and Kupper, 1999). Recruitment of effector T cells at the site of hapten painting was demonstrated in the primary ACD by reverse transcription-PCR analysis of CD8 mRNA, which was previously shown to correlate with the degree of infiltration of $CD8^+$ T cells during the elicitation phase of CHS (Akiba *et al*, 2002). $CD8^+$ T cells could be found neither in naive skin nor in the skin 4 d after hapten painting. $CD8^+$ T cells started to infiltrate the skin at day 5. This implies that the minimal time between sensitization and ability of effector cells to be recruited in the skin is 5 d. Interestingly, our results suggest that the inflammatory reaction induced by the pro-inflammatory properties of haptens, which is required for the recruitment of leukocytes in a tissue, is still present 5 d after skin painting. Activation of effector T cells was monitored by reverse transcription–PCR for IFN-γ, whose production in this system is restricted to $CD8^+$ T cells (Xu *et al*, 1996; Kehren *et al*, 1999; Akiba *et al*, 2001). Results provided evidence that infiltrating $CD8^+$ T cells in primary ACD were activated *in situ* and produced IFN-γ. Thus this new protocol could be of relevant interest to study the skin T cells recruitment and activation.

The nature of the skin cell able to present haptenated peptides to specific T cells and to induce their activation is still a matter of debate. Although DC are undoubtedly involved in hapten presentation during the sensitization phase of ACD, their role during the elicitation phase is unclear. As, in our system, CD8 effector T cells are restricted to major histocompatibility complex class I molecules (Bour *et al*, 1995; Bouloc *et al*, 1998), all major histocompatibility complex class I-expressing skin cells could be involved in hapten presentation. In this respect, we have recently shown, in murine models of ACD, that keratinocytes were the main target of $CD8^+$ CTL during the elicitation phase of CHS with early apoptosis of epidermal basal cells paralleling $CD8^+$ T cell infiltration. These data, which are in agreement with studies on human ACD (Trautmann *et al*, 2000), suggested that keratinocytes were the main hapten-presenting cells in ACD with two pathophysiologic consequences, i.e., activation of $CD8^+$ CTL and induction of keratinocyte apoptosis. Using FITC-specific primary ACD we could show that capture of FITC by epidermal cells and its retention in the skin for several days (up to day 14 after single painting) most likely accounts for the ability to develop skin inflammation at the site of primary contact even in the absence of secondary exposure. Hapten persistence allowed the two major events necessary for the development of the efferent phase of CHS: (i) recruitment of T cells in the skin under the control of inflammatory signals induced by the hapten, and (ii) activation of effector cells by hapten-presenting cells, implying that the hapten is present in an immunogenic form.

Our data illustrate the role of $CD4^+$ T cell-mediated downregulation of antigen-specific skin inflammation (Xu *et al*, 1996; Biedermann *et al*, 2001; Gorbachev and Fairchild, 2001). Indeed, mice deficient in $CD4^+$ T cells mounted an exaggerated and sustained primary ACD reaction, which was associated with massive skin infiltration with mononuclear cells, consistent with a chronic activation of $CD8^+$ effector cells. It is somehow surprising that, in normal mice, downregulation of skin inflammation started on day 8 after skin painting and was completed by day 12 despite persistence of the hapten in the epidermis. This suggests that the mechanisms involved in triggering the regulatory pathway does not preclude complete elimination of the hapten from the skin. The precise mechanisms whereby $CD4^+$ T cells exert their regulatory activity is not known (Biedermann *et al*, 2001; Bour *et al*, 1997). We have previously shown that haptenated DC are able to prime both hapten-specific $CD8^+$ and $CD4^+$ T cells and that regulation of ACD may be induced by adoptive transfer of haptenated II^+ I^- DC before epicutaneous sensitization in normal mice (Grabbe and Schwarz, 1998). It is thus possible that $CD4^+$ T cells primed with the hapten in lymphoid organs regulate expansion and/or function of $CD8^+$ effectors through as yet unidentified mechanisms that may include apoptosis through Fas/Fas ligand interaction (Piazza *et al*, 1997) or production of anti-proliferative cytokines (Fiorentino *et al*, 1991; Berg *et al*, 1995).

In conclusion, afferent and efferent phases of hapten-specific skin inflammation can be induced after a single skin contact with haptens, due to persistence of the hapten in the skin for long periods of time. The finding that the regulation of the allergic response occurs despite continuous presence of the hapten in the skin suggests that this model could be useful for understanding the mechanisms involved in the regulation of antigen-specific inflammatory responses.

REFERENCES

Adams R, Fischer T: Diagnostic patch testing. In: Adams R (ed). *Occupational Skin Disease*. Philadelphia: Saunders Company, 1990: pp 223–253

Akiba H, Ducluzeau MT, Nicolas JF: Interferon-gamma production in skin during contact hypersensitivity: No contribution from keratinocytes. *J Invest Dermatol* 117:163, 2001

Akiba H, Kehren J, Ducluzeau MT, *et al*: Skin inflammation during contact hypersensitivity is mediated by early recruitment of $CD8^+$ T cytotoxic 1 cells inducing keratinocyte apoptosis. *J Immunol* 168:3079–3087, 2002

Berg DJ, Leach MW, Kuhn R, *et al*: Interleukin 10 but not interleukin 4 is a natural suppressant of cutaneous inflammatory responses. *J Exp Med* 182:99–108, 1995

Biedermann T, Mailhammer R, Mai A, *et al*: Reversal of established delayed type hypersensitivity reactions following therapy with IL-4 or antigen-specific Th2 cells. *Eur J Immunol* 31:1582–1591, 2001

Bouloc A, Cavani A, Katz SI: Contact hypersensitivity in MHC class II-deficient mice depends on CD8 T lymphocytes primed by immunostimulating Langerhans cells. *J Invest Dermatol* 111:44–49, 1998

Bour H, Peyron E, Gaucherand M, *et al*: Major histocompatibility complex class I-restricted CD8 + T cells and class II-restricted CD4 + T cells, respectively, mediate and regulate contact sensitivity to dinitrofluorobenzene. *Eur J Immunol* 25:3006–3010, 1995

Bour H, Horand F, Krasteva M, Nicolas JF: Role of CD4 + T cells and of the CD4 molecule in contact sensitivity. *J Invest Dermatol* 108:811–812, 1997

Cavani A, Albanesi C, Traidl C, Sebastiani S, Girolomoni G: Effector and regulatory T cells in allergic contact dermatitis. *Trends Immunol* 22:118–120, 2001

Delassus S, Coutinho GC, Saucier C, Darche S, Kourilsky P: Differential cytokine expression in maternal blood and placenta during murine gestation. *J Immunol* 152:2411–2420, 1994

Enk, AH, Katz SI: Early molecular events in the induction phase of contact sensitivity. *Proc Natl Acad Sci USA* 89:1398–1402, 1992

Enk, AH, Katz SI: Contact sensitivity as a model for T-cell activation in skin. *J Invest Dermatol* 105:80–83, 1995

Fiorentino DF, Zlotnik A, Vieira P, *et al*: IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 146:3444–3451, 1991

Garrigue JL, Nicolas JF, Fraginals R, Benezra C, Bour H, Schmitt D: Optimization of the mouse ear swelling test for in vivo and in vitro studies of weak contact sensitizers. *Contact Dermatitis* 30:231–237, 1994

Gocinski BL, Tigelaar R: Roles of CD4 + and CD8 + T cells in murine contact sensitivity revealed by in vivo monoclonal antibody depletion. *J Immunol* 144:4121–4125, 1990

Gorbachev AV, Fairchild RL: Regulatory role of CD4 + T cells during the development of contact hypersensitivity responses. *Immunol Res* 24:69–77, 2001

Grabbe S, Schwarz T: Immunoregulatory mechanisms involved in elicitation of allergic contact hypersensitivity. *Immunol Today* 19:37–44, 1998

Kanerva L, Tarvainen K, Pinola A, *et al*: A single accidental exposure may result in a chemical burn, primary sensitization and allergic contact dermatitis. *Contact Dermatitis* 31:229–235, 1994

Kehren J, Desvignes C, Krasteva M, *et al*: Cytotoxicity is mandatory for CD8(+) T cell-mediated contact hypersensitivity. *J Exp Med* 189:779–786, 1999

Krasteva M, Kehren J, Horand F, Choquet G, Kaiserlian D, Nicolas JF: Dual role of dendritic cells in the induction and the down-regulation of antigen-specific cutaneous inflammation. *J Immunol* 160:1181–1190, 1997

Krasteva M, Kehren J, Sayag M, Ducluzeau MT, Dupuis M, Nicolas JF: Contact dermatitis—II. Clinical aspects and diagnosis. *Eur J Dermatol* 9:144–159, 1999a

Krasteva M, Kehren J, Ducluzeau MT, *et al*: Contact dermatitis—I. Pathophysiology of contact sensitivity. *Eur J Dermatol* 9:65–76, 1999b

Kripke ML, Munn CG, Jeevan A, Tang JM, Bucana C: Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. *J Immunol* 145:2833–2838, 1990

Macatonia SE, Edwards AJ, Knight SC: Dendritic cells and the initiation of contact sensitivity to fluorescein isothiocyanate. *Immunology* 59:509–514, 1986

Macatonia SE, Knight SC, Edwards AJ, Griffiths S, Fryer P: Localization of antigen on lymph node dendritic cells after exposure to the contact sensitizer fluorescein isothiocyanate functional and morphological studies. *J Exp Med* 166:1654–1667, 1987

Piazza C, Montani MS, Moretti S, Cundari E, Piccolella E: CD4(+) T cells kill CD8(+) T cells via Fas/Fas ligand-mediated apoptosis. *J Immunol* 158:1503–1506, 1997

Robert C, Kupper TS: Inflammatory skin diseases, T cells, and immune surveillance. *N Engl J Med* 34:1817–1828, 1999

Trautmann A, Akdis M, Kleemann D, *et al*: T cell-mediated Fas-induced keratinocyte apoptosis plays a key pathogenetic role in eczematous dermatitis. *J Clin Invest* 106:25–35, 2000

Van Der Valk SA: Epicutaneous patch testing. *Eur J Dermatol* 12:506–514, 2002

Vigan M, Girardin P, Adessi B, Laurent R: Late reading of patch tests. *Eur J Dermatol* 7:574–576, 1997

Wang B, Fujisawa H, Zhuang L, *et al*: CD4 + Th1 and CD8 + type 1 cytotoxic T cells both play a crucial role in the full development of contact hypersensitivity. *J Immunol* 165:6783–6790, 2000

Williams IR, Ort RJ, Kupper TS: Keratinocyte expression of B7-1 in transgenic mice amplifies the primary immune response to cutaneous antigens. *Proc Natl Acad Sci USA* 91:12780–12784, 1994

Xu H, DiIulio NA, Fairchild RL: T cell populations primed by hapten sensitization in contact sensitivity are distinguished by polarized patterns of cytokine production: Interferon gamma-producing (Tc1) effector CD8 + T cells and interleukin (Il) 4/Il-10-producing (Th2) negative regulatory CD4 + T cells. *J Exp Med* 183:1001–1012, 1996