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Skin Inflammation During Contact Hypersensitivity Is Mediated by Early Recruitment of CD8$^+$ T Cytotoxic 1 Cells Inducing Keratinocyte Apoptosis[1]

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Contact hypersensitivity (CHS) is a T cell-mediated, Ag-specific skin inflammation induced by skin exposure to haptens in sensitized individuals. Th1/T cytotoxic 1 cells are effector cells of CHS, whereas Th2/T regulatory CD4$^+$ T cells have down-regulating properties. We have previously shown that CHS to 2,4-dinitrofluorobenzene is mediated by specific CD8$^+$ effector cells, whose cytolytic activity is mandatory for induction of skin inflammation. In this study, using immunohistochemistry and RT-PCR analysis, we show that CD8$^+$ T cells are rapidly recruited into the skin at the site of hapten challenge before the onset of clinical and histological signs of skin inflammation. This early CD8$^+$ T cell recruitment is concomitant with: 1) transient IFN-γ mRNA expression suggesting local activation of effector cells; and 2) induction of keratinocyte (KC) apoptosis which gradually increased to a maximum at the peak of the CHS response. Alternatively, skin infiltration of CD4$^+$ T cells occurred later and coincided with the peak of the CHS reaction and the beginning of the resolution of skin inflammation. Mice deficient in CD8$^+$ T cells did not develop CHS, whereas mice deficient in CD4$^+$ T cells developed an enhanced inflammatory response with increased numbers of CD8$^+$ T cells recruited in the skin associated with massive KC apoptosis. These data show that CHS is due to the early and selective recruitment in the skin of CD8$^+$ T cytotoxic 1 effector cells responsible for KC apoptosis. ***The Journal of Immunology,*** **2002, 168: 3079–3087.**

Contact hypersensitivity (CHS)[4] is a dendritic cell (DC)-dependent T cell-mediated cell immune response elicited by epicutaneous sensitization with haptens (i.e., chemicals including metals), which become immunogenic after binding to discrete amino acid residues of proteins or peptides (1). CHS reactions in mice and humans are mediated by Th1/T cytotoxic 1 (Tc1) effector cells (2–6), and down-regulated by Th2/T regulatory (Treg) CD4$^+$ T cells (2, 3). The CHS response develops in two distinct phases. In the sensitization phase (i.e., afferent or induction phase of CHS), haptens penetrating the skin are captured by resident DC which migrate to regional lymph nodes and induce activation of specific T cell precursors. The elicitation phase (i.e., efferent phase) of CHS is induced by reexposure to the same hapten at a remote skin site. This leads within a few hours to the rapid recruitment and activation of specific T cells, and to the constitution of a local inflammatory response, which peaks at 24–48 h after challenge, and progressively decreases via active down-regulating mechanisms.

Although both clinical and experimental studies have suggested roles for CD4$^+$ and CD8$^+$ T cells as the effector T cells mediating CHS (4, 5, 7, 8), studies from this and other laboratories have indicated that: 1) CD8$^+$ Tc1 cells are effector cells in CHS responses in mice (2, 3, 5, 9, 10) and in humans (5, 11); and that 2) CD4$^+$ Th2/Treg cells regulate the magnitude of CHS (2, 3, 5, 12–15).

The mouse model of CHS to the hapten 2,4-dinitrofluorobenzene (DNFB) that we have extensively analyzed in recent years shows that CHS is exclusively mediated by CD8$^+$ Tc1 CTLs, which develop in secondary lymphoid organs upon hapten presentation by MHC class I-expressing DC (16, 17). These CD8$^+$ effector T cells migrate to the challenged site (9, 12, 18) and initiate the skin inflammation via Fas and/or perforin-mediated cytolytic function (9). Indeed, these data suggested that the development of CHS was secondary to the presentation of haptenated peptides by MHC class I-expressing skin cells to specific CD8 CTLs. However, little is known about the precise mechanisms by which the specific T cells enter the skin and are activated. For initial T cell recruitment, an important role of complement has been proposed in which C5a acts on mast cells and platelets, leading to release of mediators such as TNF-α and serotonin that activate local endothelia to facilitate T cell recruitment (19–21). Although we have shown that CD8$^+$ T cells can rapidly infiltrate the challenged skin, the recruitment kinetics of CD8$^+$ T cells and of CD4$^+$ T cells into the skin has not been analyzed. In addition, the nature of the cell type involved in hapten presentation to specific T cells during the elicitation phase of CHS remains to be determined. It has been postulated that haptens are taken up by epidermal DC which migrate to the dermis, where they can present haptens to specific T

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[4] Abbreviations used in this paper: CHS, contact hypersensitivity; Tc1, T cytotoxic 1; Treg, T regulatory; DNFB, 2,4-dinitrofluorobenzene; DC, dendritic cell; TNP, trinitrophenyl; LC, Langerhans cell; KC, keratinocyte; MCP-1, monocyte chemotactic ptotein-1; MDC, macrophage-derived chemokine; HPRT, hypoxanthine phosphoribosyltransferase.

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cells (22). However, other studies suggested that DC are not involved in hapten presentation to T cells during the elicitation phase of CHS, and that keratinocytes (KC) and other skin resident cells could be the APCs (23–25).

In the present study, we show that the development of the skin inflammation during the efferent phase of CHS is initiated by rapid recruitment of CD8$^+$ T cells in the epidermis of challenged skin, associated with the induction of KC apoptosis. CD4$^+$ T cell infiltration occurs hours later at a time where the CHS response starts to lessen. Thus, the CHS reaction is secondary to differential recruitment in the skin of effector Tc1 CD8$^+$ T cells and down-regulatory Th2/Treg CD4$^+$ T cells.

Materials and Methods

Mice

BALB/c mice (IFFA CREDO, L'Arbresle, France) were used between 6 and 10 wk of age.

Reagents

DNFB (Sigma Aldrich, St. Louis, MO) and picryl chloride (trinitrophenyl (TNP); BDH Laboratory Supplies, Poole, U.K.) were freshly prepared before CHS assays.

Abs used in in vivo experiments comprised anti-CD4 and anti-CD8 mAbs, produced respectively by the hybridoma H 35.17.2, kindly provided by G. Milon (Institut Pasteur, Paris, France), and the hybridoma GK 1.5 purchased from American Type Culture Collection (Manassas, VA). For immunohistochemistry, the following mAbs were used: anti-CD8 (KT-15 rat IgG2a, LE12 9TE, Harlan Sera-Lab, Loughborough, U.K.), anti-CD4 (GK 1.5 rat IgG2b, Leinco Technologies, Ballwin, MO), anti-MHC class II (CD311 rat IgG2b, kindly provided by A. Glasebrook, Lilly Research Laboratories, Indianapolis, IN; Ref. 26), biotinylated rabbit anti-rat IgG (H + L) (Vector Laboratories, Burlingame, CA), biotinylated alkaline phosphatase-streptavidin (StreptABComplex/AP; DAKO, Glostrup, Denmark).

Assay for CHS to DNFB

DNFB was diluted in acetone and olive oil (4/1) immediately before use. The procedure used for the CHS, i.e., the mouse ear swelling test, has been described elsewhere (27). Briefly, 25 μl of 0.5% DNFB solution was applied to a 2-cm^2 section of shaved dorsal skin. Five days later, test and control animals received on both sides of the left ear 5 μl of 0.2% DNFB or of an irrelevant hapten, 1% TNP, to test for the hapten specificity of ear swelling. The vehicle (acetone/olive oil) was applied on the right ear. Ear thickness was monitored using a micrometer (J15, Blet, Lyon, France) before challenge and every day after challenge. The ear swelling was calculated as [(T–T_o) left ear] − [(T–T_o) right ear], where T and To represent values of ear thickness before and after challenge, respectively.

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

Ab depletion of CD4$^+$ and CD8$^+$ T cells in vivo

Mice were given i.p. injections of 200 μl 1/10 diluted anti-CD4 or anti-CD8 mAb on days −1, 0, +1, and +4 of skin sensitization. Cell depletion was assessed +1 and +4 day of skin sensitization by staining for CD4 and CD8 molecules on PBMC recovered from retroorbital plexus. In all cases, specific depletion exceeded 95% on both days.

Histology and immunochemistry

Skin samples collected at different times after challenge were either paraffin embedded for histological staining with H&E, or deep frozen in OCT compound (MILES, Torrance, CA) for immunohistochemistry. Frozen sections were cut (6 μm), fixed in acetone for 10 min, and incubated in PBS-5% FCS. The sections were then sequentially incubated with: 1) rat anti-CD4, anti-CD8, or isotype-control mAb; 2) biotinylated rabbit anti-rat IgG (H + L); 3) biotinylated alkaline phosphatase-streptavidin. The labeling was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as a substrate, and the sections were counterstained with Fast Red.

For analysis of the epidermal sheets, the epidermis from the ears was peeled off the connective tissue by incubation for 90 min at 37°C in PBS supplemented with 20-mM EDTA (Sigma Aldrich). The sheets were incubated overnight at 4°C with the primary anti-mouse MHC class II mAb. Three washes in PBS were followed by the same procedure described above except for counterstain with Fast Red. Some pieces of the epidermis were subjected to TUNEL staining.

TUNEL staining

TUNEL staining was done on paraffin-embedded sections and epidermal sheets using the in situ cell death detection kit AP (Boehringer Mannheim, Mannheim, Germany) following the manufacturer's instructions. For epidermal sheets, the reaction was developed using 3-amino-9-ethylcarbazole substrate and H_2O_2 (DAKO).

RNA extraction and RT-PCR analysis of CD8 and IFN-γ mRNA

At different time points after challenge, ear samples were collected from sensitized or unsensitized mice and frozen in liquid nitrogen. The detection of RNA was conducted as described in details elsewhere (28). Briefly, total RNA was extracted using a RNAXEL kit (Eurobio, Les Ullis, France). After DNase I treatment, 1 μg of total mRNA was reverse transcribed using poly(dT)15 primers and Superscript II RT (Life Technologies, Rockville, MD; 90 min 37°C). The amount of RNA to be used for the detection was normalized using the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT) as reference. The cDNA obtained was amplified using different sets of primers, for HPRT (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′), and for CD4 (5′ primer, 5′-AGC AAC TCT AAG GTC TCT AAC C-3′; 3′ primer, 5′-AGA GTC AGA GTC AGG TTG CC-3′). The amplifications were conducted with 29 cycles for HPRT and 33 cycles for IFN-γ, CD8, and CD4 (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.

Statistical analysis

All experimental groups consisted of five mice, and all experiments were performed at least three times. The statistical significance of differences between mean values of groups was evaluated with the one-way factorial ANOVA ($p < 0.05$).

Results

CD8$^+$ T cells are effector cells, whereas CD4$^+$ T cells are involved in the down-regulation of the CHS response to DNFB in BALB/c mice

We have previously shown that CHS to DNFB in C57BL/6 (H2-b) mice was mediated by CD8$^+$ T cells and down-regulated by CD4$^+$ T cells (3). We confirm here that the pathophysiology of CHS is similar in BALB/c (H2-d) and C57BL/6 mice. Sensitized BALB/c mice developed a CHS reaction upon challenge with DNFB which peaked at 24 or 48 h, and faded away from day 3 (Fig. 1*a*). CD4$^+$ T cell-depleted mice developed an enhanced CHS reaction with a >2-fold increase in skin inflammation 48 h after challenge, confirming that CD4$^+$ T cells are necessary for the down-regulation of the CHS response. Conversely, the CHS response was inhibited in CD8$^+$ T cell-depleted mice. No ear swelling was observed when DNFB-sensitized mice were challenged with an irrelevant hapten TNP (data not shown). Histological analysis of challenged sites showed that CHS in wild-type animals was associated with vascular enlargement, dermal edema, and infiltration by mononuclear cells accompanied by a few polymorphonuclear cells (Fig. 1*f*). These histological changes were dramatically enhanced in CD4$^+$ T cell-depleted mice (Fig. 1*g*). In addition, a few eosinophilic, dyskeratotic cells were observed in the epidermis (data not shown). There were no histological changes in CD8$^+$ T cell-depleted mice (Fig. 1*h*).

Thus, the pathophysiology of CHS to DNFB in BALB/c is similar to that of C57BL/6 mice, and is mediated by CD8$^+$ T cell effectors.

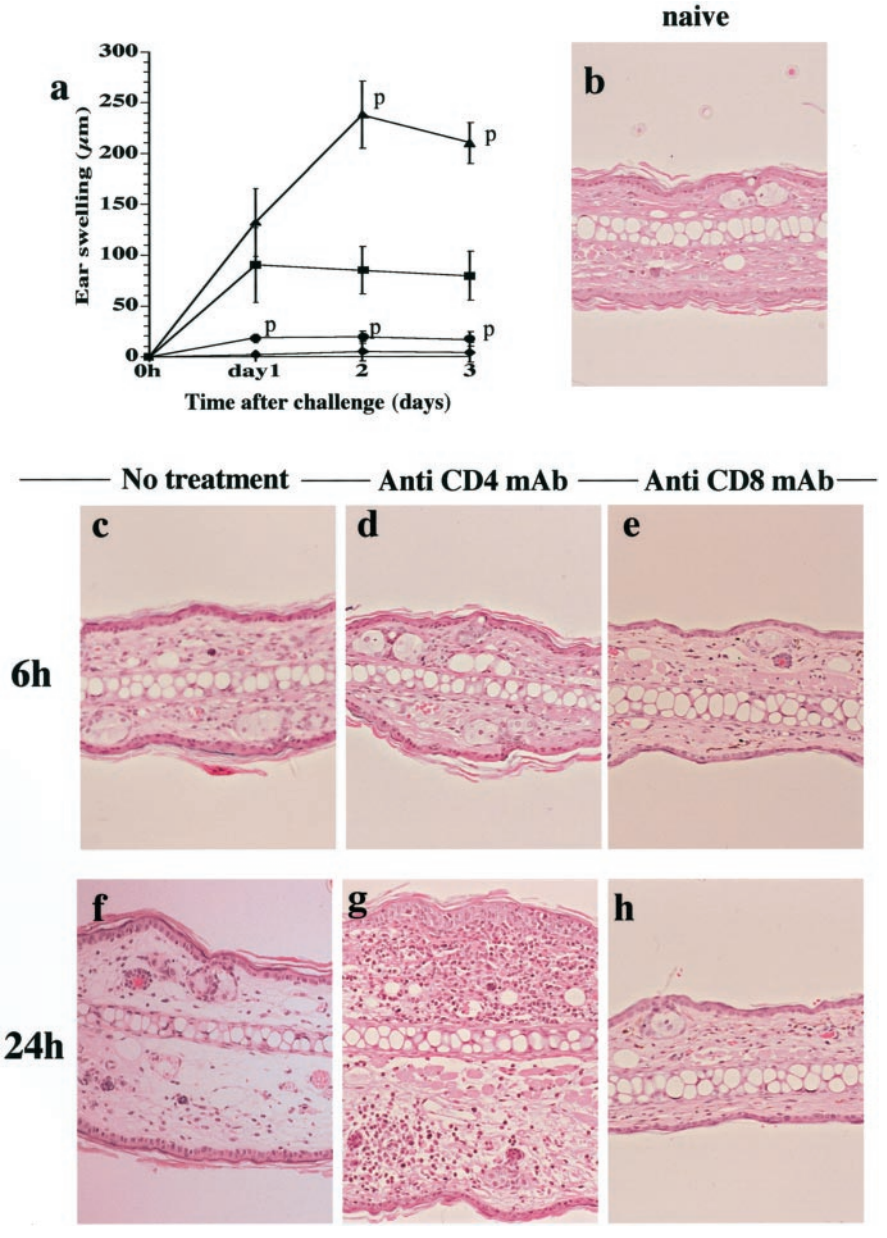

FIGURE 1. CHS reaction to DNFB in BALB/c mice. *a*, BALB/c mice were sensitized by applying DNFB 0.5% on back skin at day −5 and ear challenged with 0.2% DNFB on day 0. CHS reaction to DNFB was analyzed in mice either untreated (■), or treated with anti-CD4 (▲) or anti-CD8(●) mAb. Unsensitized challenged mice exhibited no ear swelling (♦). Results are expressed as the mean ear swelling ± SD at different time points after challenge, and are representative of three independent experiments. The letter p ($p < 0.05$) indicates statistical significance compared with untreated mice. *b–h*, Histological analysis of CHS reaction. Four-micrometer thick sections of ears recovered from either untreated (*c* and *f*), $CD4^+$ T cell-depleted (*d* and *g*), or $CD8^+$ T cell-depleted (*e* and *h*) mice at 6 h (*c–e*) and 24 h (*f–h*) after challenge were stained with H&E. At 6 h after challenge, skin sections from untreated (*c*), $CD4^+$ T cell-depleted (*d*), and $CD8^+$ T cell-depleted (*e*) mice exhibited normal histological features similar to that of naive mice (*b*). At 24 h after challenge, sections from untreated mice (*f*) displayed the characteristic features of the CHS reaction including dermal edema, mononuclear cell infiltration, and vascular enlargement, which did not appear in $CD8^+$ T cell-depleted mice (*h*). Sections from $CD4^+$ T cell-depleted mice (*g*) exhibited exaggerated inflammatory reaction, including enhanced edema, vasodilatation, and massive mononuclear cell infiltration. Magnification: *b–h*, ×200.

CHS reaction is due to early recruitment of $CD8^+$ T cells in challenged skin

Because CHS reaction is mediated by $CD8^+$ T cells and down-regulated by $CD4^+$ T cells, we hypothesized that development of the hapten-specific skin inflammation could be due to the selective and rapid recruitment of $CD8^+$ effector cells in the challenged skin. To test for this hypothesis, the kinetics of $CD8^+$ and $CD4^+$ T cell infiltration in the skin was analyzed by immunohistochemistry and RT-PCR.

Immunohistochemical analysis of ear skin from sensitized but unchallenged mice revealed a lack of $CD8^+$ cells (Fig. 2*a*), whereas some scattered $CD4^+$ cells could be found in the dermis (Fig. 2*b*). In the course of the CHS response, $CD8^+$ T cells could be detected as early as 12 h after challenge (at a time when no ear swelling was yet observed; Fig. 2*c*), while the number of $CD4^+$ cells remained unchanged (Fig. 2*d*). $CD8^+$ T cells were found mostly in the dermis, but a few were also present at the dermal-epidermal junction. At 24 h postchallenge, corresponding to the peak of the skin inflammatory response, infiltration of both $CD8^+$ and $CD4^+$ T cells was observed (Fig. 2, *e* and *f*). $CD8^+$ T cells primarily infiltrated the superficial dermis and the epidermis (Fig. 2*e*). In contrast, $CD4^+$ T cells were found only in the dermis (Fig. 2*f*). Interestingly, $CD4^+$ T cell-depleted mice, which develop an enhanced CHS reaction, exhibited increased numbers of $CD8^+$ T cells infiltrating the skin and the epidermis (Fig. 3, *a* and *b*), compared with untreated BALB/c mice (Fig. 3*c*) at 24 h postchallenge. The average number of $CD8^+$ T cells in the skin at the peak of the CHS response (24 h after challenge) was 100 and 170 cells/500 basal epidermal cells, for untreated and anti-CD4 mAb-treated mice, respectively (Fig. 3*d*).

We next examined the presence of CD8, CD4, and IFN-γ mRNA during the course of the CHS reaction to DNFB. Ear samples from previously sensitized mice were collected at different time points after DNFB challenge and subjected to mRNA extraction and semiquantitative RT-PCR analysis using HPRT mRNA as internal standard (Fig. 4, *a* and *b*). CD8 and IFN-γ mRNA were detected neither in skin of naive mice, in unsensitized challenged mice, nor in sensitized mice before challenge. During CHS, CD8

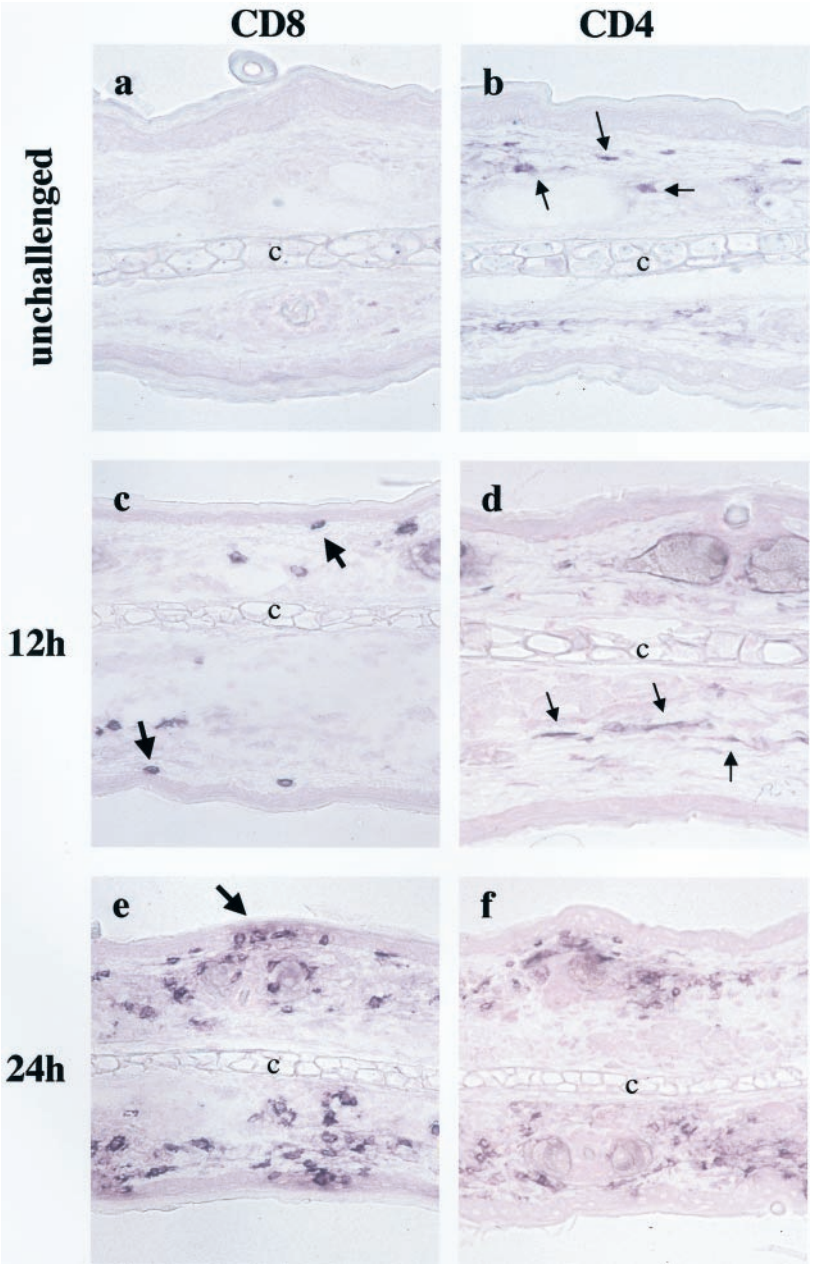

FIGURE 2. Immunohistochemical analysis of the inflammatory infiltrate during CHS to DNFB. BALB/c mice were sensitized and challenged, as described in Fig. 1. Cryostat sections from ears harvested either 1 h before challenge (*a* and *b*), or 12 h (*c* and *d*) or 24 h (*e* and *f*) after challenge were stained with anti-CD8 (*a*, *c*, and *e*) or anti-CD4 (*b*, *d*, and *f*) mAb. CD8$^+$ cells of round morphology appeared at 12 h mostly in the dermis and at the dermal-epidermal junction (arrow, *c*), and accumulated at 24 h in both dermis and epidermis (arrow, *e*). CD4$^+$ cells of irregular shape were present in the dermis of unchallenged ear (arrow, *b*) and were also present at 12 h after challenge (*d*). Accumulation of round-shaped CD4$^+$ cells was found at 24 h postchallenge in the dermis, but not in the epidermis. Magnification: *a–d*, ×400; *e* and *f*, ×200. The letter c in the figures represents ear cartilage.

and IFN-γ mRNA were first detected by 6 h after challenge, confirming that activated, IFN-γ-producing CD8$^+$ T cells infiltrate the challenged skin. CD8$^+$ mRNA increased gradually up to 48 h, whereas IFN-γ mRNA expression peaked at 24 h and decreased thereafter (Fig. 4). CD4 mRNA was present at low levels in normal skin (compatible with the rare CD4$^+$ cells found in the dermis of normal skin; Fig. 2*b*), remained stable up to 24 h postchallenge, but markedly increased thereafter (Fig. 4). These data confirmed and extended the results of immunohistological analysis and showed that early recruitment of the challenged skin by CD8$^+$ T cells during CHS precedes CD4$^+$ T cell infiltration.

CD8$^+$ T cell infiltration in the skin during CHS correlates with the appearance of KC apoptosis

We have previously shown that cytotoxicity through the Fas or the perforin pathway is mandatory for CD8$^+$ T cell-mediated CHS and associated with the development of hapten-specific, MHC class I-restricted CD8$^+$ T cells (9). Thus, we examined whether CD8$^+$ T cell recruitment in the skin at the sites of challenge is coincidental with localization of apoptosis of skin cells. We studied the kinetics of apoptosis in the skin during the course of CHS using the TUNEL-labeling method. Apoptotic cells were found neither in the ear skin of normal BALB/c mice (data not shown), nor in that of unsensitized challenged mice (Fig. 5*a*). In sensitized challenged mice, TUNEL$^+$ apoptotic cells were observed mostly in the epidermis, as early as 6 h postchallenge, a time corresponding to the onset of the skin infiltration by CD8$^+$ T cells (Fig. 5*b*). The maximum number of apoptotic cells was observed at 24 h and involved mostly epidermal cells, although scattered TUNEL$^+$ cells were found in the dermis (Fig. 5*c*). Interestingly, the number of apoptotic cells was dramatically increased in mice treated with anti-CD4 mAb, where, in some areas, almost all basal KC appeared TUNEL$^+$ within 24 h postchallenge (Fig. 5*f*). In both untreated and anti-CD4-treated mice, apoptotic KC developed with comparable kinetics, being first detected as early as 6 h postchallenge, and reaching maximal numbers at 24 h (Fig. 5*d*).

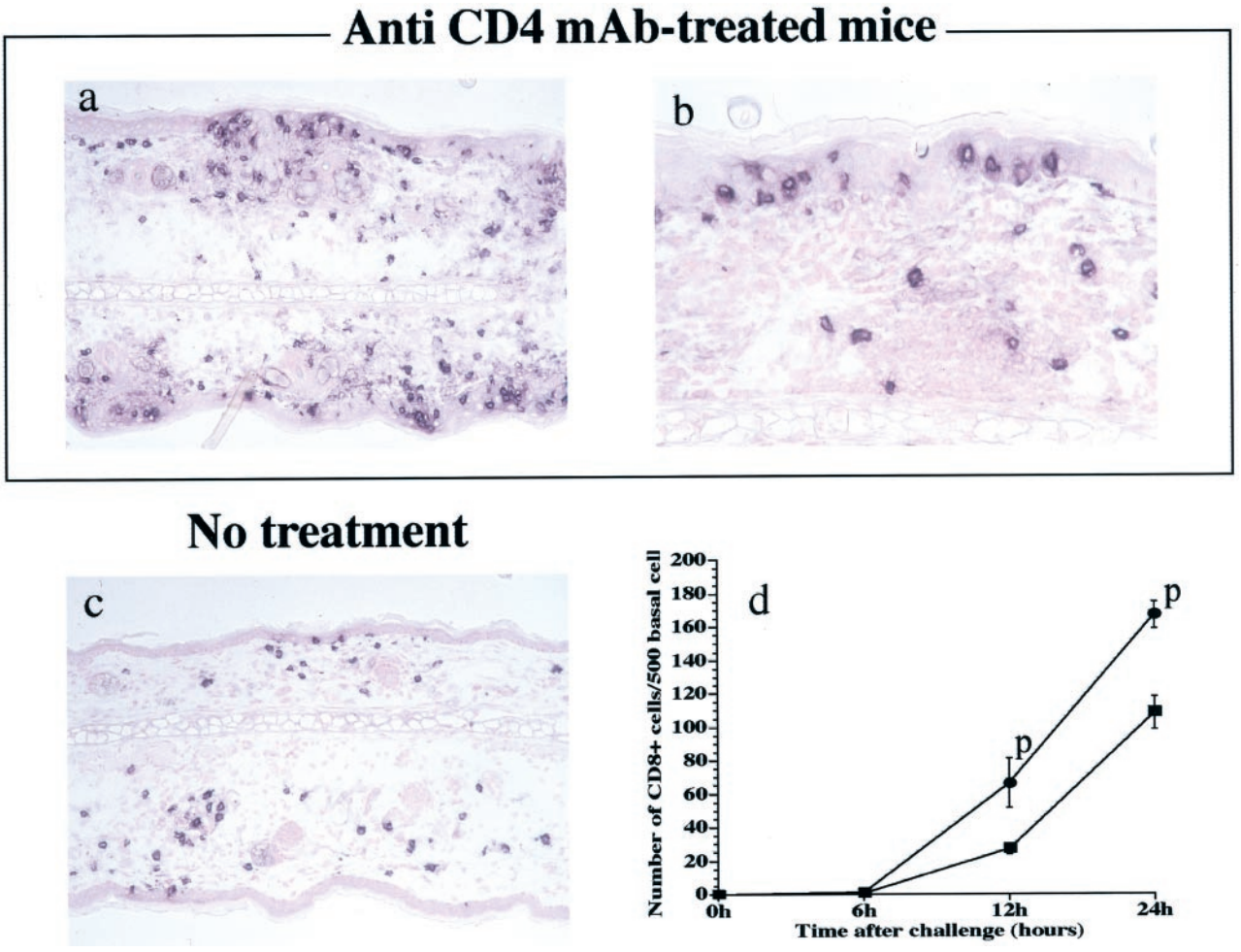

FIGURE 3. $CD8^+$ T cell infiltration in the skin of wild-type and $CD4^+$ T cell-depleted BALB/c mice. $CD4^+$ T cell-depleted (*a* and *b*) and untreated BALB/c mice (*c*) were sensitized and challenged as described in Fig. 1. Ears were removed 24 h after challenge and cryostat sections were stained with anti-CD8 mAb. Higher numbers of $CD8^+$ T cells were found in the skin of $CD4^+$ T cell-depleted mice (*a*) compared with untreated mice (*c*). $CD8^+$ T cells were localized in the superficial dermis (*a*), and in the epidermis in close contact with KC (*b*). *d*, Kinetics of infiltration of $CD8^+$ T cells in the skin of wild-type (■) and $CD4^+$ T cell-depleted BALB/c mice (●). Total number of $CD8^+$ T cell infiltrate in the skin (dermis and epidermis) was counted on a surface corresponding to 500 basal epidermal cells. Results represent the mean number of $CD8^+$ cells/500 basal cells counted in three microscopic fields from two different sections, and are representative of three experiments. The letter p ($p < 0.05$) indicates statistical significance compared with wild-type mice. Magnification: *a* and *c*, ×200; *b*, ×400.

Because epidermal apoptosis involved mainly basal epidermal cells and not suprabasal layers where Langerhans cells (LC) are located, our data suggested that KC, but not LC, were the main target of hapten-specific CTLs. This was further examined by single and double staining for MHC class II and $TUNEL^+$ cells in epidermal sheets, before and at 24 h after challenge (Fig. 6). Before challenge, the epidermis contained a network of class II^+ LC (Fig. 6*a*), but was devoid of apoptotic cells (Fig. 6*c*). Alternatively, at 24 h postchallenge, very few LC remained in the epidermis (Fig. 6*b*; consistent with previous studies which have demonstrated that epicutaneous application of haptens induces LC emigration from the epidermis; Ref. 29), whereas high numbers of apoptotic epidermal cells were found (Fig. 6*d*). Thus, an inverse correlation between the number of LC and the number of apoptotic cells was found (Fig. 6*e*). Double staining for class II and $TUNEL^+$ cells confirmed lack of class II^+ $TUNEL^+$ apoptotic LC (Fig. 6*f*). These data demonstrate that KC, but not LC, are the main targets of $CD8^+$ CTLs during the elicitation of CHS.

Discussion

The present study extends our previous findings that cytotoxicity of $CD8^+$ T cells through Fas and perforin pathways is mandatory for the CHS response (9). We demonstrate that the hapten-specific skin inflammation is initiated by the early and selective recruitment at the challenge site of cytolytic $CD8^+$ effector Tc1 cells responsible for KC apoptosis: 1) $CD8^+$ T cells were found in the skin as early as 6 h after challenge, and their numbers progressively increased up to 48 h; 2) infiltrating $CD8^+$ T cells were activated as shown by the dramatic up-regulation of IFN-γ mRNA whose production in our model is restricted to $CD8^+$ T cells (9, 30–32); 3) apoptosis of KC coincided with the appearance of $CD8^+$ T cells in the skin and gradually increased proportionally to the number of $CD8^+$ T cells infiltrating the skin.

In contrast to $CD8^+$ T cells, $CD4^+$ T cells are not recruited in the first hours following challenge. They first appeared in the challenged site at 24 h only, and their recruitment was associated with a decrease in the inflammatory reaction and a diminution of apoptotic cells in the epidermis. Thus, it is tempting to speculate that the differential kinetics in the recruitment of $CD8^+$ T cells and $CD4^+$ T cells are responsible for the typical skin inflammation of CHS. The CHS reaction can be defined as an acute Ag-specific inflammation which develops as the result of the recruitment of $CD8^+$ T cells in the absence of $CD4^+$ down-regulatory T cells. CHS decreases and resolves as soon as $CD4^+$ T cells infiltrate the

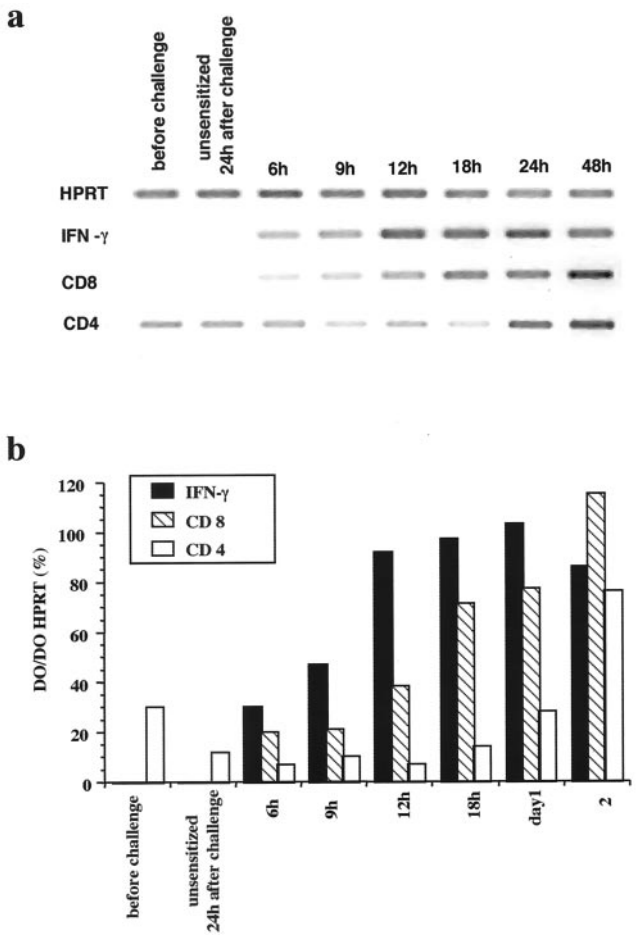

FIGURE 4. RT-PCR analysis of CD4, CD8, and IFN-γ mRNA during the course of CHS reaction to DNFB. *a*, Expression of CD4, CD8, and IFN-γ mRNA in the ears was determined by semiquantitative RT-PCR analysis at 6, 9, 12, 18, 24, and 48 h after challenge. Controls include ears from sensitized mice before challenge and ears from unsensitized mice 24 h after hapten painting. *b*, Histogram presenting CD8 (▨), CD4 (□), and IFN-γ (■) mRNA relative quantities compared with HPRT mRNA as standard. Each band of the gel of Fig. 4*a* was analyzed by densitometry, and the results are expressed as the ratios of optical densities to HPRT bands. Results are representative of three independent experiments.

inflamed skin. $CD4^+$ T cells being responsible for the down-regulation of CHS is supported by the observation that $CD4^+$ T cell-deficient mice develop an enhanced CHS reaction with a markedly increased and sustained infiltration of $CD8^+$ T cells in the skin associated with an increase in the number of apoptotic KC.

Recruitment of lymphocytes into inflamed skin is a multistep process involving recognition of vascular endothelial cells and extravasation. Haptens are able to rapidly induce expression of E- and P-selectins, VCAM-1 and ICAM-1 on endothelial cells (33–36). Several reports have shown that ~2 h after topical application of Ag to the ear of sensitized mice, there is marked edema due to local release of TNF-α, serotonin, and histamine from mast cell and platelets (19, 37–41), in which complement C5 might play an important role (20, 21). Early release of TNF-α induced expression of VCAM-1 and ICAM-1 on the luminal surface of local endothelium (36). These adhesion molecules mediate rolling, adhesion, and extravasation of blood leukocytes expressing cutaneous lymphocyte-associated Ag or P-selectin ligands into the skin. Chemokines regulate the lymphocyte traffic in part by triggering arrest of lymphocytes rolling on the endothelium (42). Among the large variety of chemokines able to attract T cells in inflamed tissues, CCL2 (monocyte chemotactic protein-1 (MCP-1), CCR2 ligand) appears pivotal for the recruitment of effector T cells in the skin during the CHS reaction (43–45). Production of MCP-1 by KC and skin cells (34, 46) occurs 4–6 h after hapten challenge, clearly before skin infiltration with T cells (34). Moreover, MCP-1 has been shown to augment lymphocyte cytotoxicity, and therefore, could be involved in both recruitment and optimal activation of hapten-specific effector CTLs in the skin (47).

The reason for the sequential infiltration of the skin by $CD8^+$ first and then by $CD4^+$ T cells is unclear. Two hypotheses may explain this observation. Activation and expansion of hapten-specific $CD8^+$ Tc1 effector cells in lymphoid organs may precede that of regulatory Th2/Treg $CD4^+$ T cells (5, 31). Indeed, in vivo priming of specific $CD8^+$ T cells is achieved within 5 days, does not require help from $CD4^+$ T cells (3), and is independent of the CD40 ligand engagement (48). Alternatively, differential expression of homing receptors and sequential expression of chemokines in the skin may account for selective attraction of Tc1 $CD8^+$ effector cells, preceding that of regulatory Th2/Treg $CD4^+$ T cells. In this respect, functional ligands for E- and P-selectins (cutaneous lymphocyte-associated Ags, P-selectin glycoprotein ligand-1) appear to be more highly expressed on Th1 cells than on Th2 cells (49), in keeping with the enhanced ability of Th1 cells to enter delayed type hypersensitivity sites, compared with Th2 cells (50). Recent studies showed that IL-12, which is mandatory for priming of Th1/Tc1 cells, is required for the expression of P-selectin ligands on both $CD4^+$ and $CD8^+$ T cells, which are able to enter inflamed tissues (51). Alternatively, Th2/T cytotoxic 2 cells primed by Ag in the presence of IL-4 and anti-IFN-γ Ab are unable to bind to P-selectin (51).

It may be postulated that $CD8^+$ T cell activation in the skin (resulting in IFN-γ production, cytotoxicity, and possibly chemokine production) provides the signals required for recruitment of $CD4^+$ T cells. Indeed, our results show that recruitment of $CD4^+$ T cells occurs several hours after that of IFN-γ-producing $CD8^+$ T cells and beginning of KC apoptosis. In several models, initial Th1 cell infiltration was followed by massive recruitment of Th2 cells (52, 53). It has been proposed that secretion of Th2 cell-attracting chemokines, such as CCL1 (I-309, CCR8 ligand) by activated Th1 cells (54) or CCL22 (macrophage-derived chemokine (MDC), CCR4 ligand) by skin cells during CHS (55) might serve to recruit CCR4/CCR8-expressing Th2 cells for down-regulating Th1-mediated inflammatory responses (56). Recent studies in humans have reported that I-309 attracted more efficiently hapten-specific regulatory $CD4^+$ T cells (Treg and Th2) than Th1 cell clones (57), consistent with the observation that CCR8 is expressed on Th2/Treg, but not on Th1 cells (58). In addition, MDC-injection in the skin allowed preferential recruitment of CCR4-expressing Th2 cells (59).

Our data showing apoptosis of KC and not of LC after hapten challenge in sensitized animals suggest that KC are the main APCs able to activate DNFB-specific $CD8^+$ T cells. Although LC are undoubtedly involved in Ag presentation during the sensitization phase of CHS, its role during the elicitation phase is unclear. The fact that LC migrate away from the site of Ag application is an argument against their active participation in T cell activation. In this respect, a model of LC-depleted mice using topical steroid application showed markedly enhanced CHS responses, suggesting that LC are not the relevant APC for activation of effector cells during the elicitation phase, but may rather provide down-regulatory signals (23). However, it cannot be ruled out that LC might be involved in hapten presentation to the early-arriving CD8 CHS effector T cells before they depart for the lymphoid organs.

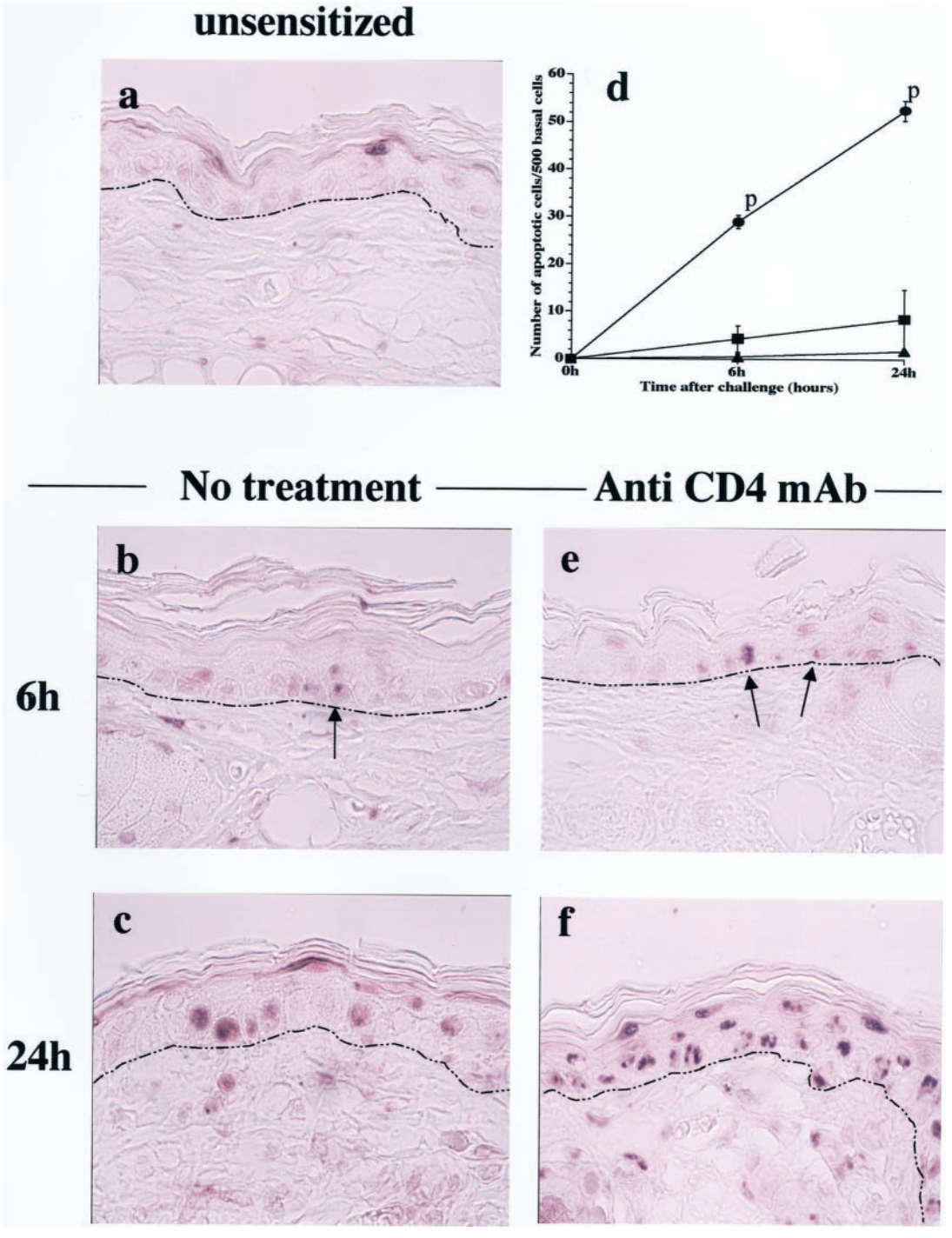

FIGURE 5. Immunohistochemical analysis of apoptosis in the skin during CHS responses. TUNEL staining was conducted on paraffin-embedded ear sections from either unsensitized challenged mice (*a*), from sensitized untreated mice at 6 h (*b*) and 24 h (*c*) postchallenge, or from anti-CD4 mAb-treated (*e* and *f*) mice. Note that apoptotic cells are present as early as 6 h postchallenge (arrow) in both untreated and anti-CD4 mAb-treated mice. *d*, Histogram showing the numbers of TUNEL$^+$ cells in the skin of untreated (■) or CD4-depleted (●) BALB/c mice at different times after challenge. The letter p ($p < 0.05$) indicates statistical significance compared with untreated mice. CD8-depleted (▲) mice represents the number of apoptotic cells in unsensitized challenged mice. Results are representative of three independent experiments and are expressed as the number of TUNEL$^+$ cells/500 basal epidermal cells. Magnification: *a–c*, *e*, and *f*, ×1000.

In summary, the results presented here support the following model for recruitment of CD8$^+$ effector Tc1 cells and CD4$^+$ Th2/Treg cells in the skin during CHS: 1) haptens initiate CHS by inducing a release of local endothelium activators and also the rapid production of MCP-1 by skin cells able to recruit CCR2$^+$ Tc1 cells; 2) hapten-specific effector Tc1 cells activated in the skin produce IFN-γ and induce KC cytotoxicity; 3) this process induces the synthesis of inflammatory cytokines and chemokines responsible for massive infiltration of mononuclear and polymorphonuclear cells and development of skin inflammation; 4) among the chemokines produced, I-309 and MDC may recruit CCR4 and CCR8-expressing Th2/Treg cells which may down-regulate the cutaneous inflammation by production of IL-10 and IL-4 immunoregulatory cytokines (5, 60).

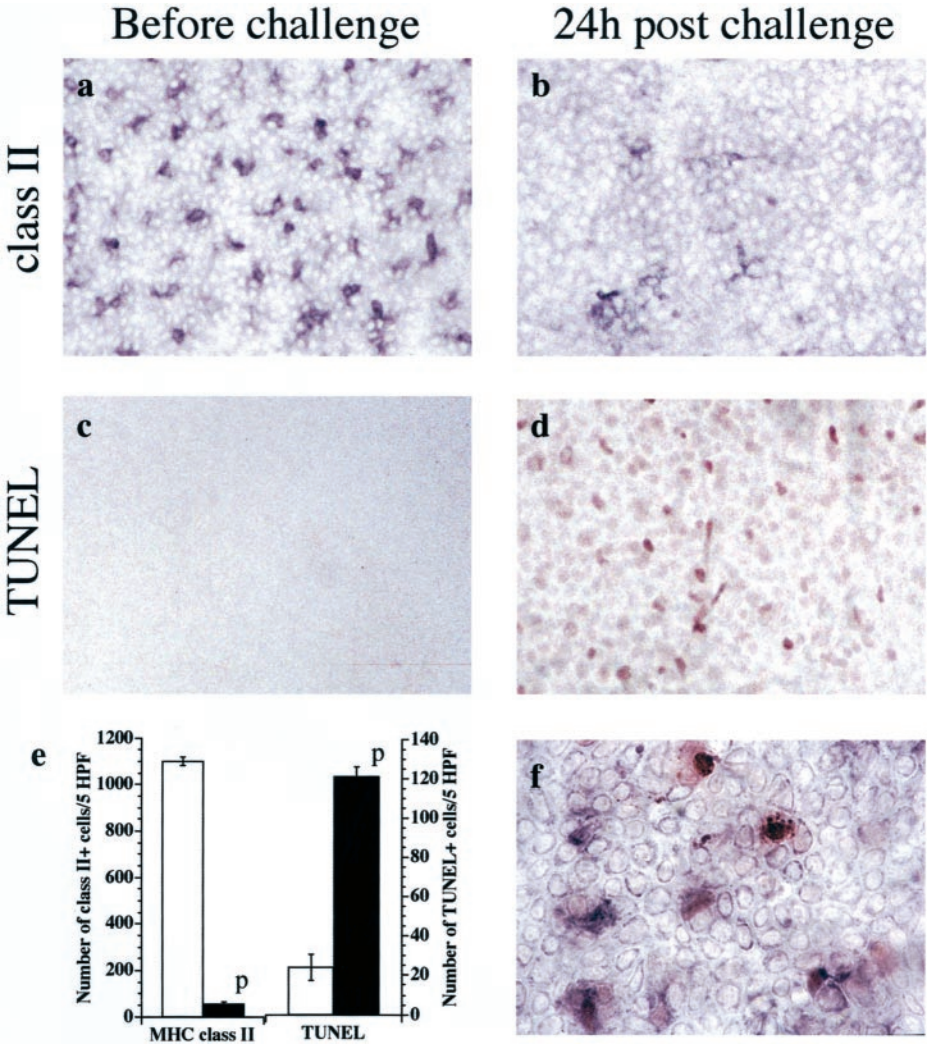

FIGURE 6. Epidermal staining of Langerhans cells and apoptotic KC. Epidermal sheets were obtained from the ear skin of sensitized BALB/c mice before (*a* and *c*) and 24 h after challenge with DNFB (*b*, *d*, and *f*). Epidermal sheets were stained for MHC class II expression (*a*, *b*, and *f*) and for apoptotic cells by the TUNEL method (*c*, *d*, and *f*). *e*, Histogram showing the number of MHC class II$^+$ or TUNEL$^+$ cells before challenge (□) and 24 h postchallenge (■). Results are expressed as number of stained cells counted in five microscopic fields analyzed at magnification ×400 (HPF, high power field). The letter p ($p < 0.05$) indicates statistical significance compared with the number of MHC class II$^+$ or TUNEL$^+$ cells before challenge. Magnification: *a–d*, ×200; *f*, ×1000.

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