Disparate Cytotoxic Activity of Nickel-Specific CD8$^+$ and CD4$^+$ T Cell Subsets Against Keratinocytes[1]

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Allergic contact dermatitis (ACD) is the result of an exaggerated immune reaction to haptens mediated by skin-homing T cells, but the effector mechanisms responsible for the tissue damage are poorly understood. Here we studied the capacity of distinct subsets of hapten-specific T cells to induce apoptosis in autologous keratinocytes. Skin- and blood-derived nickel-specific CD8$^+$ T cytotoxic 1 (Tc1) and Tc2 clones as well as CD4$^+$ Th1 and Th2 expressed the cutaneous lymphocyte-associated Ag and exhibited strong MHC-restricted cytotoxicity against nickel-coupled B lymphoblasts, as detected by the [^{3}H]TdR release assay. Both Tc1 and Tc2 clones, but not CD4$^+$ T cells, displayed a significant cytotoxic activity against resting nickel-modified keratinocytes. Following IFN-γ treatment, keratinocytes expressed MHC class II and ICAM-1 and became susceptible to Th1-mediated, but not Th2-mediated, cytotoxicity. The molecules of the two major cytotoxic pathways, Fas ligand (FasL) and perforin, were expressed by Tc1, Tc2, and Th1 cells, whereas Th2 cells expressed only FasL. Experiments performed in the presence of specific inhibitors of the perforin (concanamycin A) and FasL (brefeldin A) pathway indicated that perforin-mediated killing dominated in Tc1 and Tc2, and FasL-mediated cytotoxicity prevailed in Th2 clones, with a more heterogeneous behavior in the case of Th1 cells. Finally, perforin mRNA was expressed in ACD lesional skin, as assessed by RT-PCR analysis. In aggregate, our results indicate that keratinocytes can be target of multiple hapten-specific CTL responses, that may have distinct roles in the epidermal injury during ACD.

Allergic contact dermatitis (ACD)[3] is a common skin disease that is due to an exaggerated T cell response to highly reactive small m.w. haptens (1, 2). Sensitization occurs when haptens penetrating the skin are picked up by dendritic cells and carried to the regional lymph nodes, where MHC-hapten complexes are presented to naive T cells (3, 4). Clonally expanded hapten-specific T cells acquire a propensity to recirculate in the skin and are rapidly activated following Ag challenge. Despite considerable progress in understanding the development of hapten-specific immunity, less is known about the mechanisms responsible for the tissue injury during ACD. Characteristic histologic features of the disease include epidermal intercellular edema (spongiosis), keratinocyte damage, and the presence of an infiltrate of T cells, monocytes, and dendritic cells in the dermis and epidermis. Early ultrastructural studies of ACD revealed damaged keratinocytes in close contact to mononuclear cells (5), suggesting a role for T cell-mediated cytotoxicity in the expression of the disease, in line with other investigations on murine contact hypersensitivity (CH) (6, 7). It has been recently shown that P$^{0/0}$ *gld* mice lacking Fas ligand (FasL) and perforin genes, both of which are involved in T cell-mediated cytotoxicity, fail to mount CH reactions (8). In parallel, evidence has been provided that hapten-specific CD8$^+$ T cells have a crucial effector role in murine CH (9–11). In humans, a link between the high frequency of specific CD8$^+$ T cells in peripheral blood and the development of ACD has been suggested (12, 13), but few studies have characterized hapten-specific CD8$^+$ T cells (12–15). In contrast, in both mice and humans discrete hapten-specific CD4$^+$ T cell populations have been identified, which may play distinct roles in the course of the disease. Th1 cells, producing high amounts of IFN-γ and TNF-α, display predominant effector functions (16, 17) and may cooperate with CD8$^+$ T cells in amplifying the inflammatory response. Studies aimed at defining the role of Th2 cells, releasing IL-4 but not IFN-γ, provided conflicting results, with some indicating a suppressive (18 19) and others an enhancing or no effect on CH (20, 21). Finally, IL-10-producing CD4$^+$ T regulatory 1 lymphocytes seem to be primarily involved in the regulation of ACD by inhibiting the maturation and functions of dendritic cells (22).

Keratinocytes are profoundly involved in the elicitation and effector phase of ACD, because they secrete cytokines and chemokines that effectively activate resident dendritic cells and endothelial cells and contribute to lymphocyte recruitment into the skin (17, 23–25). Keratinocytes, under the influence of lymphocyte-derived cytokines such as IFN-γ and IL-17, also express MHC class II and adhesion molecules (ICAM-1) crucial for T cell function and retention in the epidermis (17, 26). Moreover, IFN-γ upregulates Fas expression and renders keratinocytes sensitive to Fas-mediated lysis (27, 28).

In this study we investigated the capacity of nickel-specific CD8$^+$ and CD4$^+$ T cells to induce keratinocyte apoptosis and the pathways of target cell injury. Although both type 1 and type 2 nickel-reactive CD8$^+$ and CD4$^+$ T cells were cytotoxic against B lymphoblasts, resting keratinocyte were killed exclusively by

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[3] Abbreviations used in this paper: ACD, contact dermatitis; CH, contact hypersensitivity; B-LCL, B lymphoblastoid cell line; BFA, brefeldin A; CH, contact hypersensitivity; CMA, concanamycin A; FasL, Fas ligand; Tc1, T cytotoxic 1; Tcc, T cell clone.

$CD8^+$ T lymphocytes. IFN-γ treatment rendered keratinocytes susceptible to Th1, but not Th2, cytotoxicity. These results indicate that T cell killing of keratinocytes can have an important role in mediating the epidermal damage during ACD and emphasize the role of $CD8^+$ T cells in the expression of the disease.

Materials and Methods

Patients

Patients ($n = 3$) included in the study had a history of eczematous dermatitis after contact with metals and a positive reaction to epicutaneous application of 5% $NiSO_4$ in petrolatum on the back under occlusion. They had not taken any medication for at least 15 days before skin and blood donation. Patients were enrolled in the study after written informed consent, and the study was approved by the Istituto Dermopatico dell'Immacolata ethical committee.

Abs and reagents

The mAbs anti-CD4 (SK1, IgG1) and anti-CD28 (Leu-28, IgG1) were purchased from Becton Dickinson (San Jose, CA). Anti-CD3 (UCHT-1, IgG1 azide free) mAb was obtained from Immunotech (Marseilles, France), and anti-MHC class I (W6/32, IgG1) from Dako (Glostrup, Denmark). The mAbs anti-CD8 (Leu 2a, IgG1), anti-HLA-DR (G46-6, IgG1), anti-CD54 (HA58, IgG1), anti-Bcl-2 (4D7, IgG1), anti-perforin (δG9, IgG2b), anti-Fas (ZB4, IgG1), anti-FasL (NOK-1, IgG1), and anti-cutaneous lymphocyte-associated Ag (CLA; HECA-452, rat IgM) were purchased from PharMingen (San Diego, CA). Control unconjugated mouse IgG1 and IgG2b were obtained from Becton Dickinson, and rat IgM from PharMingen (San Diego, CA). The FITC-conjugated goat anti-mouse Ig and anti-rat IgM were purchased from Southern Biotechnology Associates (Birmingham, AL) and PharMingen, respectively. Recombinant human IFN-γ and TNF-α were provided by Genzyme (Cambridge, MA). Concanamycin A (CMA) and brefeldin A (BFA) were purchased from Sigma-Aldrich (Milan, Italy).

Nickel-specific T cell lines and clones

PBMC from nickel-allergic patients were separated by centrifugation over Ficoll-Hyperpaque (Lymphoprep, Nycomed-Pharma, Oslo, Norway) and left to adhere (6×10^6 cells/ml) in petri dishes for 2 h at 37°C in RPMI 1640 supplemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-ME, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Chagrin Falls, OH; complete RPMI), and 5% human serum. The nonadherent fraction was depleted of $CD19^+$, $HLA\text{-}DR^+$, and $CD8^+$ or $CD4^+$ cells by incubation with immunomagnetic beads coated with specific mAbs (Dynabeads M450, Dynal, Oslo, Norway) to obtain >95% pure $CD4^+$ and $CD8^+$ T cells, respectively. For isolating T cells from lesional skin, biopsies from 48-h positive patch test reactions to $NiSO_4$ were extensively washed and placed in complete RPMI supplemented with 5% human serum and 30 U/ml rhIL-2 (provided by Chiron Italia, Milan, Italy). Medium was replaced every third day, and T cells emigrated from tissue samples were collected on day 12 and then cultured with irradiated autologous PBMC and 20 μg/ml $NiSO_4$ (Sigma) to enrich for nickel-specific T lymphocytes. T cell lines were cloned by limiting dilution (0.5 cells/well in 96-well U-bottom microplates) in complete RPMI plus 10% heat-inactivated FBS in the presence of 2×10^5 irradiated PBMC, 30 U/ml IL-2, and 1% PHA (Life Technologies). Clones were grown by adding IL-2 (30 U/ml) twice a week and were periodically stimulated with either 1% PHA in the presence of feeder cells or plate-coated anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml) mAbs. The nickel reactivity of both T cell lines and clones was assayed in proliferation assays as previously described (12). The pattern of cytokines released by T cell clones (Tcc) was evaluated on supernatants after 48-h activation with immobilized anti-CD3 and soluble anti-CD28, using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

Keratinocyte cultures and B cell lines

Epidermal sheets were obtained from the roof of suction blisters raised on normal skin of the forearms of nickel-allergic patients and disaggregated to single-cell suspensions using 0.5% trypsin (Biochrom, Berlin, Germany). Keratinocyte primary cultures were established by seeding epidermal cells ($1.2–2 \times 10^4$ cells/cm^2) on a feeder layer of irradiated 3T3/J2 fibroblasts (2×10^4 cell/cm^2) and were cultured in modified Green's medium as described previously (17). At 70–80% confluence, keratinocytes were detached with 0.05% trypsin and 0.02% EDTA, aliquoted, and cryopreserved in liquid nitrogen. Second- or third-passage keratinocytes were used as target cells in the cytotoxicity assay, with cells cultured in serum-free medium (keratinocyte growth medium, Clonetics, San Diego, CA) without hydrocortisone for at least 3 days before experiments were performed. Keratinocytes were treated, or not, with 300 U/ml IFN-γ alone or in combination with TNF-α (50 μg/ml) for at least 24 h and extensively washed before use in the cytotoxicity tests. Autologous B lymphoblastoid cell lines (B-LCL) were generated according to standard procedures by incubating PBMC with supernatant from the EBV-producing marmoset line B95/8 (American Type Culture Collection, Manassas, VA) in the presence of 2 μg/ml cyclosporin A for 7–15 days.

Cytotoxicity assay

To identify DNA fragmentation induced by nickel-specific Tcc in autologous B-LCL or keratinocytes the [^{3}H]TdR release assay was employed as previously described (29). B-LCL and keratinocytes were preincubated with 5 μCi/ml [^{3}H]TdR (Amersham, Little Chalfont, U.K.) at 37°C for 10 and 16 h, respectively, and seeded at $2–3 \times 10^4$/well in round-bottom microtiter plates in complete RPMI supplemented with 10% FBS. Effector T cells used in the cytotoxic assays were devoid of contaminating $CD19^+$, $CD14^+$ and $CD1a^+$ cells, as assessed by FACS analysis (data not shown). Effector cells were cocultured with target cells for 5 h in the presence or the absence of 20 μg/ml $NiSO_4$. Spontaneous release of [^{3}H]TdR by target cells was evaluated in wells containing medium alone. Cells were harvested on fiber-coated 96-well plates (Packard Instruments, Groningen, The Netherlands), and radioactivity was measured in a Topcount (Packard Instruments). The percentage of lysis was calculated as [(cpm without T cells − cpm with T cells)/cpm without T cells) × 100]. Specific lysis was determined by subtracting the percentage of lysis obtained in the absence of $NiSO_4$ from that measured in the presence of $NiSO_4$. Unspecific lysis was always <3%. For blocking experiments with mAbs, target cells were preincubated at 4°C for 30 min with the relevant mAb (anti-MHC-I and anti-HLA-DR, 1 μg/ml; anti-ICAM-1, 10 μg/ml) and then used in the [^{3}H]TdR release assay. Where indicated, Tcc were preincubated with 0–200 nM CMA or 0–50 μM BFA for 2 h and assayed for cytotoxicity in the presence of the drug as described previously (30).

Flow cytometry

Cells were examined by flow cytometry using nonconjugated primary mAbs followed by the appropriate secondary FITC-conjugated Ig. In control samples, staining was performed using isotype-matched control Ig. Expression of FasL, perforin, and Bcl-2 was determined after fixation with 2% paraformaldehyde and permeabilization with 0.5% saponin. Intracellular FasL expression directly correlates with the levels of the extracellular form, which is rapidly cleaved by metalloproteinases (31, 32) and thus hardly detected. Cells were analyzed with a FACScan equipped with CellQuest software (Becton Dickinson, Mountain View, CA).

RT-PCR analysis

Total cellular RNA was extracted from skin samples using the acid guanidinium thiocyanate-phenol-chloroform method (33). RNA (1 μg) was reverse transcribed to cDNA using oligo(dT) primers and then amplified with GeneAmp RNA PCR kit (Perkin-Elmer, Roche Molecular Systems, Branchburg, NJ) according to the manufacturer's protocol. The following synthetic oligonucleotides were used: for perforin, 5′-GTCTGCTCCTC CTGGGCATCCTTC-3′ and 5′-CGGGGGAGTGTGTACCACATGGAAA-3′ (589-bp amplification product); for IFN-γ, 5′-TGCAGGTCATTCAGATGTAG 3′, and 5′-AGCCATCACTTGGATGAGGG-3′ (306-bp amplification product); and for IL-4, 5′-GCCTGTTGTACCAGCTGGTTTTTC-3′ and 5′-TGAC CCCTGAGCATCCTGGATTAT-3′ (308-bp amplification product). As a control, the β-actin gene was used with primers 5′-TGACGGGGTCAC CCACACTGTGCCCATCTA-3′ and 5′-CTAGAAGCATTTGCGGTG GACGATGGAGGG-3′ (631-bp amplification product).

Results

Cytokine pattern and immunophenotype of nickel-specific $CD4^+$ and $CD8^+$ Tcc

$CD4^+$ and $CD8^+$ Tcc were isolated from the blood and lesional skin of patients with ACD to nickel and were characterized for Ag specificity and cytokine release as well as expression of the CLA and molecules mediating cytotoxicity (Table I). Both skin- and blood-derived $CD4^+$ and $CD8^+$ nickel-specific Tcc were positive for CLA, indicating their ability to recirculate in the skin environment (12, 23, 24), and expressed FasL upon activation, as detected by FACS analysis (Table I and Fig. 1). Perforin was constitutively

Table I. *Characteristics of the nickel-specific Tcc used in the study*

Clone	Origin	Phenotype	[^{3}H]TdR Uptake (cpm × 10^3)[a] $NiSO_4$ –	[^{3}H]TdR Uptake (cpm × 10^3)[a] $NiSO_4$ +	IFN-γ[b] (ng/ml)	IL-4 (ng/ml)	CLA[c] (ΔMFI)	Perforin (ΔMFI)	FasL (ΔMFI)
FN8.5.76	Skin	$CD8^+$	1.7 ± 0.1	18.1 ± 1.1	14.7	1.2	94	26	30
FN8.5.1	Skin	$CD8^+$	1.7 ± 0.2	24.6 ± 1.9	7.4	0.4	509	30	36
AC8.3.6	Skin	$CD8^+$	1.0 ± 0.04	25.4 ± 0.5	19.8	0.1	906	35	32
AC8.5.3	Skin	$CD8^+$	1.4 ± 0.3	26.5 ± 1.9	0.1	6.2	708	35	56
FN8.5.25	Blood	$CD8^+$	0.6 ± 0.09	9.1 ± 0.85	7.3	0.9	608	25	34
AC8.5.59	Blood	$CD8^+$	1.3 ± 0.11	7.1 ± 0.1	18.6	0.6	505	30	31
AR8.5.6	Blood	$CD8^+$	0.8 ± 0.01	8.9 ± 0.94	7.6	1	101	34	31
AR8.5.8	Blood	$CD8^+$	1.5 ± 0.05	7.9 ± 1.12	0.4	8.5	209	38	32
FN4.5.7	Skin	$CD4^+$	2.4 ± 0.01	40.4 ± 1.94	8.4	0.7	205	26	26
FN4.5.20	Skin	$CD4^+$	1.6 ± 0.2	28.6 ± 2.75	12.8	2.1	145	25	20
AC4.6.6	Skin	$CD4^+$	2.3 ± 0.41	30.3 ± 0.5	10.5	0.3	290	30	21
AR4.1.43	Skin	$CD4^+$	1.0 ± 0.05	10.3 ± 1.2	0.1	6.7	420	0	8
AC4.5.57	Blood	$CD4^+$	2.4 ± 0	39.6 ± 0.96	19.6	0.1	85	29	21
FN4.5.29	Blood	$CD4^+$	2.9 ± 0.06	10.5 ± 1.14	11	0.1	456	20	19
AR4.3.3	Blood	$CD4^+$	0.9 ± 0.04	9.9 ± 1	0.1	5.2	345	0	9
AC4.6.14	Blood	$CD4^+$	1.0 ± 0.03	49.9 ± 2.83	0.1	1.2	546	0	17

[a] Tcc were prepared from lesional skin or peripheral blood of patients with ACD to nickel. Resting Tcc were tested for Ag specificity after extensive washing to remove IL-2 and using irradiated autologous B-LCL in the presence or absence of 20 μg/ml $NiSO_4$. Data represent mean cpm ± SD of triplicate cultures.

[b] Tcc were stimulated with plate-coated anti-CD3 and soluble anti-CD28. After 48 h, supernatants were collected and cytokine content measured by ELISA. Data are expressed as mean ng/ml/10^6 cells of triplicate cultures.

[c] Expression of CLA, perforin, and FasL was determined by flow cytometry on Tcc activated with immobilized anti-CD3 or B-LCL in the presence of 20 μg/ml $NiSO_4$. Data are expressed as ΔMFI, which represents the mean fluorescence intensity of positive staining subtracted of the fluorescence of isotype-matched Ig.

expressed in all $CD8^+$ independent of their cytokine pattern and in $CD4^+$ clones with a Th1 phenotype, whereas Th2 cells were invariably negative both in resting conditions (not shown) and after activation (Fig. 1).

Both $CD4^+$ and $CD8^+$ nickel-specific clones kill autologous B-LCL, but show disparate cytotoxic capacity against autologous keratinocytes

The cytotoxic potential of both type 1 (Th1 and Tc1) and type 2 (Th2 and Tc2) nickel-specific T lymphocytes was evaluated with the [^{3}H]TdR release assay, because it detects cleaved DNA in target cells and thus directly correlates to cell apoptosis (29, 34). Both Tc1 and Tc2 clones exhibited comparable cytotoxic activity against autologous B-LCL in the presence of $NiSO_4$ (Fig. 2). Similarly, Th1 clones demonstrated strong cytotoxicity against nickel-loaded B-LCL, whereas Th2 clones were less potent (Fig. 3). Non-stimulated keratinocytes were killed less efficiently than B-LCL by $CD8^+$ cells, and IFN-γ pretreatment of keratinocytes variably enhanced the lytic activity of some Tc1, but none of the Tc2, clones (Fig. 2). In contrast, Th1 clones exhibited a poor or absent cytotoxic activity against resting keratinocytes (Fig. 3), and significant Th1-mediated apoptosis was observed only in keratinocytes previously activated with IFN-γ. Finally, Th2 clones, although being

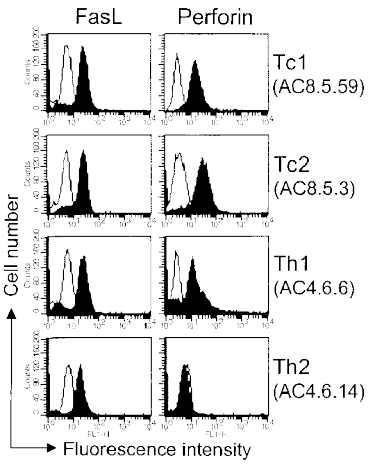

FIGURE 1. FACS analysis of perforin and FasL expression in representative nickel-specific Th1, Th2, Tc1, and Tc2 clones. Tcc were stimulated for 48 h with immobilized anti-CD3, fixed, permeabilized, and then stained with mouse anti-human perforin or anti-FasL mAb (■). □, Cells stained with isotype-matched mouse Ig.

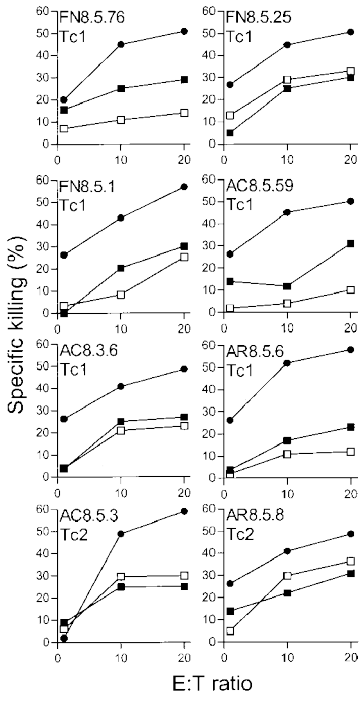

FIGURE 2. $CD8^+$ nickel-specific clones exert cytotoxic activity against B-LCL and keratinocytes. Target cells were B-LCL (●), unstimulated keratinocytes (□), and IFN-γ-pretreated keratinocytes (■). Cytotoxicity was determined using the [^{3}H]TdR release assay. Specific killing was calculated as described in *Materials and Methods*. Unspecific killing was always <3%.

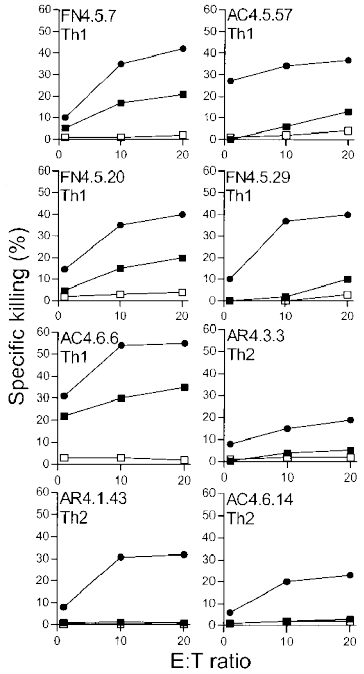

FIGURE 3. Th1 nickel-specific clones are cytotoxic for both B-LCL and keratinocytes, whereas Th2 lymphocytes kill B-LCL, but not keratinocytes. Target cells were B-LCL (●), unstimulated keratinocytes (□), and IFN-γ-pretreated keratinocytes (■). Cytotoxicity was determined using the [^{3}H]TdR release assay. Specific killing was calculated as described in *Materials and Methods*. Unspecific killing was always <3%.

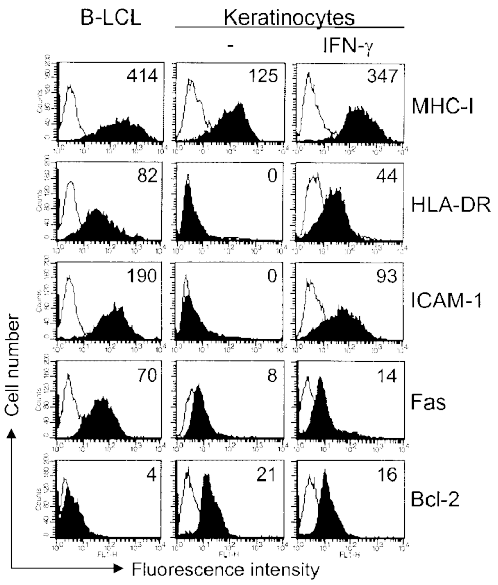

FIGURE 4. IFN-γ-treated keratinocytes show lower MHC class I/II, ICAM-1, and Fas expression and higher Bcl-2 levels compared with B-LCL. FACS analysis of B-LCL and keratinocytes was performed before and after treatment with IFN-γ. Expression in keratinocytes was assayed 48 h after stimulation with 300 U/ml IFN-γ. □, Staining with matched-isotype Ig; ■, cells stained with mAbs specific for the indicated markers. The numbers indicate the net mean fluorescence intensity.

moderately cytotoxic for B-LCL, showed no significant induction of DNA cleavage in resting or IFN-γ-treated keratinocytes in the presence of nickel (Fig. 3). However, Th2 cells could induce some Ag-independent keratinocyte killing (10–15%) when the assay was performed in the presence of PHA (data not shown). Pretreatment of keratinocytes with both TNF-α (50 μg/ml) and IFN-γ (300 U/ml) did not alter their susceptibility to the cytotoxicity induced by CD8$^+$ or CD4$^+$ clones (data not shown).

IFN-γ-treated keratinocytes show lower MHC class I/II, ICAM-1, and Fas expression, but higher Bcl-2 levels, compared with B-LCL

As we observed important differences between B-LCL and keratinocytes in the susceptibility to T cell-mediated cytotoxicity, we compared the expression of molecules involved in Ag presentation and cytotoxic pathways in these two target cells. As shown in Fig. 4, IFN-γ treatment induced de novo expression of MHC class II and ICAM-1, strongly up-regulated MHC class I, and slightly enhanced Fas expression on keratinocytes. MHC class I and class II, ICAM-1, and Fas showed higher expression on B-LCL than on IFN-γ-stimulated keratinocytes, indicating a more efficient Ag presentation to specific Tcc and an enhanced susceptibility to Fas-mediated killing. In contrast, the anti-apoptotic molecule Bcl-2 was markedly more expressed in both untreated and IFN-γ-stimulated keratinocytes than in B-LCL (Fig. 4), helping to explain the higher resistance of keratinocytes to apoptotic signals.

Heterogeneous killing pattern of nickel-specific CD4$^+$ and CD8$^+$ clones

Two main cytolytic mechanisms are used by T cells, the perforin/granzyme granule exocytosis and the Fas/FasL pathways (35, 36). To examine which mechanism prevailed in our nickel-specific clones, their cytotoxic activity was assessed in the presence of different blocking agents. CMA is known to be a specific inhibitor of the perforin pathway by altering the acidity of the lymphocyte granules, thereby promoting premature degradation of perforin (30). BFA preferentially affects the Fas/FasL pathway by preventing the egression of proteins from the endoplasmic reticulum and thus inhibiting the up-regulation of FasL (30, 37). Furthermore, it has been demonstrated that CMA and BFA affect exclusively effector, but not target, cells (30). Nickel-specific Tcc were preincubated for 2 h with different concentrations of the blocking agent and then used as effector cells against B-LCL in the [^{3}H]TdR release assay. As shown in Fig. 5, the CTL activity of all CD8$^+$ clones was markedly inhibited by CMA. BFA significantly reduced the B-LCL killing of three (FN8.5.76, AR8.5.6, and AC8.5.59) of six Tc1 clones, whereas it did not affect the Tc2 clones. With regard to the CD4$^+$ clones, CMA and BFA reduced the cytotoxic capacity of three (AC4.6.6, FN4.5.20, and FN4.5.7) and two (AC4.5.57 and FN4.5.29) Th1 clones, respectively. Finally, the Th2 clones were only inhibited by BFA. These results indicated a preferential, but not exclusive, use of the granule exocytosis pathway by nickel-specific CD8$^+$ clones and a heterogeneous pattern for CD4$^+$ clones, which showed either perforin- or Fas-dependent cytotoxicity. As expected, preincubation of target cells with anti-HLA-DR and MHC class I mAbs inhibited the cytotoxicity of CD4$^+$ and CD8$^+$ clones, respectively, ruling out an unspecific cytotoxic effect (Fig. 5). Interestingly, the anti-ICAM-1 mAb strongly inhibited (50–60% reduction of specific killing) the cytotoxic capacity of all nickel-specific CD4$^+$ clones and of those three Tc1 clones (FN8.5.76, AR8.5.6, and AC8.5.59) affected by BFA. In contrast, the remaining Tc1 and Tc2 clones were not sensitive to ICAM-1 blocking. No significant differences in the susceptibility of keratinocytes and B-LCL to the different T cell-killing mechanisms were observed. As shown in Fig. 6, the killing of two representative Tc1 and Tc2 clones was mostly inhibited by CMA independently from the target cell type, confirming the prevalent, although not exclusive (FN8.5.76), use of the perforin pathway. Th1 clones showed a disparate killing pattern also against

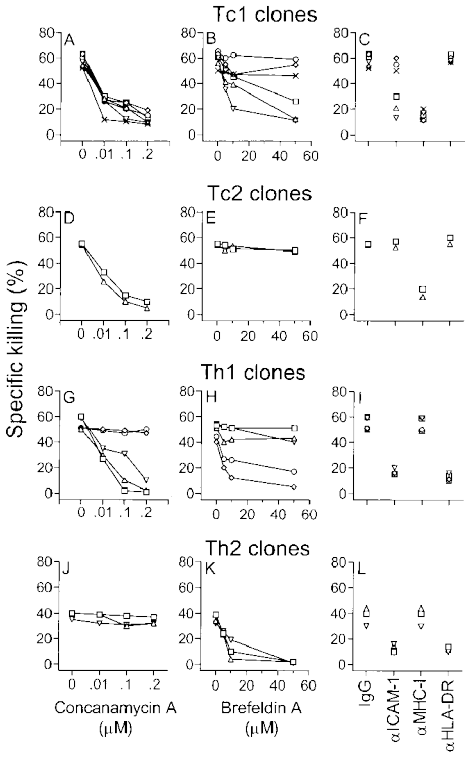

FIGURE 5. Nickel-specific $CD4^+$ and $CD8^+$ clones use disparate cytotoxic mechanisms. Tcc were preincubated for 2 h with various concentrations of CMA or BFA and then cocultured with B-LCL in the [^{3}H]TdR release assay, with the drug present during the assay. For blocking experiments with the mAbs, target B-LCL cells were preincubated with the mAb for 30 min at 4°C, and then cocultured with the effector cells. The experiments were all performed at a target/effector cell ratio of 1:10. Each symbol identifies a single nickel-specific Tcc.

keratinocytes and used either the perforin (AC4.6.6) or the Fas/FasL pathway (AC4.5.57).

Perforin mRNA is expressed in lesional ACD skin

Our in vitro results indicated that $CD8^+$ T lymphocytes have a prominent role in inducing keratinocyte apoptosis through a perforin-mediated mechanism. To investigate whether perforin was expressed during the effector phase of ACD, 48-h positive patch test reactions to nickel were analyzed by RT-PCR. Results showed that perforin mRNA was indeed expressed in ACD skin (Fig. 7). IFN-γ and IL-4 mRNA were also present in the lesional skin, indicating the involvement of both type 1 and type 2 T lymphocytes in the immune reaction. In contrast, no perforin and IL-4 and only a faint signal for IFN-γ mRNA were detected in healthy skin.

Discussion

In this study we show that skin-homing (CLA^+) nickel-specific T lymphocytes isolated from the skin and blood of patients with ACD to nickel induced cytotoxicity in autologous keratinocytes in vitro, suggesting an important pathway involved in the epidermal damage during ACD. While $CD8^+$ T cells exerted their CTL activity on both resting and IFN-γ-activated keratinocytes, Th1 cells killed exclusively keratinocytes previously exposed to IFN-γ. The mechanism by which $CD4^+$ and $CD8^+$ CTL lyse their targets has important implications for their biological functions. The perforin/granzyme pathway does not need the target cell to express specific susceptibility molecules, and thus potentially allows lysis of all cells (36). In contrast, the Fas/FasL mechanism requires the target

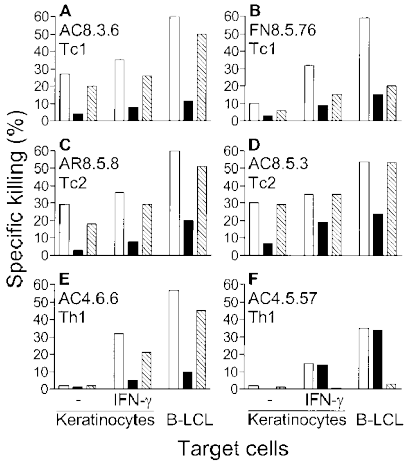

FIGURE 6. Nickel-specific T cell clones use similar cytotoxic mechanisms against B-LCL and keratinocytes. Tcc were preincubated for 2 h with 0.2 μM CMA or 50 μM BFA and then cocultured with B-LCL or keratinocytes (either resting or IFN-γ-activated) for 5 h in the [^{3}H]TdR release assay. The experiments were performed at a target:effector cell ratio of 1:10.

cell to express Fas and be sensitive to Fas-induced apoptosis (37). Nickel-specific Tc1 and Tc2 clones expressed both perforin and FasL upon activation, as described previously for $CD8^+$ T cells with different Ag specificity (38 39). The cytotoxic activity of Tc1 and Tc2 clones against B-LCL and keratinocytes was inhibited by CMA, confirming that the perforin pathway is a major killing mechanism of $CD8^+$ T cells (40, 41). In contrast, BFA blocked part of the Tc1 clones, but no Tc2 clones, indicating a minor, but significant, involvement of the Fas/FasL killing pathway in the CTL activity of some nickel-specific Tc1 clones. Our data also showed that pretreatment of B-LCL with anti-ICAM-1 mAb affected the Tc1 clones whose cytolytic functions were inhibited by the FasL-blocking agent, BFA. In contrast, the Tc2 and Tc1 clones that used only the perforin pathway were not influenced by ICAM-1 blocking. This observation is in line with the hypothesis that two types of CTL exist: type I CTL, which kill target cells through the perforin/granzyme-dependent mechanism, and type II CTLs, which require ICAM-1-derived signals to activate both Fas/FasL and perforin-dependent pathways (38). Concerning the killing potential and machinery of $CD4^+$ T cells, contrasting results have been described. Early reports on $CD4^+$ T cell-mediated cytotoxicity underlined that Fas was the major target molecule (42–44), whereas recent studies revealed the importance of perforin-dependent $CD4^+$ CTL, especially in the clearance of virus infections and in tumor rejection (45–47). Assessing this hypothesis in our system, we found that nickel-specific Th1 clones expressed both perforin and FasL and could be inhibited by either

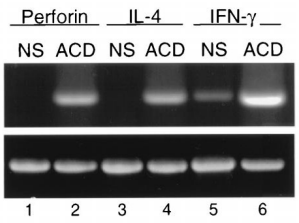

FIGURE 7. Perforin mRNA is expressed in the lesional ACD skin. Skin specimens were obtained from healthy skin and 48-h patch test reaction to $NiSO_4$ and subjected to RT-PCR analysis. Similar results were observed in biopsies from two patients.

CMA or BFA, pointing to a disparate killing pattern of Th1 cells against both B-LCL and keratinocytes. In contrast, Th2 clones uniformly expressed FasL, but were consistently negative for perforin, and their killing capacity against B-LCL was regularly inhibited by BFA, but not CMA. In summary, our findings indicate that heterogeneous populations of nickel-specific T cells can use different cytotoxic modalities to eliminate the Ag-carrying cells. Consistent with the idea that CTL have developed different strategies to obviate virus-induced resistance to cytotoxicity (45, 46, 48), CTL can exploit the very same mechanisms during immune responses to innocuous Ags, such as those inducing allergic diseases. Indeed, studies in knockout mice have demonstrated that perforin and Fas/FasL mechanisms are both necessary to mount CH reactions (8).

Keratinocytes were highly susceptible to nickel-specific cytotoxicity induced by Tc1 and Tc2 cells and, to a lesser extent, by Th1 cells, but were resistant to Th2 clones. However, Th1-mediated killing required prior treatment of keratinocytes with IFN-γ. Indeed, only keratinocytes stimulated with IFN-γ express mature MHC class II molecules and ICAM-1 (17, 26). Moreover, IFN-γ up-regulates Fas expression and renders keratinocytes susceptible to Fas-mediated cytotoxicity (27, 49), which was shown to be involved in Th1-mediated cytotoxicity. In contrast, IFN-γ treatment had variable influences on the $CD8^+$-mediated killing, with a significant enhancement of the lytic capacity for those Tc1 clones (FN8.5.76, AC8.5.59, and AR8.5.6) whose cytotoxicity was inhibited by blocking ICAM-1. The cytotoxic activity of the other $CD8^+$ clones was not significantly changed. The fact that CH responses in IFN-γ receptor-deficient mice are only partially affected indicates a nonessential role for IFN-γ in these immune responses (50). In contrast to our results, other studies indicated a higher lytic activity of herpes virus-specific $CD4^+$ T cells compared with $CD8^+$ lymphocytes against virus-infected keratinocytes (51). This finding could be the consequence of reduced MHC class I expression on keratinocytes infected with the herpes virus. The disparate cytotoxic activity of different T cell subsets against keratinocytes suggests distinct roles in the effector phase of ACD, with $CD8^+$ lymphocytes killing resting keratinocytes, and Th1 cells exerting cytotoxic functions only at later time points, when keratinocytes have already been exposed to IFN-γ released by type 1 T cells.

An interesting observation of our study was that cultured keratinocytes were, in general, more resistant than B-LCL to T cell-mediated cytotoxicity. This different susceptibility may reflect the higher expression of MHC, ICAM-1, and Fas on B-LCL as well as the absence of B7-1 and B7-2 costimulatory molecules on keratinocytes (52) (data not shown). In addition, keratinocytes expressed higher levels of the apoptosis-protective molecule Bcl-2, although the role of Bcl-2 in protecting target cells against perforin/granzyme- and Fas/FasL CTL-induced apoptosis is still a matter of debate (53–55). All nickel-specific Th2 clones were not cytolytic against keratinocytes, but efficiently killed B-LCL target cells through the Fas/FasL pathway. This may be an indication that the cytotoxicity of Th2 clones has an immunoregulatory function through the elimination of professional APC, as also proposed by others (56).

The expression of ACD mostly depends on the recruitment and expansion of hapten-specific $CD8^+$ T lymphocytes, as suggested by studies in mice deficient in distinct T cell subsets (9–11) and in the human disease (12–15). Our results clearly show that hapten-loaded keratinocytes can be target of T cell-mediated cytotoxicity. Hapten-specific $CD8^+$ T cells can exert direct cytotoxic effects on resting keratinocytes, confirming their predominant role in the initiation of epidermal damage during ACD. Among $CD4^+$ T lymphocytes, only the Th1 subset was able to kill keratinocytes, but exclusively after MHC class II induction by IFN-γ, and may thus cooperate with $CD8^+$ T cells only at a later time point in causing the tissue damage. In contrast, keratinocytes appear to be resistant to Th2-mediated cytotoxicity. Alternatively, Th1 and Th2 cells can effectively contribute to disease expression by inducing keratinocyte release of chemokines that attract T cells in the skin (17, 25) and by rendering keratinocytes more susceptible to CTL activity.

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