Patients with Allergic Contact Dermatitis to Nickel and Nonallergic Individuals Display Different Nickel-Specific T Cell Responses. Evidence for the Presence of Effector $CD8^+$ and Regulatory $CD4^+$ T Cells

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To investigate the mechanisms underlying the expression of allergic contact dermatitis, we compared the characteristics of nickel (Ni)-specific T cell responses in 10 patients with allergic contact dermatitis to Ni and in 10 healthy, nonallergic individuals. $CD4^+$ T cells purified from peripheral blood of both allergic and nonallergic subjects proliferated similarly to $NiSO_4$ *in vitro*, with the responses mostly restricted to $CD4^+$ $CD45RO^+$ memory T cells. In contrast, Ni-specific $CD8^+$ T cell responses were detected only in allergic patients. Limiting dilution assay confirmed a high frequency of Ni-specific $CD4^+$ T cells in both individual categories, and of Ni-specific $CD8^+$ T cells in allergic patients, but not in nonallergic persons. Ni-specific $CD4^+$ T cell clones prepared from nonallergic subjects displayed lower interferon-γ and higher interleukin-10 production compared with T cell clones from allergic patients. The T cell skin-homing receptor, cutaneous lymphocyte-associated antigen, was expressed on the large majority of specific $CD4^+$ clones from both the groups. Finally, Ni-specific $CD8^+$ clones prepared from patients also expressed the cutaneous lymphocyte-associated antigen receptor, and released high interferon-γ and no interleukin-4. In aggregate, the results suggest that the presence of specific $CD8^+$ T cells and a distinct pattern of cytokine release (e.g., an augmented production of interleukin-10) by $CD4^+$ T cells can be important elements in determining whether a hapten induces allergy or a silent immune response.

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Allergic contact dermatitis (ACD) is the result of a T cell mediated immune response to haptens applied onto the skin (Grabbe and Schwarz, 1998). Haptens are highly reactive, lipophilic small chemicals that acquire immunogenicity by coupling to peptides loaded on major histocompatibility complex (MHC) molecules (Martin *et al*, 1992; Cavani *et al*, 1995). Haptens penetrating the skin are picked up by dendritic cells, which then migrate to regional lymph nodes where the hapten-peptide complexes are presented in a MHC-restricted fashion to specific naïve T cells (Cruz *et al*, 1989; Hauser, 1990; Girolomoni *et al*, 1993; Bacci *et al*, 1997). Cutaneous challenge of sensitized subjects with the relevant hapten causes a rapid recruitment and expansion of hapten-specific T cells into the skin, and gives rise to the inflammatory reaction. Skin homing of T lymphocytes is mainly regulated through the expression of the cutaneous lymphocyte-associated antigen (CLA), a highly sialylated adhesion molecule that binds to E-selectin on activated endothelium (Picker *et al*, 1991; Santamaria Babi *et al*, 1995; Fuhlbrigge *et al*, 1997). In murine models, evidence exists that cytokines have a profound influence on contact hypersensitivity (CH) to haptens, which is promoted by interleukin (IL)-12 and type 1 cytokines (Muller *et al*, 1995; Riemann *et al*, 1996), and downregulated by IL-10, and to a lesser extent by IL-4 (Gautam *et al*, 1992; Enk *et al*, 1994; Ferguson *et al*, 1994; Berg *et al*, 1995; Asada *et al*, 1997). Additionally, it has been suggested that the magnitude of murine CH depends upon the balance between hapten-specific $CD8^+$ T cells, playing primarily an effector role, and $CD4^+$ T lymphocytes, including IL-10 and IL-4 producing subsets, with prominent regulatory functions (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Xu *et al*, 1996).

In humans, whether ACD expression could be regulated through the activity of Th1/Th2 or $CD4^+/CD8^+$ T cell subsets is largely unknown. In particular, ACD to nickel (Ni) has been linked to the presence of specific $CD4^+$ T cells belonging to the Th1 subset, which can be isolated from both peripheral blood and the site of cutaneous challenge (patch test) of allergic patients (Sinigaglia *et al*, 1985; Kapsenberg *et al*, 1987, 1992). More recently, however, Ni-specific Th2 cell clones have been isolated from the skin of patients (Werfel *et al*, 1997). In nonallergic individuals, Ni-specific T cell responses have been described as well (von Blomberg-van der Fliers *et al*, 1987), although most of the authors interpreted such responses as the consequence of a nonspecific mitogenic effect of the metal on T cells (Pappas *et al*, 1970; Svejgaard *et al*, 1978; Räsänen and Tuomi, 1992). Instead, these findings may suggest that nonallergic subjects can mount immune responses to Ni, and that regulatory mechanisms may prevent disease expression. Therefore, in this study we compared the characteristics of Ni-specific T cell responses in patients with ACD and in healthy, nonallergic individuals. Our results appear to indicate that

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Abbreviations: ACD, allergic contact dermatitis; CH, contact hypersensitivity; CLA, cutaneous lymphocyte-associated antigen; Ni, nickel; PBMC, peripheral blood mononuclear cells.

$CD8^+$ T cells are crucial for the expression of ACD to Ni, because Ni-specific $CD8^+$ T cell responses were detected in patients with ACD to Ni, but not in nonallergic individuals. In contrast, both subject categories harbor strong memory $CD4^+$ T cell responses to Ni, with Ni-specific $CD4^+$ CLA^+ T cell clones from nonallergic individuals producing higher amounts of IL-10 and lower interferon (IFN)-γ. Thus, distinct mechanisms may contribute to maintain clinically silent ongoing T cell immune responses to Ni.

MATERIALS AND METHODS

Patients and nonallergic individuals Ten nonatopic patients (median age, 31 y; nine females and one male) with ACD to Ni and 10 nonatopic, age-matched healthy volunteers (median age, 30 y; six females and four males) without history of cutaneous allergy to metals were included in the study. Both patients with ACD to metals and control subjects were tested with a standard patch test series, including $NiSO_4$ 5% in petrolatum (International Contact Dermatitis Research Group Series). Patch tests were applied under occlusion with Finn Chambers (Alpharma, Norgesplaster, Norway) on the upper back, and evaluated after 48 and 72 h. The reactivity was graded as follows: –, no reaction; +, redness, edema; ++, redness, edema, and papules; +++, redness, papules, and vesicles. To increase patch test sensitivity and definitively exclude contact sensitivity to the metal, healthy controls were also selected on the basis of a negative patch test to 5% $NiSO_4$ performed after 24 h pretreatment of the skin with 0.1% sodium lauryl sulfate (Seidenari *et al*, 1996). Both patients and controls were not taking any medication for at least 15 d before blood donation. Peripheral blood samples were obtained after informed consent.

T cell purification and proliferation assays T cell proliferation assays were performed in RPMI 1640 complemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-mercaptoethanol, 100 U penicillin per ml, 100 μg streptomycin per ml (all from Life Technologies, Chagrin Falls, OH), and 5% pooled homologous plasma (complete medium). Peripheral blood mononuclear cells (PBMC) were separated by centrifugation over Ficoll-Hypaque (Lymphoprep, Nycomed-Pharma, Oslo, Norway) and left to adhere (6×10^6cells per ml) in Petri dishes for 2 h at 37°C in complete medium. After extensive washing, adherent cells were incubated for 30 min in 0.2% ethylenediamine tetraacetic acid, removed from dishes by scraping, X-irradiated, and then used as antigen presenting cells (APC) at 10^5 cells per well. The nonadherent fraction was depleted of $CD19^+$, $HLA\text{-}DR^+$, and $CD4^+$ or $CD8^+$ cells by incubation with immunomagnetic beads coated with specific monoclonal antibodies (MoAb) (Dynabeads M450, Dynal, Oslo, Norway). After two rounds of bead depletion, >98% pure $CD8^+$ or >95% pure $CD4^+$ T cells were obtained, as confirmed by flow cytometry analysis. In selected experiments, $CD4^+$ T cells were further purified into $CD45RA^+$ and $CD45RO^+$ T cells by negative selection. To this end, purified $CD4^+$ T cells were incubated with anti-CD45RO (UCHL-1) or anti-CD45RA (L48) MoAb (Becton Dickinson, San Jose, CA) for 1 h at 4°C, followed by anti-mouse IgG-conjugated immunomagnetic beads. Purified T cell populations were used as

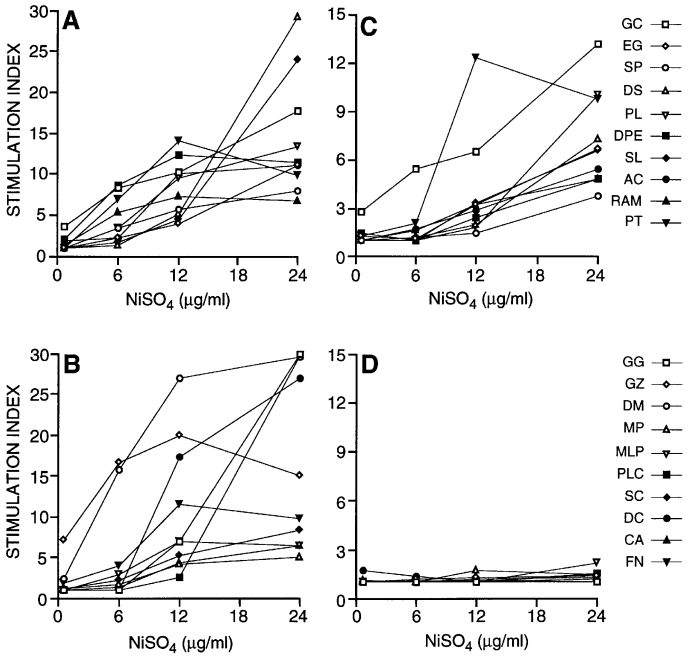

Figure 1. Ni-specific $CD4^+$ T cells from allergic and nonallergic individuals proliferates *in vitro* to $NiSO_4$, whereas only allergic patients show Ni-specific $CD8^+$ T cell responses. $CD4^+$ T cells (*A*, from allergic patients; *B*, from nonallergic individuals) and $CD8^+$ T cells (*C*, from allergic; *D*, from nonallergic) were purified from peripheral blood by negative selection with immunomagnetic beads, and used as responders (1.5×10^5 cells per well) in proliferation assay together with autologous adherent cells (10^5 cells per well). Results are shown as stimulation index, which represents the ratio between the mean [^{3}H]thymidine incorporation of triplicate cultures tested in the presence and in the absence of $NiSO_4$. Differences in $CD4^+$ T cell proliferation between allergic and nonallergic subjects were not significant ($p > 0.05$) at each $NiSO_4$ concentration.

Table I. Characteristics of the subjects included in the study and results of purified $CD4^+$ and $CD8^+$ T cell proliferation to $NiSO_4$ *in vitro*[a]

Subjects	Sex	Age	ACD to Ni	Patch test reactivity	$CD4^+$ T cells		$CD8^+$ T cells	
					–	$NiSO_4$	–	$NiSO_4$
1. GC	F	31	+	++++	1.70 ± 0.38	30.24 ± 2.80	1.24 ± 0.16	16.49 ± 1.97
2. DS	F	26	+	+++	1.18 ± 0.39	34.48 ± 1.68	0.71 ± 0.04	5.26 ± 0.14
3. PL	F	32	+	++	3.44 ± 1.92	45.79 ± 6.19	1.25 ± 0.07	12.62 ± 0.15
4. DPE	F	22	+	+++	2.40 ± 0.17	29.47 ± 1.82	1.58 ± 0.32	4.23 ± 0.89
5. SL	F	31	+	+++	2.20 ± 0.42	50.24 ± 0.88	3.50 ± 0.38	8.91 ± 0.01
6. EG	F	36	+	+	4.14 ± 0.23	41.21 ± 1.49	1.46 ± 0.24	6.72 ± 0.36
7. PT	F	32	+	+++	2.18 ± 0.05	31.73 ± 0.51	0.80 ± 0.51	9.97 ± 1.21
8. SP	F	29	+	+	1.40 ± 0.20	8.91 ± 0.44	2.07 ± 0.74	7.99 ± 1.20
9. AC	M	34	+	++	4.39 ± 0.38	44.71 ± 1.10	2.98 ± 0.79	19.88 ± 3.10
10. RAM	F	31	+	+++	2.31 ± 0.37	15.52 ± 1.61	2.84 ± 0.26	9.93 ± 0.86
1. CA	F	32	–	–	0.54 ± 0.11	3.57 ± 0.17	3.28 ± 0.01	3.65 ± 0.36
2. GG	M	39	–	–	0.70 ± 0.34	21.15 ± 0.29	2.57 ± 0.19	2.60 ± 0.86
3. DM	F	24	–	–	2.77 ± 0.14	81.91 ± 7.34	3.37 ± 1.10	3.41 ± 0.92
4. DC	M	39	–	–	0.51 ± 0.10	13.89 ± 3.39	3.38 ± 1.21	3.25 ± 0.41
5. GZ	F	39	–	–	1.83 ± 0.21	46.13 ± 4.98	1.80 ± 0.21	2.50 ± 0.43
6. SC	F	28	–	–	3.93 ± 0.47	29.33 ± 3.40	4.12 ± 0.76	5.73 ± 0.12
7. MP	F	27	–	–	3.01 ± 0.91	13.25 ± 3.51	1.04 ± 0.15	1.84 ± 0.15
8. MLP	M	31	–	–	1.06 ± 0.42	6.65 ± 1.56	0.32 ± 0.02	0.73 ± 0.01
9. PLC	M	29	–	–	0.75 ± 0.15	22.49 ± 6.22	0.58 ± 0.09	0.94 ± 0.06
10. FN	F	24	–	–	0.84 ± 0.07	9.62 ± 0.10	0.19 ± 0.034	0.25 ± 0.01

[a]$CD4^+$ and $CD8^+$ T cells were purified from nonadherent PBMC by immunomagnetic depletion with specific MoAb, and used as responder cells (1.5×10^5 cells per well) in a proliferation assay together with peripheral blood adherent cells (10^5 cells per well) as APC, in the absence or in the presence of the optimal $NiSO_4$ concentration (12–24 μg per ml). [^{3}H]Thymidine incorporation was measured after 5 d of culture. Results are given as mean cpm $\times 10^3$ ± SD of triplicate cultures.

responder cells in proliferation assays performed in triplicate at 1.5×10^5 cells per well in flat-bottom 96 well plates without or with 0.6–24 μg $NiSO_4$ per ml (Sigma, St. Louis, MO). After 5 d, cocultures were pulsed with 5 μCi [^{3}H]thymidine per ml (Amersham, Little Chalfont, U.K.) for about 16 h at 37°C, and then harvested onto fiber coated 96 well plates (Packard Instruments, Groningen, The Netherlands). Radioactivity was measured in a beta counter (Topcount, Packard Instruments). Results are given as the stimulation index, which represents the ratio of [^{3}H]thymidine uptake in the presence and the absence of $NiSO_4$. MHC restriction of $CD4^+$ and $CD8^+$ T cell reactivity to $NiSO_4$ was evaluated by incubating APC with 10 μg per ml of pooled anti-HLA-DR, -DP, and -DQ MoAb, anti-HLA class I MoAb, or mouse IgG (all from Becton Dickinson) for 30 min at 4°C and then for additional 30 min at 37°C prior to their use in proliferation assays.

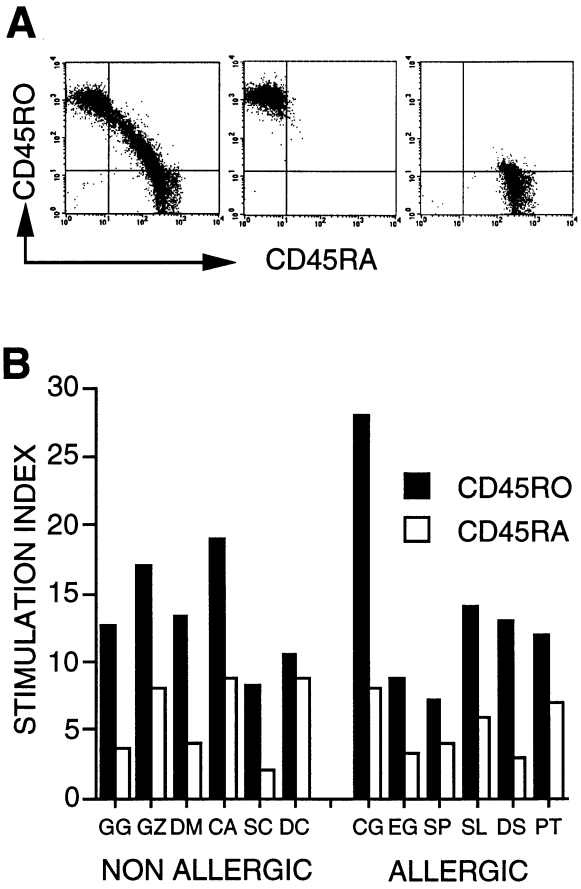

Figure 2. Both allergic patients and nonallergic subjects harbor Ni-specific memory $CD4^+$ $CD45RO^+$ T lymphocytes. $CD4^+$ T cells were purified by indirect immunomagnetic separation using UCHL-1 and L48 MoAb, and then used in proliferation assay. (*A*) Representative $CD4^+$ T cell staining before (*left panel*) and after (*middle and right panels*) purification. Cells were labeled with fluorescein isothyocyanate-conjugated anti-CD45RA and phycoerythrin-conjugated anti-CD45RO. (*B*) Proliferative response of $CD4^+CD45RO^+$ and $CD4^+CD45RA^+$ T cell subsets from six nonallergic and six allergic individuals in the presence of 12 μg $NiSO_4$ per ml using adherent cells as APC.

Frequency of Ni-specific T cells Frequency of Ni-specific T cells was assessed by limiting dilution of purified $CD4^+$ and $CD8^+$ T cells (500–20,000 cells per well) in the presence of 6 μg $NiSO_4$ per ml and 10^4 irradiated autologous PBMC in U-bottomed 96 microplates in complete medium. For each dilution, 24 replicate cultures in the presence of $NiSO_4$ were compared with 12 replicate cultures performed in the absence of $NiSO_4$. [^{3}H]Thymidine incorporation was measured after 5 d of culture. Ni-stimulated cultures in which the cpm exceeded the mean cpm plus 3 SD of the 12 corresponding antigen-free cultures were considered as positive. The distribution of Ni-reactive T cells was analyzed by plotting the fraction of the nonresponding cultures on the *y* axis and the cell input on the *x* axis. The frequency of Ni-specific T cells could be roughly calculated as the inverse of the number of T cells giving rise to 37% negative wells, as described by Lefkovitz and Waldman (1979).

T cell cloning, immunophenotype, cytokine production $CD4^+$ and $CD8^+$ short-term T cell lines were cloned by limiting dilution in the presence

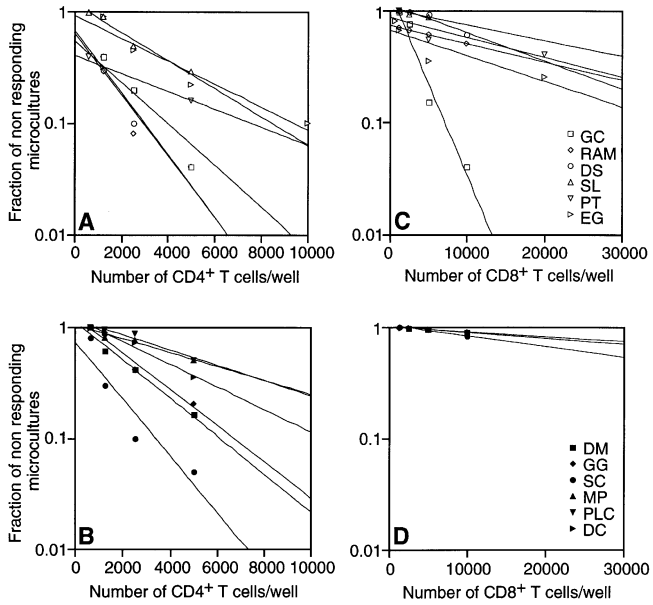

Figure 3. Frequency of Ni-specific $CD4^+$ and $CD8^+$ T cells. Results of the limiting dilution assays performed with purified $CD4^+$ and $CD8^+$ T cells from six allergic (*A*, $CD4^+$; *C*, $CD8^+$) and six nonallergic individuals (*B*, $CD4^+$; *D*, $CD8^+$). T cells (500–20,000 cells per well) were plated with 10^4 irradiated adherent PBMC in U-bottomed microplates in the presence of 6 μg $NiSO_4$ per ml, and evaluated for [^{3}H]thymidine uptake as described in *Materials and Methods*. For each Ni concentration, 24 replicates in the presence of $NiSO_4$ and 12 without $NiSO_4$ were plated. Wells showing [^{3}H]thymidine incorporation exceeding the mean ± 3 SD of the controls were considered positive. The estimated frequencies roughly correspond to the condition showing 37% of negative replicates, and are shown in **Table II**.

Table II. Allergic patients and nonallergic individuals show similar frequency of Ni-specific $CD4^+$ T lymphocytes, but differ in the frequency of Ni-responsive $CD8^+$ T lymphocytes

	Frequency of Ni-specific T cells			Frequency of Ni-specific T cells	
Allergic	$CD4^+$	$CD8^+$	Nonallergic	$CD4^+$	$CD8^+$
1. GC	1:2669	1:2660	1. DM	1:2439	1:80402
2. RAM	1:2841	1:25988	2. SC	1:1697	1:107555
3. SL	1:3440	1:31149	3. MP	1:7446	NC[a]
4. DS	1:2756	1:17278	4. PLC	1:6216	1:45817
5. PT	1:5434	1:24933	5. GG	1:2669	NC
6. EG	1:4220	1:19734	6. DC	1:4250	NC
mean	1:3560	1:20290	mean	1:4119	ND[b]

[a]NC, not calculable.
[b]ND, not determined.

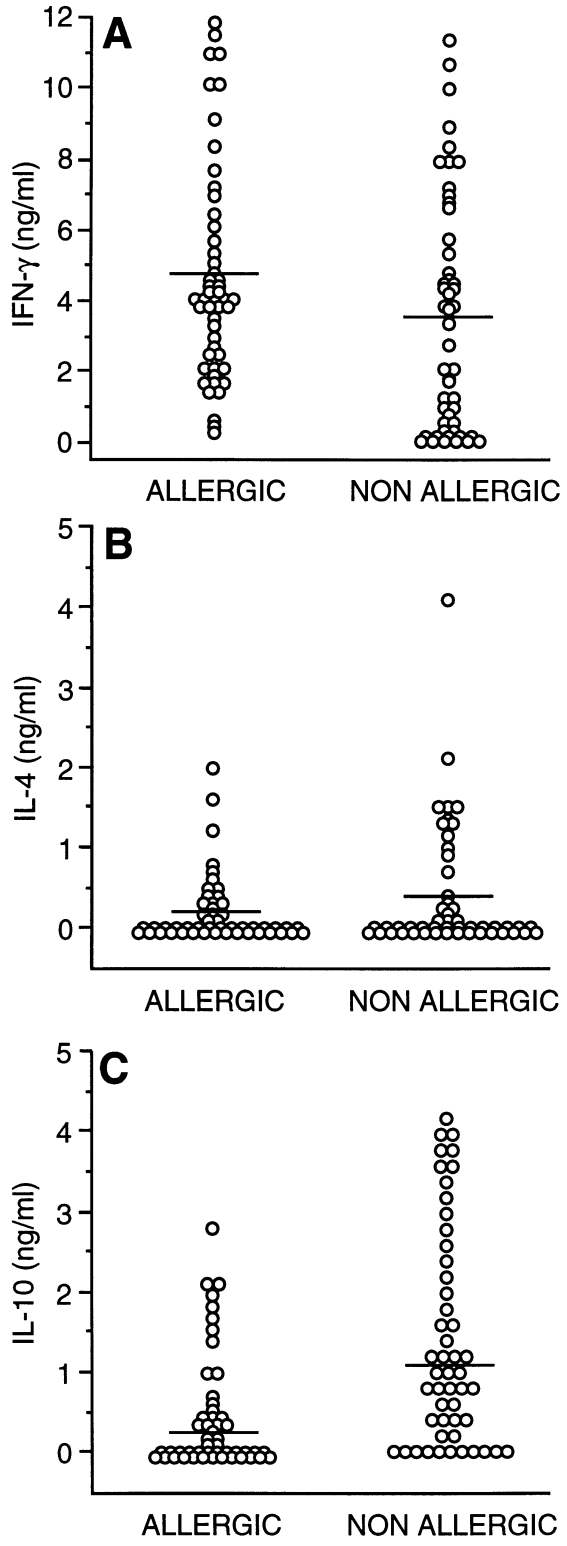

Figure 4. Ni-specific $CD4^+$ T cell clones from allergic patients and nonallergic individuals display a distinct pattern of cytokine release. Culture supernatants were collected 48 h after stimulation with 10 ng PMA per ml and 1 μg ionomycin per ml, and content of IFN-γ (*A*), IL-4 (*B*), and IL-10 (*C*) was measured by enzyme-linked immunosorbent assay. Ni-specific T cell clones from nonallergic donors (n = 50) released a lower amount of IFN-γ ($p = 0.03$) and higher IL-10 ($p = 0.006$), compared with T cell clones from allergic patients (n = 50). No significant differences were observed for IL-4 release.

of 2×10^5 PBMC, 20 U rIL-2 per ml (kindly provided by Chiron Italia) and 1% phytohemagglutinin (Life Technologies) in U-bottomed 96 well microplates in complete medium containing 10% fetal bovine serum (Hyclone, Logan, UT). Clones were grown with rIL-2 (20 U per ml) and stimulated once a month with 1% phytohemagglutinin in the presence of feeder cells. Antigen specificity of T cell clones was assessed using autologous EBV-transformed B cell lines or PBMC as APC and 12 μg $NiSO_4$ per ml. Immunophenoytpe of the clones was evaluated by double-color flow cytometry analysis using anti-CD4, anti-CD8, anti-TCRαβ, and anti-TCRγδ fluorescein isothyocyanate- or phycoerythrin-conjugated MoAb (Becton Dickinson). CLA expression was studied using the HECA-452 MoAb (kindly provided by Dr. Louis J. Picker, Laboratory of Experimental Pathology, Department of Pathology, University of Texas South-western Medical Center, Dallas, TX) or control rat IgM, followed by phycoerythrin-conjugated anti-rat IgM (Pharmingen, San Diego, CA). Cells were analyzed with a FACScan equipped with Cell Quest software (Becton Dickinson, Mountain View, CA). Supernatants from T cell clones (10^6 cells per ml) stimulated in 24 wells with 10 ng phorbol 12-myristate 13-acetate (PMA) per ml and 1 μg ionomycin per ml (Sigma), or with $NiSO_4$ (12 μg per ml) plus 10^6 autologous irradiated PBMC, were collected after 24–48 h culture, filtered, and stored at −80°C. IL-2, IL-4, IL-10, and IFN-γ content was measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN), following the manufacturer's instructions.

Statistical analysis Data were compared using the Mann–Whitney rank sum test (SigmaStat, Jandel, San Rafael, CA); p values ⩽ 0.05 were considered significant.

RESULTS

$CD4^+$ T cells from both allergic patients and healthy controls proliferate *in vitro* in response to $NiSO_4$ Patients included in our study had a positive history of eczematous dermatitis after contacting metals and a positive patch test to 5% $NiSO_4$ (**Table I**). Nonallergic individuals had no history of dermatitis to metals and a negative patch test to $NiSO_4$ even after pretreatment of the skin with 0.1% sodium lauryl sulfate, a procedure that increases the sensitivity of the patch test (Seidenari *et al*, 1996). Neither the patients nor the healthy individuals had personal or family history of atopic diseases, and serum IgE were within normal limits. To assess the Ni-specific T cell response to $NiSO_4$, pure $CD4^+$ T cell populations prepared from Ni-allergic and nonallergic subjects were compared in proliferation assays in the presence of autologous adherent cells and serial dilution of $NiSO_4$. As shown in **Table I** and **Fig 1**, both allergic and nonallergic displayed a strong $CD4^+$ T cell proliferation to $NiSO_4$, without significant differences at each $NiSO_4$ concentration tested. $NiSO_4$ concentrations above 24 μg per ml were toxic, and drastically reduced T cell proliferation. $CD4^+$ T cell responses to Ni were markedly inhibited by blocking MHC class II receptors on APC with pooled anti-HLA-DR, -DP, and -DQ MoAb, but not with mouse IgG or anti-class I MoAb (not shown). Among nonallergic and allergic donors, both high and low responders were identified, without correlation either with the severity of the disease or with the patch test reactivity in the latter individual group (**Table I**). Peripheral blood samples were obtained either prior to or 2 wk after patch testing, but patch testing did not influence per se the magnitude of the $CD4^+$ T cell responses, as no significant differences in the extent of the Ni-specific T cell proliferation were observed between assays performed before and after patch testing (not shown).

The $CD45RO^+$ memory T cell subset mainly accounts for $CD4^+$ T cell proliferation to $NiSO_4$ To exclude the possibility that the $CD4^+$ T cell responses observed in nonallergic subjects could have depended on *in vitro* T cell priming, $CD4^+$ T cell proliferation to $NiSO_4$ was examined using purified $CD45RO^+$ $CD45RA^-$ (memory) and $CD45RA^+$ $CD45RO^-$ (naïve) subsets as responders. Separation of $CD45RO^+$ and $CD45RA^+$ cells was performed by negative selection using an indirect immunomagnetic technique with L48 and UCHL-1 MoAb, respectively, followed by anti-IgG-conjugated magnetic beads, and purification was assessed by two-color fluorescence-activated cell sorter analysis (**Fig 2*A***). Indeed, $CD4^+$ $CD45RO^+$ T cells from nonallergic individuals proliferated significantly to $NiSO_4$, demonstrating the presence of Ni-specific $CD4^+$ memory T cells in nonallergic individuals (**Fig 2*B***). A similar pattern of $CD4^+$ T cell

Table III. Ni-specific CD4$^+$ T cell clones from both allergic and nonallergic individuals display a similar pattern of cytokine release in response to different stimuli[a]

		PMA + ionomycin			APC + $NiSO_4$		
T cell clones		IFN-γ	IL-4	IL-10	IFN-γ	IL-4	IL-10
DM35[b]	Th1	7776	2	530	3581	1.4	376
DM44[b]	–	32	0.7	2817	30	0.5	2120
SC1[b]	Th1/Th0	8000	2100	310	5240	1900	230
SC21[b]	–	250	0	3800	240	0	2450
MLP8[b]	Th1	3900	0	0	3850	0	0
GC20[c]	Th1	3300	0	0	1720	42	0
GC34[c]	Th1	2000	71	0	2091	21	0
AC29[c]	–	108	1.4	3046	30	7	1211
AC60[c]	Th1	10100	0	0	5712	0	0
PL45[c]	Th1	3500	400	1500	2801	311	982

[a]Culture supernatants were collected 48 h after stimulation with 10 ng PMA per ml and 1 μg ionomycin per ml, or autologous APC and 12 μg per ml $NiSO_4$. IFN-γ, IL-4, and IL-10 were measured by enzyme-linked immunosorbent assay. Data are expressed as pg per ml.
[b]Ni-specific CD4$^+$ T cell clones from nonallergic individuals.
[c]Ni-specific CD4$^+$ T cell clones from allergic patients.

responses were observed in allergic patients. CD4$^+$ CD45RA$^+$ T cells from both allergic and nonallergic individuals proliferated to $NiSO_4$ as well, although to a lesser extent. When CD4$^+$CD45RO$^+$ and CD4$^+$CD45RA$^+$ T cells from the same subjects where tested with the recall antigen, tetanus toxoid, a strong proliferative response of CD4$^+$CD45RO$^+$ T cells and a much lower, but still significant, proliferation of CD4$^+$CD45RA$^+$ T cells were observed (not shown). It has recently been demonstrated that in the absence of antigenic stimulation memory T cells may reacquire an unprimed (CD45RA$^+$ CD45RO$^-$) phenotype, suggesting that the CD45RA/CD45RO switch is not unidirectional (Rothstein *et al*, 1991; Hamann *et al*, 1996). Therefore, it is possible that the Ni-specific proliferation of CD4$^+$ CD45RA$^+$ T cells may depend on reversal of CD45 isoform switching of resting Ni-specific T cells rather than on *in vitro* T cell priming.

CD8$^+$ T cells from Ni-allergic patients, but not from healthy individuals, proliferate *in vitro* in response to $NiSO_4$ CD8$^+$ T cells are thought to be critical in the development of murine CH (Gocinski and Tigelaar, 1990; Bour *et al*, 1995; Xu *et al*, 1996). Hapten-specific CD8$^+$ T cell clones have been isolated from patients with ACD to urushiol (Kalish and Johnson, 1990), and, more recently, from patients with ACD to Ni (Vollmer *et al*, 1997; Werfel *et al*, 1997), suggesting their contribution to the expression of the human disease as well. Thus, purified CD8$^+$ T cells obtained from both allergic and nonallergic individuals were compared in terms of proliferation to $NiSO_4$. Results showed that CD8$^+$ T cells from all allergic individuals proliferated to Ni (stimulation index > 3), whereas nonallergic subjects failed to respond to the hapten (**Fig 1**, **Table I**), suggesting that the presence of Ni-specific CD8$^+$ T cells is required for the expression of the disease.

The frequency of Ni-specific CD4$^+$ T cells is comparable in allergic and nonallergic subjects, whereas the frequency of CD8$^+$ T cells is higher in patients The frequency of peripheral blood derived Ni-specific T cells was determined by limiting dilution analysis. In agreement with the proliferation data, the estimated frequency of Ni-specific CD4$^+$ T cells from allergic and nonallergic individuals did not show significant differences, ranging in both subject groups from 1:1700 to 1:6200. In contrast, the frequency of Ni-specific CD8$^+$ T cells ranged from 1:2600 to 1:31,000 in allergic patients, and was extremely low or undetectable in nonallergic subjects (**Fig 3**, **Table II**).

Ni-specific CD4$^+$ T cell clones derived from peripheral blood of allergic and nonallergic individuals are both CLA$^+$ but display a different pattern of IL-10 and IFN-γ release In the following experiments, a large number of Ni-specific T cell clones were prepared from peripheral blood of allergic patients and nonallergic donors and compared for the pattern of cytokine release and CLA expression. These clones were all CD4$^+$, CD8$^-$, TCRαβ$^+$, and TCRγδ$^-$ (not shown). Ni-specific CD4$^+$ T cell clones ($n = 50$) from nonallergic individuals displayed lower IFN-γ (mean 3.56 *vs* 4.72 ng per ml, $p = 0.03$), and increased IL-10 release (1.04 *vs* 0.4 ng per ml, $p = 0.006$) when compared with T cell clones ($n = 50$) prepared from allergic patients, whereas IL-4 production did not differ significantly (0.4 *vs* 0.2 ng per ml, nonallergic and allergic, respectively) (**Fig 4**). Comparing the resulting pattern of cytokine release, CD4$^+$ clones from nonallergic individuals showed a lower percentage of Th1 clones (58% *vs* 76%) and a similar number of Th0 clones (26% *vs* 24%), whereas only 4% of T cell clones from nonallergic subjects, and none from allergic subjects, showed a clear cut Th2 pattern. Interestingly enough, IL-10 was the predominant cytokine produced by 15 of 50 (30%) and by three of 50 (6%) of the Ni-specific CD4$^+$ T cell clones isolated from nonallergic and allergic individuals, respectively. These T cell clones exhibited high IL-10 production and very low or undetectable IFN-γ or IL-4 release, and could not fulfil the criteria for the Th1 or Th2 pattern. Representative IL-10^{high} producing T cell clones are shown in **Table III**. In the remaining T cell clones from both individual groups, IL-10 release did not segregate with IFN-γ or IL-4 production, and was detected in either Th2, Th0, as well as Th1 subsets, as previously reported (Del Prete *et al*, 1993). The same pattern of cytokine release was observed when T cell clones were stimulated with PMA and ionomycin or autologous APC and $NiSO_4$, although lower amounts of cytokines were released with the latter stimulation (**Table III**).

CLA is thought to be the major homing receptor for T cell targeting to the skin, being selectively expressed on the majority of skin-infiltrating T cells during ACD (Picker *et al*, 1991; Bos *et al*, 1993; Santamaria Babi *et al*, 1995). Therefore, differences in the CLA expression on Ni-specific T cells could be crucial in determining the clinical outcome of the immune response to the hapten. **Figure 5** shows that the large majority of Ni-specific CD4$^+$ T cell clones prepared from peripheral blood of both allergic and nonallergic donors were CLA$^+$, thus possessing the potential for recirculating through the skin.

Ni-specific CD8$^+$ T cell clones from allergic individuals express a Tc1 phenotype and are CLA$^+$ To better characterize the Ni-specific CD8$^+$ T cell responses, 20 Ni-specific CD8$^+$ T cell clones (CD8$^+$, CD4$^-$, TCRαβ$^+$, and TCRγδ$^-$) isolated from five allergic patients were examined for cytokine release and nine of them for CLA expression. Results indicated that the great majority of hapten-specific CD8$^+$ T cell clones were CLA$^+$ and produced IFN-γ but not IL-4, thus belonging to the Tc1 subset (**Fig 6**). Several attempts to isolate CD8$^+$ T cell clones from nonallergic subjects failed, in agreement with their very low frequency in these individuals.

DISCUSSION

In murine models, it has been demonstrated that the expression of CH to haptens is primarily regulated through the activities of distinct T

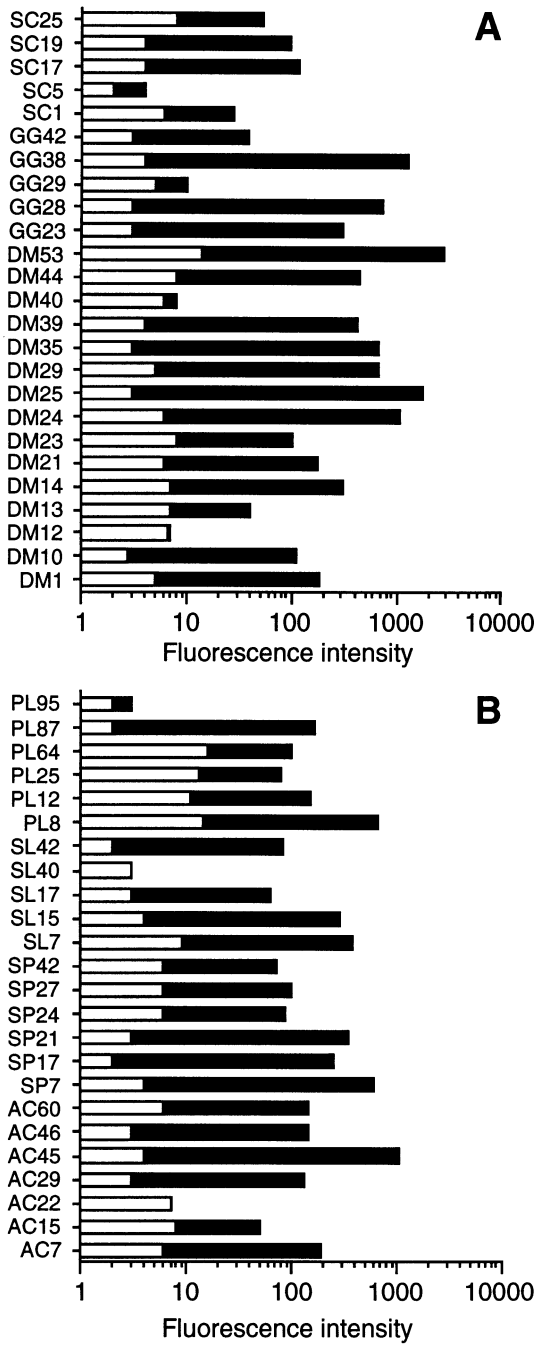

Figure 5. Ni-specific CD4$^+$ T cell clones from both allergic and nonallergic individuals express the CLA skin homing receptor. T cell clones were stained with rat IgM (*white bars*) or HECA-452 MoAb (*black bars*) followed by phycoerythrin-conjugated anti-rat IgM, and examined by flow cytometry. Results are shown as mean fluorescence intensity.

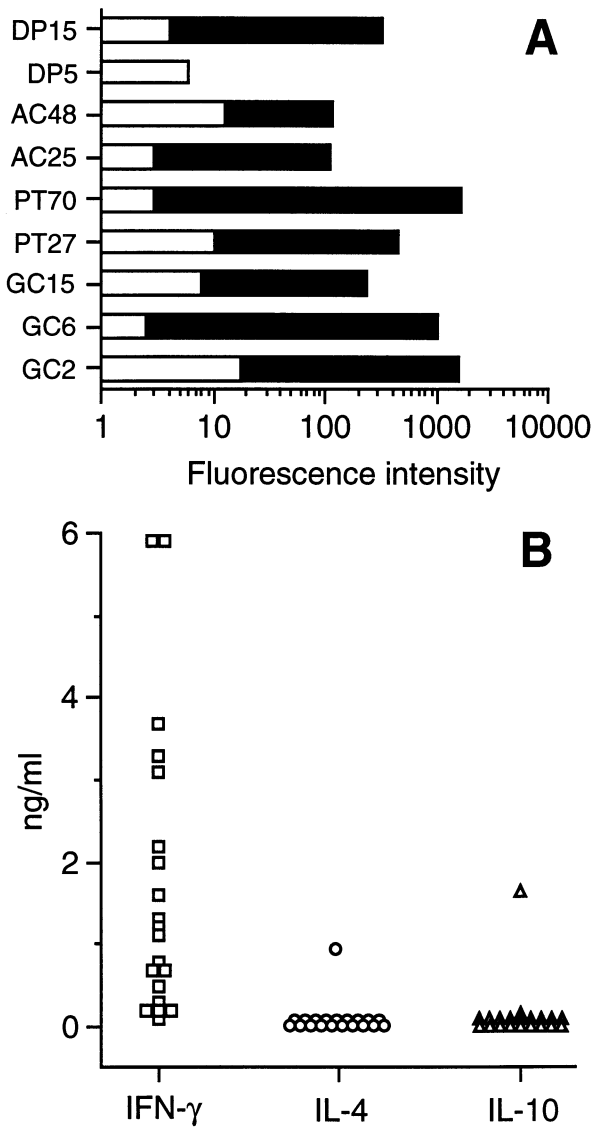

Figure 6. Ni-specific CD8$^+$ T cell clones from Ni-allergic patients are CLA$^+$ and belong to the Tc1 subset. (*A*) CLA expression was evaluated as described in **Fig 5**. (*B*) Cytokine release was measured by enzyme-linked immunosorbent assay in culture supernatants 48 h after stimulation with PMA and ionomycin.

cell subsets. Using CD4$^+$- or CD8$^+$-Ab-depleted mice (Gocinski and Tigelaar, 1990) and MHC class II or class I knockout mice (Bour *et al*, 1995), it has been shown that hapten-specific IFN-γ-producing CD8$^+$ T cells are crucial for the development of CH and exert a major effector function. In contrast, CD4$^+$ T cells appear to perform a predominant regulatory activity by releasing type 2 cytokines and IL-10 (Xu *et al*, 1996). So far no studies have compared the reciprocal role of CD4$^+$ and CD8$^+$ T cells in human disease, but indirect evidence suggests an important role for CD8$^+$ T cells in the cutaneous reactions to haptens. In particular, ACD to Ni has been documented in patients with CD4$^+$ idiopathic lymphopenia (Goodrich *et al*, 1993) and in HIV$^+$ patients with very low CD4$^+$ T cell counts (Viraben *et al*, 1994). Moreover, hapten-specific CD8$^+$ T cells have been isolated from peripheral blood of patients with ACD to urushiol, a strong sensitizer contained in poison ivy (Kalish and Johnson, 1990), and, more recently, from the peripheral blood and the skin of patients with ACD to Ni (Vollmer *et al*, 1997; Werfel *et al*, 1997).

Ni is a widely diffused metal and it is the most common cause of ACD (Peltonen, 1979); however, in spite of the large diffusion of the hapten and the extremely high possibility of contacting Ni-releasing materials in professional settings and in everyday life, most people are apparently protected from the development of ACD to Ni. To assess whether the expression of the disease could be related to the expansion of distinct T cell subsets and/or a different pattern of cytokine release, we compared the characteristics of Ni-specific T cell responses in Ni-allergic patients and in nonallergic, healthy individuals. Our results showed that Ni-specific CD8$^+$ T cell responses could be detected only in allergic patients. Ni-specific CD8$^+$ T cell clones prepared from these patients displayed a Tc1 pattern of cytokine release and were CLA$^+$, suggesting their active intervention in the expression of the dermatitis. The lack of functional CD8$^+$ T cell responses in nonallergic individuals could have different explanations. First, it might be the consequence of a reduced capacity to generate MHC class I-restricted hapten-epitopes. Because haptens recognition by CD8$^+$ T cells depends

on the interaction of the chemical with MHC class I-loaded peptides (Martin *et al*, 1992), a different repertoire of bound peptides could greatly influence the generation of appropriate numbers of antigenic stimuli. Ni-allergy, however, has not been definitely associated with particular HLA class II or class I alleles (Emtestam *et al*, 1993). Alternatively, the expansion of effector $CD8^+$ T cells might be prevented by regulatory mechanisms, including hapten-specific suppressor T cells. In contrast to $CD8^+$ T cell responses, both allergic and nonallergic individuals were shown to harbor in the peripheral blood Ni-specific $CD4^+$ T cells belonging to the $CD45RO^+$ memory subset. Both the frequency of Ni-specific $CD4^+$ T cells and the magnitude of the Ni-specific $CD4^+$ T cell responses to different $NiSO_4$ concentrations were similar in allergic and nonallergic subjects. These findings suggest that $CD4^+$ T cell priming to Ni occurs also in nonallergic subjects, and can be related to the continuous exposure to this ubiquitous metal. In keeping with our results, it has been shown that T cell responses to *Dermatophagoides farinae* and *Parietaria judaica* can be detected in both allergic and nonallergic subjects (Cavaillon *et al*, 1988; Sallusto *et al*, 1993). In such a case, the expression of the allergic disease has been related to the cytokine pattern of allergen-specific $CD4^+$ T cells that were shown to belong to the Th2 subset in atopic individuals and to the Th1 subset in healthy subjects (Wierenga *et al*, 1991).

Our results showed that the great majority of Ni-specific $CD4^+$ T cells form both allergic and nonallergic subjects were CLA^+ and thus they do not differ in their capacity to target to the skin. In contrast, a distinct pattern of cytokine release was observed. Ni-specific $CD4^+$ T cell clones from nonallergic subjects exhibited lower IFN-γ production and augmented IL-10 release compared with T cell clones from allergic patients. In contrast to our data, no differences in IFN-γ production were observed by Kapsenberg *et al* (1992) when Ni-specific T cell clones obtained from two allergic patients and from a single nonallergic donor were compared. In 15 of 50 (30%) Ni-specific $CD4^+$ T cell clones obtained from nonallergic individuals, and in three of 50 (6%) clones from patients with ACD to Ni, a unique pattern of cytokine release was detected, consisting of high IL-10 and low or undetectable IFN-γ, IL-4, and IL-2 release. These IL-10^{high} Ni-reactive $CD4^+$ T cell clones closely resembled a newly identified $CD4^+$ T cell subset, named T regulatory cells 1 (Tr1), generated *in vitro* in the presence of IL-10 from both human peripheral blood and murine spleen cells. Tr1 cells produce a high amount of IL-10 and low quantities of IL-2, IL-4, and IFN-γ, and display an immunosuppressive function both *in vitro* and *in vivo* (Groux *et al*, 1997). Work is currently in progress in our laboratory to better define the functional role of these Ni-specific IL-10^{high} T cell clones. In humans, IL-10 has been identified as a potent anti-inflammatory cytokine produced by both Th1 and Th2 $CD4^+$ T cell clones (Del Prete *et al*, 1993), which inhibits T cell proliferation of both subsets, and regulates the development of Th1 cells by blocking IL-12 driven Th1 responses (D'Andrea *et al*, 1993). The immunosuppressive effects of IL-10 mostly depend on the inhibitory activity on the APC functions of dendritic cells and macrophages (Fiorentino *et al*, 1991; Enk *et al*, 1993; Hsieh *et al*, 1993). Strong evidence exists demonstrating that IL-10 provides fundamental regulatory mechanisms for both the induction and the effector phases of murine CH. IL-10 mRNA is enhanced in the skin of allergic patients challenged with $NiSO_4$ (Szepietowski *et al*, 1997) as well as in the skin of sensitized mice challenged with the relevant hapten (Ferguson *et al*, 1994). Neutralization of IL-10 with local injections of specific MoAb prolongs murine CH beyond its natural course (Ferguson *et al*, 1994); IL-10 injection during the sensitization process or before challenge with the hapten markedly decreases CH responses, and induces hapten-specific unresponsiveness (Enk *et al*, 1994). Finally, CH to haptens is enhanced in mice with targeted disruption of the IL-10 gene (Berg *et al*, 1995). Whether Ni-specific IL-10 producing $CD4^+$ T cells could have a direct role in preventing clinical expression of ACD, e.g., by downregulating Ni-specific $CD8^+$ responses, needs to be further investigated. In keeping with this hypothesis, it has been shown that IL-10 inhibits murine as well human $CD8^+$ T cell proliferation and IFN-γ production (Bejarano *et al*, 1992; Macatonia *et al*, 1993). Also IL-4 has been described as an important regulatory cytokine in murine CH (Gautam *et al*, 1992; Asada *et al*, 1997), but other experiments using IL-4 knockout mice have produced contrasting data, with CH reactions either depressed (Weigmann *et al*, 1997) or similar (Berg *et al*, 1995) when compared with CH reactions in littermate controls. Finally, it has been shown that skin infiltrating Ni-specific $CD4^+$ T cells produced higher amount of IL-4 than Ni-specific T cell clones derived from peripheral blood (Werfel *et al*, 1997). Although we could not detect significant differences in IL-4 production between Ni-specific $CD4^+$ T cell clones from allergic and nonallergic individuals, we cannot exclude a contribution of IL-4 in the modulation of ACD.

In conclusion, our results indicate that the expansion of Ni-specific $CD8^+$ T cells is directly correlated to the expression of ACD to Ni, whereas Ni-specific $CD4^+$ T cells may have a predominant regulatory function, possibly via release of IL-10. Strategies to selectively target these distinct T cell populations should prove to be helpful in immunotherapeutic approaches to ACD.

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