Activation of Nickel-Specific CD4$^+$ T Lymphocytes in the Absence of Professional Antigen-Presenting Cells

Francesca Nasorri, Silvia Sebastiani, Valentina Mariani, Ornella De Pità,* Pietro Puddu,* Giampiero Girolomoni, and Andrea Cavani

Laboratory of Immunology and *Department of Immunodermatology, Istituto Dermopatico dell' Immacolata, IRCCS, Rome, Italy

Allergic contact dermatitis ensues from exaggerated T cell responses to haptens. Dendritic cells are required for the initiation of hapten sensitization, but they may not be necessary for disease expression. Here we investigated the antigen-presenting cell requirement of nickel-specific CD4$^+$ lymphocytes isolated from the blood of six allergic individuals. A significant proportion (42 out of 121; 35%) of the T cell clones proliferated *in vitro* to nickel also in the absence of professional antigen-presenting cells, suggesting a direct T–T hapten presentation. Antigen-presenting-cell-independent T cells showed a predominant T helper 1 phenotype. Nickel recognition by these T cells was major histocompatibility complex class II restricted, not influenced by CD28 triggering, independent from their state of activation, and did not require processing. The capacity of this T cell subset to be directly stimulated by nickel was not due to unique antigen-presenting properties, as both antigen-presenting-cell-dependent and antigen-presenting-cell-independent clones displayed comparable levels of HLA-DR, CD80, and CD86, and were equally capable of presenting nickel to antigen-presenting-cell-independent clones. In contrast, neither T cell types activated antigen-presenting-cell-dependent T lymphocytes. T–T presentation induced T cell receptor downregulation, CD25, CD80, CD86, and HLA-DR upregulation, and interferon-γ release, although to a lesser extent compared to those induced by dendritic cell–T presentation. Following T–T presentation, the clones did not undergo unresponsiveness and maintained the capacity to respond to dendritic cells pulsed with antigen. In aggregate, our data suggest that antigen-presenting-cell-independent T cell activation can effectively amplify hapten-specific immune responses. ***Key words: allergy/antigen presentation/nickel/skin/T lymphocytes. J Invest Dermatol 118:172–179, 2002***

Allergic contact dermatitis (ACD) is a common T-cell-dependent disease due to exaggerated immune responses to haptens applied onto the skin (Grabbe and Schwarz, 1998; Cavani *et al*, 2001). Skin dendritic cells (DC) are essential in the development of hapten-specific T cell responses. Upon hapten exposure, maturing Langerhans cells and dermal DC migrate towards regional lymph nodes (Kripke *et al*, 1990; Förster *et al*, 1999; Rennert *et al*, 2001), where the hapten–peptide complexes are presented to naive T cells in a major histocompatibility complex (MHC) restricted manner. In sensitized individuals, hapten challenge determines the rapid recruitment into the skin of specific memory T lymphocytes, which upon activation mediate the tissue damage (Traidl *et al*, 2000). The role of DC in the expression phase of ACD is less defined, and they may not be required. In particular, the expression of murine contact hypersensitivity is increased when the hapten is applied on skin depleted of Langerhans cells (Grabbe *et al*, 1995). Indeed, the need for professional antigen-presenting cells (APC) in the activation of memory/effector T lymphocytes is less stringent compared to that of naive T cells (Dubey *et al*, 1996; Iezzi *et al*, 1998; Lanzavecchia and Sallusto, 2000). Consistent with this notion, the effector phase of contact hypersensitivity is less dependent on T cell costimulation than the sensitization phase (Nuriya *et al*, 2001). Various skin cell types can serve as APC for memory/effector T cells during the efferent phase of skin immune responses, including endothelial cells, fibroblasts, and keratinocytes (Kündig *et al*, 1995; Ma and Pober, 1998; Traidl *et al*, 2000).

Following *in vivo* or *in vitro* activation, human T lymphocytes express MHC class II and costimulatory molecules, and they thus have the potential to act as APC. It has been previously shown that T–T presentation occurs for peptides or denatured proteins, resulting either in functional T cell activation (Gerrard *et al*, 1985; Hewitt *et al*, 1989; LaSalle *et al*, 1991; Barnaba *et al*, 1994) or T cell anergy (Lamb *et al*, 1983; Celis and Saibara, 1992; Sidhu *et al*, 1992; Marelli-Berg *et al*, 1997; Taams *et al*, 1999). In the case of hapten-specific T cell responses, the occurrence of T–T presentation has not been investigated yet. In this study, we show that a significant proportion of nickel-specific CD4$^+$ T cells undergo activation when exposed to the metal in the absence of professional APC, and may be importantly involved in the amplification of immune response to haptens.

MATERIALS AND METHODS

Patients Six non-atopic patients (median age, 28 y; three females and three males) with ACD to nickel were enrolled in the study. Patients

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Reprint requests to: Dr. Andrea Cavani, Laboratory of Immunology, Istituto Dermopatico dell'Immacolata, IRCCS. Via Monti di Creta 104, 00167 Roma, Italy. Email: cavani@idi.it

Abbreviations: ACD, allergic contact dermatitis; APC, antigen presenting cell; DC, dendritic cell; PFA, paraformaldheyde.

had a history of eczematous dermatitis after contacting metals and a positive patch test to 5% $NiSO_4$. They were not taking any medication for at least 15 d before blood donation. Peripheral blood samples were obtained after informed consent.

Antibodies The following mouse antihuman monoclonal antibodies (MoAb) were used: pure anti-HLA-DP (B7/21, $mIgG_1$), anti-HLA-DR (G46-6, $mIgG_{2a}$), and anti-CD28 (CD28.2, $mIgG_1$), and fluorescein isothiocyanate (FITC) conjugated anti-CD3 (Leu-4, $mIgG_1$), anti-CD4 (SK3, $mIgG_1$), anti-CD25 (2A3, $mIgG_1$), anti-CD14 (MΦP9, $mIgG_{2a}$), anti-HLA-DR (L243, $mIgG_{2a}$); anti-CD1a (HI149, IgG_1), anti-CD83 (Hb15a, IgG_{2a}), anti-CD80 (L307.4, $mIgG_1$), and anti-CD86 (2331, $mIgG_1$) were purchased from BD PharMingen (San Diego, CA). Pure anti-CD3 (UCHT1, IgG_1) MoAb was provided by Immunokontact (Boggio, Switzerland). Pure anti-HLA-DQ (SPVL3, $mIgG_{2a}$) and phycoerythrin-conjugated anti-T cell receptor (TCR) α/β (BMA031, IgG_{2b}) were obtained from Immunotech (Marseille, France). FITC-conjugated control mouse Ig was from BD PharMingen.

Nickel-specific T cell lines and clones Short-term nickel-specific $CD4^+$ T cell lines were obtained from the peripheral blood of nickel-allergic patients, as previously described (Cavani *et al*, 2000). Briefly, peripheral blood mononuclear cells (PBMC) were separated by centrifugation over Ficoll-Hypaque (Lymphoprep, Nycomed-Pharmacia, Oslo, Norway) and left to adhere (6×10^6 cells per ml) in Petri dishes for 2 h at 37°C with 5% CO_2 in RPMI 1640 complemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% nonessential amino acids, 0.05 mM 2-mercaptoethanol, 100 U per ml penicillin, and 100 μg per ml streptomycin (all from Invitrogen Italia, San Giuliano Milanese, Italy) (complete RPMI) and 5% autologous plasma. $CD4^+$ T lymphocytes were positively selected from the nonadherent fraction by incubation with immunomagnetic beads coated with specific MoAb (Dynabeads M450, Dynal, Oslo, Norway). Blood-derived T cell lines were cloned by limiting dilution (0.5 cells per well in 96-well U-bottomed microplates) in complete RPMI plus 10% fetal bovine serum (FBS; Hyclone, Logan, UT) in the presence of 3×10^5 allogeneic feeder cells, 1% phytohemagglutinin (Life Technologies), and 30 U per ml recombinant human interleukin-2 (IL-2) (kindly provided by Chiron Italia). T cell clones were grown with IL-2 and stimulated monthly with 1% phytohemagglutinin and feeder cells or with plate-coated anti-CD3 plus soluble anti-CD28 (both at 0.5 μg per ml). Antigen specificity of T cell clones was assessed using irradiated PBMC or autologous Epstein–Barr virus (EBV) transformed B cell lines with or without 10 μg per ml $NiSO_4$ (Sigma-Aldrich, Milan, Italy) in complete RPMI supplemented with 5% autologous plasma. After 32 h, cocultures were pulsed with 5 μCi per ml ^{3}[H]thymidine (Amersham, Little Chalfont, U.K.) for 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 (Packard Instruments).

Preparation of monocyte-derived DC and B cell lines Adherent PBMC were depleted of $CD2^+$, $CD19^+$, and $CD56^+$ cells by negative selection with MoAb-coated immunomagnetic beads (Dynal). The resulting population (>90% $CD14^+$ cells) was cultured in complete RPMI supplemented with 10% FBS, 200 ng per ml rhGM-CSF (Mielogen, Schering-Plough, Milan, Italy), and 200 U per ml rhIL-4 (R&D Systems, Minneapolis, MN) at 37°C with 5% CO_2 for 6–7 d. Lipopolysaccharide from *Escherichia coli* (Sigma) was added at 50 μg per ml for the last 24 h of culture. This procedure gave >95% pure $CD1a^+$ $CD14^-$ $CD83^+$ mature DC preparations. Autologous B cell lines were generated by incubating PBMC with supernatant from the EBV-producing marmoset line B95/8 (American Type Culture Collection, Rockville, MD) in the presence of 2 μg per ml cyclosporine A for 7–15 d.

Flow cytometry analysis Resting and activated $CD4^+$ T cell clones were washed in phosphate-buffered saline with 2% FBS and 0.01% NaN_3, and then stained with FITC- or phycoerythrin-conjugated MoAb. Staining with matched isotype control Ig was included. Cells were analyzed with a FACScan equipped with Cell Quest software (Becton Dickinson, Mountain View, CA). The kinetics of TCR downregulation was evaluated in the presence or the absence of professional APC and 10 μg per ml $NiSO_4$. Cells were cultured at 37°C with 5% CO_2, and at selected time points they were collected, fixed with 2% paraformaldheyde (PFA) for 15 min on ice, and stained with anti-TCR MoAb.

Antigen presentation assays DC (5000 cells per well) or EBV-transformed B cells (50,000 cells per well) were cocultured with autologous T cells (50,000 cells per well) in flat-bottomed 96-well plates with or without $NiSO_4$. Alternatively, T cells were incubated with or without $NiSO_4$ in the absence of DC or B cells. In selected experiments, DC, B cells or T cells were pulsed with 20 μg per ml $NiSO_4$ for 2 h at 37°C, washed with complete RPMI with 20% FBS, and then used to stimulate responding T lymphocytes. In blocking experiments, APC were incubated with 1 μg per ml of MoAb or control IgG for 30 min on ice followed by 30 min at 37°C before the addition of $NiSO_4$. To define the requirement of antigen processing, B cells were fixed with 2% PFA for 10 min at room temperature, resuspended in 0.15 M glycine for 1 min, and finally washed twice before use. Fixation was performed either before or after pulsing with $NiSO_4$. Incorporation of ^{3}[H]thymidine was measured 12–96 h after stimulation. Cultures were carried out in triplicate for each condition.

T cell cytokine release Supernatants from T cell clones (10^6 cells per ml) stimulated in 24-well plates with or without autologous DC or EBV-transformed B cells in the presence of 10 μg per ml $NiSO_4$ were collected after 12–96 h culture, filtered, and stored at –80°C. IL-4, IL-10, and interferon-γ (IFN-γ) content was measured by enzyme-linked immunosorbent assay (ELISA; Duoset Elisa System Kit, R&D Systems) following the manufacturer's instructions. Cultures were carried out in triplicate for each condition.

T cell anergy assay T cells were stimulated with or without DC in the presence or the absence of $NiSO_4$ (first stimulation). After 24 h of coculture, cells were collected, washed extensively, and plated in 24 wells with low dose IL-2 (10 U per ml) to maintain cell viability (Barnaba *et al*, 1994; Steinbrink *et al*, 1997). After 8 d, T cells (50,000 per well) were transferred in 96-well plates with DC (5000 per well) in the presence or not of $NiSO_4$ (second stimulation). T cell proliferation was evaluated after the first and second stimulation as described above.

RESULTS

A subset of nickel-specific T cells activates in the absence of professional APC Nickel-specific T cell clones were generated from the peripheral blood of six allergic subjects and characterized for their antigen specificity and cytokine expression profile. To determine their APC requirement, proliferation to nickel was assessed in the presence or the absence of autologous EBV-transformed B cells. To avoid contamination with other cell populations, T cell clones were expanded with plate-bound anti-CD3 plus soluble anti-CD28 MoAb in the absence of feeder cells. Moreover, carry-over of contaminating monocytes, DC, or B cells was excluded by fluorescence-activated cell sorter analysis, which demonstrated the absence of $CD14^+$, $CD19^+$, or $CD1a^+$ cells in the T cell cultures. All the clones proliferated *in vitro* in the presence of autologous B cells and 10 μg per ml $NiSO_4$. A significant number of these clones, however, could be activated by nickel in the absence of professional APC (APC-independent clones), suggesting a direct T–T hapten presentation. In contrast, the activation of the remaining clones strictly depended on the presence of professional APC (APC-dependent clones). **Table I** shows the results from one patient, whereas **Table II** summarizes the results on 121 clones obtained from the six subjects studied. The frequency of APC-independent clones ranged from 30% to 47% (average 35%). Both the APC-dependent and APC-independent clones showed a predominant T helper (Th) 1 cytokine profile, with high release of IFN-γ. Less than 10% of both clone types was Th0. On the other hand, among APC-independent clones a higher percentage of Th2 was detected (14.2% *vs* 7.6%). In contrast, T regulatory (Tr) lymphocytes, characterized by high IL-10 production and a potent immunosuppressive activity (Cavani *et al*, 2000; Sebastiani *et al*, 2001), were less represented among the APC-independent clones (11.9% *vs* 21.5%).

T–T nickel presentation is MHC class II restricted, uninfluenced by CD28 triggering, and occurs independently from the state of activation of the T cells To better understand the mechanisms of T–T nickel presentation, blocking experiments were performed with specific anti-MHC class II, -CD80, or -CD86 MoAb. T cell proliferation was strongly inhibited by anti-HLA-DR in all but two T clones, which were blocked instead by anti-HLA-DQ MoAb (**Fig 1*A*, *B***). In contrast, anti-CD80 or anti-CD86 pretreatment did not significantly affect T

Table I. A portion of nickel-specific T cell clones from the peripheral blood of a nickel-allergic patient proliferates in the absence of professional APC[a]

T cell clone	APC + T cells + $NiSO_4$	APC + T cells	T cells + $NiSO_4$	T cells	IFN-γ^b (ng per ml)	IL-4^b (ng per ml)	IL-10^b (ng per ml)
FN4.1.2	11.46	1.53	0.34	0.24	2.1	0.1	2.2
FN4.1.3	3.88	0.97	0.28	0.35	2.2	0	0
FN4.1.4	3.48	1.00	0.84	0.63	0.4	0	5.3
FN4.1.5	4.27	1.10	0.37	0.47	0.8	0	6.4
FN4.1.6	3.71	0.73	0.34	0.58	0.5	0	6.1
FN4.1.8	3.99	1.05	0.52	0.45	0.6	0	2.3
FN4.1.9	3.12	1.29	0.24	0.24	0	0.1	2.4
FN4.1.11	43.33	1.29	31.45	0.51	6.1	0	1.5
FN4.1.12	33.43	1.15	30.41	0.53	7.1	0.1	2.9
FN4.1.13	51.18	1.98	35.78	0.38	6.4	0.1	2.6
FN4.1.14	15.27	0.93	0.13	0.27	2.5	0.2	0.1
FN4.1.15	12.19	0.96	0.09	0.16	4.2	1.7	1
FN4.1.17	15.69	0.72	0.08	0.17	3.8	0.2	1.7
FN4.1.18	3.01	0.63	3.28	0.43	0.2	0.1	3.8
FN4.1.19	48.86	2.07	31.11	0.50	0	0	2.9
FN4.1.20	40.60	1.88	26.31	0.47	0.2	1.7	3
FN4.1.22	21.03	0.99	0.81	0.19	0.8	0	4.3
FN4.1.23	38.17	0.71	11.43	0.28	3	0	0.2
FN4.1.24	9.150	1.05	6.43	0.40	2.9	8.9	8.5
FN4.1.25	30.98	2.28	0.24	0.23	0.2	0	0.6
FN4.1.34	10.83	0.70	0.22	0.03	0.1	0.4	0
FN4.1.43	26.18	1.86	0.19	0.07	2.5	0	1.4
FN4.1.48	29.75	2.18	0.05	0.04	2.9	0.2	2.6
FN4.1.59	27.22	1.28	0.41	0.29	4.9	0	2.7
FN4.1.64	20.88	0.36	0.22	0.26	1	0	7.2
FN4.1.66	44.38	0.98	31.99	0.43	6.7	0	2.6
FN4.1.67	49.78	1.83	12.97	0.17	4.2	0	0.7

[a]T cells were stimulated in the presence or absence of EBV-transformed B cells and/or 10 μg per ml $NiSO_4$. ^{3}[H]thymidine uptake was measured after 48 h of culture. Results are given as mean cpm $\times 10^3$ of triplicate cultures.
[b]Cytokine release was evaluated by ELISA on supernatants of triplicate cultures stimulated 48 h with B cells plus nickel. SD were < 15% of mean values, and have been omitted for simplicity.

Table II. About one-third of nickel-specific T cell clones are APC independent, and show a predominant Th1 cytokine expression profile

Subject	T cell clone type	Number (%)	Th1 No (%)	Th0 No (%)	Th2 No (%)	Tr No (%)
AL	APC dependent	8 (53)	3 (37.5)	1 (12.5)	3 (37.5)	1 (12.5)
	APC independent	7 (47)	2 (28.5)	1 (14.2)	4 (57.1)	0
FN	APC dependent	17 (63)	7 (41.1)	1 (5.8)	1 (5.8)	8 (47)
	APC independent	10 (37)	6 (60)	1 (10)	1 (10)	2 (20)
LC	APC dependent	7 (70)	6 (85.7)	0	0	1 (14.3)
	APC independent	3 (30)	2 (66.6)	0	1 (33.3)	0
GC	APC dependent	20 (66)	18 (90)	2 (10)	0	0
	APC independent	10 (33)	9 (90)	1 (10)	0	0
KK	APC dependent	6 (66)	2 (33.3)	0 (16.6)	1	3 (50)
	APC independent	3 (33)	1 (33.3)	1 (33.3)	0	1 (33.3)
AC	APC dependent	21 (70)	14 (66.6)	2 (9.5)	1 (4.7)	4 (19)
	APC independent	9 (30)	7 (77.7)	0	0	2 (22.3)
Total	APC dependent	79 (65)	50 (63.3)	6 (7.6)	6 (7.6)	17 (21.5)
	APC independent	42 (35)	27 (64.2)	4 (9.5)	6 (14.2)	5 (11.9)

cell responses. It has been shown that the capacity of T cells to present nominal antigens is heavily influenced by their activation state, which affects the endocytic capacity and the neo-synthesis of MHC class II molecules (Barnaba *et al*, 1994). Thus, we compared resting (2 wk after anti-CD3/CD28 stimulation; CD25 negative) and activated (6 d after anti-CD3/CD28 stimulation, CD25 positive) APC-independent and APC-dependent clones in their ability to respond to nickel in the absence of professional APC. Both resting and activated APC-independent clones proliferated to nickel, although activated clones responded earlier and less potently than resting clones (**Fig 1*C***). In contrast, neither activated nor resting APC-dependent clones proliferated to nickel in the absence of professional APC.

APC-independent T cells show a distinct antigen recognition capacity We next investigated whether the unique behavior of APC-independent clones was due to distinct antigen-presenting or antigen recognition capacities. Resting, CD25 negative, APC-independent T cell clones showed a high expression of MHC class II molecules and variable (moderate to high) expression of the costimulatory molecules CD80 and CD86, which were strongly upregulated upon TCR triggering with anti-

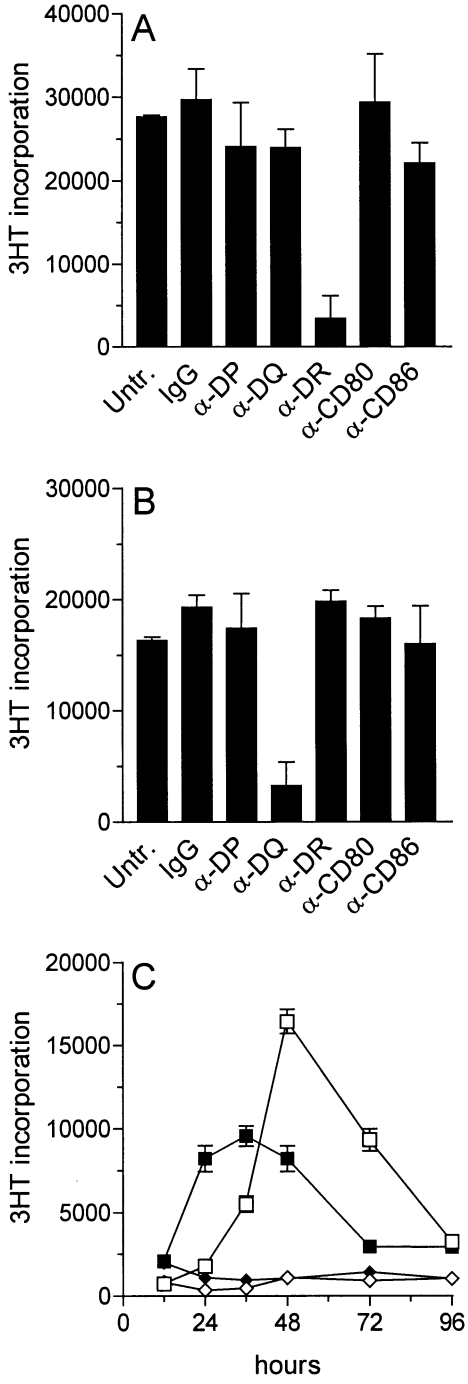

Figure 1. T–T nickel presentation is MHC class II restricted, uninfluenced by CD28 triggering, and occurs independently of the state of activation of T cells. The APC-independent T cell clones FN4.1.12 (*A*) and AC4.1.7 (*B*) were preincubated 30 min on ice with the indicated MoAb and then directly stimulated with 10 μg per ml $NiSO_4$. The figure shows the results obtained with two representative clones out of 65 examined. (*C*) APC-independent (clone AC4.1.7, *squares*) and APC-dependent (clone AC4.1.19, *diamonds*) clones either resting (2 wk after stimulation, *open symbols*) or activated (6 d after stimulation, *filled symbols*) were compared in their nickel-specific response in the absence of added APC. Results are expressed as mean cpm ± SD of triplicate cultures.

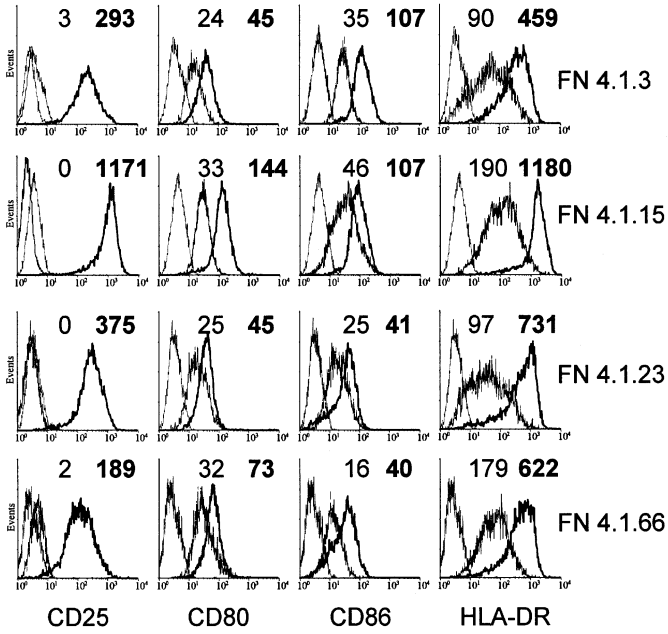

Figure 2. APC-dependent and APC-independent T cell clones express comparable levels of MHC class II and costimulatory molecules both in the resting state and upon activation. APC-dependent (FN4.1.3, FN4.1.15) and APC-independent (FN4.1.23, FN4.1.66) clones were stained with specific MoAb or control Ig (*dotted histograms*) both in the resting condition (*continuous thin histograms*) or 48 h after anti-CD3 activation (*continuous bold lines*), and examined by flow cytometry. Left and right numbers represent the net mean fluorescence intensity of resting and activated T cells, respectively. Results are representative of 55 T cell clones examined.

CD3 MoAb (**Fig 2**). The levels of MHC class II and costimulatory molecules did not differ significantly from those present on APC-dependent clones, both in resting and activated state. To further sustain the hypothesis that T–T nickel presentation was not associated with increased antigen-presenting functions of the T cells, cross-experiments were performed using nickel-pulsed APC-independent or APC-dependent T cells as APC and responding T cells. Irradiated APC-dependent or APC-independent T cell clones pulsed with $NiSO_4$ were equally capable of stimulating APC-independent, but not APC-dependent T cells (**Fig 3**). These findings demonstrated that T–T nickel activation was not due to a particular antigen-presenting function of APC-independent clones, but rather was correlated with a distinct antigen recognition capacity.

As peptides but not complex proteins can be presented by T cells (Gerrard *et al*, 1985; Hewitt *et al*, 1989; LaSalle *et al*, 1991) in the next set of experiments we evaluated whether antigen processing was required for nickel-specific T cell activation. To address this issue, B cells were fixed with 2% PFA either after (pulsed/fixed APC) or prior to (fixed/pulsed APC) pulsing for 2 h with 20 μg per ml $NiSO_4$, and finally were used as stimulators for T cell clones. All the 30 APC-independent T cell clones responded to the antigen when presented by pulsed/fixed APC or fixed/pulsed APC, indicating that processing was not necessary for proper T cell recognition. In contrast, a more variable behavior was observed among APC-dependent T cells, with some (17 of 30) responding to both pulsed/fixed and fixed/pulsed APC, and others (13 of 30) responding only to pulsed/fixed APC (**Fig 4**). These results indicated that the APC-independent T cell clones recognized only nickel epitopes not requiring processing. Not all the T cell clones that recognized a processing-independent nickel epitope could be activated through a direct T–T presentation of the metal, however.

T–T hapten presentation results in IFN-γ release, TCR downregulation, CD25, CD80, and CD86 upregulation, and is not followed by T cell anergy The fate of T cells after T–T antigen presentation has been previously investigated with

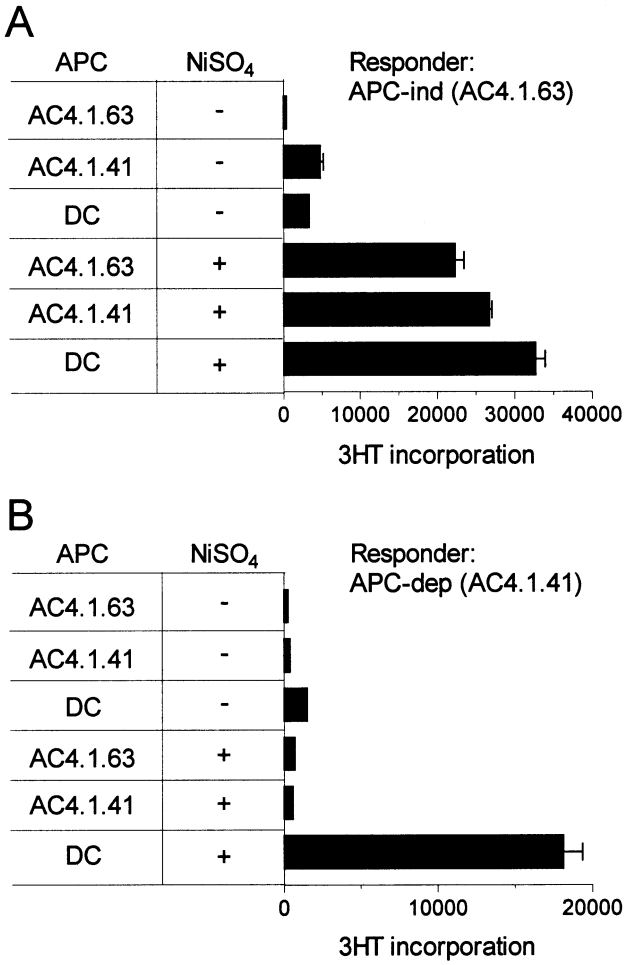

Figure 3. APC-independent T cell clones proliferate to both nickel-pulsed APC-independent and APC-dependent T cells. APC-independent (AC4.1.63) or APC-dependent (AC4.1.41) T cells were used either as stimulator or responding cells in cross-experiments. Mature DC were used as control APC. Stimulator T cells were pulsed 2 h with 20 μg per ml $NiSO_4$, washed, and irradiated before use. Data are expressed as mean cpm ± SD of triplicate cultures. Results are representative of experiments performed with six T cell clones of both groups.

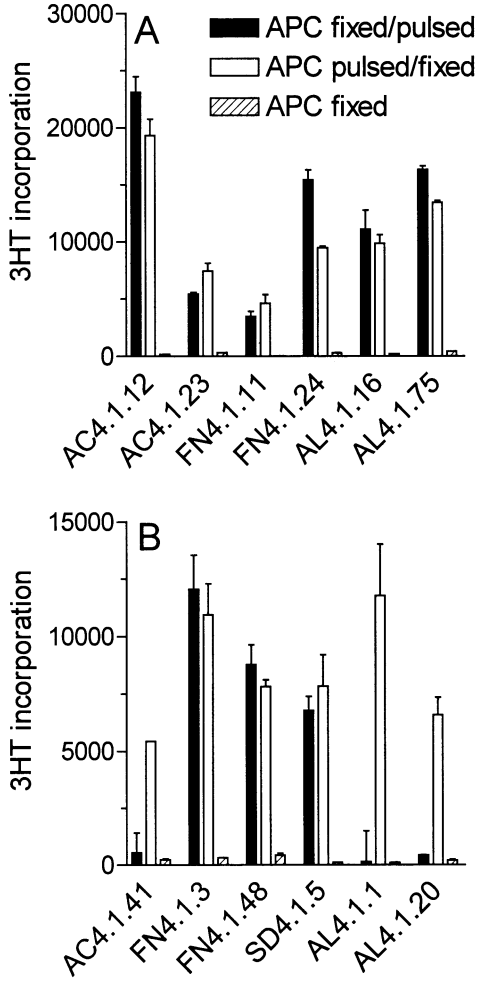

Figure 4. T–T nickel presentation does not require processing. EBV-transformed B cells were either pulsed for 2 h with 20 μg per ml, washed, and then fixed in 2% PFA (pulsed/fixed APC) or fixed before the pulsing procedure (fixed/pulsed APC). B cells were irradiated and used as stimulators in antigen presentation assays with either APC-independent (*A*) or APC-dependent (*B*) T cell clones. Data are expressed as mean cpm ± SD of triplicate cultures. Results of six T cell clones of each group out of 30 examined are shown.

controversial results (Lamb *et al*, 1983; Celis *et al*, 1992; Sidhu *et al*, 1992; Barnaba *et al*, 1994; Marelli-Berg *et al*, 1997; Taams *et al*, 1999). T–T nickel presentation was followed by cell proliferation and IFN-γ release comparable to those induced by B-cell–T presentation. DC–T presentation resulted in significantly higher cell proliferation and IFN-γ secretion, and required a lower nickel concentration for optimal T cell activation (**Fig 5*A*, *B***). These differences were not the consequence of a diverse kinetics of the T cell response as in both T–T and DC–T nickel presentation maximal response was achieved after 48 h of coculture (data not shown). Antigen recognition determines a rapid internalization of the CD3–TCR complex, which is proportional to the degree of TCR stimulation induced by peptide–MHC complexes (Valitutti *et al*, 1995). T–T nickel presentation induced a rapid TCR–CD3 downregulation in APC-independent, but not APC-dependent, T cells, reaching a plateau at 6 h after addition of nickel (**Fig 5*C***). TCR downregulation, as well as upregulation of CD25, HLA-DR, CD80, and CD86 (**Fig 5*D***), induced by T–T presentation was less prominent, however, than those determined by DC–T presentation, and, in the case of TCR downregulation, also slightly delayed in time. All these findings confirmed that T–T nickel presentation led to a functional T cell activation, although less conspicuous than that induced by professional APC.

Finally we tested whether T–T presentation in APC-independent cells was followed by anergy. After the first stimulation, T cell clones were maintained in culture with minimal IL-2 concentration (10 U per ml) for 8 d, and then further assayed in their capacity to respond to the hapten in the presence or absence of DC. Results indicated that T cells previously activated through either a T–T or a DC–T nickel presentation maintained a comparable attitude to proliferation in response to DC and nickel (**Fig 6**), suggesting that T–T presentation did not result in T cell anergy.

DISCUSSION

Recently activated T lymphocytes express MHC class II and functional B7 molecules both *in vivo* and *in vitro* (Azuma *et al*, 1993; Barnaba *et al*, 1994; Hollsberg *et al*, 1997; Jeannin *et al*, 1999), and may thus potentially act as APC for memory/effector T cells engaged in peripheral immune responses. Our work provides evidence that a significant number of nickel-specific T cells isolated from allergic subjects can be directly activated by the metal in the absence of professional APC. T–T nickel presentation was MHC

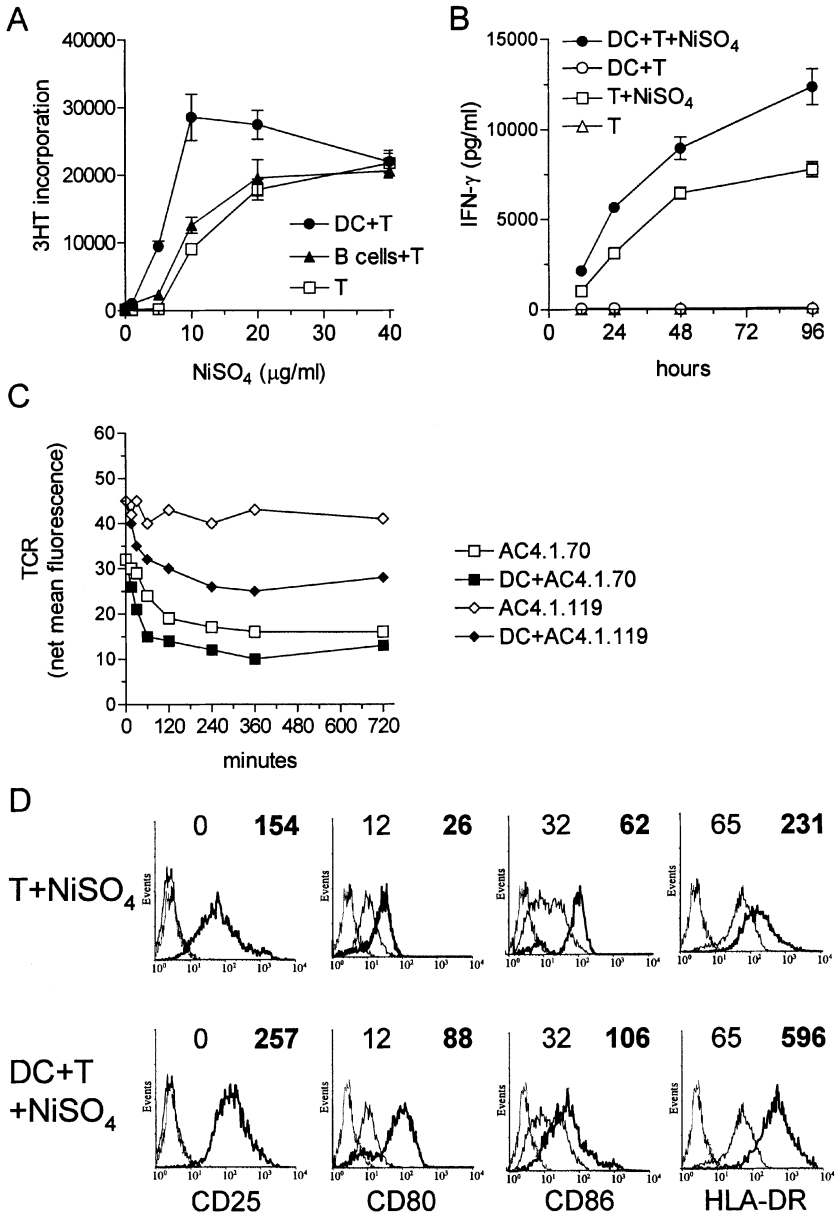

Figure 5. T–T presentation is less effective in promoting proliferation, IFN-γ release, TCR downregulation, and upregulation of CD25, CD80, CD86, and MHC class II compared to DC-T presentation. (*A*) DC or B cells were incubated with grading doses of $NiSO_4$, irradiated, and used to stimulate the APC-independent T cell clone AC4.1.70. Alternatively, $NiSO_4$ was added directly to AC4.1.70 culture. Proliferation was evaluated by adding ^{3}[H]thymidine for the last 12 h of coculture. Data are expressed as mean cpm ± SD of triplicate cultures. (*B*) Supernatants were collected at 12–96 h of DC–T and T–T cocultures and evaluated for IFN-γ content by ELISA. Data are expressed as mean ± SD pg per ml of triplicate cultures. (*C*) To evaluate TCR downregulation in responding T cells, DC–T cell cocultures or T cells alone were incubated with 10 μg per ml $NiSO_4$ at 37°C and at serial time points cells were fixed with PFA 2%, stained with anti-TCR MoAb, and examined by flow cytometry. Results are given as the net mean fluorescence intensity. (*D*) The APC-independent T cell clone AC4.1.2 was activated with 10 μg per ml of $NiSO_4$ with or without autologous DC. At day 0 or after 48 h, T cells were collected, stained with the specific MoAb, and evaluated by flow cytometry gating CD3 positive cells. Data are given as mean fluorescence intensity at day 0 (*continuous histograms* and *normal characters*) and at 48 h (*bold histograms* and *characters*) subtracted from the isotype control (*dotted histograms*).

class II restricted, independent from CD28 triggering, and was followed by CD25, CD80, CD86 upregulation, cytokine release, and cell proliferation. Indeed, a common immunohistochemical finding of ACD is the presence of a relevant number of HLA-DR$^+$ T lymphocytes (Ralfkiaer and Wantzin, 1984). HLA-DR expressed by T lymphocytes can derive from neo-synthesis or absorption of cell surface MHC–peptide complexes from the APC membrane during APC–T cell interactions (Arnold *et al*, 1997; Huang *et al*, 1999; Hwang *et al*, 2000). It has been previously shown that T cells may present peptides and denatured protein antigens. Resting T lymphocytes have a limited endocytic capacity, however, and are relatively inefficient in processing soluble proteins in the absence of specific membrane receptors (Hewitt and Feldman, 1989; LaSalle *et al*, 1991; Franco *et al*, 1992). T cell activation encompasses both augmented liquid phase pynocytosis and MHC class II synthesis, allowing T lymphocytes to internalize and process complex proteins (Barnaba *et al*, 1994). In the case of nominal antigens, differences among T lymphocytes concerning their capacity to respond to a T–T antigen presentation have not been described. Our data indicate that only a subset of nickel-specific T lymphocytes respond to the hapten in the absence of professional APC. This unique attribute of APC-independent T cells to be activated following T–T presentation does not depend upon an enhanced antigen-presenting capability. In fact, APC-independent and APC-dependent T cell clones expressed comparable levels of MHC class II molecules both in resting and activated states. Additionally, APC-dependent and APC-independent clones pulsed with nickel were equally efficient in promoting activation of APC-independent T cells, but they were both incapable of stimulating APC-dependent T cell clones in cross-experiments. The degree of T cell activation did not significantly affect T–T nickel presentation in either APC-independent or APC-dependent clones, and the latter were unresponsive to nickel at any state of activation when stimulated in the absence of professional APC. These findings indicate that the epitope recognized by APC-independent T cells may be generated and exposed on the surface of both T cell types, whereas APC-dependent T cells recognize one or more epitopes generated only by professional APC. In addition, APC-independent T cells stimulated by professional APC and nickel showed a higher proliferation compared to APC-dependent clones. In these conditions, the selective block of MHC class II on APC-independent T cells prior to culture with professional APC

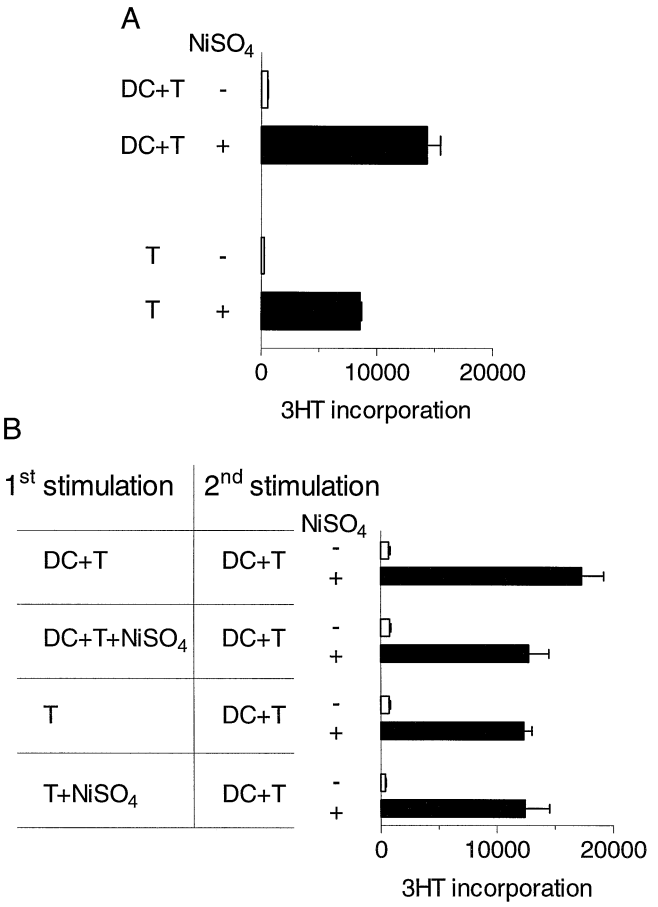

Figure 6. APC-independent T cell clones maintain the capacity to respond to nickel after T–T presentation. (*A*) The APC-independent T cell clone FN4.1.13 was incubated or not with DC in the presence or absence of 10 μg per ml of $NiSO_4$, and ^{3}[H]thymidine incorporation was measured after 48 h (first stimulation). (*B*) T cells from parallel wells were washed and cultured with low dose IL-2 (10 U per ml) for 8 d. Thereafter, T cells were activated with DC ± 10 μg per ml of $NiSO_4$ (second stimulation) for 48 h, and ^{3}[H]thymidine uptake was determined. Data are expressed as mean cpm ± SD of triplicate cultures.

reduced T cell proliferation by 30%–35% (data not shown). This finding suggests that APC-independent T cells can recognize hapten–class II complexes on both T cells themselves and professional APC.

Haptens are visible to the immune system as hapten–peptide complexes bound to MHC molecules (Martin *et al*, 1993; Cavani *et al*, 1995). Metals behave as haptens in many respects, and nickel can directly interact with peptides in the groove of MHC class II molecules forming square-planar structures frequently involving histidine residues (Sinigaglia, 1994). Both nickel and urushiol interaction with APC give rise to processing-independent and processing-dependent epitopes, the latter deriving from internalization of hapten molecules or hapten–protein complexes (Kalish *etal*, 1994; Moulon *et al*, 1995). Moreover, the existence of distinct populations of nickel-specific T cells differing in their APC requirements has been suggested by previous work. In particular, some T cell clones have been reported to be activated by both DC and B cells, and others exclusively activated by skin-derived DC (Kapsenberg *et al*, 1987). Finally, it has been shown that a significant portion of nickel-specific T cell clones proliferated in the continuous presence of the metal but were unresponsive when stimulated by nickel-pulsed APC (Moulon *et al*, 1995). To better understand the nature of the epitopes recognized by APC-independent T cell clones, we performed experiments with PFA-fixed APC. The results demonstrate that the epitopes recognized by APC-independent T cell clones do not require processing. Thus, in T–T presentation the epitopes were generated by a direct interaction of the hapten with MHC class II molecules expressed on T cells, in agreement with the data previously described for nominal antigens. Nevertheless, not all the processing-independent clones belonged to the APC-independent subtype, suggesting that independence from APC processing was necessary but not sufficient for T–T presentation. It is reasonable to hypothesize that fewer epitopes are generated by T cells upon interaction with nickel, whereas professional APC may display a broader spectrum of nickel epitopes.

The role of T–T hapten presentation in the expression of peripheral immune responses may vary depending on the outcome of the T cell activation. It has been recently reported that T cell self-presentation determines a more sustained and prolonged TCR downregulation compared to that induced by APC–T cell presentation, fails to upregulate OX40 and B7, and leads ultimately to a state of T cell unresponsiveness (Taams *et al*, 1999). Thus, T–T presentation has been proposed as an efficient regulatory mechanism involved in the maintenance of peripheral T cell tolerance (Lamb *et al*, 1983; Celis *et al*, 1992; Sidhu *et al*, 1992; Marelli-Berg *et al*, 1997; Taams *et al*, 1998). Other reports did not reveal any difference in the quality of the T cell activation after T–T antigen presentation, however, and did not show induction of anergy (Barnaba *et al*, 1994). T–T nickel presentation in our APC-independent T cell subset induced TCR downregulation, CD80, CD86, HLA-DR, and CD25 upregulation, as well as IFN-γ release and cell proliferation, indicating a full-competent activation of the T cells, although these modifications were quantitatively more limited than those induced by mature DC. Importantly, following T–T nickel activation, APC-independent T cells maintained their capacity to respond to the metal both in a T–T- and in an APC–T-dependent manner, indicating that although T–T nickel presentation induced a lower grade of T cell activation, it was not followed by induction of T cell anergy. Nickel-specific Th cells are very heterogeneous with respect to their cytokine expression profile, with Th1 playing a predominant effector role and Tr cells exerting primarily a regulatory activity by inhibiting DC maturation and functions (Cavani *et al*, 2001). APC-independent clones secreted large amounts of IFN-γ and were predominantly Th1, similar to the APC-dependent clones. On the other hand, a higher and lower percentage of Th2 and Tr clones, respectively, was detected among APC-independent T cells. Although this result is potentially very interesting, it deserves further confirmation on a larger series of clones. The fact that APC-dependent T cells are less polarized towards the Tr cell subset may suggest that they are mainly involved in effector/inflammatory activities.

In conclusion, our data suggest that, in the efferent phase of ACD, T lymphocytes can simultaneously act as effector cells and APC. In particular, the subset of APC-independent lymphocytes could have a role in the initiation and rapid amplification of the skin allergic reaction, representing a ready-to-go subset of nickel-reactive T cells not requiring professional APC for complete functional activation.

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