Immune-Mediated Destruction of Melanocytes in Halo Nevi Is Associated with the Local Expansion of a Limited Number of T Cell Clones

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The β-chain repertoire of the T cells that infiltrate spontaneously regressing nevi (the halo nevus phenomenon) was studied. In addition to the infiltration of the halo nevi by cutaneous lymphocyte-associated Ag-positive lymphocytes, oligoclonal expansion of T cells was observed in all halo nevi of all patients. T cells using the same TCR β-chain were observed in distinct halo nevi of the same patient but not in his peripheral blood, demonstrating a local expansion of common clones that are most likely activated by the Ag(s) shared by independent halo nevi of the same patient.

Melanomas are usually infiltrated by lymphocytes. Despite a well-documented melanoma-specific T cell response, most malignant melanocytic tumors escape immune control. However, spontaneous, immune-mediated regression of melanomas occasionally occurs. The understanding of the mechanisms underlying this phenomenon is of primary importance for the immunotherapy of melanomas. In this respect, the regression of benign nevi, known as the halo nevus phenomenon, offers a convenient in vivo model. In the halo nevus phenomenon, an otherwise normal, benign nevus regresses due to a combination of immunological factors. The mechanisms responsible for the halo nevus phenomenon are not known with certainty. CD4+ and CD8+ T cells infiltrate the halo nevi (4), and T cells have been implicated in the destruction of the nevus melanocytes primarily because the peripheral T cells of a patient with a halo nevus are able to lyse the melanocytes of a normal nevus (5) and because activated CD8+ T cells are predominant in the halo nevi (6). However, the molecular targets of the cytotoxic T cells, the nature of the T cells, or the way in which the T cells are activated remain unknown.

The identification of the T cells undergoing local expansions (i.e., the T cells activated locally by nevi Ags) is thus of primary importance in the analysis of the halo nevus phenomenon. A previous analysis of the TCR of lymphocytes infiltrating the halo nevi revealed a local proliferation of T cell clones (7). The present work is aimed at approaching the in vivo diversity of the β-chains of the TCR of the T cells present in nevi undergoing immunemediated regression, which avoids the biases introduced by the in vitro cloning of T cells or the cloning of PCR products. For this purpose, we have used a PCR-based technology to describe the Vβ, Jβ, and complementarity-determining region 3 (CDR3) length usages of the T cells that infiltrate 13 halo nevi removed from eight patients. Two distinct populations of T cells infiltrating the halo nevi can be distinguished: 1) nonactivated or polyclonally activated T cells and 2) clonally expanded T cells. The β-chains of the TCR of the T cells expanded in a halo nevus of a patient were also found to be associated with the T cells expanded in other halo nevi of the same patient but were not found in the blood. Finally, the amino acid sequences of the Ag recognition regions of the TCR β-chains of the expanded T cells shared common amino acid patterns, suggesting that they may recognize the same or closely related antigenic epitope(s).

Materials and Methods

Patients

Eight Caucasian patients presenting 1–30 halo nevi were prospectively included in the present study. The patients showed no evidence of any overt disease. After approval of the research program by the local ethics committee, written informed consent was obtained from each patient. The diagnosis of halo nevus was confirmed by a histological analysis of skin biopsies. A total of 13 halo nevi (diameters ranging from 0.5 to 1 cm) were collected. Peripheral blood was taken from each patient, and PBLs were prepared by the Ficoll-Hypaque procedure. HLA class I and class II typing were performed. The patients showed no evidence of any overt disease.

Immunohistochemical staining

A fragment of each biopsied halo nevus was frozen at −80°C immediately following surgery. Frozen sections were analyzed for the expression of CD3, CD4, CD8, S100, and HLA class II using mAbs purchased from Dako (Copenhagen, Denmark), Fas and Fas ligand mAbs (purchased from Immunotech, Marseille, France), perforin and granzyme B mAbs (purchased from Pharmingen, San Diego, CA), and HLA class I mAb (purchased from Sigma, St. Louis, MO) according to conventional procedures. Apoptotic cells were revealed using the terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate nick-end labeling procedure (Boehringer Mannheim, Mannheim, Germany).

RNA extraction and cDNA preparation

Total RNA was extracted as described previously (8) from 10⁷ blood T cells or from the entire nevi, except for the fragment used for histology.

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² Abbreviations used in this paper: CDR, complementarity-determining region; CLA, cutaneous lymphocyte-associated Ag.
mRNA was reverse-transcribed from PBLs and halo nevi using a technique described elsewhere (9).

**TCR β-chain analysis**

The “immunoscope” technique has been extensively described elsewhere (10). Briefly, standardized amounts of cDNA (i.e., the product of the reverse transcription of 10 μg of total RNA) were PCR amplified using each of the 24 Vβ-specific probes and a common Cβ-specific probe. All primers used in the present study have been described elsewhere (11). Each Vβ-Cβ PCR product was analyzed by electrophoresis in an agarose gel. For the analysis of CDR3 length diversity, PCR-amplified products were subjected to five cycles of primer extension using an internal, fluorescent, Cβ-specific probe (11). The labeled material was loaded on a sequencing gel and analyzed using an automatic sequencer (Applied Biosystems, Foster City, CA) equipped with a computer program (immunoscope), which enables the determination of the intensity of fluorescence of each band as well as its actual size. The results are depicted as peaks with surfaces that are proportional to the amount of material; the location of the peaks was determined by the length of the CDR3 region. Vβ-Cβ PCR products were also subjected to five cycles of primer extension using each of the 13 fluorescent JB-specific probes (1.1–2.7). The resulting products were analyzed as described above. The size distribution of Vβ-Cβ and Vβ-Jβ is Gaussian in the case of nonactivated or polyclonally activated lymphocytes, whereas Ag-dependent proliferating T cells generated a non-Gaussian distribution, with expanded peaks corresponding to cells using a definite CDR3 length within a Vβ-Jβ combination (12).

**Detection of melanoma-associated Ag mRNAs**

The cDNA was PCR amplified with primers specific for the MAGE, BAGE, GAGE, and RAGE families, respectively, according to conventional procedures (13–16). PCR products were analyzed by electrophoresis on an agarose gel. The mRNA coding for tyrosinase was used as an internal positive control.

**Direct DNA sequencing**

After separation of the PCR products by electrophoresis in a 2% agarose gel and electro-elution of the DNA fragments, direct sequencing of PCR products was conducted using the United States Biochemical-Amersham kit (Arlington Heights, IL).

**Results**

**Histology of the nevi under analysis**

A fragment of each halo nevus studied was sectioned and analyzed. None of the nevi showed signs of malignancy; however, all showed histological signs of regression that are associated with infiltration by lymphocytes, occasional macrophages, and neutrophils. Consequently, the regressing nevus retained for RT-PCR analysis fit the definition of halo nevus (3). Further immunohistochemical analysis confirmed earlier reports (4, 6), indicating that the infiltrate was composed of CD4+ T cells and CD8+ T cells. The CD4+/CD8+ ratio was in the 0.5–3 range depending upon the biopsy. CD8+ T cells were usually predominant. The majority of the T cells were granzyme B+, perforin+, and Fas ligand+ and were thus likely endowed with cytotoxic activity. Nearly all of the T cells present in the nevi were CLA+, a cell surface protein associated with skin homing (17). Melanocytes strongly expressed MHC class I but not class II molecules and underwent apoptosis as shown using the terminal deoxynucleotidyl transferase-mediated uridine 5’-triphosphate nick-end labeling technique. Normal nevi did not show cell infiltration or apoptosis of melanocytes.

**TCR repertoire of the αβ T cells that infiltrate the halo nevi is biased**

Few T cells infiltrate the halo nevi (105 T cells/halo nevus on average), and they cannot be recovered easily from halo nevi, preventing direct FACS analysis. Thus, Vβ usage was studied by RT-PCR. A total of 13 halo nevi were surgically removed from eight patients (A, K, C, G, L, B, P, and Y). Total RNA was immediately extracted from the samples and reverse transcribed. The resulting cDNAs were first amplified by PCR using each of the 24 Vβ-specific probes and a common Cβ-specific probe. PCR products were analyzed first by agarose gel electrophoresis. No Vβ-Cβ sequences could be amplified from normal nevi or from nevi C1 and L. In the other nevi, the different Vβ-Cβ sequences that could be amplified varied with the patients. They were Vβ 1, 2, 3, 4, 5, 6B, 7, 8, 10, 14, 15, 16, 17, 18, 21, and 23 in nevus A1; Vβ 1, 2, 3, 4, 5, 6A, 6B, 7, 8, 9, 14, 15, 16, 17, 18, and 19 in nevus K1; the same Vβ in nevus K2 as in nevus K1, except Vβ8; the same Vβ in nevus K3 as in nevus K1, plus Vβ 11 and 21; Vβ8 in nevus C2; Vβ7 in nevus C3; and Vβ 6A and 7 in nevus G. Vβ7 was used in nevus B. All Vβ except for Vβ12 and 13A were used in nevus P; Vβ 3, 4, 6A, 7, 8, 14, and 18 were used in nevus Y. The absence of some Vβ in the halo nevi cannot be attributed to sampling problems, because a complete Vβ-Cβ analysis can be conducted on as few as 1000 T cells (18). This absence is instead due to the extent of regression of the nevi studied. As a control of primer efficiencies and of the presence/absence of β-chains, all 24 Vβ-Cβ families could be amplified out of the control PBMCS of each patient.

The Vβ-Cβ PCR products prepared from 10 halo nevi of five patients (halo nevi A1, A2, K1, K2, K3, C1, C2, C3, G, and L) were further analyzed for the distribution of CDR3 size by running primer extensions on the Vβ-Cβ PCR products, using a common, fluorescently labeled, internal Cβ-specific primer. The labeled primer extension products were separated on an automated sequencer. The results are depicted by the pictograms of Fig. 1. The Vβ-Cβ patterns were found to be Gaussian in the blood of all patients. In contrast, the Vβ-Cβ PCR products prepared from halo
nevi displayed diverse patterns. Most Vβ-Cβ combinations yielded a Gaussian signal that was indicative of an absence of specific stimulation of the corresponding T cells; other combinations (Vβ15 in nevi A1, A2, and K1; Vβ1 in nevi K1, K2, and K3; Vβ6A in nevus G; Vβ6B in nevus K3; Vβ7 in nevi K1, K2, and K3; Vβ17 in nevi K1, K2, and K3; and Vβ21 in nevus K3) yielded isolated peaks with a unique CDR3 length, revealing the presence of Ag-driven T cell proliferation. Thus, the immune response to halo nevi is complex, and a limited number of T cell clones is expanded, presumably due to the recognition of specific Ag(s).

Expanded T cells using the same TCR β-chain are observed in several halo nevi removed from the same patient

A frequent feature of the halo nevus phenomenon is the simultaneous regression of several nevi in the same patient. To determine whether the same T cell repertoire is observed in distinct nevi of the same patient, we have determined the Vβ-(CDR3)-Jβ repertoire of the T cells present in several halo nevi by running primer extensions on the Vβ-Cβ PCR products that yielded single peaks in the immunoscope analysis. The procedure was conducted using each of the 13 Jβ-specific, fluorescently labeled primers and was followed by an analysis of the products on a sequence gel and the subsequent determination of the nucleotide sequence of the PCR products.

Thus, we have analyzed the Vβ-CDR3-Jβ repertoire of the T cells infiltrating the two halo nevi removed from patient A, the three halo nevi removed from patient K, and the three halo nevi removed from patient C. The halo nevi of patients A and K presented a dense lymphocyte infiltrate, whereas the infiltrate in patient C had a very poor lymphocyte count.

In patient A, a single peak was detected in the Vβ15-Cβ PCR products of the two halo nevi. Upon further analysis of the Vβ15-Cβ PCR products using the 13 Jβ-specific probes, Jβ2.3 was found to be predominantly used in the two halo nevi studied; it was associated with a 10-aa long CDR3 region. In contrast, the Vβ15-Jβ2.3 repertoire of the peripheral blood T cells of patient A showed a Gaussian pattern (Fig. 2). The nucleotide sequence of the Vβ15-Jβ2.3 PCR products obtained from the two halo nevi of patient A was determined by direct sequencing (Fig. 3). The two nucleotide sequences were found to be identical. Thus, T cells sharing the same β-chain have undergone expansion in two physically distinct halo nevi, a finding that is strongly suggestive of a proliferative response to the same antigenic peptide(s) presented by the same restriction element(s). A probe specific for the Vβ15-Jβ2.3 CDR3 region of the β-chain of the TCR characteristic of the T cell population expanded in patient A (clonotypic primer) was designed and used to search for T cells sharing the same TCR β-chain in the PBLs of patient A and also to determine the relative frequency of these T cells in the halo nevi. Using the clonotypic primer in run-off experiments on Vβ15-Cβ PCR products, a single peak was observed in both halo nevi. The peak was superimposable with that observed in the Vβ15-Jβ2.3 analysis (Fig. 4, solid line). No signal was observed in the Vβ15-Cβ PCR products prepared from the PBLs of patient A (Fig. 4, broken line). The relative frequency of the T cells detected by the clonotypic primers was assessed by determining the ratio of the area of the peak of interest to the total area of all peaks (19). The T cells bearing this particular β-chain represented 16% and 11% of the total T cells in the A1 and the A2 halo nevi, respectively, and thus were largely predominant among other T cells present in the halo nevi. Hence, they are expanded in the two halo nevi but not in blood. Consequently, the expansion of T cells in the two halo nevi studied in patient A must have occurred locally, within the nevus, and not at the periphery due to some antigenic stimulation that would be irrelevant to the immune-mediated regression of the nevi, such as an infection.

The 3 halo nevi taken out of the 30 halo nevi of patient K were also studied. The description of the Vβ usage in the three halo nevi is given in Fig. 1 (lines K1-K2 and K3). The Vβ-Cβ repertoire showed that, among otherwise polyclonal profiles, the three halo nevi shared three expanded T cell populations. Upon further Vβ-Jβ analysis, three expanded T cell populations were identified in each halo nevus: the first was characterized by a Vβ17-Jβ2.1

**FIGURE 2.** CDR3 size distribution and Jβ usage in the Vβ15+ T cells of patient A. Top panel, Vβ15-Cβ PCR products of the two halo nevi (A1, A2) and the blood (b) of the patient at the time the nevi were biopsied were subjected to run-off reactions with fluorescent primers specific for each of the 13 Jβ segments (abscissas). The CDR3 size profiles are symbolized as described in Fig. 1. Bottom panel, Vβ15-Jβ2.3 fluorescence profiles in the two halo nevi and in blood. Profiles are expressed as fluorescence intensity on the y-axis and as CDR3 size in amino acids on the x-axis.

**FIGURE 3.** Nucleotide and deduced amino acid sequences of the CDR3 region of the T cells recurrently found expanded in the different halo nevi of patients A and K. The Dβ region is underlined.
rearrangement and a 6-aa CDR3 length, the second by a V\textsubscript{b}17-J\textsubscript{b}2.3 rearrangement and a 10-aa CDR3 length, and the third by a V\textsubscript{b}7-J\textsubscript{b}1.2 rearrangement and a 8-aa CDR3 length (Fig. 5). We have determined the nucleotide sequence of the V\textsubscript{b}7-J\textsubscript{b}1.2, V\textsubscript{b}17-J\textsubscript{b}2.3, and V\textsubscript{b}17-J\textsubscript{b}2.1 PCR products prepared from each of the three halo nevi of patient K. The three V\textsubscript{b}7-J\textsubscript{b}1.2 nucleotide sequences were found to be identical, as were the three V\textsubscript{b}17-J\textsubscript{b}2.3 sequences and the three V\textsubscript{b}17-J\textsubscript{b}2.1 sequences (Fig. 3). Thus, three different T cell populations characterized by usage of the same \(\beta\)-chain are found in the three halo nevi of the patient, presumably indicating a local expansion of each population. A unique peak was also observed in the V\textsubscript{b}1-C\textsubscript{b} combination of the three halo nevi of patient K. However, the surface of the peak was very small compared with that of the V\textsubscript{b}7-C\textsubscript{b} peak. Upon J\textsubscript{b} analysis of the PCR products, no significant peaks were observed. Finally, the analysis of the T cell repertoire of the rare T cells infiltrating the three halo nevi of patient C showed either a preferential usage of V\textsubscript{b}7 (C3) or a preferential usage of V\textsubscript{b}8 (C2). No material could be amplified from the third halo nevus (C1). Primer extension analysis of the PCR products using C\textsubscript{b}- and J\textsubscript{b}-specific primers yielded a Gaussian distribution. No peaks were detected, and the signals were beyond the limits of detection. It is worth noting that the halo nevi of patient C had been excised at the end of the regression process and were associated with a very poor lymphocytic infiltrate.

TCR \(\beta\)-chains of the selectively expanded T cell clone do not recognize classical melanoma tumor Ags

Some halo nevi exhibit moderate atypia (20). The abnormal expression in the halo nevi melanocytes of melanoma Ags that are not expressed in normal nevi could possibly explain their regression, as in spontaneously regressing melanomas. Consequently, we have searched for the expression of such Ags in the halo nevi on the basis of RT-PCR analysis of total mRNA. None of the halo nevi

![FIGURE 4.](image-url) Usage of a clonotypic primer to investigate the presence or absence of the A1, A2 clone in the blood of patient A. A fluorescent clonotypic primer encompassing the CDR3 sequence of the clone (sequence fam-GTATCTGTGATCCCGCTAGTTGT) was used to screen the V\textsubscript{b}15-C\textsubscript{b} PCR products of nevi A1 and A2 and of the V\textsubscript{b}15-C\textsubscript{b} PCR products from the blood of patient A.

![FIGURE 5.](image-url) CDR3 size distribution and J\textsubscript{b} usage in V\textsubscript{b}7 and 17 transcripts from the halo nevi K1, K2 and K3 of patient K. The V\textsubscript{b}7-C\textsubscript{b}, and V\textsubscript{b}17-C\textsubscript{b} PCR products displaying a restricted CDR3 size distribution (one peak) in the K1, K2 and K3 halo nevi were subjected to run-off reactions with fluorescent primers specific for each of the 13 J\textsubscript{b} segments Three J\textsubscript{b} (namely J\textsubscript{b}2.1, J\textsubscript{b}2.3 and J\textsubscript{b}1.2) were used in all three nevi. Their profiles are plotted as full lines vs profiles derived from the corresponding V\textsubscript{b}-J\textsubscript{b} in blood (dotted line). x- and y-axes are as in Fig. 1.
nevi were found to express the melanoma-associated protein members of the MAGE, BAGE, GAGE, and RAGE families. Tyrosinase mRNA, used as an internal positive control, was amplified in all samples studied (data not shown). Thus, the peptide(s) involved in the halo nevus phenomenon are different from the tumor-associated Ags of the melanoma cells identified thus far.

Antigenic peptides presented by classical MHC class I or class II molecules are primarily recognized by the CDR3 loop of the α- and β-chains of the TCR, with little contribution from the CDR1 and CDR2 regions (21). The diversity of the α-chain could not be studied for lack of sufficient material. The CDR3β sequences associated with all of the T cells expanded in the halo nevi were found to use the DJβ2.1 region. The in-frame use of part of the DJβ2.1 region in sequences A, K1, and K3 resulted in the presence of a recurrent motif (TS or TSG) in the CDR3β of these three clones (Table I). A TSG motif was also found in the opposite direction, in the CDR3β region of clone K2, which used an out-of-frame DJβ2.1 segment. The recurrence of the TS (TSG) motif may indicate that the T cells under study recognize identical or closely related peptides in the same or similar restriction context. In this respect, the two patients shared the HLA-A1 allele: patient A was A1 (33), Cw6 (8), DR3 (10), and DQB1 (0205); patient K was A1, Cw7, DR1 (4), and DQ (5,3). Finally, none of the β-chains of the nearly 400 TCR β-chains identified in the melanoma-infiltrating T cells or recognizing melanoma Ags (MAGE, BAGE, GAGE, RAGE, MART-1, glycoprotein 100, tyrosinase, etc.) was found to share any amino acid sequence homology with the A, K1, K2, or K3 sequences.

**Discussion**

Normal nevi do not contain infiltrating lymphocytes. By contrast, halo nevi are infiltrated by CLA⁺, CD4⁺ T cells, and CD8⁺ T cells, are most likely endowed with cytotoxic activity, and are known to be activated (4, 6). To characterize the in vivo diversity of the efficient immune response against these self-melanocytic tumors, we have used a PCR-based technology that allows the determination of the β-chain repertoire and excludes the biases introduced by cell culture and cloning (22). We have found that the T cells that infiltrate the halo nevi fall into two main families: 1) Most of the T cells that infiltrate the halo nevi were found to be either quiescent or to have undergone polyclonal activation and have most likely been selected out of the circulating lymphocyte population due to the expression of CLA, a protein involved in skin homing (17); 2) A small number of T cell clones characterized by their Vβ-CDR3β-β usage proliferate in situ. A limited number of different T cells proliferate in the halo nevi of each patient, most likely as the result of the specific recognition process of a small number of Ags (12, 23, 24). These T cells are not found to be expanded in blood, which implies that their expansion is triggered by the Ags of the tumor and not by some irrelevant antigenic stimulation such as a viral infection. Our results confirm and extend the results obtained by others, who showed preferential Vβ region usage and a higher frequency of certain nucleotide sequences among the PCR products of the α-chain that is suggestive of clonal T cell expansions (7). The results are also similar to the β-chain repertoire in melanomas: a strictly local and clonal expansion of T cells has been reported in regressive melanomas (2), and an expansion and accumulation of specific T cells has been found in melanomas (25); the β-chain repertoire was found to be associated to clonal and oligoclonal responses in progressive melanomas (26) and to different clones in the regressive and progressive regions of primary malignant melanomas (27).

In addition to the oligoclonality of the T cell response in the halo nevi, a few expansions of T cells sharing the same Vβ-CDR3β-β nucleotide sequence were recurrently found in several halo nevi of the same patient. Regardless of whether these cells share the same rearranged TCR α-chain, these proliferating T cells derive from a small number of the same αβ T cell clone(s) at most and consequently probably recognize the same molecular target at different sites in the skin. Because the nevi are not clonal in nature (28), the Ag(s) are likely to be shared by all halo nevi of a given patient. In addition, the recurrence of the TS or TSG motif in the CDR3 sequences of the expanded T cells in different patients may be suggestive of the recognition of related antigenic peptides. Aside from whether a unique Ag or several Ags are recognized by this family of T cells, the nature of the halo nevi Ag(s) remains unknown; however, it does not belong to the proteins that are known thus far to be specifically expressed by melanoma cells. The halo nevus Ag(s) might be an autoimmune-like process mediated by an oligoclonal T cell response.

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**References**


