Predominant Expression of CD44 Splice Variant v10 in Malignant and Reactive Human Skin Lymphocytes

Stephan N. Wagner, Christine Wagner, Uwe Reinhold,* Renate Funk, Margot Zöller,† and Manfred Goos

Department of Dermatology, Medical School, University of Essen, Germany; *Department of Dermatology, Medical School, University of Homburg/Saar, Germany; †Department of Immune Regulation and Tumor Defense, German Cancer Research Center, Heidelberg, Germany

The remarkable functional diversity of the cell surface receptor CD44 may be due to expression of multiple variant isoforms generated by alternative splicing of variant exons. Functional and correlative data implicate a role of CD44 variant isoforms in adhesion dependent processes such as lymphocyte recirculation and tumor progression and metastasis. We have analyzed 25 primary cutaneous lymphomas and 35 reactive lymphoid cell skin infiltrates or T cell-mediated skin diseases for the expression of CD44 variant isoforms. Irrespective of histologic typing, staging, and grading, cutaneous lymphomas as well as nonmalignant skin-infiltrating $CD3^+CD4^+$ and $CD8^+$ T and $CD19^+$ B lymphocytes exhibited a strong expression of CD44v10 and a moderate expression of CD44v3 as determined by immunohistochemistry, immunofluorescence microscopy, and mRNA analysis. Expression of v5, v6, v7, and v9-containing CD44 variant isoforms was not detected. Furthermore, flow cytometry revealed expression of CD44v10 on a significant proportion of peripheral blood lymphocytes from Sézary's syndrome patients and a remarkable coexpression with cutaneous lymphocyte antigen. These results indicate a distinct CD44 variant isoform expression pattern associated with skin-homing lymphocytes different to lymphatic cells at noncutaneous sites. This differential expression pattern of CD44 variant isoforms may contribute to the development of lymphocyte skin infiltrates and/or the unique biologic behavior of cutaneous lymphomas. ***Key words: cutaneous lymphomas/$CD4^+$ T cells/$CD8^+$ T cells/skin-homing lymphocytes. J Invest Dermatol 111:464–471, 1998***

CD44 represents a heterogenous class of glycosylated proteins with molecular masses ranging from 80 to over 250 kDa in size. In its most widely expressed 80–90 kDa "standard or hematopoietic" form (CD44s/CD44H), CD44 has been shown to represent the principle cell surface receptor for hyaluronan (Aruffo *et al*, 1990; Miyake *et al*, 1990; Bartolazzi *et al*, 1994), and to mediate binding to laminin, major histocompatibility complex class II invariant chain, MIP-1β, serglycin, collagen XIV (undulin), and osteopontin/eta-1 (Jalkanen and Jalkanen, 1992; Ishii *et al*, 1993; Naujokas *et al*, 1993; Tanaka *et al*, 1993; Toyama-Sorimachi *et al*, 1995; Ehnis *et al*, 1996; Weber *et al*, 1996) with lower affinity. CD44 has been implicated originally as a lymph node homing receptor, directing lymphocyte adhesion to high endothelial venules and the extracellular matrix (Jalkanen *et al*, 1986), and may also participate in multiple adhesion-dependent cellular processes such as prothymocyte homing, macrophage and T cell activation, natural killer cell-mediated cytotoxicity, hemopoiesis, and tumor metastasis (Haynes *et al*, 1989; Underhill, 1992; Günthert, 1993; Lesley *et al*, 1993; Günthert *et al*, 1995).

This remarkable functional diversity of CD44 may be due either to post-translational modifications like glycosylation (Katoh *et al*, 1995; Lesley *et al*, 1995), phosphorylation of the cytoplasmic domain (Pure *et al*, 1995), and binding of CD44 to the cytoskeleton (Bourguignon *et al*, 1993), or to multiple CD44 isoforms generated by alternative splicing of at least 10 "variant" exons (designated as v1–v10) inserted into a specific site within a membrane-proximal segment of the extracellular domain (Screaton *et al*, 1992). These so-called "variant" isoforms (CD44v) are generated by selective usage of different variant exons or exon combinations. The subsequently inserted peptide sequences are remarkably hydrophilic and provide further glycosylation and chondroitin sulfate attachment sites, which may endow the CD44 molecule with additional binding properties (Bennett *et al*, 1995b; Günthert *et al*, 1995).

Generation of CD44v isoforms may provide an additional molecular basis for the functional diversity of the CD44 molecule as indicated by experimental and correlative data in tumor progression and metastasis. Thus, CD44v isoforms containing variable exon v4–7 or v6–7 have been shown to confer metastatic behavior in a rat pancreas adenocarcinoma cell line *in vivo* (Günthert *et al*, 1991; Rudy *et al*, 1993), and lymphogenic spread of this cell line could be prevented by anti-CD44v6 antibody (Reber *et al*, 1990; Seiter *et al*, 1993). Similarly, transfection of CD44v8–v10 into a murine fibrosarcoma cell line increased tumor cell growth and metastasis formation (Dougherty *et al*, 1992), and transfection of CD44v3 into lymphocytic cells resulted in bone marrow tumor formation *in vivo* (Bartolazzi *et al*, 1995). Expression of some variant exon combinations has been linked to defined tissues (i.e., CD44v8–v10 to epithelial tissue) (Stamenkovic *et al*, 1991; Günthert, 1993), and in some human malignancies CD44v isoform expression profiles are correlated with malignant phenotype, tumor progression, or prognosis (Fox *et al*, 1993; Günthert *et al*, 1995; Zöller, 1995). In a similar fashion, activation as well as infiltrative or aggressive behavior of lymphocytes or lymphoid neoplasms may be related to

Manuscript received May 22, 1997; revised February 24, 1998; accepted for publication May 13, 1998.

Reprint requests to: Dr. Stephan N. Wagner, Hautklinik, Universitätsklinikum Essen, Hufelandstr.55, D-45122 Essen, Germany.

Abbreviations: CD44s, CD44 standard isoform; CD44v, CD44 variant isoform; CL, primary cutaneous lymphoma; CLA, cutaneous lymphocyte-associated antigen/carbohydrated P-selectin glycoprotein ligand-1; TCL, T cell lymphoma.

CD44v isoform expression as suggested by CD44v6 expression during antigenic stimulation of T lymphocytes (Arch *et al*, 1992; Koopmann *et al*, 1993; Stauder *et al*, 1995) and correlation of CD44v6 expression with grading and prognosis in primary nodal non-Hodgkin's lymphomas (Koopmann *et al*, 1993; Ristamäki *et al*, 1995; Stauder *et al*, 1995).

Infiltration of a specific subset of peripheral blood lymphocytes into the skin requires a specific multistep process of lymphocyte adhesion to and migration through vascular endothelium (Butcher, 1991; Springer, 1994; DeGrendele *et al*, 1996; Uksila *et al*, 1997). Furthermore, primary cutaneous lymphomas represent clinicopathologic entities with a biologic behavior significantly different to primary nodal lymphomas (Willemze *et al*, 1997). Because adhesion properties of lymphocytes as well as tumor cell proliferation, growth, and metastasis have been shown to be determined significantly by the composition of variant exons spliced into the CD44H frame work (Stamenkovic *et al*, 1991; Sy *et al*, 1991; Dougherty *et al*, 1992, 1994; Bartolazzi *et al*, 1995; Droll *et al*, 1995; Jackson *et al*, 1995), the expression pattern of CD44v isoforms on cutaneous reactive or malignant lymphocytes may not necessarily correlate with that observed in nodal lymphomas or on activated peripheral blood lymphocytes. In this study, we provide evidence that skin-infiltrating lymphocytes lack CD44v6 expression but strongly upregulate CD44v10 expression during both the manifestation of an immune response and the development of cutaneous lymphoid malignancies.

Table I. Clinicohistopathologic features of primary cutaneous lymphomas

Clinicopathologic classification	n	Grading	Typing	Staging
Patch/plaque-stage mycosis fungoides	1	indolent	T cell	I A
Patch/plaque-stage mycosis fungoides	5	indolent	T cell	I B
Patch/plaque-stage mycosis fungoides	1	indolent	T cell	II A
Tumor-stage mycosis fungoides	5	indolent	T cell	II B
Erythroderma-stage mycosis fungoides	1	indolent	T cell	III
Large cell anaplastic ($CD30^+$) TCL	2	indolent	T cell	II B
Large cell anaplastic ($CD30^+$) TCL	1	indolent	T cell	IV A
Lymphomatoid papulosis	3	indolent	T cell	
Subcutaneous panniculitis-like TCL	1	aggressive	T cell	II B
Sézary's syndrome	2	aggressive	T cell	IV A
Large cell pleomorphic ($CD30^-$) TCL	1	aggressive	T cell	II B
Immunocytoma	1	indolent	B cell	II B
Immunocytoma	1	indolent	B cell	IV B

MATERIALS AND METHODS

Preparation of tissues and blood samples Human tissue specimens represented 25 primary cutaneous lymphomas (CL; including two cases of Sézary's syndrome with respective lymph node samples), 10 lymphocyte skin infiltrates of atopic dermatitis and allergic contact dermatitis, respectively, 25 tissue samples of diverse reactive or T cell-mediated cutaneous inflammatory diseases, including a pseudolymphoma, and two cases of inflammatory skin-draining lymph nodes (**Tables I** and **II**). Extirpated samples were halved, one part being immediately frozen in liquid nitrogen for immunohistochemical staining and RNA extraction. The other part was fixed in 10% buffered formalin (pH 7.0) and embedded in paraffin wax for histologic examination. Diagnosis was made independently by two of the authors according to standard histologic criteria (Willemze *et al*, 1997), and confirmed by immunophenotyping (CL) and intralocus T cell receptor γ rearrangement analysis [T cell lymphoma (TCL)].

Peripheral blood mononuclear cells were isolated from whole blood by Ficoll-Hypaque density centrifugation. T cell enriched populations were obtained from peripheral blood mononuclear cells by passage through a nylon wool column (Wako, Neuss, Germany). The proportion of residual $CD14^+$ monocytes and $CD19^+$ B cells was <1%.

Immunohistochemistry, double labeling immunofluorescence microscopy, and flow cytometry Immunohistochemistry was performed as described previously (Heider *et al*, 1993; Koopmann *et al*, 1993). Briefly, 8 μm cryosections were fixed in acetone for 10 min, washed in phosphate-buffered saline, and preincubated with normal goat serum (10% in phosphate-buffered saline). After incubation with the primary antibody for 1 h, immunoreaction was visualized by APAAP staining (Cordell *et al*, 1984). All primary antibodies were titrated to give optimal results. Immunostaining of human skin keratinocytes, which express variant exon sequences v2–v10 (Hudson *et al*, 1995), served as internal quality standard. Negative controls included omission of the first, second, and/or third antibody layer and substitution of the primary antibody by rabbit preimmune or nonimmune IgG or monoclonal mouse IgG of irrelevant specificity. Primary antibodies included variant exon-specific monoclonal BBA11 (R&D Systems, Wiesbaden, Germany) directed against v3, VFF8 (v5), VFF7 (v6), VFF9 (v7), VFF16 (v10), SFF2 directed against CD44 frame work (Bender, Vienna, Austria), and FW11.24 (anti-v9, kindly provided by U. Günthert, Basel Institute for Immunology, Basel, Switzerland).

For double labeling immunofluorescence microscopy, cryosections were fixed in acetone for 10 min at room temperature and blocked with phosphate-buffered saline containing 1% (wt/vol) bovine serum albumin. Incubation of tissue sections with monoclonal antibody VFF16 recognized by dichlorotriazinyl amino fluorescein-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) was followed by incubation with monoclonal mouse antibodies directed against antigens (clones) CD1a (Na1/34), CD3 (UCHT1), CD4 (MT310), CD8 (DK25), CD16 (DJ130c), CD19 (HD37), and CD68 (KP1) (DAKO, Hamburg, Germany) detected by biotinylated rabbit anti-mouse

Table II. Expression of CD44v on lymphocytes within various tissue samples

Lymphocytes within	n	s	v3	v5	v6	v7	v9	v10
Primary cutaneous lymphomas								
Patch/plaque-stage mycosis fungoides	7	+++[a]	++	–	–	–	–	+++
Tumor-stage mycosis fungoides	5	+++	++	–	–	–	–	+++
Erythroderma-stage mycosis fungoides	1	+++	++	–	–	–	–	+++
Large cell anaplastic ($CD30^+$) TCL	3	+++	++	–	–	–	–	+++
Large cell pleomorphic ($CD30^-$) TCL	1	+++	++	–	–	–	–	+++
Subcutaneous panniculitis-like TCL	1	+++	++	–	–	–	–	+++
Lymphomatoid papulosis	3	+++	++	–	–	–	–	+++
Immunocytoma	2	+++	++	–	–	–	–	+++
Sézary's syndrome	2	+++	++	–	–	–	–	+++
Pseudolymphoma	1	+++	++	–	–	–	–	+++
Allergic contact dermatitis	7	+++	++	–	–	–	–	+++
Atopic dermatitis	3	+++	++	–	–	–	–	+++
Psoriasis vulgaris	4	+++	++	–	–	–	–	+++
Lichen planus	3	+++	(+)	–	–	–	–	++
Alopecia areata	2	+++	(+)	–	–	–	–	++
Toxic epidermal necrolysis	4	+++	+	–	–	–	–	+++
Pityriasis lichenoides et varioliformis acuta	1	+++	(+)	–	–	–	–	++
Granuloma anulare	2	+++	(+)	–	–	–	–	++
Dermatitis herpetiformis	1	+++	+	–	–	–	–	+++
Lupus erythematodes	4	+++	+	–	–	–	–	+++
Malignant melanoma	3	+++	(+)	–	–	–	–	++
Lymph node, inflammatory skin draining	2	+++	++	–	(+)	–	–	+++

[a] –, negative; (+), very weak/equivocal; + , weak; ++, moderate; +++, strong immunostaining.

immunoglobulins (DAKO) and subsequently labeled with streptavidin-Texas Red (Amersham Buchler, Braunschweig, Germany).

Flow cytometric analyses were performed by one- or two-color immunofluorescence using fluorescein isothiocyanate- and phycoerythrin-conjugated antibodies. For indirect immunofluorescence, 5×10^5 cells were incubated with unconjugated primary antibody followed by fluorescein isothiocyanate- or phycoerythrin-conjugated affinity purified goat anti-mouse IgG $F(ab')_2$ fragments (Coulter-Immunotech, Hamburg, Germany). Stained cells were subsequently subjected to flow cytometry on a Coulter Epics XL (Coulter-Immunotech) and fluorescence emissions collected by selective filtration (530 nm for fluorescein isothiocyanate, 575 nm for phycoerythrin). Each antibody was titrated to obtain optimal results, staining with irrelevant appropriate isotype-matched antibodies served as control. Primary antibodies used were SFF2, VFF16, and directly fluorescein isothiocyanate-conjugated anti-CLA (cutaneous lymphocyte-associated antigen/carbohydrated P-selectin glycoprotein ligand-1) HECA-452 (Pharmingen, San Diego, CA).

Northern blot analysis Total cellular RNA was prepared from tissue specimens by guanidinium thiocyanate extraction and cesium chloride centrifugation (Chirgwin *et al*, 1979). RNA samples (10 μg total cellular RNA) were size fractionated on 1% agarose, 2 M formaldehyde gels and transferred to nylon membranes by vacuum blotting. Membranes were UV-cross-linked (Stratalinker, Stratagene, Heidelberg, Germany) and hybridized to random primed [α-^{32}P] dATP-labeled cDNA probes at 42°C as described (Wagner *et al*, 1992). After high stringency washing, finally two times for 60 min in 0.1 × sodium citrate/chloride buffer, 0.1% sodium dodecyl sulfate at 55°C for CD44 and 68°C for β-actin, filters were subjected to autoradiography for 12–48 h. The probe used comprised nucleotides from position 270–761 of the published human CD44 cDNA sequence (Stamenkovic *et al*, 1989). The probe was radiolabeled to a specific activity of 2×10^9 cpm per μg using a T7 DNA polymerase random priming kit (Stratagene, Heidelberg, Germany).

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) Five micrograms total cellular RNA of each case were subjected to reverse transcription using random hexanucleotide primers and mouse mammary leukemia virus reverse transcriptase (Promega, Heidelberg, Germany). First strand cDNA syntheses were carried out for 1.5 h at 42°C in a total volume of 50 μl. Five microliters of first strand cDNA were amplified by Taq polymerase (Stratagene, Heidelberg, Germany) essentially as described by Mackay *et al* (1994). Oligonucleotide primers used were AAGGAGAAGCTGTGCTACGT-CG, 5′ sequence, and ATCCACACGGAGTACTTGCG, 3′ sequence, for β-actin, and GCACAGACAGAATCCCTGCTACC, 5′ sequence, and GGGGTGGAATGTGTCTTGGTCTC, 3′ sequence, according to positions 759–781 and 805–783 (directly flanking CD44 variant exons), respectively, of the published human CD44 cDNA sequence (Stamenkovic *et al*, 1989). PCR reaction parameters were 94°C, 1 min; 55°C, 1 min; 72°C, 1 min over 22 cycles for β-actin. Based on densitometric scanning of β-actin RT-PCR products, the amount of cDNA for CD44v RT-PCR reaction was adjusted. PCR reaction parameters for CD44 were 96°C, 5 s; 58°C, 15 s; 72°C, 1 min over 30 cycles. Under these conditions the increase of PCR products was confirmed to be linear in both PCR reactions. Furthermore, titration experiments excluded competitive inhibition of generation of large PCR products. Controls included the omission of reverse transcriptase and the replacement of cDNA by water in the reverse transcription and the amplification reaction mixture, respectively. Ten microliter aliquots of the 50 μl reaction were seperated on a 2.5% agarose gel and the amplification products visualized under UV-light after staining of the gel with ethidium bromide.

Sequencing of RT-PCR products For sequencing of PCR products, the amplificates of 251, 197, and 173 base pairs in size were purified by agarose gel elution (Mermaid Kit, Dianova, Hamburg, Germany). Sequencing of PCR products was performed directly according to the dideoxy chain termination method (Sanger *et al*, 1977) with PCR primers used as sequencing primers.

RESULTS

CD44 standard and variant isoform expression in human skin Previous studies have indicated strong expression of CD44s in various human epithelial tissues including the skin and dermal appendages (Heider *et al*, 1993; Mackay *et al*, 1994; Terpe *et al*, 1994), whereas expression of CD44v isoforms has been reported to be restricted to the epidermis (Heider *et al*, 1993). By use of variant exon-specific antibodies we detected intense immunostaining for CD44v3, v5, v6, v7, v9, and v10 encoding isoforms in the stratum basale and stratum spinosum of human epidermis, whereas stratum granulosum stained only sporadically and stratum corneum was consistantly negative (**Fig 1**). Stratum basale and stratum spinosum of human epidermis also stained brightly with CD44s (**Fig 1**). As described previously (Heider *et al*, 1993; Mackay *et al*, 1994) by immunoreaction with blood vessels, fibroblasts, and connective tissue, CD44s immunostaining of subepidermal tissue was different from that observed for CD44v isoforms (not shown).

Northern blot analysis of different samples from human skin revealed a consistent pattern of CD44 transcripts. Seven major transcripts were observed, which according to their size may well represent the 1.6, 2.2, and 5.0 kb species of the hematopoietic isoform, the 2.0, 2.6, and 5.4 kb species of the epithelial isoform, and a variable minor transcript of 3.6 kb as previously reported for epithelial tumor cell lines (Goldstein *et al*, 1989; Harn *et al*, 1991; Stamenkovic *et al*, 1991) (**Fig 2**). By RT-PCR analysis with variant exon flanking primers a variety of transcripts with different lengths was observed (**Fig 3**).

CD44 standard and variant isoform expression on cutaneous lymphomas Twenty-five primary cutaneous lymphomas including B and T cell lymphomas of different histologic subtypes, different grading, and different stages of disease progression (**Table I**) were analyzed for CD44v isoform expression. By use of immunohistochemistry, 25 of 25 CL were found to intensely express CD44v10- and CD44s-encoded epitopes and to moderately express CD44v3. No immunoreactivity was observed for CD44 variant exons v5, v6, v7, and v9 (**Fig 1**, **Table II**). By double labeling immunofluorescence microscopy the main proportion of CD44v10-expressing cells could be identified as $CD3^+CD4^+$ and $CD8^+$ T cells or $CD19^+$ B cells. $CD16^+$ natural killer cells and some scattered $CD68^+$ macrophages showed a weak CD44v10 immunoreactivity, whereas intraepidermal $CD1a^+$ dendritic cells and fibroblasts could not be detected to express CD44v10.

Northern blot analysis of CL revealed the presence of three major transcripts with a preferential expression of the suggested 5.0 kb species of hematopoietic transcripts and a weaker expression of the suggested 1.6 and 2.2 species (Stamenkovic *et al*, 1989) (**Fig 2**). In subepidermal tissue-infiltrating CL [including large cell anaplastic ($CD30^+$) TCL, large cell pleomorphic (CD30) TCL, and subcutaneous panniculitis-like (TCL)] these three transcripts were present almost exclusively. Comparable results were obtained for epidermal tissue-infiltrating cutaneous TCL including patch/plaque-, tumor-type, and erythroderma-stage mycosis fungoides (**Fig 2**). These cutaneous TCL characteristically infiltrate the epidermis, thus the presence of the so-called epithelial 2.0, 2.6, 5.4, and 3.6 kb isoforms in these cases may be due to contamination with epidermal tissue.

Three major transcripts were also detected by semiquantitative RT-PCR analysis using primers complementary to variant exons 5′ and 3′ flanking standard sequences. Subepidermal tissue-infiltrating CL consistently expressed three transcripts of 251, 197, and 173 base pairs in size, whereas epidermis-infiltrating cutaneous TCL contained some additional transcripts at significant reduced amounts corresponding to those observed in epidermal tissue (**Fig 3**). Sequence analysis identified the 251 bp product as complete exon v10 cDNA and the 173 bp amplificate as complete exon v3. The 197 bp transcript represented a 150 bp splice variant of exon v10. A comparable CD44v10 splice variant has been previously described in murine tissue (Günthert, 1993).

CD44 standard and variant isoform expression on reactive lymphoid cell skin infiltrates of various skin diseases The uniform and intense expression of CD44v10 and the moderate expression of CD44v3 in CL, irrespective of histologic subtype, grading, and stage of disease progression, prompted us to study the expression of CD44s and CD44v epitopes on reactive lymphocyte skin infiltrates of atopic dermatitis and allergic contact dermatitis, diverse inflammatory and T cell-mediated skin diseases, pseudolymphoma, and inflammatory skin-draining peripheral lymph nodes. By means of immunohistochemistry, northern blot analysis, and RT-PCR sequence analysis, in 10 of 10 reactive lymphocyte skin infiltrates of atopic dermatitis and allergic contact dermatitis the expression pattern of CD44s and CD44v isoforms was identical to malignant lymphoid cell skin infiltrates with high expression levels of v10 and standard epitopes and moderate expression of CD44v3 (**Figs 1–3**; **Table II**). Interestingly, the very same expression pattern was noted on lymphocytes of diverse inflam-

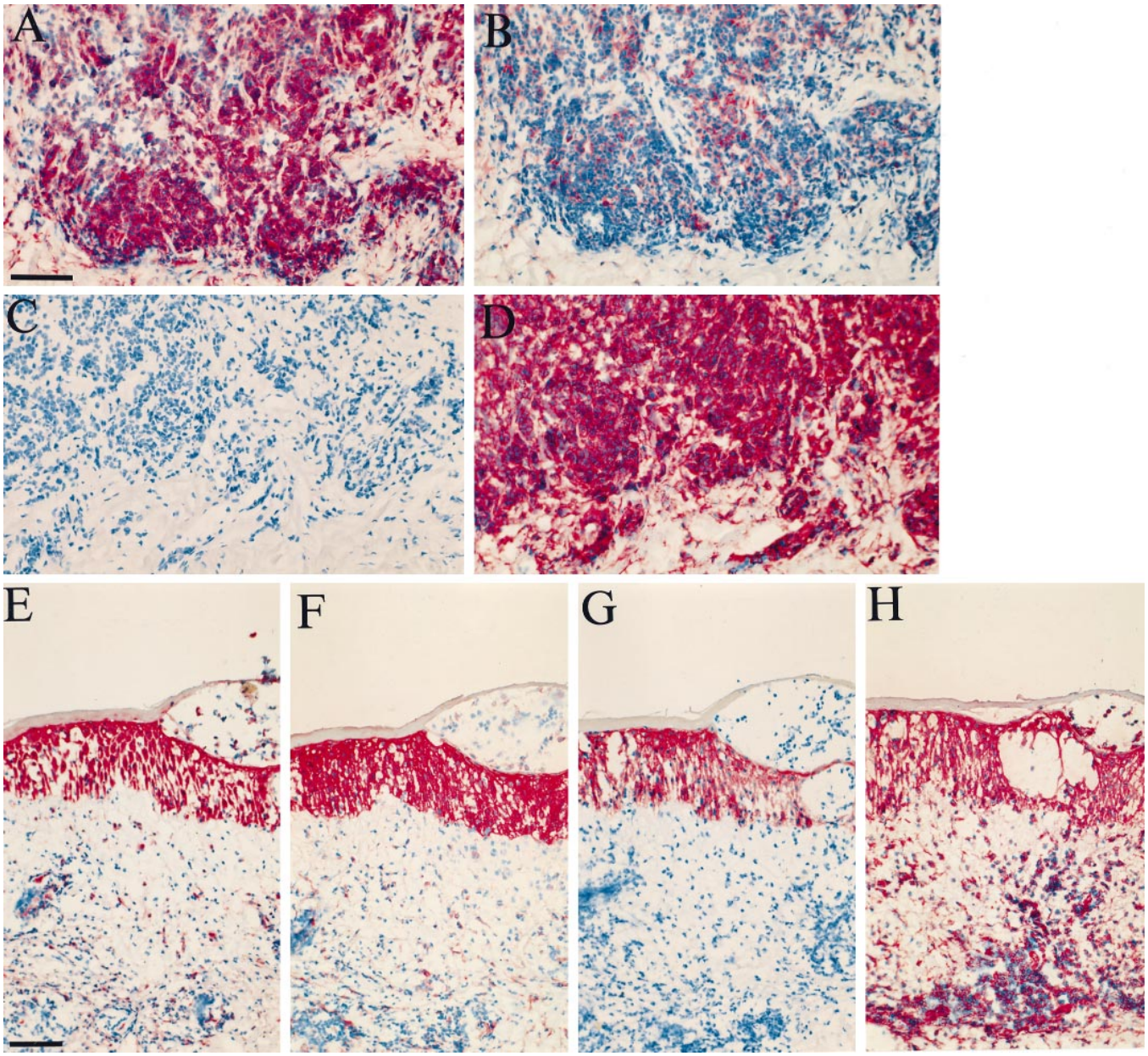

Figure 1. Expression of CD44 v10- and v3-containing isoforms on malignant and reactive skin-infiltrating lymphocytes. Tissue samples of tumor-type mycosis fungoides (*A–D*) and allergic contact dermatitis (*E–H*) were stained with anti-CD44v10 (*A*, *E*), anti-CD44v3 (*B, F*), anti-CD44v6 (*C*, *G*), and anti-CD44s (*D*, *H*) antibodies. Strong immunoreactivity of lymphocytes with anti-CD44v10 and anti-CD44s antibodies, moderate immunostaining for CD44v3. Note negative immunostaining with CD44v6-reactive antibody. Cryostat sections, APAAP method. *Scale bars*: 50 μm.

matory and T cell-mediated skin diseases (**Table II**). Again, double labeling immunofluorescence microscopy demonstrated strong immunostaining for CD44v10 on $CD3^+CD4^+$ and $CD8^+$ T cells or $CD19^+$ B cells (**Fig 4**), weak CD44v10 immunoreactivity on $CD16^+$ natural killer cells and some scattered $CD68^+$ macrophages, but no detectable CD44v10 immunoreactivity on intraepidermal $CD1a^+$ dendritic cells and dermal fibroblasts (not shown).

Although normal lymphoid tissue is thought to express CD44s and to lack CD44 variant isoforms (Heider *et al*, 1993; Koopmann *et al*, 1993; Terpe *et al*, 1994), CD44v6 expression was described upon antigenic activation (Koopmann *et al*, 1993). In peripheral lymph node tissues draining inflammatory skin, however, there was a remarkable expression of CD44v10 and, to a lesser extent, v3 isoforms identical to the expression pattern on malignant and reactive lymphocyte skin infiltrates (**Table II**).

CD44 standard and variant isoform expression on Sézary cells of skin, lymph nodes, and peripheral blood Among the 25 CL analyzed, two cases of Sézary's syndrome were included, from which skin biopsies, lymph node tissues, and peripheral blood samples were available. As described already, Sézary cells infiltrating the skin exhibited an immunoreactivity pattern as observed in other CL. CD44v10- and CD44s-encoded epitopes were strongly expressed, whereas only a moderate immunoreactivity for CD44v3 and lack of staining for CD44 variant exons v5, v6, v7, and v9 could be observed (**Fig 5**, **Table II**).

A similar immunostaining pattern was observed on Sézary cells involving the respective lymph node tissues. Again, there was a remarkable expression of CD44v10 and, to a lesser extent, v3 isoforms identical to the expression pattern observed in Sézary cells infiltrating the skin (**Fig 5**, **Table II**).

Peripheral blood involvement of Sézary's syndrome patients was

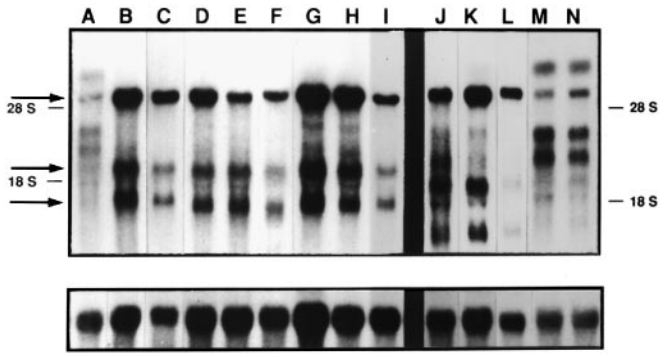

Figure 2. CD44 isoform expression in tissue samples of malignant and reactive lymphoid cell infiltrates different to normal skin as obtained by northern blot hybridization. *Lanes A*, *M*, *N*, unaffected skin; *lanes B*, *D*, mycosis fungoides, tumor stage; *lanes C*, *E*, mycosis fungoides, patch/plaque stage; *lane F*, large-cell anaplastic ($CD30^+$) TCL; *lanes G*, *H*, mycosis fungoides, erythroderma stage; *lane I*, large-cell ($CD30^-$) pleomorphic TCL; *lanes J*, *K*, allergic contact dermatitis; *lane L*, immunocytoma. *Upper panel*: 10 μg of the respective total RNA were analyzed by hybridization with a cDNA complementary to standard human CD44 cDNA sequences. The migration positions of 18S rRNA and 28S rRNA are indicated. Note predominant expression of the 1.6, 2.2, and 5.0 kb hematopoietic isoforms (*arrows*) in malignant and reactive lymphoid cell infiltrates as compared with normal skin. *Lower panel*: control hybridization with β-actin cDNA probe.

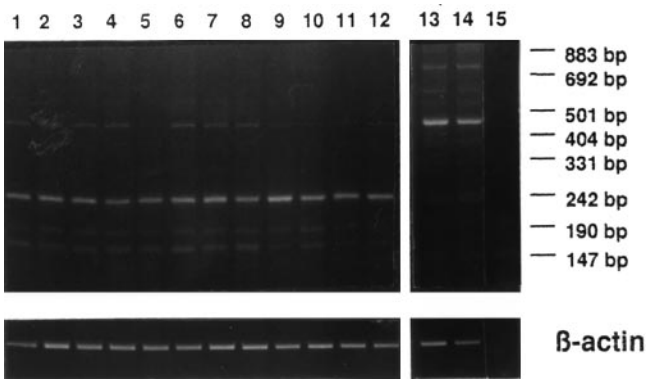

Figure 3. Semiquantitative RT-PCR analysis reveals preferential expression of distinct CD44v transripts in malignant and reactive lymphoid cell infiltrates. *Lanes 1*, *2*, large-cell anaplastic ($CD30^+$) TCL; *lane 3*, large-cell ($CD30^-$) pleomorphic TCL; *lane 4*, mycosis fungoides, erythroderma stage; *lane 5*, subcutaneous panniculitis-like TCL; *lanes 6–8*, mycosis fungoides, patch/plaque stage; *lane 9*, immunocytoma; *lanes 10–12*, mycosis fungoides, tumor stage; *lanes 13–14*, normal skin; *lane 15*, negative control. Quantitation of cDNA was performed by standardizing for equal amounts of β-actin cDNA. RT-PCR products were separated on agarose gels. *Upper panel*: preferential expression of the 251, 197, and 173 bp CD44v transcripts in malignant and reactive lymphoid cell infiltrates as compared with unaffected skin. The additional transcript in *lanes 1–4* and *6–12* was identified as the epithelial isoform of CD44 (v8–v10, due to contamination with epidermal keratinocytes) by dideoxysequencing. *Lower panel*: β-actin equilibration of tissue samples. The mobilities of the molecular weight marker are indicated.

confirmed by cytologic analysis and immunophenotyping of peripheral blood samples. In contrast to the reported relative lack of CD44v isoform expression on normal peripheral blood lymphocytes (Pals *et al*, 1989; Horst *et al*, 1990; Jalkanen *et al*, 1991; Joensuu *et al*, 1993; Koopmann *et al*, 1993; Mackay *et al*, 1994), 19.9% and 29.2%, respectively, of peripheral blood lymphocytes of Sézary's syndrome patients exhibited immunoreactivity for CD44v10 as determined by flow cytometry. In both these cases, a significant proportion of peripheral blood lymphocytes also exhibited expression of CLA (11.9% and 29.9%, respectively). Two-color flow cytometry revealed that 95.8% and 85.3%, respectively, of CLA-positive lymphocytes coexpressed CD44v10 (**Fig 6**).

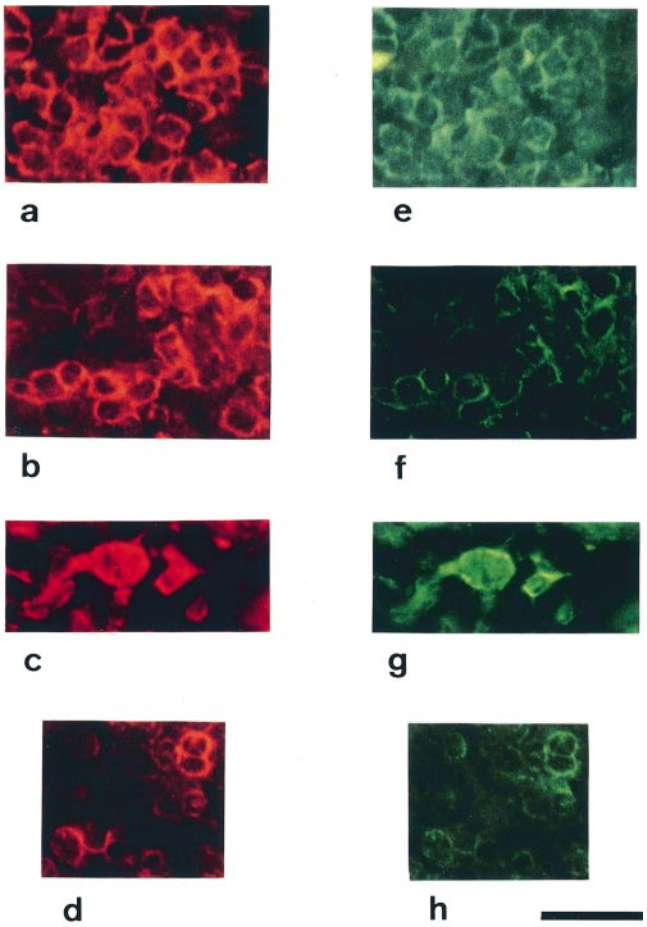

Figure 4. $CD3^+CD4^+$ and $CD8^+$ T lymphocytes and $CD19^+$ B lymphocytes express CD44v10. Double labeling immunofluorescence microscopy of cryostat tissue sections of a reactive lymphoid cell skin infiltrate with anti-CD3 (*a*), anti-CD4 (*b*), anti-CD19 (*c*), and anti-CD8 (*d*) antibodies and corresponding immunofluorescent staining for CD44v10 (*E–H*). Streptavidin-Texas Red-labeling (*a–d*) and dichlorotriazinyl amino fluorescein-labeling (*e–h*) of cryosections. Note CD44v10 immunofluorescence on $CD3^+CD4^+$ and $CD8^+$ T lymphocytes as well as on $CD19^+$ B lymphocytes. *Scale bar*: 25 μm (*a*, *b*, *d–f*, *h*), 10 μm (*c*, *g*).

DISCUSSION

Peripheral blood lymphocytes are considered to consist of different subpopulations with tissue selective homing, with one subset representing skin-associated lymphocytes defined by the expression of CLA/carbohydrated PSGL-1 (Fuhlbrigge *et al*, 1997; Picker *et al*, 1990, 1991). In this study, we have analyzed the expression of CD44v isoforms on this specialized subset, i.e., reactive and malignant skin-infiltrating lymphocytes of 25 primary cutaneous malignant lymphomas and 35 reactive lymphoid cell infiltrates or T cell-mediated skin diseases. By means of immunohistochemistry and double labeling immunofluorescence microscopy we could clearly demonstrate that malignant and reactive skin-infiltrating lymphocytes strongly express CD44v10-containing isoforms and moderately express CD44v3 containing isoforms. CD44v10 expression was observed predominantly on lymphatic cells such as $CD3^+CD4^+$ and $CD8^+$ T cells as well as $CD19^+$ B cells and to a lesser extent on natural killer cells. Other CD44v isoforms containing exons v5, v6, v7, and v9 were not detectable. Because semiquantitative RT-PCR is considered to be a useful addition to immunohistochemical evaluations (Mackay *et al*, 1994; Günthert *et al*, 1995; Stauder *et al*, 1995), we additionally performed CD44v transcript analysis. By use of unaffected skin tissue samples as control specimens in this RT-PCR approach, as well as in the northern blot analysis, we could clearly demonstrate CL to exhibit a different CD44v isoform expression pattern at the mRNA level. By sequencing of RT-PCR products we could identify the predominantly

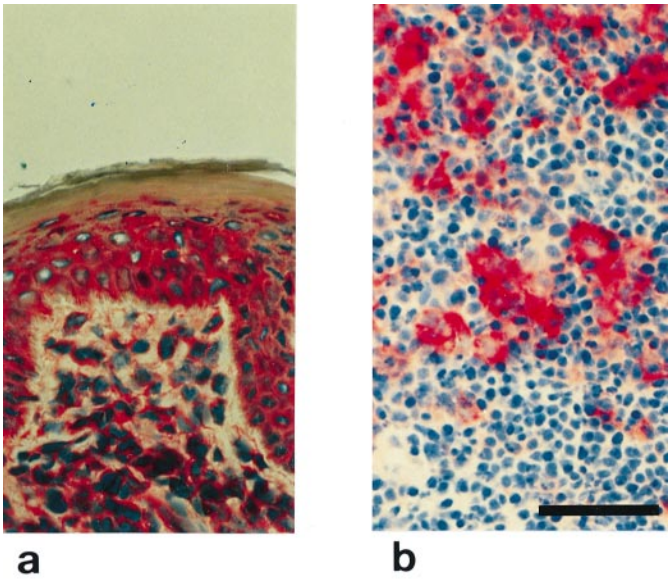

Figure 5. Expression of CD44v10-containing isoforms on skin- and lymph node-infiltrating Sézary cells. Tissue samples of Sézary cells involving the skin (*a*) and lymph node tissue (*b*) were stained with anti-CD44v10 antibody. Strong and consistent immunoreactivity of Sézary cells with anti-CD44v10 antibody in both skin and lymph node infiltrates. Cryostat sections, APAAP method. *Scale bar*: 50 μm.

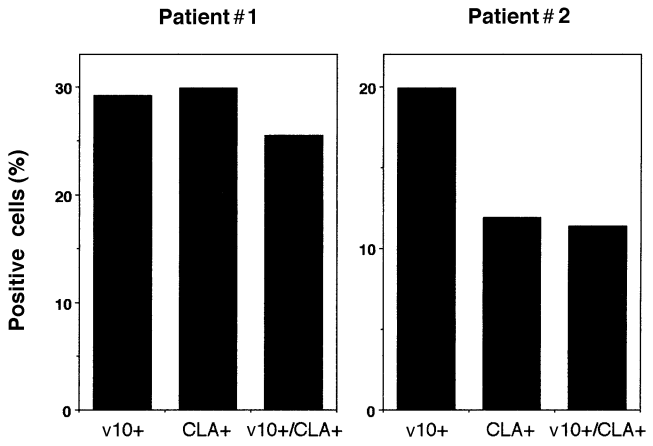

Figure 6. Two color fluorescence analysis. CLA^+ T cells from peripheral blood of Sézary's syndrome patients coexpress CD44v10. Both patients exhibited Sézary cells > 10% of circulating peripheral blood lymphocytes and an elevated CD4/CD8 ratio > 5.0.

expressed mRNA species to represent CD44v10-containing isoforms constituting of solely exon v10 embedded into the CD44 frame work.

Data presented by Dommann *et al* (1995), who reported on the presence of CD44v6 on a small series of CL, are not supported by our results at the protein or at the mRNA level. Furthermore, neither Orteu *et al* nor Bennet *et al* could demonstrate CD44v6 immunoreactivity in cutaneous lymphomas and T cell-mediated skin diseases, respectively (Bennett *et al*, 1995a; Orteu *et al*, 1997). The strong staining of the strata spinosum and basale of normal epidermis and in a small series of primary nodal non-Hodgkin's lymphoma (not shown), does also exclude insufficient immunoreactivity of the CD44v6 antibody used in this study. Moreover, we established a CD44 cDNA mini-library from a microdissected primary tissue sample of tumor-stage mycosis fungoides by cloning of PCR products obtained with primers corresponding to the 5′ untranslated region and to the 3′ polyA tail of the CD44 framework sequence. In the meantime, we have sequenced more than 200 single clones and observed the preferential expression of CD44s- and v10-containing isoforms. Within these 200 sequenced clones CD44v6-containing isoforms were not detected (S.N. Wagner *et al*, manuscript in preparation).

Sézary's syndrome is characterized by the occurrence of neoplastic T cells (Sézary cells) in skin, lymph nodes, and peripheral blood. In this study, we were able to perform CD44v isoform expression studies on Sézary cell infiltrates in the skin and lymph nodes as well as in the peripheral blood compartment of two Sézary's syndrome patients. Again, we observed predominant expression of CD44v10 on Sézary cells involving the skin as well as lymph node tissue and found a significantly increased proportion of CD44v10-expressing lymphocytes in the peripheral blood of these patients. The lack of CD44v expression on normal peripheral blood lymphocytes (Pals *et al*, 1989; Horst *et al*, 1990; Jalkanen *et al*, 1991; Joensuu *et al*, 1993; Koopmann *et al*, 1993), on the majority of normal bone marrow cells (Ghaffari *et al*, 1995), or on extracutaneous lymphatic tissues (Heider *et al*, 1993), the predominant upregulation of CD44v3- and v6-containing isoforms upon activation of peripheral lymphocytes (Koopmann *et al*, 1993; Stauder *et al*, 1995), and the predominant expression of CD44v3 and v6 in primary nodal non-Hodgkin's lymphomas (Koopmann *et al*, 1993; Ristamäki *et al*, 1995; Stauder *et al*, 1995), indicate differential CD44v expression on skin-associated lymphocytes and the majority of activated peripheral blood lymphocytes/nodal lymphomas. The association of CD44v10 expression with skin-associated lymphocytes is further supported by the remarkable coexpression with CLA/carbohydrated PSGL-1, the so-called skin homing receptor, on peripheral blood lymphocytes of Sézary's syndrome patients.

It has been proposed repeatedly that the multiple isoforms of CD44 are the correlate of a multitude of functions. The upregulated expression of CD44v10 on CL and reactive lymphocyte infiltrates of the skin provides an example of distinct and divergent expression of individual variant exons. Both CD44v3 and v10 constitute exons that may modify the CD44 molecule post-translationally. Thus, exon v3 contains a serine-glycine-serine-glycine motif that confers additional ligand binding specificities to CD44, i.e., a heparan sulfate attachment side, which can bind and present a number of cytokines (Bennett *et al*, 1995a). This may constitute an important contribution to the inflammatory conditions at the sites of lymphocyte skin infiltrates. The role of this particular modification in CL, however, remains to be established. What could be the function of CD44v10 upregulation on skin-infiltrating lymphocytes? Exon v10 contains a chondroitin sulfate attachment site that may induce new binding properties. Thus, cell surface chondroitin sulfate proteoglycans antagonize cell adhesion to extracellular matrix proteins such as fibronectin, collagen, and laminin (Knox and Wells, 1979; Rich *et al*, 1981; Rosenberg *et al*, 1985; Yamagata *et al*, 1989), regulate motility and invasiveness of murine melanoma cells when attached to CD44 (Faassen *et al*, 1992), and mediate binding by other CD44 molecules (Droll *et al*, 1995). This is further supported by experimental models using $CD44v10^+$ Namalwa transfectants demonstrating increased homotypic and comparable heterotypic cell–cell adhesion properties as well as increased invasion into types I and III collagen matric when compared with $CD44s^+$ transfectants.[1] Thus, one may speculate that CD44v10-expressing malignant and reactive $CD3^+CD4^+$ and $CD8^+$ T as well as $CD19^+$ B lymphocytes may represent lymphocyte subsets with specific migration and homing properties. *In vivo* models of skin infiltration and lymphocyte activation by appropriately transfected lymphoid cell lines shall help to elucidate the functional consequences of CD44v10 expression on lymphocytes in more detail.

We acknowledge Drs. K. Fellbaum and H. Höfler (Institut für Pathologie, Technische Universität München) for kindly providing control tissue specimens. We thank M. Schröder for excellent technical assistance and H. Apel for photographic work. This work was supported by a grant of the Deutsche Krebshilfe (10–1150-Wa 1).

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