Treatment with an Anti-CD44v10-Specific Antibody Inhibits the Onset of Alopecia Areata in C3H/HeJ Mice

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A murine CD44v10-neutralizing antibody has been reported to impair delayed-type hypersensitivity reactions. Because alopecia areata is characterized by a delayed-type hypersensitivity-like T cell mediated immune response, we addressed the question whether an anti-CD44v10-antibody influences the onset of alopecia areata. Therefore, we used the C3H/HeJ mouse model with the induction of alopecia areata in unaffected mice by the grafting of lesional alopecia areata mouse skin. Six grafted mice were injected (intraperitoneally) with anti-CD44v10, six grafted mice with anti-CD44standard, and six with phosphate-buffered saline only. After 11 wk phosphate-buffered saline injected animals on average had developed alopecia areata on 36.8% of their body. The onset of hair loss was slightly delayed and its extent reduced to 17.2% of their body in anti-CD44standard-treated mice. By contrast, five of six anti-CD44v10-treated mice did not show any hair loss and one mouse developed alopecia areata on only 1% of the body. Immunohistochemical examination revealed a marked reduction of perifollicular $CD8^+$ lymphocytes and, to a lesser degree, $CD4^+$ cells as well as a decreased expression of major histocompatibility complex class I on hair follicle epithelium in anti-CD44v10-treated mice as compared with phosphate-buffered saline or anti-CD44 standard-treated mice. Our data show that anti-CD44v10 is able to inhibit the onset of alopecia areata in C3H/HeJ mice. This might be accomplished by an anti-CD44v10-triggered impairment of immune cell homing (e.g., $CD8^+$ T cells), resulting in a decrease of their number in target tissues. ***Key words: autoimmunity/CD4/CD8/CD44s. J Invest Dermatol 115:653–657, 2000***

The standard form of the cell surface receptor CD44 (CD44s) was originally described as a lymphocyte homing receptor (Jalkanen *et al*, 1986); however, it is now known to be widely expressed in many different tissues and cells (Naor *et al*, 1997). By alternative splicing of at least 10 variant exons, which are inserted into a specific site within the extracellular domain of CD44s, multiple variant isoforms of CD44 are generated (Jackson *et al*, 1992; Screaton *et al*, 1993). CD44s and CD44 splice variants have been shown to be involved in various autoimmune disorders (Gunthert *et al*, 1998).

The splice variant v10 (CD44v10) is expressed on leukocytes in several autoimmune and other inflammatory skin diseases and it is thought to be involved in the process of leukocyte homing in the skin (Seiter *et al*, 1998; Wagner *et al*, 1998). Because alopecia areata (AA) is generally considered to represent an autoimmune disorder (Baadsgaard, 1991), it is not surprising that CD44v10 is also expressed on T cells in lesional AA skin (Wagner *et al*, 1998). Recently, a murine CD44v10-neutralizing antibody (anti-CD44v10) has been reported to impair delayed-type hypersensitivity (DTH) reactions (Rösel *et al*, 1997). Because AA and the DTH reaction are both characterized by a T cell mediated immune response with a Th1 cytokine profile, we addressed the question whether an anti-CD44v10-antibody may influence the onset of AA.

To answer this question we used the C3H/HeJ mouse model of AA (Sundberg *et al*, 1994; Tobin *et al*, 1997; Freyschmidt-Paul *et al*, 1999). AA-like hair loss occurs spontaneously in up to 20% of C3H/HeJ mice, and it can also be induced in unaffected littermates by autologous transplantation of alopecic skin from affected C3H/HeJ mice (McElwee *et al*, 1998). This technique provides an improved animal model for the analysis of the AA immune cascade, because (i) the time of onset of AA can be roughly determined in advance, and (ii) the influence of various pathogenetic factors on the development of the disease can be studied.

Manuscript received July 13, 1999; revised March 23, 2000; accepted for publication July 17, 2000.

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Abbreviations: AA, alopecia areata; CD44s, standard form of the cell surface receptor CD44; CD44v10, splice variant v10 of the cell surface receptor CD44.

MATERIALS AND METHODS

Antibodies The following antibodies were used *in vivo*: anti-CD44v10 (clone K926, rat IgG2a) (Rösel *et al*, 1997), anti-CD44s [clone IM7, rat IgG2b, American Type Culture Collection (ATCC), Manassas, VA]. As primary antibodies for immunohistochemistry the same anti-CD44v10 and anti-CD44s antibodies were used as well as anti-CD4 (clone RM4–5, rat IgG2a, Pharmingen, San Diego, CA), anti-CD8 (clone 53–6.7, rat IgG2a, Southern Biotechnology, Birmingham, AL), anti-major histocompatibility

complex (MHC) class I (clone ER MP 42, rat IgG2a, BMA, Augst, Switzerland), anti-MHC class II (clone ER-TR 3, rat IgG2b, BMA), and anti-ICAM-1 (clone KAT-1, rat IgG2a, ATCC).

As a secondary antibody for immunohistochemistry a biotinylated goat anti-rat IgG (Dianova, Hamburg, Germany) was used.

Animals and treatment C3H/HeJ mice with AA-like hair loss (Sundberg *et al*, 1994) and C3H/HeJ mice without hair loss were provided by The Jackson Laboratory (Bar Harbor, ME). From five mice with AA, alopecic skin was grafted on to the back of 19 unaffected C3H/HeJ mice according to the technique as previously described (McElwee *et al*, 1998). After grafting, three mice were injected intraperitoneally twice a week for 11 wk with 200 μg anti-CD44v10 in 200 μl phosphate-buffered saline (PBS), three mice with 200 μg anti-CD44s in 200 μl PBS, and three mice with 200 μl PBS only. One PBS injected mouse died during the study. In a second series, using lower concentrations, three mice were injected with 150 μg anti-CD44v10 in 200 μl PBS, three mice received 150 μg anti-CD44s in 200 μl PBS, and four mice 200 μl PBS twice a week for 11 wk.

Assessment of hair loss The hair status was examined and documented daily. Photographs were taken once a week. The extent of hair loss was assessed quantitatively by means of an image analysis software (Sigma Scan Pro, Jandel Scientific, San Rafael, CA) based on the photographs taken at the end of the study. Briefly, photographs were scanned and areas of alopecic skin, with the exception of the graft, were outlined using the software. The total area of alopecic skin was calculated for both the ventral and dorsal surface and this was converted to a percentage of the total skin surface area visible in the photographs.

Statistical analysis Differences in the median area of alopecic skin between (i) PBS-treated and anti-CD44s-treated mice, and (ii) PBS-treated and anti-CD44v10-treated mice, were investigated with the one-sided exact Mann–Whitney test at the 5% test level. Adjustments for multiple testing were performed according to Bonferroni–Holm.

Immunohistochemistry Frozen vertical sections of grafted skin, unaffected host skin, and alopecic host skin were fixed in acetone and air dried. Between all incubation steps, sections were washed with Tris-buffered saline (0.05 M, pH 7.6). Non-specific binding was blocked by application of an avidin–biotin blocking kit solution (Vector Laboratories, Burlingame, CA) and by 2% bovine normal serum and 2% goat normal serum in Tris-buffered saline. Subsequently, the slides were incubated with the primary antibody, diluted in Tris-buffered saline containing 2% normal bovine serum, at room temperature for 1 h. After washing, sections were incubated with the secondary antibody diluted in Tris-buffered saline, containing 4% mouse normal serum and 2% goat normal serum, for 30 min at room temperature. After washing, a routine staining method for avidin–biotin complex labeled with alkaline phosphatase was used (Vector Laboratories) and counterstained with Mayer's hematoxylin. Negative controls included replacement of primary antibody by normal rat IgG.

The number of perifollicular and intrafollicular infiltrating $CD4^+$ and $CD8^+$ T cells was assessed semiquantitatively using three categories: no cells (–), single cells (+), moderately dense infiltrate (++), and dense infiltrate (+++). The expression of MHC class I, MHC class II, and ICAM-1 on hair follicle epithelium was evaluated according to the following scoring system: no staining (–), weak staining (+), moderate staining (++), and intense staining (+++). This analysis was performed blinded by two independent investigators on two sections of grafted and host skin of each mouse, and the average per group was calculated for PBS-treated mice, anti-CD44s-treated mice, and for anti-CD44v10-treated mice.

RESULTS

An anti-CD44v10-specific monoclonal antibody inhibits the onset of AA After 3–5 wk, PBS-injected mice showed initial patchy hair loss (**Fig 1**). After 11 wk PBS-injected mice had developed AA-like hair loss in a mean ± SD of 36.8% ± 21.5% of total skin surface area (**Fig 2*a***). In anti-CD44s-injected mice hair loss was first observed after 4 up to 7 wk (**Fig 1**). At the end of the study the extent of hair loss measured 17.17 ± 24.68% (mean ± SD) (**Fig 2*b***). By contrast, three of three mice that had received 200 μg anti-CD44v10 did not show any hair loss in host skin. In the group of mice that had received only 150 μg anti-CD44v10, two mice did not show any hair loss, and one mouse developed hair loss after 9 wk on 1% of total skin surface (**Figs 1** and **2*c***). The graft remained bald in all mice. Thus there is a significant difference in the median area of alopecic skin between (i) PBS and anti-CD44s-treated mice (adjusted $p = 0.0433$), and (ii) between PBS and anti-CD44v10-treated mice (adjusted $p = 0.0011$).

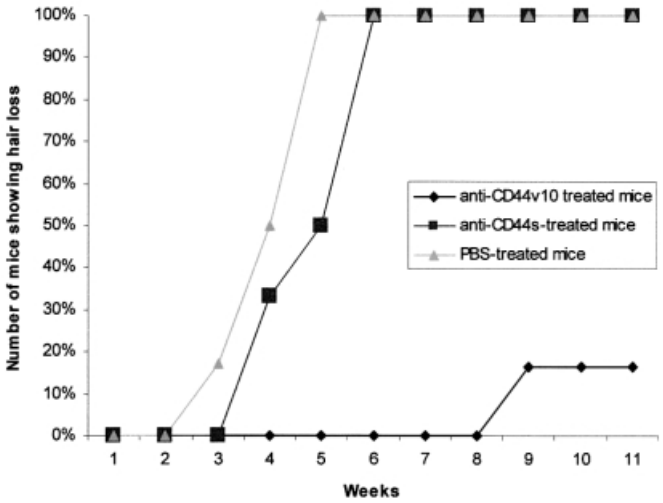

Figure 1. The onset of AA is delayed in anti-CD44s-treated mice and inhibited in five of six anti-CD44v10-treated mice. Time of onset of hair loss in control mice, anti-CD44s, and anti-CD44v10-treated mice shown as weeks after grafting.

Skin of anti-CD44v10-treated mice shows a reduced number of infiltrating $CD8^+$ cells and a decreased MHC class I expression on hair follicle epithelium The graft and alopecic host skin of PBS-injected mice showed a dense perifollicular and intrafollicular infiltrate of $CD8^+$ cells and only moderately dense $CD4^+$ cells. $CD8^+$ cells were primarily localized in the center of the infiltrate in close vicinity to, or within, the hair follicle epithelium, whereas $CD4^+$ cells were more numerous in the periphery of the infiltrate. An intense staining for MHC class I, MHC class II, and ICAM-1 was found on bulbar, suprabulbar, and upper parts of the hair follicle epithelium.

In grafted skin of anti-CD44v10-treated mice, there was a striking decrease of $CD8^+$ T cells and, to a lesser degree, of perifollicular $CD4^+$ T cells as compared with controls. MHC class I expression on hair follicle epithelium was reduced (**Fig 3*c***, ***g***, ***k***, **Tables I** and **II**). Unaffected anagen skin of anti-CD44v10-injected mice did not show any perifollicular infiltrate of $CD4^+$ and/or $CD8^+$ T cells, and the number of $CD8^+$ T cells, but not of $CD4^+$ T cells, was reduced throughout all parts of the skin. Hair follicle epithelium only showed a faint staining for MHC class I (**Fig 3*d***, ***h***, ***l***, **Tables I** and **II**).

In the mice treated with anti-CD44s a slight reduction of $CD8^+$, but not of $CD4^+$ T cells and MHC class I expression, was observed in the graft and alopecic host skin as compared with PBS-injected mice (**Fig 3*b***, ***f***, ***j*,** **Tables I** and **II**).

No differences in MHC class II and ICAM-1 expression on hair follicle epithelium could be found between controls and anti-CD44s or anti-CD44v10-treated mice (**Table II**).

CD44v10 was expressed on keratinocytes but not on infiltrating lymphocytes CD44v10 was strongly expressed on keratinocytes of the epidermis and hair follicles (matrix and outer root sheath) in all mice, whereas no other cell types in the skin, in particular no lymphocytes, showed staining for CD44v10. Staining for CD44s showed no differences between the three groups of mice. In all sections, lymphocytes of the inflammatory infiltrate showed a strong staining for CD44s, and CD44s expression on hair follicles was observed on epithelial cells of the matrix and outer root sheath.

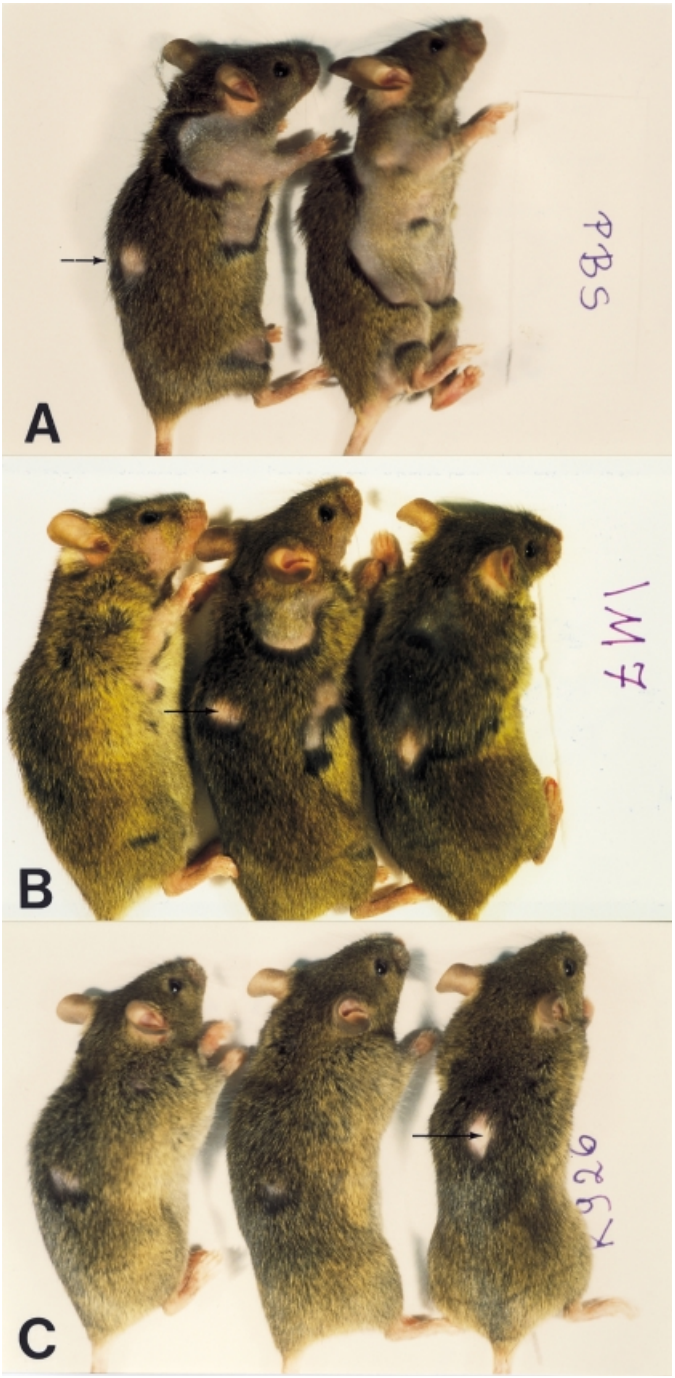

Figure 2. The onset of hair loss is inhibited by anti-CD44v10 treatment in AA-grafted C3H/HeJ mice. Extent of hair loss in AA-grafted C3H/HeJ mice after injection of (*A*) 200 μl PBS: AA in 28.8% (left) and 53.7% (right) of the body; (*B*) 200 μg anti-CD44s: AA in 4.1% (left), 10.3% (middle) and 4.4% (right) of the body; and (*C*) 200 μg anti-CD44v10: AA in host skin. The graft (*arrow*) remained bald in all mice.

DISCUSSION

Development of hair loss in all control mice demonstrates that AA can be reliably induced by the described graft technique. This is in line with the results obtained by McElwee *et al* (1998) who induced AA by the described technique in 15 of 15 unaffected C3H/HeJ mice 7–11 wk after grafting.

Our results show that treatment with anti-CD44s slightly delayed the onset of AA in C3H/HeJ mice and reduced its extent with statistical significance (adjusted $p = 0.0433$). A similar effect of anti-CD44s has been reported for arthritis in mice, with a delayed onset and decreased severity of the disease (Mikecz *et al*, 1995; Verdrengh *et al*, 1995).

Furthermore, our results show that treatment with anti-CD44v10 does not only mitigate the disease but inhibits the development of AA in C3H/HeJ mice with statistical significance (adjusted $p = 0.0011$) in a concentration-dependent manner. Anti-CD44v10 also mitigates experimental autoimmune encephalomyelitis, a mouse model of multiple sclerosis, but only in combination with anti-CD44v6 and anti-CD44v7 (Laman *et al*, 1998). Our data provide evidence that in C3H/HeJ mice, anti-CD44v10 alone is able to inhibit the development of AA, which represents an autoimmune disorder of the hair follicle (Baadsgaard, 1991).

To elucidate the mechanisms whereby anti-CD44v10 inhibited the onset of AA and by which anti-CD44s delayed and reduced hair loss, our immunohistochemical studies focused on anagen hair follicles that most likely represent the immunologic target in AA. In analogy to human AA, perifollicular and intrafollicular infiltrates of $CD4^+$ and $CD8^+$ T cells with a predominance of the latter and an abnormal expression of MHC class I, MHC class II, and ICAM-1 on the epithelium of anagen hair follicles characterize the AA-like hair loss in C3H/HeJ mice (Sundberg *et al*, 1994; Freyschmidt-Paul *et al*, 1999). The presence of these immunohistochemical changes in grafts and alopecic host skin of control mice, as observed in our study, further confirms the concept that hair loss induced by the performed graft technique is true AA.

In anti-CD44v10-treated mice, the most striking change, as compared with control mice, was a reduction of skin-infiltrating $CD8^+$ T cells. The finding that AA did not develop in skin virtually devoid of $CD8^+$ T cells but still containing $CD4^+$ T cells, suggests that $CD8^+$ T cells play an essential pathogenetic role in AA that is inhibited by anti-CD44v10. A key role of $CD8^+$ T cells in AA is also supported by three recent studies: Induction of hair regrowth in C3H/HeJ mice with AA by treatment with a contact sensitizer was accompanied by a reduction of perifollicular and intrafollicular $CD8^+$ T cells (Freyschmidt-Paul *et al*, 1999). *In vivo* depletion of $CD8^+$ T cells restored hair growth in the Dundee experimental bald rat model for AA (McElwee *et al*, 1996). Gilhar *et al* (1999) showed that AA could be transferred to human scalp explants on severe combined immunodeficiency mice by injection of $CD8^+$ T cells, but not by $CD4^+$ T cells.

We interpret our observation in the sense that anti-CD44v10 prevents the development of AA by inhibiting the action of $CD8^+$ T cells. Because CD44v10 expression on lymphocytes has been described to be important for migration of lymphocytes towards, and infiltration into dermal injury (Rösel *et al*, 1997), we hypothesize that anti-CD44v10 inhibits the autoimmune attack against the hair follicle by inhibiting the migration of $CD8^+$ T cells into the skin. This interpretation is in line with a recent observation of CD44v10 expression on peripheral blood leukocytes in various skin-associated autoimmune diseases (Seiter *et al*, 1998) and on peripheral blood leukocytes of rheumatoid arthritis patients (Endo *et al*, 1998). Finally, the absence of CD44v10-expressing lymphocytes in the skin of C3H/HeJ mice with AA points towards a requirement of the molecule restricted to the process of migration or extravasation and a subsequent loss of CD44v10 expression after the lymphocytes have entered target tissue; however, discrepant results have been obtained considering the latter aspect, e.g., in humans CD44v10-expressing lymphocytes have been found in lesional AA skin (Wagner *et al*, 1998). In other inflammatory skin diseases discrepant findings on CD44v expression have been reported, too (Seiter *et al*, 1998; Wagner *et al*, 1998). At least some of these differing staining patterns might be explained by hidden epitopes (Seiter *et al*, 1996). Despite these heterogeneous findings, our results support the concept that CD44v10 functions as a receptor for the homing of lymphocytes into the skin.

Whereas the expression of MHC class II or ICAM-1 on hair follicle epithelium remained unaffected by anti-CD44v10, the number of infiltrating $CD4^+$ T cells and the degree of MHC class I

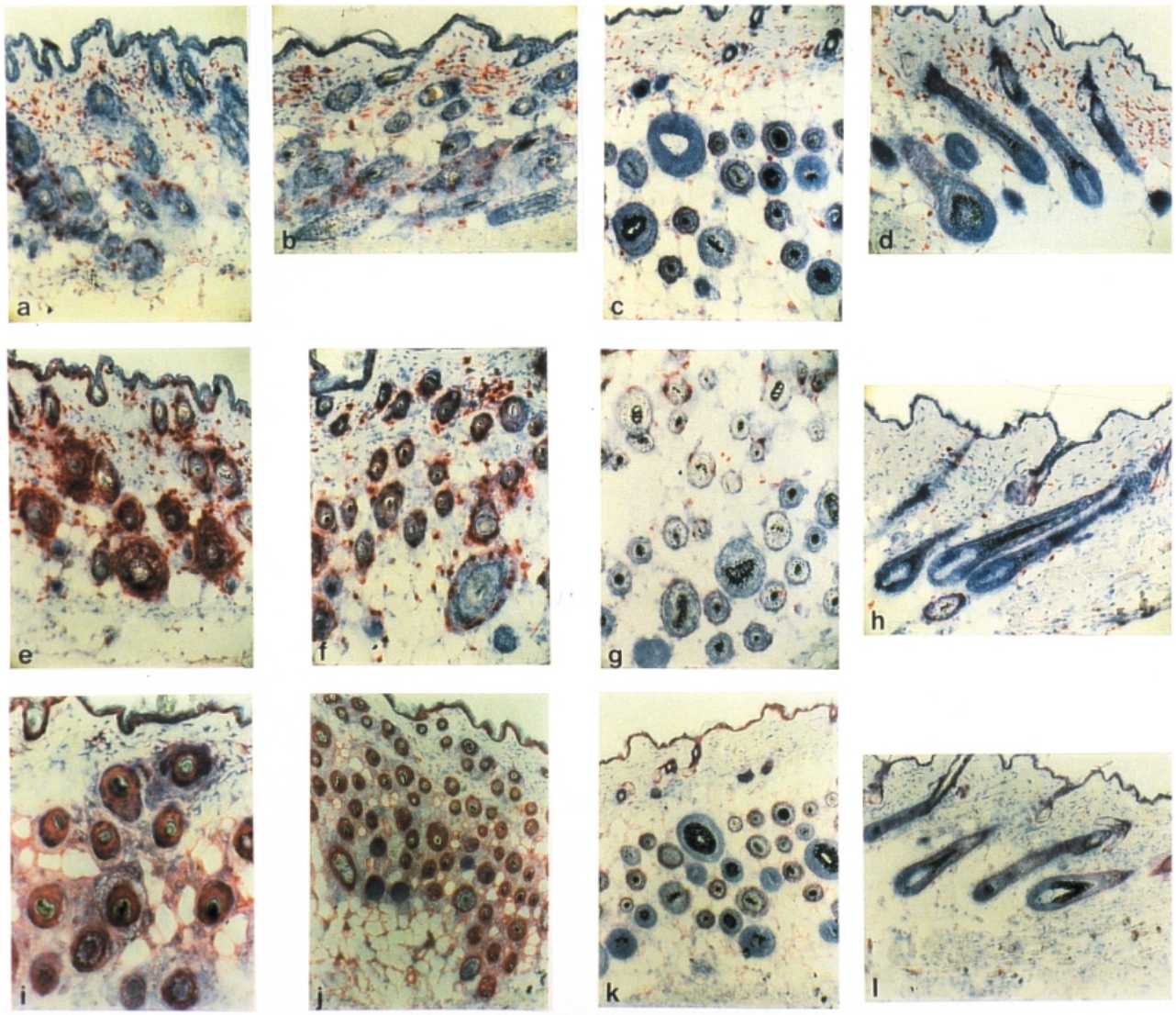

Figure 3. Decrease of CD8$^+$ and perifollicular CD4$^+$ cells and reduced MHC class I expression on hair follicle epithelium in anti-CD44v10-treated mice. Immunohistochemical staining for CD4 (*a–d*), CD8 (*e–h*), and MHC class I (*i–l*) of AA skin in PBS-treated control mice (*a*, *e*, *i*), AA skin in anti-CD44s-treated mice (*b*, *f*, *j*), grafted skin in anti-CD44v10-treated mice (*c*, *g*, *k*) and unaffected skin in anti-CD44v10-treated mice (*d*, *h*, *l*). (*a*) Perifollicular CD4$^+$ T cells in control mice; (*b*) no reduction of CD4$^+$ T cells in anti-CD44s-treated mice; (*c*) reduced number of perifollicular CD4$^+$ T cells in grafts of anti-CD44v10-treated mice; (*d*) no perifollicular CD4$^+$ T cells in unaffected skin of anti-CD44v10-treated mice; (*e*) dense perifollicular CD8$^+$ T cells in control mice; (*f*) slight reduction of perifollicular CD8$^+$ T cells in anti-CD44s-treated mice; (*g*) marked reduction of perifollicular CD8$^+$ T cells in grafted skin of anti-CD44v10-treated mice; (*h*) no perifollicular CD8$^+$ T cells in unaffected skin of anti-CD44v10-treated mice; (*i*) aberrant MHC class I expression on hair follicle epithelium in control mice; (*j*) no decrease of MHC class I expression in anti-CD44s-treated mice; (*k*) reduced MHC class I expression in grafted skin of anti-CD44v10-treated mice; (*l*) faint staining for MHC class I on hair follicle epithelium in unaffected skin of anti-CD44v10-treated mice.

Table I. Density of perifollicular and intrafollicular CD4$^+$ and CD8$^+$ T cells in skin of control mice, anti-CD44s and anti-CD44v10-treated mice

	Control graft	Control-induced AA	Anti-CD44s graft	Anti-CD44s-induced AA	Anti-CD44v10 graft	Anti-CD44v10 unaffected skin
CD4$^+$	++[a]	++	++	++	+	–
CD8$^+$	+++	+++	++	++	+	–

[a]No cells (–), single cells (+), moderately dense infiltrate (++), dense infiltrate (+++). Data are shown as the average per group.

expression on hair follicle epithelium was moderately reduced in anti-CD44v10-treated mice. Whether this reduction can be taken as an epiphenomenon due to the absence of the initial inflammatory infiltrate of CD8$^+$ T cells, remains to be explored.

In anti-CD44s-treated mice only a slight reduction of infiltrating CD8$^+$ T cells was observed, whereas the other immunohistochemical features remained unchanged. The weak influence of anti-CD44s on the number of infiltrating CD8$^+$ T cells, resulting in a

Table II. Expression of MHC class I, MHC class II, and ICAM-1 on hair follicle epithelium of control mice, anti-CD44s, and anti-CD44v10-treated mice

	Control graft	Control-induced AA	Anti-CD44s graft	Anti-CD44s-induced AA	Anti-CD44v10 graft	Anti-CD44v10 unaffected skin
MHC class I	+++[a]	+++	+++	+++	+	+
MHC class II	+++	+++	+++	+++	+++	+++
ICAM-1	+++	+++	+++	+++	+++	+++

[a]No staining (–), weak staining (+), moderate staining (++), intense staining (+++). Data are shown as the average per group.

delayed onset and reduced extent of hair loss, can be explained by the following theory: CD44s and CD44v10 might fulfill different functions during the autoimmune process leading to AA, whereby the function of CD44s might be substituted by other mechanisms. This explanation is supported by previous reports that demonstrated an inhibitory effect of antibodies directed against CD44 variant isoforms, but not of those directed against CD44s, on experimental autoimmune encephalomyelitis (Laman *et al*, 1998), or on trinitrobenzenesulfonic acid-induced colitis (Wittig *et al*, 1997, 1998). In accordance with our results, the lymphocytic infiltrate remained unchanged by anti-CD44s treatment in trinitrobenzene-sulfonic acid-induced colitis and also in murine arthritis, despite the mitigating effect of anti-CD44s on the latter disease (Verdrengh *et al*, 1995).

In conclusion, our findings demonstrate a pivotal role for CD44v10 in the development of AA as anti-CD44v10 efficiently inhibits the development of AA in C3H/HeJ mice, most likely by abrogating the immigration of $CD8^+$ T cells.

This study was supported by grants of the Deutsche Forschungsgemeinschaft (Ho 1598/1–3) to R.Ho. and (Zo40–5/3) to M.Z and the National Institute of Health (AR 43801) to J.P.S.

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