

## INVESTIGATIVE REPORT

# Analysis of the Expression of Cutaneous Lymphocyte-associated Antigen on the Peripheral Blood and Cutaneous Lymphocytes of Alopecia Areata Patients

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Alopecia areata has been reported to be accompanied by abnormal autoimmune dysfunction. We examined the expression of cutaneous lymphocyte-associated antigen (CLA), which is a skin-specific lymphocyte homing receptor, in the peripheral blood lymphocytes and skin of patients with alopecia areata. In the patients' peripheral blood, the percentage of CLA-positive CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes, was significantly higher than that of normal controls. The patients with severe or progressive alopecia areata showed a much higher CLA-positivity compared to patients recovering from the disease. A chronological study showed that the percentage of CLA-positive peripheral blood lymphocytes, CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes decreased in parallel with the patients' good clinical course. The CLA-positivity in peripheral blood lymphocytes, CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes of patients with alopecia areata who did not respond to oral corticosteroid therapy remained higher than in those who responded well to the treatment. In the affected scalp skin, many infiltrating lymphocytes around the hair follicles, which were CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes, expressed CLA. These findings suggest that the CLA-positivity correlates with clinical activity and that CLA-positive CD4<sup>+</sup> or CD8<sup>+</sup> lymphocytes may play an important role in alopecia areata. **Key-words:** alopecia areata; lymphocyte-associated antigen; lymphocytes.

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The cause of alopecia areata (AA) is not fully understood, but indirect evidence suggests that an autoimmune abnormality may be involved in the pathogenesis. For example, helper T cells or Langerhans' cells infiltrate surrounding hair follicles in patients with AA (1, 2). While Ledesma & York (3) reported a highly significant decrease in the suppressor T-cell population of patients with AA, Imai et al. (4) reported that the mean percentages of HLA-DR + CD3<sup>+</sup> cells and CD57-CD16<sup>+</sup> cells among peripheral blood

lymphocytes (PBL) in patients with AA were significantly higher than those of normal controls. Baadsgaard & Lindskov (5), however, reported that the median percentage of circulating CD3, 4, 8 and 57 positive cells among PBL was normal in patients with AA. In addition, recent reports have shown that autoimmune abnormalities exist in patients with AA (6, 7) and that there is a more frequent incidence of hair-follicle-specific antibody (6) and anti-nuclear antibodies (7) in the sera of patients with AA than in those of normal controls.

Cutaneous lymphocyte-associated antigen (CLA), recognized by the monoclonal antibody HECA-452, is a putative skin homing receptor and one of the ligands of E-selectin (8). Inflammatory lesions of the skin, such as contact dermatitis, contain 85% CLA-positive T lymphocytes, whereas in non-cutaneous inflammatory lesions only 5% of T cells are CLA-positive (9). Recently, the up-regulation of CLA expression on CD68-positive dendritic epidermal cells was observed in various skin diseases (10). Furthermore, CLA expression on T lymphocytes can be up-regulated by IL-6, IL-12 and TGFβ1 *in vitro* (11). Thus, CLA expression is probably involved in the function of immunological disorders.

We examined CLA expression on both PBL and affected scalp skin of patients with AA and compared this with normal healthy controls in order to investigate the immune abnormalities of AA.

## MATERIALS AND METHODS

### Patients

Fifty-eight patients with AA participated, including 36 (9 males and 27 females) with progressive or continuously severe AA (including 17 cases of alopecia totalis and universalis) and 22 (5 males and 17 females) with recovering AA who had visited the Department of Dermatology, Faculty of Medicine, University of Tokyo. Patients' ages ranged from 17 to 69 years in severe AA (mean 34.9 years) and from 13 to 63 years in recovering AA (mean 36.1 years). The age at onset of alopecia ranged from 3 months to 60 years in severe AA and from 6 months to 10 years in recovering AA. None of the subjects were clinically diagnosed as having connective tissue diseases, thyroiditis, or allergic dermatitis. Twenty-five non-allergic healthy volunteers (6 males and 19 females, mean 31.4 years) were also examined as normal controls. The study was approved by the Ethics Committee of Tokyo University Hospital. The patients gave their informed consent.

## Methods

**Collection of PBL.** Peripheral blood mononuclear cells (PBMC) of patients with AA and healthy volunteers were isolated from citrate-anticoagulated whole blood by dextran sedimentation and Ficoll-Paque centrifugation. PBL was selectively collected from PBMC using the gating function of FACS (see below).

**Staining and FACS analysis of PBL.** The analysis of PBL for the expression of cell surface markers was performed using FACSscan (Becton Dickinson & Co., Mountain View, Calif.) and CellQuest software (Becton Dickinson & Co.). Two-colour analysis was performed. Fresh PBMCs were washed twice and resuspended at 106 cells/ml in phosphate-buffered saline (PBS) with 1% fetal calf serum (FCS), and then incubated on ice for 30 min with FITC-conjugated anti-human CLA monoclonal antibody (mAb) (rat IgM; PharMingen, San Diego, Calif.), Cy-chrome-conjugated anti-human CD4 or Cy-chrome-conjugated anti-human CD8mAb (mouse IgG1; PharMingen, San Diego, Calif.). FITC-conjugated rat IgM and Cy-chrome-conjugated mouse IgG (PharMingen, San Diego, Calif.) were used as isotype controls; negative results were always obtained using these isotype controls. After washing twice with PBS, the PBL samples were selected from PBMC by gating with cell size, and the expressions of CLA, CD4 and CD8 were analysed among  $4 \times 10^4$  PBL.

**Biopsy of AA affected scalp skin.** Actively affected scalp skin samples were obtained from two patients with AA. Both patients were diagnosed as having progressive alopecia universalis. Normal skin was obtained at surgery of benign skin tumours. Skin samples were immediately frozen and stored at  $-80^\circ$  until use.

**Immunohistochemical staining.** Six-micrometre-thick cryostat sections were fixed in acetone for 20 min at  $-20^\circ$  and washed with PBS. The sections were blocked, then incubated first with rat anti-human CLA mAb (HECA-452, rat IgM; PharMingen, San Diego, Calif.), mouse anti-human CD4 (mouse IgG1) or mouse anti-human CD8 (mouse IgG1) overnight at  $4^\circ$ , followed by treatment with the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif.) in accordance with the manufacturer's recommendations. Rat IgM or mouse IgG1 (PharMingen, San Diego, Calif.) were used as negative controls. The immunoreactivity of sections was visualized by diaminobenzidine (Wako, Tokyo, Japan). The isotype-matched control antibodies always showed negative staining.

## Statistical analysis

P-values were calculated by an ANOVA with a post-hoc test (among severe AA, recovering AA and normal controls) or Welch's *t*-test (between total AA and normal controls). The data were shown as mean  $\pm$  SD (%). The accepted level of significance was  $p < 0.05$ .

## RESULTS

### CLA-positive percentage in PBL, CD4+ and CD8+ lymphocytes from peripheral blood of patients with AA

The numbers of PBL in patients with AA and normal controls proved to be almost the same by peripheral blood examination (data not shown). We analysed CLA-positivity in  $4 \times 10^4$  PBL per sample. As shown in Fig. 1, FACS analysis showed higher CLA-positivity in patients with AA ( $n = 58$ ) than in normal controls ( $n = 25$ ) ( $5.6 \pm 2.5\%$  vs  $3.3 \pm 1.2\%$  for PBL). The mean  $\pm$  SD values of CLA positivity among CD4+ or CD8+ lymphocytes in patients with AA and normal controls

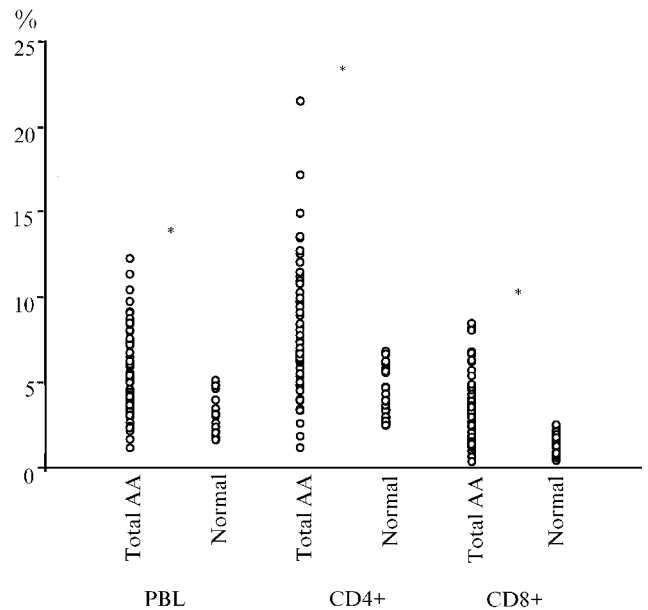


Fig. 1. Cutaneous lymphocyte-associated antigen (CLA) positivity in peripheral blood lymphocytes (PBL) and CD4+ or CD8+ lymphocytes from patients with alopecia areata (AA; total group) and normal controls. (\* $p < 0.01$  by Welch's *t*-test).

were as follows: CD4+, AA:  $8.2 \pm 3.8\%$ , normal:  $4.7 \pm 1.5\%$ ; CD8+, AA:  $3.5 \pm 2.1\%$ , normal:  $1.6 \pm 0.6\%$ . When the patients with AA were sub-grouped according to the activity of the disease (Table I), CLA-positivity in CD4+ and CD8+ lymphocytes in severe AA was significantly higher than in normal controls or recovering AA ( $p < 0.01$  for both). However, there was no significant difference of CLA-positivity in PBL, CD4+ or CD8+ lymphocytes between recovering AA and normal controls. We next chronologically examined CLA-positivity in PBL, CD4+ or CD8+ lymphocytes of patients with AA. We selected 8 patients with severe AA and with a relatively high CLA-positivity in PBL. All patients showed good hair re-growth by combination therapies such as liquid nitrogen application, topical corticosteroid, oral corticosteroid, topical calpronium chloride, diphenylcyclopropanone administration within 10 months. The CLA-positivity in PBL, CD4+ and CD8+ lymphocytes decreased in parallel with their improved clinical response (data not shown).

Table I. Percentage of CLA-positivity in subpopulations of lymphocytes in patients with alopecia areata (AA) of different severity and in healthy controls (mean  $\pm$  SD).

Subjects	PBL (% positivity)	CD4+ (% positivity)	CD8+ (% positivity)
Severe AA ( $n = 36$ )	$6.4 \pm 2.6^*$	$9.4 \pm 4.0^*$	$4.1 \pm 2.1^*$
Recov. AA ( $n = 22$ )	$4.2 \pm 1.8$	$6.2 \pm 2.5$	$2.4 \pm 1.5$
Controls ( $n = 25$ )	$3.3 \pm 1.2$	$4.7 \pm 1.5$	$1.6 \pm 0.6$

CLA = cutaneous lymphocyte-associated antigen; PBL = peripheral blood lymphocytes. \* $p < 0.01$  for difference compared to controls and recovering AA.

*Change of CLA-positivity in PBL, CD4+ and CD8+ lymphocytes in patients with AA during oral corticosteroid therapy*

Because oral corticosteroid therapy is usually effective in AA (12–15), although some patients are resistant to this therapy, we compared CLA-positivity in PBL, CD4+ and CD8+ lymphocytes between steroid-responsive (Group A; n=9) and resistant (Group B; n=7) patients after oral corticosteroid treatment. CLA-positivity in PBL, CD4+ and CD8+ lymphocytes was  $4.0 \pm 1.6\%$ ,  $6.1 \pm 2.6\%$  and  $2.0 \pm 1.0\%$  in Group A, and  $7.6 \pm 3.5\%$ ,  $11.3 \pm 5.7\%$  and  $5.0 \pm 2.7\%$  in Group B. The CLA-positivity in Group A was not significantly different from that in normal controls, but Group B showed a significantly higher CLA positivity in PBL, CD4+, CD8+ lymphocytes compared to normal controls and Group A ( $p < 0.01$  for all).

*Immunohistochemical stainings of the lesional skin of patients with AA using anti-human CLA mAb*

Immunohistochemical stainings of the lesional scalp skin of patients with AA showed infiltration of mononuclear cells around the hair follicles. Many of these cells were CD4+ or CD8+ lymphocytes, and among them many CLA-positive lymphocytes were detected (Fig. 2a–c). There were only a few CLA-positive lymphocytes around the hair follicles of normal controls (data not shown).

## DISCUSSION

There have been several studies (3–5, 21) investigating the character of lymphocytes in AA. Only one report (22) has shown that T lymphocytes of patients with AA that had been cultured with hair follicle homogenate, along with antigen-presenting cells of the patients, were able to induce the typical changes in AA. The changes included hairloss and perifollicular infiltrates of T lymphocytes, which suggests that CLA-positive T lymphocytes reacting with hair follicle antigens may exist in the affected scalp skin or PBL of AA patients. We therefore examined the percentage of CLA positivity in circulating lymphocytes among different clinical stages of AA.

We found that patients with severe AA showed a significantly higher CLA-positivity in PBL, CD4+ and CD8+ lymphocytes compared with that of normal controls. On the other hand, the recovering AA patients did not show any significant differences of CLA positivity from normal controls. These and other results suggest that CLA-positivity correlates closely with the clinical severity of AA and that both CD4 and CD8 are important in the pathogenesis of AA as CLA-positive cells.

It has been reported that oral corticosteroid therapy is effective for AA, especially severe types (12–15),

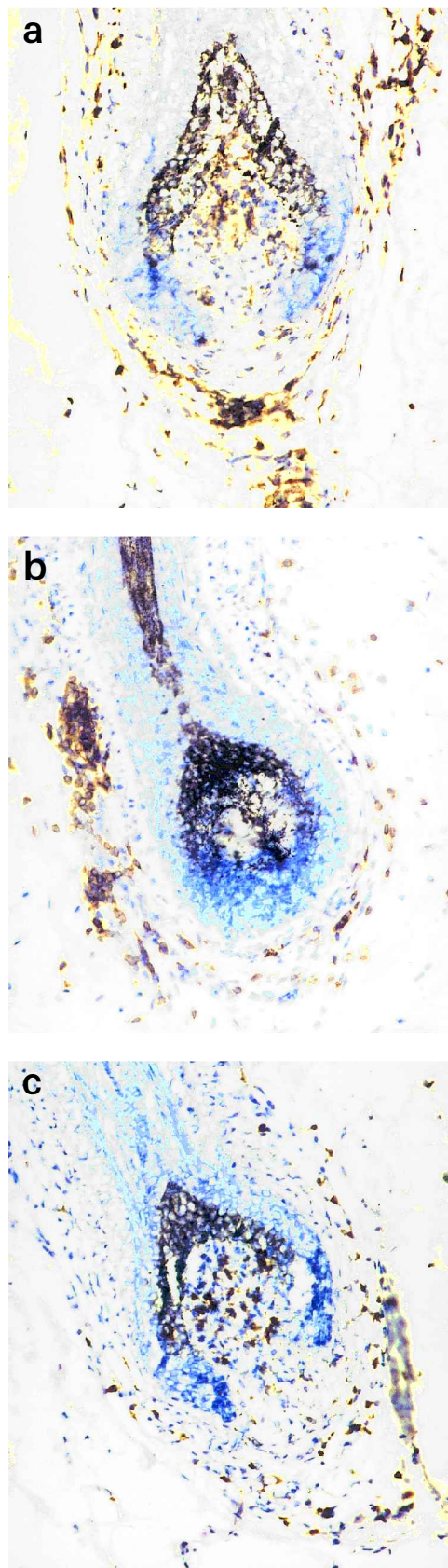


Fig. 2. Immunohistochemical staining of the affected scalp of an alopecia areata patient. (a) CLA, (b) CD4, (c) CD8 (original magnification,  $\times 400$ ).

although the indication of the preventive use of systemic steroids in AA is still a matter of discussion (23, 24). We used oral prednisolone 10–30 mg/day for some patients with severe AA who were relatively young adults and had no systemic disease. Our results showed that CLA-positivity in PBL, CD4+ and CD8+ lymphocytes decreased among the patients who responded well to oral corticosteroid therapy, but remained high among those who were resistant. Corticosteroids inhibit the immunological function of lymphocytes as well as the function of hair bulb cells, and it is speculated that these drugs inhibit the function of infiltrating CLA-positive lymphocytes or PBL in AA. As for the steroid-resistant patients who showed a high CLA-positivity, some other mechanisms, such as a dysfunction of steroid receptors or the enhanced secretion of cytokines (IL-6, IL-12, TGF $\beta$ 1) (11), may modulate CLA expression leading to worsening of AA lesions.

We detected the infiltration of CLA-positive lymphocytes around the hair follicles of patients with AA by immunohistochemistry. The lymphocytes around the AA hair follicles were also CD4 or CD8 positive. CD19- or CD20-positive B lymphocytes were few (data not shown). There was almost the same number of CLA+, CD4+ or CD8+ cells around the hair follicle of the AA patients. These results suggest that most of the lymphocytes infiltrating around the hair follicles of AA patients were CLA-positive T lymphocytes.

## REFERENCES

- Perret C, Wiesner-Menzel L, Happle R. Immunohistochemical analysis of T-cell subsets in the peribulbar and intrabulbar infiltrates of alopecia areata. *Acta Derm Venereol* 1984; 64: 26–30.
- Wiesner-Menzel L, Happle R. Intrabulbar and peribulbar accumulation of dendritic OKT-6 positive cells in alopecia areata. *Arch Dermatol Res* 1984; 276: 333–334.
- Ledesma GN, York KK. Suppressor cell decrease in alopecia areata. *Arch Dermatol Res* 1982; 274: 1–8.
- Imai R, Takamori K, Ogawa H. Changes in populations of HLA-DR+CD3+ cells and CD57-CD16+ cells in alopecia areata after corticosteroid therapy. *Dermatology* 1994; 188: 103–107.
- Baadsgaard O, Lindskov R. Circulating lymphocyte subsets in patients with alopecia areata. *Acta Derm Venereol* 1986; 66: 266–268.
- Tobin DJ, Orentreich N, Fenton DA, Bystryn JC. Autoantibodies to hair follicles in alopecia areata. *J Invest Dermatol* 1994; 102: 721–724.
- Yano S, Ihn H, Nakamura K, Okochi H, Tamaki K. Antinuclear and antithyroid antibodies in 68 Japanese patients with alopecia areata. *Dermatology* 1999; 199: 191.
- Picker LJ, Kishimoto TK, Smith CW, Warnock RA, Butcher EC. ELAM-1 is an adhesion molecule for skin-homing T cells. *Nature* 1991; 349: 796–799.
- Picker LJ, Michie SA, Rott LS, Butcher EC. A unique phenotype of skin-associated lymphocytes in humans. *Am J Pathol* 1990; 136: 1053–1068.
- Nakamura K, Yasaka N, Asahina A, Kato M, Miyazono K, Furue M, et al. Increased numbers of CD68 antigen positive dendritic epidermal cells and upregulation of CLA expression on these cells in various skin diseases. *J Dermatol Sci* 1998; 18: 170–180.
- Picker LJ, Treer JR, Ferguson-Darnell B, Collins PA, Bergstresser PR, Terstappen LMM. Control of lymphocyte recirculation in man. II. Differential regulation of the cutaneous lymphocyte-associated antigen, a tissue-selective homing receptor for skin-homing T cells. *J Immunol* 1993; 150: 1122–1136.
- Kern F, Hoffman WH, Hambrick GW, Blizzard RM. Alopecia areata: immunologic studies and treatment with prednisone. *Arch Dermatol* 1973; 107: 407–412.
- Virginia C, Fiedler MD. Alopecia areata: a review of therapy, efficacy, safety, and mechanism. *Arch Dermatol* 1992; 128: 1519–1529.
- Whiting DA. The treatment of alopecia areata. *Cutis* 1987; 40: 247–250.
- Sharma VK. Pulsed administration of corticosteroids in the treatment of alopecia areata. *Int J Dermatol* 1996; 35: 133–136.
- Duijvestijn AM, Horst E, Pals ST, Rouse BN, Steere AC, Picker LJ, et al. High endothelial cell differentiation in human lymphoid and inflammatory tissues defined by monoclonal antibody HECA-452. *Am J Pathol* 1990; 130: 147–155.
- Horst E, Meijer CJ, Duijvestijn AM, Hartwig N, Van der Harten HJ, Pals ST. The ontogeny of human lymphocyte recirculation: high endothelial cell antigen (HECA-452) and CD44 homing receptor expression in the development of the immune system. *Eur J Immunol* 1990; 20: 1483–1489.
- van der Valk P, Mullink H, Huijgens PC, Tadema TM, Vos W, Meijer CJ. Immunohistochemistry in bone marrow diagnosis: value of a panel of monoclonal antibodies on routinely processed bone marrow biopsies. *Am J Surg Pathol* 1989; 13: 97–106.
- van Dinther-Janssen AC, Pals ST, Scheper R, Breedveld F, Meijer CJ. Dendritic cells and high endothelial venules in the rheumatoid synovial membrane. *J Rheumatol* 1990; 17: 11–17.
- Meijer CJ, Beljaards R, Horst E, Willemze P, van der Valk P, Pals ST. Differences in antigen presentation between primary cutaneous and node-based lymphomas. *J Invest Dermatol* 1989; 92: 479.
- Ogg BGS, Dunbar PR, Romedo P, Chen J, Cerundolo V. High frequency of skin-homing melanocyte-specific cytotoxic T lymphocytes in autoimmune vitiligo. *J Exp Med* 1998; 188: 1203–1208.
- Gilhar A, Ullmann Y, Berkutzi T, Assay B, Kalish RS. Autoimmune hair loss (alopecia areata) transferred by T lymphocytes to human scalp explants on SCID mice. *J Clin Invest* 1998; 101: 62–67.
- Fisher DA. Desideratum dermatologica-wanted: a dependable and safe means to prevent alopecia areata progression to the totalis/universalis state. *Int J Dermatol* 1998; 37: 497–499.
- Nelson DA, Spielvogel RL. Alopecia areata. *Int J Dermatol* 1985; 24: 26–34.