Immunohistochemical Analysis of T-cell Subsets in the Peribulbar and Intrabulbar Infiltrates of Alopecia areata

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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 (Stockh) 1984; 64: 26–30.

In 11 patients with untreated alopecia areata in the progressive stage of the disease, an in situ analysis of the inflammatory infiltrate of the hair bulbs was performed by means of different monoclonal antibodies. Most of the peribulbar cells reacted with the pan T-cell antibodies OKT 3 and Lyt 3. Staining for T-cell subsets revealed that the proportion of OKT 4^+ cells was about fourfold higher than that of OKT 8^+ cells. Almost all of the T cells were OKIa1$^+$, indicating that they were in an activated state. In four of the 11 cases, both subsets of T lymphocytes were also found to infiltrate the hair matrix itself. These results would appear to be consistent with the assumption that alopecia areata is caused by a T cell mediated autoimmune mechanism. *Key words: Hair disease; Histophathology; Immunoperoxidase method; Monoclonal antibodies; T cell subpopulations.* (Received April 5, 1983.)

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Alopecia areata is a common hair disease of unknown etiology. Recent therapeutic studies and laboratory findings suggest that the disease is caused by autoimmunity (1–5). In view of the fact that autoantibodies against constituents of the hair follicle could so far not be demonstrated (6, 7), it has been postulated that the underlying autoimmune mechanism is mediated by T cells rather than by B cells (8).

In a previous communication we reported results of an in situ analysis performed in 5 patients with alopecia areata, demonstrating that the peribular infiltrates consist predominantly of T cells (9). It was so far not possible, however, to determine the ratio of T cell subsets within these infiltrates. The present study includes 11 patients in whom we were able to analyse the inflammatory infiltrate of one and the same hair bulb with different monoclonal antibodies.

PATIENTS AND METHODS

Excisional scalp biopsy samples were obtained from 24 patients with untreated alopecia areata in the progressive stage of the disease. The biopsies were taken from the margin of a bald area. Routine histological examination revealed that 14 of these patients showed very marked peribulbar infiltrates (Fig. 1*a*), and these cases were selected for immunohistochemical analysis. Using serial sections stained with Mayer's haemalum, we ensured that at least three or four slides with unstained cryostat sections of a given hair bulb were available for analysis. The sections were air-dried and fixed in acetone. Subsequently, the following steps were performed at room temperature in a moist chamber, with washing twice in phosphate-buffered saline (PBS, pH 7.2) after each step:

1. incubation with undiluted normal goat serum (NGS);
2. application of one of the monoclonal antibodies Lyt 3 (NEN, charge no. FPA 491), OKT 3 (Ortho Pharmaceuticals, charge no. 11 J 041), OKT 4 (Ortho Pharmaceuticals, charge no. 11 A 007), OKT 8

(Ortho Pharmaceuticals, charge no. 11 A 009) and OKIa 1 (Ortho Pharmaceuticals, charge no. 11 M 050), at a dilution of 1 : 20 in PBS/10% NGS, using 20 μl/section, applied for 30 min;
3. incubation with peroxidase-conjugated goat-anti-mouse-IgG (Medac) as a second antibody, at a dilution of 1 : 20 in PBS/10% NGS for 30 min;
4. visualization of peroxidase activity by incubation with a saturated and filtered solution of diaminobenzidine (Serva) in PBS containing 0.01% H_2O_2, performed for 10 min;
5. darkening and fixation with a 1% solution of osmium tetroxide, applied for 20 min.

As a control, one slide of a given series was incubated with PBS instead of the monoclonal antibody.

Due to technical difficulties, 3 cases could not be analysed. In the remaining 11 cases, the inflammatory infiltrates of the hair bulbs were photographed at a fixed magnification (×40), and the reacting cells within a given area were counted.

RESULTS

The two pan T-cell antibodies OKT3 and Lyt3 both gave identical results. Most of the inflammatory peribulbar cells were positive (Fig. 1 *b*). After application of the OKT4 antibody, 65–70% of the peribulbar cells were positive (Fig. 1 *c*), whereas after incubation with the OKT8 antibody the proportion of positive cells was 15–20% (Fig. 1 *d*). Thus, the OKT4/OKT8 ratio was approximately 4 : 1, and this preponderance of OKT 4^+ cells was a rather constant finding.

Incubation with the OKIa 1 antibody resulted in a positive reaction of almost all of the bulbar and peribulbar cells.

In 4 of the 11 cases we found a scattered intrabulbar infiltrate of round cells bearing T-cell markers (Fig. 2 *a–d*). Both the OKT4 and OKT8 subtypes were present. In view of the limited number of positive cells, we were so far unable to determine the exact ratio of the two subsets within the intrabulbar infiltrate, but it was our general impression that this ratio did not differ from that of the peribulbar infiltrate.

DISCUSSION

The results show that in the progressive stage of alopecia areata, the bulk of the peribulbar infiltrate consists of T cells with a 4 : 1 ratio of OKT 4^+ to OKT 8^+ cells. Apparently, the number of B lymphocytes and macrophages is very low within the round-cell infiltrate, at least in this stage of the disease, but this obviously does not exclude the possibility of an important functional role for these cells in the pathomechanism of alopecia areata.

The fact that we were able to demonstrate T lymphocytes of both the OKT4 and OKT8 subsets infiltrating the hair matrix confirms previous reports of the presence of intrabulbar round cells in this disease (10, 11). The infiltration of the zone of regeneration of hair follicles is certainly of importance in the pathogenesis of this disease. As it is so far not possible, however, to distinguish suppressor cells from cytotoxic cells, the precise role of the intrabulbar T lymphocytes remains unclear.

Almost all of the inflammatory cells surrounding and infiltrating the hair bulbs stained with the OKIa 1 antibody. This antibody reacts with 90% of B cells and monocytes and 20% of 0 cells as well as with activated T lymphocytes. We did not perform a double tracing analysis of T cell markers and Ia-positive cells. In view of the fact, however, that we have demonstrated in serial sections of the same hair root the T cell nature of the bulk of the peribulbar and intrabulbar infiltrates, the reaction with the OKIa 1 antibody suggests that most of these cells are activated T lymphocytes. Further research should show whether this pattern of reaction is observed in all patients with alopecia areata, or whether these findings are characteristic of the progressive stage of the disease.

The results of our *in situ* analysis are consistent with the findings of those authors who

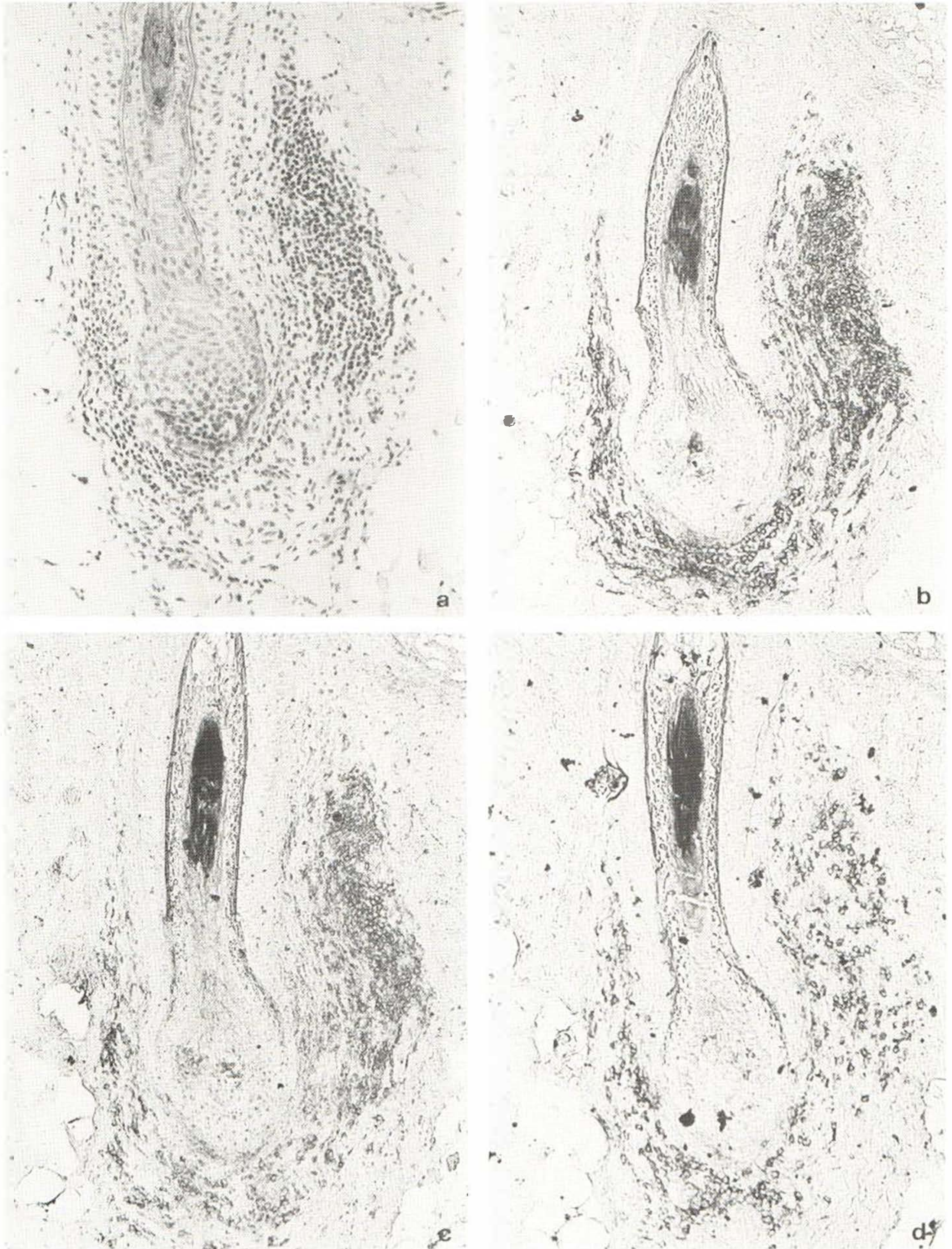

Fig. 1. Alopecia areata. Four cryostat sections of the same hair bulb. (*a*) Staining with Mayer's haemalum shows a dense peribulbar infiltrate. (*b*) Lyt 3 antibody reacts with 80–90% of peribulbar cells. (*c*) OKT 4 antibody reacts with 65–70% of the peribulbar cells. (*d*) OKT 8 antibody reacts with 15–20% of peribular cells.

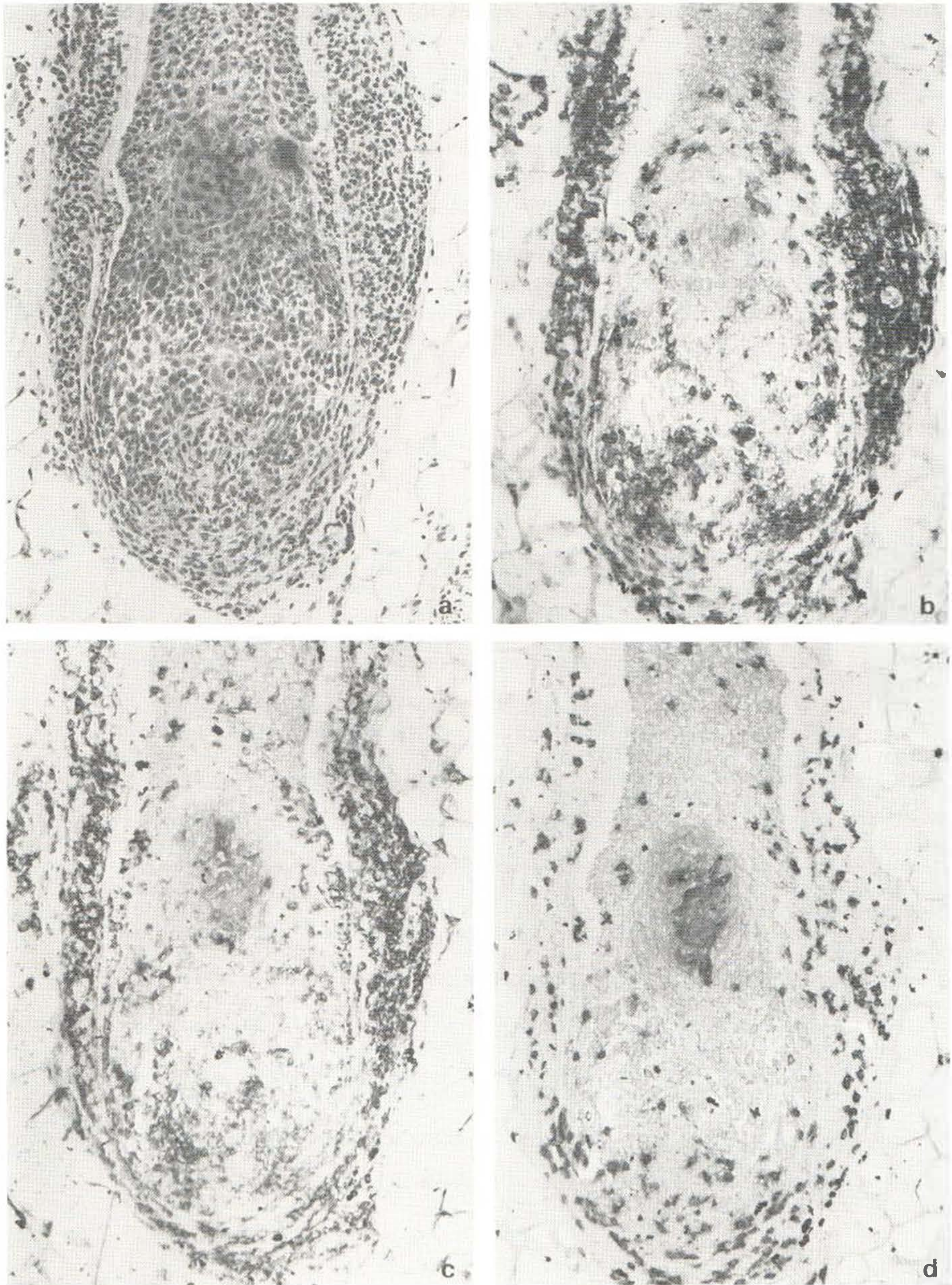

Fig. 2. Alopecia areata. Demonstration of intrabulbar T lymphocytes of both subsets. Their ratio seems to be similar to that of peribulbar T cells. Four cryostat sections of the same hair bulb, stained with (*a*) Mayer's haemalum, (*b*) Lyt 3 antibody, (*c*) OKT 4 antibody, and (*d*) OKT 8 antibody.

reported a decreased proportion of OKT 8⁺ cells in the peripheral blood of patients suffering from alopecia areata as compared with the normal ratio which is 2 : 1 at the most (12), or a decrease in peripheral T_γ cells which are known to exert suppressor activity (3). Other authors, however, found an increase of peripheral OKT 8^+ cells (13) or T_γ cells (4) in their patients. In the present study we did not examine the blood lymphocytes, but our preliminary findings obtained in other patients affected with alopecia areata have not shown an increase of OKT 8^+ cells in the blood.

In conclusion, this immunohistochemical analysis of untreated alopecia areata provides further evidence favouring the hypothesis that the disease is caused by an autoimmune mechanism mediated by T lymphocytes. In a further study we shall compare the present results with those obtained after induction of hair growth with an allergic contact dermatitis, in order to see whether this mode of treatment affects the OKT 4/OKT 8 ratio of the inflammatory infiltrate of the hair bulbs.

ACKNOWLEDGEMENT

This study was supported by the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen (grant no. FA 8684).

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