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Evidence for a Complement-mediated Inhibition and an Antibody-dependent Cellular Cytotoxicity of Dermal Fibroblasts in Alopecia Areata

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Immunological mechanisms have long been suggested to mediate hair loss in alopecia areata. In this process hair bulb melanocytes and dermal papilla fibroblasts are believed to be primarily involved. In the present study we further investigated the role of humoral factors in alopecia areata. Three different experiments were performed on normal human epidermal melanocytes as well as normal human dermal fibroblasts: (i) incubation with medium containing 2, 10, or 20% alopecia areata serum ($n = 12$ patients) for 16 h, (ii) incubation with medium supplemented with preheated alopecia areata serum (1 h at 56°C) and healthy human fresh serum as a complement source (1:1) and (iii) incubation with 2, 10 or 20% alopecia areata serum but, in addition, containing peripheral blood mononuclear cells from healthy subjects (effector/target ratio, 50:1). As controls, normal human fibroblasts and normal human epidermal melanocyte cultures were also incubated with serum from healthy individuals ($n = 5$) under the same culture conditions. The results showed that alopecia areata serum exerted a significant stimulation of proliferation of both normal human fibroblasts ($p > 0.05$ at 2%, $p > 0.05$ at 10%, $p < 0.05$ at 20%), and normal human epidermal melanocytes ($p > 0.05$ at 2%, $p < 0.05$ at 10%, $p > 0.05$ at 20%). Interestingly, however, alopecia areata serum induced a significant dose-dependent proliferation inhibition of normal fibroblasts, when there were preheated and supplemented with a complement source ($p < 0.05$ at 2%, $p < 0.05$ at 10%, $p < 0.01$ at 20%), and also when peripheral blood mononuclear cells were added ($p < 0.05$ at 2%, $p < 0.05$ at 10%, $p > 0.05$ at 20%). In both conditions, however, no significant influence was found after incubation of normal human epidermal melanocytes with alopecia areata serum ($p > 0.05$). Our data suggest that dermal fibroblasts may somehow be involved in the pathogenesis of alopecia areata and that two mechanisms could possibly contribute to their inhibition and damage: (1) a complement-mediated and (2) an antibodydependent cellular cytotoxicity. The role of hair bulb melanocytes remains to be further delineated. ***Key words: epidermal melanocytes; humoral factors; pathogenesis.***

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Alopecia areata (Aa) is a common disease of the hair follicle, affecting about 2% of new patients attending dermatology clinics (1). Although several hypotheses have been proposed, the exact pathogenesis of the hair loss is not clear. The common association of Aa with autoimmune thyroiditis (2), pernicious anemia (3), Addison's disease, vitiligo (4), discoid and systemic lupus erythematosus (5) and autoimmune atrophic gastritis (6) indicates that hair loss might also be mediated by autoimmune mechanisms. In further support of this concept are the frequently reported peripheral T-cell dysregulation (7, 8), the increased frequency of circulating organ-specific autoantibodies (9) and the presence of peribulbar T-lymphocytic infiltrate (10, 11). In addition, the immunosuppressive and/or immunomodulatory effects of most treatment modalities inducing hair regrowth, such as PUVA (12), intralesional and topical steroids (13), topical cytotoxic drugs (14) and contact allergens (15) are in favour of this concept. Nevertheless, no specific antibody to any part of the hair apparatus has consistently been demonstrated, and the exact cell target in the affected hair follicle is not known (1).

In this report, evidence for cytolytic antibodies in Aa patients is put forward. Furthermore, our results show that dermal fibroblasts undergo inhibition or damage by a complement-mediated and an antibody-dependent cellular cytotoxicity mechanism (ADCC) in vitro. We believe that these findings provide a preliminary clue supporting the immune-mediated pathogenesis of hair loss in Aa.

MATERIAL AND METHODS

Twelve patients with active Aa lesions were included in the present study (3 males and 9 females, mean age 26.5 years). The clinical data of the patients are summarized in Table I. Peripheral venous blood (20 ml) was withdrawn from each patient under sterile conditions and sera were separated and stored immediately until use at −70°C. Sera obtained from 5 healthy hospital employees (2 males, 3 females, mean age 29 years) served as control group.

Melanocyte culture

Normal human epidermal melanocytes (NHMel) were cultivated from two different donors by modification of the method by Gilchrest et al. (16). Epidermal cells were grown in tissue culture flasks (Falcon 75 cm^2, Becton & Dickinson, Heidelberg, Germany) in 5% CO_2, at 37°C in a humidified atmosphere. The medium used was modified MCDB 153 (Seromed, Heidelberg, Germany) with added amino acids and supplemented with the following substances: 2 mM Ca^{++}, 10 μg/ml transferrin, 5 μg/ml insulin (Sigma, Deisenhofen, Germany), 0.4% whole bovine pituitary extract (Clonetics Inc., San Diego, CA, USA), 2 ng/ml bovine basic fibroblast growth factor (bFGF) (Boehringer Mannheim, Germany), 10^{-9} M cholera toxin (Calbiochem), 5×10^{-7} M hydrocortisone (Serva, Heidelberg, Germany) and 100 IU/ml penicillin, 100 μg/ml streptomycin (both Seromed). Ten per cent fetal calf serum was added within the first 2 days of the primary culture and the first 24 h of each subculture. Pure melanocyte cultures were obtained, as could be seen by positive staining with the melanocyte markers S 100- (Dakopatts, Hamburg, Germany) and K 1.2.58- (Cell Diagnostika GmbH, Münster, Germany) monoclonal antibodies (MoAbs) using the APAAP (alkaline-phosphatase anti-alkaline-phosphatase) method.

Fibroblast cultures

Normal human dermal fibroblasts (NHFib) were obtained from the dermis trimmed at processing the same foreskin used for melanocyte

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Table I. *Clinical characteristics of patients* (n = *12*) *and controls* (n = *5*)

All patients were in an acute phase of alopecia areata (Aa) and without any treatment

N° of pat./sex	Age (years)	Aa type	Initial start of disease
Aa-group			
1/F	14	Aa patchy	8 years
2/M	44	Aa patchy	15 years
3/F	22	Aa universalis	8 months
4/M	25	Aa ophiasis type	9 months
5/F	16	Aa patchy	14 months
6/F	12	Aa subtotalis	12 months
7/F	21	Aa patchy	12 months
8/M	28	Aa universalis	10 years
9/F	26	Aa patchy	9 years
10/F	36	Aa patchy	15 months
11/F	41	Aa subtotalis	13 years
12/F	34	Aa totalis	8 years

culture by the explant method. Dermal tissues were incubated in fibroblast growth medium consisting of Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum, 1.7 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin (all Seromed). Pure fibroblast culture was obtained as evident from negative labelling with S 100 and K 1.2.58 MoAbs, and positive labelling with vimentin. Second passage NHFib were maintained in melanocyte growth medium for at least one week prior to incubation with Aa sera.

Proliferation and cytotoxicity assays

The proliferation and cytotoxicity assays were performed in tissue culture cluster plates (96 flat-bottomed wells) (Becton & Dickinson, Heidelberg, Germany) by incubating 4×10^3 cells/well in 0.2 ml of media. Second passage NHMel and NHFib were used in this experiment; they were incubated with 2%, 10%, and 20% fetal calf serum for 48 h prior to incubation with sera of Aa patients and controls. The NHMel and NHFib were divided into 3 groups. The first group was incubated with Aa sera at 2%, 10% and 20% concentrations. The second group was incubated with sera preheated at 56°C for one hour to inactivate the intrinsic complement followed by supplementation with a pool of fresh sera (1:1 ratio) from normal individuals as a complement source, at the concentrations mentioned above. The third group was incubated with sera from Aa patients at the concentrations mentioned earlier together with peripheral blood mononuclear cells (PBMC) obtained from two different healthy donors. The ratio of NHFib and NHMel to PBMC (T/E) was 1:50. Each target cell group was taken in duplicate; one group was incubated with Aa sera and the second with control sera. Proliferation and cytotoxicity assays were performed after 16 h of incubation by a fluorometric microassay using 4-methylumbelliferyl heptanoate (4-MUH) (Sigma). Fluorescence values were measured by a Titertec Fluoroskan II (Flow Lab., Meckenheim, Germany) as previously described (17, 18).

Evaluation and statistical analysis

Statistical differences of the data were evaluated by the two-sided Student's *t*-test. The fluorescence units obtained reflect the number of living cells and correlate well with 3H-thymidine uptake. The percentage of cytotoxicity was calculated from the formula:

$$\% \text{ cytotoxicity} = 100 \times \frac{(C-D) - (E-C1)}{C}$$

where C represents the fluorescence values of the control cultures that received no effectors; Cl is the fluorescence of the effectors remaining in control wells after 3 washes with phosphate-buffered saline; D is the fluorescence of the attached targets in the C cultures after three washes; and E is the fluorescence values of the samples that received PBMC.

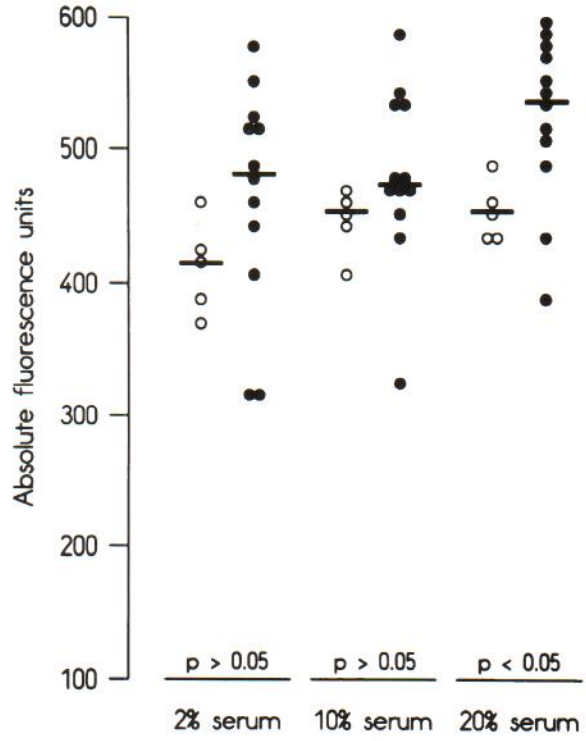

Fig. 1. Stimulatory effects of sera from alopecia areata patients on normal human dermal fibroblasts. ○ Normal sera, ● Alopecia areata.

RESULTS

Proliferation and cytotoxicity assays

Two different NHMel and NHFib cultures maintained in melanocyte growth medium and prestimulated with fetal calf serum at

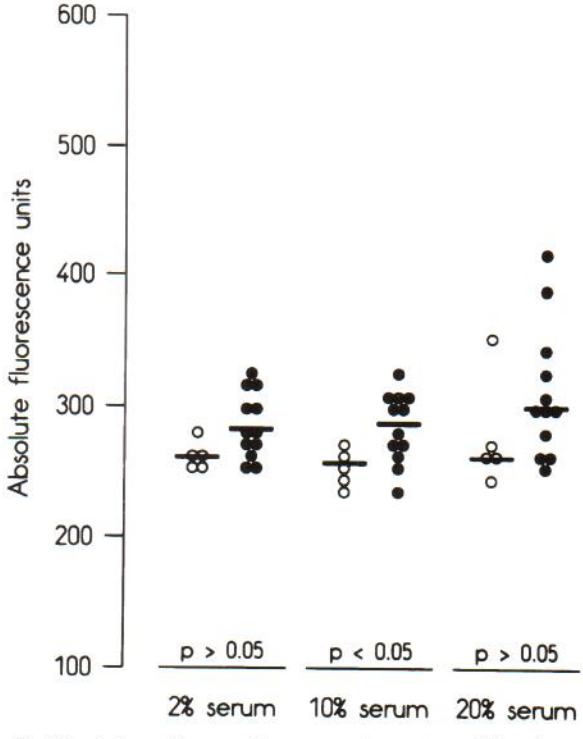

Fig. 2. Stimulation of normal human melanocyte proliferation after incubation with alopecia areata sera. ○ Normal sera, ● Alopecia areata.

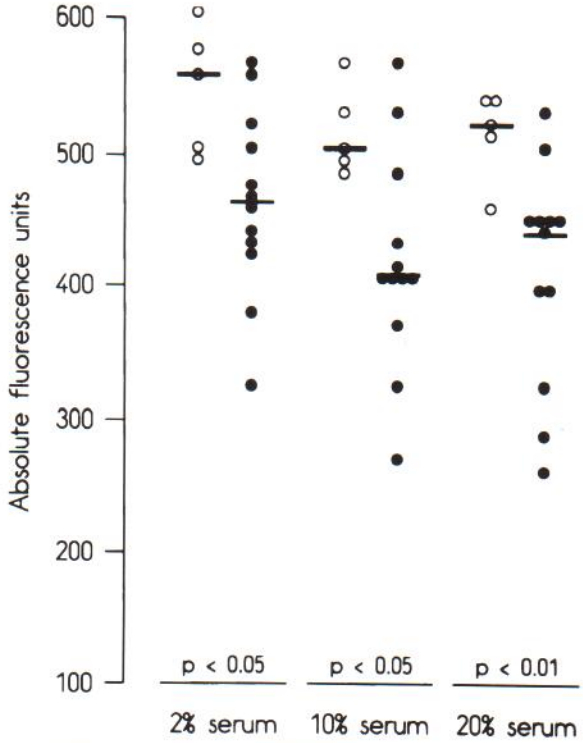

Fig. 3. Dose-dependent inhibitory effects on normal human dermal fibroblasts after incubation with preheated Aa serum combined with a complement source. ○ Normal sera, ● Alopecia areata.

the same concentrations as the test sera, i.e. at 2%, 10% and 20%, were examined for their susceptibility to inhibition or killing by Aa sera, decomplemented Aa sera plus a complement source and Aa sera together with PBMC. Sera of Aa patients alone induced a mild but significant stimulation of proliferation of NHFib, particularly at high serum concentrations ($p > 0.05$ at 2%, $p > 0.05$ at 10%, $p < 0.05$ at 20%) (Fig. 1). Similarly, NHMel proliferation was stimulated with Aa sera ($p > 0.05$ at 2%, $p < 0.05$ at 10%, $p > 0.05$ at 20%) (Fig. 2).

On the other hand, decomplemented Aa sera that were supplemented with complement resulted in a significant dose-dependent proliferation inhibition of NHFib ($p < 0.05$ at 2%, $p < 0.05$ at 10%, $p < 0.01$ at 20%) (Fig. 3), whereas the same sera induced either no effect or a mild significant stimulation of NHMel proliferation ($p < 0.05$ at 2%, $p > 0.05$ at 10%, $p > 0.05$ at 20% serum concentration) (data not shown). Although equal numbers of target cells were used at time 0 of the assay, higher fluorescence units were measured after 16 h in wells containing decomplemented sera plus complement, in comparison with wells incubated with target cells and sera alone.

Sera of Aa patients together with normal PBMC resulted in a mild significant decrease in the number of living NHFib, particularly at high serum concentrations ($p < 0.05$ at 2%, $p < 0.05$ at 10%, $p > 0.05$ at 20%). The estimated percentage of cell lysis was 11.8% at 2%, 14.7% at 10% and 19.7% at 20% (Fig. 4). No significant inhibition of proliferation or increased cellular cytotoxicity was measured with NHMel at any of the added serum concentrations ($p > 0.05$ at 2%, $p > 0.05$ at 10%, $p > 0.05$ at 20%) (data not shown).

In general, fluorescence units of NHFib wells were higher than those of NHMel after 16 h of incubation in all the experiments carried out in this study, although equal numbers of target cells were initially given to each well.

DISCUSSION

The possible role of circulating antibodies in inducing hair loss in Aa has been suspected for many years, but results of experimental work in this field are inconsistent. Bystryn et al. (19) and Safai et al. (20) have demonstrated deposition of $Complement_3$ and occasionally IgG and IgM in affected hair follicles of patients with Aa. In another study, however, there was no significant difference in the frequency of immune complex deposition between normal and affected scalp of Aa patients (21). Other studies have failed to show any immunoglobulin or complement deposition in Aa (22). Animal studies were also inconclusive. Complete hair regrowth in grafts obtained from lesions of Aa and transplanted onto nude mice has been observed, suggesting that humoral factors might be responsible for the hair loss (23). However, injection of human Aa sera has failed to inhibit hair growth in transplants of human scalp skin grafted onto nude mice (24). Differences in disease activity, different methods of evaluation and differences in the effectiveness of complement system between mice and humans provide some explanations for these apparent controversies.

The significance of inhibition and damage of NHFib in our study is not known. This reaction, however, seems to be selective, as NHMel are not significantly affected and also specific as vitiligo sera exerted similar reactions on NHMel, as already reported in separate studies carried out by us and others (25, 26). Dermal papillae, mainly composed of a specialized subpopulation of fibroblasts, are critical for the development of hair

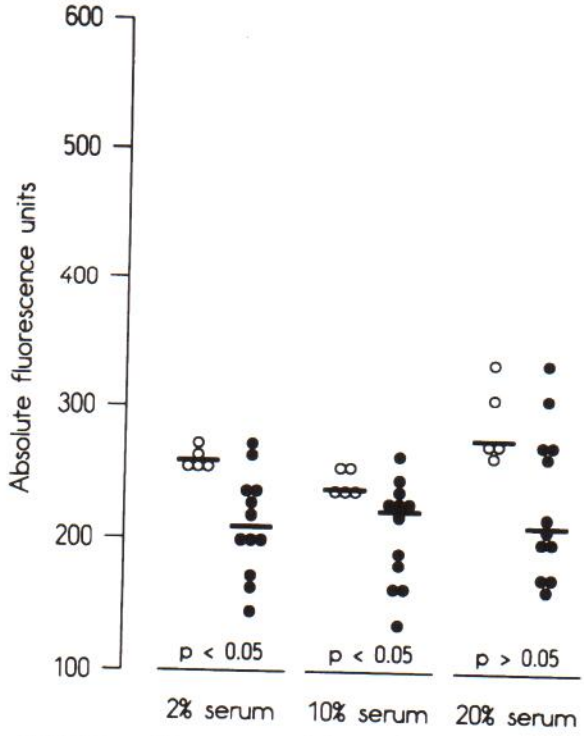

Fig. 4. Inhibitory effects on normal human dermal fibroblasts after incubation with Aa sera together with PBMC from healthy individuals. ○ Normal sera, ● Alopecia areata.

(27). It can be postulated that anti-dermal papilla fibroblast antibodies in Aa sera cross-react with NHFib that have been cultivated in vitro. In support of this assumption are the nail changes commonly observed in association with Aa, largely regarded as due to involvement of nail matrix cells (28, 29).

At present the significance of the stimulatory effect of Aa sera on NHFib and NHMel is not known, but a similar effect has also been observed, albeit with NHMel, with sera of vitiligo patients (26). The high fluorescence values measured with preheated sera may be due to the effect of heat or the addition of fresh serum as a complement source. The higher fluorescence values obtained from all wells containing NHFib in comparison with NHMel are most probably due to the higher proliferative capacity of fibroblasts versus melanocytes, particularly in the presence of a serum-containing medium (16). Fluorescence values obtained in an ADCC reaction cannot be compared with those of sera alone or sera plus complement, since wells in this reaction contained two types of cells, namely PBMC and target cells rather than target cells alone.

Bulbar melanocytes have frequently been suggested to play a role in the pathogenesis of Aa. Aa often spares depigmented hairs; regrowing hairs are initially depigmented, and retinal and epidermal melanocytes may also be involved in the disease process (1). Our results did not show any evidence of significant NHMel inhibition or killing by Aa sera, although cells were intentionally maximally stimulated with several mitogens and fetal calf serum to detect any minor inhibitory effects. Nevertheless, the role of bulbar melanocytes in Aa cannot be ruled out. Our experiments have been carried out using epidermal melanocytes which might be antigenically different from those of hair bulbs. Indeed, further investigations have to be carried out to elucidate the role of bulbar melanocytes and dermal papilla cells as target cells in Aa.

Although these results seemingly provide clear evidence for autoimmune mechanisms in Aa, the question whether circulating antibodies are the effect or the initial cause of hair follicle injury is still not resolved. Nonetheless, the reported aberrations of peripheral T-cell subpopulations, the demonstration of significant T-cell infiltrate around the hair follicle and the presence of circulating antibodies suggest that hair loss in Aa might be, at least in part, immunologically mediated.

REFERENCES

1. Alopecia areata workshop. J Invest Dermatol 1991; 96: 76S-90S.
2. Klein U, Weinheimer B, Zaun H. Simultaneous occurrence of alopecia areata and immunothyroiditis. Int J Dermatol 1974; 13: 116–118.
3. Allison IR, Curtis AC. Vitiligo and pernicious anemia. Arch Dermatol 1955; 72: 407–408.
4. Cunliffe WJ, Hall R, Newell DJ, Stevenson CJ. Vitiligo, thyroid disease and autoimmunity. Br J Dermatol 1968; 80: 135–139.
5. Lerchin E, Schwimmer B. Alopecia areata associated with discoid lupus erythematosus. Cutis 1975; 15: 87–88.
6. Zauli D, Veronesi S, Fusconi M, Lama L, Meloni M, Tosti A, et al. Nonorgan specific autoimmunity in alopecia. Dermatologica 1985; 171: 12–15.
7. Todes-Taylor N, Turner R, Wood GS, Stratte PT, Morhenn VB. T-cell subpopulation in alopecia areata. J Am Acad Dermatol 1984; 11: 216–223.
8. Lutz G, Fritsche C, Bauer R, Kreysel HW. Zirkulierende T-Lymphozytengruppen bei Alopecia areata. Aktuel Dermatol 1988; 14: 222–226.
9. Main RA, Robbie RB, Gray ES, Donald D, Horne CHW. Smooth muscle antibodies and alopecia areata. Br J Dermatol 1975; 92: 389–393.
10. Perret C, Wiesner-Menzel L, Happle R. Immunohistochemical analysis of T-cell subset in the peribulbar and intrabulbar infiltrates of alopecia areata. Acta Derm Venereol (Stockh) 1984; 64: 26–30.
11. Gollnick H. Das zelluläre Infiltrat am anagenen Follikel bei Alopecia areata. Eine immunhistologische Studie an über 60 Patienten. Z Hautkr 1988; 15: 161–162.
12. Claudy AL, Gagnaire D. PUVA treatment of alopecia areata. Arch Dermatol 1985; 119: 975–978.
13. Unger WP, Schemmer RJ. Corticosteroids in the treatment of alopecia totalis. Arch Dermatol 1978; 114: 1486–1490.
14. De Prost Y, Teillac D, Paquez F, Garrugi L, Bachelez H, Touraine R. Placebo-controled trial of topical cyclosporine in severe alopecia areata. Lancet 1986; ii: 803–804.
15. Macdonald Hull S, Norris JF. Diphencyprone in the treatment of long-standing alopecia areata. Br J Dermatol 1988; 119: 367–374.
16. Gilchrest BA, Vrabel MA, Flynn E, Szabo G. Selective cultivation of human melanocytes from newborn and adult epidermis. J Invest Dermatol 1984; 83: 370–376.
17. Stadler R, Detmar M, Stephanek K, Bangemann C, Orfanos CE. A rapid fluorometric assay for the determination of keratinocyte proliferation in vitro. J Invest Dermatol 1989; 93: 532–534.
18. Krüger-Krasagakes S, Garbe C, Kossman P, Orfanos CE. A rapid and sensitive fluorometric microassay for determining cell-mediated cytotoxicity to adherent growing cell lines. J Immunol Methods 1992; 156: 1–8.
19. Bystryn JC, Orentreich N, Stengel F. Direct immunofluorescence studies in alopecia areata and male pattern alopecia. J Invest Dermatol 1979; 73: 317–320.
20. Safai B, Orentreich N, Good RA. Immunological abnormalities in patients with alopecia areata. Clin Res 1979; 27: 244.
21. Igarashi R, Morohashi M, Takeuchi S, Sato Y. Immunofluorescence studies on complement components in the hair follicles of normal scalp and scalp affected by alopecia areata. Acta Derm Venereol (Stockh) 1981; 61: 131– 135.
22. Herzer P, Czarnetzki BM, Holzman H, Lemmel EM. Immunological studies in patients with alopecia areata. Int Arch Allergy Appl Immunol 1979; 58: 212–218.
23. Gilhar A, Krueger GG. Hair growth in scalp grafts from patients with alopecia areata and alopecia universalis grafted onto nude mice. Arch Dermatol 1987; 123: 44–50.
24. Gilhar A, Pillar T, Assay B, David M. Failure of passive transfer of serum from patients with alopecia areata and alopecia universalis to inhibit hair growth in transplants of human scalp skin grafted onto nude mice. Br J Dermatol 1992; 126: 166–171.
25. Norris DA, Kissinger MR, Naughton GM, Bystryn JC. Evidence for immunologic mechanisms in human vitiligo: patients' sera induce damage to human melanocytes in vitro by complement-mediated damage and antibody-dependent cellular cytotoxicity. J Invest Dermatol 1988; 90: 783–789.
26. Abdel-Naser MB, Krueger-Krasagakes S, Krasagakis K, Gollnick H, Orfanos CE. Further evidence for involvement of both cell mediated and humoral immunity in generalized vitiligo. Pigment Cell Res 1994; in press.
27. Jahoda CAB, Horne HA, Oliver RF. Induction of hair growth by implantation of cultured dermal papilla cells. Nature 1984; 311: 560–562.
28. Runne U, Orfanos CE. The human nail. Structure, growth and pathological changes. Curr Probl Dermatol 1981; 9: 102–119.
29. Laporte M, Andre J, Stouffs-Vanhoof F, Achten D. Nail changes in alopecia areata: light and electron microscopy. Arch Dermatol Res 1988; 280: 85–89.