American Journal of Pathology, Vol. 144, No. 4, April 1994

Chemotherapy-Induced Alopecia in Mice

Induction by Cyclophosphamide, Inhibition by Cyclosporine A, and Modulation by Dexamethasone

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We introduce cyclophosphamide-induced alopecia (CYP-IA) in C 57 BL-6 mice as a clinically relevant model for studying the biology of chemotherapy-induced alopecia and for developing anti-alopecia drugs. One injection of CYP to mice with all back skin follicles in anagen VI induces severe alopecia that strikingly reproduces the follicle response, recovery, and histopathology seen in human CYP-IA. CYP dose-dependently induces abnormal follicular melanogenesis and dystrophic anagen or, in more severely damaged follicles, dystrophic catagen. Both dystrophy forms are followed by an extremely shortened telogen phase, but differ in the associated hair loss and in recovery patterns, which determines hair regrowth. This follicular response to CYP can be manipulated pharmacologically: systemic cyclosporine A shifts it toward a mild form of dystrophic anagen, thus retarding CYP-IA and prolonging "primary recovery". Topical dexamethasone, in contrast, forces follicles into dystrophic catagen, which augments CYP-IA, but accelerates the regrowth of normally pigmented hair ("secondary recovery"). (Am J Pathol 1994, 144:719–734)

Induction of massive hair loss (alopecia) by many chemotherapeutic drugs ranks among the psychologically most devastating undesirable effects of cancer treatment. So far, no fully satisfactory remedy for suppressing chemotherapy-induced alopecia (CIA) in man is at hand. Physiologically and clinically relevant models for studying, modulating, and blocking CIA are therefore urgently required. With the help of such models, it is critically important to define the patterns of follicle response and recovery to design more effective strategies for interfering with the ill-studied biology of CIA.

CIA results from induction of follicle dystrophy[1–3] and/or the premature induction of follicle regression (catagen) in growing (anagen) hair follicles. The complexity of hair growth and cycling prevents utilization of cell culture systems and mandates study of CIA in intact mammalian skin *in vivo* or in organ culture (cf. refs. 4 to 7). We postulate that to be considered "clinically relevant", such models should meet several criteria. They should employ the main target-hair follicles of CIA: mature, pigmented terminal hair follicles that have already traversed some postnatal hair growth cycles. At the time of drug delivery, these follicles should be in the most vulnerable stage of the hair cycle, anagen VI,[8] because this is the predominant population of scalp hair follicles shedding their hair shafts during CIA.[2,3] Ideally, the type of hair loss and the follicle histopathology as well as the course of follicle response and recovery seen in the model should resemble that of CIA with the same alopecia-inducing drug in man. Finally, the predictive value of such an animal model for the human system improves if the employed test drug is already appreciated for its hair growth-modulatory properties in man.

No model meeting these criteria has been available until now. A recently reported rat model for blocking CIA only works in neonatal animals during a narrow window of postnatal follicle development,[9–13] whereas CIA in humans occurs at a much later stage. After drug delivery, these rats loose lanugolike fur in an ill-defined stage of the hair cycle, not pigmented hair shafts produced by terminal hair follicles in anagen.[9–13] The histological features of CIA in the neonatal rat have not yet been systematically com-

Supported in part by grants from Deutsche Forschungsgemeinschaft (Pa 345/3-1) and Sandoz Pharma Ltd.

Accepted for publication December 7, 1993.

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pared with the pathology, clinical course, and recovery phase of human CIA caused by the corresponding cytostatic drugs; and only agents not appreciated as hair growth-modulatory in man were reported to block CIA in this model.[9–13]

Here, we report an alternative model that meets the above criteria: cyclophosphamide-induced alopecia (CYP-IA) in the adolescent C 57 BL-6 mouse. In this model, we define basic patterns of follicle response and recovery and show how cyclosporine A (CsA) and dexamethasone-21-acetate (DEX) modulate both. Only CYP-IA was studied because CYP is one of the most widely used cytostatic agents in clinical medicine and because several studies on CYP-IA in man are available for comparison (eg, refs. 22 to 25). CsA was administered because the induction of unwanted hair growth is one of the most common side effects of CsA-therapy in man[2,4] and because CsA suppresses the hair loss associated with alopecia areata, which—like CIA—is characterized by premature induction of catagen and by dystrophic anagen follicles.[1,2,26] In addition, we have shown that CsA, in the dose and administration schedule employed here, induces anagen[4] and inhibits catagen development in C 57 BL-6 mice.[28] Potent topical corticosteroids have long been used for the management of human hair loss,[2,26] and exert profound hair growth-modulatory effects in mice, including the inhibition of anagen development[20] and the induction of normal-appearing catagen.[27]

Materials and Methods

Animal Model

Adolescent, 6- to 8-week-old, female, syngeneic C 57 BL-6 mice (15 to 20 g) with normal, black fur (ie, heavily melanized terminal hair shafts) were purchased from Charles River, Hannover, Germany. Mice were housed in community cages with 12-hour light cycles at the UKRV Animal Facilities of the FU Berlin and were fed mouse chow and water *ad libitum*. In contrast to the mosaic cycling of human hair follicles, these mice display a unique hair cycle synchronization, which makes them a most productive model for hair research (cf. refs. 8, 14 to 17). Only mice in the resting stage of the hair cycle (telogen) were used for these studies. In the truncal skin of mice, all melanocytes reside in hair follicles, and pigment production (melanogenesis) occurs exclusively in anagen follicles. Thus, telogen C 57 BL-6 mice can be recognized reliably by the homogeneously pink color of their back skin.[4,5,8,18]

Study Design

Anagen was induced in the back skin of telogen mice by depilation. This was done so that, at the start of pharmacological manipulation, all hair follicles in the depilated back skin area of all mice were in exactly the same stage of anagen development (predictable and highly synchronized anagen development can only be achieved with anagen induction by depilation, as opposed to spontaneous anagen development [cf. refs. 4, 5, 18, 20]). As soon as anagen VI had been reached, all mice were injected once with a high dose of CYP (test) or with vehicle (control). In separate experiments, DEX or CsA or appropriate vehicle controls were administered in addition (shortly before *and* after the CYP injection) so as to manipulate CYP-IA. In all experiments, control and test mice were then compared for signs of hair loss, for skin color changes indicating the effect of test drugs on hair cycling and follicle melanogenesis, and for hair regrowth. Mice were not shaven at any time so that the observed patterns of hair loss and regrowth would only reflect the effects of the test drugs employed. At different time points after CYP injection, selected mice were sacrificed to correlate visible hair phenomena with a histological profile of follicle response and recovery (morphometry). Together, this allowed defining of the pattern of hair follicle response to, and recovery from, pharmacological manipulation.

Anagen VI Induction

Under ketaminhydrochloride-induced general anesthesia, telogen mice that had gone through several postnatal hair cycles were induced to enter anagen by depilation of all telogen hair shafts. This was done by applying a melted wax/rosin mixture to the back skin and by peeling off this mixture after hardening, as described.[5,18] By this technique, all depilated telogen hair follicles immediately begin to transform into anagen follicles (stages I to VI; cf. refs. 8, 14 to 16) with their associated melanogenesis (cf. ref. 18). This predictably results in progressive skin pigmentation and thickening within 5 to 6 days, in the development of mature anagen VI follicles and a gray to black skin color within 8 to 9 days, and the subsequent appearance of new, uniformly black hair shafts.[8,18,29] Histologically, functionally and macroscopically, depilation-induced anagen VI follicles are indistinguishable from spontaneously developing anagen follicles. Under reconversion of the skin color from

black to pink, follicle regression (catagen) and its associated cessation of melanogenesis occur spontaneously 17 to 20 days after anagen induction without loss of hair shafts.[8,18–20]

Induction of Alopecia

As soon as early anagen VI had been reached by these induced anagen follicles, ie, on day 9 postdepilation (p.d.), a single intraperitoneal injection of CYP was given (10 to 150 mg/kg body weight, freshly dissolved in distilled water, ENDOXAN, Asta Medica). Control mice received vehicle only (distilled water). The LD_{50} for CYP in mice is 275 mg/kg (intravenous administration, lethal toxicity after 14 days).[21] A total of 45 test mice (150 mg CYP/kg) and 36 control mice (vehicle) were studied in eight separate experiments, and data from these separate experiments were pooled. Between these experiments, data were highly reproducible. By histology, we had verified that depilated back skin on day 9 p.d. contains almost exclusively anagen follicles corresponding to the early stage of anagen VI.[5,8,18]

Pharmacological Manipulation of CYP-IA: DEX, CsA

CYP-IA was manipulated pharmacologically in two ways: a) DEX (0.1% in propylene glykol; Sigma Chemical Co., St. Louis, MO) was liberally administered once daily on days 9 to 13 p.d. with a brush to the back skin of anagen-induced, CYP-injected mice (control: propylene glykol only); and b) test mice received a total of three intraperitoneal (i.p.) injections of CsA (3 × 250 mg/kg CsA, ie, approximately 5 mg/mouse in 0.5 ml corn oil, SANDIMMUNE, Sandoz Pharma Ltd.) on days 7, 9, and 11 p.d. (controls: 0.5 ml corn oil).

A total of 25 test mice with co-administration of CYP+CsA were studied in three separate experiments that yielded highly comparable results (data pooled), alongside the eight experiments with mice injected with CYP alone (control). In a second control ($n = 10$, two separate experiments), we determined that mice injected i.p. with both vehicles (distilled water on day 9 p.d., corn oil on days 7, 9, 11) showed exactly the same macroscopic and microscopic reaction patterns as mice injected with the CYP-vehicle alone or anagen-induced mice without any additional applications. The effects of topical DEX or i.p. CsA on CYP-IA, skin pigmentation, hair regrowth, and follicle histology were analyzed as indicated below for normal CYP-IA. Particular attention was paid to any retarding or enhancing effects on the degree of alopecia, to the hair regrowth pattern, and to the pigmentation of regrowing hair shafts.

Analysis of CYP-IA

After the CYP injection on day 9 p.d. the back skin of test and control mice was examined daily for a period of up to 32 days for signs of alopecia and color conversion from black to pink, indicating the progression of anagen follicles in the hair cycle via catagen to telogen. This color change results from the abrupt termination of melanogenesis in follicular melanocytes very early during catagen development.[8,19,23,26] Visible changes were recorded and photodocumented. Starting 1 week after CYP injection, the skin was examined daily for signs of hair regrowth, skin repigmentation, and the quality of hair shaft pigmentation.

In C 57 BL-6 mice of this age group, depilation-induced anagen follicles spontaneously enter catagen between days 17 and 20 p.d., mostly around day 18.[19] The development of catagen follicles is indicated macroscopically by a change in skin color from black to light gray and occurs in large waves, appearing first in the neck region, then on the flanks, until finally the tail region enters catagen. Within 1 to 2 days after catagen induction, the follicle enters telogen, as indicated by a skin color change from gray to pink (cf. refs. 8, 18, 19). Therefore, the alopecia and skin color changes (expressed as percentage of mice with alopecia or in catagen) were recorded separately for two distinct skin regions: region 1: lower quarter of paravertebral back skin above the insertion of the tail; region 2: upper quarter of paravertebral back skin (neck region).

Histological morphometry was performed to check whether the visible changes in skin pigmentation truly reflected changes in hair follicle cycling and not only isolated drug effects on melanogenesis, and to classify and quantify the follicle subpopulations in a given skin area during the course of experimentation. For histology, back skin from defined skin regions was harvested from test and control mice sacrificed by CO_2 inhalation during defined time points after injection of test drugs or vehicle. The time points of harvesting, the number of mice studied, and the skin regions selected for tissue-harvesting are indicated in the figure legends. The selection of mice sacrificed for histological analysis was non-random and was guided by the degree of alopecia, respectively skin depigmentation: in all groups, those mice that had developed most alopecia or skin depigmentation were sacrificed first. To obtain longitudinal sections

through the hair follicles, full-thickness skin was harvested at the level of the subcutis perpendicular to the paravertebral line. Skin was fixed in 5% buffered formaldehyde and processed for routine histology (paraffin embedding, Giemsa stain).

Morphometrical analysis and photography of Giemsa-stained skin sections were performed at 100 to 400× magnification under a Leitz microscope. Follicles were classified according to their hair cycle stages following the criteria of Chase[8] and Straile et al,[19] and the percentage of follicles in normal anagen VI or normal catagen was recorded. Because CYP administration induces both premature catagen development and dystrophic forms of anagen and catagen in humans,[22,23] the percentages of both dystrophic follicle phenotypes were also assessed. Dystrophic anagen (dys A) was defined by an unusually small amount and abnormal distribution of melanin in anagen hair bulbs, together with melanin clumping, melanin incontinence, and/or bulging of the dermal papilla.[22,23] Dystrophic catagen (dys C) was defined by catagen follicles with melanin distribution and clumping in areas that are normally melanin-free.[19,22,23] The back skin regions and the number of test and control mice studied by morphometry are indicated in the figure legends.

Due to the purposely non-random selection of animals for histology and the progressively decreasing number of animals in the test and control groups during the course of experimentation, statistical analysis of the *in vivo* observations was considered unhelpful and possibly misleading, so that only absolute data are shown. Statistical analysis of the morphometric observations was done by subjecting the data to five consecutive calculations following accepted methods[30]: 1) data transformation for relative data (P = arcsin (square-root [p]); 2) Friedman two-way analysis of normal variance test for testing differences between the various skin regions; 3) Levene test for testing homogeneity of variances; 4) Kruskall-Wallis test for testing significance between experimental groups; 5) Mann–Whitney U-Wilcoxon rank sum test for calculating P values. P values < 0.05 were accepted as significant, P values < 0.005 as highly significant.

Results

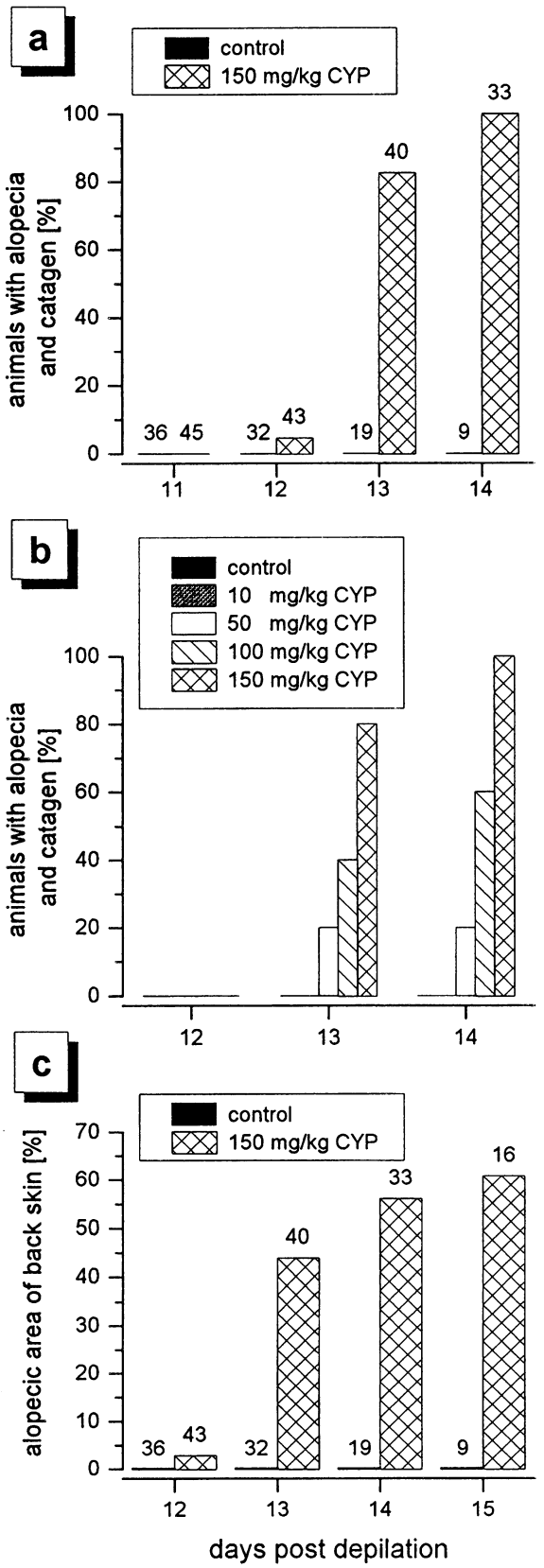
CYP rapidly and reproducibly caused premature termination of anagen and significant hair loss. This diffuse alopecia showed regional differences and was reversible. Regrowing hair shafts often were abnormally pigmented. Histologically, this was based on CYP-induced follicle dystrophy, including disruption of follicle melanogenesis, in two patterns: induction of dys A or of dys C. CYP-induced alopecia and follicle pathology strikingly resembled that already described for human CYP-IA. Dys A and dys C follicles differed in their patterns of recovery and, consequently, hair shaft regrowth and repigmentation. Coadministration of CsA or DEX shifted the follicle response either to dys A (CsA) or dys C (DEX), thus significantly altering the time course and degree of CYP-IA as well as the recovery from it: CsA retarded CYP-IA and prolonged "primary recovery". DEX, in contrast, augmented CYP-IA, but accelerated the regrowth of normally pigmented hair ("secondary recovery").

CYP Terminates Anagen Prematurely and Causes Severe Alopecia

Figures 1a and 2 show that a single i.p. injection of 150 mg CYP/kg on day 9 p.d. sufficed to prematurely terminate anagen by day 14 p.d. in all test mice. As judged by the visible change in the back skin color from black to gray, almost all anagen follicles in the neck region had left the anagen phase of the hair cycle and had entered catagen (compare Figure 2, a and c). As expected from the literature,[19] all vehicle-injected control animals stayed in anagen until catagen developed spontaneously (days 18 to 20 p.d.). In a given back skin region of test mice, it took only 1 to 2 days from the first visible signs of premature catagen induction until all anagen follicles had entered catagen, whereas the last patches of CYP-induced catagen anywhere over the entire back had developed by day 15 p.d.

These CYP-induced color changes, indicating anagen termination and catagen induction, were associated with substantial alopecia, which occurred at the earliest by day 12 p.d. and maximally by day 14 p.d.: patches of bald back skin became visible, and hair shafts all over the back could be pulled out just by rubbing the skin gently, which was impossible in the control mice that had all remained in anagen (Figure 2). Quantitative analysis showed that by day 14 p.d. all mice injected with 150 mg CYP/kg had developed alopecia (as opposed to none of the control animals) (Figure 1, a and b). Starting from day 12 p.d., the alopecic back area of test mice rapidly enlarged, until it covered 60% of the anagen-induced back skin by day 15 p.d. (Figure 1c).

Like catagen development, the occurrence of alopecia was dose-dependent (Figures 1b and 2, b and c), and required the injection of at least 50 mg/kg CYP, whereas the highest dose tested (150 mg/kg) produced maximal alopecia (Figures 1b and 2, b and c).



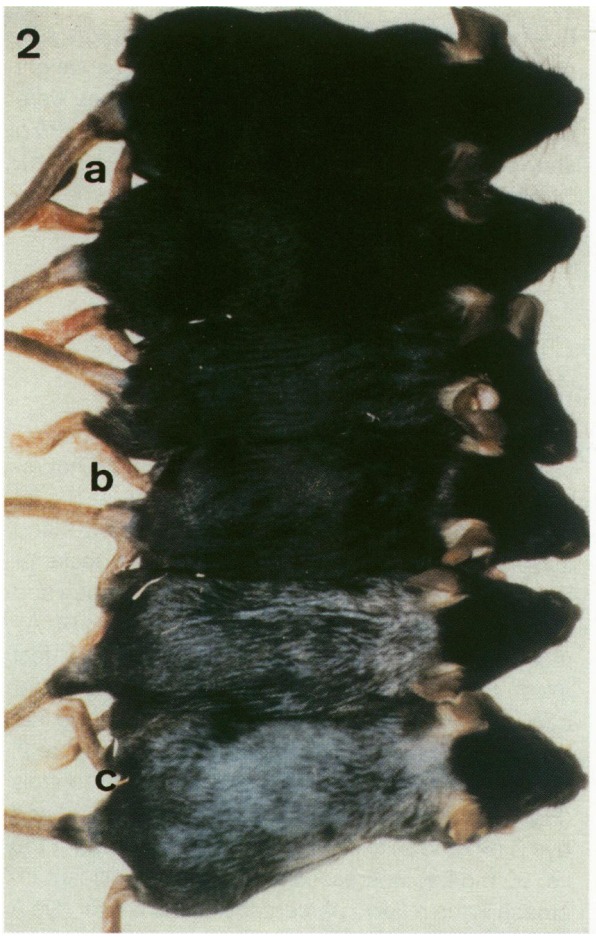
Even with this high dose (LD_{50} for mice: 275 mg/kg[21]), no overt signs of systemic toxicity such as weight loss, behavioral, or neurological abnormalities were observed. Test mice injected with 10 mg CYP/kg showed no differences from control mice (Figure 1b). Six-week-old mice showed a more pronounced alopecia to a lower drug dose (100 mg/kg) than 8-week-old animals.

CYP-IA Results from the Induction of dys A or dys C

Morphometric analysis confirmed that the macroscopic color changes after CYP-injection did not result from selective, CYP-induced disturbances of melanogenesis without alteration of hair growth and cycling. Instead, the color change and alopecia reflect the induction of dystrophic forms of anagen and catagen in anagen VI follicles (Figures 5 and 6). Almost no normal catagen follicles were found in test mice. This also explains the loss of hair shafts, because normal anagen or catagen follicles do not loose their shafts in the manner depicted in Figure 2. Figure 6 shows that, compared to vehicle controls (Figure 6, c and f), CYP induces dys A (6a and d) and dys C (6b and e) follicles. A higher dose of CYP (150 vs. 50 mg/kg) was associated with a significant increase in the relative percentage of dys C over dys A ($P < 0.05$) (Figure 5).

Routine light microscopy revealed notable disturbances of follicular melanogenesis after CYP injection, such as clumping, incontinence and abnormal follicular distribution of melanin granules (compare Figure 6, d and f). Dys C follicles showed substantial remnants of melanin granules in the proximal, regressing hair bulb (Figure 6e, left follicle), as opposed to the normal, largely depigmented catagen follicles found in vehicle-treated control mice between day 18 and 21 p.d. (Figure 6g). In addition to these pigmentary disorders, dys A follicles from test mice exhibited bulging of the dermal papilla and distension of the follicular canal (Figure 6, d and b). Exactly this distinct

Figure 1. *Macroscopic effects of CYP: alopecia and catagen induction.* a *Percentage of animals with signs of both alopecia and catagen (as defined by color change from black to gray) between days 11 and 14. CYP injection: 150 mg/kg on day 9 p.d. Given are the means from pooled data from eight separate experiments representing a total of 45 test mice and 36 control mice (only absolute data listed, as no normal distribution: daily several mice were sacrificed for histology, as indicated by the decreasing* n *listed over each bar).* b *Dose-dependency of alopecia and catagen induction. Representative experiment* (n = *5 per group, repeated twice with highly comparable results).* c *Progression of alopecia over time after CYP injection. 150/mg CYP injected at day 9. Indicated is the minimal percentage (mean) of back skin with considerable, visible alopecia (estimated by an independent observer, numbers above bars: see* a).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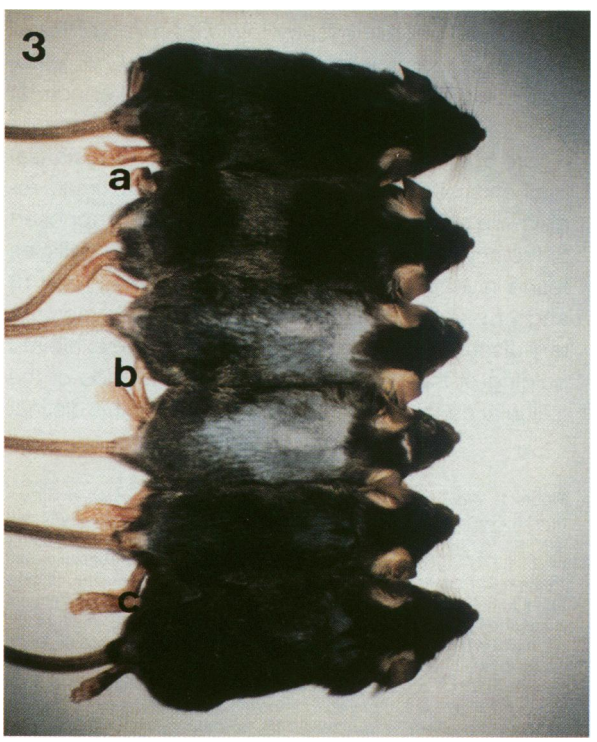
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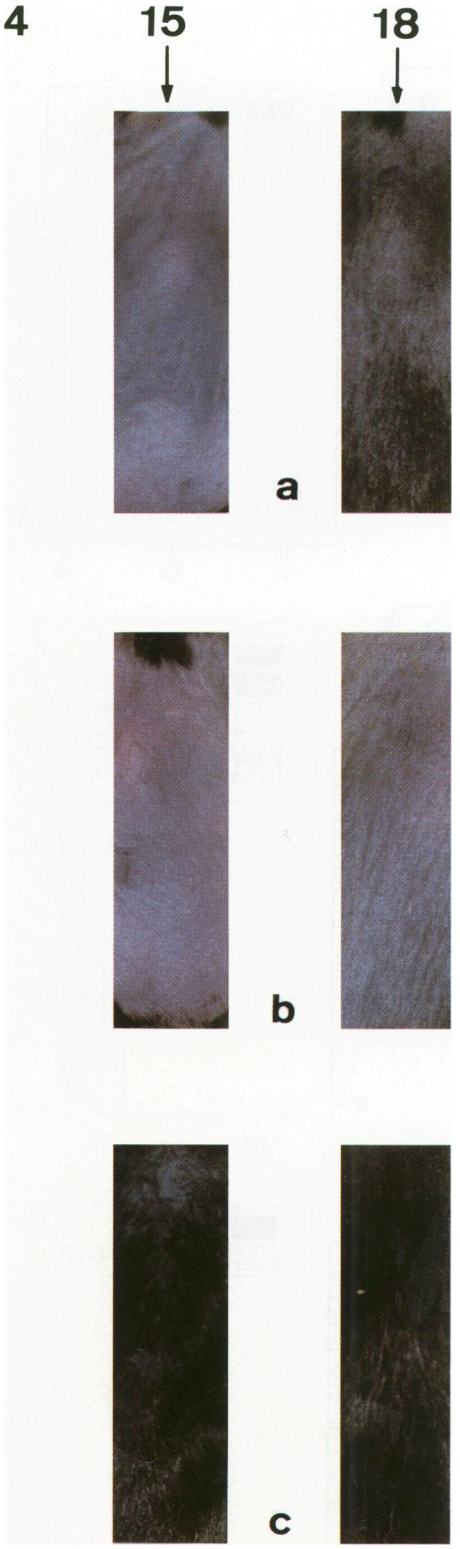
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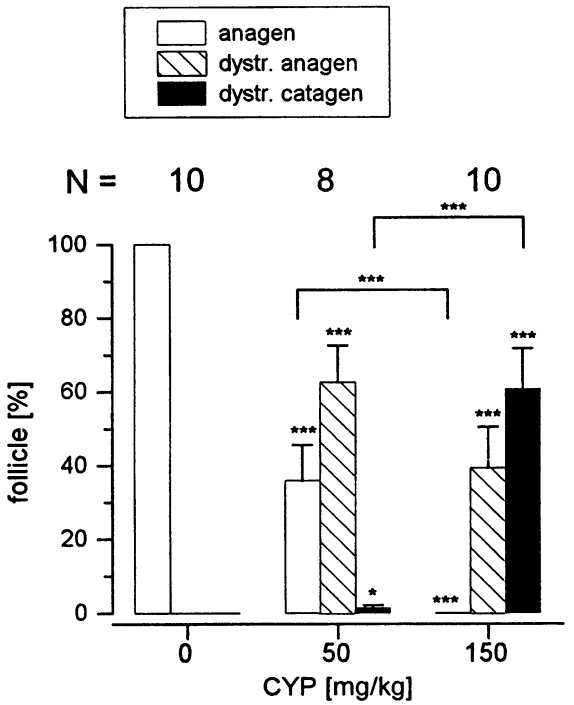

Figure 5. *Morphometric analysis of CYP-induced dystrophic anagen versus dystrophic catagen.Eight to 10 mice per group were injected with 0, 50, or 150 mg/kg CYP on day 9 p.d. On day 13 p.d. (ie, in the damage response phase), skin samples from the neck region* (2) *of each mouse were compared with respect to the degree and type of follicle damage. This was assessed morphometrically by classifying the follicles seen in a total of at least 100 microscopic fields (100× magnification) per group. Given are the means ± 1 SE; level of significance compared to controls and between the two test groups (horizontal bars): * = P < 0.05, *** = P < 0.005.*

follicle pathology has been described as characteristic for CYP-IA in humans.[22]

The Response of Anagen Follicles to CYP Damage Differs between Back Skin Regions

Signs of premature anagen termination and alopecia were always detected first in the neck region (region 2). Corresponding color changes and alopecia in the flanks and over the cephalad 3/4 of the back skin developed within a lag period of 0.5 to 2 days after the neck region. A small area of anagen skin with marginal hair loss usually remained in the lower 1/4 of the back, particularly directly above the insertion of the tail (region 1, see Figures 2c and 3b). No hair loss was noted in skin regions other than the back where anagen VI had been induced. This is to be expected because only in the depilated back skin area almost all follicles were in the highly drug-sensitive anagen stage of the hair cycle.

These regional, dose-dependent differences in the response to CYP injection were quantified morphometrically on day 13 p.d. In the tail region (1), 50 mg/kg CYP left 70% of anagen follicles morphologically intact, as opposed to only 40% of anagen follicles in the neck region (2). 150 mg/kg CYP induced dys C in only 30% of the anagen follicles of the tail region (1), but in 60% of follicles in the neck region (2) (data derived from pooling the data of at least 100 microscopic fields per region, based on a total of 7 to 10 mice studied). These regional differences were significant ($P < 0.05$) and are important, because the percentage of dys A versus dys C follicles determines the course of follicle recovery and thus the regrowth of a pigmented fur coat in a given skin area (see below).

The Recovery from CYP-IA Follows Defined Patterns: "Primary" and "Secondary Recovery"

Macroscopically, the following sequence of events after CYP injection was observed, after maximal alopecia had developed: first visible signs of follicle recovery, the reappearance of new hair shafts, became apparent on days 16 to 17 p.d. (ie, 7 to 8 days after CYP injection). By day 18 p.d., the skin pigmentation in all test mice had darkened again, and terminal hair

Figure 2–4. *Macroscopic effects of CYP, CYP+DEX, and CYP+CsA. Representative test and control animals from the experiments summarized in Table 1.*

Figure 2. *Alopecia and skin color change on day 14 p.d. (ie, 5 days after CYP injection). Note the dose dependency.* a = *vehicle injection;* b = *50 mg/kg CYP i.p. on day 9 p.d.;* c = *150 mg/kg CYP i.p. on day 9 p.d. Note mice treated with a lower dose of CYP showed only slight skin color changes and hair loss* (2b), *as opposed to the severe alopecia and dramatic skin color changes seen in mice treated with the standard CYP dose* (2c). *The figure also demonstrates that alopecia generally was more severe in the neck than in the tail area of murine back skin.*

Figure 3. *Inhibition of CYP-IA by CsA (day 15 p.d.)* a = *vehicle control (distilled water + corn oil);* b = *150 mg/kg CYP on day 9 p.d.;* c = *150 mg/kg CYP on day 9 p.d. + 250 mg/kg CsA i.p. on days 7, 9, 11 p.d. Co-administration of high doses of CsA i.p. before and after CYP injection* (3c) *significantly retarded the occurrence of CYP-induced alopecia* (3b).

Figure 4. *Comparison of macroscopic effects of CYP vs. CYP+DEX vs. CYP+CsA on days 15 and 18 p.d.* a = *CYP (as in* 3b*);* b = *CYP+DEX (once daily 0.1% dexamethasone-21-acetate on days 9 to 13 p.d.);* c = *CYP + CsA (as in* 3c*). Note the degree and velocity of hair shaft regrowth in mice treated with CYP alone* (4a) *or with CYP+CsA* (4c) *as well as the significantly enhanced and better pigmented hair regrowth in the CsA-treated group* (4c). *This rapid hair regrowth in both groups of mice represents "primary recovery" of dystrophic anagen follicles that have not yet entered the next hair cycle (cf. Table 1). CYP+DEX-treated mice* (4b), *in contrast, show now primary recovery; however, the increase of skin pigmentation between day 15 and day 18 attests to the development of a new, synchronized wave of anagen follicles. This reflects "secondary recovery" of dystrophic catagen follicles that already have traversed the CYP-induced, premature catagen and telogen stages. The underlying histological changes are summarized in Table 1.*

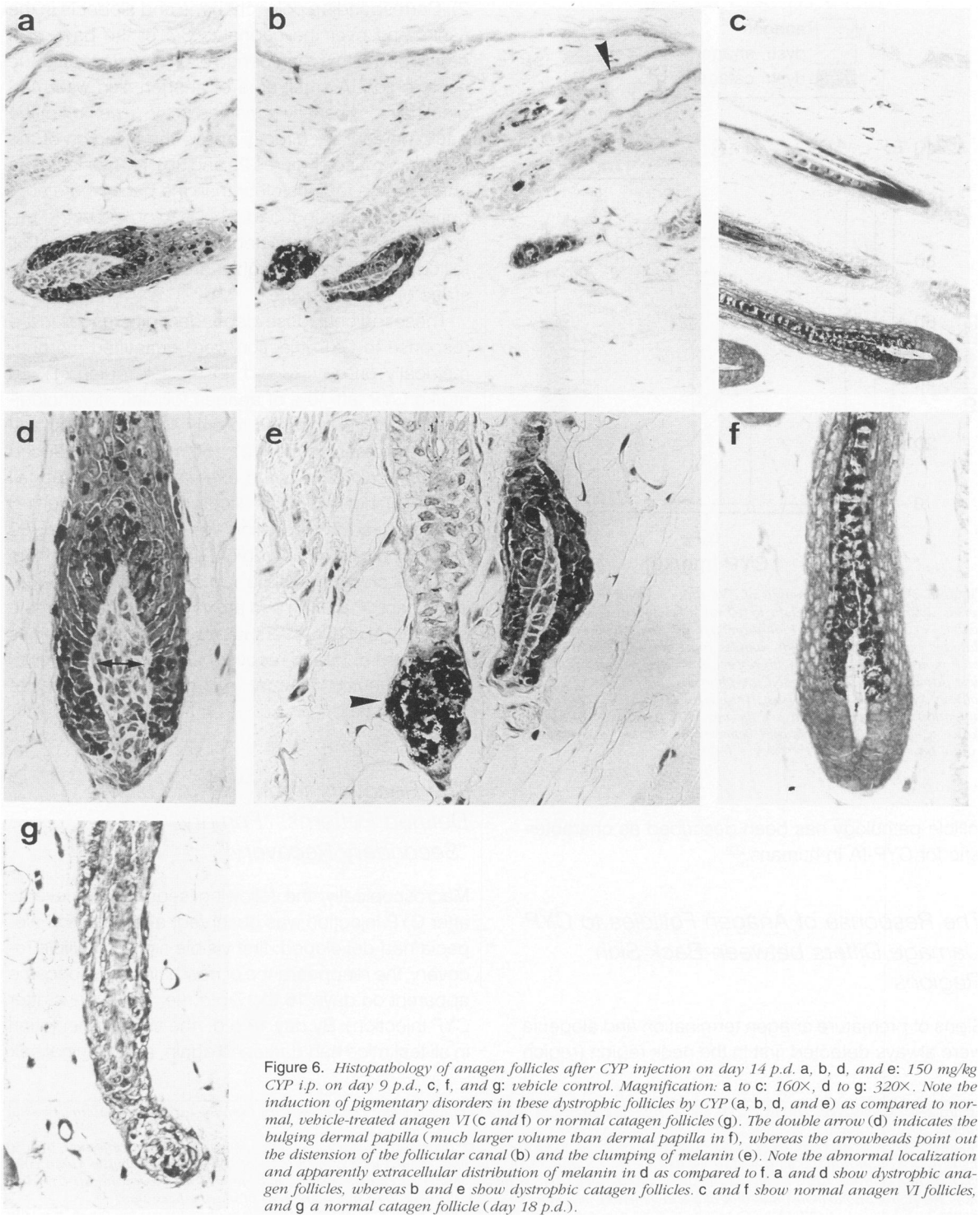

Figure 6. *Histopathology of anagen follicles after CYP injection on day 14 p.d.* a, b, d, *and* e: *150 mg/kg CYP i.p. on day 9 p.d.,* c, f, *and* g: *vehicle control. Magnification:* a *to* c: *160×,* d *to* g: *320×. Note the induction of pigmentary disorders in these dystrophic follicles by CYP* (a, b, d, *and* e) *as compared to normal, vehicle-treated anagen VI* (c *and* f) *or normal catagen follicles* (g). *The double arrow* (d) *indicates the bulging dermal papilla (much larger volume than dermal papilla in* f), *whereas the arrowheads point out the distension of the follicular canal* (b) *and the clumping of melanin* (e). *Note the abnormal localization and apparently extracellular distribution of melanin in* d *as compared to* f. a *and* d *show dystrophic anagen follicles, whereas* b *and* e *show dystrophic catagen follicles.* c *and* f *show normal anagen VI follicles, and* g *a normal catagen follicle (day 18 p.d.).*

shafts reappeared in most previously bald skin areas (Figure 4a). By day 30 p.d., the entire back skin of all test mice was covered by hair shafts again, yet most of the regrown furcoat was depigmented (Figure 7c). The speed of follicle recovery depended on the dose of CYP used for inducing alopecia and was most pronounced in test mice with the lowest dose of CYP still associated with alopecia (50 mg/kg) (not shown). Extensive histological analysis suggested that CYP-damaged follicles recover in two logical patterns, de-

pending on whether dys A or dys C has been induced by CYP (Table 1). These distinct patterns explain the hair regrowth phenomena described above.

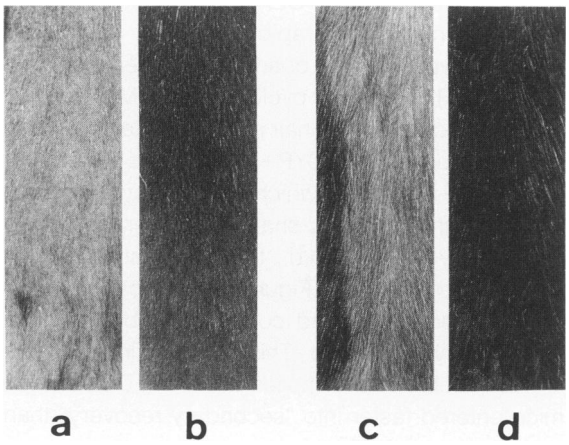

Figure 7. *Visible hair shaft regrowth in CYP- versus CYP+DEX-treated mice. Visible hair shaft regrowth in mice treated with CYP* (a *and* c) *or CYP+DEX* (b *and* d) *by day 25* (a *and* b) *and 30 p.d.* (c *and* d). *Note the considerably denser fur coat in CYP+DEX-treated mice versus the CYP controls at day 25* (a *and* b) *and the much more pronounced, persistent hair pigmentation disorder in mice not treated with DEX on day 30 p.d.* (c *versus* d). *As summarized in Table 1, this reflects that CYP-treated mice primarily show dys A follicles, which produce abnormally pigmented hair shafts during their "primary" recovery, and do not produce normally pigmented hair shafts until their secondary recovery (some time after day 30 p.d.). DEX-treated mice, in contrast, display predominantly dys C follicles, which are already well into their secondary recovery phase by day 25, thus generating mainly normally pigmented hair shafts.*

Dys A follicles recover quickly and resume hair shaft production ("primary recovery"): normal anagen VI follicles are detectable again by day 18 p.d., which then leave this first experimental anagen phase and enter telogen around day 25 p.d. Surprisingly, these normal-appearing telogen follicles reenter spontaneously into the next hair cycle very shortly thereafter. Already on day 30 p.d., many normal anagen IV follicles are seen. This is remarkable, inasmuch as the normal duration of telogen for mice of this age group is 2 to 3 weeks, and means that CYP-IA is associated with a significant shortening of the subsequent telogen phase. Due to the substantial CYP-induced damage of follicular melanogenesis, largely depigmented new hair shafts are generated during primary recovery. By day 30, in their secondary recovery phase, former dys A follicles have not yet developed into anagen VI follicles so they have not had the chance to produce a new, normally pigmented hair shaft at that time (Table 1), but are about to do so within a few days.

Dys C follicles, in contrast, have no "primary recovery" and enter into a pathological form of telogen already around day 18 p.d. This telogen, too, is much shorter than normal telogen. By day 25 p.d., former dys A follicles are well into their "secondary recovery", show no substantial morphological abnormalities

Table 1. *Histological Profile of the Response Patterns of Murine Anagen VI Follicles to CYP, CYP + DEX, or CYP + CsA Administration*

	A-induction ↓		CYP ↓								
days p.d.	0	1	9	12	13	14	16	18	20	25	30
Control	T	A I	A VI					C	T		
CYP	T	A I	A VI	→	dy C			dy T		A II [2]	A VI
					dy A			A VI [1,3]		T	A IV
CYP + DEX	T	A I	A VI	dy C		dy T [3]	A II [2]	A IV		A VI	
CYP + CsA	T	A I	A VI			mostly dy A		A VI [1,3]		*n.a.*	

first cycle, anagen

second cycle, anagen

All telogen follicles in the back skin of C 57 BL-6 mice were induced by depilation to enter into the anagen phase of the hair cycle and had reached the early stage of anagen VI by day 9 p.d. At this time all test animals received 150 mg/kg CYP. Vehicle-treated controls (distilled water i.p. +/– corn oil i.p. or topical propylene glykol) showed no differences from untreated, depilation-induced animals. In two experimental series, in addition to CYP, test mice were treated with either topical DEX or intraperitoneal CsA (as indicated below) to modulate CYP-IA. Per time point and experiment indicated, at least 100 microscopic fields (100 to 400×) from 6 to 10 different mice derived from at least three separate experiments were examined. The table summarizes the observations qualitatively by indicating the dominant follicle population at various days after anagen-induction by depilation (p.d.).

T = telogen; A = anagen; C = catagen; dy = signs of follicle dystrophy; n.a. = not assessed; p.d. = post depilation (days after anagen induction by depilation); CYP = one i.p. injection of 150 mg/kg cyclophosphamide on day 9 p.d.; DEX = topical dexamethasone-21-acetate (0.1%) once daily on days 9 to 13 p.d.; CsA = three i.p. injections of 250 mg/kg cyclosporine A (ie, ca. 5 mg/mouse) on days 7, 9 and 11 p.d.

1 = primary recovery (ie, a damaged anagen VI follicle resumes hair shaft production without having progressed into the next anagen cycle); 2 = secondary recovery (ie, production of a new hair shaft not by the old, damaged anagen follicle, but by its successor in the subsequent anagen phase after having traversed catagen and telogen); 3 = only discrete signs of follicle dystrophy.

anymore, and have reached anagen VI with its associated production of normally pigmented hair shafts around day 30. The macroscopic appearance of the regrown furcoat after CYP-IA, therefore, entirely depends on the (dose-dependent) ratio of dys A versus dys C follicle induced by chemotherapy. This ratio is significantly altered by co-administering DEX or CsA with CYP.

DEX Augments CYP-IA and Retards Hair Regrowth

Alopecia was more extensive in CYP+DEX-treated mice and lasted longer than in CYP-treated mice, because very few follicles remained in dys A, thus excluding the possibility of "primary recovery" (Table 1). When DEX (0.1%) was administered topically once daily to CYP-injected mice from days 9 to 13 p.d., this significantly enhanced the CYP-induced skin color changes and alopecia (Figure 4b). Color change and alopecia were accelerated by 1 day (day 12 p.d.) and lasted significantly longer than in mice treated with CYP alone (compare Figure 4, a and b). Also, the mice treated with CYP+DEX showed substantial areas of completely pink skin next to gray spots, suggesting the rapid induction of telogen (via catagen) (Figure 4b). This was confirmed histologically: by day 14 p.d., CYP+DEX-treated mice showed predominantly telogen follicles, which were abnormally melanized as a sign of relatively mild follicle dystrophy (Figure 8). The small minority of dys C follicles detectable next to these dystrophic telogen forms displayed the same follicle pathology as depicted in Figure 6, b and e.

The combination of DEX+CYP thus terminates anagen VI much more rapidly than CYP alone, and forces the vast majority of anagen follicles to rapidly move on to the next hair cycle (Table 1). Mice treated with CYP alone showed hair regrowth at latest by day 18 p.d., as opposed to CYP+DEX-treated mice (compare Figure 4, a and b), which even by day 22 p.d. still had no regrowth of hair shafts. However, beginning around day 17 to 18 p.d., the previously pink skin color darkened again (Figure 4b), indicating that a new anagen phase had commenced by that time ("secondary recovery"). This was confirmed histologically (Table 1) and implies that CYP+DEX-treated mice entered faster into "secondary recovery" than any other experimental group.

DEX Accelerates Normal Pigmentation of Regrowing Hair Shafts

When hair shaft regrowth finally occurred in CYP+DEX-treated mice by day 25 p.d., most of the newly generated hair shafts were normally pigmented, as opposed to control mice (CYP alone), whose back fur displayed largely depigmented hair shafts and was much more sparse (Figure 7, a and b). By day 30 p.d., the difference in hair pigmentation of CYP- versus CYP+DEX-treated mice was even more

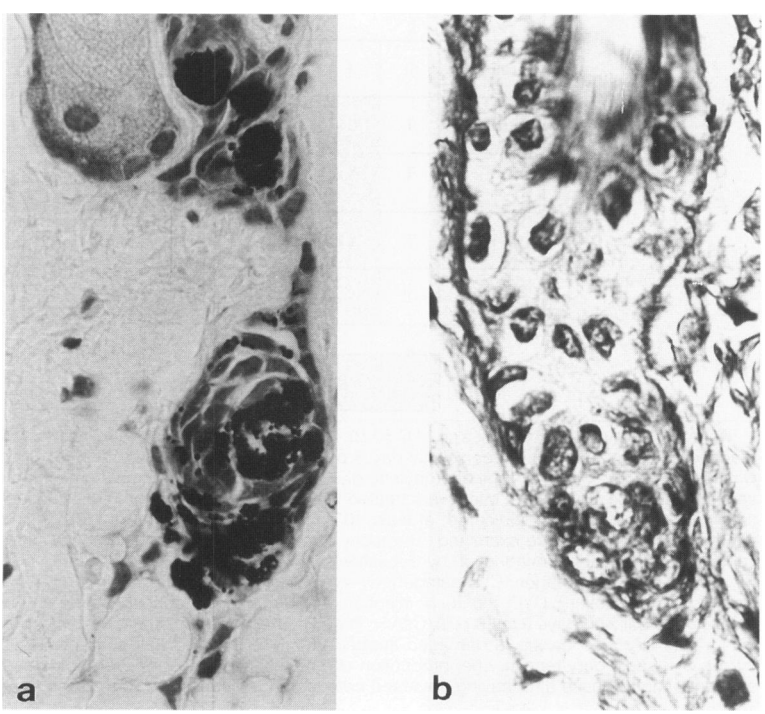

Figure 8. *CYP+DEX: rapid induction of dystrophic telogen follicles. This figure shows a representative dystrophic telogen follicle* (**a**)*, which dominates histologically on day 14 p.d. after topical co-administration of 0.1% DEX. Note the abnormal presence, distribution and clumping of melanin, compared to a normal, non-pigmented telogen follicle* (**b**)*. Most notably, the duration of dystrophic telogen is abnormally short (see Table 1).*

obvious (Figure 7, c and d). Histologically, most hair follicles in the CYP+DEX-treated mice were already in anagen II to III by day 16 p.d., whereas on day 18 p.d. anagen IV had been reached. Considering that the development of anagen IV from a telogen follicle takes 4 to 5 days in these mice,[5,8,18,20] this means that the significant shortening of the telogen period observed in CYP-treated mice (see above) was also seen in the CYP+DEX group, where telogen was shorter than in any other group (Table 1).

CsA Retards the Occurrence of CYP-IA and Diminishes the Severity of Follicle Dystrophy

When CsA was co-administered i.p. with CYP, CYP-IA developed 2 days later than in controls (CYP alone), the alopecic area was smaller, and the CYP-induced morphological follicle damage was greatly reduced. Figure 3 shows that, on day 15 p.d., CYP+CsA-treated mice (3c) looked almost like vehicle-control mice (3a), although hair shafts could be pulled out more easily in CYP+CsA- than in vehicle-treated mice. CYP-treated mice had developed substantial alopecia and catagen induction by that time (3b). Quantitative analysis revealed that, on day 13 p.d., no alopecia had developed in CYP+CsA-treated mice as opposed to CYP-controls, and the back skin area displaying alopecia one day later was markedly smaller in CYP+CsA-treated mice (18%) than that of CYP-controls (56%) (Figure 9a). By day 18 p.d., CYP+CsA-treated mice had regrown a dense, yet sparser than normal, mostly regularly pigmented fur coat (Figure 4c), whereas mice treated with CYP alone displayed a significantly sparser and more depigmented fur coat than the CYP+CsA group (Figure 4a).

Morphometry revealed that, on day 15 p.d., a significantly higher percentage of follicles was in dys A, and a highly significantly lower percentage was in dys C in the CYP+CsA group than in the CYP group (Figure 9b): the vast majority of CsA-protected follicles were in dys A, and even about 10% normal anagen follicles were found, despite injection of a very high dose of CYP. Histologically, this protective effect of CsA on CYP-induced follicle damage was evident

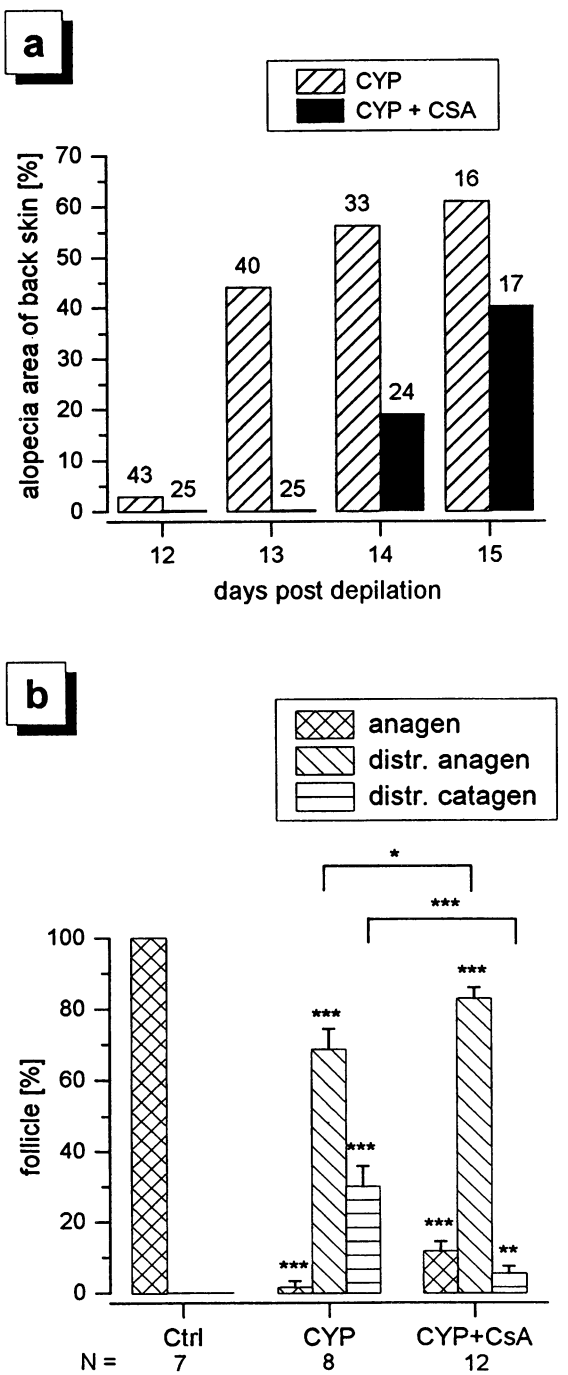

Figure 9. *Cyclosporine retards CYP-IA and diminishes follicle dystrophy.* **a**: *CYP+CsA: alopecia later and less extensive. This panel demonstrates that alopecia in mice receiving CYP+CsA developed hair loss later and less extensively than mice treated with CYP alone (administration details see Table 1). Means from pooled data (figure above bars: number of mice studied per group; the decreasing number of animals studied reflects successive sacrification of mice for histology).* **b**: *CYP+CsA: less dystrophic catagen follicles than with CYP. This panel compares the percentage of normal anagen, dys A, and dys C follicles on day 15 p.d. after the injection of vehicle (Ctrl), 150 mg/kg CYP (CYP), or CYP+ 3 × 250 mg/kg cyclosporine A (CYP+CsA). Per group, at least 100 microscopic fields from 7 to 12 animals per group (derived from two separate experiments) were assessed by morphometry. Data were pooled, and means ± 1 SE and levels of significance were calculated. Note that the co-administration of CsA increased the relative percentage of normal and dys A follicles, as opposed to CYP-treated mice, where dys C follicles dominated. Level of significance compared to Ctrl, or between test groups (horizontal bars): ***P < 0.001, **P < 0.01, *P < 0.05.*

from the substantially lower degree of follicle dystrophy (eg, only discrete abnormalities of melanogenesis) (compare Figure 10, a to c). The faster and more pronounced hair regrowth seen in CYP+CsA-treated mice reflects that these mice had a substantially prolonged "primary recovery" phase (Table 1). Due to the apparent anti-dystrophy and melanogenesis-protective properties of CsA, the hair shafts regrowing during primary recovery of CYP+CsA-treated follicles were mainly well-pigmented, in contrast to those produced by CsA-unprotected dys A follicles during primary recovery. Due to the length of primary recovery and the fact that all mice were sacrificed for histology by day 30 p.d., the secondary recovery of CYP+CsA-treated mice could not be assessed in these studies.

Discussion

We report a simple, yet instructive and "clinically relevant" animal model for the study and pharmacological manipulation of CIA. Systemic CYP administration to C 57 BL-6 mice with mature, pigmented terminal hair follicles in anagen VI induces the acute onset of diffuse, reversible alopecia. This is accompanied by skin color changes reflecting CYP-induced abnormalities of follicular melanogenesis and hair cycling and is followed by two distinct waves of hair regrowth ("primary" and "secondary recovery"), which restore the fur coat. Histologically, CYP induces two types of follicle dystrophy. More severely damaged anagen follicles are transformed into dys C, with rapid shedding of the hair shaft and abrupt cessation of follicular melanogenesis. Less severe damage induces dys A, with variable hair shaft shedding and continued, but abnormal follicular melanogenesis. Both dys A and dys C follicles run through a significantly shortened telogen phase and display very different recovery patterns.

The follicle response to, and recovery from, CYP in this model strikingly reproduces CYP-IA in man, including hair regrowth and hair pigmentation disorders

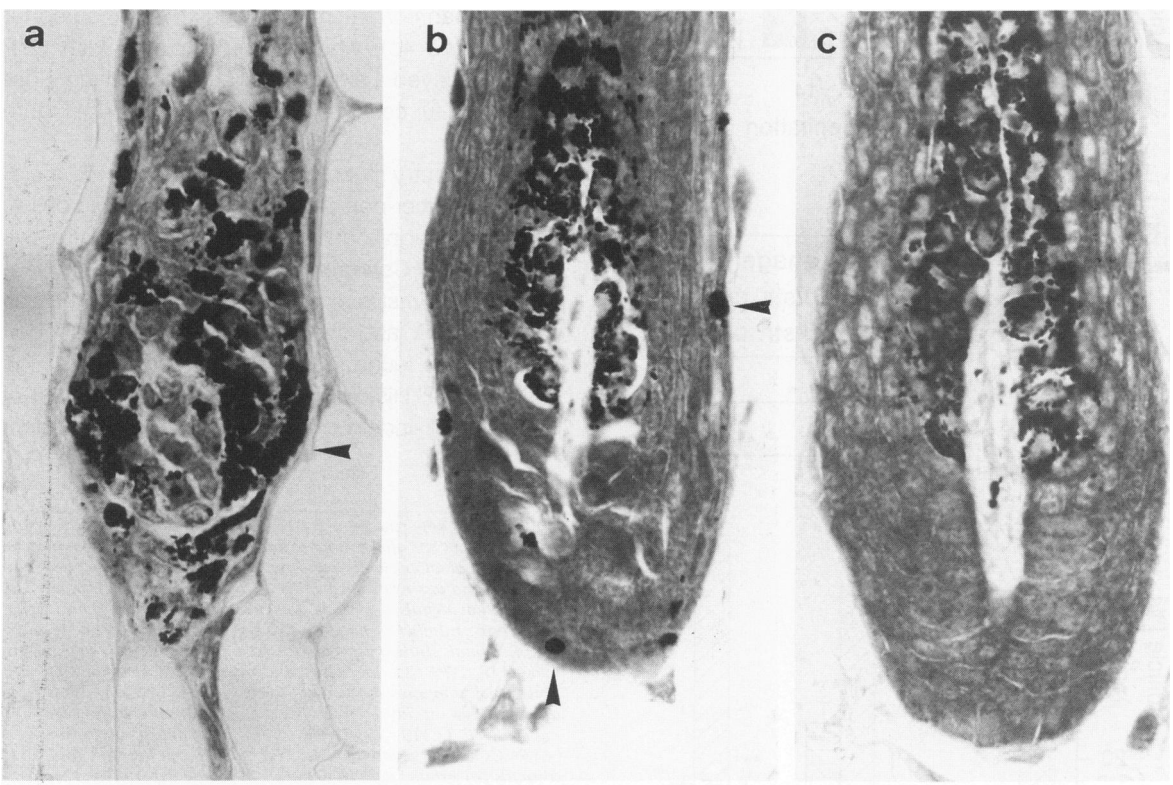

Figure 10. *Protective effect of CsA co-administration on anagen VI follicles. Back skin follicles on day 16 p.d. after injection of* a = *CYP alone (150 mg/kg) → dys C;* b = *CYP+CsA → dys A (mild);* c = *vehicles (distilled water + corn oil) → normal anagen VI. Note that most follicles in the CYP group at this time are dys C follicles like the representative follicle shown here* (a), *reflecting the severe follicle damage caused by CYP injection on day 9 p.d. In contrast, the CsA co-administration maintains a majority of CYP-damaged follicles in a dys A stage* (b) *that, in addition, shows much more discrete signs of follicle dystrophy than the dys A follicles also found in the CYP group (cf. Figure 6d). The arrowheads denote clumping and abnormal follicular distribution of melanin, compared to the normal intrabulbar melanin distribution in an anagen VI follicle* (c).

seen frequently in human CIA. Most notably, CYP-treated follicles show the same characteristic histopathology (clumping, incontinence, and abnormal distribution of melanin, bulging of dermal papilla, distension of pilar canal), and the typical, dose-dependent mixture of dys C and dys A induction described for human CIA due to CYP.[22,23] (cf. ref. 36). As in human CIA, where massive loss of scalp hair is contrasted with marginal loss of facial and body hair, murine CYP-IA reveals regional differences in the follicular response to chemotherapy. In addition, we show that murine CYP-IA is augmented by two drugs that effectively modulate human hair growth (DEX, CsA; cf. refs. 2, 20, 26) and that DEX treatment accelerates the regrowth of normally pigmented hair shafts. This corresponds well to the reported enhancement of pigmented hair regrowth in human CIA after steroid administration.[25]

The light-microscopic histopathology of follicular melanogenesis after CYP-injection corresponds ultrastructurally to enlarged or fragmented melanosomes with abnormal intra- and extracellular localization, and biochemically to a sharp decline of the normal anagen VI-associated activity of two key enzymes of melanogenesis, tyrosinase and DOPAchrome tautomerase (Paus et al, manuscript in preparation). Because melanin is a potent scavenger molecule and binds many drugs[32,33] the presence or absence of melanin in a follicle may affect its vulnerability to chemical injury (cf. refs. 29, 33). Given that melanogenically active anagen follicles are the prime target of CIA, the use of normally pigmented anagen hair follicles may be an important feature of a good animal model for CIA.

The cumulative dose of CYP administered clinically during chemotherapy generally is higher than the single CYP dose necessary for alopecia induction in this model, whereas the daily CYP doses used clinically are lower. It is notoriously difficult to reliably judge which drug dose in an animal model is truly "equivalent" to a certain dose in the human system, but an experimental dose may be considered roughly equivalent to a corresponding human dose, if it produces the same characteristic spectre of drug effects in the animal, as is the case in this model (characteristic follicle pathology, alopecia and hair regrowth, associated with little overt drug toxicity). Thus, the high dose of CYP used does not detract from the clinical relevance of this model.

Using this model, we have identified basic patterns of follicle response to and recovery from chemotherapy (see Table 1). Whether CYP-damaged anagen follicles enter into the dys A or into the dys C pathway determines the velocity and quality of hair regrowth, as both types of dystrophic follicles follow a separate path of recovery. Dys A follicles actually stay longer in anagen than normal anagen VI follicles and recover quickly the capacity to produce hair shafts ("primary recovery") until anagen is switched off and a new hair cycle is entered. Due to CYP damage of melanogenesis, the first wave of regrowing hair shafts during primary recovery shows severe pigmentation disorders; yet, the second wave of hair shafts generated during the "secondary recovery" is normally pigmented. Dys C follicles, in contrast, stay much shorter in anagen than dys A or normal anagen follicles and do not have a primary recovery. They rapidly run through a dystrophic telogen period, and produce new hair shafts only during the next hair cycle. However, this secondary recovery phase occurs much earlier than in dys A follicles and generates mainly well-pigmented hair shafts (Table 1).

Drugs that modulate CYP-IA operate via favoring one type of follicle response over another, thus profoundly affecting the degree of alopecia as well as hair regrowth. DEX co-administration stops anagen VI earlier than even the highest dose of CYP alone and forces almost all follicles along the dys C pathway, thus rapidly inducing total alopecia. DEX leads to a second anagen cycle at a time when normal and dys A follicles are still in the first anagen phase of the experiment. Having traversed an almost normal catagen-telogen-anagen transition with only discrete signs of dystrophy, damaged melanogenesis is restored rapidly, and secondary recovery sets in very early. Thus, mainly normally pigmented hair shafts quickly replace the lost hair shafts (Table 1).

CsA co-administration, in contrast, shifts the response fully toward dys A (and a milder form of dys A), inasmuch as CsA diminishes follicle dystrophy in general and retards the onset of catagen. This is associated with less disturbance of follicle melanogenesis and an exceptionally long primary recovery. In consequence, alopecia is immediately followed by significant hair regrowth already during primary follicle recovery, and regrowing hair is largely well-pigmented, whereas CsA-unprotected dys A follicles produce less, and mainly depigmented, hair during primary recovery. Thus, CYP+DEX-treated mice have the fastest onset of secondary recovery, whereas CYP+CsA-treated mice have the longest and most efficient primary recovery phase. These DEX and CsA effects correspond well to our previous findings that CsA induces anagen and inhibits catagen in C 57 BL-6 mice[4,28] and that potent topical corticosteroids block induced anagen development[20] and induce a

normal-appearing catagen when administered to anagen VI follicles.[27]

One important aspect of follicle recovery apparent from these studies is that the anagen follicle repairs severe damage via a two-step process: follicle involution (catagen), followed by almost immediate construction of a new anagen follicle. As soon as (and only when!) CYP-damaged follicles have entered into their second experimental anagen phase ("secondary recovery"), no more signs of morphological follicle damage are detected, and follicular melanogenesis appears normal, as judged by histology and the production of normally pigmented hair shafts—quite in contrast to the pathology still displayed by anagen VI follicles during "primary recovery" (Table 1). This regenerative strategy of the anagen follicle is remarkable, in that this complex mini-organ repairs damage by rapid induction of terminal differentiation and apoptosis (ie, catagen [cf. refs. 15–17, 19, 34]), immediately followed by rapid morphogenesis (anagen).

Our study highlights that, of all hair cycle stages, only anagen VI and telogen are extremely variable in length, whereas anagen I to V and catagen seem to run a predetermined time course, once initiated. The drastically shortened telogen phase after CYP or CYP+DEX treatment was the most unexpected finding. Because a shorter telogen phase entails more rapid initiation of the next growth phase, this helps to understand why recovery from CIA in man commonly is very fast (cf. ref. 3). CYP is the first drug ever demonstrated to abruptly shorten telogen of a follicle it has damaged during its preceding anagen, thus precipitating the follicle into the next phase of active growth. We propose that this is part of the regenerative strategy of the follicle, enabling it to traverse the depicted two-step repair process with maximal speed. If toxic follicle damage by CYP is *not* a prerequisite for telogen abbreviation, this might actually be exploited for treating telogen effluvium and androgenetic alopecia[2,31] by nontoxic, telogen-shortening CYP-metabolites or -analogs.

Knowledge of these distinct patterns of follicle response and recovery now allow better targeted management of CIA. The identified patterns invite following three general strategies: 1) protection from or reduction of follicle dystrophy as the initial response to drug-damage (eg, by topically effective immunophilin ligands with little systemic effects, but fully retained hair growth-modulatory potential; cf. for protective effects of CsA); 2) increase of the ratio of dys A over dys C follicles so as to enlarge the follicle population capable of primary recovery (eg, by dose reduction or fractionation); and 3) promotion and acceleration of secondary recovery: if follicle damage is too severe to suppress alopecia effectively, initial enhancement of alopecia by induction of almost 100% dys C follicles (eg, by co-administration of topical dexamethasone) may produce a significantly faster and cosmetically more satisfactory regrowth of hair.

The C 57 BL-6 model can be utilized to explore and characterize further these strategies for managing CIA, and for developing anti-alopecia drugs, although it remains to be explored whether this model is also a useful tool for analyzing CIA by agents other than CYP. One advantage of this model is that it can be followed up on the *in vitro* level in a complementary organ culture assay, which utilizes C 57 BL-6 anagen follicles in long-term culture of intact skin on gelatin gels.[6] Before launching clinical studies with potential anti-alopecia agents, these can be evaluated *in vitro*, namely in long-term organ culture of intact human scalp skin.[7] In addition, the molecular and ultrastructural pathology underlying CIA as well as the molecular and cellular repair mechanisms after chemical injury can be dissected in this system, such as CYP-induced changes in cell and hair shaft ultrastructure, follicle protein synthesis, and gene expression.

This murine model also is of interest to pigment cell research and the study of epithelial cell apoptosis: the CYP-inducible disorders of melanogenesis, and their manipulation by DEX and CsA, may be utilized for manipulating follicular melanogenesis *in situ*. Because normal melanogenesis associated with the C 57 BL-6 hair cycle has been well-defined,[18] corresponding physiological data are available for comparison. Apoptosis of hair follicle keratinocytes is a prominent characteristic of normal catagen development and seems to be tightly regulated (cf. refs. 34, 37); enhanced apoptosis of hair bulb keratinocytes is the standard response to follicular injury and a recognized feature of CYP-IA[24] (cf. ref. 34). It is conceivable that glucocorticosteroids known to induce thymocyte apoptosis[35,39] further enhance programmed cell death in the hair bulb, whereas CsA, which inhibits thymocyte apoptosis,[39,40] may suppress it. CYP-induced apoptosis of follicle keratinocytes and its manipulation by DEX and CsA can, therefore, be employed as physiologically relevant assay system for dissecting the still obscure regulation of epithelial cell apoptosis (cf. 34, 38, 39).

In summary, CYP-IA in the C 57 BL-6 mouse is the first animal model to fulfill the proposed requirements of a "clinically relevant" model with reasonable predictive value for the human system. It is a versatile experimental tool for dissecting not only the ill-understood biology of CIA, but also for studying the control of epithelial cell apoptosis and follicular mel-

anogenesis *in situ*. Most notably, it points to novel strategies for the management of CIA and serves as a sensitive screening assay for the development of drugs that inhibit CIA or enhance recovery from it.

Acknowledgments

The excellent technical assistance of R. Pliet, and M. Gesche, as well as the statistical advice of Dr. M. Giurfa are gratefully acknowledged.

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