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Animal Model

Harlequin Ichthyosis (*ichq*)

A Juvenile Lethal Mouse Mutation with Ichthyosiform Dermatitis

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The harlequin ichthyosis (ichq) mouse mutation arose spontaneously in 1989 in a colony of BALB/cJ mice at The Jackson Laboratory. Affected mice developed thick skin due to formation of compact, orthokeratotic scales that fractured over articular surfaces, secondary to bending. Harlequin ichthyosis mice on the inbred BALB/cJ background died between 9 and 12 days of age. Onset of the clinical phenotype corresponded with emergence of hair fibers from follicles at 5 days of age. There was marked proliferation of the root sheaths of anagen hair follicles, limited to the region within the dermis. Sebaceous glands were present but small compared with those of littermate controls. Emerging hair fibers were surrounded by a thick, compact sheath of cornified cells. Mutant skin contained large mitochondria with lamellar-shaped, electron-dense structures at the ultrastructural level. Keratohyalin granules were smaller and less pleomorphic than those in control mice. Lamellar bodies were not evident in either mutant or littermate control mice. Using a panel of antibodies to evaluate changes in keratinocyte differentiation, mouse-specific keratin 6 was overexpressed in the suprabasilar, hyperplastic epidermis. Loricrin expression, within the cytoplasm of cells in the stratum granulosum, decreased rapidly postmortem, unlike that in normal mice where it was stable for over 24 hours postmortem. Filaggrin expression, within granules of cells in the stratum granulosum, was prominent, corresponding to hypergranulosis evident by light microscopy in mutant mouse skin. Skin grafts from harlequin ichthyosis mice grafted onto immunodeficient nude mice maintained the phenotype for the 10-week observation period. The mutant gene locus mapped to the proximal end of mouse chromosome 19 and is inherited as a fully penetrant autosomal recessive gene. The harlequin ichthyosis mouse mutation is very similar to human type 2 harlequin ichthyosis for which it may be a good model. (Am J Pathol 1997, 151:293–310)

Ichthyosis represents a group of inherited human diseases characterized by an excessive amount of superficial scale on the skin, resembling the scales of a fish.[1–3] One rare form, known as harlequin fetus or harlequin ichthyosis, is particularly difficult to study because most affected infants die within a few weeks of birth.[4] Harlequin ichthyosis is considered by many investigators to be the most severe form of autosomal recessive, nonbullous, congenital ichthy-

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osiform erythroderma/lamellar ichthyosis.[5–7] A recessive mode of inheritance has been suggested based upon the observation of many single-case reports, some with affected siblings,[8–14] reports of parental cosanguinity without disease in either parent,[12,15] and absence of affected individuals in successive generations. Prenatal diagnosis is now possible using sonography[16] or fetal skin biopsy.[11,12]

The mechanisms involved in harlequin ichthyosis that produce abnormal epidermal proliferation are unknown; however, a number of abnormalities of keratinization have been identified suggesting that this might be a heterogeneous disease.[17–19] Detailed studies have classified harlequin ichthyosis into three subtypes based on histological features and keratin expression profiles.[20] Whether these are in fact different diseases or represent variations in a single genetic defect modified by other cellular genes is unknown. It is not easy or practical to approach this question in outbred species, such as humans or even most domestic animals.

Ichthyosis-like congenital diseases also occur in other species, including cattle, llamas, and dogs.[21–24] Unfortunately, as with human beings, these reports present single cases from which affected lines were not established. A form of ichthyosis (not harlequin ichthyosis) has been reported in chickens due to an autosomal recessive mutation.[25–27]

A mouse mutation with an ichthyosiform phenotype on an inbred background would be a valuable tool to determine the genetic mutation and biochemical mechanisms of disease as well as to provide a test system for novel pharmacological or genetic therapies. Among the large number of mutations with alopecia there are some characterized by abnormalities of cornification or keratinization.[28–30] For example, the ichthyosis mutation (gene symbol *ic*) was discovered in 1950[31] and has been proposed as a model for several of the human ichthyosiform diseases.[32,33] However, the *ic* mutation was re-evaluated and found to more closely resemble a form of alopecia termed trichothiodystrophy,[34] a disease that does have a mild ichthyosiform component. Flaky skin (*fsn*) is another mutation with alopecia and thick, flaking scales that increase in severity with age.[35,36] Mice homozygous for *fsn* have a dermal inflammatory cell infiltrate, alterations in keratin expression, and other biochemical abnormalities that are more typical of psoriasis vulgaris than ichthyosis.[36] An important observation made with the *fsn* mutation is that the morphological criteria and severity of the cornification abnormality can be modified to resemble various forms of psoriasis by changing the background genotype. This suggests that the heterogeneous disease known as psoriasis may be a single disease modified by other genes.[36] Many others exist and are reviewed elsewhere,[37] but none resemble harlequin ichthyosis.

We report here a spontaneous, autosomal recessive, juvenile lethal, mouse mutation that has morphological and biochemical similarities to the human disease, harlequin ichthyosis. The mutant locus maps to the proximal end of mouse chromosome 19.

Materials and Methods

Mice

The animals used in these studies were BALB/cJ and CAST/Ei inbred and CByB6F1/J - *Hfh11^{nu}/Hfh11^{nu}* hybrid nude mice raised at The Jackson Laboratory, Bar Harbor, ME. The *ichq* mutation was maintained on the inbred strain (BALB/cJ) on which it arose by test mating normal littermate mice (+/+ or +/*ichq*, hereafter referred to as +/?). Tested mice were those +/*ichq* that produced mutant progeny.

Three to five weaned mice, depending on body weight, were housed in polycarbonate cages (324 cm^2). For breeding colonies, a pair of mice and their offspring were housed in these cages. The number of young mice per mating was limited to 12 suckling mice, 2 weeks of age or older. Young mice were weaned between 21 and 32 days of age depending upon growth weight. Cages were mechanically washed weekly and filled with an appropriate amount of sterilized white pine shavings. Water bottles were mechanically washed weekly and filled with acidified water (pH 2.8 to 3.2). Food hoppers are built into the cage lid and were filled at least once a week with Rodent Diet NIH-31 with 6% fat (Agway, Syracuse, NY). Cage lids are covered with a nonwoven polyester filter. Animal rooms were equipped with controlled light cycles of 12:12 (light:dark) hours.

Tissue Collection

A minimum of 5 each of *ichq/ichq* and +/*ichq* mice were necropsied at daily intervals from 4 through 9 days of age. Mice were euthanized by CO_2 asphyxiation and decapitated using American Veterinary Medical Association approved guidelines. Skin was collected from the dorsal and ventral trunk, eyelids, ear, muzzle, tail, and footpads. Both vertical and horizontal sections were prepared for dorsal and ventral skin.[38–40] Organs collected included the skull, vertebrae, spinal cord, skeletal muscle, brown

and white fat, femur, stifle joint, mammary gland, brain, liver, spleen, pancreas, intestines (Swiss rolls), stomach, kidney, adrenal, lung, urinary bladder, trachea, thyroid gland, esophagus, genital tract, heart, tongue, and lymph nodes. All organs were fixed by immersion in Fekete's acid-alcohol-formalin solution. Skulls and long bones were decalcified after fixation. Tissues were fixed overnight, transferred into 70% ethanol, trimmed, processed, and embedded routinely in paraffin. Blocks were sectioned at 5 μm and stained with hematoxylin and eosin for microscopic evaluation. Skulls were sectioned in three planes to evaluate the pituitary gland, eyes, ears, teeth, and other structures. Serial sections of skin were stained with Masson's trichrome (for scarring), Verhoeff-van Gieson (for elastic fibers), or periodic acid-Schiff (for fungi, glycogen, glycoproteins, and glycolipids), and Von Kossa (for mineralization) stains. Dorsal skin was also frozen from two 9-day-old mutant and control mice, and frozen sections were prepared and stained with oil red O (for lipids).

For gene mapping studies, spleen, liver, and kidneys were removed at necropsy and frozen in liquid nitrogen for subsequent DNA extractions. In addition, the ventral skin, tail, and/or brain were removed from 0- to 6-day-old mice and frozen in liquid nitrogen. Frozen tissues were stored at −70°C until use.

Epidermal Kinetics

Six mutant and three control female mice at 9 days of age were injected intraperitoneally with 1 μCi/g body weight of tritiated thymidine (specific activity, 20 μCi/mol/L: New England Nuclear, Boston, MA) to label cells entering the S phase of the cell cycle. After 1 hour, mice were euthanized by CO_2 asphyxiation, and necropsies were performed. Skin was collected and fixed as described above, sectioned, covered with emulsion, incubated for 30 days, developed, and counterstained as previously described.[41,42] The number of positive nuclei (four or more silver grains over the nucleus) were counted in the interfollicular epidermis or in the outer root sheaths of the hair follicles only within the region limited on the surface by the junction with the interfollicular epidermis and deep by the junction of the dermis and hypodermis. Positive cells were counted in a 40× field with three fields per slide counted. The comparative kinetics between mutant and control mice were determined by counting the number of positive cells divided by either the number of hair follicles per field or the epithelial area within the dermis per field.

Cutaneous Morphometric Analyses

Epidermal thickening was grossly and microscopically evident in the *ichq/ichq* mice. The interfollicular epidermal thickness, length of hyperplastic outer root sheath, dermal thickness, and full skin thickness were measured using an image analyzer (Quantimet 600HR Image Analysis System, Leica, Deerfield, IL) as previously described.[43] Data were analyzed using the Excel computer program (Microsoft Corp., Redmond, WA). Skin was measured from dorsal and ventral truncal regions of all mice used in the longitudinal study. Data were pooled per group between 6 and 9 days of age and analyzed using a Student *t*-test.

Ultrastructural Evaluation

Transmission electron microscopy was done on skin from nine *ichq/ichq* and four +/? mice at 9 days of age. Dorsal skin was fixed for 18 hours at 4°C in 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4 (CB), and post-fixed for 18 hours in aqueous 1% osmium tetroxide in CB. An additional four mutant and three control mice had skin collected as above but post-fixed in 0.2% rhuthenium tetroxide in CB rather than osmium tetroxide.[44] All tissues were stained *en bloc* with 2% uranyl acetate in 10% ethanol and further dehydrated in a graded ethanol series. Samples were infiltrated and embedded in Quetol 657 resin (EMS, Fort Washington, PA) and polymerized at 65°C for 48 hours. Ultrathin sections were collected, stained with uranyl acetate and Reynold's lead citrate, and examined in a JEOL 100CXII transmission electron microscope.[45]

Scanning electron microscopy was done on nails, skin punches, and skin grafts. A 1-cm square sample of mouse skin was removed from mutant (n = 9) and control (n = 4) mice or from nude mice with grafts (n = 2), avoiding the subcutaneous fat layer. The samples were placed, connective tissue side down, on dry nylon mesh and immersed in cold 2.5% glutaraldehyde in CB. In addition, during necropsy, the left front and rear feet from three mutant and three control mice were amputated at the carpus/tarsus and prepared in a similar manner. After overnight fixation at 4°C, samples were washed twice with CB and post-fixed in 0.5% osmium tetroxide in CB. Some of the skin samples were then fractured in liquid nitrogen to evaluate cross sections and then processed further with the rest of the tissues. Samples were subsequently dehydrated in a series of graded ethanols to 100%. After three changes in 100% ethanol, the samples were critical point dried,

attached to aluminum stubs with silver adhesive, and sputter coated with 15 nm of gold. Samples were examined in a JEOL 35C scanning electron microscope.[45]

Immunohistochemistry

Serially sectioned dorsal skin was screened with rabbit affinity-purified rabbit polyclonal antibodies directed against mouse-specific keratins K1, K5, K6, K10, K13 and K14 as well as filaggrin and loricrin (gift of Dr. D. R. Roop, Baylor College of Medicine, Houston, TX). Immunohistochemical analysis with these antibodies was performed as previously described.[46] Briefly, deparaffinized 5-μm serial sections were incubated in 3% H_2O_2 in methanol for 15 minutes to block endogenous peroxidase. A 30-minute incubation in 10% ovalbumin in phosphate-buffered saline (PBS, pH 7.6) was used to block nonspecific antibody adsorption. Slides were incubated overnight in primary antibody at 4°C and then washed three times in PBS. The reaction was detected using a modification of the avidin-biotin complex method (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) with diaminobenzidine (Sigma Chemical Co., St. Louis, MO) as the chromogen. Hematoxylin was used as the counterstain.

Expression of these epidermal markers in dorsal skin from four BALB/cJ +/+ and six *ichq/ichq* mice was assessed in skin removed at necropsy, fixed immediately and at 1, 2, 3, 4, 5, and 24 hours postmortem. Tissues were left out at room temperature to evaluate the effects of postmortem autolysis. Skin was processed as described above.

Skin Grafts

Skin graft donor mice consisted of five BALB/cJ-*ichq/ichq* mice, two females and three males, and five BALB/cJ +/? littermate control mice that were 7 to 8 days old. In addition, unmanipulated *ichq/ichq* and +/? mice at 7 to 8 days of age were necropsied for comparison with the donor mice. Recipients consisted of 10 17-week-old nude (CByB6-F1/J-*Hfh11^{nu}/Hfh11^{nu}*) mice, 4 females and 6 males, obtained from production colonies of The Jackson Laboratory.

Skin grafts were performed routinely.[41,43] Briefly, donor mice were euthanized by CO_2 asphyxiation and then decapitated. Circular pieces of skin (epidermis and dermis), approximately 1 to 1.5 cm in diameter, were aseptically removed from the dorsal, cervical, and lumbar regions of the donor mice and placed in sterile PBS until the recipient mouse was ready to receive the graft. Recipient mice were anesthetized with tribromoethanol (0.2 ml/10 g body weight; Aldrich Chemical Corp., Milwaukee, WI). A circular piece of skin (epidermis and dermis), approximately 1 to 1.5 cm in diameter, was aseptically removed from the left and right flanks of *Hfh11^{nu}/Hfh11^{nu}* recipients. To these sites, donor skin was transplanted. Nexaban (Veterinary Products Laboratory, Phoenix, AZ) was used on the opposing edges of the graft site to improve adherence. Grafts were covered with pieces of sterile Telfa Pad (Kendall Co., Boston, MA) held in place with micropore surgical tape (3M Medical-Surgical Division, St. Paul, MN). Each recipient received one skin graft from an *ichq/ichq* and one from a +/? littermate control mouse. Each donor provided grafts for transplantation to two *Hfh11^{nu}/Hfh11^{nu}* recipients.

Genetics

The genetic basis of this mutation was established by crossing a female C.B-C^{+}*Hbb^{s}*−+/+ with a male carrier of the new mutation (+/*ichq*). The F1 progeny and offspring of F1 × F1 intercrosses were sexed and phenotyped to determine whether this was a recessive or dominant mutation and whether or not it was sex linked.

An intersubspecific cross (F2) was set up between BALB/cJ +/*ichq* and CAST/Ei (*Mus musculus castaneus*). The F1 progeny from this cross were intercrossed, and the F2 progeny were typed for the *ichq* gross phenotype. Ninety-nine affected intercross F2 mice (*ichq/ichq*) were typed for simple sequence length polymorphisms distributed throughout the genome.

Genomic DNA was isolated from spleen with a QIAmp tissue kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocols. Polymorphic microsatellites previously published[47,48] were analyzed using the polymerase chain reaction (PCR). Primers from the Massachusetts Institute of Technology were purchased from Research Genetics (Huntsville, AL). Amplifications were performed with a volume of 15 μl having a final concentration of 2.25 mmol/L $MgCl_2$, using the buffer provided by the manufacturer of the *Taq* DNA polymerase (Promega, Madison, WI). Thirty-nine PCR cycles, consisting of 20 seconds at 94°C, 40 seconds at 55°C, and 10 seconds at 72°C preceded by an initial denaturation step of 3 minutes at 94°C, were performed in microtiter plates. PCR products were all analyzed on 4% agarose gels (NuSieve 3:1 agarose, FMC Bioproducts, Rockland, ME) and stained with ethidum bromide to visualize the bands.

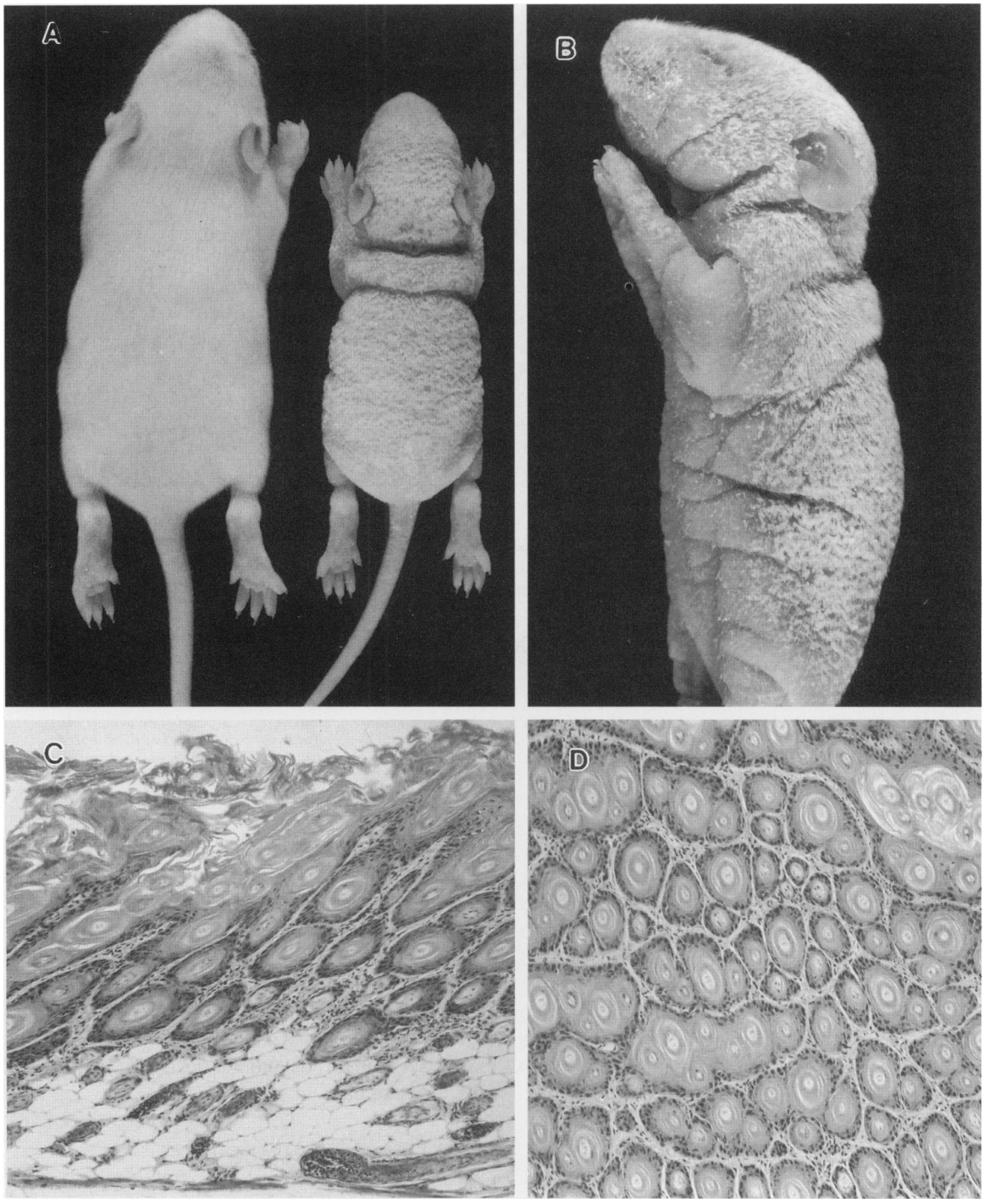


Figure 1. A: *Harlequin ichthyosis mouse* (**right**) *is runted, covered with white scales, and lacks a thick hair coat compared with a littermate control* (**left**). **B:** *Lateral view of a mutant mouse reveals the dorsal skin is more seriously affected than the ventral.* **C** *and* **D:** *Oblique* (**C**) *and transverse* (**D**) *sections exacerbate the hair follicle involvement. Magnification,* ×*125.*

Linkage between the *ichq* mutation and the molecular markers, genetic distances, and confidence intervals were calculated using the Gene-Link program.[49] Markers were ordered by minimizing double recombinants.

Results

History

A pair of neonatal BALB/cJ mice were submitted to The Jackson Laboratory Diagnostic Laboratory for

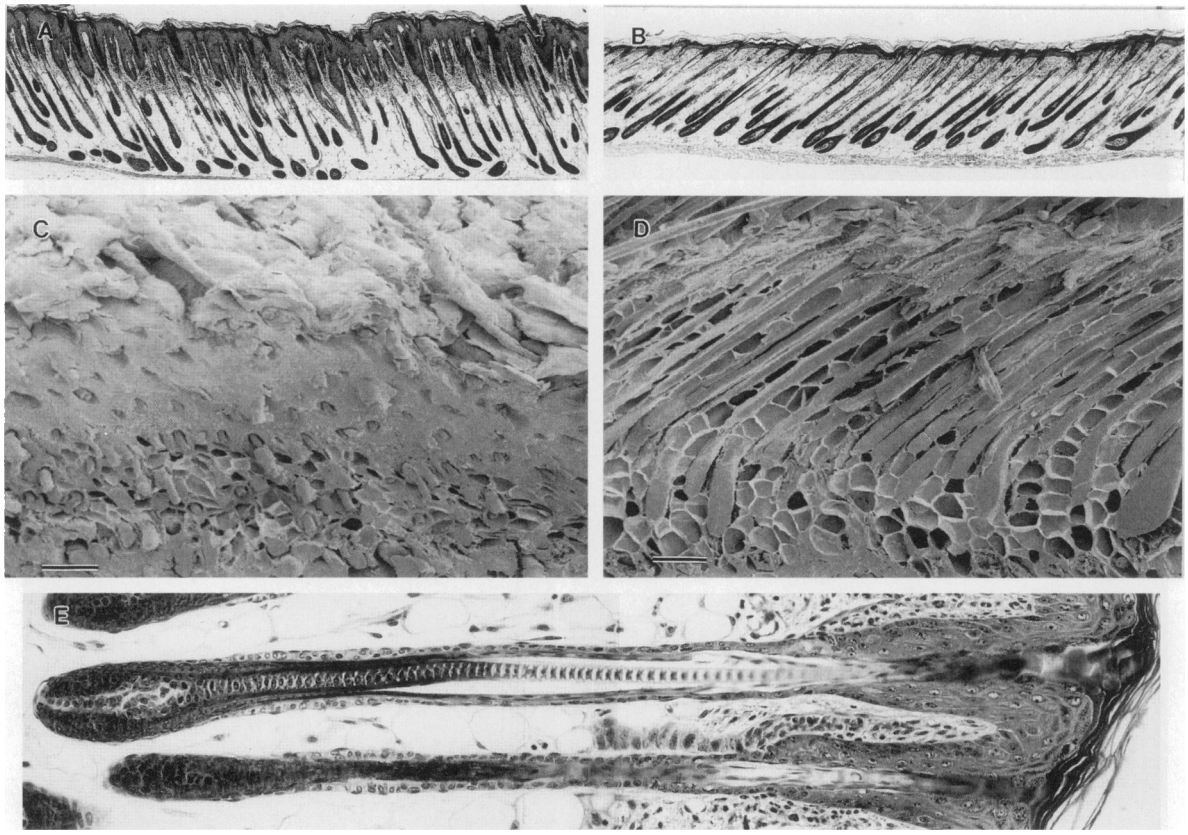


Figure 2. **A** *and* **B**: *Harlequin ichthyosis mouse skin exhibits hyperplasia of the interfollicular epidermis and root sheaths of follicles within the region of the dermis* (**A**) *compared with a control* (**B**). *Magnification,* ×*50,* **C** *and* **D**: *These features along with the highly compact nature of the scale are evident in freeze-fracture scanning electron micrographs of mutant* (**C**) *and control* (**D**) *skin (bar, 100* μ*m).* **E**: *The infundibula of anagen hairs are hyperplastic. Note the mild infiltration of neutrophils and mast cells between the follicles. Magnification,* ×*312.*

examination in 1989. One of the mice was of normal size and just beginning to develop its first hair coat. The second mouse, a littermate, was runted, dead on arrival, and covered with thick white scales and few hairs. Parents and related adults were obtained from the BALB/cJ production colony and crossed. Affected offspring were obtained from one breeding pair that was expanded to form the current stable breeding colony. The mutation was given the name harlequin ichthyosis and the gene symbol *ichq*.

Clinical History and Phenotypic Description

Offspring from tested heterozygote breeding pairs yielded 25% affected offspring with an equal sex ratio, the expected ratio for an autosomal recessive mutation. Homozygote mutant pups were indistinguishable from heterozygote and wild-type pups until 5 days of age when the skin of *ichq/ichq* mice became thick and scaly compared with littermate controls (Figure 1A). A fold of skin appeared on the anterior, dorsal, truncal skin, just behind the head in mutant mice, associated with skin thickening. Fine scales developed at this time that formed a thick, white coating by 9 days of age. The dorsal skin was primarily affected (Figure 1B), although all other skin sites appeared to be affected at the gross level to various degrees. The oldest mutant mouse in our inbred BALB/cJ colony lived to 12 days of age. Mutant mice were runted and did not develop a normal first hair coat as evidenced by the fact that they remained pink instead of developing the normal, fine, white hair.

Microscopic Description

Dorsal skin sections, cut slightly oblique to the plane of the hair follicles, distorted the degree of the mutant phenotype causing it to appear to have massive, diffuse orthokeratosis involving follicular ossia to the level of the dermis (Figure 1, C and D). Hair fiber remnants extended into the stratum corneum. This orientation was virtually identical to published photomicrographs of human harlequin ichthyosis patients.[11,20,50] This phenotype was also evident when skin was sectioned in the plane of the follicles (Figure

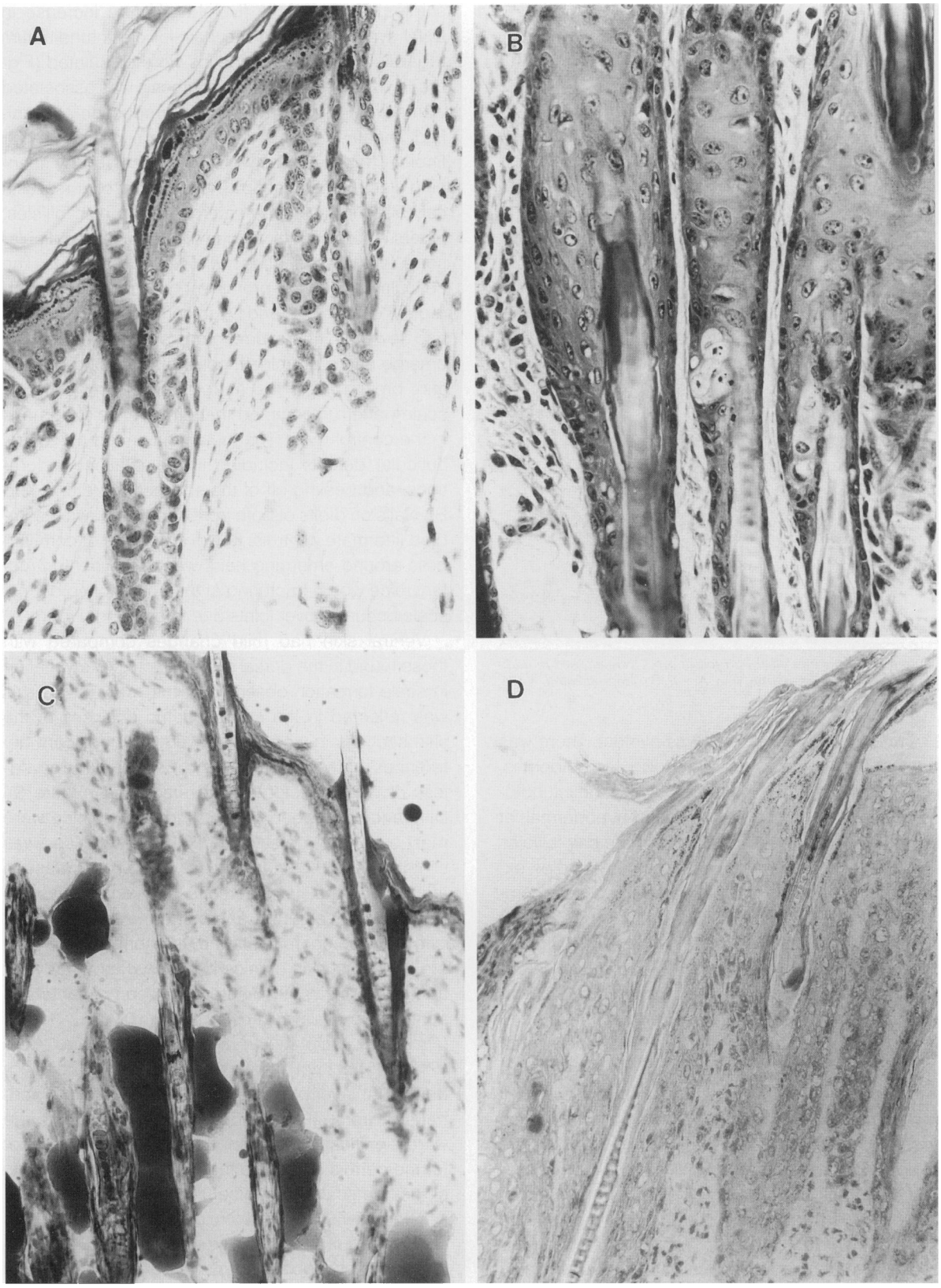


Figure 3. A *and* B: *Sebaceous glands were prominent in control follicles* (A) *but difficult to find in harlequin ichthyosis follicles* (B). *Magnification,* ×*500.* C *and* D: *Oil red O stains revealed an abundance of lipids in control* (C) *but not mutant* (D) *skin. Magnification,* ×*312.*

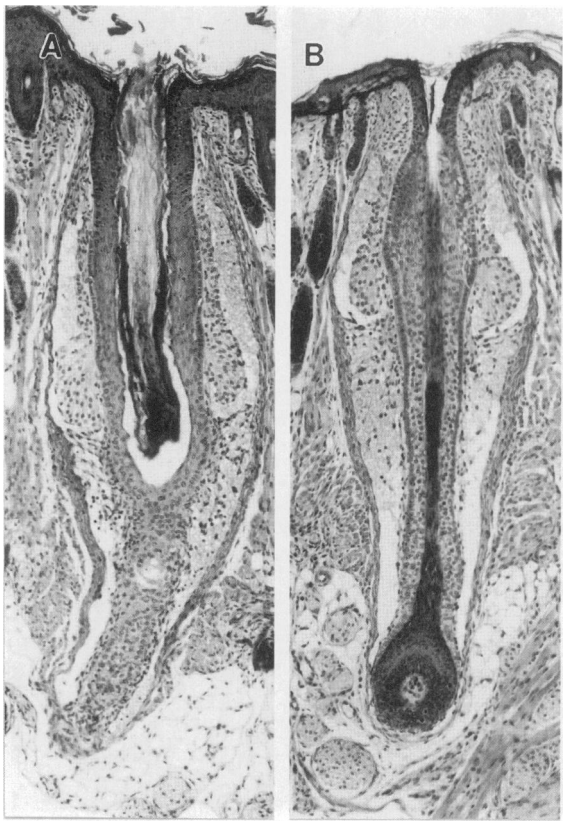


Figure 4. *Vibrissae and other hair follicle types were affected in addition to those of the pelage* (ichq/ichq, **A**; +/?, **B**). *Magnification,* ×*125.*

2, A and B), but more detail was evident. There was marked thickening of the interfollicular epidermis, including the stratum corneum, but also the infundibula to the junction of the dermis and hypodermal fat layer. Below this junction, anagen-stage hair follicles appeared to be normal. Freeze-fracture studies exacerbated this effect and illustrated the dense, compact nature of the scale (Figure 2, C and D). Figure 2 illustrates this feature as well as the mild infiltration of neutrophils and mast cells in the dermis. Sebaceous glands were small, infrequent, difficult to find, and immature (Figures 2E and 3, A and B). Oil red O stains of frozen sections revealed an abundance of lipid in sebaceous glands and surrounding emerging hair fibers in littermate control but not mutant mice (Figure 3, C and D). All other hair follicle types appeared to be involved as well as those of the pelage (Figure 4). The number of hair follicles per field were not significantly different from age- and sex-matched controls. Hair fibers that did emerge broke off near the surface in mutant mice. The interfollicular epidermis and follicular infundibular region became hyperplastic associated with a significant increase in keratinocyte proliferation ($P<0.001$), as determined by tritiated thymidine pulse labeling studies. There was a fivefold or more increase in DNA synthesis within the hair follicle infundibulum regardless of how the kinetics were calculated (Figure 5). The increased DNA synthesis was associated with severe orthokeratosis and production of cornified sheaths around the emerging, defective hair fibers. These features were best observed by scanning electron microscopy. Normal control dorsal skin had all four hair types emerging from hair follicles. Fibers emerged with various diameters and cuticular scale features reflecting the different hair types. The cuticular scale was prominent, sharp, smooth, and regular (Figure 6, C and D). In contrast, *ichq/ichq* skin had few hair fibers emerging, and those that did emerge had a prominent collar surrounding a short, thin, broken fiber. The fiber diameter and cuticular scale pattern resembled that of normal zigzag hairs in the control mice. The mutant hairs had numerous cuticular defects including linear striation, perforations, and pealing off of the scales (Figure 6, A and B). Nails on digits of both front and rear paws resembled littermate controls; however, collars of cornified cells around emerging hairs were prominent in mutant mice where fracturing of the compact epidermal scale occurred over joints due to bending (Figure 7).

Ventral skin had mild changes compared with those found in the dorsal skin, reflecting the variation in scale formation observed at the gross level. This was reflected in the morphometric analyses of the skin sections in which there were no significant differences between mutant and control skin regardless of what structures were measured (Figure 8). Differences between mutant and control mice were most evident in dorsal skin. Full skin thickness was slightly greater in mutant mice due to a decrease in the hypodermal fat layer offset by an increase in thickness of the dermal and epidermal layers in *ichq/ichq* mice compared with the +/? control mice (Figure 8). Dorsal skin of mice between 6 and 8 days of age was significantly different between mutants and controls for full thickness ($P = 0.005$), dermis ($P<0.001$), malphighian layer ($P<0.001$), and scale ($P<0.005$) but not significantly different for the hypodermal fat layer or follicle length. Dorsal *versus* ventral skin from mutant mice from 6 to 9 days of age were significantly different for all categories but not for the dermis and scale for mice 5 to 9 days of age or just the scale from mice 6 to 8 days of age.

Affected F2 intercross mice used in the genetic studies had many similar gross and histological features to those observed in the BALB/cJ *ichq/ichq* mice. The mutant offspring from these hybrid crosses developed grossly evident phenotypic changes over a period of several days within any

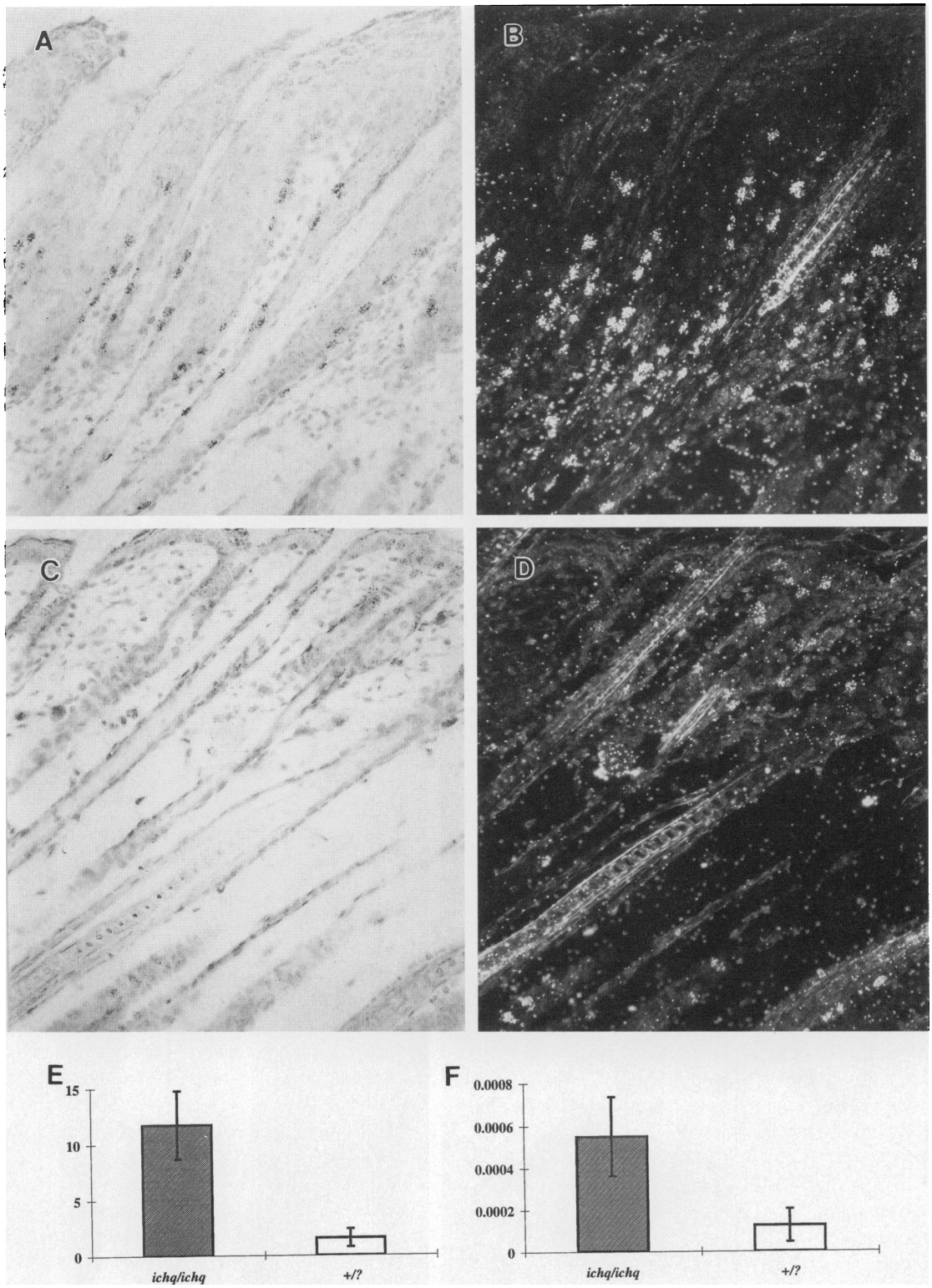


Figure 5. *Autoradiographs of tritiated thymidine pulse-labeled skin reveals high DNA synthesis within the infundibular epithelium of* ichq/ichq (**A** *and* **B**) *but not +/? control mice* (**C** *and* **D**) **A** *and* **C**: *Light field.* **B** *and* **D**: *Dark field. Magnification, ×312. DNA synthesis was significantly elevated* ($P<0.001$) *in* ichq/ichq *mice* versus *+/? controls regardless of whether the number of positive cells were measured per follicular infundibulum* (**E**) *or epithelial area* (μm^2) *within the dermis* (**F**).

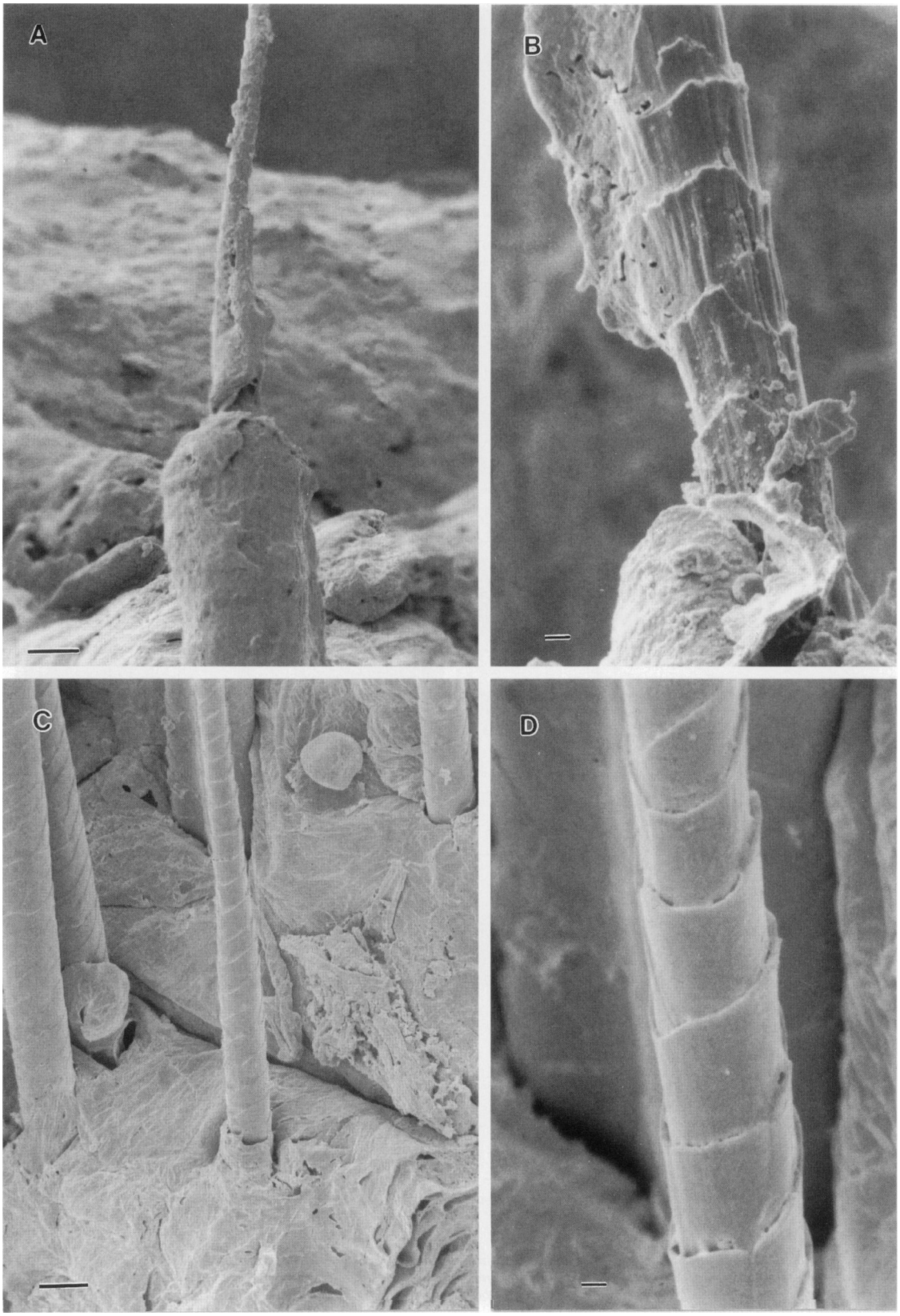


Figure 6. *Scanning electron microscopy of dorsal skin demonstrated a dense sheath of cornified cells around emerging fibers* (A) *with deformities of the fibers themselves* (B) *in* ichq/ichq *mice. In contrast, +/? controls had numerous normal hair fibers emerging from follicles* (C) *with normal features of the fiber shaft* (D). *Bar, 10 μm* (A *and* C) *and 1 μm* (B *and* D).

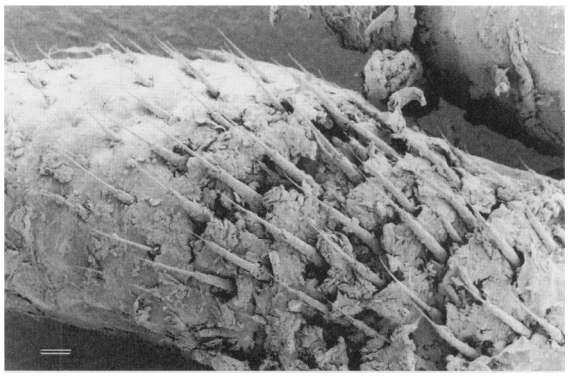


Figure 7. *Scanning electron micrograph illustrates uniform production of compact sheaths around emerging fibers on digits and fracturing of the scale over a joint secondary to bending. Bar, 100 μm.*

given litter (Figure 9A) compared with those on the BALB/cJ inbred background in which abnormalities were consistently evident by 5 days of age. Some hybrid mice lived 20 days or longer compared with the rare BALB/cJ *ichq/ichq* mouse that lived to 12 days of age. Microscopically, the phenotypic changes were similar except for 8/16 F2 hybrid *ichq/ichq* mice examined histologically that had focal parakeratosis around the follicular os (Figure 9B).

Immunohistochemistry

A panel of antibodies was used to evaluate expression of mouse-specific epidermal keratins, filaggrin, and loricrin. Suprabasilar terminal differentiation proteins K1 and K10 were strongly positive in normal +/? littermates. In contrast, *ichq/ichq* mice had decreased to patchy expression with areas of no expression in more hyperplastic biopsies. Keratins 5 and 14 were expressed in basal cells, hair follicles, and sebaceous glands in similar patterns in both mutant and control mice. Keratin 6 normally localizes within the inner layer of the outer root sheath of hair follicles, which it did in the control mice. However, in *ichq/ichq* mice, the hyperplastic epidermis exhibited a marked positive signal. Keratin 13 was not identified in either mutant or control skin as it is a marker of oral stratified squamous cells. Filaggrin was expressed in keratohyalin granules in both mutant and control mice. This was more prominent in the mutant mouse skin because of moderate to marked hypergranulosis. Loricrin was not expressed in biopsies from the first mutant mouse obtained before establishment of a breeding colony. That particular mouse was submitted dead. To determine whether the lack of expression was simply due to postmortem changes, skin from four normal BALB/cJ mice was collected, placed on an index card, and left at room temperature, and samples were placed into fixative every hour for the first 5 hours and then 24 hours after necropsy. All keratin and filaggrin expression patterns were identical in wild-type mice in both fresh and autolytic skin. However, skin from six *ichq/ichq* mice processed in an identical manner exhibited a loss of expression of loricrin but not other markers as tissues underwent autolysis.

Transmission Electron Microscopy

By transmission electron microscopy, mitochondria in mutant mouse skin were larger than those of littermate controls. Mitochondria in *ichq/ichq* mice were vesiculated and contained lamellar-like structures that became electron dense and homogeneous as they increased in size (Figure 10, A–D). Keratohyalin granules were present in both mutant and control skin. Those in control mice were more pleomorphic in size and shape than those in mutant mice (Figure 11, A and B). No lamellar bodies were observed in either mutant or control mice epidermis. The stratum corneum contained an abundance of vacuoles or degenerating organelles (Figure 11B). The dermis contained a mild increase in mast cells and neutrophils between hair follicles in mutant mice, which was also observed by light microscopy (Figure 2E).

Skin Grafting

Full-thickness skin grafts of both *ichq/ichq* and +/? donors retained their phenotypes on nude mouse recipients during the 10-week observation period. Various degrees of scarring were evident in the graft sites, but it did not affect the follicular features. In *ichq/ichq* grafts, interfollicular epidermis and follicular root sheaths remained hyperplastic. The compact sheath of cornified cells surrounded hair fibers, as was the case in donor skin. Control graft sites resembled normal adult mouse skin. By scanning electron microscopy, cornified material poured out of the follicular os and desquamated, resembling a rosebud opening. Hair fibers of various pelage types emerged and appeared to be normal, unlike the original donor tissue (Figure 12).

Genetics

Harlequin ichthyosis in the mouse was determined to be an autosomal recessive mutation by crossing a female C.B-C^{+}*Hbb*^{s} −+/+ with a male carrier of the new mutation (+/*ichq*). All F1 progeny were normal. One intercross mating of F1 × F1 yielded 7 affected and 27 normal offspring, which was not significantly

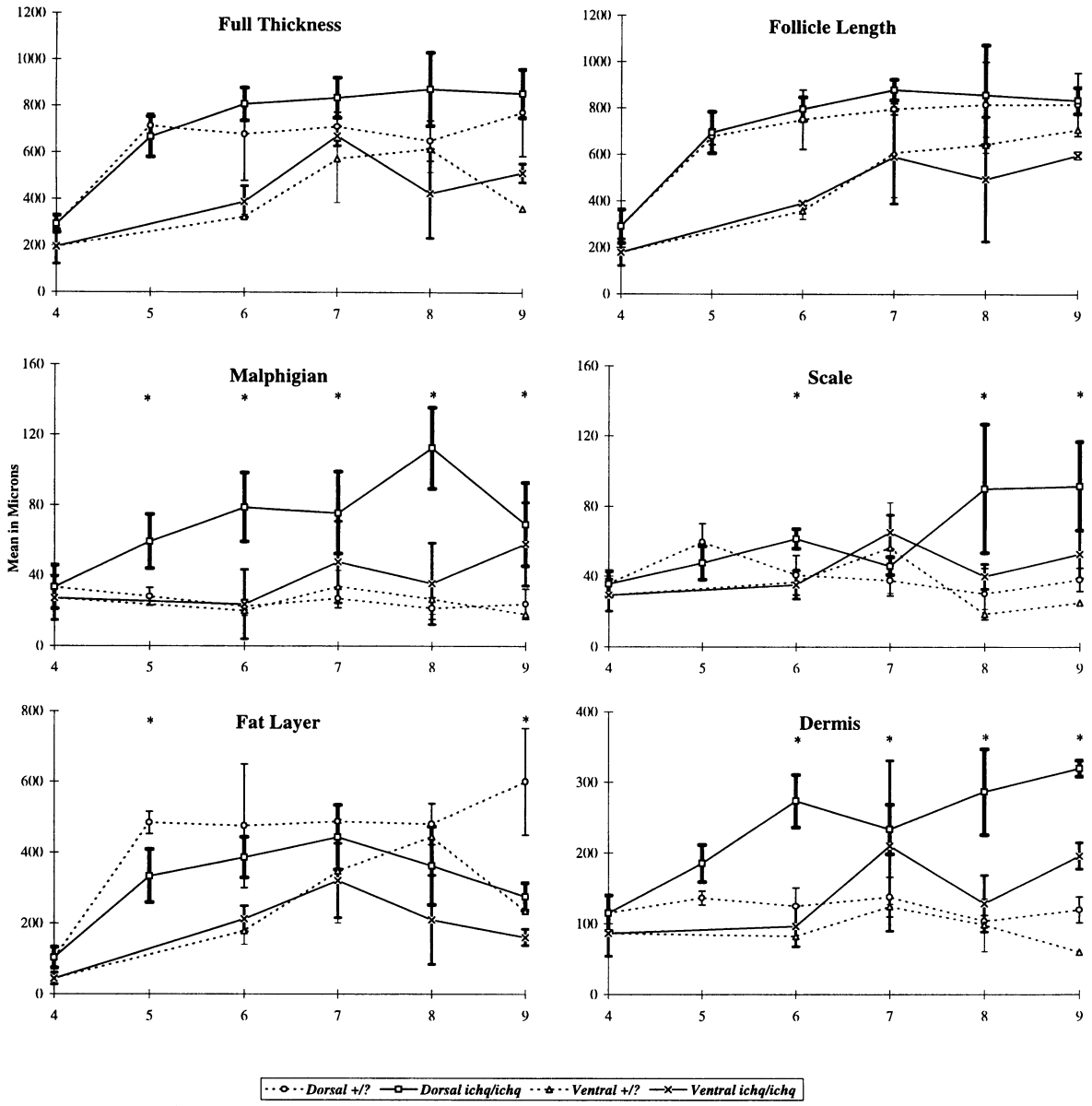


Figure 8. *Graphs summarize changes in thickness of the entire skin (full thickness), malphighian layer, hypodermal fat layer, follicle length, scale of the stratum corneum, and dermis of dorsal and ventral skin. The* y *axis is thickness/length in microns and the* x *axis is age in days.*

different (χ^2 = 0.34; $P > 0.6$) from the 1/4 ratio expected for a recessive mutation.

Affected F2 intercross mice were systematically tested for recombination with simple sequence length polymorphism markers using Massachussetts Institute of Technology primers, covering the entire mouse genome. Significant linkage was detected between the harlequin ichthyosis mutation and *D19Mit68* and *D19Mit29*. To refine the map location, markers flanking *D19Mit68* and *D19Mit29* were also tested for recombination, and the results indicated that the mutant gene locus was 4.0 cM distal from the centromere on mouse chromosome 19 (Figure 13).

Discussion

Harlequin ichthyosis is an extremely rare congenital, often neonatal, lethal disease in humans. As such, it is extremely difficult to investigate. We report here a spontaneous mouse mutation that appears to closely resemble human harlequin ichthyosis and offers a

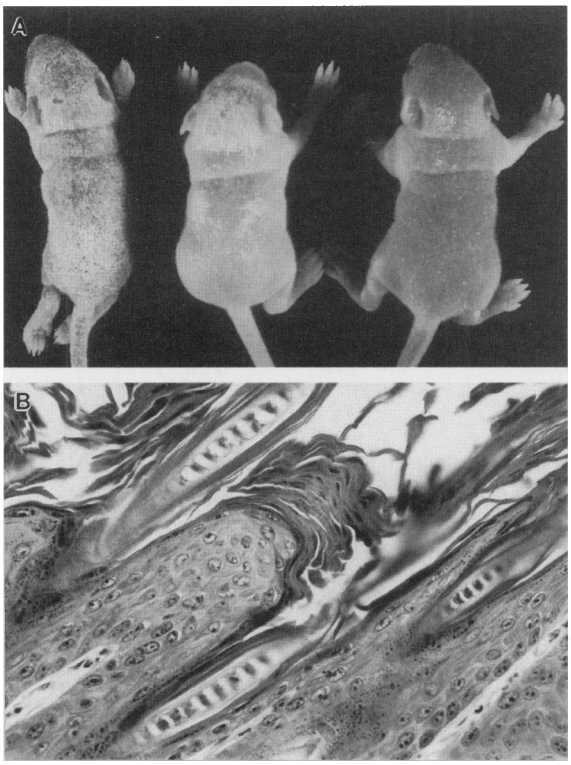


Figure 9. A: *Affected F2 hybrids from a cross between BALB/cJ−+/*chq *and CAST/Ei inbred mice reveals a variation in onset and severity of disease within one litter.* B: *One-half of the hybrids had prominent, focal parakeratosis. Magnification, ×500.*

potential model to investigate the underlying genetics and biochemical mechanisms of disease.

Human harlequin ichthyosis can be diagnosed prenatally between 20 and 22 weeks of gestation. This corresponds to the time when hair fibers emerge from follicles. Characteristic changes cannot easily be made in biopsies taken earlier.[51] Furthermore, ectropion can be a grossly evident feature in human fetuses with this disease.[50] Mice do not develop the harlequin ichthyosis phenotype *in utero* nor is it evident at birth, suggesting that these are not really similar diseases. However, the mouse cannot complete development in a 21-day gestation period. This is evident by the lack of hair fiber emergence until 5 days postpartum and eyelid opening until 12 days postpartum. As such, the clinical phenotypes that first appear in mice with the harlequin ichthyosis phenotype become evident at the same developmental time as human fetuses, except the development period corresponds to 5 days postpartum in the mouse. These types of observations as well as those of hair cycle development differences between the first (postpartum) and subsequent cycles[30] support this observation of postpartum completion of development.

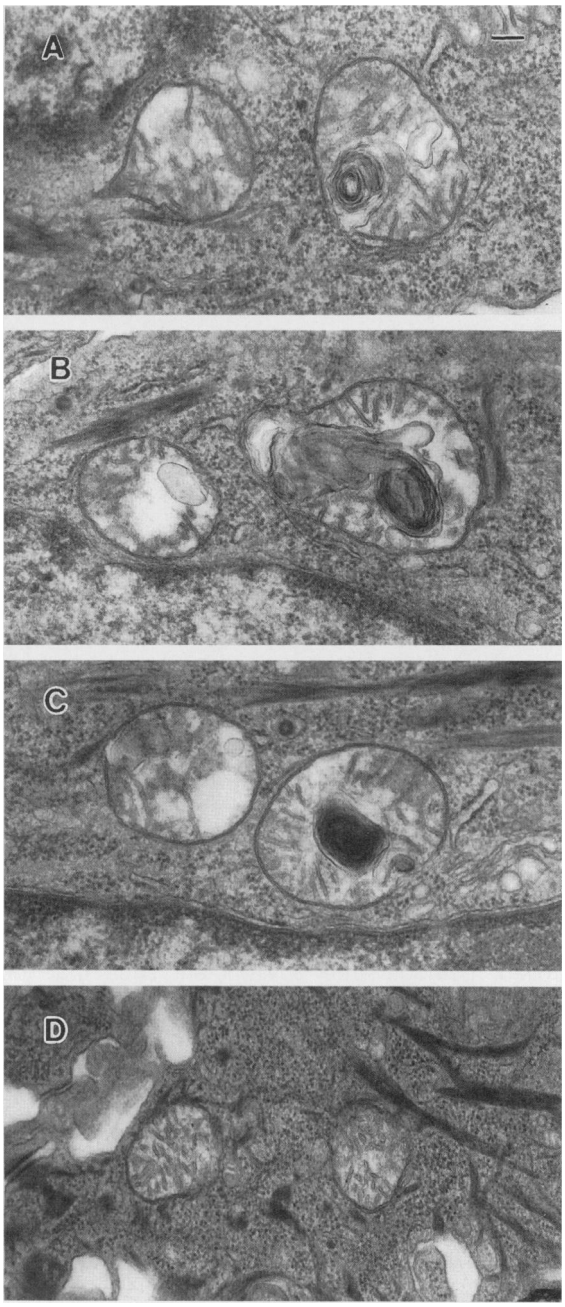


Figure 10. A *to* C: *Mutant mice had mitochondria that were large, contained vesicular structures, and developed lamellar structures that became homogeneous and electron dense.* D: *In contrast, control mice had smaller mitochondria with regular cristae. Bar, 100 nm.*

Human harlequin ichthyosis, not surprisingly, is a pleomorphic disease consisting of at least three subtypes.[20] At the ultrastructural level, common features found in many human cases and the mouse mutation resembling harlequin ichthyosis include abnormal, large mitochondria with vesicular changes and absence of lamellar granules.[50–52] The number, size, and distribution of keratohyalin granules varies in

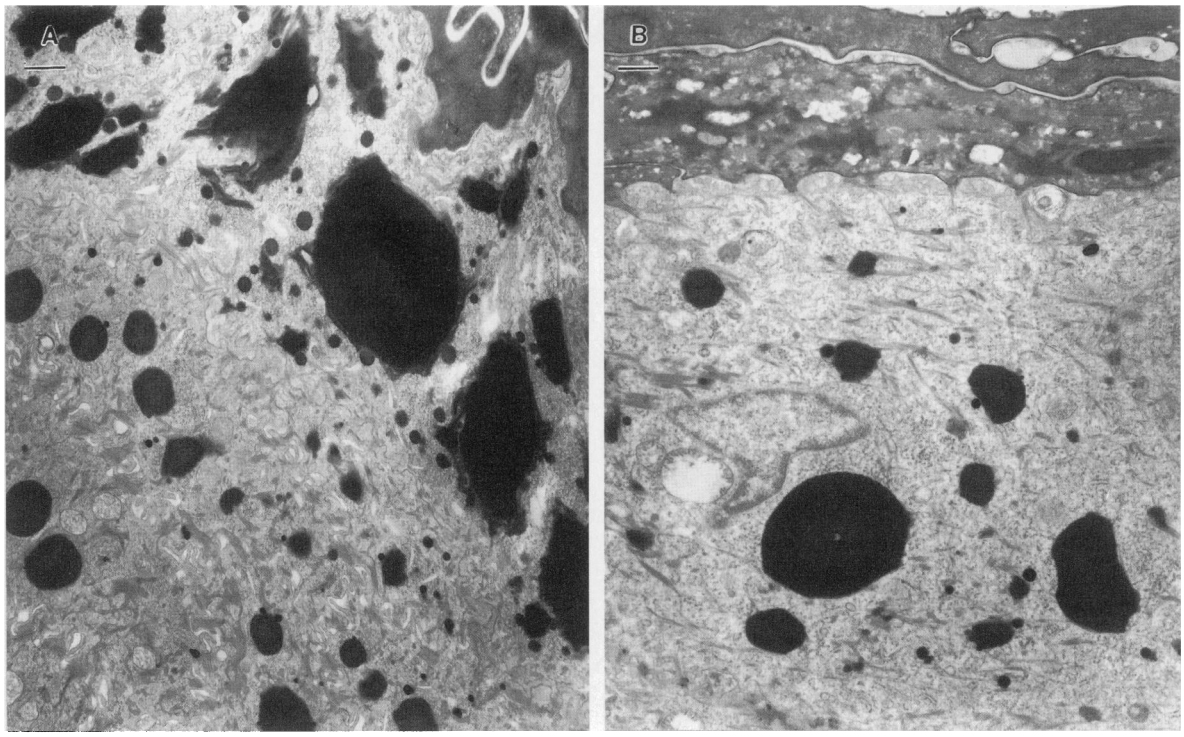


Figure 11. *Keratohyalin granules were more pleomorphic is size and shape in +/?* (**A**) *compared with* ichq/ichq (**B**) *mice. Bar, 0.5 μm. Note uniform* (**A**) versus *vacuolated* (**B**) *stratum corneum in control and mutant, respectively.*

human cases. Because of the variability in these features in human cases, direct correlation with the mouse mutation has little value. The mouse mutation is maintained on an inbred background in a controlled, pathogen-free environment such that the phenotype will be highly reproducible unless the genetic background or husbandry conditions are changed.[53] This was evident in F2 hybrids created by intercrosses between BALB/cJ and CAST/Ei strains resulting in development of focal parakeratosis at the microscopic level. More detailed studies of histological and ultrastructural changes of the skin from *ichq/ichq* mice on various congenic backgrounds will probably generate mutants that exhibit many of the ranges of changes reported in the scattered human case reports.

Scaly skin phenotypes in mice have been arbitrarily divided into the psoriasiform (inflammatory-based) and ichthyosiform (non-inflammatory-based) dermatoses.[54] As more of these types of mutations are defined, this distinction becomes more one of degree than an absolute separation. Mild infiltration of primarily neutrophils and mast cells were observed in the dermis of *ichq/ichq*. If the noninflammatory definition for ichthyosis is strictly adhered to, this would not make *ichq* a candidate for this group of diseases. However, similar types of infiltrates are reported in human harlequin ichthyosis cases.[51] The role of these cells in the scaly skin phenotype can only be speculated on. It may be possible to address it by grafting skin from *ichq/ichq* mice onto severe combined immunodeficiency (*scid/scid*) mice that are immunodeficient and will accept the grafts if these recipients are also homozygous for the beige mutation (*bg/bg*), in which granulocyte function is abnormal,[37] or other mutations that have defects in these and other cell types.

Formation of electron-dense bodies within mitochondria was a feature found in the harlequin ichthyosis mouse epidermal cells. Similar changes have been observed in hyperplastic mouse skin that is either induced with irritant chemicals[55] or in some genetically based hyperproliferative skin diseases such as flaky skin and chronic proliferative dermatitis.[45,56] Although it is tempting to consider this change to be a species-specific alteration of mitochondria in hyperproliferative skin, neither infectious causes of epidermal hyperplasia[57] nor spontaneous, genetic-based hyperproliferative disease in nude mice have these changes (Sundberg, unpublished observation).

Absence of lamellar granules in the epidermis has been reported in human harlequin ichthyosis.[50–52] This finding implicates a defect in lipid metabolism as the potential primary defect. Harlequin ichthyosis mice also lack lamellar granules. Mice selected as

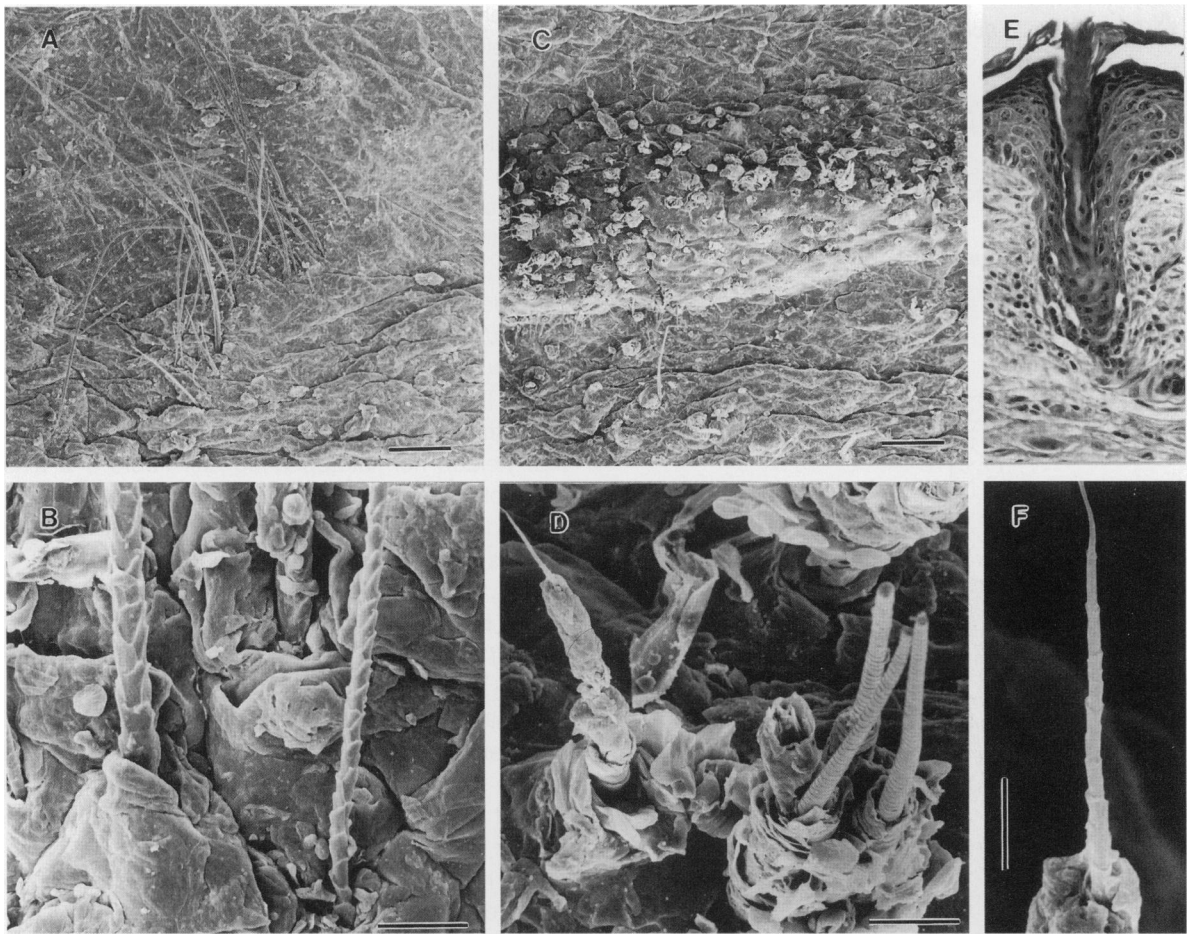
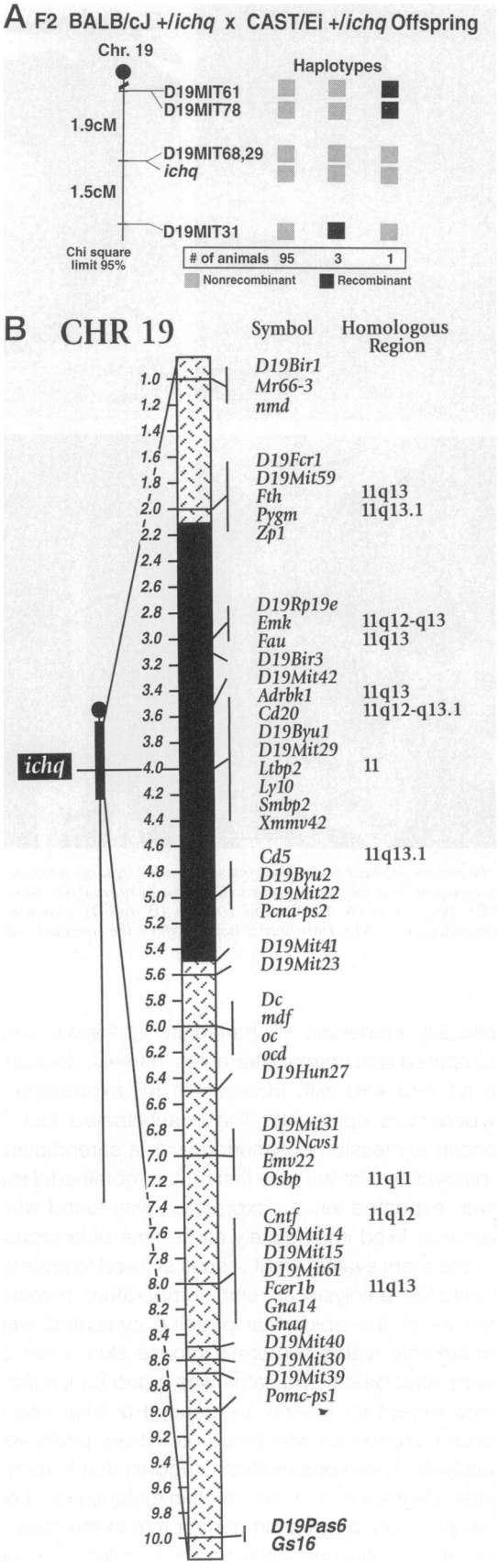


Figure 12. *Scanning electron microscopy of skin grafts onto nude mice reveal the presence of hair fibers emerging surrounded by large amounts of cornified material in* ichq/ichq (C) *mice. Mutant hair fibers of various types are emerging and the fibers appear to be relatively normal* (D). *Normal +/? skin grafted to the same mice resembled that of normal controls* (A *and* B). *Bar, 1 mm.* (A *and* C) *and 100 μm,* (B *and* D). *Histological examination revealed cornified cell collars around emerging hair fibers* (E). *Magnification, ×312. Hair fiber is normal above the cornified collar. Bar, 10 μm* (F); *higher magnification of fiber below* (D).

controls were normal-appearing, age-matched littermates. These controls also lacked lamellar granules. Specimens sent to Dr. P. Elias for lipid and more detailed ultrastructural studies were used to confirm the lack of lamellar granules in both mutant and control mice. Wild-type (+/+) BALB/cJ mice will need to be screened to rule out a strain mutation on heterozygous effect if the controls had one copy of the mutant gene.

Freeze-fracture studies of *ichq/ichq* mouse skin demonstrated the tightly packed and very thick stratum corneum. This feature is similar to human harlequin ichthyosis. Other mouse mutations with scaly skin have separation of scales into individual squamae,[45] very different from *ichq/ichq* mice.

Keratin, filaggrin, and loricrin expression follow predictable patterns in mouse mutations[29,41,43] or carcinogen-induced cutaneous hyperplasia or neoplasia.[46] Similar patterns were identified in the interfollicular epidermis of harlequin ichthyosis mice compared with normal littermates, namely, decrease in K1 and K10 with increase in K6 expression in hyperplastic epidermis. The unanticipated lack of loricrin expression postmortem was a serendipitous observation with the very first mutant obtained. However, expected loricrin expression was found when skin was fixed immediately at the time of necropsy. Subsequent evaluation of tissues allowed to undergo controlled autolysis at room temperature revealed that all of the epidermal proteins evaluated were remarkably stable in normal mouse skin, even 24 hours after death. The same was found for *ichq/ichq* mice except for loricrin. Decreased or total loss of loricrin expression was found as tissues underwent autolysis. These observations suggest that loricrin is either degraded or cross-linked in mutant mice. Loricrin probably plays an important role in the cutaneous abnormality and water barrier functions in harle-



quin ichthyosis. Three other mouse mutations with hair defects (soft coat, *soc*; matted, *ma*) or scaly skin (flaky tail, *ft*) map near the loricrin gene on mouse chromosome 3.[37,58] If the scaly skin mutation proves to be due to a primary defect in loricrin, functions of loricrin in these types of phenotypic abnormalities may be defined. The mechanisms involved are not currently understood, although they are under investigation.

To determine whether the cutaneous phenotype of harlequin ichthyosis resolved with age, skin from both mutant and control mice was grafted onto nude mice. The hair fiber cuticular defects observed in intact mutant mice resolved in the 10-week-old grafts that yielded normal-appearing hair fibers and also were of different pelage hair types. However, the marked abnormal cornification process within the follicles remained a feature during the observation period. The lower portion of anagen follicles, the region below the dermis in the hypodermal fat layer, in *ichq/ichq* mice appeared to be normal. These grafting studies suggest that the lower portion of the hair follicle is uninvolved in the mutant phenotype and can produce a normal hair fiber if the mutant mouse survives. These studies also suggest that the mutant phenotype is limited to the infundibular region of the follicle.

The harlequin ichthyosis mutation was mapped to the proximal end of mouse chromosome 19. Although the locus for the human harlequin ichthyosis mutation has not been mapped, the homologous region of the human genome is chromosome 11q12–13, which is a likely region to investigate.[59] Over 20 genes have been mapped in the region of mouse chromosome 19 between 1.0 and 7.0 cM. Of these, potential candidates for the *ichq* mutation include adrenergic receptor kinase-β1 (*Adrbk1*), proliferating cell nuclear antigen pseudogene 2 (*Pcna-ps2*), phospholipase Cβ3 (*Plcb3*), latent transforming growth factor-β binding protein 2 (*Ltbp2*), and oxysterol binding protein (*Osbp*).[59] As the resolution of this map is further refined, the list of candidate genes can be reduced to make identification and/or positional cloning of novel genes a realistic goal.

Another mouse mutation, asebia (*ab*), located on mouse chromosome 19, which is not a juvenile lethal, has hyperplastic interfollicular epidermis, long, mildly hyperplastic anagen hair follicles, and small, hypoplastic sebaceous glands. Mutations at the ase-

Figure 13. **A:** *Mouse chromosome 19 genotypes and resulting genetic map for 99 F2* ichq/ichq *mice (198 meioses) from an intersubspecific cross.* **B:** *Harlequin ichthyosis mapped to the proximal end of mouse chromosome 19, 4.0 cM from the centromere. Human Chromosome 11q12–13 has many homologous sequences.*[59]

bia locus[37] are not alleles of harlequin ichthyosis. The asebia locus is 39 cM from the centromere, very distal from *ichq*, which maps only 4.0 cM from the centromere.[59]

The harlequin ichthyosis mouse mutation represents a powerful new tool to investigate the pathophysiology and genetics of this rare, deforming, juvenile lethal disease found in many species of mammals, including humans.

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