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# A New Murine Model for Mammalian Wound Repair and Regeneration

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**Regeneration is generally considered to be a phenomenon restricted to amphibians in which amputated limbs reform and regrow. We have recently noted a strain of mouse, the MRL, which displays a remarkable capacity for cartilagenous wound closure and provides an example of a phenomenon previously considered to be a form of regeneration. Specifically, through-and-through ear punches rapidly attain full closure with normal tissue architecture reminiscent of regeneration seen in amphibians as opposed to scarring, as usually seen in mammals. Histologically, we have demonstrated normal cell growth and microanatomy, including angiogenesis and chondrogenesis, as opposed to control C57BL/6 mice which have ear holes that contract minimally but do not close. Finally, this phenomenon is a genetically definable quantitative trait.** 

***Key Words:*** **mice; wound healing; regeneration.**

## INTRODUCTION

The biological response to traumatic injury in higher organisms falls into two categories: regeneration and wound repair. Regeneration involves the gross replacement and restoration of adult tissue mass with normal architecture and function and, in the extreme case, full organs, whereas wound repair involves the migration of fibroblasts to the wound site, formation of granulation tissue, and the laying down of collagen in a disorganized fashion with the formation of scar tissue. Rarely do hair follicles and sweat glands return and normal architecture and function is not fully restored. It is generally observed that the capacity for tissue regeneration in mammals is limited, especially compared to that in amphibians, where entire limbs can be regenerated after amputation (1). This apparent lack of mammalian regenerative capacity has largely directed the focus of study toward wound repair, the archetypal response to injury in mammals.

While the above describes the generic mode of wound healing in mammals, there do exist several instances of limited regeneration reminiscent of that occurring in amphibians (2). They include the regrowth of the tips of fingers (3), antlers (4), and an example of rabbit ear hole closure (5). This suggests that the capacity for regeneration has not been completely lost.

There are, of course, cells and tissues in mammals that are continually being replaced. This occurs either (a) by cell duplication producing, for example, liver (6, 7) and blood vessels through the process of angiogenesis (8) or (b) by the generation and differentiation of cells from stem cells, a process that produces new epithelial cells (9, 10) and lymphoid cells (11). However, in these cases, the architectural scaffolding is often maintained and hence there is no gross tissue replacement, i.e., regeneration. On the other hand, certain cells and tissues exist throughout life without being replaced and do not proliferate. These include nerve cells, heart tissue, the eye lens, and the cartilage of the nose and ear (12).

We describe herein a mouse model of wound repair and regeneration which has allowed us to begin to explore the molecular, cellular, and genetic bases of this phenomenon. Full-thickness through-and-through ear punches of about 2 mm in diameter have generally been used as a means of long-term identification of mice kept in colonies. This is based on the fact that these ear punch holes generally do not close over the lifetime of the animal and provide a convenient and permanent marker. We noted in the case of the MRL mouse strain, however, that these earpunches were transient, with full closure occurring within 4 weeks with normal tissue architecture and without scarring. That this is probably not only wound repair but regeneration is indicated by the presence of normal dermal regrowth with organized extracellular matrix (ECM) deposition, normal vasculature, and cartilage regrowth that is similar to that in the normal ear. In contrast, ear hole wounds in control C57BL/6 mice (and for that matter, in every other strain of mouse we are aware of) never close completely. Histological differences can be seen between the MRL and the C57BL/6 mice as early as day 2, and these differences include rate of reepithelialization, annular swelling, rapid connective tissue proliferation, angiogenesis, and chondrogenesis.

The MRL mouse has been intensively studied as a model for systemic lupus erythematosis (SLE), and, in its mutant form, the lpr/lpr strain displays gross and rapid lymphoproliferative disease, serum autoantibod-



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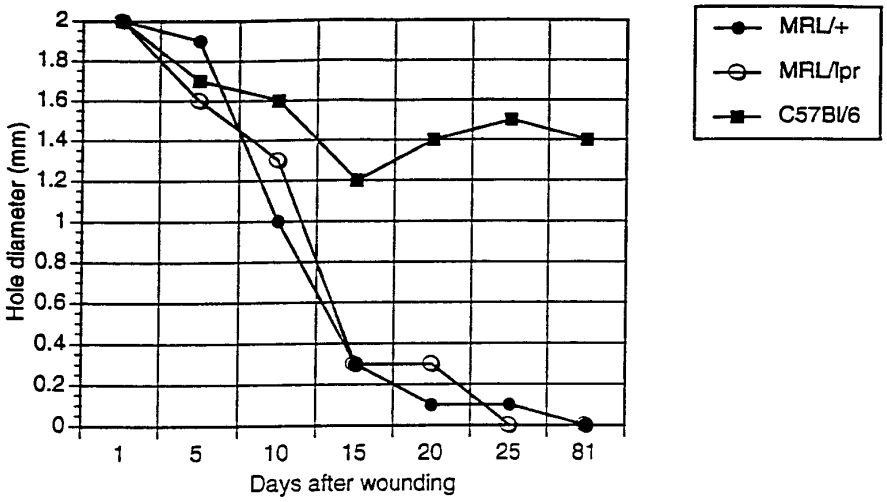



**FIG. 1.** The kinetics of ear punch hole closure. Two-millimeter holes were punched in ears on day 0 and, for each strain of mouse, holes were measured at days indicated on the horizontal axis. Average hole diameters are shown ($n = 4$).

ies, and autoimmunity as it ages (13–15). This lpr defect is due to a mutation in the fas gene which leads to an inability to mediate apoptosis of lymphocytes (16, 17). While this defect might suggest that an unchecked growth of cells due to lack of apoptosis is the underlying cause of the regenerative capacity, both MRL/lpr and MRL/+ mice, with and without a defective fas molecule, in fact have the same regenerative capacity. Breeding studies involving the generation of F1 and backcross strains between MRL and C57BL/6 mice have shown that there is a genetic basis for this wound repair/regeneration trait.

## METHODS

*Animals.* MRL/Mp−+/+ (MRL/+) mice and MRL/Mp−lpr/lpr (MRL/lpr) mice were obtained from The Jackson Laboratories (Bar Harbor, ME). C57Bl/6 (B6) mice were obtained from Taconic Laboratories (Germantown, NY). The animals were then bred and maintained under standard conditions at the Wistar Institute Animal Facility.

*Phenotyping.* A 2-mm through-and-through hole was made in the center of the cartilagenous part of both ears of 6-week-old mice using a metal ear punch (Fisher Scientific, Pittsburgh, Catalog No. 01-337B). The holes were measured at the time of wounding and followed for wound closure using a grid-etched reticle (Bausch and Lomb, 7×).

*Histology.* Ears were removed with scissors by cutting at the base of the pinna. They were fixed overnight in 10% buffered formalin. To facilitate sectioning, they were held flat during fixation by inverting the lid of a processing cassette on the base and sandwiching the flattened ear by applying gentle pressure with a rubber band.

Once fixed, ears were bissected across the widest point of the hole using a dissecting microscope and a No. 10 scalpel blade. The two halves were then glued together with collodion, again using the dissecting microscope to obtain perfect alignment of the cut edges and hole margins. Because the collodion would most likely dissolve in reagents used to prepare specimens for paraffin embedding, the ears were sutured together using 5-O silk on a 1 $\frac{1}{2}$-in. straight Keith Abdominal cutting needle with a triangular point.

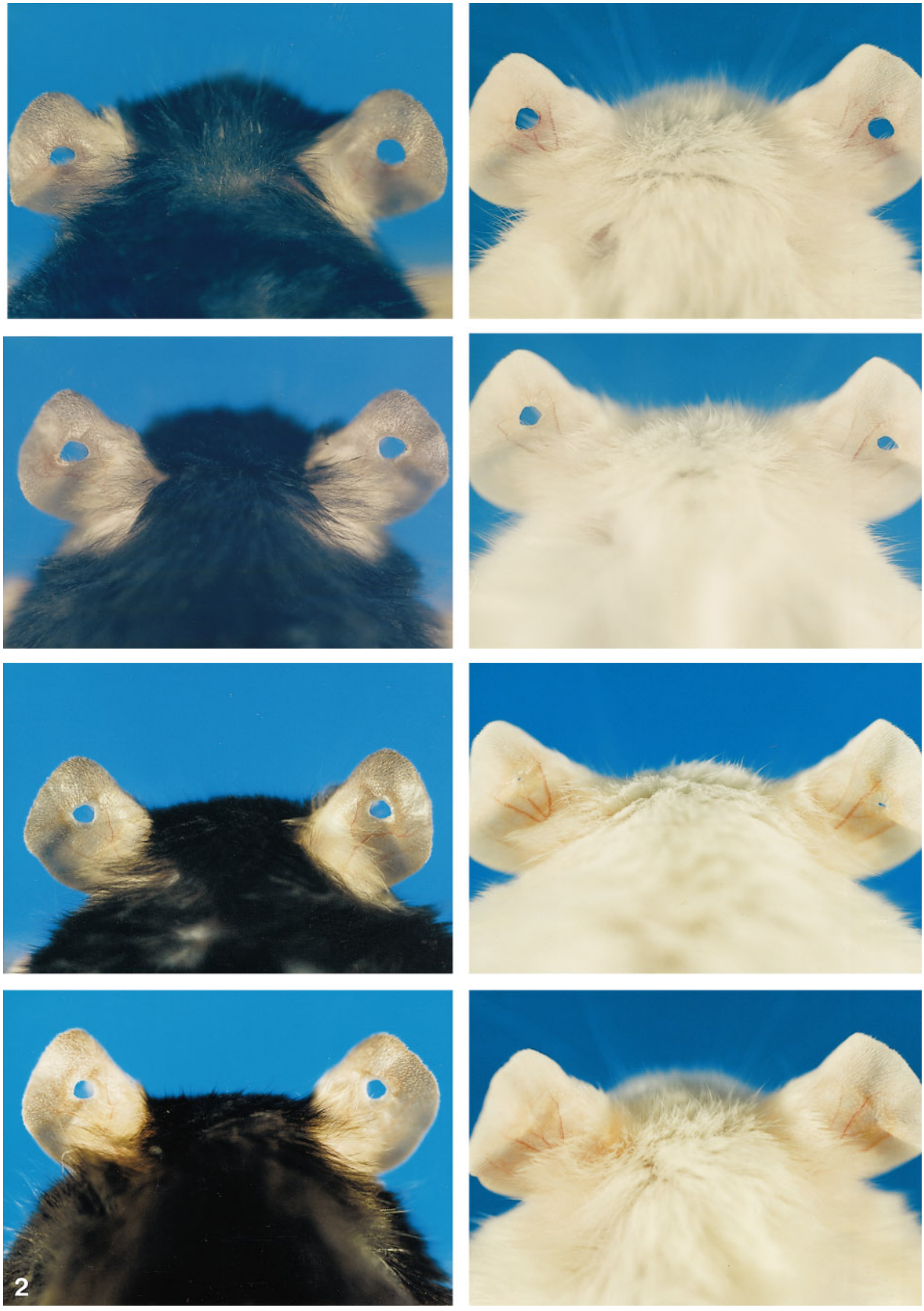
Tissues were embedded in paraffin and sectioned so that the cut edges containing the hole margins were in the plane of the section. Slides were stained with hematoxylin and eosin or with Gomori trichrome strain.

## RESULTS

### *The Wound Healing Phenotype: Kinetics and Gross Aspects*

C57BL/6 and MRL mice were ear punched using a standard metal ear punch to create a well-circum-

**FIG. 2.** Photographs of the healing ear wounds. C57BL/6 (left) and MRL/lpr (right) ears were punched bilaterally in the center of the ears creating a 2-mm through-and-through wound and followed for 33 days. From top to bottom, one can see the progression of hole closure from day 1, day 9, day 20, to day 33.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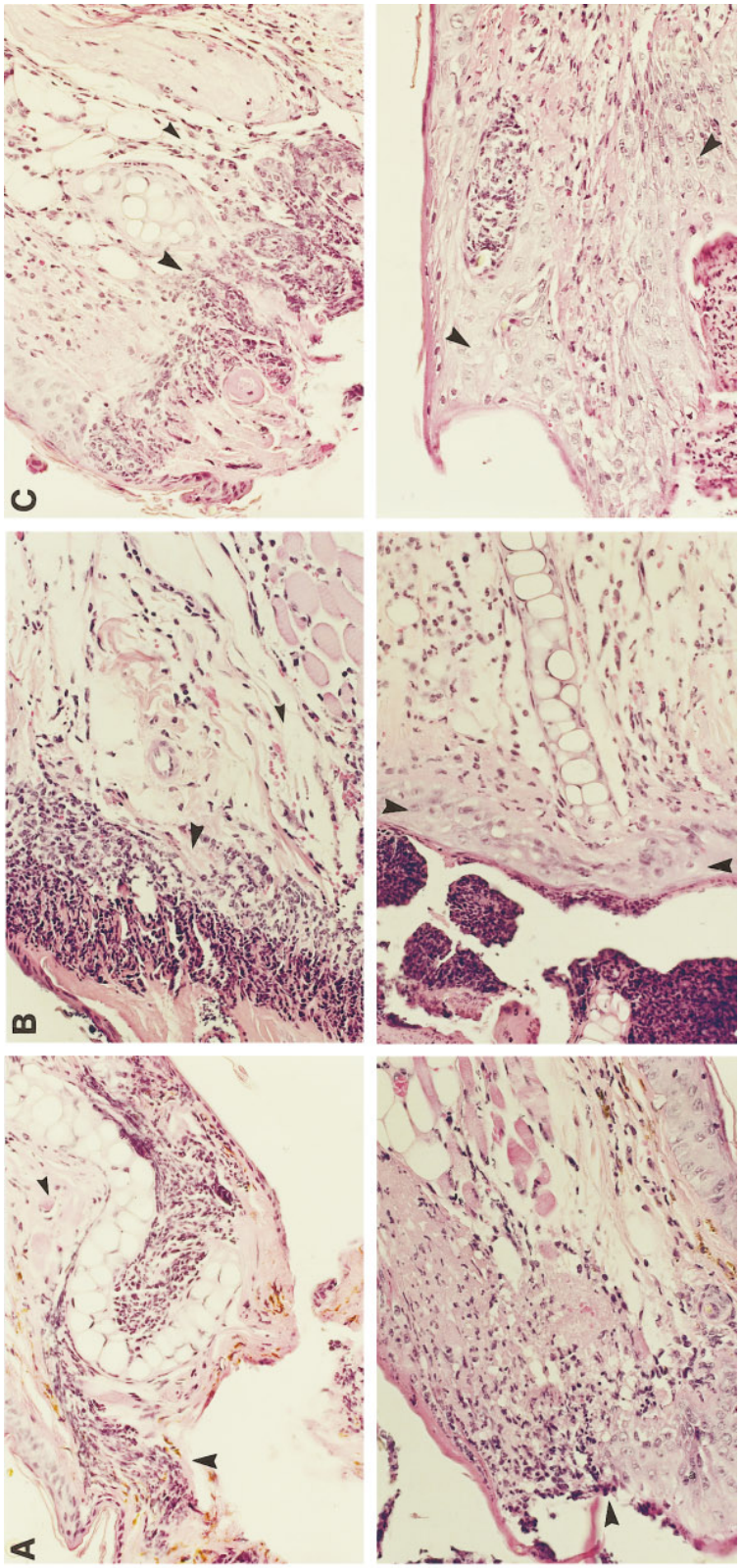


**FIG. 3.** Histological examination of early events in ear hole closure. The first 2 days after wounding of C57BL/6 ears (column A) and MRL/+ (column B) and MRL/lpr ears (column C) were examined. On day 1, these sections show more swelling at the wound site for the MRL tissue (smaller arrowheads). Eschar and inflammatory cellular infiltrate at the wound margin is similar for both strains and reepithelialization is not seen (larger arrowheads). On day 2, The C57BL/6 wound is partially covered (arrowhead) by eschar with migrating epithelium not yet covering the wound (9 of 14 edges examined did not close) while the MRL wound is completely covered (arrowheads) with epithelium (10 of 14 edges examined completely closed). Magnification, 40×, tissue is stained with hemotoxylin and eosin.

scribed circular surgical wound of about 2 mm in diameter at a site at which the thickness of the ear is considerably less than a millimeter. The animals used initially were within the 8- to 12-week-old young-adult age range so that early developmental considerations would not be an issue. As can be seen in Fig. 1, by day 15 maximal closure was achieved in the C57BL/6 with a 30% reduction in the original hole diameter, and this remains stable. In contrast, the MRL achieves an 85% reduction in hole diameter by day 15 with complete closure by day 25. Reexamination of the ears on day 81 showed no further changes. In Fig. 2, the closed MRL wound is evident and it is difficult to identify the original site of the hole since there is no fibrosis or scarring.

### *The Wound Healing Phenotype: Histological Aspects*

One possibility considered for the complete hole closure in MRL mice was that there was a defect in the ability of their epithelium to migrate across the cut edge of the dermis and cartilage, thereby allowing uninhibited connective tissue proliferation. Histologic sections of healing ear punch holes for the first 2 days were examined (Fig. 3). This possibility was rejected when it was observed that not only did epithelium promptly migrate across the MRL wounds, but that this change occurred 1 day earlier for MRL mice (day 2) than for C57BL/6 mice (day 3, not shown). Indeed, epithelium completely covered virtually all wounds examined after day 1 for MRL mice. Epithelium covered all C57BL/6 wounds examined after and including day 3, except one wound from day 5 (Fig. 4a) which showed the continued presence of eschar with migrating epithelium failing to bridge the cut edge.

As ears were prepared for histology, two grossly observable differences were noted between C57BL/6 and MRL. The first was that, for all time points, the tissue surrounding the wounds in the MRL ears was severely hyperemic when compared to that of the C57BL/6 wounds. Also, starting on day 4, and continung on each succeeding day, a prominent annular swelling was observed around the MRL wounds that was absent for C57BL/6 wounds.

Consistent with the grossly observable differences between the wounds, histological examination at each timepoint (Figs. 4a, 4b, and 4c; days 5, 10, and 20) showed a marked difference in the degree of angiogenesis, cell proliferation, connective tissue matrix formation, fibroblast migration, and ECM deposition occurring in the two strains. Also, the presence of hair follicles with accompanying sebaceous glands within the healing wounds was noted for both MRL and C57BL/6 wounds but appeared more prominent and numerous in MRL than in C57BL/6 wounds.

At all of these time points, C57BL/6 wounds have shown limited progression beyond the cut cartilage margins and have a distinct paucity of epidermal hair follicles and sebaceous glands. In contrast, the MRL wounds show marked progress toward full closure due to extensive dermal proliferation and are well supplied with hair follicles and sebaceous glands in the new growth zone. ECM is laid down so as to preserve normal architecture, the underlying connective tissue is hyperplastic, and the epidermis is rich and thick. The ear cartilage layer has not significantly extended into the wound site beyond the initial cut margin.

In Fig. 5, C57BL/6 and MRL ears are shown on day 81 after wounding. Here numerous ingrowths of cartilage that are absent from the C57BL/6 ear can be seen in the MRL ear. The cartilage ingrowths are surrounded by numerous adipocytes which normally make up the minor subcutaneous or hypodermal layer connecting ear cartilage to dermis. It is not clear why fat cells have come to be such a prominent cell type by this time point.

### *The Pattern of Inheritance Seen in Wound Healing is Quantitative*

Our initial findings on the hereditary nature of the wound healing trait can be seen in Fig. 6 (top). In these studies, mice were ear punched at 6 weeks of age and were examined at 2 and 4 weeks after ear punching. The 4-week ear hole size of the MRL mice ranged from 0 to 0.4 mm, while the ear hole size of the C57BL/6 mice ranged from 1.2 to 1.6 mm. These two healing phenotypes were nonoverlapping. Fifteen F1 mice bred from MRL $\times$ C57BL/6 had ear holes intermediate between those of the two parents (ranging from 0.4 to 1.1 mm).

In an initial experiment, two backcross populations were created by using (MRL female $\times$ C57BL/6 male) F1 females and mating them to the parental males (Fig. 6, bottom). The backcross population to MRL displayed a curve skewed to MRL-type healing. In the backcross population to C57BL/6, the progeny showed a curve with its mean displaced to C57BL/6-type (i.e., poor) healing. The healing thus appears to be a quantitative trait.

## DISCUSSION

### *A Curious Result*

These studies arose from the serendipitous finding that the numbering by ear punching of MRL mice proved to be ineffective as several weeks after punching, the ear holes disappeared. Perhaps the most striking characteristic was that the ear hole would close without detectable scarring. Ear punching, in which a well-circumscribed through-and-through circular wound of about 2 mm in diameter at a site in which

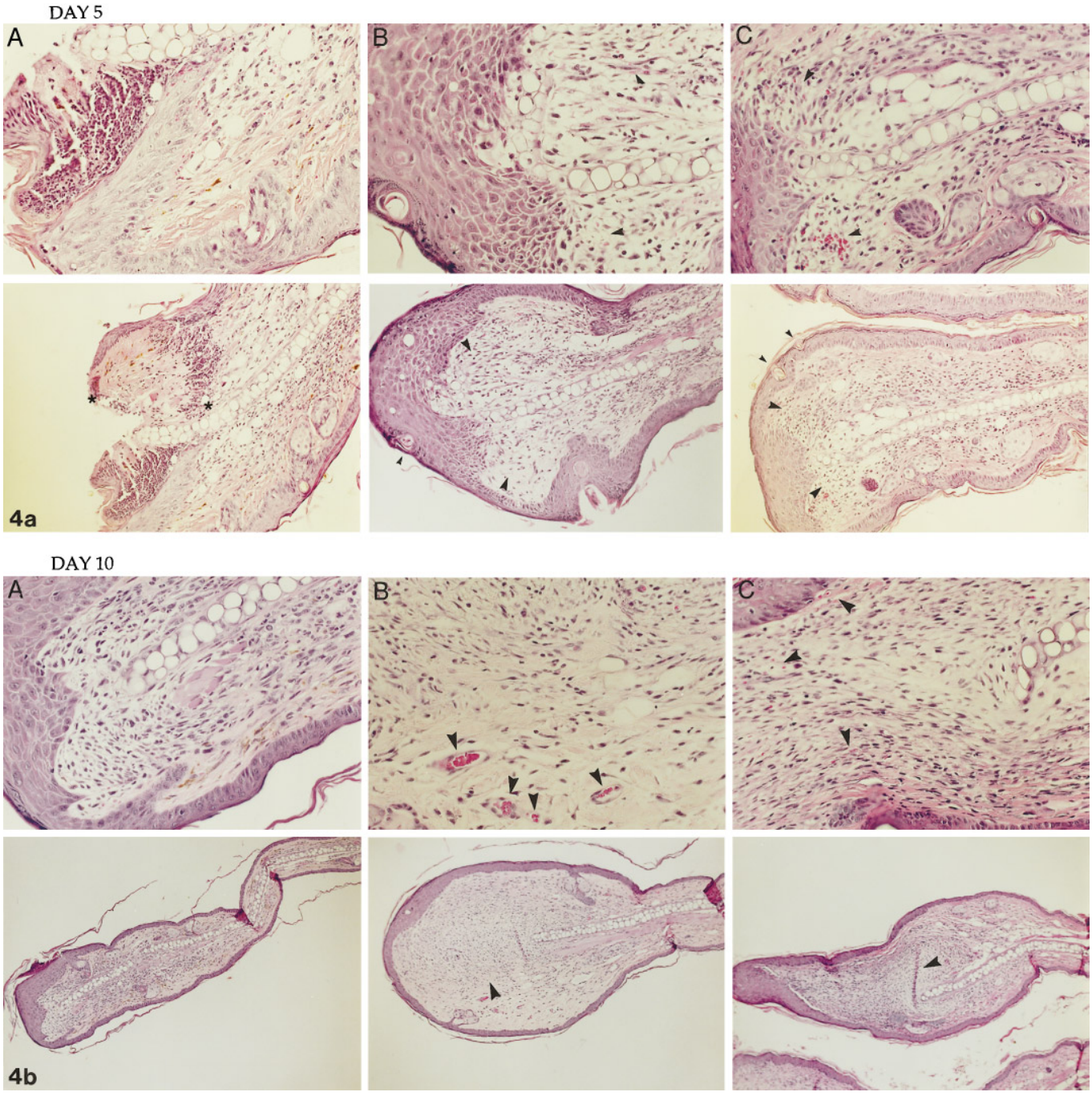


**FIG. 4.** Day 5, 10, and 20 wounds. For all days indicated for C57BL/6 tissue (A), MRL/+ tissue (B), and MRL/lpr tissue (C). All sections are stained with hemotoxylin and eosin (pictures are representative of four edges examined). (a) Day 5 (A, B, C: 40×, top; and 20×, bottom): Swelling at the MRL wound site is extensive with neovascularization (40×, arrowheads) and marked dermal fibroblast proliferation (20×, large arrowheads). Adnexae in the new epithelium can be seen here (20×, small arrowheads). A C57BL/6 wound that has failed to epithelialize is shown here, although this is not typical (20×, *). (b) Day 10 (A, B, C: 40×, top; and 10×, bottom): There is marked neovascularization (40×, arrows) and fibroblast proliferation (10×, arrows) seen in the MRL wound extending out beyond the borders of the wound where the cut cartilage edges are seen. Note the extent to which dermal cells have migrated out beyond the wound margin marked by the cartilage edge for the two MRL ears compared to the C57BL/6 ear. (c) Day 20 (A, C:20×; B:40×-upper panels and 5×-lower panels): The prominent proliferation of fibroblasts in the dermis and the appearance of a blastema-like structure has led to significant closure of the MRL wound as originally marked by the cut edges of cartilage at the right and left margins of the photograph (5×-between arrows). By comparison, there is little extension of C57BL/6 tissue into the wound space. The homogeneity of fibroblast proliferation and ECM deposition is most striking in MRL (B:40×-upper panel).

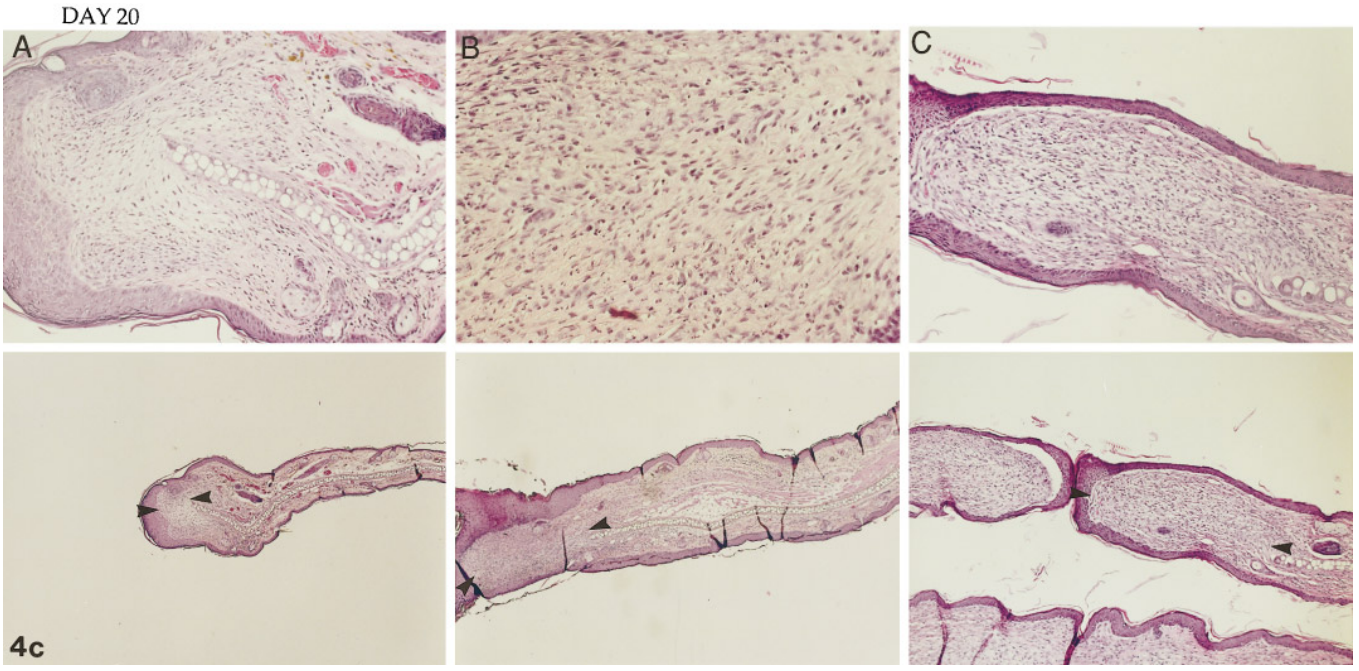


**FIG. 4**—*Continued*

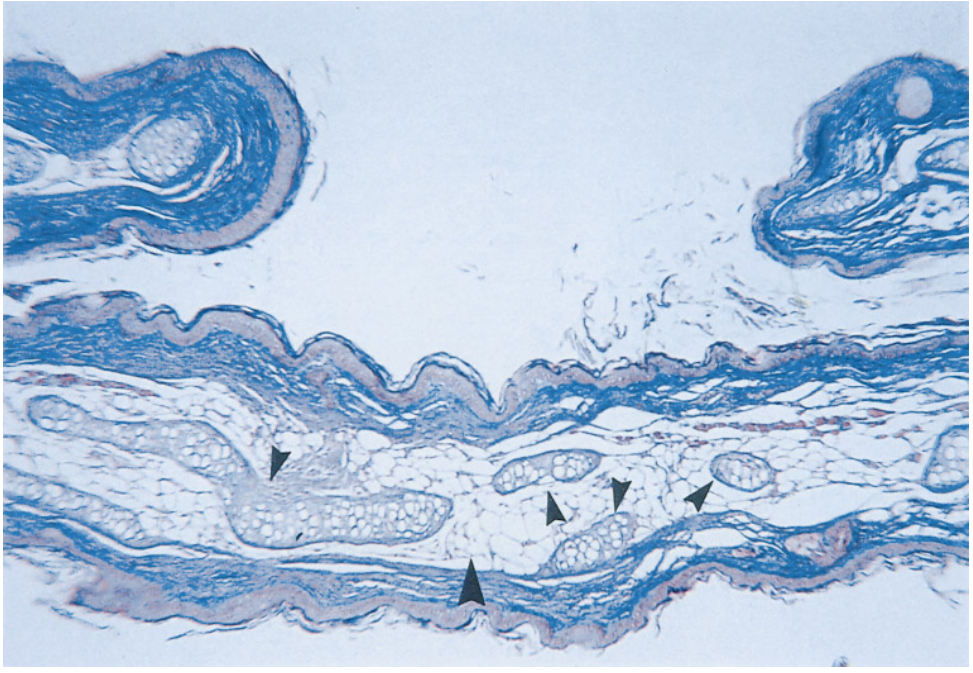

**FIG. 5.** Late-stage ear wound closure. Wound sites ($n$ = 3) 81 days after wounding for C57BL/6 (top) and MRL/lpr (bottom) mice were aligned using a dissection microscope and sutured together to ensure sectioning through the former wound for MRL/lpr tissue. Cartilagenous islands (small arrowhead) present throughout the MRL/lpr section are surrounded by prominent adipocytes (large arrowhead). These features are absent from the C57BL/6 tissue. The tissue sections are stained with Gomori trichrome. Magnification, 10×.

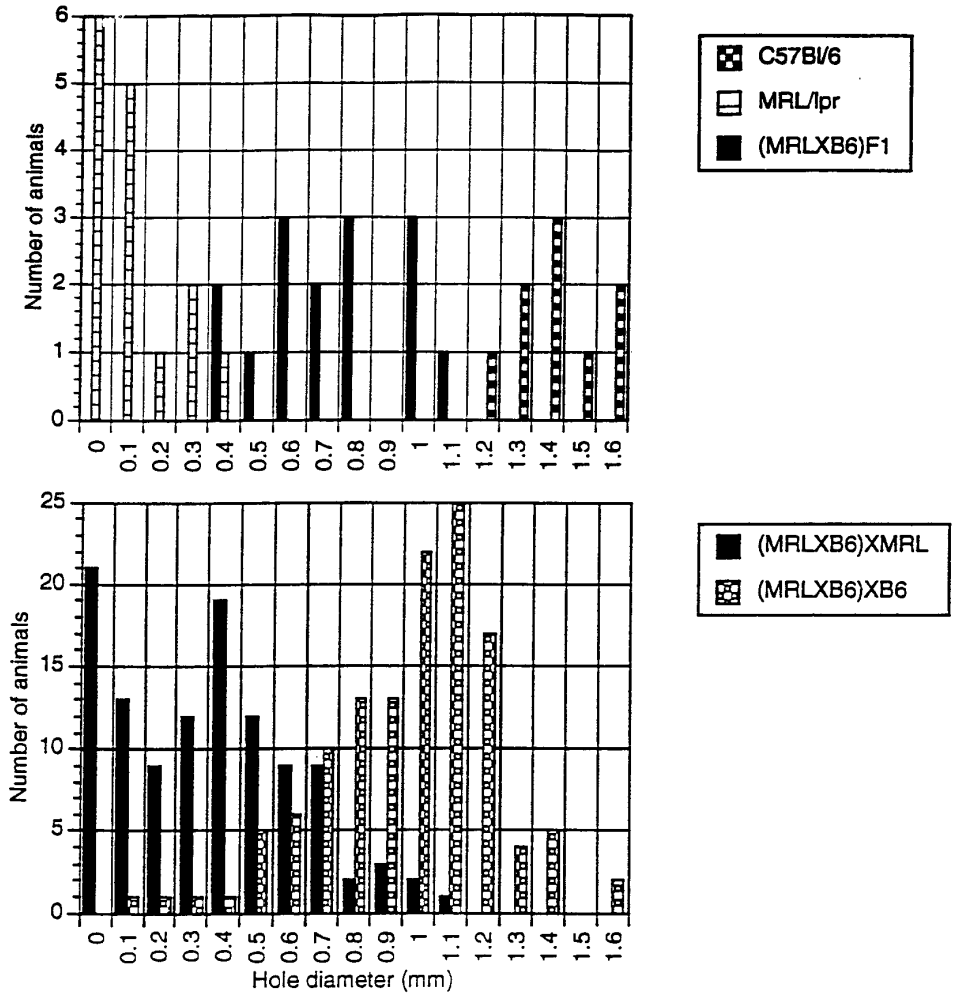


**FIG. 6.** The analysis of frequencies of wound closure on day 30 in parental and crossbred populations. Histograms of day 30 earpunch hole diameters can be seen for the following genotypes: C57BL/6 and MRL/lpr parental mice and (MRLxB6)$F_1$ mice (top) and the first backcross to each parental strain (bottom).

the thickness of the ear is considerably less than a millimeter, is a general method of identifying and numbering mice over a long period of time. In all other mouse strains, these ear punches do not heal shut and generally lead to sharp and well-defined circular holes with circumferential healing. This is unlike what is seen with rodent trunk excision wounds which show contracture of the wound bed and scarification (18). Rodent body skin is loose, unlike mouse ear skin, which is connected to a layer of cartilage. Thus, wound contracture is all but impossible in our study where there is no wound bed and the adjacent tissue is firmly attached to the underlying cartilage.

Histological studies showed that normal tissue ingrowth and remodeling with epithelial and fibroblast proliferation were the cause of the closure. In the MRL strain, new epithelium closed the circumferential wound margin within 2 days and became stratified and cornified. ECM was laid down in an orderly fashion, and underlying connective tissue was hyperplastic and appeared to form a blastema-like structure. The blastema has been described in terms of amphibian limb regeneration. In the adult animal, it is the beginning of new tissue mass derived from adult mesenchymal tissue at the limb stump where proliferation, dedifferentiation, and redifferentiation occur as part of the process of regeneration (19, 20). The major cell types that undergo dedifferentiation include dermis and cartilage (21). With the new dermal growth seen in the MRL mouse, angiogenesis was seen as early as day 4. Hair follicles and sebaceous glands were seen in this new growth area but it is not clear whether these adnexae migrated, were pulled into the new epithelial growth, or actually represented newly regenerated structures. In the first 3 weeks, there was minimal evidence for the cartilage extending into the new growth area. However, examination of the wound site at 3 months showed new cartilage filling the wound site in what

appears to be the process of reorganization. This is in contrast to what is seen in the C57BL/6 wounds where closure is minimal.

Viewing hole closure from a purely geometric perspective, we note that ingrowth of tissue must be accompanied by continuous remodeling. Otherwise, the tissue would bunch up and become irregularly enfolded. What we observe, however, is a grossly normal flat tissue layer. Wound repair/regeneration studies done from distal extension wounds, such as fingertip regrowth, do not address this issue (3). There have been extensive studies addressing the rate of closure of skin wounds in mammals dating back at least to the classic work of Carrel on guinea pig and human skin (22). Interestingly, the proportional rate of closure is similar and occurs within 1 month, though in the case of skin there is a wound bed, upon which a provisional matrix forms and epithelium regrows, and wound contracture dominates. In the work reported here, there is new tissue mass which extends into the wound with no guidance or support from an underlying layer of tissue and no realistic possibility of contracture. Interestingly, Carrel and Hartmann (22) noted that the rate of closure seen in mammalian skin wounds was the same as that seen in salamander regeneration.

### *The MRL Mouse*

The MRL mouse (H-2k) is derived from an interbreeding of the LG mouse (75%; H-2d/f), the AKR mouse (12.6%; H-2k), the C3H mouse (12.1% h-2k), and the C57Bl/6 mouse (0.3% H-2b) (13) and was selected originally for its large size. MRL/lpr, a mutant derived from this colony, showed enlarging spleen and lymph node with age, lymphoproliferation with aberrant control of apoptosis in germinal centers, and a high susceptibility to autoimmune disease with autoantibodies, an arthritis-like syndrome, and glomerulonephritis. This was shown to be the direct result of a retrotransposon insertion into the second intron of the fas gene in the lpr strain (16, 17, 23). However, the rapid and complete wound closure that we describe here is unrelated to fas since the MRL/+ mouse has the same healing characteristics. Furthermore, wound closure is unlinked to the lympadenopathy ($R = 0.4$) associated with lpr mice and the autoantibodies made to histone proteins by these animals (data not shown) (13–15, 24). This lack of fas involvement has been confirmed in mapping studies using MRL/lpr and C57BL/6 backcross mice showing a clear genetic basis for this regeneration trait, unlinked to the fas genetic locus (McBrearty *et al.*, submitted for publication). One characteristic of the MRL mouse is its large size; however, there is no evidence that this trait is linked to adult body weight ($R = 0.12$).

### *Wound Repair versus Regeneration—Gross and Microanatomic Aspects*

The type of cell replacement seen in wound healing can generally be divided into two categories: wound repair and regeneration. Wound repair, after injury, begins with a fibrin clot which provides a provisional matrix for cell migration and release of chemotactic factors leading to recruitment of inflammatory cells. Epithelial cells migrating from the wound margin express integrins which can recognize the matrix components, thus propelling the cells forward. Collagen is laid down in an irregular fashion and the provisional matrix is replaced by a collagenous scar (25, 26). As concisely stated by J. Gross,

> "Mammals in contrast to some amphibians (but not all) substitute repair for regeneration to close full thickness traumatic wounds. The basic mechanisms involve pulling the wound edges over the excised area and simultaneously filling in the defect with a temporary cellular, vascular, and extracellular matrix-containing granulation tissue which, with time, is largely absorbed or converted to an avascular, sparsely cellular fibrous scar. The relative degree to which each of these processes is used is dependent on species and region of the body. For example, in the human excision wound, there is slow, or no, centripetal movement of the dermal edges in many areas of the body; granulation tissue which gradually converts to scar fills the defect. The final fibrotic patch in human skin and the minimal residual scar in most mature animal species lacks the normal dermal collagenous pattern, and the epidermis covering the scar fails to develop appendages such as hair and the several types of glands." (1).

Regeneration, on the other hand, involves the gross replacement and restoration of tissue mass with normal architecture and, in the extreme case, full organs. Renegeration can be seen in vertebrates such as urodeles (salamanders and newts) but is not seen widely in mammals. The main thrust of these studies, done primarily in amphibian species, has focused on the blastema—the tissue structure that contains undifferentiated mesenchymal progenitor cells. A covering of wound epithelium is formed by the migration of epidermal cells around the wound (27). It is from this structure that the new limb regenerates.

A noted study of mammalian regeneration involves the closure of through-and-through (1 cm diameter) rabbit ear holes (5). Rabbit ears, like mouse ears, have a central cartilage layer and the gross and microanatomic sequelae observed are similar to what we have seen in the MRL mouse, i.e., the rabbit exhibits complete closure with normal skin architecture and chrondrogenesis. Ear wounds in dog and sheep showed incomplete hole closure as seen in the C57BL/6 mouse. The rabbit ear has been used to study the effects of locally applied growth factors (28, 29). Wounds were created down to the cartilage layer and recombinant human fibroblast growth factor applied to the wound led to relatively greater amounts of granulation tissue,

neovascularization, and an increase in the ECM. Interestingly, the same experiment done in mice showed little or no effects of this growth factor (30).

Following up on reports of regrowth of amputated fingertips in children, wherein anatomically normal fingertips including nailbeds have sporadically been observed, Borgens examined the effect of precision surgical amputations on the foretoes of B6C3H mice (3). Briefly, it was observed that amputations distal to the last phalangeal joint led to predictable regrowth of toe tips, but, in most cases the nail did not grow back or when it did was abnormal. Amputations as little as 100 $\mu$m proximal (into the middle phalange) led to no regrowth, and an obvious blastema was not present even when regrowth occurred.

Though it does appear that a regeneration blastema is formed in this murine wound closure model, it is not clear that the same events taking place during limb regeneration in the amphibian are occurring here. What is useful in this case is that we have identified a mouse strain, unlike other mouse strains, in which regeneration is readily seen and quantified and which has allowed a genetic dissection of this trait. In a second study (31), we have made use of an extensive microsatellite map in the mouse to determine the underlying genetic basis of this trait and have identified multiple loci with an array of potentially interesting candidate genes. A minimum of seven loci were identified at a 20-cM resolution, none of which corresponds to any previously identified locus involved in MRL autoimmunity (17). One candidate gene of interest is the retinoic acid receptor $\gamma$. Retinoic acid (RA) is an initiator and important component in development and regeneration and acts on epithelial cells, binding to retinoic acid $\gamma$ (RAR$\gamma$) receptors in the cytoplasm (32). In amphibians, RAR$\delta$ (the RAR$\gamma$ homologue) is highly abundant in the limb regeneration blastema (32, 33) and RA is known to activate the sonic hedgehog, hox, msx, and wnt genes also activated in mammalian embryonic development (34).

## ACKNOWLEDGMENTS

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