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Eyelid development and fusion induced by cortisone treatment in mutant, lidgap-Miller, foetal mice. A scanning electron microscope study

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SUMMARY

In normal mice the eyelids grow across the eye and fuse together during days 15 and 16 of gestation, and the mice are born with their eyes closed. In mutant lg^{Ml}/lg^{Ml} (lidgap-Miller) foetuses this growth and fusion does not occur and the mice are born with their eyes open. A single prenatal treatment with cortisone on day 14 of gestation masks the genetic defect, and the mice are born with their eyes closed. In the present study, a scanning electron microscope was used to investigate: (1) whether eyelid closure in cortisone-treated lg^{Ml}/lg^{Ml} foetuses differs from that in normal foetuses of the CBA/J and ICR/Ml strains and, if so, how; and (2) how developing eyelids of untreated lg^{Ml}/lg^{Ml} foetuses differ from normal.

The induced closure differs from normal. During their growth, eyelids of treated mutant foetuses have a deficiency of rounded periderm cells at their margins; the margin cells agglomerate and flatten prematurely. In closed eyes of treated mutants there is a gap between periderm cells of the upper and lower eyelids along part of the fusion line. Fusion is completed late, during day 17. Maturation of the head periderm is advanced in treated lg^{Ml}/lg^{Ml} foetuses on day 16, relative to normal CBA/J and untreated lg^{Ml}/lg^{Ml} foetuses.

Untreated lg^{Ml}/lg^{Ml} foetuses differ from normal in that the eyelids never cover the eye and lack rounded periderm cells at their perimeter. The periderm cells present form a flattened band along the eyelid margin rather than, as in normal eyelids, along the fusion line.

The results of the cortisone study suggest that the route to eyelid closure in mice may not be narrowly canalized either in cell morphology or number, or in timing, and that closed eyes may be achieved after cortisone treatment in lg^{Ml}/lg^{Ml} foetuses as part of an induced maturation of the periderm.

INTRODUCTION

Studies of several mutations in the mouse have contributed to an understanding of some of the requirements for normal development of the face (Juriloff & Harris, 1983; Flint, 1980; Fitch, 1961; Gluecksohn-Waelsch, Hagedorn & Sisken, 1956; Juriloff, Sulik, Roderick & Hogan, 1985; Seegmiller & Fraser, 1977; Herring & Lakars, 1981). Several mutations are known that interfere with normal development of the eyelids (Juriloff, Harris & Miller, 1983); at least some of these mutations have no other obvious deleterious effect. Lidgap-Miller (lg^{Ml}) is one such mutant.

Key words: eyelid, mouse, birth defects, cortisone, prevention, mutant, lidgap-Miller, scanning electron microscope.

Normally, at the end of the embryonic period in mammals, i.e., between day 14 and day 16 of gestation in mice, the upper and lower eyelids cover the eye and fuse tightly with each other, remaining fused until approximately 12 to 14 days *postpartum* when the connection breaks down and the eyes open (Theiler, 1972). Developing eyelids consist of loose mesenchyme covered by an epithelial sheet – the epidermis on the outer eyelid surface and the conjunctiva on the inner. Covering the epidermis is an additional cell layer, the periderm. Eyelid fusion involves only the peridermal and epidermal layers, and contains true fusion elements such as gap junctions and desmosomes (Anderson, Ehlers, Matthiessen & Claesson, 1967). The mesenchymal layers of upper and lower eyelids remain separate (see Pei & Rhodin, 1970, figs 8 and 10). It is not known which of the cell layers (mesenchyme, epidermis, periderm) provides the impetus for the eyelids to cover the eye and fuse, but because changes have been observed in the periderm during all temporary fusions in the mouse foetus – eyelids to each other, digits to each other, pinnae to the scalp (Maconnachie, 1979) – the focus in the present study is on the periderm.

In mice, the periderm forms as an outer layer of the epidermis on days 10 and 11 of gestation (Weiss & Zelickson, 1975*a*; Nakamura & Yasuda, 1979). It is one-cell thick and covers the outer surface of the embryo/foetus from day 12 until it is shed during day 17 of gestation (Weiss & Zelickson, 1975*b,c*), i.e., it is present during the entire period of eyelid growth and fusion. During this period the underlying epidermis is proliferating and differentiating rapidly to become a stratified, keratinized epithelium. One probable role of the periderm is to provide a permeability barrier for the differentiating skin (Hayward & Kent, 1982). Recent evidence suggests that it may also be a secretory tissue (Janzen, Van Blerkom & Runner, 1984). The surface features of the periderm cells covering the apical ectodermal ridge have been shown to indicate normal or abnormal development of the underlying limb bud (Nakamura & Yasuda, 1979; Yasuda & Nakamura, 1983).

In a previous study of normal mice (ICR/Ml strain) the SEM was used to study the changing patterns of cell shape and distribution on the surfaces of developing eyelids (Harris & McLeod, 1982). As the eyelids move across the eye, many periderm cells in a band next to the gap differentiate, i.e. they change from flat, polygonal cells with sparse microvilli to rounded cells with a profusion of surface projections. These rounded cells are present in clumps around the eyelid margins (see figs 4, 5, 8–10 in Harris & McLeod, 1982). It is not clear whether the rounded cells that almost fill the gap in the final stage of eyelid closure are all of peridermal origin or are of mixed peridermal and epidermal origin. When the eyelids fuse together many of these rounded cells are extruded and become flattened along the fusion line (see fig. 6 in Harris & McLeod, 1982), to be sloughed off with the periderm before birth (fig. 7 in Harris & McLeod, 1982).

In mutant lg^{Ml}/lg^{Ml} embryos the eyelids fail to come together and fuse. Almost all lg^{Ml}/lg^{Ml} newborn mice have both eyes open. The open-eyed phenotype of the lg^{Ml}/lg^{Ml} genotype can be prevented by prenatal treatment with cortisone; treated foetuses are born with their eyes closed (Watney & Miller, 1964), apparently

normally. This remains one of the few examples in which foetuses that are genetically programmed to be born with a morphological defect can as a result of prenatal treatment be born 'normal'. The optimum time of treatment (with a single subcutaneous injection of cortisone acetate to the dam) is between days 13 and 15 of gestation. The response is dose dependent to approximately 60 mg kg^{-1}, at which 94 % of mutant foetuses have both eyes closed; the percentage cured drops at higher, apparently toxic, doses (Harris, Juriloff & Biddle, 1984). Histologically no differences in eyelid growth and fusion have been found between cortisone-treated lg^{Ml}/lg^{Ml} and normal foetuses (Harris & Fraser, 1968). The mechanism by which cortisone restores apparently normal eyelid development in lg^{Ml}/lg^{Ml} mutant foetuses and the reason that the eyes do not close in untreated lg^{Ml}/lg^{Ml} mutant foetuses are not known. Recently the list of substances that can cure the lg^{Ml}/lg^{Ml} phenotype has been expanded to include two other glucocorticoids (hydrocortisone acetate, Nakatsu, Ihara & Miller, 1984; and corticosterone, Nakatsu *et al.,* personal communication) and thyroxine (T4, Juriloff, 1985).

The present work may be one example of a general phenomenon of reversing a mutant phenotype back to normal by prenatal treatment. Important questions, from both the clinical and experimental biological points of view, are whether or not the apparently normal development is really normal, and whether the normal end point can be arrived at in development in more than one way.

In the present study, the detailed surface observations possible with the scanning electron microscope (SEM) have been used to investigate: (1) how the cortisone-induced eyelid closure in lg^{Ml}/lg^{Ml} foetuses takes place, and whether it follows the normal pattern of development, and (2) what developmental steps, in terms of the patterns of cell shape and distribution on the surface of the eyelids, are abnormal or lacking in untreated mutant lg^{Ml}/lg^{Ml} foetuses, compared with normal ICR/Ml (Harris & McLeod, 1982) and CBA/J strains. The developmental timing of eyelid closure in treated mutant foetuses as compared with normal strains also has been ascertained.

MATERIALS AND METHODS

Mice

Two inbred strains of mice, LM/Bc (mutant) and CBA/J (normal), were used in this study. The mutant mice, all homozygous for lg^{Ml} (lidgap-Miller), were of the same inbred strain of mixed genetic origin used in previous studies. The full history of LM/Bc can be found in Harris *et al.* (1984). The LM/Bc mice were in the F44 to F46 generations of inbreeding. The CBA/J mice were F3 generation descendants of mice obtained from the Jackson Laboratory, Bar Harbor, Maine. CBA/J mice were known to have late eye closure (unpublished observations) relative to the previously studied normal strain, ICR/Ml (Harris & McLeod, 1982). Based on the hypothesis that the morphology and timing of eyelid development may vary among normal strains, CBA/J was used to extend the normal range to which LM/Bc would be compared.

All females were nulliparous and 2 to 5 months old at the time of breeding. The mice were housed in the Zoology Research Unit 1, Animal Care Centre, U.B.C. The animal rooms were on a 12 h light (7:00 to 19:00 h) and dark cycle. The mice were given Purina Laboratory Chow and tap water *ad libitum*.

Timed matings were obtained by putting one to three females with singly caged males for 3–4 h between 8:00 and 13:00 h. At the end of this period the females were checked for the presence of vaginal plugs. 10:00 h on the day of the plug was called day 0, 0 h (0/0) of development.

Treatment

On day 14/2·5 (±1·5 h) of gestation each female to be treated was weighed and 50 mg kg^{-1} body weight of cortisone acetate (Cortone, 50 mg ml^{-1}, Merck-Frosst Laboratories) was injected subcutaneously into its interscapular region. Day 14 was chosen because it is the day of maximum response. 50 mg kg^{-1} was used because based on previous observations (Harris *et al.* 1984) it is far from the observed toxic dose range but should produce 90 to 95 % closed eyes.

Scanning electron microscopy (SEM)

Pregnant females were killed by cervical dislocation at specific times on day 16 or 17 of gestation. The uterus was removed and immersed in cold (4 °C) Sorensen's phosphate buffer (pH 7·3). The foetuses were dissected out and their eyes scored with a dissecting microscope for degree of closure. The closure categories have been illustrated previously (Harris *et al.* 1984). Representative whole foetuses were immersed in cold 2·5 % glutaraldehyde in Sorensen's phosphate buffer and stored at 4 °C. The following steps were all done at 4 °C. Between ½ h and 2 days later the foetuses were decapitated and the heads put into fresh fixative where they remained for from 1 to 7 days. The heads were transferred to the phosphate buffer for trimming, whence they were returned to fixative or left in buffer for processing on one of the next two days. The specimens were rinsed twice in buffer (20 min each), postfixed in 2 % osmium tetroxide in phosphate buffer for 1–1½ h, rinsed three times in buffer (20 min each), and dehydrated in a graded series of ethanols (15 min each) to 100 % ethanol (three times, 20–25 min each), during which they returned to room temperature. They were critical-point dried from liquid CO_2 in a Ladd Critical Point Dryer, mounted on stubs using double-sided tape and colloidal silver, and stored in a vacuum desiccator. They were sputter-coated *in vacuo* with gold–palladium in a Hummer VI and examined a few hours or days later with a Cambridge Stereoscan 100 SEM operated at 15 kV.

Treated and untreated LM/Bc litters were collected at the same developmental times on the same or adjacent days. Heads from treated and untreated foetuses were matched for gestational age and, where possible, were processed and viewed together. Several LM/Bc eyes were examined at each developmental time: day 16/2 (2 treated, 2 untreated), day 16/5 (4, 2), day 16/7 (4, 0), day 16/9 (7, 7), day 16/13 (6, 6), day 17/1 (9, 5), day 17/6 (2, 2), day 17/8 (6, 0).

Untreated CBA/J litters were collected on day 16/8 and day 16/12; five eyes of the former and six of the latter were examined by SEM.

RESULTS

Timing of eyelid closure after cortisone

Table 1 shows the number of eyelids closed and the number of eyelids in various other degrees of closure in treated LM/Bc and untreated CBA/J foetuses at each developmental time studied. In LM/Bc foetuses, almost no eyes were closed by the middle of day 16. On day 17/1, 53 % (19/36) of the eyes had a pinhole-sized gap or were closed. All eyes were closed on day 17/6. The two eyes with small gaps on day 17/8 would probably have remained open. The CBA/J data are presented to show the range of eyelid development present in the samples used for SEM, but as only one litter at each time point was used, the data are not reliable for comparison of eyelid closure time with that of LM/Bc. Information on the timing of eyelid closure is published for another normal strain in the same facility,

ICR/Ml (Harris & McLeod, 1982). Some ICR/Ml eyes were almost closed on day 16/0 and all were tightly fused by day 17/0.

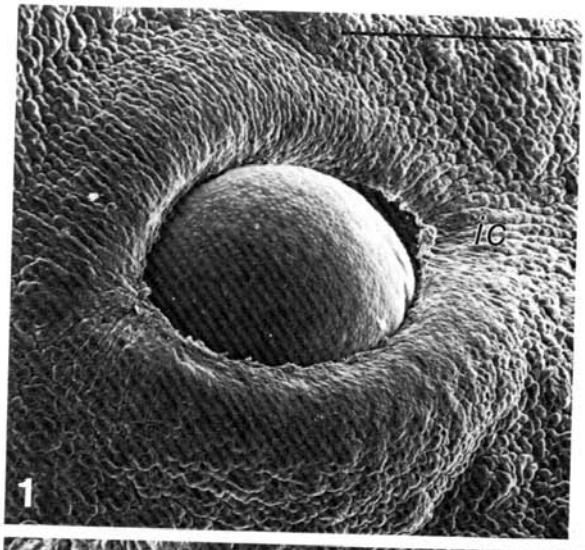
How normal are eyelids of cortisone-treated LM/Bc foetuses?

The external detail of closed eyelids in cortisone-treated LM/Bc foetuses is strikingly uniform, and different from normal. All eight closed LM/Bc eyes examined from day 17/1 and 17/6 foetuses looked like the eye in Fig. 2. The eyelids appear to be firmly fused along the entire fusion line. From the outer canthus (the outer corner of the opening) to the middle of the closure, cells are piled along the outer surface of the fusion line. These cells are flattened in a manner similar to those seen along fused eyelids of normal ICR/Ml foetuses (Harris & McLeod, 1982, fig. 6). However, toward the inner canthus there is a space between the surface cells of the upper and lower eyelids. The cells of the exposed surface are similar to those seen in the sheath in the inner canthus earlier in eyelid growth (see Fig. 16); because of their elongated shape and orientation parallel to the fusion line they are almost certainly eyelid rather than corneal cells. No eye from either of the normal strains examined, ICR/Ml or CBA/J, had such an exposed area in the fusion line. The CBA/J eye, just closed (Fig. 3), has a large cluster of rounded cells in the region where fusion occurred last, and some flattened cells along the fusion line towards the outer canthus. Eyelids of ICR/Ml 17-day foetuses have flattened cells covering the length of the fusion line (Harris & McLeod, 1982). Hence, although the eyes of LM/Bc foetuses after cortisone treatment are closed, the nature of the closure is not entirely normal.

Some of the eyelids of treated LM/Bc foetuses examined on day 17/1 had covered most of the eye and fused much of the way, but had not completed the closure process (Figs 4, 5). As in the eyes described above, toward the outer canthus there are flattened cells apparently attached along, and spreading to either side of, the fusion line. Also, toward the inner canthus the surface cells of the

Table 1. *Timing of eyelid closure of lidgap-Miller foetuses after cortisone, and of untreated CBA/J foetuses*

		Number of eyes				
	Time (d/h)	Wide gap	Medium gap	Small gap	Pinhole	Closed
LM/Bc	16/2	3	7	2	0	0
	16/5	4	7	5	0	0
	16/7	3	11	0	0	0
	16/9	5	22	8	2	1
	16/13	0	7	7	0	0
	17/1	0	8	9	5	14
	17/6	0	0	0	0	32
	17/8	0	0	2	0	16
CBA/J	16/8	8	0	0	0	0
	16/12	0	0	1	3	4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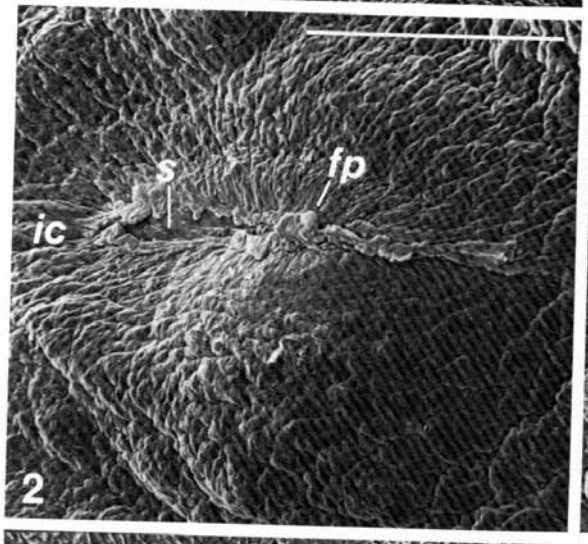
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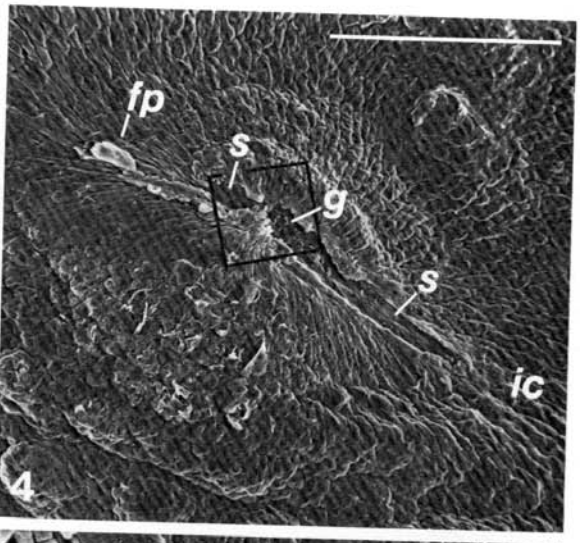
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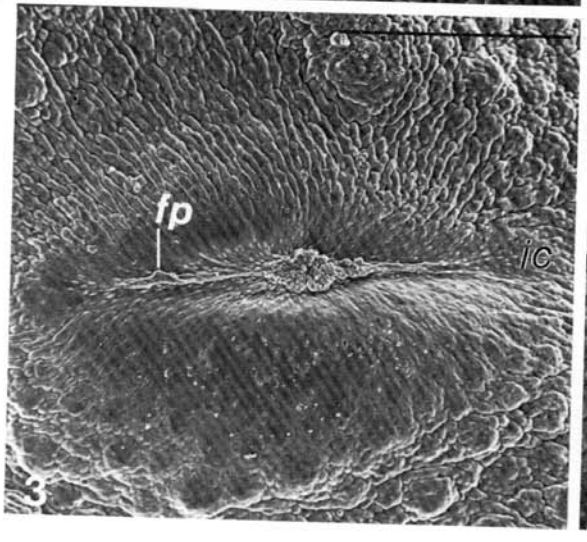
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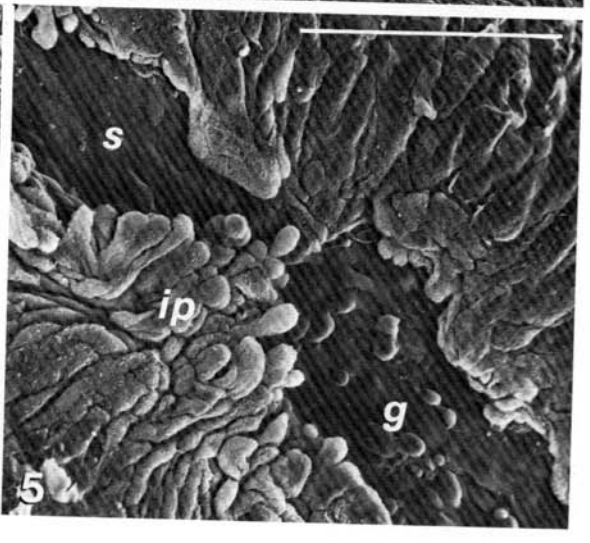
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upper and lower lids have a gap between them; the eyelids are apparently fused together in this region by the underlying layers of cells. The eye shown differs from the closed eyes of treated foetuses in that there is a region in the centre of the fusion line where the cells of the upper and lower eyelids have not quite met, leaving a pinhole-sized opening over the cornea. A cluster of bulbous and elongated eyelid cells (Fig. 5) appears to have attempted to bridge the remaining gap. The patchy distribution and irregular shapes of these cells contrast with the more uniform clustering and roundness of cells in CBA/J eyelids that have just closed (Fig. 3) or are almost closed (Figs 8, 14, 17).

Differences between normal (CBA/J and ICR/Ml) and treated LM/Bc eyelids are also apparent earlier in the growth and fusion process. Figs 7, 10, 13, 16 show treated LM/Bc eyes and Figs 8, 14, 17 show a CBA/J eye, all at intermediate stages of closing. A similar intermediate stage of ICR/Ml closure is shown in Harris & McLeod (1982, fig. 4). Many of the elements of normal eyelid development are present in treated mutant eyes. Some fusion has occurred from both canthi, and piles of surface cells surround the remaining opening (Figs 7, 10). However, at the inner canthi, normal CBA/J (Fig. 17) and normal ICR/Ml (Harris & McLeod, 1982, figs 4 and 5) foetuses have a sheath of cells framed and largely overlain by clumped, rounded cells forming an approximate 'V' diverging from the area of completed fusion. In the treated mutant, most of the sheath is bare (Fig. 16) and outlined by long stringy cells and occasional clumps of rounder ones.

Other differences are seen across the whole of the edges of both eyelids. In normal eyelids, even with the profusion of rounded cells surrounding the opening, there is an appearance of order. In treated mutant eyelids there is not. The difference is perhaps most striking in Figs 13 (treated mutant) and 14 (normal) of the outer canthi. In the normal, most of the cells surrounding the gap are rounded, many with tails radiating away and quite far back from the gap, along the surface of the periderm. By contrast, in the treated mutant (Fig. 13) many of the periderm cells overlying the outer canthus and rimming the gap appear to have agglomerated to form large irregular shapes, which have begun to flatten. In the normal, very little agglomeration or flattening of cells occurs until after eyelid closure is complete.

At higher magnification the greater uniformity of the CBA/J eyelid perimeter cells during the closure process (Fig. 21) relative to those of the treated mutant

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Figs 1–5. External SEM views of whole eyes of 16- and 17-day foetuses. Upper eyelid is at top of Figure. *fp*, flattened periderm cells along fusion line; *g*, gap where eyelids have not fused; *ic*, inner canthus; *ip*, irregular periderm cells partially bridging gap; *s*, space where underlying cells have fused and periderm cells have not. Figs 1–4 bar represents 500 μm; Fig. 5 bar represents 100 μm.

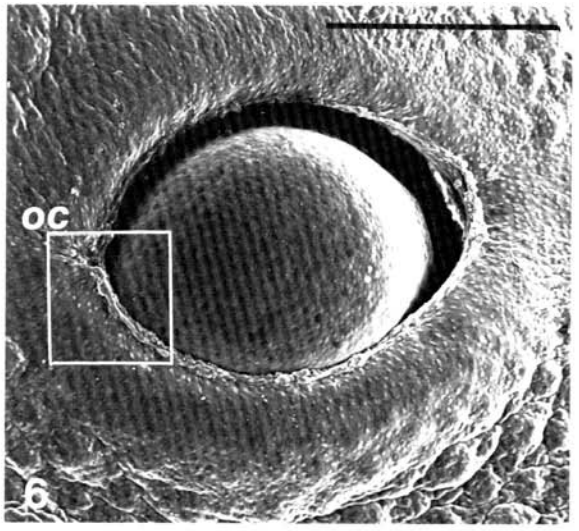
Fig. 1. Untreated LM/Bc, day 17/1, wide open eye.

Fig. 2. Cortisone-treated LM/Bc, day 17/1, closed eye.

Fig. 3. CBA/J, day 16/12, just-closed eye.

Fig. 4. Cortisone-treated LM/Bc, day 17/1, not-quite-closed eye.

Fig. 5. Higher magnification of part of eyelid fusion zone in Fig. 4.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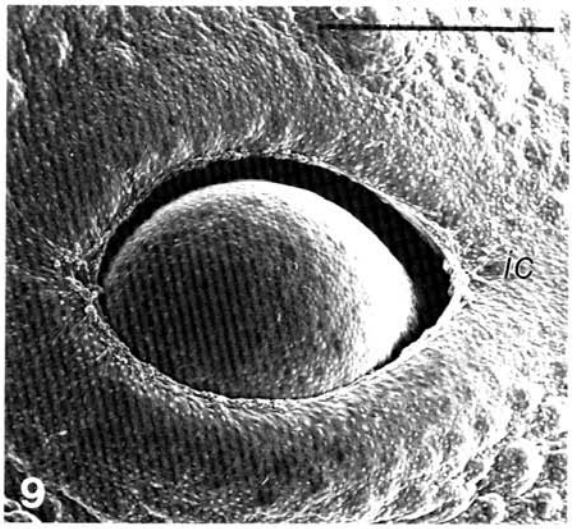
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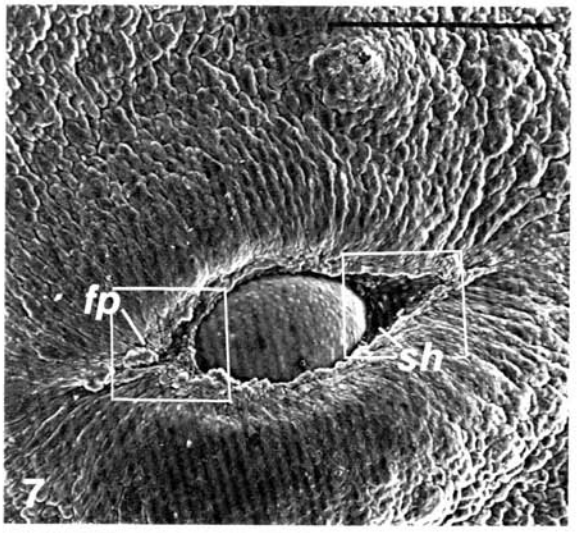
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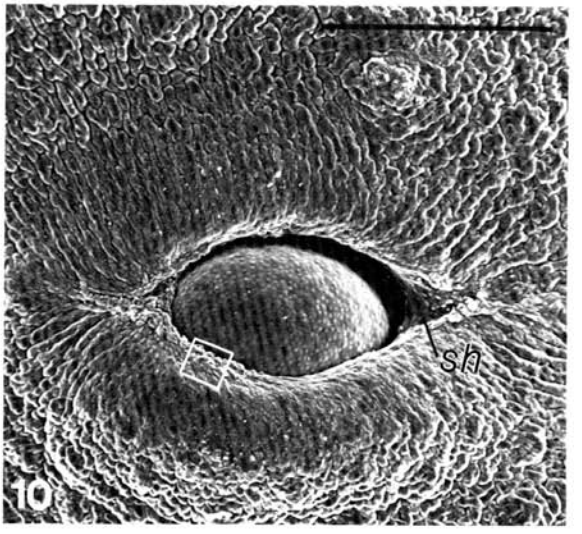
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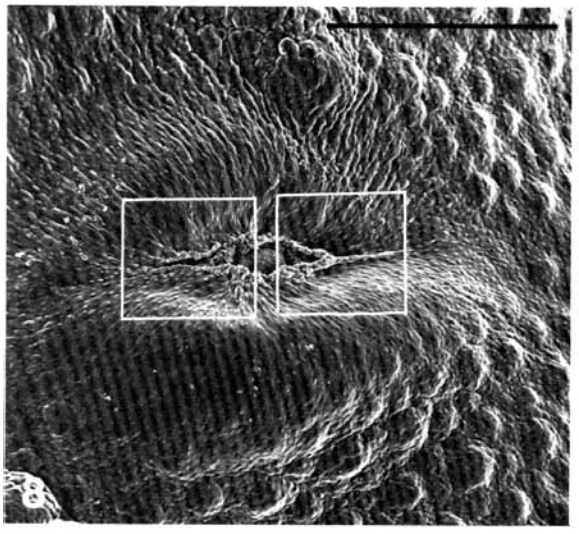
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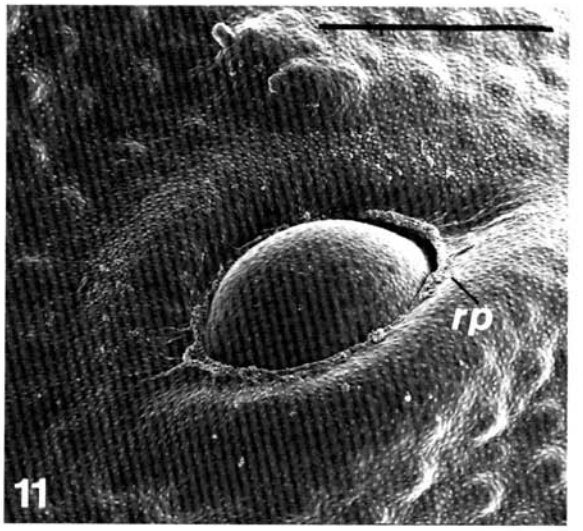
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(Fig. 20) is again striking. Most of the CBA/J cells are covered with microridges, little raised from the cell surfaces; the margins of individual cells are distinct. On the other hand, in the treated mutant many of the cell boundaries are indistinct; many cells are covered with blebs that protrude from their surfaces, and blur the spaces between cells, giving this region a much fuzzier appearance than in the normal. Also, in the treated mutant the orientation of cells is roughly parallel to the margin of the gap, whereas in the normal it is roughly perpendicular.

In order to confirm that the rounded periderm cells at the leading tips of the developing eyelids are indeed cells, the histological material that had been prepared in our laboratory for several normal strains (SWV/Bc, BALB/cGaBc, ICR/Ml) in the course of other studies was reviewed. A representative eyelid, of a 15-day SWV/Bc foetus, is shown in Fig. 15. Cell nuclei can be seen plainly in the rounded cells at the leading eyelid tips. Also, nuclei of some of the flattened periderm cells are visible near the tips of the outer and inner surfaces of the eyelids (see arrows).

Differentiation of the periderm and underlying skin

The facial skin of cortisone-treated LM/Bc foetuses appears more mature than that of untreated LM/Bc foetuses of the same age, and of CBA/J foetuses with a similar degree of eyelid closure. In the least mature stage shown here, the eyelid surface of untreated LM/Bc (Fig. 9) and CBA/J (Fig. 11) foetuses contains evenly spaced small bumps (likely cell nuclei). Developing hair follicles appear as large mounds (e.g. bottom right of Fig. 11). By contrast, the skin surface of the cortisone-treated LM/Bc foetus (Fig. 10), of identical age to (day 16/5), collected on the same day as, and processed simultaneously with the untreated LM/Bc foetus (Fig. 9), has already lost its pattern of individual bumps, and is much more and deeply grooved. The skin of untreated LM/Bc foetuses reaches this grooved stage almost a day later than does that of the treated foetuses (day 17/1, Fig. 1). The skin of CBA/J foetuses reaches the grooved stage at about the time that the eyelids finish fusing (day 16/12, Fig. 3).

The skin of the treated LM/Bc foetuses has advanced to a rougher stage by day 17/1 (Fig. 2). On day 17/8, no good views could be obtained of the fusion line of any of the five treated mutant eyes examined by SEM because in all of these eyes a superficial sheet of cells had lifted from the head during processing and remained

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Figs 6–11. External SEM views of whole eye of 16-day foetuses. Upper eyelid is at top of Figure. Bar represents 500 μm. Inner canthus is on right.

Fig. 6. Untreated LM/Bc, day 16/9, wide open eye. *oc*, outer canthus.

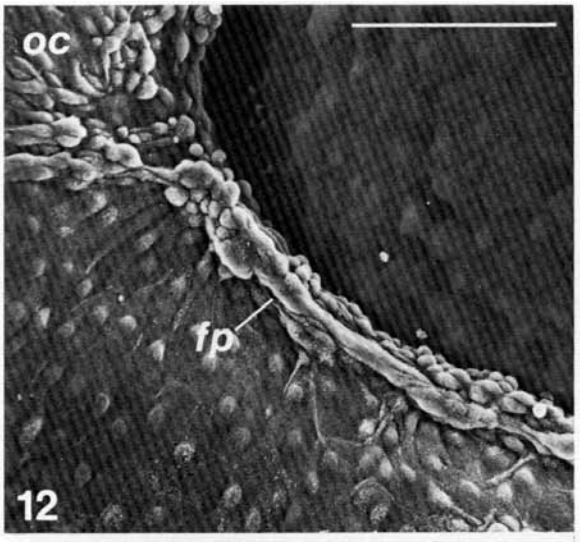
Fig. 7. Cortisone-treated LM/Bc, day 16/9, partly-closed eye. Note flattened periderm cells (*fp*) at outer canthus, and sheath (*sh*) at inner canthus.

Fig. 8. CBA/J, day 16/12, almost-closed eye. Note profusion of rounded cells at margin of remaining gap.

Fig. 9. Untreated LM/Bc, day 16/5, wide open eye. *ic*, inner canthus.

Fig. 10. Cortisone-treated LM/Bc, day 16/5, partly-closed eye. *sh*, sheath.

Fig. 11. CBA/J, day 16/8, wide open eye. Note rounded periderm cells (*rp*).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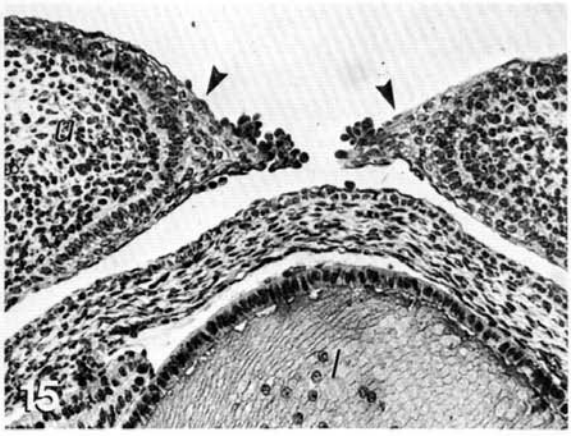
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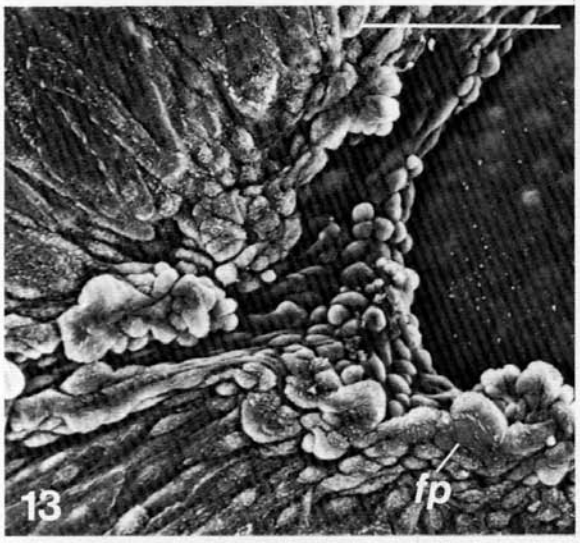
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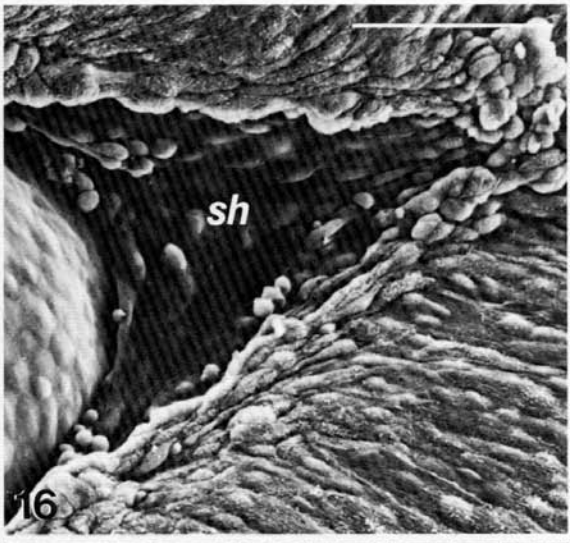
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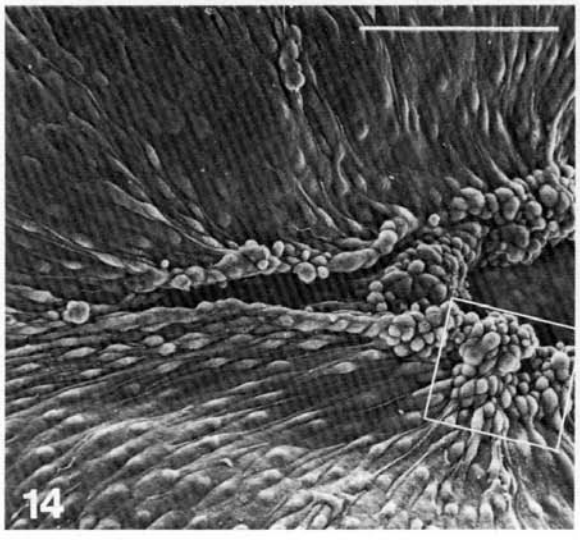
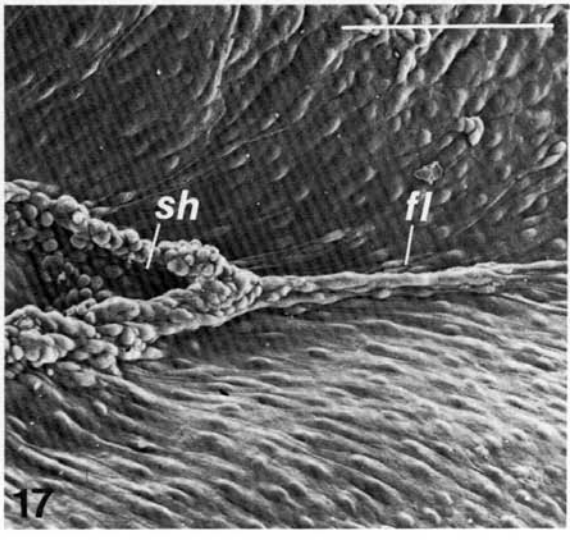

14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attached only along the eyelid fusion line. This sheet is likely the periderm, which is normally shed during day 17 (Weiss & Zelickson, 1975*c*).

Abnormality in untreated lidgap-Miller eyelids

The eyelids of untreated LM/Bc foetuses change remarkably little during the time in which the eyelids of CBA/J and cortisone-treated LM/Bc foetuses are growing across the eye and fusing shut. The eyes in Figs 1, 6, and 9 are almost interchangeable, but span 20 h of development. The eyelids never advance across the eye. However there is a considerable bulge of eyelid tissue surrounding the eye, and the inner and outer canthi are distinguishable. There appears to be a small attempt at fusion in the outer canthus, but none in the inner. On day 16, eyes of untreated LM/Bc foetuses (Figs 9, 12) have some rounded cells along the perimeter of the gap, but not nearly as many as surround the wide gap in CBA/J eyes (Figs 11, 14), nor as many as surround the partly closed eyes of treated LM/Bc foetuses (Figs 7, 10, 13), although many cells of the latter are flattened.

The outer canthus and portion of lower eyelid margin of an untreated 16-day LM/Bc eye (Fig. 12) contrasts with a partially closed eye of a treated LM/Bc foetus of identical age (Fig. 13) or with a partially closed eye of a normal foetus (Harris & McLeod, 1982, fig. 4). In the untreated LM/Bc eye there are fewer surface cells at the perimeter, most of which have agglomerated and flattened into a long string parallel to the gap margin, a configuration that is found in normal eyelids (CBA/J or ICR/Ml) only after fusion has occurred.

A higher magnification of cells of the eyelid margin in an untreated LM/Bc 16-day foetus (Fig. 19) shows a few rounded cells at the edge, several cells that lack clear grooves between them, and three or four rows of elongated cells oriented parallel to the eyelid margin. The cells are covered with microridges. Some of them have blebs. Overall the cells are neither as rounded and distinct as those of the normal (Fig. 21) nor as fuzzy as those of the treated mutant (Fig. 20).

DISCUSSION

Cortisone prevents the phenotypic expression (open eyes at birth) of the lidgap-Miller mutant gene. Eyelid growth and fusion in lg^{Ml}/lg^{Ml} (LM/Bc) foetuses

Figs 12–17.

Figs 12–14, 16, 17. External SEM views of inner and outer canthi of 16-day foetuses. Upper eyelid is at top of Figure. Bar represents 100 μm.

Fig. 12. Untreated LM/Bc, day 16/9, outer canthus (*oc*) and part of lower eyelid of eye in Fig. 6. Note flattened periderm cells (*fp*) along eyelid rim.

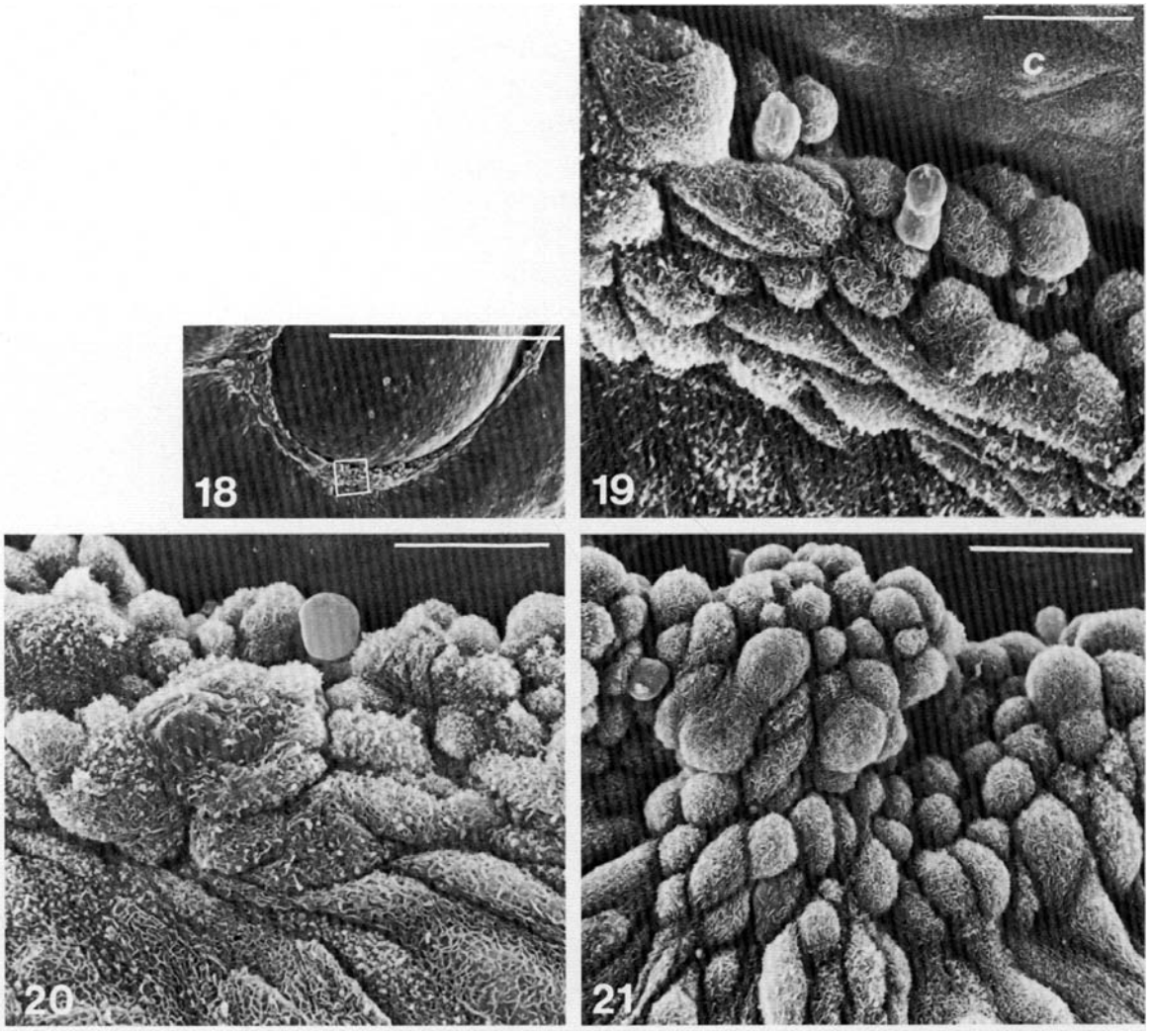
Fig. 13. Cortisone-treated LM/Bc, day 16/9, outer canthus and eyelid margin cells of eye in Fig. 7. Note many agglomerated and flattened periderm cells.

Fig. 14. CBA/J, day 16/12, outer canthus of eye in Fig. 8. Note cell streaming to eyelid margin.

Fig. 15. Histological cross section of eye and eyelids of a 15-day SWV/Bc foetus. Haematoxylin and eosin. ×180. *u*, upper eyelid, *l*, lens. Arrows indicate nuclei of flattened periderm cells.

Fig. 16. Inner canthus of eye in Fig. 7. Note bare sheath (*sh*).

Fig. 17. Inner canthus of eye in Fig. 8, with fusion line (*fl*) and sheath (*sh*).



Figs 18–21. Cells at margin of lower eyelid near outer canthus. Bar represents 500 μm in Fig. 18, 20 μm in Figs 19–21.

Fig. 18. Untreated LM/Bc, day 16/9, for location of Fig. 19.

Fig. 19. Untreated LM/Bc, day 16/9, from Fig. 18. Note long cells parallel to eyelid margin, and paucity of rounded cells. *c*, cornea.

Fig. 20. Cortisone-treated LM/Bc, day 16/9, from Fig. 10. Note agglomerated and irregularly shaped cells with furry appearance.

Fig. 21. CBA/J, day 16/12, from Fig. 14. Note profusion of rounded cells and orientation perpendicular to eyelid margin.

treated with 50 mg kg^{-1} of cortisone on day 14 of gestation is functionally normal near birth, i.e., the eyelids of 18-day foetuses are fused closed and cannot be pulled apart (unpublished observation). The process of closure induced by cortisone differs from the normal process in a number of ways which are summarized in Table 2. The later steps of eyelid development in the mutant after

cortisone treatment, including reopening, which normally occurs on about day 12 to 14 after birth, have not yet been studied.

How cortisone acts to mask the expression of the lg^{Ml}/lg^{Ml} genotype is not known. Some possibilities are that: (1) glucocorticoids are required for normal

Table 2. *Comparison of untreated LM/Bc, cortisone-treated LM/Bc, and normal eyelid development*

Feature	Untreated LM/Bc*	Cortisone-treated LM/Bc	Normal ICR/Ml or CBA/J
Percent eyes closed by day 18 of gestation	8 %†	90–98 %‡	100 %§
Fusion line of recently fused eyelids	No fusion	Part of line covered by flattened cells. Gap in superficial layer of cells at inner canthus	Entire line covered by ridge of cells. Cell shape ranges from rounded at central recent fusion area to elongated and flattened elsewhere
Inner canthus during eyelid closure	No inner sheath.	Inner sheath almost bare and bounded by a few elongated periderm cells	Inner sheath well covered and bounded by rounded periderm cells
Eyelid margins during eyelid closure	No closure. A few rounded cells, a few elongated, agglomerated and flattened cells parallel to margins	Some rounded cells, some agglomerated and flattened cells, with disorderly orientation	Abundant rounded cells at margins. Cells perpendicular to margin and uniform in appearance
Cell surface features at margins of closing eyelids	No closure. Moderately distinct cell boundaries; cell surfaces densely covered with microridges; some blebs	Indistinct cell boundaries; microridges obscured by abundant blebs	Distinct cell boundaries; cell surfaces densely covered with microridges; few blebs
Maturation of periderm during eyelid closure	No closure. Specimens age-matched to treated and normal show same appearance as normal	Maturing; wrinkled and deeply grooved	Immature; relatively smooth with uniformly 'pebbled' appearance and shallow grooves

* Development described for eyes that do not close.
† Harris *et al.* (1984).
‡ Table 1.
§ Unpublished observations.

eyelid closure and cortisone replaces a deficiency in glucocorticoids; (2) the *lg* gene product, deficient or defective in the mutant, can be induced to sufficient activity levels by cortisone (which acts by inducing gene transcription, Harrison, 1983); (3) cortisone induces activity of genes that compensate for the *lg* lesion; or (4) cortisone induces a second abnormality that incidentally allows the eyelids to close. The possibility that cortisone induces a second, compensating, defect has been excluded previously by a histological study of cortisone-treated lg^{Ml}/lg^{Ml} and normal eyes (Harris & Fraser, 1968) in which all of the structures in and around the eye of treated mutant foetuses were found to be of normal size, shape, and composition.

The question addressed in the present study is whether the eyelid closure of cortisone-treated LM/Bc mice is normal at the level of SEM examination of the external surface. There were several possible outcomes, including: (1) completely normal development; (2) normal types and locations of surface cells, but quantitative differences in one or more cell types; (3) a normal but delayed sequence; (4) a sequence that results in eyelid fusion but involves cells that are not normal in morphology and/or distribution; or (5) some combination of the above.

The conclusion of the present study is that cortisone induces eyelid closure in the mutant via a mechanism that involves cells that are not entirely normal in morphology or distribution, and later in development than is normal (Table 2). It might be argued that these abnormalities are due to either too little cortisone, or too much. However, at the dose used, $50\,mg\,kg^{-1}$, most eyelids (about 90%) would be expected to close, and the dose is well below the observed toxic range (Harris *et al.* 1984). The differences between the normal and cortisone-induced eye closure in the mutant suggest that the action of cortisone is not simply the replacement of a deficiency of glucocorticoid. The possibility that the lg^{Ml}/lg^{Ml} foetuses are deficient in glucocorticoids has been considered previously. Tuan, Rekdal & Burton (1971) showed that lidgap-Miller foetuses do not differ from several normal strains of mice in their total body corticosterone concentration on day 17 of gestation.

The maturity of the head periderm, surrounding and including the eyelids, is clearly more advanced on day 16 of gestation in cortisone-treated LM/Bc foetuses than in normal CBA/J or untreated LM/Bc foetuses. Although it is possible that this premature maturation is a secondary effect of cortisone, unrelated to its eye-closing action (which might, for example, be stimulation of cell division or alteration of the constitution of extracellular matrix in the mesenchyme), the most likely hypothesis for the action of cortisone to produce closed eyelids in LM/Bc foetuses seems to be that it stimulates these eyelids to cover the eye and fuse together as part of a general hastening of periderm differentiation. Whether or not the *lg* gene itself is one of a group of genes induced in this maturation cannot be determined from the data in the present experiment.

Glucocorticoids are known to enhance maturation and differentiation of other developing mammalian tissues such as lung, liver, and small intestine (Sugimoto, Kojima & Endo, 1976). Tye & Burton (1980) observed in foetal mice that

dexamethasone, a synthetic corticoid hormone, induced on day 14 changes in thymidine, uridine and leucine uptake that are normally observed between days 15 and 18, and they concluded that the normal pattern of development was accelerated. In the rat, Greengard (1975) has shown that several of the cluster of hepatic enzymes that normally appears in late foetal life can be induced prematurely by cortisol administration. Also, in normal development, glucocorticoids are first secreted immediately prior to the appearance of this hepatic enzyme cluster (Greengard, 1975). On another front, Salomon & Pratt (1978) have shown that low doses of glucocorticoids stimulate growth *in vitro* of maxillary mesenchymal cells from mouse embryos. Both the secondary palate and the lower eyelid are derived from the maxillary process of the embryonic mammalian face. In a review of the role of glucocorticoids in secondary palate formation in mice, they (Salomon & Pratt, 1979) conclude that physiological levels of glucocorticoids along with other growth factors may be required for normal growth and differentiation of palatal epithelium and mesenchymal cells. In sum, there is evidence that glucocorticoids are required for normal growth and maturation of at least some foetal tissues and that administration of glucocorticoids can induce this maturation prematurely.

It is interesting to note that the closed eyelids of thyroxine-treated LM/Bc foetuses, examined by SEM, are remarkably similar to those treated with cortisone. The head periderm also matures prematurely. Like cortisone, thyroxine is known to have a role in maturation of foetal tissues (discussed by Juriloff, 1985).

The timing of eyelid closure and fusion in cortisone-treated LM/Bc foetuses is delayed relative to any normal strains or hybrids studied (B6.CBA, Theiler, 1972; CBA/J, Table 1 and unpublished observations; ICR/Ml, Harris & McLeod, 1982; BALB/cGaBc and SWV/Bc, unpublished observations). This suggests that cortisone is not simply restoring a necessary factor in a system poised and stalled at the brink of initiation of eyelid closure. Instead, it seems that cortisone has either pushed an extremely delayed step back 'on scale' or is acting through agents that are not the major normal agents, and that become available later in development than normal closure usually takes place.

An important observation, based on comparing the treated and untreated mutant with normal, is that some developmental steps are linked and some are not. In the untreated mutant, the eyelids do not move across the eye and rounded cells do not pile up on the leading edges of the eyelids. In the treated mutant the eyelids do move across the eye and there is some congregation of rounded cells, although not to the normal extent, at the eyelid margins. While the two phenomena (eyelids covering the eye and presence of rounded cells) are linked, it is not clear whether there is a causal relationship between them. However it appears that eyelid fusion is not necessary for the flattening of periderm cells that normally are found flattened on the surface of the fusion line. In the untreated mutant, the periderm cells near the eyelid margins do flatten on eyelids that remain far apart. As periderm cells of 16-day mouse embryos have been shown to secrete glycoprotein (Janzen *et al.* 1984) it is interesting to speculate that some

similar secretion induces the flattening of the eyelids over the eye and that the defect in lidgap-Miller is the deficiency of periderm cells on the leading edges of the eyelids.

Conclusions

(1) Eyelid closure induced by cortisone treatment in lg^{Ml}/lg^{Ml} (LM/Bc) foetuses differs from normal in surface cell morphology and distribution, based on SEM observations. It is also delayed. These observations show that a closed-eye phenotype at birth can be achieved in more than one way, and suggest that the canalized route (Waddington, 1940, 1975) to eyelid closure has some width in terms of cell morphology and number, and of developmental timing.

(2) The untreated mutant is lacking in more than a single step of eyelid closure. In addition to failure of eyelid movement over the eye it lacks most accompanying normal surface cell changes except the flattening of the periderm cells that normally occurs after fusion. That is, two of the developmental features are apparently linked and a third is independent.

(3) We suggest that cortisone may be inducing eyelid closure in LM/Bc foetuses as part of an induced maturation of the periderm.

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