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Chemical blockade of olfactory perception by N-methyl-formimino-methylester in albino mice. II. Light microscopical investigations

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Abstract. Evidence is given that N-methyl-formimino-methylester (MFM), a highly volatile substance, causes olfactory receptor cell degeneration in mice. The time course of this degeneration and morphological changes in the compartment of receptor cell progenitor cells are described. Due to the morphological appearance of the progenitor cells and their dynamic transformation after exposure to MFM, two different types of progenitor cells can be distinguished: (a) light-staining globose basal or blastema cells, which are thought to be real progenitor cells and to remain in the mitotic cycle for generating new sensory cells; and (b) dark-staining basal cells with condensed chromatin, which are quiescent. The results agree with electrophysiological data indicating temporary inhibition of olfactory perception after MFM.

Introduction

Olfactory sensory epithelia are unique among the sensory epithelia with primary neurons in that their neuronal cells are continuously replaced (e.g., Moulton, 1975; Graziadei and Monti Graziadei, 1978a). A number of techniques (e.g., olfactory bulb ablation, olfactory nerve sectioning, destruction of the sensory olfactory epithelium by $ZnSO_4$-irrigation, and blockade of mitosis by colchicine) have been applied to study the turnover rate of the receptor cells and indicate that the replacement of olfactory sensory cells of adult warm blooded vertebrates occurs about every 30 days (Clark and Warwick, 1946; Sen Gupta, 1967; Alberts and Galef, 1971; Takagi, 1971; Powers and Winans, 1973; Matulionis, 1975; Breipohl *et al.*, 1976; Harding and Wright, 1979; Monti Graziadei and Graziadei, 1979; Booth *et al.*, 1981; Simmons and Getchell, 1981a, 1981b; Simmons *et al.*, 1981). In fish and tiger salamanders the time span between olfactory bulb ablation or olfactory nerve sectioning and complete reconstitution in the population of receptor cells in the olfactory epithelium seems to be considerably longer than in mammals (Breipohl and Ohyama, 1981; Simmons and Getchell, 1981a, 1981b; Simmons *et al.*, 1981). Nevertheless, 7 days following the ablation of the olfactory bulbs of goldfish, at least a temporary enhancement of cell mitosis has been noted (Breipohl and Ohyama, 1981). The question of whether the rapid cell renewal within the olfactory epithelium is due to the stimulation of quiescent and/or normally and continuously dividing progenitor cells is not clear. To help answer this question, studies on the epithelial composition of a layer of supporting cells, sensory cells, and basally located progenitor cells were undertaken in the present work under normal and experimental conditions.

Preliminary investigations on the effect of N-methyl-formimino-methylester

(MFM) on electrophysiologically measured olfactory thresholds of mice suggested to us that MFM may cause degeneration of olfactory sensory cells and that cell degeneration may be responsible for the obtained temporary inhibition of olfactory perception (Schmidt *et al.*, in preparation). In the present study, the morphological alterations in the olfactory sensory epithelium of mice after MFM-application are described. Special attention has been paid to the time course of olfactory nerve cell alteration and to changes in the overall composition of the sensory epithelium.

Methods

White adult male mice (NMRI strain) served as experimental animals. As described in detail elsewhere (Schmidt *et al.*, 1981), saturated gaseous MFM was applied, with the help of a syringe, at room temperature, for approximately 0.5 s (2 – 3 inhalations). In addition to the controls, 3 experimental animals were sacrificed at each of 8 intervals after the application of MFM (1.5 h; 1, 3, 7, 14, 21, 35 and 56 days). The nasal cavity was superfused with a 0.1 M 3% glutaraldehyde solution in sodium cacodylate buffer (pH 7.4) at room temperature for 3 – 5 min. After opening the nasal cavity, the olfactory epithelium was fixed for another 24 h at 4°C in the same fixative. The sensory epithelia were dissected out, washed in 2% sucrose in sodium cacodylate buffer (pH 7.4), postfixed in buffered 1% osmiumtetroxide (pH 7.35), dehydrated, and finally embedded in Epon. The light microscopical investigations were performed on 1 – 2 μm sections stained with 1% azure II-methylene blue. In the experimental animals, 3 subsequent 500 μm pieces of olfactory epithelium were sectioned and every 20th section of such a specimen block was used for semiquantitative counting of mitoses (Diagram 2). The counts of mitoses were calculated per 100 μm^2.

Results

As in other mammalian species (Moulton and Beidler, 1967; Moulton, 1975b), the olfactory epithelium of mice is mainly composed of sensory cells, supporting cells, and basally located progenitor cells. The most apical compartment of the epithelium contains a peripheral zone relatively free of nuclei and a single layer of supporting cell perikarya, together accounting for about 1/7 to 1/3 of the height of the epithelium (Figure 1; Diagram 1). Beneath this apical compartment is the broad layer of sensory cell perikarya comprising about 2/3 of the epithelium height. Sensory cell perikarya often show a columnar arrangement. Necrotic nucleic occur only rarely. From the sensory cell perikarya, dendrites extend up to the epithelium surface and end in the olfactory knobs. A distinct cellular compartment exists basal to the sensory cell perikarya, and comprises 1/10 to 1/6 of the height of the epithelium. The most prominent feature of this small band are the flat, elongated dark-staining basal cells which form a thin, more or less continuous layer immediately above the basement membrane. Interspersed between these typical basal cells and the most basally located sensory cell perikarya are lighter staining cells with a taller perikaryon, the so-called blastema cells (Andres, 1965) or globose basal cells (Graziadei and Monti Graziadei, 1978b), which herein are called progenitor cells since they are precursors for olfactory receptor cells

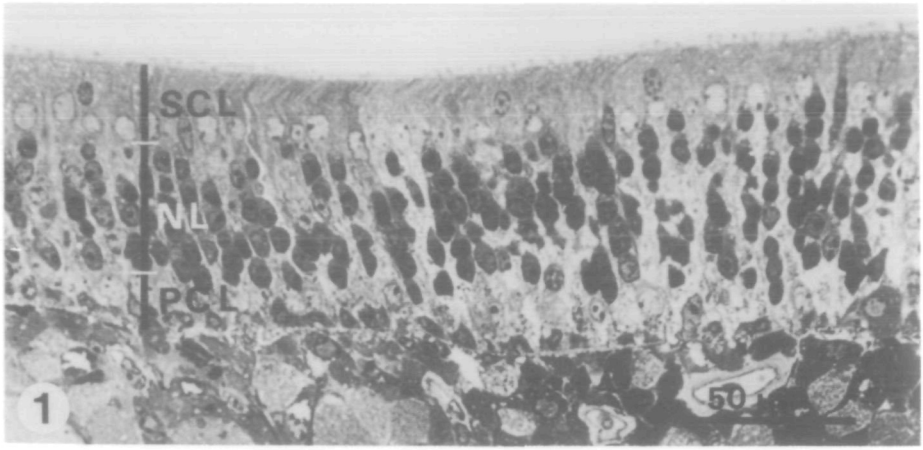


Fig. 1. Olfactory epithelium of control mouse. SCL: apical epithelial compartment with supporting cell perikarya; NL: layer of sensory cell perikarya; PCL: compartment of progenitor cells.

(Andres, 1965; Graziadei and Monti Graziadei, 1978b). Mitoses are regularly observed in the layer of progenitor cells, and rarely in the layer of supporting cell perikarya. Mitotic figures have been observed only exceptionally in the middle of the epithelium occupied by the perikarya of the sensory cells and the interspersed duct cells of Bowman glands.

When compared with the controls, no gross morphological alterations can be seen in the sensory cell layer of the epithelium 1.5 h and 24 h after the application of MFM (Figures 2 and 3). However, mitotic figures in the progenitor cell layer occur more frequently (Diagram 2). The demarcation between the progenitor cells and the most basally located sensory cell perikarya is less sharp than in controls. Three days after MFM application no obvious morphological alterations are observed in the supporting cell layer, but enlarged numbers of pycnotic cells appear in the sensory cell layer (Figure 4). The compartment of the progenitor cells is enlarged. Occasionally distinct irregular indentations of the blastema cell band into the layer of the sensory cell perikarya are observed. The number of mitotic figures in the layer of progenitor cells exceeds that in the controls, but is lower than that 24 h after MFM application (Diagram 2). Moreover, a discontinuation in the thin band of the typical basal cells is repeatedly observed. Irregular shapes of these cells, rather than flat and elongated configurations as in the controls, become more frequent.

Seven days after MFM application the morphological alterations in the sensory epithelium are obvious (Figure 5). Though the overall composition of the epithelium is not altered, one finds more conspicuous indentations of the progenitor cell layer into the sensory cell layer and a more irregular demarcation between the sensory cell layer and the compartment of the progenitor cells (Diagram 1). After 7 days, the number of mitotic figures is greater than in all earlier stages (Diagram 2). Flat basal cells only occur infrequently (Figure 5). The morphological appearance of the layer of supporting cell perikarya is unaffected.

The morphological alterations in the overall composition of the sensory

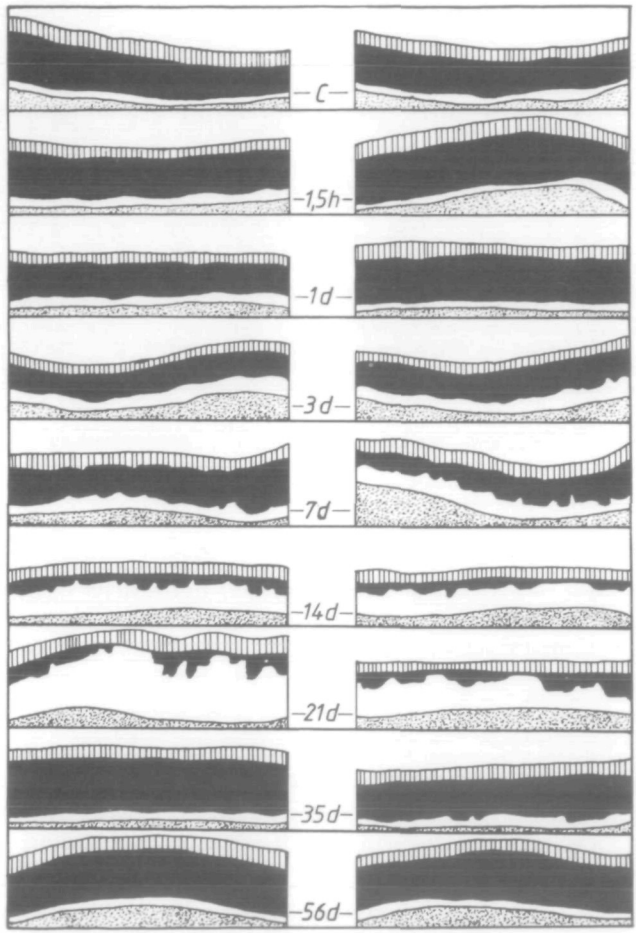


Diagram 1. Schematic representation of MFM-induced changes in the overall epithelium composition of an apical compartment with supporting cell perikarya (hatched), a middle layer of neuronal perikarya (dark), and a progenitor cell compartment (white) at different survival times (1.5 h – 56 days). Subepithelial connective tissue is stippled. Two examples are drawn per survival time.

epithelium are more pronounced 14 and 21 days after the application of MFM and the height of the epithelium is considerably reduced. Only a thin band of receptor cell perikarya is typically observed between the supporting cell layer and the increased compartment of progenitor cells (Diagram 1). The dark-staining basal cells, if present at all, are often triangular in shape and have apical extensions (Figures 6, 7 and 8). The number of mitoses is higher than at earlier stages and reaches values which are about 5 to 6 times those of the controls (Diagram 2). Mitotic figures are not restricted to the direct vicinity of the basement membrane but appear also deeper in the layer of blastema cells.

Five weeks after exposure to MFM, the morphological alterations are less conspicuous than before and the samples again show structural features resembling those of the controls (Diagram 1). However, signs of thinning out of the sensory cell perikarya compartment and an irregular demarcation between this cellular

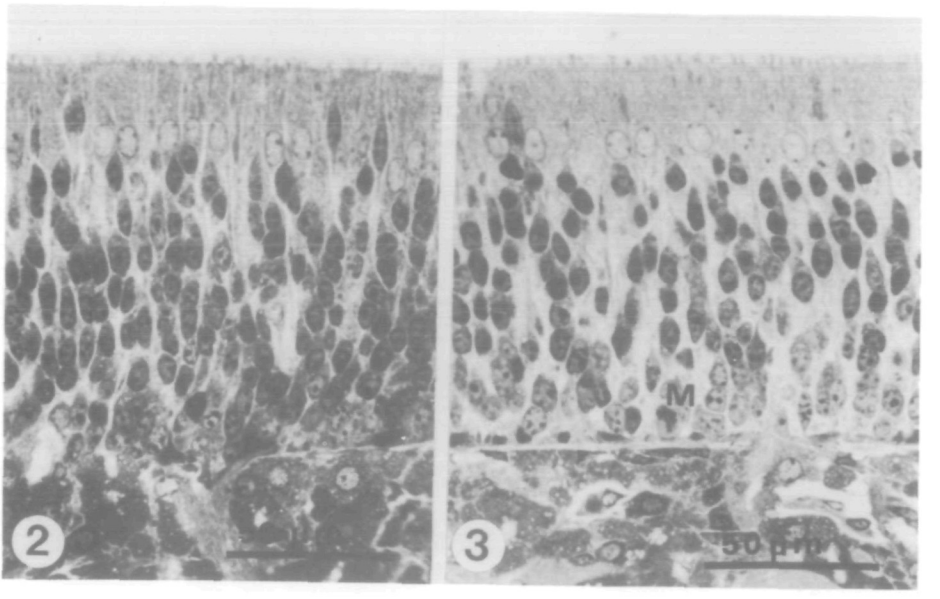


Fig. 2. Olfactory epithelium of mouse 1.5 h after exposure to MFM. Demarcation between the layers of sensory and progenitor cells is less clear than in Fig. 1.

Fig. 3. Olfactory epithelium of mouse one day after exposure to MFM. Demarcation between progenitor cell compartment and neuronal cell compartment is not sharp. M: mitosis.

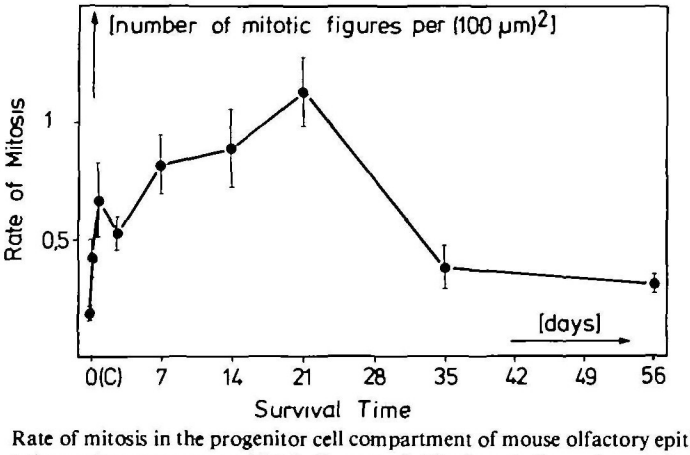


Diagram 2. Rate of mitosis in the progenitor cell compartment of mouse olfactory epithelium at different survival times after exposure to MFM. C: control. The bars indicate the standard deviations.

band and the compartment of progenitor cells can still be observed. In the latter, the thin flat layer of typical dark staining basal cells reappeared almost completely. The rate of mitoses decreased considerably and is lower than in all the preceding experimental stages. Nevertheless, it is still higher than in the controls (Diagram 2). However, samples with a clearly reduced number of olfactory knobs at the epithelial surface can still be detected.

Eight weeks after MFM exposure no obvious structural differences can be seen in the olfactory sensory epithelium when compared with controls (Diagram 1;

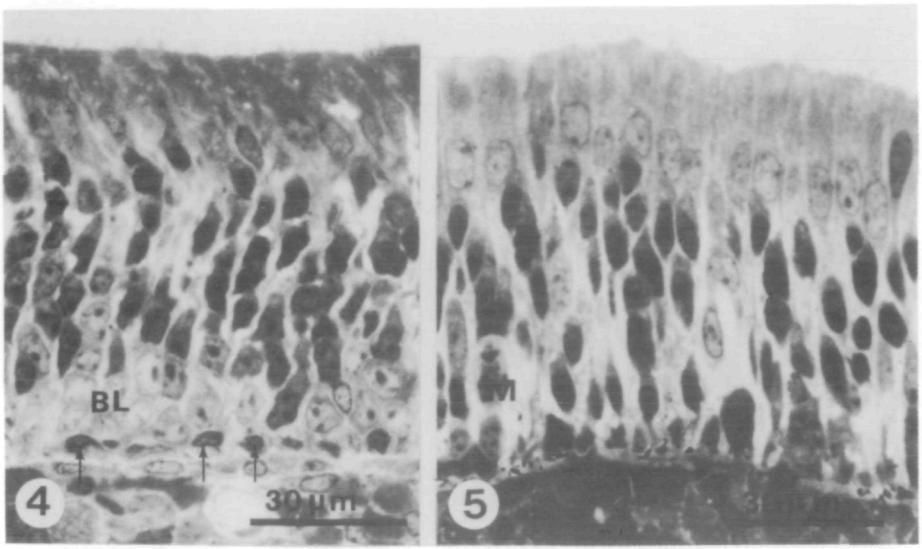


Fig. 4. Mouse olfactory epithelium 3 days after exposure to MFM. No principal differences in comparison to controls. BL: light staining "globose basal cells". Note row of typical dark staining basal cells (arrows).

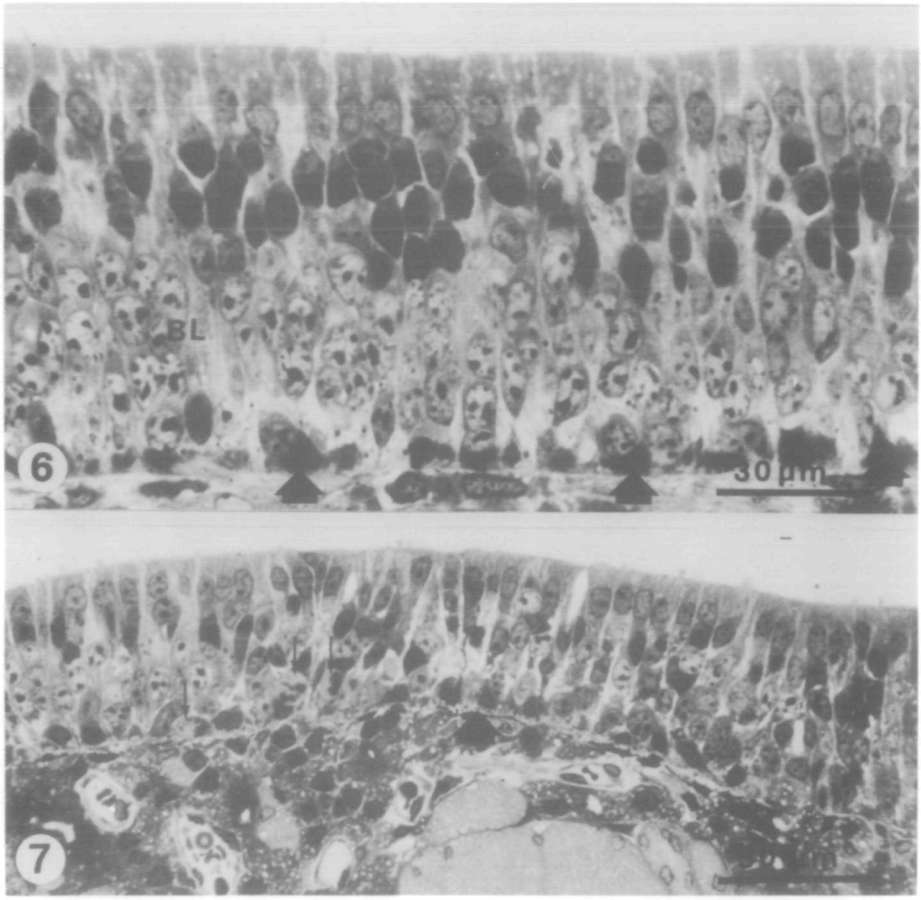
Fig. 5. Mouse olfactory epithelium 7 days after exposure to MFM. No sharp demarcation exists between neuronal cell layer and progenitor cell compartment. M: mitosis.

Figure 9). The number of mitoses decreased further but is still slightly higher than in untreated animals (Diagram 2).

Discussion

1) MFM, a highly volatile substance, causes degenerative alterations in the olfactory epithelium of the nasal septum of mice.
2) Only sensory cells show degeneration after the above described treatment with MFM.
3) In addition to the necroses of receptor cells and the irregular thinning out of the receptor cell perikaryal layer, morphological alterations are observed in the progenitor compartment. Degenerative and reactive alterations become less evident from 5 weeks onwards after MFM exposure.
4) The rate of mitoses increases a few hours after MFM treatment, then decreases (3 days) and then increases again to reach values 5 or 6 times that of controls after about 3 weeks. After 5 weeks, the rate of mitoses gradually approaches that of the controls.
5) A temporal blocking of olfactory function after MFM application, as suggested from electrophysiological investigations (Schmidt *et al.*, 1981), may be due to the morphological alterations described here. However, nothing can be said about the mechanism of degeneration due to MFM.

The function of the olfactory sensory epithelium is related to an exposure to airborne chemicals. These chemicals may have to pass through the mucus embedding the receptive structures. However, airborne chemicals have not been used as



Figs. 6 and 7. Mouse olfactory epithelium 14 days after exposure to MFM. The layer of neuronal cell perikarya is markedly reduced. The progenitor cell compartment is enlarged due to an accumulation of globose basal cells. The demarcation between these compartments has an irregular course. Dark staining basal cells occasionally show transition forms to somewhat triangular shaped cells (thick arrows). Thin arrows indicate mitoses. Note the reduction of olfactory knobs at the epithelium surface.

yet to study the well established regenerative capacity of the receptor cells at a morphological level. Many airborne chemicals are known to cause inhibition of olfactory perception. Among these substances MFM is a very potent olfactory blocker (e.g., Herberhold, 1975). Previous investigations showed that an exposure of only 0.5 s to MFM causes a considerable blocking of the electrophysiological activity in the olfactory bulbs of mice as inferred from the EEG. The olfactory threshold of geraniol, for example, increased by a factor of 10^6 after MFM application, was maintained at this level of 3 weeks and only became normal from 5 weeks onwards (Schmidt *et al.*, 1981). Moreover, MFM causes complete anosmia upon inhalation (Effenberger, personal observation). As demonstrated herein, MFM may act as a rather specific toxic substance to the olfactory receptor cells. In spite of massive degeneration of the sensory cells bet-

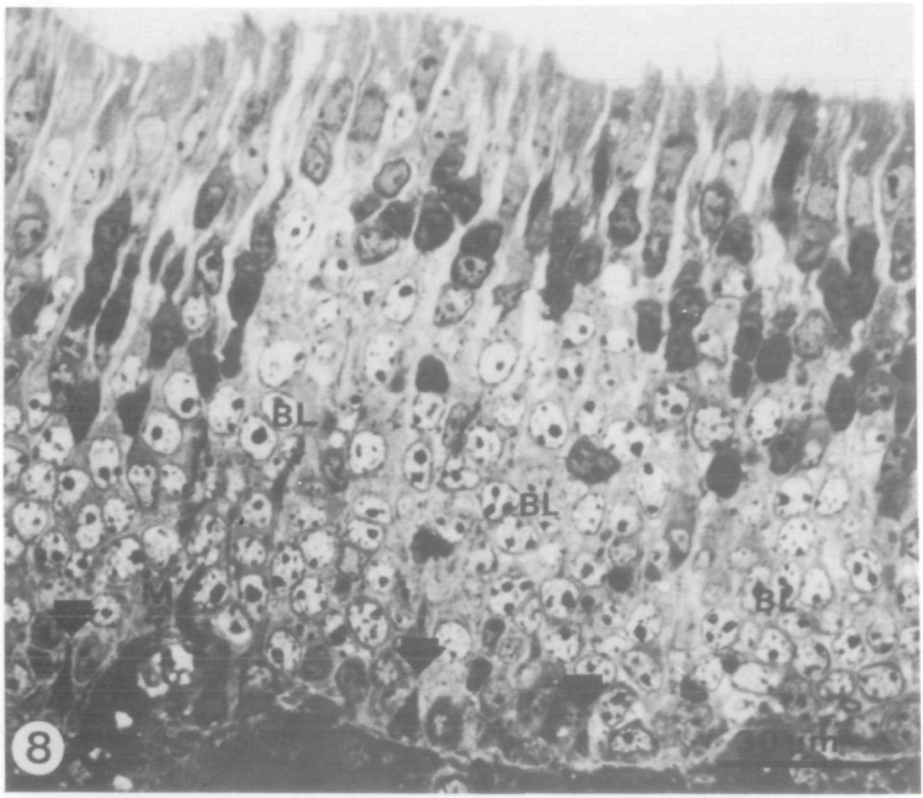


Fig. 8. Mouse olfactory epithelium 21 days after exposure to MFM. Similar epithelium configuration as 14 days after exposure (c.f. Figs. 6 and 7). Note triangular-shaped dark-staining basal cells (thick arrows). M: mitosis.

ween one and 3 weeks after MFM exposure, the other cellular components of the olfactory epithelium remained light microscopically unaffected (supporting cells) or showed an increased activity (progenitor cells). The above mentioned electrophysiological data can therefore be explained by a reduced number of receptor cells being active from one week onwards. These data match the electrophysiological data (Schmidt *et al.*, 1981). A comparable relationship between a functional recovery and a morphological repair of the olfactory epithelium has been reported for the tiger salamander (Simmons and Getchell, 1981a, 1981b; Simmons *et al.*, 1981) after olfactory nerve section.

However, cessation of the electrical activity of the olfactory bulb and an increase of the thresholds for different odorants (unpublished) occurs several hours after MFM treatment (Schmidt *et al.*, 1981). This indicates that, in spite of a rather unchanged morphology at the level of the light microscope, the sensory cells are already affected in this early phase. It is very likely that a toxic effect of MFM on the membranes of the sensory cell terminals leads only gradually to necrosis of receptor cells. Further transmission electron microscopic investigations may support this hypothesis.

As well as reduction of the sensory cell compartment, a considerable increase of the progenitor cell compartment was found between 3 days and 5 weeks after

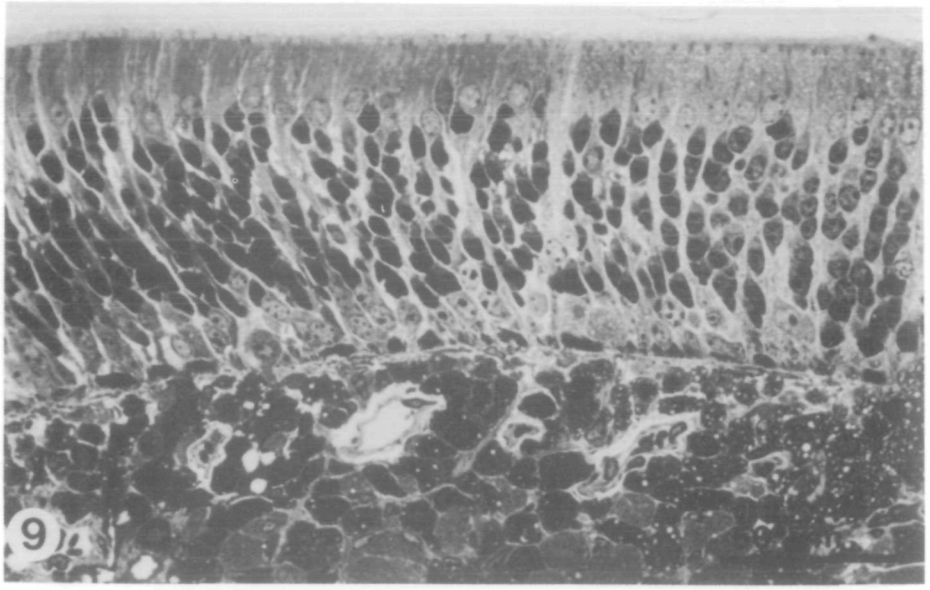


Fig. 9. Mouse olfactory epithelium 56 days after exposure to MFM. No principal differences can be seen between these and the controls.

exposure to MFM. This was mainly due to an increase in the number of "globose basal cells" (Graziadei and Monti Graziadei, 1978b). At the same time, the layer of dark-staining basal cells became discontinuous and their shapes showed transitional forms to blastema cells. From this it was concluded that these dark, intermediate, and light basal cells may have four different pathways but may still have a common ancestor (Diagram 3) i.e., (i) they are on their way to differentiate into receptor cells; (ii) they are typical blastema cells and continue with cell division; (iii) they are daughter cells, which differentiate into the flat and elongated dark-staining basal cells; or (iv) they represent transitional stages between such dark-staining flat and elongated basal cells and dividing cells.

Moulton and Fink (1972) estimated that about 90% of the basal daughter cells migrate to the periphery and differentiate into receptor cell neurons and supporting cells, while about 10% continue with mitotic activity. The present results support these findings and those of Andres (1965) related to the progenitor function of blastema cells, and suggest that some of the progenitor cells do not take part in the normal course of the receptor cell generation cycle but behave as quiescent cells.

The decline in the rate of mitoses around day 2 after MFM-exposure (Diagram 2) may be explained by a temporary exhaustion of mitotic-active blastema cells due to a pronounced transformation of their daughter cells into receptor cells (c.f. Breipohl and Ohyama, 1981). From 3 days onwards the progenitor cells, which up to the MFM-treatment have been in a "quiescent stage" (c.f. Leblond and Walker, 1956; Moulton, 1975b), reenter the regeneration cycle and participate in the increase of mitotic activity (Diagram 2). The lighter staining nuclei of the transitional cells indicate despiralization of the chromatin and a higher

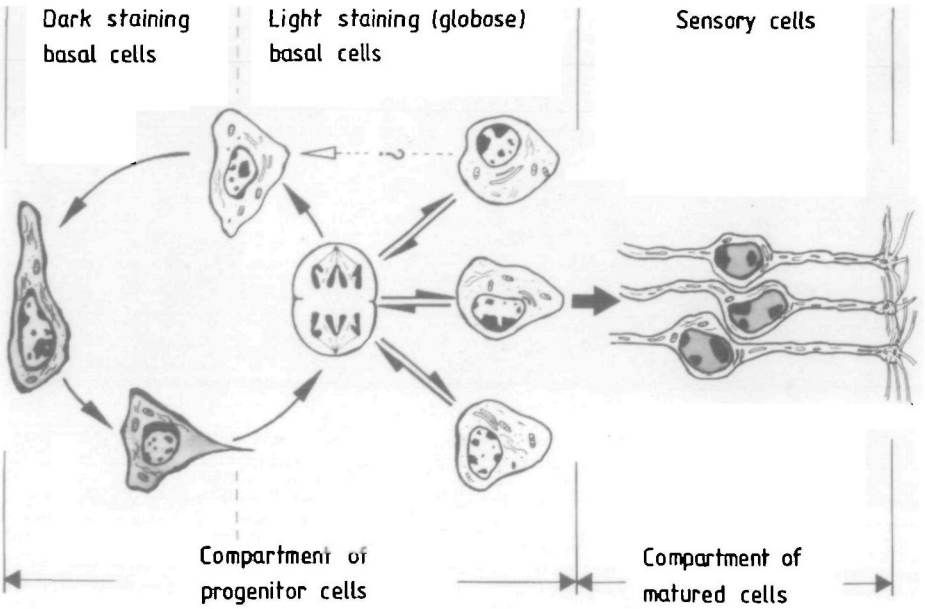


Diagram 3. Hypothetic transformation pathways between progenitor cells and olfactory receptor cells. The origin of new supporting cells is not included because it is not mentioned in this paper.

metabolic activity, as is necessary, for example, for DNA-reduplication in the S-phase of the generation cycle and later on for mitosis. Once a nearly normal population of sensory cells has reestablished, quiescent dark-staining basal cells should reappear, as seen in our material from 5 weeks onwards after MFM exposure.

The regulatory mechanism for establishing a constant number of sensory cells in the olfactory epithelium is presently not understood. It is possible that the size of the receptor cell population is only limited because of the fact that individual receptor cells die after a species-specific life span. Some authors assume that under normal conditions there are matching rates of basal cell division and of sensory cell necrosis (Moulton, 1975b; Simmons and Getchell, 1981b). Extended dying of the receptor cells may lead to an enhanced mitosis and overhasty transformation of progenitor cells into sensory cells (Monti Graziadei, 1979; Breipohl and Ohyama, 1981). Different levels of steroid hormones may also affect mitosis and transformation of progenitor cells as reported by Balboni and Vannelli, 1981, who found not only considerable differences in basal cell numbers between male and female rodents but also changes during the course of the ovarial cycle. Moreover, Vannelli and Balboni (1982) and Stumpf and Sar (1982) have evidence that the olfactory epithelium of rodents serves as a target organ for estrogens. Whether cyclic changes in olfactory sensitivity (Schmidt, 1978, 1979) in mice may be correlated with cyclicity in cell turnover of sensory cells needs further study.

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