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Fibroblast cell proliferation in the mouse molar periodontal ligament

K. A. S. PERERA* AND C. H. TONGE†

* *Department of Oral Biology, School of Dentistry, University of Leeds, Leeds LS2 9LU and* † *Department of Oral Anatomy, The Dental School, University of Newcastle upon Tyne, Newcastle upon Tyne NE2 4BW*

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INTRODUCTION

During the early stages of tooth development cellular proliferation and differentiation adjacent to the growing rootsheath give rise to periodontal fibroblasts which are important in determining the structure of the periodontal ligament (Ten Cate, 1972; Ten Cate, Mills & Solomon, 1971). A substantial amount of work has been done in the past on teeth of continuous eruption concerning periodontal fibroblast cell proliferation and tooth eruption. However detailed studies have not been carried out to study the part played by periodontal fibroblasts in teeth of limited eruption, such as rodent molars, the results of which might form an experimental model directly applicable to human tooth eruption. For instance Chiba (1965, 1968), Melcher (1967), Beertsen (1973), Beertsen, Everts & Hooff (1974) have shown a shift of fibroblasts and fibres in an occlusal direction within the rodent incisor periodontal ligament, while Ness & Smale (1959) and Ness (1964, 1967) considered that the fibroblasts in the periodontal ligament act as the prime mover in tooth eruption. More recently Zajicek (1974), using [^{3}H]thymidine, has shown a migration of fibroblasts in different layers (shells) and at different velocities, thereby implying an active migration of periodontal ligament fibroblasts with tooth eruption in the continuously erupting rat incisor.

The intention of this paper is to study the proliferation of periodontal fibroblasts in relation to molar tooth eruption.

MATERIALS AND METHODS

Animals

Sixty four young mice, in four groups aged 10, 12, 16 and 20 days, respectively, were used. The 16 animals in each group were approximately the same weight. Animal groups were housed separately and fed water and standard laboratory pellets (BOCM) *ad libitum*, during the period of the experiment.

Tritiated thymidine, specific activity 1·9 Ci/mM, was given by intraperitoneal injection in doses of 1 μCi/g body weight. Injections were given (whenever possible) between 9 am and 10 am. One animal from each group was killed at 1, 3, 5, 7, 9, 12, 16, 24, 36, 48, 60, 72, 84 and 96 hours after the injection using an overdose of pentobarbitone Na. A non-labelled animal was used as a control for each of the four groups.

* Reprint requests to Dr Perera.

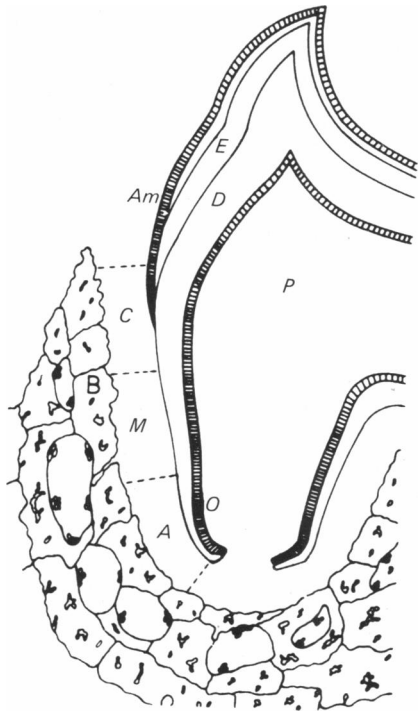


Fig. 1. Areas assessed. Diagram illustrating the three arbitrary zones (apical, middle and cervical) into which the molar periodontal ligament was divided for quantitative estimation of labelling indices. *A*, apical zone; *B*, alveolar bone; *Am*, ameloblasts; *C*, cervical zone; *D*, dentine; *E*, enamel; *M*, middle zone; *O*, odontoblasts; *P*, pulp.

Histology

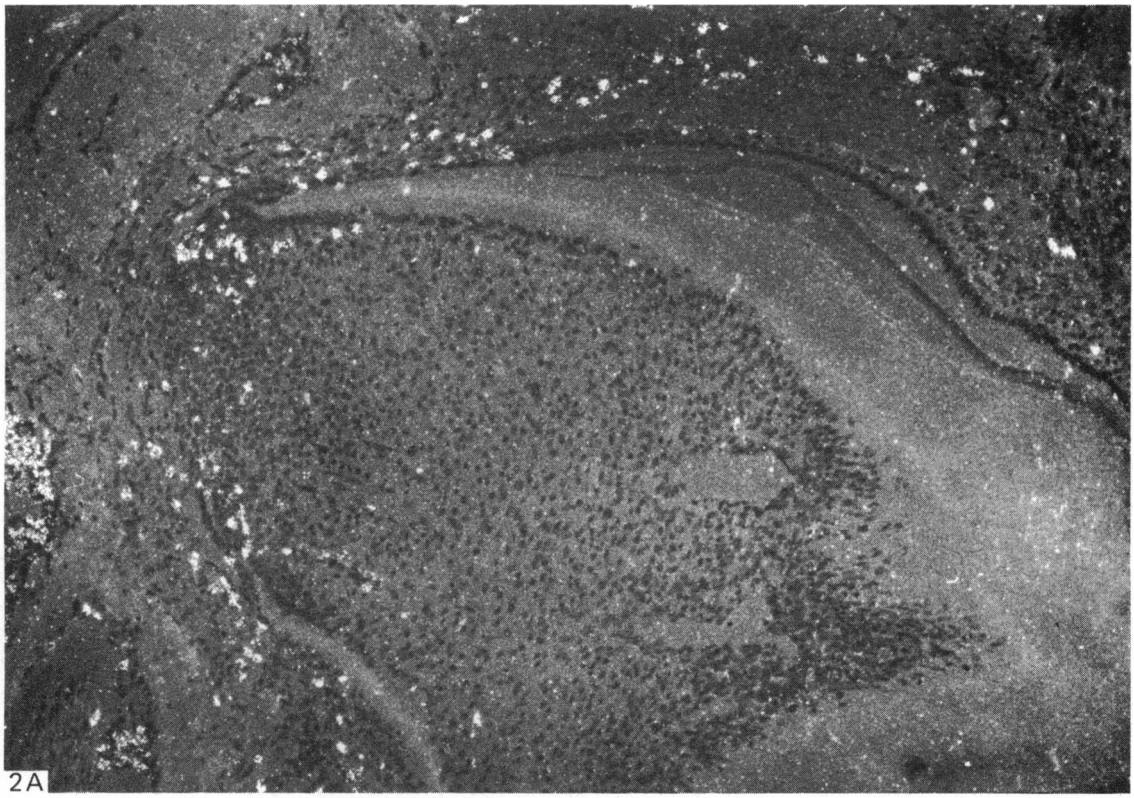
Immediately after death, the mandible of each animal was removed by dissection, fixed in 10% formalin (buffered with phosphate at pH 7) and decalcified in 10% EDTA pH 6·9 for 2–3 days. Adequate decalcification was confirmed by radiological examination of randomly selected specimens. They were then trimmed and washed in running tap water for 4–8 hours before processing and paraffin wax embedding. Serial sections were cut in the mesiodistal plane at 6 μm from the periodontal ligament of the mandibular first molars, one section in five being retained.

Autoradiography

Autoradiographs of both experimental and control sections were prepared according to the liquid emulsion dipping technique (Rogers, 1973). After dipping in Ilford K2 solution diluted with distilled water and drying, the slides were placed in exposure

Fig. 2. (A) Autoradiograph: D–G reflected light. A low power autoradiograph of the first molar periodontal ligament, 8 hours after the injection of ^{3}H-thymidine and viewed under dark ground reflected light. The distribution of labelled fibroblasts within the periodontal ligament is quite evident. Labelled cells are also seen in the pulp among the undifferentiated pulp cells. ×35.

(B) Same autoradiograph: transmitted light. The same above autoradiograph but viewed under transmitted light for detailed tissue and cell identification. Labelled cells are evident as dark dots. *Am*, ameloblasts; *D*, dentine; *E*, enamel: *LF*, labelled fibroblasts; *P*, pulp.



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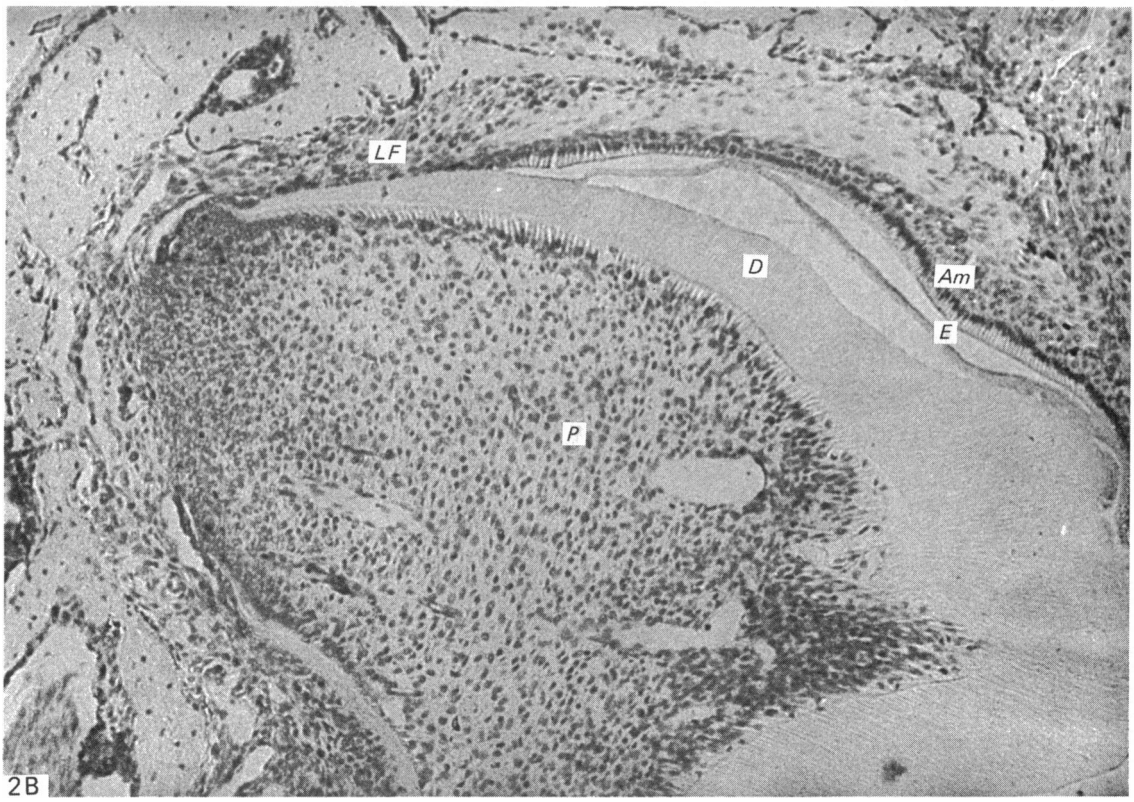

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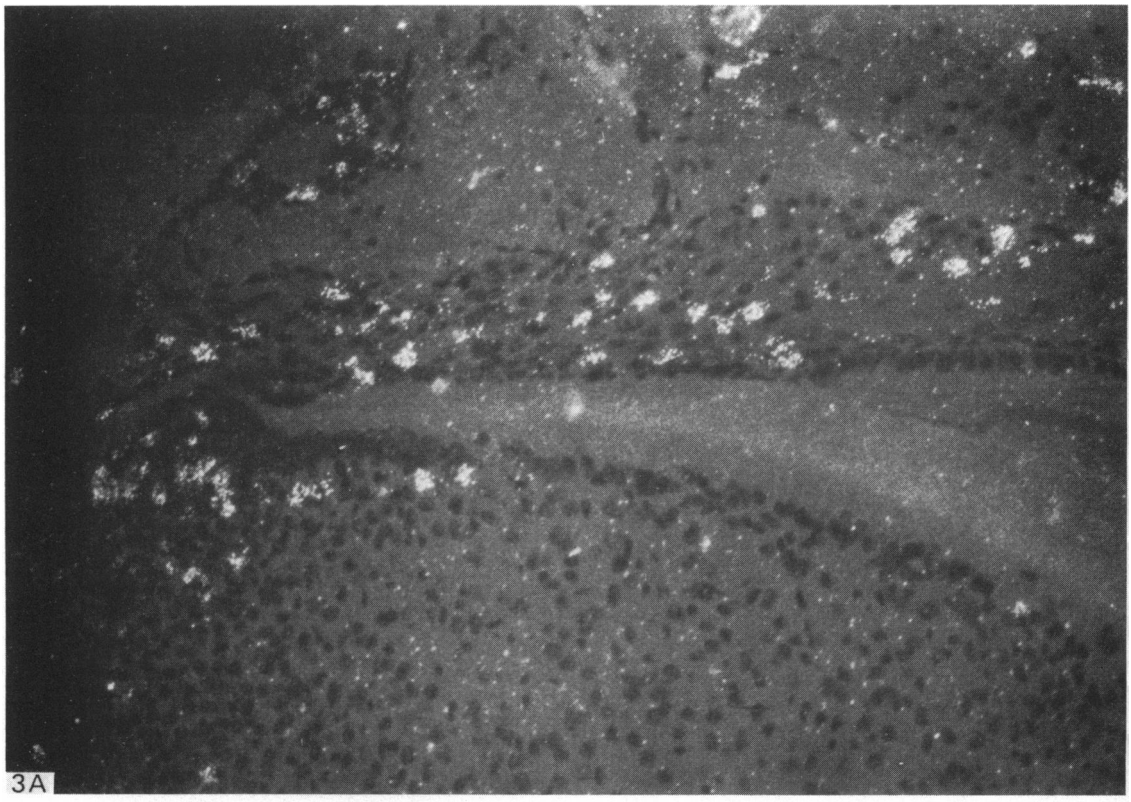

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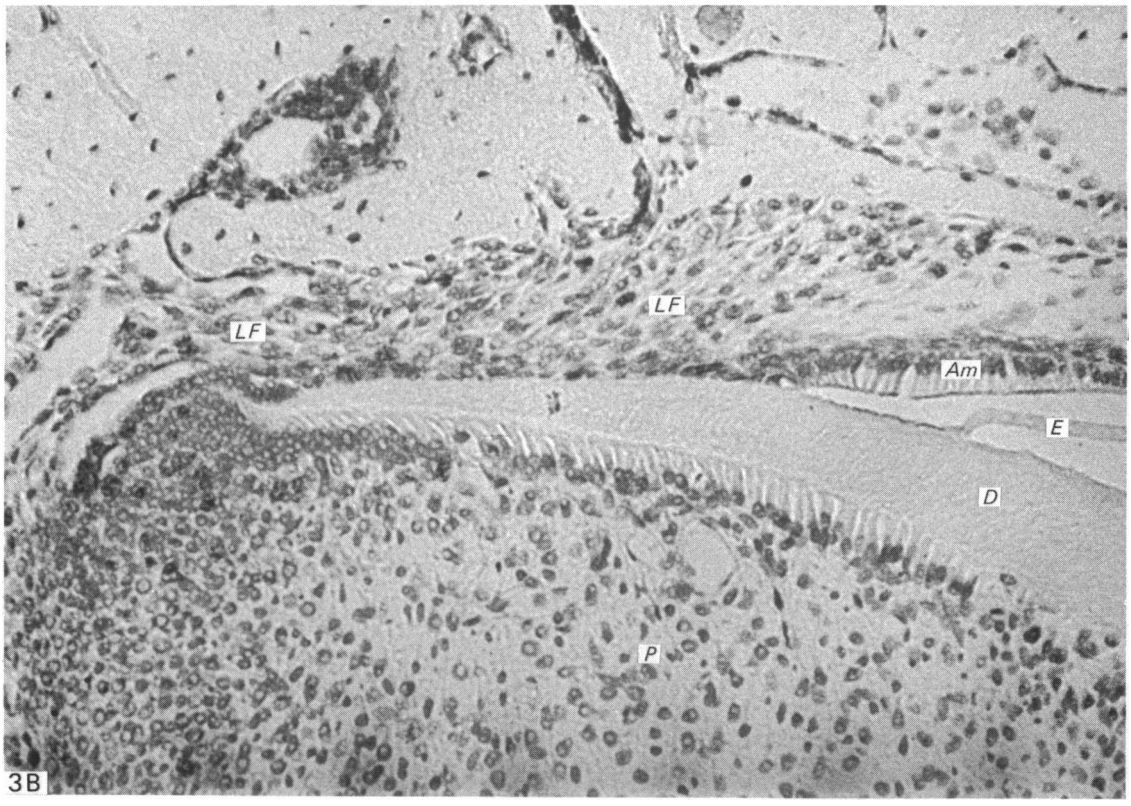


3B

boxes, with the lids off, in a dessicator over dried silica gel overnight at room temperature. The boxes were then closed and stored in a refrigerator at 4 °C for 4–5 weeks. Control non-radioactive sections were processed identically and simultaneously with the labelled sections, and two fogged experimental slides were included in each exposure box. This provided a check for both latent image fading and positive and negative chemography (Rogers, 1973) which helped to establish that the silver grains seen over the experimental tissue were due to radioactivity and were not artefactual. The autoradiographs were developed at the same time in Kodak D19 Developer for 5 minutes at 20 °C, fixed in Kodak Unifix (30 % sodium thiosulphate) at 18–20 °C for 10 minutes and subsequently washed in running tap water for about 10–15 minutes. Finally the sections were stained with haematoxylin and eosin.

Labelling index of periodontal ligament fibroblasts

The molar periodontal ligament was divided into three zones (apical, middle and cervical) by drawing two lines at right angles to the tooth surface at equal intervals between the epithelial attachment and the root apex (Fig. 1). The normal but limited development of the periodontal ligament in the 10 days group made it difficult to recognise these three zones. Consequently, periodontal ligaments in this group were divided into two equal zones (apical and cervical).

The distribution of labelling indices of periodontal ligament fibroblasts with respect to post-injection times of [^{3}H]thymidine was studied in the selected zones and evaluated for all four age groups. The specific pattern of proliferation, maturation and migration of periodontal ligament fibroblasts was identified for each age group.

Analysis

Quantitative analysis of the autoradiographs (Figs 2, 3) was achieved by counting labelled cells and determining the labelling indices. During the counting of labelled cells no attempt was made to differentiate periodontal ligament fibroblasts from pre-osteoblasts, pre-osteoclasts and/or precementoblasts, since it was realised that these cells were morphologically indistinguishable at this early stage of periodontal ligament development. In making counts, a cell was considered to be labelled if there were at least 4 silver grains overlying the nucleus. Background levels were much lower than this (1 or 2 grains). Using an eyepiece grid, labelled cell counts were made in all three zones of each of the sections using an oil-immersion × 100 objective lens. In each zone an area of a square of side 0·07 mm was subdivided into four squares with sides of 0·035 mm; cell counts were made using these squares as basic units. Three such areas were counted in each zone per section. Over 20 sections were used for each experimental stage. For a selected zone the labelling index (percentage labelled cells) per section was determined by dividing the number of labelled cells by the total

Fig. 3. (A) Autoradiograph: D-G reflected light. A high power view of the previous autoradiograph (Fig. 2) of the developing periodontal ligament (mesial aspect) of the first molar tooth, viewed under dark-ground reflected light. Labelled and dividing cells are quite evident within the periodontal ligament. Labelled and dividing cells are also seen within the pulp adjacent to the root. × 220.

(B) Same autoradiograph: transmitted light. The same autoradiograph (Fig. 3) but viewed under transmitted light for detailed tissue and cell identification. Labelled and dividing cells are seen with dark silver grains. *Am*, ameloblasts; *D*, dentine; *E*, enamel; *LF*, labelled and dividing fibroblasts; *P*, pulp.

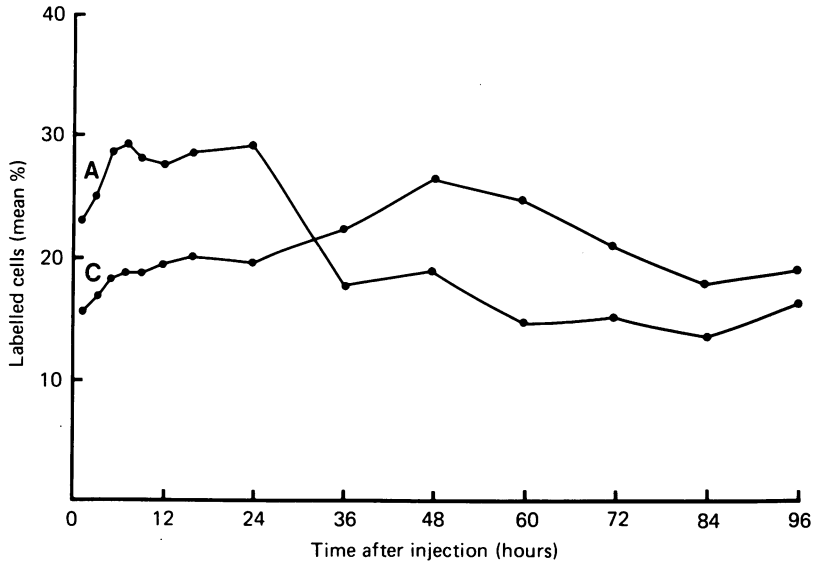


Fig. 4. L.I. curves: 10 days old group. Curves of mean percentage labelled cells (labelling indices) for apical (A) and cervical (C) zones of the molar periodontal ligament of 10 days old mice during normal development and eruption of teeth with time after the administration of [^{3}H]-thymidine.

number of periodontal ligament fibroblasts present in that zone and multiplying by 100. Using these values, the mean percentage labelled cells and their standard errors were computed for the selected zone.

RESULTS AND INTERPRETATION

Proliferative indices in the periodontal ligament of 10, 12, 16 *and* 20 *days old mice*

Tables showing the computed mean percentage labelled cells with the standard errors ($I_L \pm$ S.E.) for different post-injection times, in the three zones (apical, middle and cervical) of the four groups of mice are available, on request from the authors. The zones selected for counting labelled cells were arbitrary, since there is no anatomical or cytological arrangement demarcating these zones in the periodontal ligament.

10 *days old group*

Figure 4 shows the labelling index distribution curves for the apical zone (A) and for the cervical zone (C) with time after [^{3}H]thymidine administration. In the apical zone (A) over the first 7 hours there was a rapid increase in the labelling index (from $23{\cdot}03 \pm 2{\cdot}85$ % to $29{\cdot}21 \pm 2{\cdot}86$ %) but thereafter there was a plateau. After 24 hours the labelling index decreased rapidly during the next 12 hours and then more slowly, reaching a minimum value of $13{\cdot}33 \pm 2{\cdot}91$ % after 84 hours. However, in the cervical zone the situation was different, the labelling index showing a gradual but exponential increase over the first 48 hours and reaching a maximum value of $26{\cdot}26 \pm 2{\cdot}46$ %. Thereafter, the labelling index decreased gradually, reaching a minimum value of $17{\cdot}72 \pm 3{\cdot}71$ % after 84 hours.

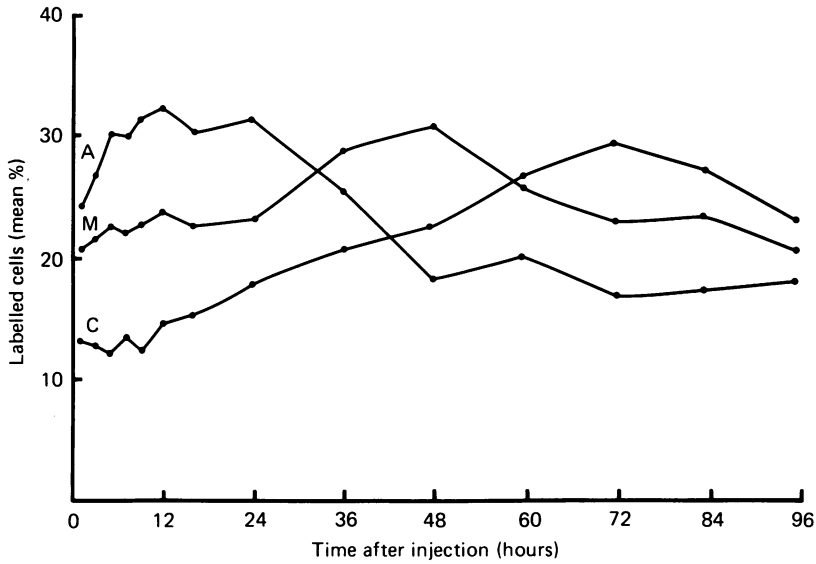


Fig. 5. L.I. curves: 12 days old group. Curves of mean percentage labelled cells (labelling indices) for apical (A), middle (M) and cervical (C) zones of the molar periodontal ligament of 12 days old mice during normal development and eruption of teeth, with time after the administration of ^{3}H-thymidine.

12 *days old group*

The labelling index distribution curves for the 3 zones (apical, A, middle, M, and cervical, C) of this group are shown in Figure 5. During the first 12 hours there was a rapid increase from 24·16 ± 4·77 % to 32·25 ± 3·81 % in the apical zone (A), but thereafter the curve described a plateau with marked fluctuations for a further 12 hours, and after 24 hours there was a rapid decrease to a value of 18·18 ± 2·79 % at 48 hours and to a minimum value of 16·67 ± 2·56 % at 72 hours.

The labelling index curve for the middle zone (M) increased gradually over the first 48 hours, from 20·75 ± 3·86 % at 1 hour to 30·55 ± 2·67 % at 48 hours and decreased gradually over the next 48 hours to a minimum of 20·22 ± 2·31 % at 96 hours.

The situation in the cervical zone was quite different; the labelling index distribution curve (C), after some initial fluctuations, showed an exponential increase over the period of the first 72 hours (from 13·14 ± 1·79 % at 1 hour to 29·01 ± 2·65 % at 72 hours) and thereafter fell to a value of 22·72 ± 1·63 % at 96 hours.

16 *days old group*

In this group (Fig. 6), the labelling index distribution for the apical zone (A) showed a small but rapid increase in the labelling index count over the first 12 hours from 18·06 ± 2·61 % to 25·25 ± 2·33 %; thereafter the labelling index count decreased somewhat rapidly to a value of 11·11 ± 1·31 % at 36 hours, and thereafter the values fell gradually to 6·51 ± 1·01.

Labelling index distribution for the middle zone (M) increased from an initial value of 14·21 ± 2·53 % at 1 hour to a peak value of 25·01 ± 2·91 % at 48 hours, and thereafter decreased gradually to a minimum of 12·49 ± 1·96 % at 96 hours.

Labelling index values for the cervical zone (C) showed an exponential increase

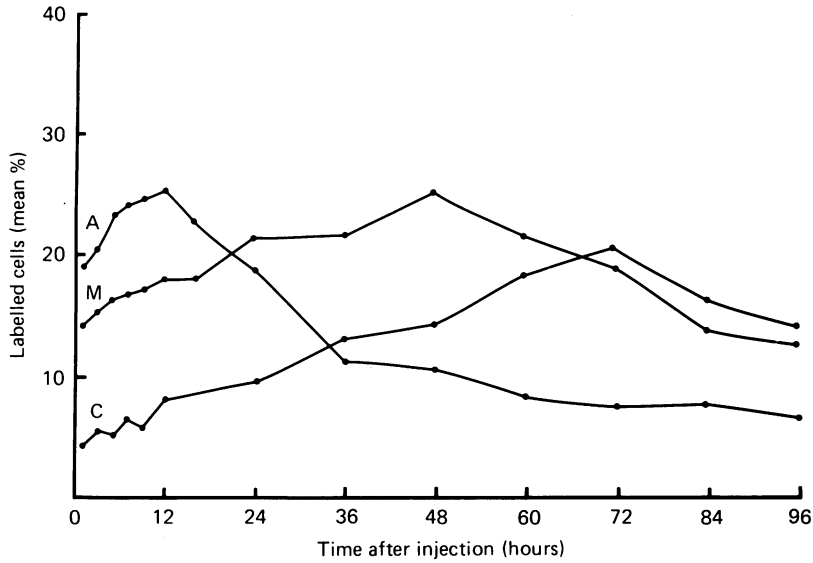


Fig. 6. L.I. curves: 16 days old group. Curves of mean percentage labelled cells (labelling indices) for apical (A), middle (M) and cervical (C) zones of the molar periodontal ligament of 16 days old mice during normal development and eruption of teeth, with time after the administration of ^{3}H thymidine.

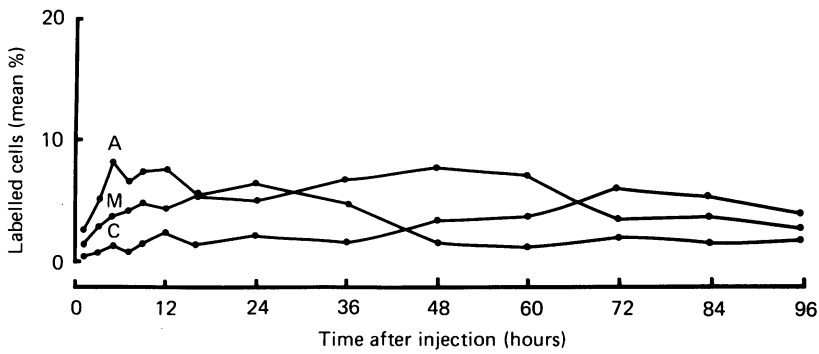


Fig. 7. L.I. curves: 20 days old group. Curves of mean percentage labelled cells (labelling indices) for apical (A), middle (M) and cervical (C) zones of the molar periodontal ligament of 20 days old mice during normal development and eruption of teeth, with time after the administration of ^{3}H-thymidine.

Figs. 4–7. *Proliferative index distribution curves compared within groups*

from $4{\cdot}23 \pm 0{\cdot}73$ % to $20{\cdot}33 \pm 2{\cdot}16$ % over the period of the first 72 hours and thereafter decreased to a value of $13{\cdot}98 \pm 1{\cdot}60$ % at 96 hours.

20 *days old group*

Figure 7 shows the labelling index distribution curves for the apical (A), middle (M) and cervical (C) zones respectively for the 20 days old group. At this stage the labelled cell counts were not significantly high, although there was a slight increase over the first 12 hours ($2{\cdot}51 \pm 1{\cdot}61$ % to $7{\cdot}51 \pm 1{\cdot}71$ %) in the apical zone, over the first

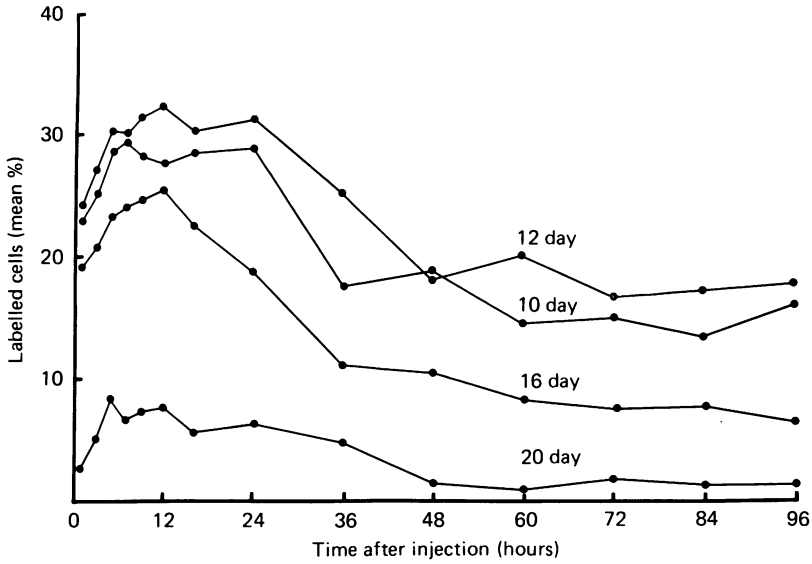


Fig. 8. L.I. curves: apical zones of 10, 12, 16 and 20 days old groups. Curves of mean percentage labelled cells (labelling indices) for apical zones of the molar periodontal ligament of 10, 12, 16 and 20 days old mice, during normal development and eruption of teeth, with time after the administration of ^{3}H-thymidine.

48 hours (1·51 ± 0·91 % to 7·59 ± 1·36 %) in the middle zone, and over the first 72 hours (0·55 ± 0·14 % to 5·76 ± 1·12 %) in the cervical zone.

Apical zones of 10, 12, 16 *and* 20 *days old groups compared*

When the labelling index distribution curves over the apical zones of the four groups are compared (Fig. 8) the rapid increase in the labelling index (i.e. rate of entry into DNA synthesis phase) in the apical zone was most evident in the 12 days group, where it reached a maximum of 32·25 % at 12 hours after the injection. A similar pattern of the percentage labelled *s*-phase cells was evident in the 10 and 16 days old groups but the increase was not as high as that in the 12 days old group. In the 20 days old group, although a slight increase in the percentage labelled cells was noticeable over the first 24 hours, thereafter it reached a minimum of 1 % at 60 hours. All curves diminished after 24 hours, reaching a minimum at or after 72 hours.

Middle zones of 12, 16 *and* 20 *days old groups and cervical zone of* 10 *days old group compared*

Comparing the labelling index distribution curves over the middle zones of 12, 16 and 20 days old groups, peak values were evident 48 hours after the injection of [^{3}H]-thymidine. The values decreased after 60 hours and, as in the apical zone, the 12 days old group showed the maximum peak value although similar but a lower peak value for the percentage labelled *s*-phase cells was evident in the 16 days old group. In the 20 days old group, the relative increase in the percentage labelled cells was markedly less.

The normal but limited development of the periodontal ligament in the 10 days old group precluded the selection of a middle zone. However the labelling index

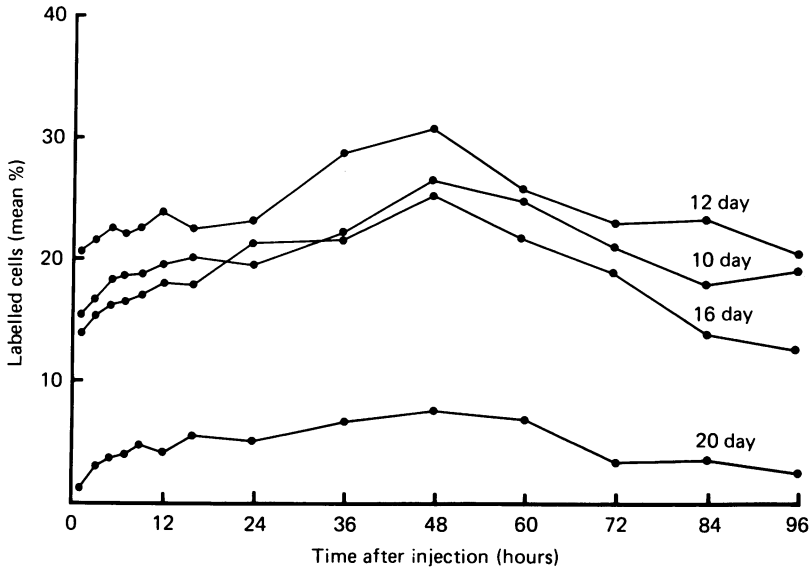


Fig. 9. L.I. curves: Middle zones of 12, 16 and 20 days old groups and cervical zone of 10 days old group. Curves of mean percentage labelled cells (labelling indices) for middle zones of the molar periodontal ligament of 12, 16, 20 days and cervical zone of 10 days old mice during normal development and eruption of teeth with time after the administration of ^{3}H-thymidine.

curve for the cervical zone in this group followed a very similar sequence to the middle zones in the other groups (12, 16 and 20 days old groups) reaching a peak value at 48 hours and decreasing 60 hours after the injection. Because of this similarity the labelling index curve for the cervical zone in the 10 days old group was compared with the middle zones of other groups (Fig. 9).

Cervical zones of 12, 16 *and* 20 *days old groups compared*

The labelling index distribution in the cervical zones of the 12, 16 and 20 days old groups (Fig. 10) reached peak values 72 hours after the injection of [^{3}H]thymidine. Again, as in other zones, a maximum peak value was seen in the 12 days old group (13·14 % at 1 hour to a peak level of 29·01 % at 72 hours). The peak value in the 16 days old group was lower than that in the 12 days old group. The increase in the 20 days old group was relatively insignificant. The labelling index in the cervical zone of the 10 days old group reached a peak value 48 hours after the injection but not after 72 hours and consequently was compared with middle zones (Fig. 9).

In summary the labelling index in various zones of the periodontal ligament of the four groups studied at different times after [^{3}H]thymidine administration has highlighted the following points:

1. All zones of all groups showed an initial level of mitotic activity; highest in the 12 days old, high in the 10 days old, low in the 16 and very low in the 20 days old groups.

2. This proliferative activity increased and reached maximum peaks after the injection of [^{3}H]thymidine at 24, 48 and 72 hours for the apical, middle and cervical zones respectively. This labelling index distribution sequence was evident in all the groups. In the 10 days old group, peak values were seen at 24 and 48 hours after the injection.

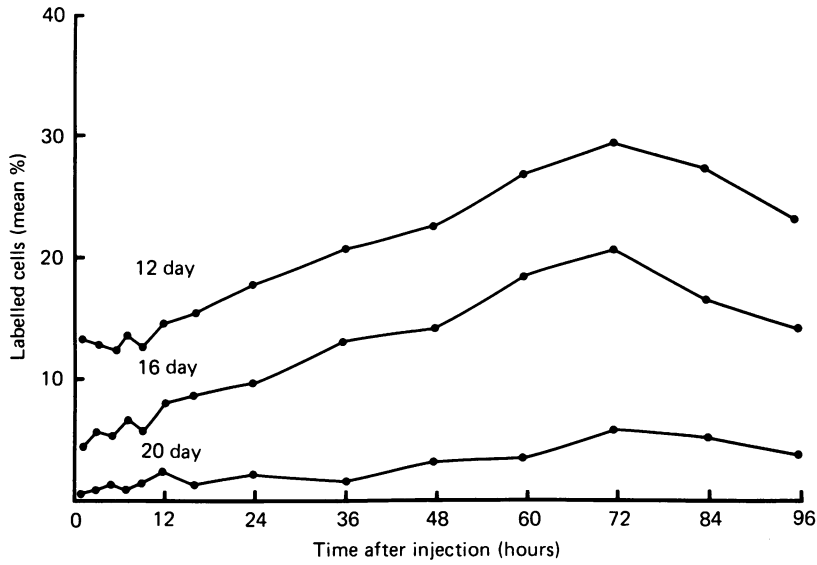


Fig. 10. L.I. curves: cervical zones of 12, 16 and 20 days old groups. Curves of mean percentage labelled cells (labelling indices) for cervical zones of the molar periodontal ligament of 12, 16 and 20 days old mice, during normal development and eruption of teeth, with time after the administration of ^{3}H-thymidine.

Figs. 8–10. *Proliferative index distribution curves compared between groups*

3. The 24, 48 and 72 hours sequence of peak values for the labelling indices was highest in the 12 days old group, although similar, but lower, peak values were apparent in the 10 and 16 days old groups. In the 20 days old group, these peaks were less marked.

DISCUSSION

Fibroblast cell proliferation in the periodontal ligament of teeth of limited eruption has demonstrated a precise pattern of developmental behaviour and several tentative conclusions concerning the mode of cell renewal within the developing molar periodontal ligament can be made:

1. The labelling index at different depths (zones) in the periodontal ligament has shown a variation in proliferative activity. The distribution of labelling indices increased in the apical zone and supports the concept that this is a progenitor zone of the periodontal ligament. However, the middle and cervical zones also demonstrated measurable labelling indices within the first few hours after the injection of [^{3}H]-thymidine. These varying proliferative rates in the middle and cervical zones might be capable of maintaining structural homeostasis.

2. The early increase during the first 12 hours to a peak of the labelling index in the apical zone, followed by a similar increase in the middle zone (during the first 48 hours) and finally in the cervical zone (during the first 72 hours) suggested that fibroblasts migrate from apical zone to middle and subsequently to cervical zones.

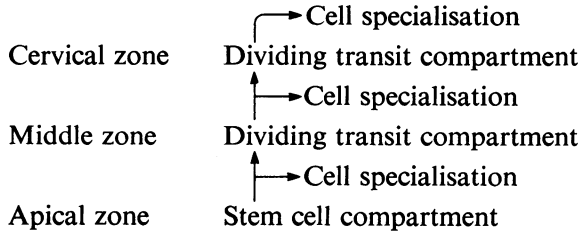
3. The definite sequence of proliferative and migratory behaviour of periodontal ligament fibroblasts was established in the different groups studied. However, proliferation and migration varies in extent; these were highest in the 12 days old group, high in the 10 days old group, low in the 16 days old group and virtually

absent in the 20 days old group, which suggested that the fibroblast proliferation and migration within the molar periodontal ligament is a time-limited process.

The cellular origin of the developing fibroblasts is well documented (Van Winkle, 1967*a*, *b*; Melcher & Eastoe, 1969). It has been thought that periodontal ligament fibroblasts, like fibroblasts elsewhere in the body, have either a haematogenous origin or arise locally from undifferentiated mesenchymal cells in loose connective tissue, particularly from those cells which are associated with blood vessels. However recent work (Ten Cate *et al.* 1971; Ten Cate & Mills 1972; Ten Cate, 1972) has demonstrated the development of the periodontal ligament fibroblasts from the perivascular cells derived from the cells of the ectomesenchymal investing layer, rather than or in addition to maturation of pre-existing cells within the dental follicle. Therefore, in the periodontium a haematogenous origin of the fibroblasts is very unlikely.

In a kinetic study which explored the fibroblast cell proliferation of the incisor periodontal ligament, Zajicek (1974) demonstrated the existence of a velocity profile throughout the ligament, with cells in different layers (shells) migrating at different velocities. His explanation for this peculiar velocity profile was that periodontal ligament fibrocytes, by active migration, pull the tooth from its socket. Although this explanation seems plausible, at least in the continuously growing incisor, there is as yet no experimental confirmation or rejection by other workers of the possible existence of this velocity profile. Ten Cate (1972), in a short qualitative, autoradiographic study of cell division within the periodontal ligament of rat molar teeth, demonstrated proliferation in a progenitor zone and maturation in a transitional zone and suggested a migration of the fibroblasts.

This study has demonstrated the existence of a progenitor area with proliferating fibroblasts in the apical zone and migration of these cells into other zones (middle and cervical). The increase in the percentage of labelled fibroblasts in the upper zones (middle and cervical) takes place mainly after a period of about 48 hours and 72 hours respectively, with a concomitant reduction of the labelled cells in the corresponding lower zones (apical and middle). The cellular dilution observed in these zones is interpreted as being mainly due to fibroblasts migrating from lower zones to upper zones which are also proliferating. Such a sequence might be expressed:



Proposed apico-occlusal migratory model

Proliferation takes place in the apical progenitor zone, the cells migrating from the apical zone to middle and cervical zones with a decreasing degree of proliferative activity. If we accept this migration concept, then, by definition, apical cells would have no input and may be regarded as stem cells. The ectomesenchymal origin of these stem cells was discussed earlier. The middle zone may be regarded as a dividing transit compartment, receiving cells from the stem cell compartment and subsequently feeding the cervical zone. Cells in these zones then specialise to provide

secretory fibroblasts, and possibly osteoblasts and cementoblasts, as Ten Cate & Mills (1972) suggested, but we must regard these as dividing transit compartments since there was a measureable proliferative rate over the first few hours of the injection of [^{3}H]thymidine. The migratory mechanism, however, in the case of the molar periodontal ligament, is a time-limited phenomenon. Since the maximum proliferative and migratory behaviour was seen in the 12 days old group it would appear that the periodontal ligament behaves and functions as a 'stimulated system' during this period. This is interesting because 12 days is the approximate age of tooth eruption in the mouse.

Beertsen *et al.* (1974), in their study into the fine structure of fibroblasts, have demonstrated the existence of an ultrastructural system that could account for the active migration of fibroblasts. In these cells, they have observed the existence of microfilaments and microtubules arranged in networks as well as in bundles in the peripheral part of the system. They claim that these microfilaments, possibly in co-ordination with microtubules, might be associated with cell locomotion. There is also evidence for the migratory behaviour of fibroblasts in other experimental situations (Weiss, 1961; Ross, 1968; Vasiliev *et al.* 1970; Abercrombie, Heaysman & Pegrum, 1971).

Although there is substantial evidence to implicate the migration of periodontal ligament fibroblasts with incisor tooth eruption, and the results of this investigation provide evidence in favour of a close relationship between fibroblast cell migration and eruption of molar teeth, it is not possible to interpret these results as having a causal relationship.

Future work should attempt to establish whether there is a cytokinetic basis for the proliferation and migration seen in this study.

SUMMARY

Cytogenesis of the developing molar periodontal ligament was studied by determining labelling indices in 4 groups of young mice. Autoradiographs were made using paraffin sections of demineralised specimens from 10 days, 12 days, 16 days and 20 days old mice allowed to survive a varying period (1–96 hours) after the administration of [^{3}H]thymidine. Labelled fibroblasts in number per unit area were counted over three different zones (apical, middle and cervical) of the sections and labelling index distribution curves were drawn.

The present study has demonstrated the existence of a progenitor area with proliferating fibroblasts in the apical zone and a migration of these cells into other zones (middle and cervical). However, it has also shown that a measurable proliferative rate is present at all levels of the molar periodontal ligament in all ages of animals investigated. Such an observation is compatible with fibroblasts migrating from the apical zone in an occlusal direction and maintaining a decreasing degree of proliferative activity as they progress. Consequently, a tentative apico-occlusal migratory model for the periodontal ligament fibroblast proliferation and migration is proposed. Accordingly, fibroblast proliferation takes place in the apical progenitor zone, and cells migrate from the apical zone to middle and cervical zones, but maintaining a decreasing degree of proliferative activity as they migrate. Peak proliferative and migratory activity is seen in the 12 days group which is also the time that tooth eruption takes place in the mouse. Thus, a close relationship exists between fibroblast cell proliferation, migration and tooth eruption.

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