A Radioautographic Study of Chondrocytic Proliferation in Nasal Septal Cartilage of the Prenatal Rat

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Six pregnant female Sprague-Dawley rats were labeled with *tritiated thymidine* and killed, one each day for the final six gestational days prior to birth. *Nasal* septal cartilages of thirty pups (five from each litter) were monitored *radioautographically* for cellular proliferation. It was found that *septal growth* was greatest on prenatal day 17 and least on day 21. There was no specific area in the septal cartilage that could be identified as a growth center.

The mechanisms governing facial morphogenesis are unknown. Numerous studies (Babula et al., 1970; Kremenak and Searls, 1971; Latham and Scott, 1970; Ohyama, 1969; Searls and Kinser, 1972; Searls, 1975, and Siegel, 1976) have undertaken to determine precise controlling factors in development of the maxillofacial complex. Many data have been accumulated thus far. Among the more interesting of these data are those concerned with the nasal septum (Figure 1) and its contribution to the overall mystery of facial growth.

Moss et al. (1968), reporting on their work on 20-day-old rats, and Stenstrom and Thilander (1970, 1972) after monitoring facial growth following septal extirpation in young guinea pigs have concluded that septal cartilage makes nothing more than a passive contribution to growth of the midface. However, Kvinnsland and Breistein (1973) noted the development of *marked* facial deformities after septal removal in very young rats as did Sarnat and Wexler (1967, 1968) subsequent to partial septal resection in 29–48-day-old rabbits.

The data reported by these various investigating teams seemed to conflict. The question was, why? There could be many possible reasons for such a diversity of results but the most obvious appeared to be the *timing* of the surgery. Could it be that different investigators were reporting results at variance with one another not because of surgical methods but because of the *age* of the laboratory animal at the time of septectomy? Was there a time in the development of the facial structures when the entire complex was more susceptible to surgical insult than at some other time? If so, what might that time be?

It seemed reasonable to assume that the greatest deviation in development

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would occur if growth mechanisms were interrupted during periods of greatest growth activity. But during what phase of development did these periods occur? In order to find out, it was necessary to determine those times during development when growth was at its peak. The question was, how?

In 1968, Long, Greulich, and Sarnat reported on septal growth in young rabbits using radioactive labeling as a laboratory tool. It was evident that, with some modifications, this technique would be useful in answering our questions.

In 1972, the first attempts to monitor septal growth radioautographically were made. Since then, three studies have been reported from this laboratory, all utilizing radioactive thymidine as the labeling agent. The first study involved nasal septal cartilaginous growth in the 10-day-old rat (Searls and Kinser, 1972). The second concentrated on similar growth in the 5-day rat (Searls, 1975), and the third (submitted) described septal development in the newborn rat.

Finally, the present project was undertaken to follow septal cartilaginous development prenatally (Figure 2) from its inception during the twelfth day of gestation to its last prepartal day (21st day of gestation). However, because specific septal outlines could not be discerned with certainty until several days after the septal primordium first began to develop, the first data could not be generated until the 16th gestational day.

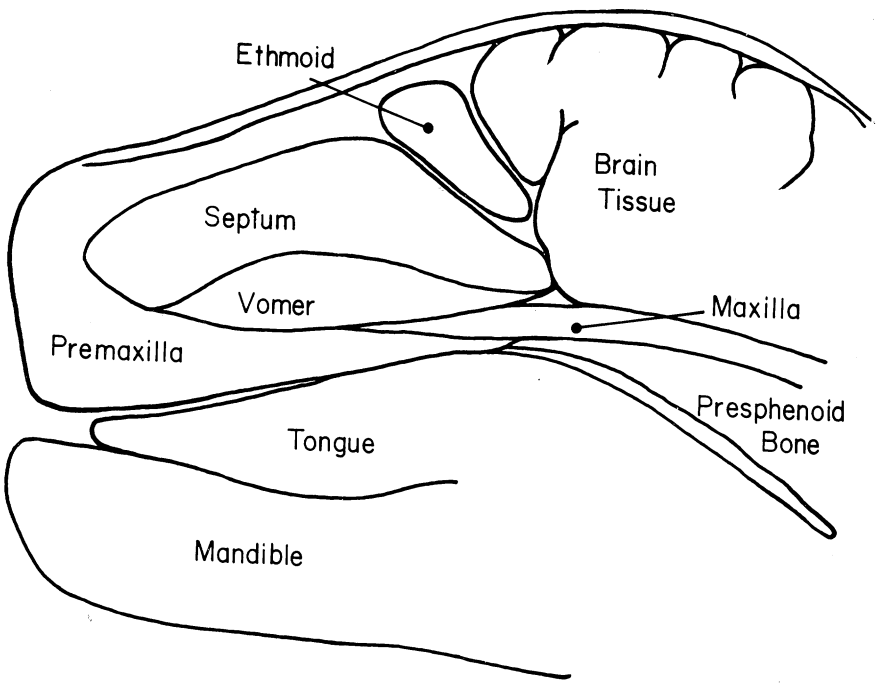

FIGURE 1. Midsagittal section of 18-day (in utero) rat head shows relationship of cartilaginous nasal septum to contiguous skeletal structures.

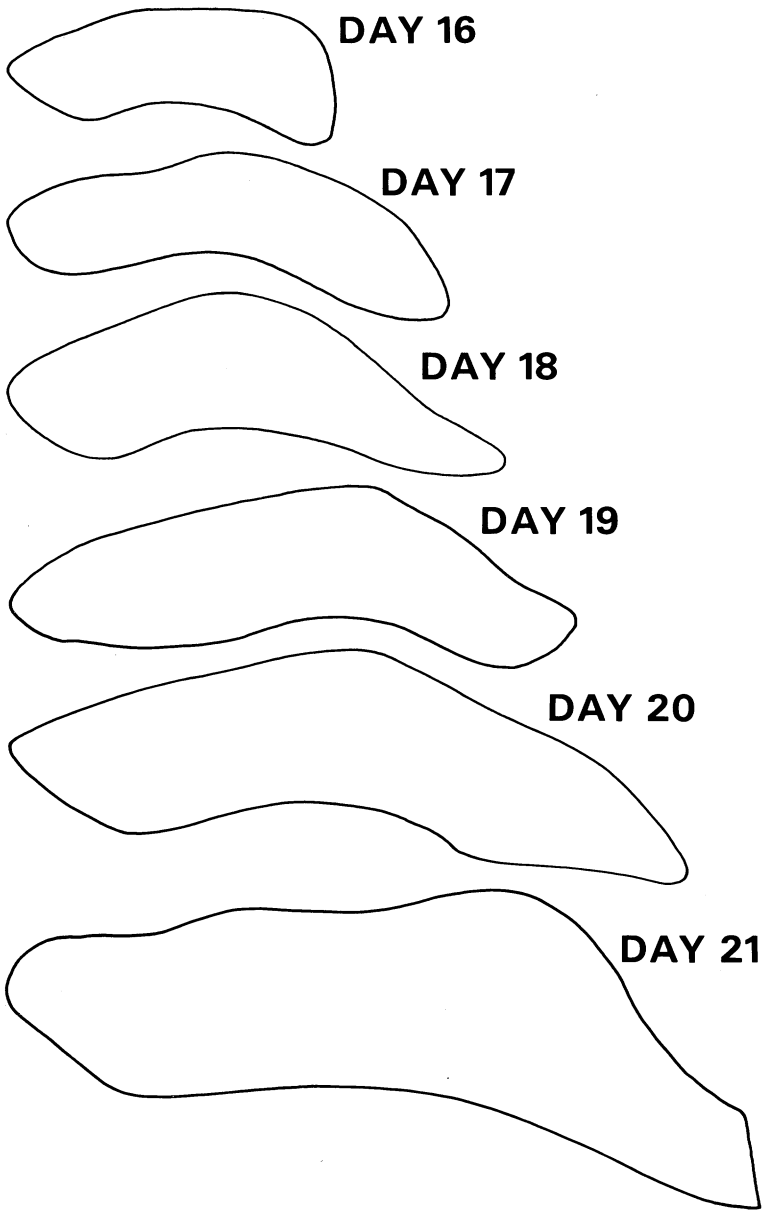

FIGURE 2. Illustration showing growth of rat nasal septum over final 6 gestational days.

Materials and methods

The laboratory animals used in this investigation were unborn pups of pregnant female albino rats[1]. Timed pregnancies were induced in six females of the Sprague-Dawley strain, and all were sacrificed in sequential, 24-hour time periods during the last six days of pregnancy, which are the 16th, 17th, 18th,

[1] Sprague-Dawley Co., Madison, Wisconsin.

19th, 20th and 21st in-utero days. On the day of sacrifice, each gravid female was weighed, and a dose of tritiated thymidine[2] in the concentration of one microcurie (specific activity 6.7 Ci/mM) per gram of body weight was injected into the long saphenous vein.

One hour after administration of the radioactive label, each mother was anesthetized by ether inhalation and an incision was made in the anterior abdominal wall so that the two uterine horns could be manipulated for further surgery. A longitudinal cut was made in each horn, exposing the amniotic sac of each developing fetus. All fetuses within each horn were removed and immersed immediately in a vial of 10% buffered formalin[3]. The number of viable fetuses per mother varied from nine to 13, but all were saved until it could be determined which were to be utilized in this study.

After a fixation period of 48 hours, the fetuses were removed from the formalin and weighed. The five pups exhibiting the most uniform size and weight from each litter were selected for histologic workup and ultimate radioautographic evaluation. Each animal was decapitated, and the heads were washed in tapwater for four hours and demineralized (chelated) in ethylenediamine tetraacetic acid (EDTA) for 36 hours. Subsequently, each head was washed in running tapwater, rinsed in distilled water, run through a series of graded alcohols (50% to absolute), cleared in xylene, and embedded in Paraplast[4].

The resultant blocks were oriented so that each head could be sectioned rostrocaudally (i.e., coronally) at six μm on a rotary microtome. Every fifth section was mounted, four sections per slide, so that each slide represented a 120-μm section of head. After the sections were mounted on slides, each was dip-coated with photographic emulsion[5] in a darkroom illuminated by a Wratten Series No. 2[6] Safelight at a distance of three feet. After two weeks' exposure time, the slides were developed, fixed, allowed to dry, and stained metachromatically with toluidine blue. An arbitrary decision was made to do counting procedures on the first and third sections of each slide only. Thus, for each fetus, the sections on which counting was done were approximately 60 μm apart.

The above steps were performed on five fetuses in each litter, and there were six litters examined (one litter for each prenatal day, 16 through 21). Consequently, the total number of radioactive fetal heads involved in this study was 30.

The counting of the labeled septal cells within the 30 fetal heads was done on a Leitz[7] binocular microscope using the same optics and the same techniques described previously (Searls, 1975), (Figure 3).

Findings

Figure 4 represents midsagittal reconstructions (from the types of coronal sections seen in Figure 3) of 16th, 17th, and 18th day prenatal septa while

[2] New England Nuclear Corp., Boston, Massachusetts.
[3] Calcium acetate, 20 gms/liter.
[4] Fisher Scientific, Chicago, Illinois.
[5] NTB-3, Eastman Kodak Co., Rochester, New York.
[6] Eastman Kodak Co., Rochester, New York.
[7] Ernst Leitz, GMBH, Wetzlar, West Germany.

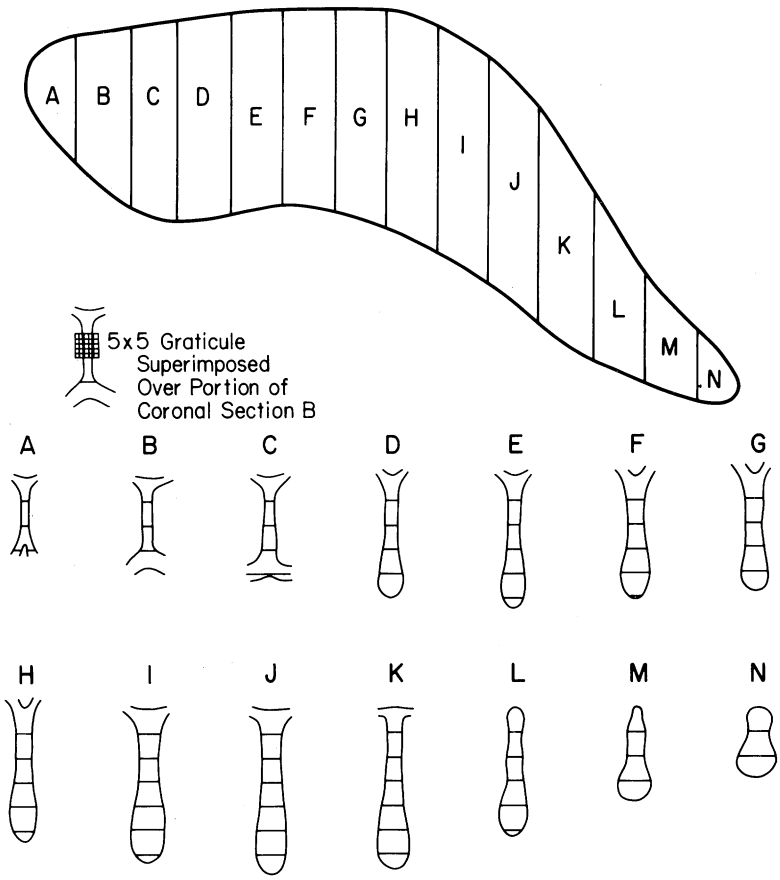

FIGURE 3. For purposes of this study, the cartilaginous nasal septa of each of the six groups of gestational rats were divided arbitrarily into 14 separate parts (labeled A through N) as seen in the top part of this figure. Coronal sections in lower part represent average sizes and shapes of sections taken from corresponding portions of midsagittal septum seen above.

Figure 5 represents a similar reconstruction for days 19, 20, and 21. Mean data and ranges derived from the labeled cell counts of the thirty septa are tabulated. Figures 6 and 7 are graphic representations of the same data.

The purpose of these on-going studies in our laboratory is to determine the areas of growth within the cartilaginous nasal septum of the growing rat. An assumption has been made that those areas of highest chondrocytic mitotic activity are also those areas of greatest septal growth. However, it is an obvious fact that the bulk of the cartilaginous septum is not its cellular component but rather its intercellular component, i.e., that part composed of collagenous fibers and sulfated mucopolysaccharides. Therefore, to determine the role played by the non-cellular portion of the septum in its overall growth, a pilot study was undertaken two years ago in which radioactive sulfur (S-35) was used to monitor matrix production.

Five-day-old rats were injected, in various concentrations, with S-35 and the labeling of the septal cartilaginous matrix was observed microscopically. It was found that labeling was so intense, even in moderate dosages, that grain counts could not be carried out. However, at low power microscopic examination, it was obvious that the radioisotope was generally evenly distributed with no one area

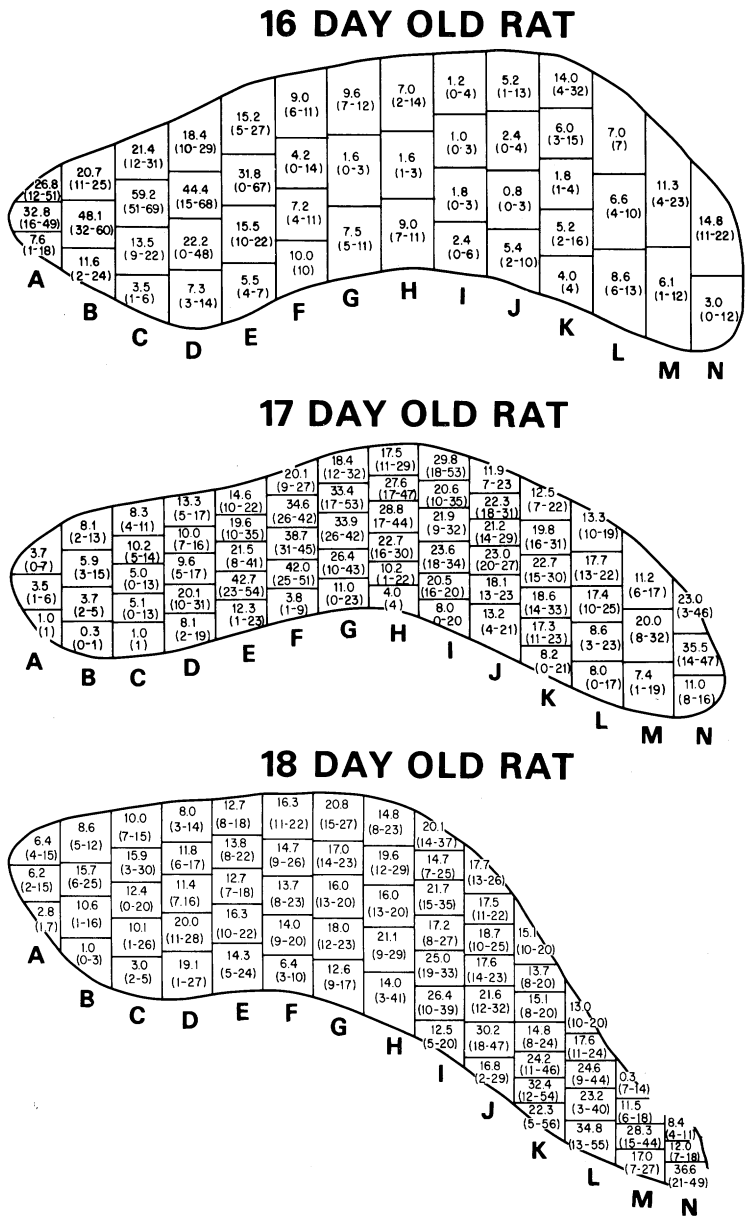

FIGURE 4. Mean counts of labeled cells in representative fields of the cartilaginous septa of 16-, 17-, and 18-day gestational rats; range is shown in parentheses.

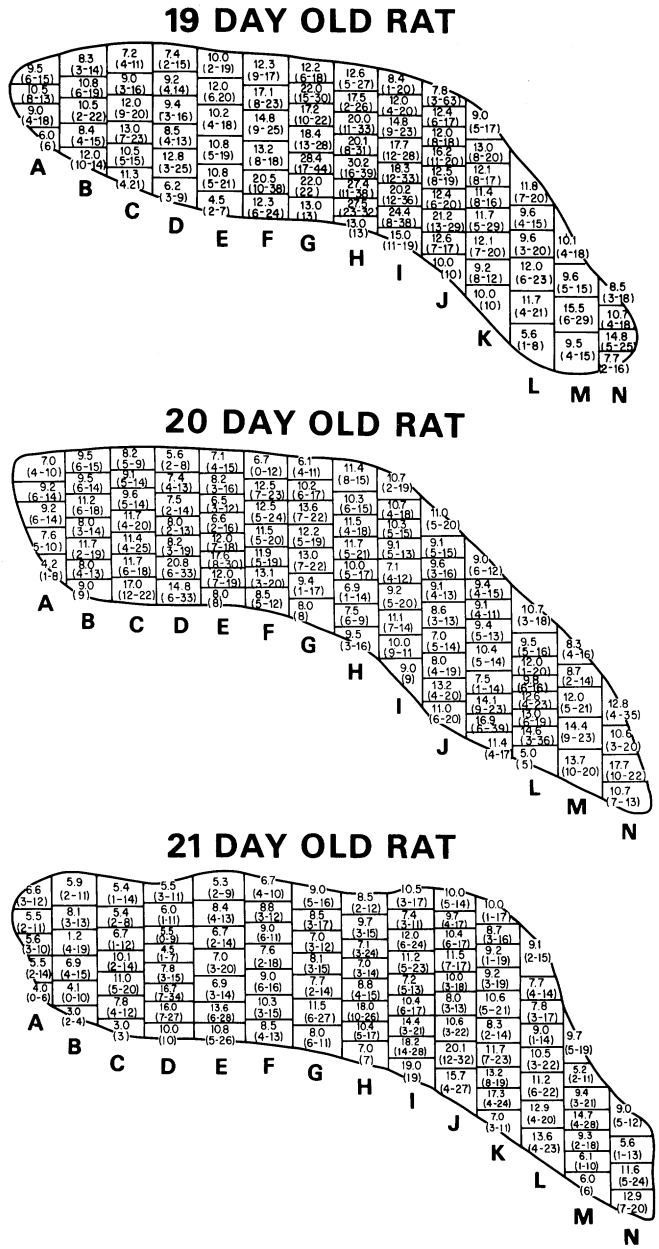

FIGURE 5. Mean counts of labeled cells in representative fields of the cartilaginous septa of 19-, 20-, and 21-day gestational rats; range is shown in parentheses.

showing appreciably higher concentration than any other. On the basis of the foregoing, it is assumed that all chondrocytes generate matrix pretty much uniformly and that it is probably more valid to identify areas of growth by determining those sites of greatest cellular mitotic activity than by identifying those areas active in matrix synthesis.

In the present study, in order to identify areas of high cellular proliferation, which we assumed were also the areas of greatest growth, we labeled pregnant rats in various stages of gestation with tritiated thymidine.

It was hoped that one anatomical location within the cartilaginous septum might be found in which mitoses were concentrated and from which the bulk of

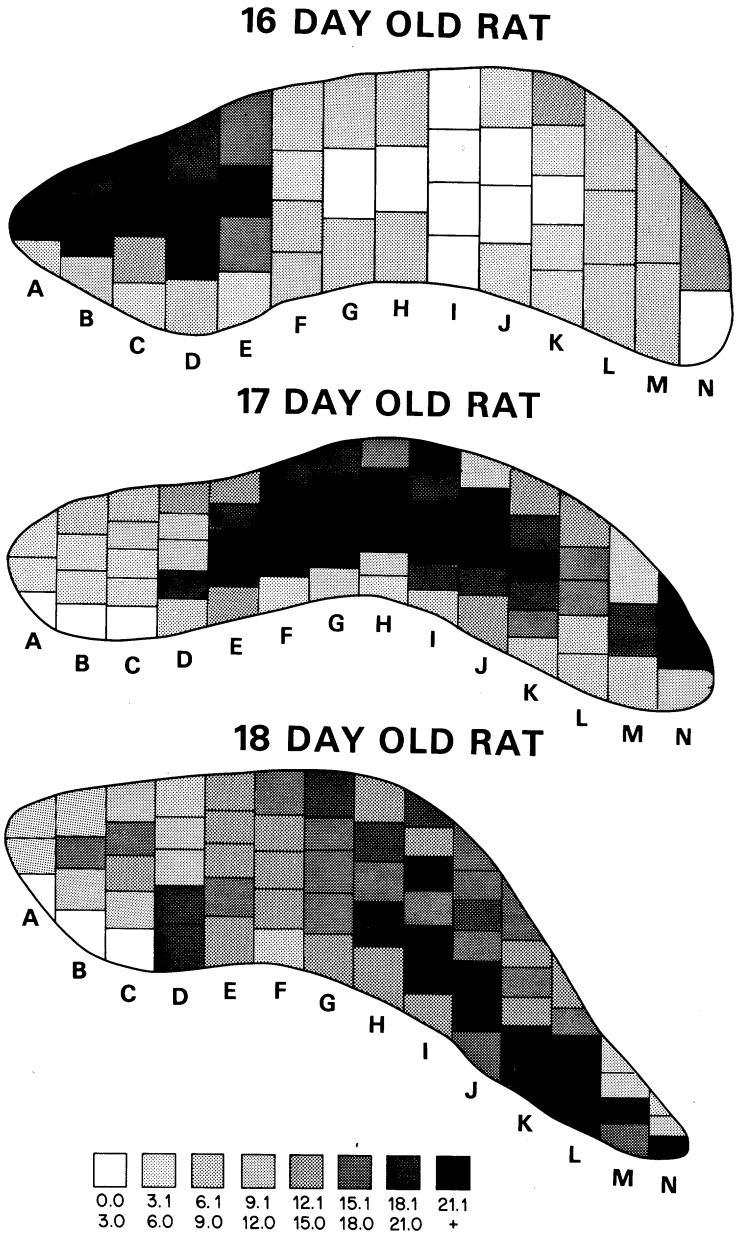

FIGURE 6. Graphic midsagittal reconstruction of Figure 3 showing relative label concentration within 16-, 17-, and 18-day gestational septa. Numbers at bottom of figure indicate range of average numbers of labeled cells per microscopic field counted.

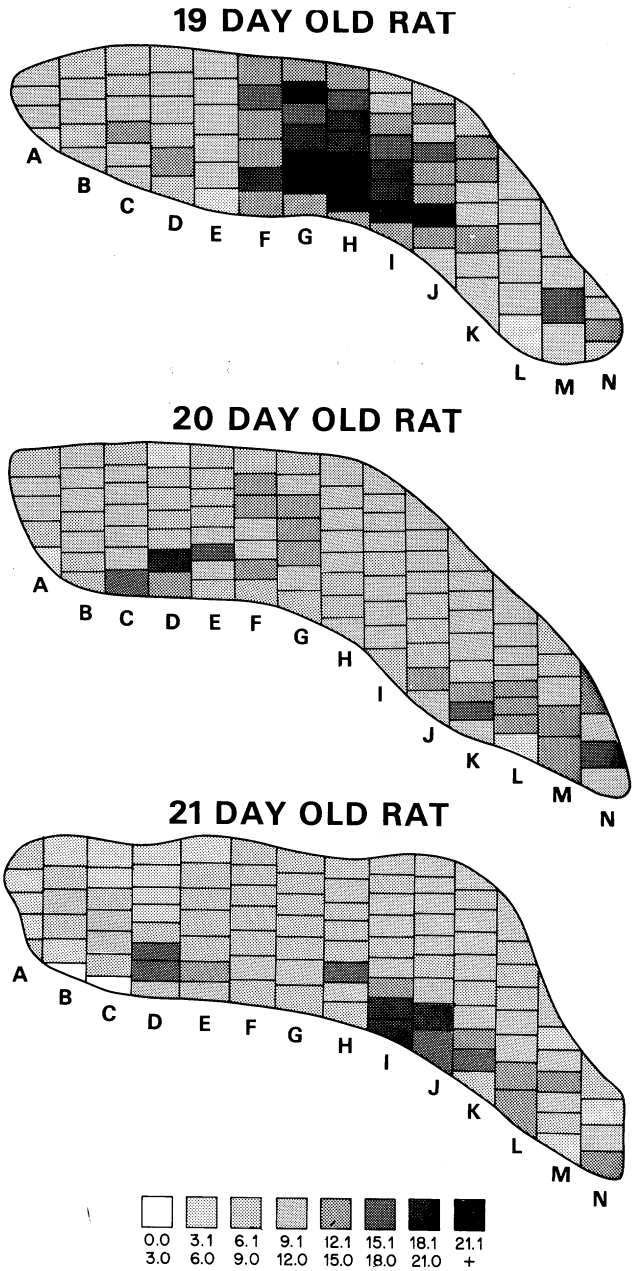

FIGURE 7. Graphic midsagittal reconstruction of Figure 4 showing relative label concentration within 19-, 20-, and 21-day gestational septa. Numbers at bottom of figure indicate range of average numbers of labeled cells per microscopic field counted.

activity within the growing septum was generated. It was even postulated that the septoethmoidal junction might turn out to be that area. However, a close perusal of Figures 4 through 7 will show that this was not the case.

On day 16 (in utero), the areas of greatest mitotic activity were limited to the

anterior one-third of the septum. In sections A through E (Figures 4 and 6), the average grain count per microscopic field was 22.53. In contrast, the remaining nine sections (F–N) showed only 5.84 counts per field, indicating a significant increase in proliferative activity in the nasal tip of the septum. It will be noted, however, that this mitotic activity was not present on the vomeral surface either anteriorly or elsewhere in the septum.

On day 17, labeling became considerably less intense at the tip and more highly concentrated in the middle two-thirds of the septum. The average count in the anterior five sections dropped from 22.53 (day 16) to 10.35. If only the first four sections (where the count was markedly reduced) are considered, the disparity becomes even greater (6.88 grains/field). The entire posterior two-thirds of the septum (sections E–N) would have to be identified as an area of high proliferation since grain counts averaged 19.8 per field there. However, it will be noted again that the vomeral surface was relatively free of concentrated activity.

Twenty-four hours later (day 18), another shift in mitotic activity was observed, this time to the posterior one-half of the septum, including intense labeling in the presphenoidal region. The posterior eight sections (G–N) showed 18.88 grains per field while the average count in the anterior six sections was 11.04.

Day 19 showed a general, overall diminution in radioactive labeling (Figures 7 and 8). Again, however, there was a shift in the active zone, this time back to

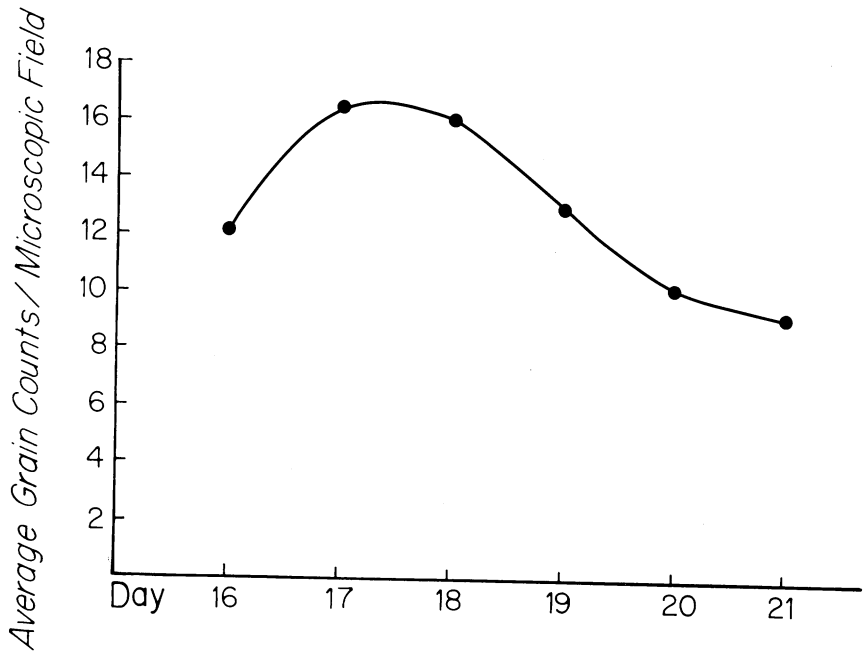

FIGURE 8. Graph showing changes in mitotic activity as fetus approaches parturition. After moderate activity on day 16 (12.1 grains/field), mitoses increase sharply and reach a peak on the 17th day (16.5). Day 18 brings about a modest decline (16.1) followed by a marked drop on both the 19th (13.1) and 20th (10.3) days. The 21st day shows a leveling off at 9.3 grains/field.

the middle one-third, a picture somewhat similar to that seen on day 17. The area of highest intensity was limited to sections F through J, the average grain count in this region being 15.88. For the portion of the septum anterior to this, 8.62 counts/field were recorded, while, for the posterior portion, the count was 10.69.

Two days prior to birth (day 20), there was such a marked reduction in cellular activity (an average of 10.3 grains/field) that it is impossible to identify an active zone anywhere within the septum. Furthermore, all areas were more or less uniformly labeled (Figures 5 and 7), the range being restricted generally to between six and 15 grain counts per field. This is in contrast to day 17 (day of highest activity) where the range varies from 0.3 to 42.7 counts/field.

Day 21 (last gestational day) continued the same general trend seen in day 20. There was a further diminution in grain count (Figures 5 and 7). Again, no one area could be identified as one of marked chondrocytic proliferation. The inferior surface of the presphenoidal tail (sections I through L, Figure 7) might be called an active zone since counts were somewhat elevated there, but generally the grain count throughout the rest of the septum remained quite constant (Figures 5 and 7).

Discussion

As stated earlier, the hypothesis for this study was that one specific location might be identified within the nasal septum that was responsible for the growth of the entire structure. Based on previous work (Searls and Kinser, 1972), it was postulated that the septoethmoidal junction might be the site of this activity.

Instead, it was found that on prenatal day 16 (the first day in which the cartilaginous septal outline could be recognized) mitotic activity was limited almost exclusively to the *anterior* tip, a location just opposite to the one predicted. Twenty-four hours later there occurred a reversal of active zones. The tip became relatively quiescent and the middle and posterior regions began to demonstrate intensification of activity.

A similar pattern held true for day 18. The tip showed relatively few mitoses; there was generation of greater activity in the mid-portion, but the zone of highest activity was found in the presphenoidal tail, especially its inferior portion. This activity in the posterior septum was lost 24 hours later, the highest number of grain counts again being found within the center of the septum. By days 20 and 21, areas of high proliferation were insignificant or nonexistent.

It must be concluded from the foregoing that no single area can be designated as a primary growth site for the developing nasal septum. This still, however, does **not** rule out the possibility that the septum *as a whole* may act as a prime growth center for the midfacial skeleton. It merely points up the fact that the zones of cellular proliferation are not stationary and confined to one location as previously thought but are in a state of flux, shifting from one area to another with each twenty-four hour period of growth.

Whether the growth sites identified in this study create an active, thrusting force in orofacial development or are merely compensatory responses to midfacial movement of surrounding structures is a question which still remains unresolved.

Summary

Six pregnant female Sprague-Dawley rats were labeled with tritiated thymidine and killed, one each day, during the terminal six days of pregnancy. Nasal septal cartilages of five pups from each litter (a total of thirty pups in all) were monitored radioautographically for cellular proliferation.

It was found that chondrocytes were moderately active mitotically on gestational day 16, showing 12.1 grains per microscopic field. Activity increased sharply the following day (16.5 grains/field) then declined slightly on day 18 to a 16.1 count. There was a marked drop on both the 19th and 20th days (13.1 and 10.3 grains/field respectively) followed by a leveling off on the 21st day (9.3 grains/field).

No specific location in the septal cartilage could be identified as a growth center. The area of highest proliferative activity varied from day to day, being seen in the nasal tip on day 16, centered in the middle two-thirds of the cartilage twenty-four hours later, then moving caudally to the posterior one-half the following day (day 18). On day 19, a final shift back to the middle of the septum was observed. It was impossible to identify an active zone anywhere within the septum of either the 20- or the 21-day prenatal rat.

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