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A study of the regeneration of olfactory neuron populations in Rana pipiens

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A STUDY OF THE REGENERATION OF OLFACTORY NEURON POPULATIONS IN RANA PIPIENS

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A Study of the Regeneration of Olfactory Neuron Populations in *Rana pipiens*

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to my parents

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INTRODUCTION

Olfactory recognition is mediated by the olfactory epithelium, a 100-200μm thick pseudostratified columnar framework consisting of three types of cells: olfactory receptor cells, supporting epithelial (sustentacular) cells, and basal epithelial cells. The olfactory receptor cells are of interest, since they are true neurons whose axons project to the olfactory bulb and create synapses with the mitral, or tufted cells in the glomerular region (1). This property is distinct from the receptors for taste because these cells do not form axons and therefore do not directly connect with the central nervous system.

Olfactory receptor neurons are also unique in vertebrate physiology in that they represent one of the few constituents of the nervous system that can regenerate from a population of stem cells, as old cells are continually shed off into the nasal cavity. (Nottebohm and Goldman have recently observed that the song control nuclei in zebra finches also undergo neurogenesis during adulthood (2).) As early as 1940, Nagahara had proposed a "resting cell" in the olfactory epithelium, which could provide the source of new neurons when old ones degenerated following the section of the olfactory nerves in mice (3). In a review of regeneration, Takagi (1971) concluded with caution that there existed some evidence that primary olfactory neurons could regenerate (4). Authors cited included Andres (1965, 1966) who postulated a "blastema cell" in young animals that might produce new neurons even after birth. Experiments by Thornhill (1967) in the lamprey and Moulton (1971) in the mouse used autoradiography to demonstrate that tagged cells were present in the normal adult olfactory mucosa, and suggested that they could be

neurons (5, 6). In 1971, Graziadei and Metcalf published results showing that the basal cells of the neuroepithelium acted as the stem cells of the functional receptor neuron population (7). None of these studies, however, proved that receptor neurons regularly degenerated and were replaced.

In 1973, Graziadei performed important regeneration studies using *Rana pipiens* and *Rana catesbeiana* (8). With the help of autoradiography and electron microscopy, he showed that severing the olfactory nerve affects the sensory epithelium in the following ways: 1) it leads to a complete and accelerated rate of degeneration in all receptor cells; 2) it stimulates the basal cells to divide, demonstrated by thymidine uptake during the first 10 days postoperatively; and 3) it induces the differentiation of a large number of the dividing basal cells into a new population of functional receptor neurons. The time course of these changes was as follows: in the first three days following surgery, there were no significant organizational or morphological differences between the operated and unoperated sides of the animals. Starting on the fourth day after surgery, the epithelium showed progressive signs of degeneration, and by the end of the second week after surgery, all of the previously functional neurons had degenerated. The maturation of new receptors took around 4-6 weeks, and by the 60th day after surgery, Graziadei reported that the epithelium of the operated side "almost without exception resembles in all ways the epithelium of the unoperated side."

Further studies by Graziadei and DeHan (1973) demonstrated that the newly formed axons of the new receptor neurons actually crossed the surgical gap created by olfactory nerve axotomy in the frog and reestablished "normal" synaptic contacts with the glomeruli of the

olfactory bulb (9). Later work in mice showed that the presence of the olfactory bulbs was not even necessary for reconstitution (10). The new axons created after bulbectomy projected past their normal synaptic area into the anterior cerebral cortex and even formed "typical" glomerular structures, confirmed by immunohistology using antisera against olfactory protein. Ultrastructural examination showed a familiar glomerular pattern with synaptic contacts to unidentified cortical dendrites. These findings were supplemented later in transplant studies using *Xenopus laevis* (11). Stout and Graziadei surgically removed olfactory placodes from larvae at various stages of development and transplanted them onto the heads of host larvae. Their results showed the great plasticity of olfactory tissue and its unique ability to induce localized hyperplasia in the form of anatomically normal glomerular structures in regions of the central nervous system normally inappropriate for such activity.

Later Graziadei (1979) extended his examination of the stages of differentiation and maturation of the olfactory neurons with morphological and autoradiographic methods into mammals (12). These studies agreed with other animal systems showing a turnover of receptor cells, and suggested it "very likely that [olfactory neuron turnover] also occurs in man." During the same year Graziadei added to his morphological findings in mice with degeneration and reconstitution studies (13). In this work, he reports that following olfactory nerve axotomy, the appearance of new neurons in the epithelium begins after only eight days, and that after 30 days, the epithelia of the unoperated and operated sides appear identical to controls. On the basis of autoradiography these new neurons were found to arise entirely from the basal cells of the neuroepithelium.

The morphological and radiographic evidence for receptor neuron

turnover was combined and strengthened with electrophysiologic correlates of functional recovery after axotomy in the olfactory epithelium of the salamander (*Ambystoma tigrinum*) by Simmons and Getchell (14). In their work they found that: 1) evoked transepithelial voltage transients by several odor stimuli decreased in magnitude during the first 7 days following unilateral nerve section and could not be seen at 10 days post-surgery, suggesting a retrograde degeneration of sensory neurons. Unoperated sides remained normal. 2) Progressive recovery of voltage responses was demonstrated at several time points, showing the same characteristic wave form as controls. After 100 days, response magnitudes were again up to control levels. 3) Visual inspection showed an intact olfactory nerve with connections to the olfactory bulb region. Simmons and Getchell concluded that physiological recovery correlated with morphological studies, that functional neuronal elements in the olfactory epithelium underwent a gradual replacement process following olfactory nerve section. In addition, their work revealed that the physiological function of receptor cells preceded the actual synaptic connection of the cells with the olfactory bulb. This showed that the ability of the receptor cells to respond to odors was intrinsic to the epithelium itself, a result that can be compared with Graziadei's plasticity experiments (10, 11).

The precise location of the primary olfactory receptor site has been an intriguing and controversial question. Not until 1980 was there convincing evidence that the odorant recognition sites resided in the cilia of the olfactory neuron. In that year, Rhein and Cagan performed the first efficient preparation of isolated olfactory cilia, obtained from the fish olfactory organ using a "calcium shock" technique (15). Their binding

studies using [^{3}H] labeled odorant amino acids showed that the isolated cilia from olfactory rosettes of rainbow trout were functionally active. The ciliary polypeptides involved in binding, however, remained uncharacterized.

In work by Chen and Lancet using gel electrophoresis, olfactory cilia from the frog, *Rana ridibunda* , contained several specific proteins, as compared to respiratory cilia (16). Four of these polypeptides were glycosylated, whereas respiratory ciliary membranes had few, if any glycoproteins, suggesting that one or more of the glycopeptides was directly involved in the chemoreceptive process.

The most important goal of the biochemical work on olfaction has been the direct isolation of the active receptor proteins. Recently Pace and Lancet have made strides in this field by providing support for the notion that olfactory responses are mediated by a high-affinity GTP-binding protein similar in composition to that found in the visual, hormonal, and brain signal transducing systems (17). This protein seems to be involved in the activation of a highly active odorant-sensitive adenylate cyclase system, found in the cilia of vertebrate olfactory neurons.

In the search for understanding the remarkable specificity of the olfactory system for thousands of different scents, Fujita et al generated a library of mouse monoclonal antibodies against a homogenate of the rabbit olfactory bulb (18). In their work they obtained two types of monoclonals, one of which appeared to selectively label most olfactory nerves in the lateral portion of the olfactory epithelium and relatively few in the medial portion. This they took to demonstrate a "molecular heterogeneity among olfactory receptor cells, which may represent certain functional specificities."

Later studies by the same group used immunohistochemical techniques to show two subgroups of vomeronasal olfactory fibers as evidenced by selective staining by different monoclonals (19). These fibers in turn showed an anatomical projection specificity, one group projecting to the rostrolateral portion of the accessory olfactory bulb, the other to the caudomedial portion, though both groups of fibers were intertwined as they coursed along the vomeronasal nerve. These experiments introduced the potential that immunohistochemistry could have in sorting out the subclasses of receptor cells that could exist in the olfactory epithelium. For a current review of the function of vertebrate olfactory sensory cells, the reader is referred to the discussion by Lancet (20).

The chief methods of analysis employed previously to observe the changing surface of the epithelium as it evolves during regeneration have been autoradiography with [^{3}H] thymidine and electron microscopy. This study, however, will investigate the degeneration and renewal of the olfactory epithelium using the recently developed techniques of immunohistochemistry, by staining the olfactory epithelium with specific antibodies against marker proteins at several time points following unilateral olfactory nerve section. The histological results, as seen under fluorescence microscopy, will then be compared to the unoperated side to serve as controls.

METHODS

Animals

Nine healthy Northern leopard frogs (*Rana pipiens*) were obtained from Charles D. Sullivan of Nashville, TN. They were kept at room temperature (23° C) and fed meal worms twice a week. For all surgical procedures, the animals were anesthetized using a 10% solution of urethane by injection, and placed on an ice bath.

Surgical Procedures

The frogs were anesthetized and covered with ice, leaving the anterior portion of the head exposed. An incision was made midline, starting approximately between the eyes and extending anteriorly, avoiding the pineal gland, and ending around 2mm proximal to the opening of the external nares. A small hole was made in the skull just posterior to the cribiform plate, exposing the left olfactory nerve, which was completely transected, including the sheath, slightly distal to its bulbar attachment. The incision was then sutured together, and the animals allowed to recover from anesthesia, which generally took around 20 minutes. Following the operation, the activity level and feeding of the frogs appeared normal.

Tissue Isolation

The frogs were sacrificed over a period of time starting at 5 days after surgery and extending as follows: 10, 16, 20, 30, 36, 46, 60, and 90 days. Following decapitation, olfactory tissue was isolated from both sides of the animal and fixed initially in a solution of 4% paraformaldehyde in phosphate buffer solution (PBS), pH 7.6. This was followed by postfixation

in 2% DMSO in PBS, followed by embedding in OCT tissue medium in preparation for cryostat slicing.

Antibody Isolation

Antibodies were obtained by injecting white BALB/c mice with olfactory cilia isolated from frog olfactory epithelium. After allowing three weeks for immunologic induction, the mice were given a booster injection of the same preparation. Three days later, the mice were sacrificed and their spleens recovered. Antibody secreting cells in the mouse spleen then were fused with SP/0 cells (20). Hybrids were selected for in HAT (hypoxanthine, aminopterin, thymidine) medium, screened by immunofluorescence against 10μm sections of frog olfactory epithelium, subcloned, and eventually expanded in tissue culture. In this study, three specific antibodies were isolated, and shall be referred to as antibodies A, B, and J.

Antibody Staining

In preparation for frozen sectioning, glass slides were washed in 100% ethanol and treated with a solution of 0.5% chrom-alum and 0.5% gelatin. Following cryostat sectioning of the olfactory tissue in OCT onto the pretreated slides, the epithelium was first incubated for 40 minutes with a solution of 10% fetal calf serum (FCS) and 1% bovine serum albumin (BSA) in PBS to block nonspecific antibody binding. This was followed by incubation with a diluted antibody supernatant in Dulbecco's modified Eagle's medium containing 10% fetal calf serum for one hour, followed by three two minute rinses with PBS. Next, biotinylated goat anti-mouse antibody was diluted 1:100 in a 5% FCS, 0.5% BSA solution in PBS and placed on the slides for one hour with three subsequent rinsings of PBS. Then, FITC avidin was diluted 1:250 in a solution of 5% FCS, 0.5% BSA in

0.2M sodium carbonate, pH 8.6, and added to the samples for 10 minutes, followed by four 5 minute rinses of PBS. The slides were then dehydrated stepwise, first in 70% ethanol, then twice in 95% ethanol, twice in xylene, and finally fixed in permount for viewing under fluorescence microscopy.

RESULTS

In histological section of the normal frog olfactory epithelium (Figure 1) it is difficult to distinguish the different cell types. From ultrastructural studies, however, the nuclei of sustentacular cells occupy the uppermost stratum, those of the receptor cells the middle stratum, and the basal cells lie close to the basement membrane (7, 22). On the surface of the epithelium can be seen a tangled meshwork of cilia, the presumed sites of interaction between odiferous substances and receptor cells, and microvilli from the supporting epithelial cells. At the luminal surface, the plasma membranes of the sustentacular and receptor cells are bound by typical junctional complexes. Bowman's glands, found in the loose connective tissue vascular layer, produce a watery mucoid secretion which is thought to dissolve substances for recognition (7, 9).

The antibodies used in this study to monitor the process of reconstitution show different patterns of staining in control olfactory tissue. Antibody A appears to have high affinity for a protein found at the base of cilia as they emerge from the surface of the epithelium (Figure 2). This area is referred to, due to its appearance under electron microscopy, as the olfactory "knob." In addition, antibody A stains the rest of the luminal portion of the epithelium with a lower level affinity, giving rise to a more or less widespread surface fluorescence.

Antibody B stains cilia with a more diffuse pattern and appears to show a variable affinity for the olfactory knobs that is difficult to quantify due to the high level fluorescence of the cilia (Figure 3). This could suggest an affinity for a protein that is more diffuse in its

distribution along the length of the epithelium.

Lastly, antibody J has a very interesting and unique staining pattern that lights up olfactory knobs, as does A, but only a distinct subpopulation of them (Figure 4). Under electron microscopy using ferritin-conjugated antibody J, one can see the clear affinity for one knob, with no apparent recognition at all for the adjacent knob (Figure 5). Antibody J also does not appear to have as much affinity as antibody A for the rest of the surface epithelium.

At five days, the first time point examined following olfactory nerve section, the operated side of the epithelium has undergone some rather striking structural changes. Antibody A lights up only isolated knobs on the epithelium and shows less widespread binding than on control tissue (Figure 6). The receptor cilia on the luminal surface are reduced in number and the epithelium itself appears thinner.

Antibody B also seems to lose most of its binding sites after five days and gives a very similar picture to A, lighting up only very small discrete areas of tissue (Figure 7).

Antibody J, however, is most curious in its staining pattern. Whereas previously it showed a predilection for a subpopulation of olfactory knobs on control epithelia, at five days post-surgery, it demonstrates a more widespread activity, lighting up large regions of the surface olfactory epithelium (Figure 8).

At ten days after section, the degenerative process seems to be complete, and under H&E stain, the epithelium lacks cilia, and microvilli from the supporting cells are all that can be seen on the entire olfactory surface (Figure 9). The antibody staining of A and B at this time shows little fluorescence, while J continues to light up more diffuse areas of the

outer epithelium, although olfactory knobs are not evident.

The situation at 15, 20, 30, and 36 days shows little signs of change from 10 days. A and B continue to show little or no staining, and J still has an extended distribution of activity on the epithelium.

At 45 days after nerve section, the first signs of departure from the picture at five days become evident. Antibodies A and B start to light up small patches of epithelium, but J retains its diffuse fluorescence.

At 60 days, under H+E, the luminal surface no longer appears bare. Short and stubby-looking cilia join the microvilli of the supporting cells, and A and B increase their staining activity, while J seems to have decreased its somewhat. In addition, the thickness of the central portion of the epithelium appears to have increased, possibly reflecting a progressive return of the receptor cell population.

At 90 days, the epithelium still reveals the olfactory cilia to be far from fully returned, lacking the wafted floating appearance of cilia extending out from the surface of a control animal (Figure 10). Upon sacrificing the animal, the unoperated olfactory nerve still measures about twice as large in diameter as its reconstituted counterpart. On the other hand, the staining specificities of antibodies A, B, and even J, seem to have returned almost entirely back to what they were on a control animal. Antibody A again shows a wide distribution of activity on the surface epithelium, with highest affinity for the olfactory knobs (Figure 11); B has returned to its staining pattern of ciliary affinity (Figure 12); and J comes back with an affinity for selected olfactory knobs, as before (Figure 13). Clearly, though, the number of cilia and knobs is still considerably less than in an intact, unoperated epithelium.

In summary, then, the qualitative changes in the evolution of a new

receptor cell population demonstrate a cyclic process, consistent with a theory of basal cell renewal of receptor neurons.

DISCUSSION

This study was undertaken for the purpose of using immunohistochemistry to trace the regenerative phenomena of the vertebrate olfactory epithelium. Previous work in olfaction has only employed morphological evidence to measure regeneration. This study, however, follows the course of biochemical markers of differentiated vertebrate olfactory tissue in healthy live animals. Observations of the staining results from control epithelia and in epithelia from animals at various times following olfactory nerve section show the specificity of antibodies A, B, and J for certain proteins that change in quantity and distribution during reconstitution. Antibody A shows its highest staining activity in control tissue, especially around the olfactory knobs. Following nerve section, it gradually loses its affinity for the epithelial surface, falling to a minimum after around 10-15 days. Antibody B, with its affinity for olfactory cilia, likewise stains strongest in control tissue and decreases in overall staining after axotomy, falling to a minimum within 2 weeks. After 45 days, these antibodies slowly begin to regain fluorescent activity, and at the last time point observed, 90 days, the structures revealed by both of these antibodies are clearly present, though not at control levels. These antibodies, then, might correlate directly with the level of functional neuronal receptors in the epithelium, showing a maximum level of effectiveness both before surgery and after allowing sufficient time for a population of basal cells to mature into a functional set of receptor neurons.

In control tissue, antibody J displays a selectivity for a subpopulation

of neurons dispersed along the epithelium. From 5 to 60 days following nerve axotomy, though, J shows a heightened degree of affinity for significantly larger portions of tissue. If, as Graziadei has suggested, there exists "a dramatic regenerative response by basal cells when the population of receptor cells is artificially diminished," (7) then perhaps J recognizes a protein or epitope that is normally present in low levels in normal cycling olfactory epithelium, but that becomes expanded following a stimulatory event, such as the severance of the nerve. This protein, then, could represent an immature component of a receptor neuron which might normally be present in an epithelium that experiences an ongoing turnover of receptor cells. Following the "artificial" destruction of the entire functional neuronal population, though, this protein would be present in higher concentration. If J does recognize a kind of immaturity marker protein, it will be a useful probe for measuring the strength of a given replacement response by a basal cell group.

Immunohistochemistry, then, could be a useful tool for tracing the reconstitution and normal development in the olfactory epithelium. Most observations made correlate well with previous regeneration studies, adding support for the notions 1) that there exists a regular turnover of olfactory receptor neurons, with old neurons degenerating to be renewed by a continuously differentiating population of basal cells, and 2) that severing the olfactory nerve leads to a rapid degeneration of functional receptor neurons, causing the induction of proliferation and differentiation by the basal cells.

The time course of these events following axotomy in general agrees well with previous studies (7, 8, 13). Graziadei (1973) reported that degeneration of the functional neuron population was complete within two

weeks, and that new receptors in the epithelium took between 4 and 6 weeks to become evident. However, Graziadei also stated that after 60 days the epithelium of the operated side completely resembled that of the unoperated side. In our results, the thickness of the epithelium does not appear to return completely to control levels. Even after 90 days, the nerve bundles on the operated side are visibly smaller than their unoperated counterparts. This data, then, does not suggest a total reconstitution of the olfactory epithelium to receptor cell levels present preoperatively, within the time frame examined.

Work is now being performed in this laboratory to study the effect of ambient temperature upon the regeneration process. Results have shown that a warmer environment leads to greatly increased rates of tissue renewal (23).

In summary, immunohistochemistry has been utilized in an attempt to better understand the events involved in neuron regeneration in the olfactory epithelium of *R. Pipiens*. This technique may be useful in revealing some of the secrets involved in epithelial renewal and could potentially yield valuable knowledge in our search for better comprehending the vertebrate nervous system.

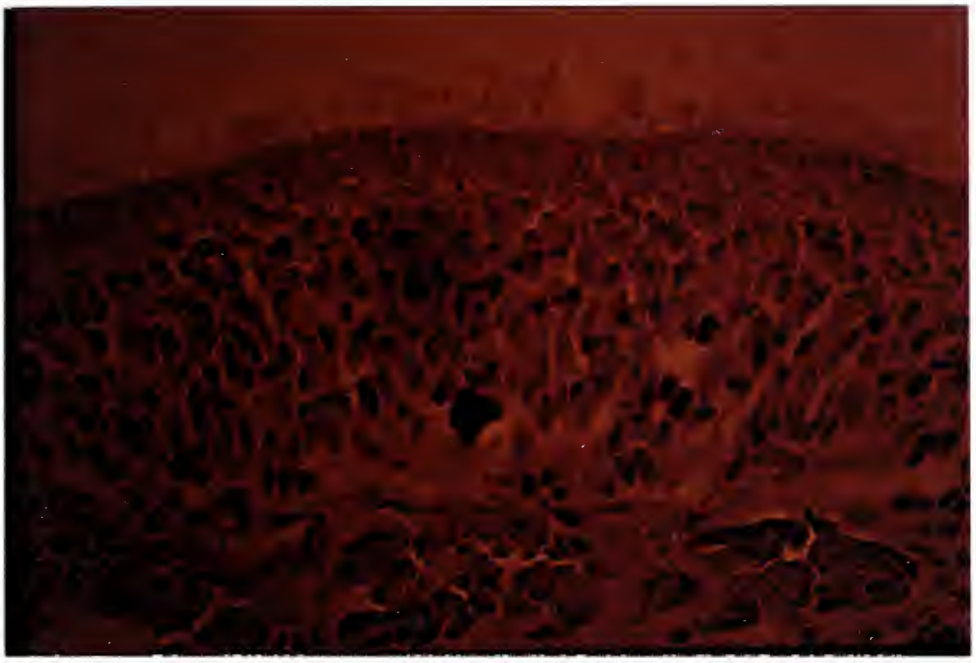

Figure 1: control *R. Pipiens* epithelium, H&E stain, 40X magnification, light microscopy.

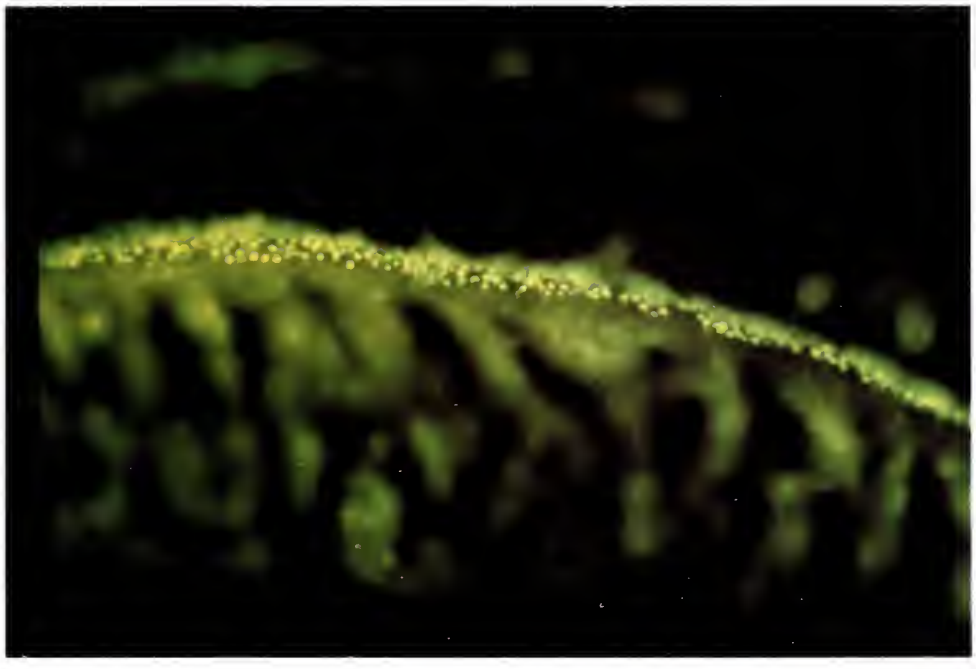

Figure 2: control *R. Pipiens* epithelium, stained with Antibody A, 40X magnification under oil, fluorescent microscopy.

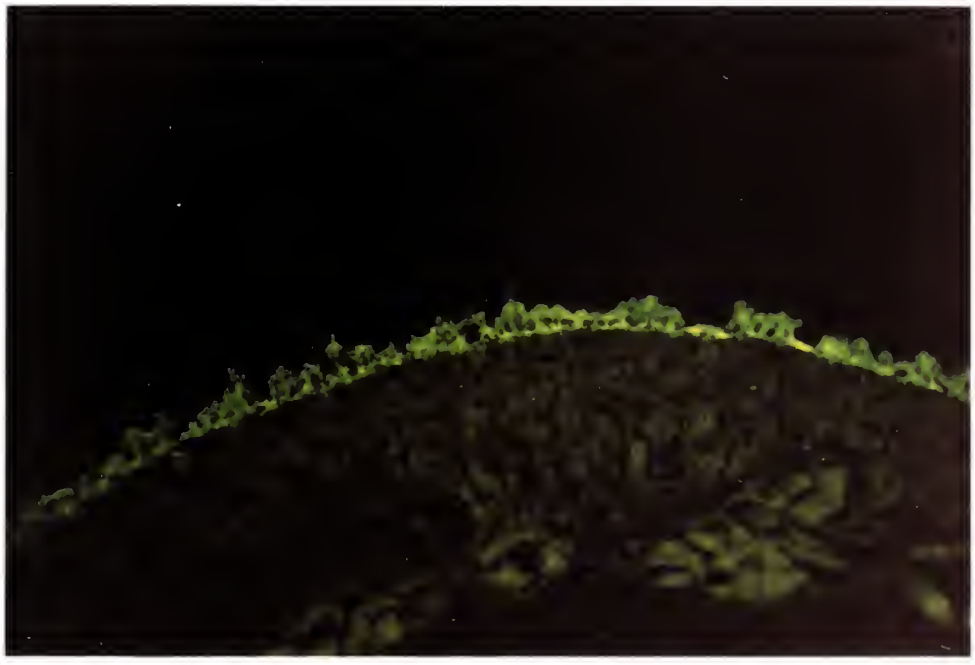

Figure 3: control *R. Pipiens* epithelium, stained with Antibody B, 20X magnification, fluorescent microscopy.

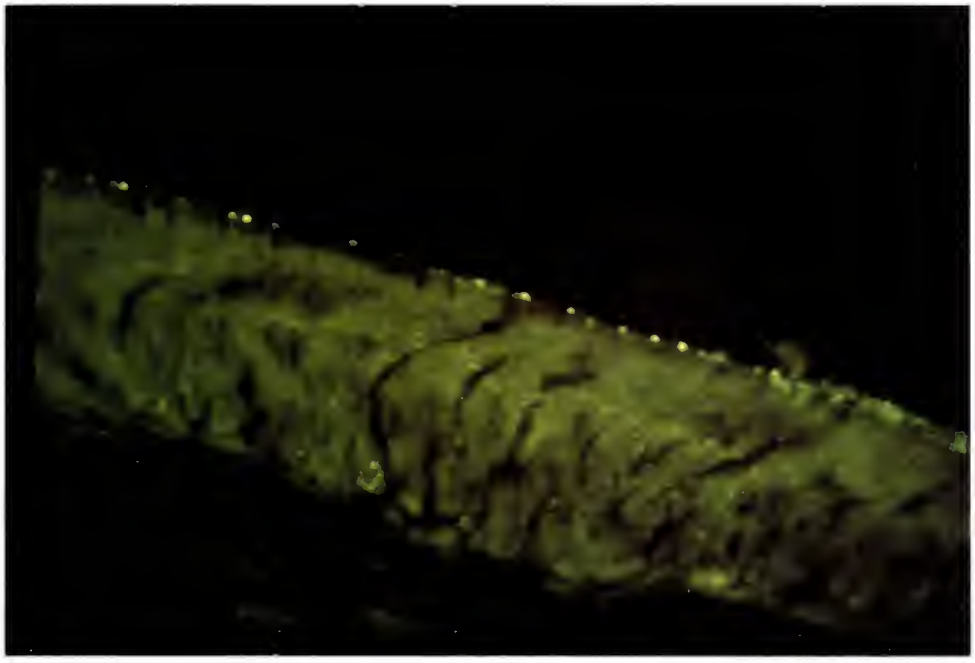

Figure 4: control *R. Pipiens* epithelium, stained with Antibody J, 40X magnification under oil, fluorescent microscopy.

Figure 5: control *R. Pipiens* epithelium, stained with ferritin-conjugated antibody J, electron microscopy.

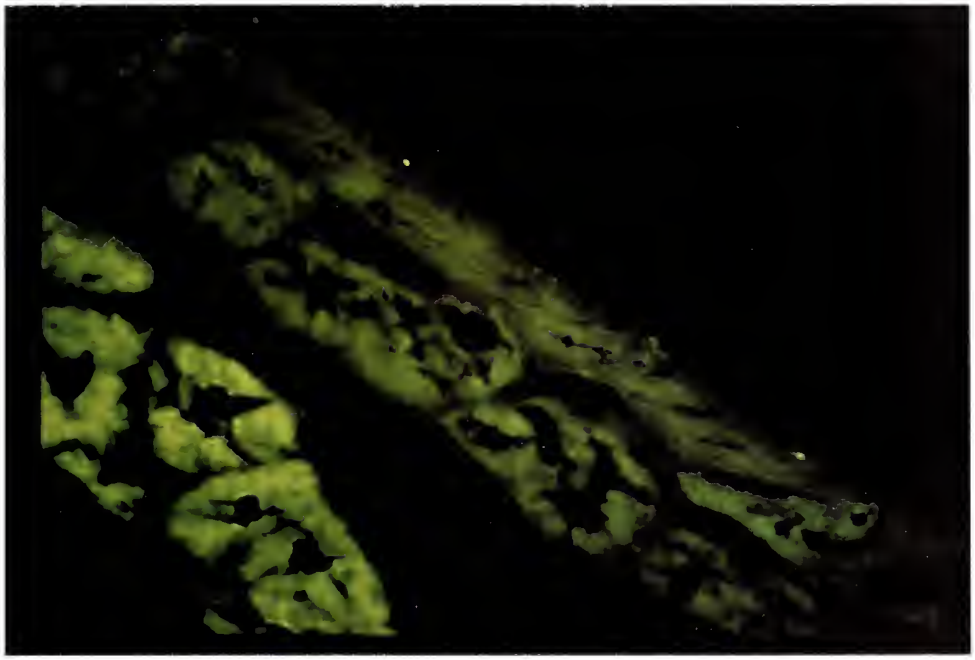

Figure 6: 5 day operated *R. Pipiens* epithelium, stained with Antibody A, 20X magnification, fluorescent microscopy.

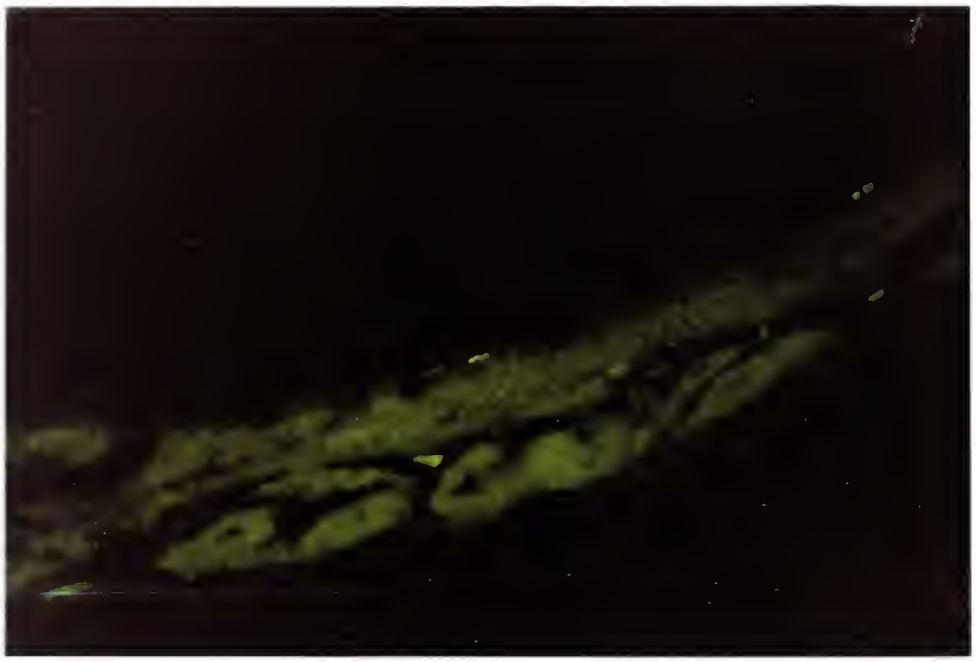

Figure 7: 5 day operated *R. Pipiens* epithelium, stained with Antibody B, 20X magnification, fluorescent microscopy.

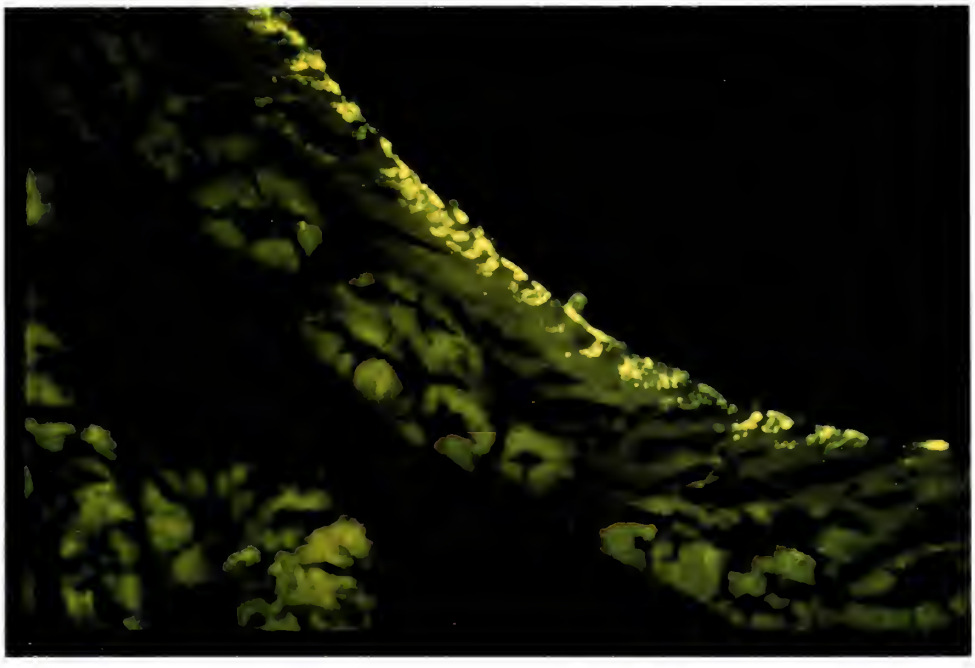

Figure 8: 5 day operated *R. Pipiens* epithelium, stained with Antibody J, 20X magnification, fluorescent microscopy.

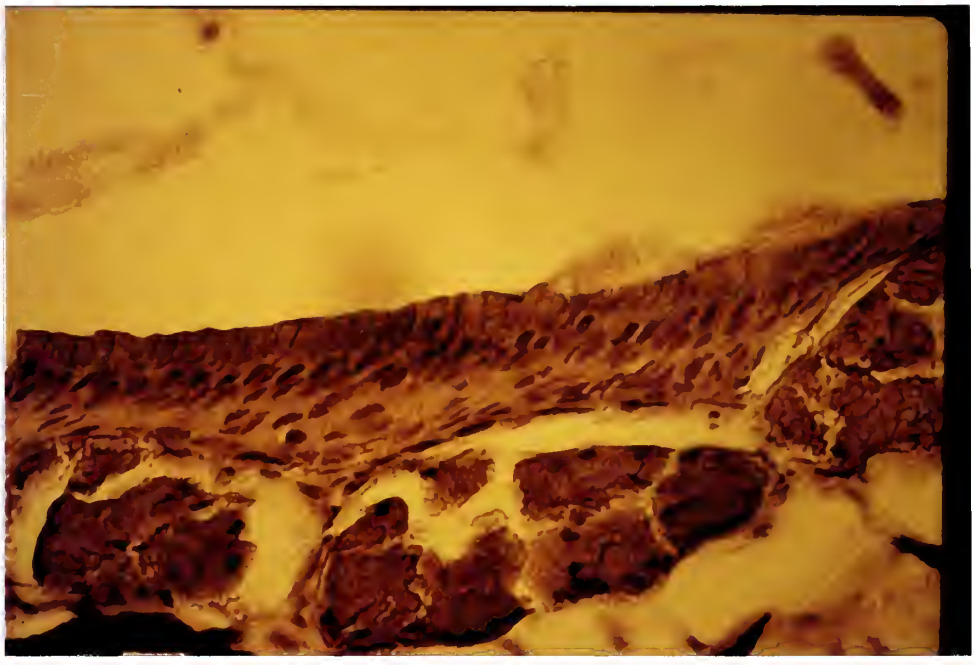

Figure 9: 10 day operated *R. Pipiens* epithelium, H&E stain, 40X magnification, light microscopy.

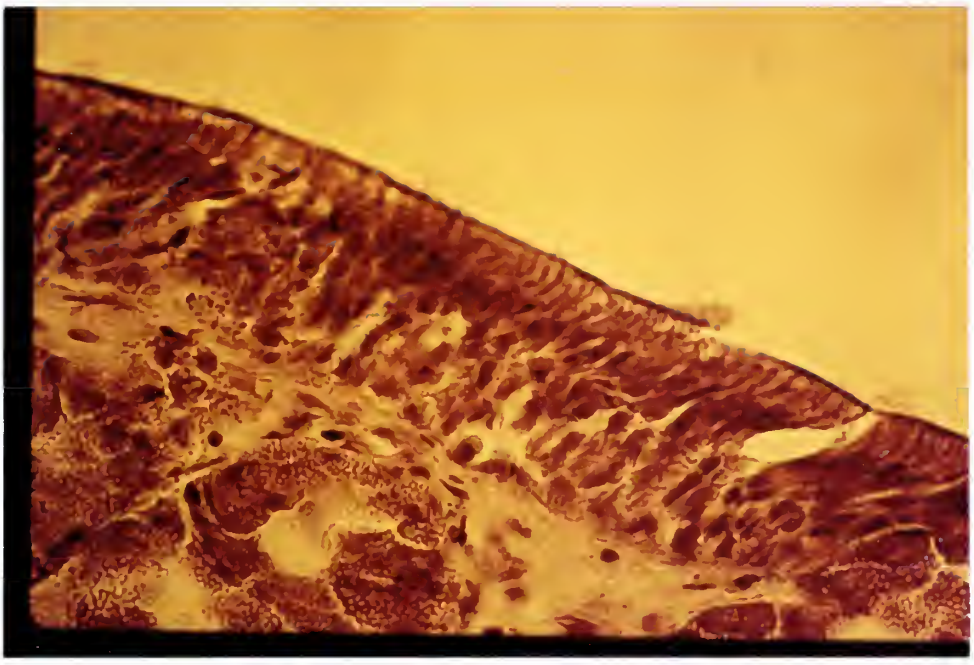

Figure 10: 90 day operated *R. Pipiens* epithelium, H&E stain, 40X magnification, light microscopy.

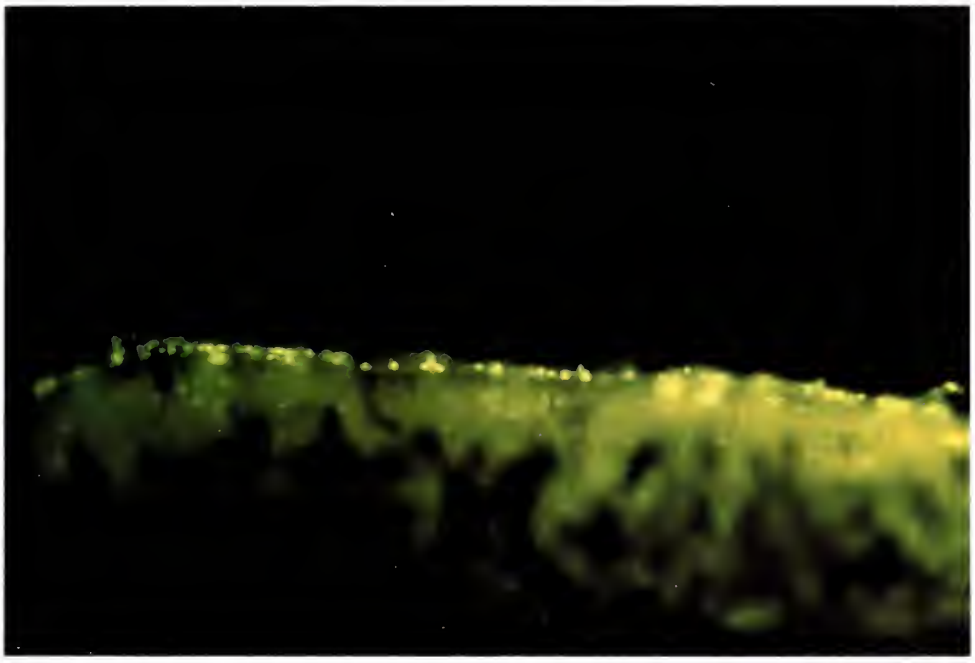

Figure 11: 90 day operated *R. Pipiens* epithelium, stained with Antibody A, 40X magnification under oil, fluorescent microscopy.

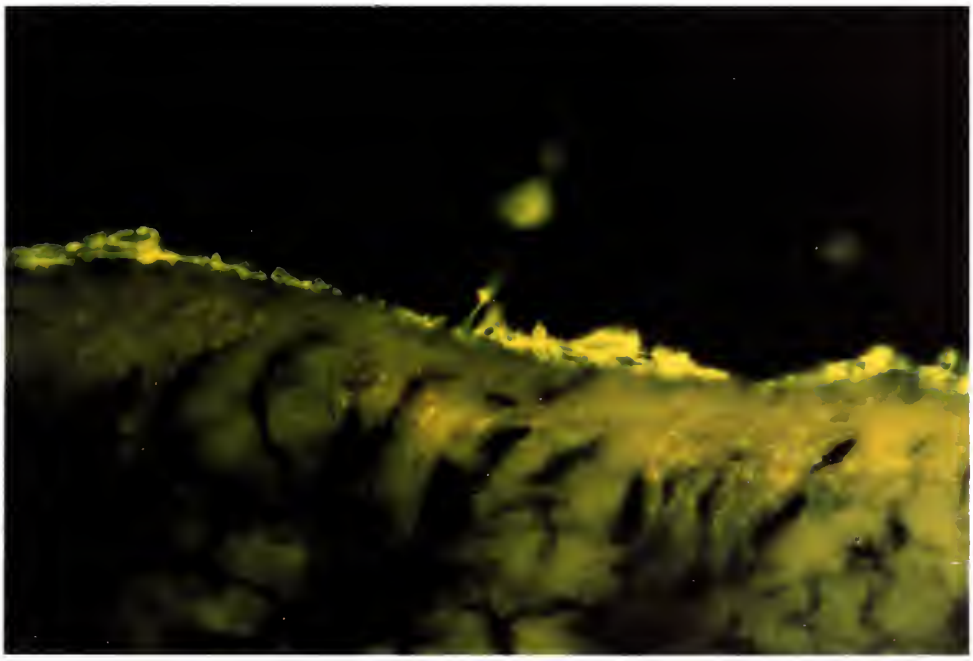

Figure 12: 90 day operated *R. Pipiens* epithelium, stained with Antibody B, 40X magnification under oil, fluorescent microscopy.

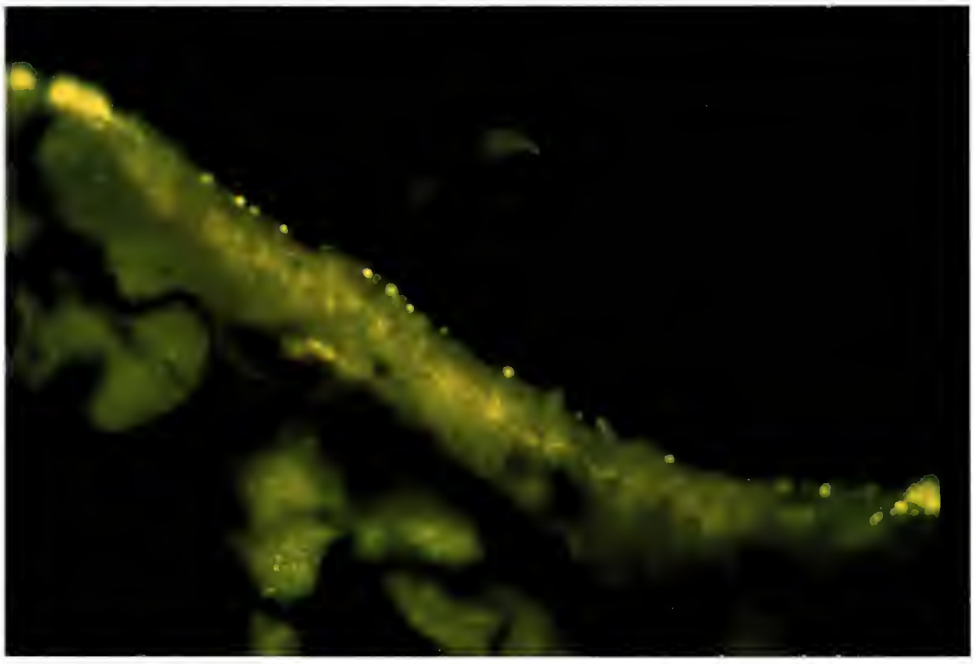

Figure 13: 90 day operated *R. Pipiens* epithelium, stained with Antibody J, 40X magnification under oil, fluorescent microscopy.

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