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PSEUDOBLASTEMA GROWTH DURING WOUND HEALING IN THE LEECH *HIRUDO MEDICINALIS*

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RESUM

S'estudia el mecanisme d'increment cel·lular durant el desenvolupament del pseudoblastema, tap cel·lular que es forma durant el procés de regeneració de ferides en els hirudinis. A *Hirudo medicinalis,* el pseudoblastema està format per cèl·lules vasocentrals provinents del teixit vasofibrós. La detecció de proliferació cel·lular per incorporació de BrdU mostra la capacitat de dividir-se de les cèl·lules vasocentrals en espècimens immadurs. Els espècimens madurs tenen una gran dificultat per incorporar BrdU i no se n'han obtingut resultats. Les observacions histològiques mostren la importància de la migració cel·lular, l'empobriment en cèl·lules vasocentrals al teixit vasofibrós en ferides severes i el reemplaçament posterior, presumiblement per proliferació cel·lular, de les cèl·lules vasocentrals perdudes.

RESUMEN

Se estudia el mecanismo de incremento celular durante el desarrollo del pseudoblastema, un tapón celular que se forma durante el proceso de regeneración de heridas en los hirudíneos. En *Hirudo medicinalis*, el pseudoblastema está formado por células vasocentrales provenientes del tejido vasofibroso. La detección de proliferación celular por incorporación de BrdU muestra la capacidad de dividirse de las células vasocentrales en especímenes inmaduros. Los especímenes maduros tienen una gran dificultad para incorporar BrdU y no se han obtenido resultados. Las observaciones histológicas muestran la importancia de la migración celular, el empobrecimiento en células vasocentrales en el tejido vasofibroso en heridas severas y el posterior reemplazamiento, presumiblemente por proliferación celular, de las células vasocentrales perdidas.

ABSTRACT

How cells are provided during the development of the pseudoblastema, a cell plug formed during wound healing in Hirudinea, was studied. In *Hirudo medicinalis,* the pseudoblastema is formed by vasocentral cells arising from the vasofibrous tissue. Detection of cell proliferation by incorporation of BrdU shows the ability of vasocentral cells to proliferate in immature specimens. Mature specimens present great difficulties for incorporating BrdU so no data was obtained. Histological observations show the importance of cell migration in the pseudoblastema growth, the impoverishment in vasocentral cells of the vasofibrous tissue in long term severe wounds and the replacement, presumably by proliferation, of loose vasocentral cell in later stages.

Key word: Cell proliferation, *Hirudo medicinalis*, leech, pseudoblastema, vasocentral cell, wound healing.

INTRODUCTION

The origin of cellular regeneration is a central matter in wound healing. Two cell sources should be considered: cell growth and cell migration.

During leech wound healing, one of the first events is the formation of a cell plug at the wound site. This cell plug, named pseudoblastema, grows progressively during the first stages of the wound healing. Cells that clump to form the pseudoblastema are connective cells that migrate from the so called "vasofibrous tissue", a thread-like network of fibrils located mainly in the peridigestive region, consisting of pigmented vasofibrous cells surrounding small groups of clear vasocentral cells (Huguet and Molinas, 1994).

Since no data is available concerning cell proliferation in cell plugs in Hirudinea, our purpose was the study of the mechanisms of the pseudoblastema growth. In Polychaeta and Oligochaeta the cell plug formed in the wound site, with no regenerating activity, is later replaced by other cells forming the blastema that show a high degree of cell proliferation (Burke, 1974; Chapron, 1964; Douglas, 1970; Jamieson, 1981). The absence of cell division in the initial cell plug that precedes the blastema is reported in Polychaeta (Douglas, 1970). In Hirudinea cell proliferation has been detected in the regenerating epidermis of *Helobdella stagnalis* (Cornec, 1990), but not in the cell plug. In previous works (Huguet and Molinas, 1988, 1992, 1994) no mitotic figures have been detected in healing tissues of *Hirudo medicinalis,* even following a colchicine treatment (unpublished data).

Timidine analogs, respectively 3H timidine and 5-bromo 2'deoxiuridine, have been widely used to study mitotic activity. However, the incorporation of nucleotide analogs is apparently difficult in freshwater invertebrates as reported by Baguñà *et al.* (1989) in planarians and Cornec (1990) in the leech *Helobdella stagnalis*. We discuss the usefulness of 5-bromo 2'deoxiuridine in Hirudinea and provide detailed descriptions of protocols used.

MATERIALS AND METHODS

Living material

Specimens of *Hirudo medicinalis* L. from lagoons in Alt Empordà (Girona, Spain) and one-month-old specimens kindly provided by Biopharm (U.K.) were used. They were acclimatized to laboratory conditions before being used for experiments. The temperature of the water in the aquariums containing the leeches was about 18 °C. In these conditions some adult specimens produced fertile cocoons from which young leeches were obtained.

To perform this work, the animals were anaesthetized with 8 % ethanol for 15 minutes and disinfected superficially with ethanol. Two different types of wounds were performed, incision wounds and NO_3Ag impregnations. Incisions were made on the ventral surface of the leeches, between annuli 65 to 85, affecting epidermal, dermal and muscle layers of the body wall. NO_3Ag impregnations were made with a cotton moistened with a saturated solution of NO_3Ag applied during two seconds on the same body region. Recovery took place in 2% sodium p-toluol sulfocloramide

(chlorine) solution to avoid infection.

Series of NO_3Ag wounds were performed and the animals were killed 10 min., 4 hours, 24 hours, 48 hours, 6 days, 8 days, 15 days, 30 days, 36 days and 63 days post-injury for conventional histological observations.

The exemplars used in the different proliferation detection tests were:

- Tests 1 to 5: series of mature exemplars with incision wounds, killed: 8 hours, 24 hours, 48 hours, 3 days, 4 days, 5 days, 7 days, 10 days, 14 days, 25 days and 60 days post-injury, for every test.
- Tests 6 and 7: immature exemplars born in our laboratory, about one month old and with a single feed consisting of frog blood, with 5 days incision wounds.
- Tests 8 and 9: immature exemplars about one month old provided by Biopharm, fed with mammalian blood. In test number 8, incision wounds were made and exemplars were killed 48 hours, 3 days, 7 days, 14 days and 25 days post injury. In the test number 9, NO_3Ag treatments were made and exemplars were killed 7 days and 14 days post injury.

In each test, two exemplars for each post-injury period were used. Controls with *Mus musculus* were also tested.

Methods

For conventional histological observations, the specimens were fixed in 4 % formaldehyde in phosphate buffer, pH 7.2. Samples were embedded in glycol-methacrylate (Bonet and Molinas, 1983) and stained with hematoxylin/eosin.

In the study of cell proliferation two different commercial kits were used: "Cell Proliferation RPN-20" (Amersham) and "5-Bromo-2'-deoxy-uridine Labeling and Detection Kit I" (Boehringer), both based on the same principle: the incorporation of BrdU in newly synthesized DNA and its posterior detection with antibodies. Simultaneously with primary antibody incubation a nuclease digestion of DNA was used to allow antibody access. The first kit used a peroxidase labeled secondary antibody and in the second one it was fluorescein labeled.

After anaesthesia, the specimens were injected with dilutions of BrdU (see table I) in the Ringer's solution for leeches: NaCl, 115 mM; $CaCl_2$, 1.8 mM; KCl, 4 mM; Tris maleate buffer, 10 mM (pH 7.4) (Sawyer, 1986). Depending on the size of the leech, 0.2-0.4 ml were injected into two different points of the body. Frequently during the injection the fluid flows into the gut. In every test, two exemplars were injected without BrdU.

In tests 3 to 9, besides injection, 0.6 mg BrdU / ml was added to the jar water (Table I).

After different periods of BrdU incubation (see table I) the specimens were killed with long term anaesthesia, and fixation subsequently took place. Tissues were processed for paraffin or criotom techniques and immunodetection was made (Table I).

Different strategies were used to optimize the results (Table I). To solve the problem of non-incorporation of BrdU, higher concentrations and longer periods of BrdU incubation were tested. Incubation with hydrogen peroxide 2 % in methanol just after deparaffination and rehidratation was tested in order to reduce endogen

peroxidase activity. To solve troubles with the binding of the primary antibody with BrdU, more accurate deparaffination, complementary DNA digestion with ClH 2 N for 30 minutes followed by neutralization with borate buffer 0,13 M (pH 8,5), and longer periods of antibody incubation were tested.

RESULTS

After incision wounds, cells from the vasofibrous tissue migrate to form a compact plug on the wound surface, the pseudoblastema (Figs. 1,2). In our experimental conditions, the first migratory cells are observed 10 minutes post-injury. One hour later, cells have reached the wound limit, and 4 hours post-injury, a developed pseudoblastema is present. The pseudoblastema shows a high growth rate until 48 hours wich subsequently decreases. Finally, between days 10 and 14 post-injury, the pseudoblastema begins to disintegrate and 5 weeks post-injury the disintegration is complete.

In NO_3Ag treatments, argentic salts penetrate into the body's wall tissues affecting the epidermis, the dermis and the external layers of the musculature (Fig. 3). The wound affects a very large area, about 2-3 X 4-4.5 mm. About hour 24, vasocentral cells accumulate just below the impregnated tissues, and later (days 3-5) all the impregnated tissue mass splits out, leaving a surface covered by the pseudoblastema (Fig. 4). After day 15, little growth of the pseudoblastema can be observed, although abundant migratory vasocentral cells are found in the pseudoblastema vicinity. The pseudoblastema is present during long periods, and disintegration begins after 30 days.

Cell proliferation

Of all the experiments tested only test number 6 gives positive results in leech tissues. Controls with tongue and intestinal tissues of *Mus musculus* are positive as expected. The bacteria usually found in leech nephridia are labeled in all the different tests as well. Inespecific labeling with primary antibody is observed in vasofibrous cell granules, inclusively without BrdU incubation.

No BrdU labeling is observed in regenerating tissues or in the neighboring tissues of adult specimens. In tissues with active proliferation expected, i.e. intestinal epithelium or germinal line, no labeling is either observed.

In an immature specimen, corresponding to test number 6, with an incision wound of 5 days, labeling is observed. Labeling is located in intestinal epithelial cells, vasofibrous tissue of the intestinal vicinity, pseudoblastema cells, and vasocentral cells near the pseudoblastema (Figs. 5 to 8). Nearly all the intestinal epithelial cells are positive, but no labeling is detected in intestinal caecums (Fig. 5 and 6). Concerning the vasofibrous tissue, we cannot distinguish whether the labeling is in the vasofibrous cells or in the vasocentral cells. The pseudoblastema presents labeled cells in the basal zone (Fig. 7).

The other exemplar of test number 6 died 24 hours before the planned sacrifice and subsequent fixation. Although tissues were poorly conserved, labeling can be observed in nuclei distributed in the connective tissue.

Vasofibrous tissue during the regeneration process

In the incision wounds, no changes have been observed in the vasofibrous tissue, but they are clearly detectable in the NO_3Ag treatments. Those changes can be clearly observed in the vasofibrous tissue located around the nerve cord (Figs. 9 to 12).

In the stages previous to tissue splitting, vasocentral cells separate from the vasofibrous tissue and migrate to the affected area. Migration becomes progressively more important (Fig. 10). A short time after the beginning of migration and during the first stages of the regeneration process (4 hours to 6 days), the vasofibrous tissue of the wound vicinity becomes poor in vasocentral cells. Later, between 8 and 36 days, an increment of vasocentral cells in the vasofibrous tissue can be observed, even over normal levels, especially around day 15 (Fig. 11). Once the pseudoblastema has been disintegrated (63 days), vasofibrous tissue shows a normal aspect (Fig. 12).

DISCUSSION

During the pseudoblastema evolution, growth can be observed, mainly during the first stages. This growth may be only due to migration of vasocentral cells arising from the vasofibrous tissue or, besides this mobilization, to proliferation of vasocentral cells.

The proliferation detection experiments are not conclusive. Like other invertebrates (Baguñà et al., 1989; Cornec, 1990), *Hirudo medicinalis* seems to present a notable difficulty for incorporating nucleotide analogs.

We postulate that the maturation level is important in the label incorporation process, since only immature specimens with a single feed consisting of frog blood do incorporate BrdU. On the other hand, one-month-old specimens fed with mammalian blood do not show any labeling. Even if they are as old as the specimens of the anterior group, they exhibit a later maturation level, possibly due to their more complete feeding.

Labeling in the intestinal epithelium may be due to the accessibility of BrdU, which usually flows into the gut during injection. The fact that only the vasofibrous tissue located near the intestine incorporates BrdU may be due to the same reason.

The presence of labeled cells in the pseudoblastema does not necessary indicate that cells proliferate in the pseudoblastema. They could be labeled in their original tissues (vasofibrous tissue) and subsequently migrate to the wounded area. Two facts point out that, in this specimen, BrdU incorporation took place in the perintestinal vasofibrous tissue. First, only the basal zone of the pseudoblastema (where presumably migrating cells are added) contains labeled cells, and, second, the presence of migrating labeled vasocentral cells near the pseudoblastema. This does not reject the possibility that pseudoblastema cells do proliferate. Experiments allowing accessibility of BrdU to the pseudoblastema together with short incubation periods may solve this question.

Despite the capability of vasocentral cells to proliferate, the continuous affluence of vasocentral cells to the pseudoblastema during the pseudoblastema growth induces us to think that migration is the principal mechanism of growth.

After NO_3Ag treatments, in which there is a massive and lasting migration, vasofibrous tissue becomes severely poor in vasocentral cells. The increment of vasocentral cells observed in the subsequent stages may be attributed to a putative proliferation process occurred to satisfy the claim of vasocentral cells during the long regeneration periods.

In conclusion, besides migration, proliferation may occur during the evolution process of the pseudoblastema. Despite the fact that our experiments are not conclusive, they show that vasocentral cells have proliferative ability during wound healing at least in immature exemplars, and probably, as NO_3Ag wound healing observations indicate, in mature exemplars as well.

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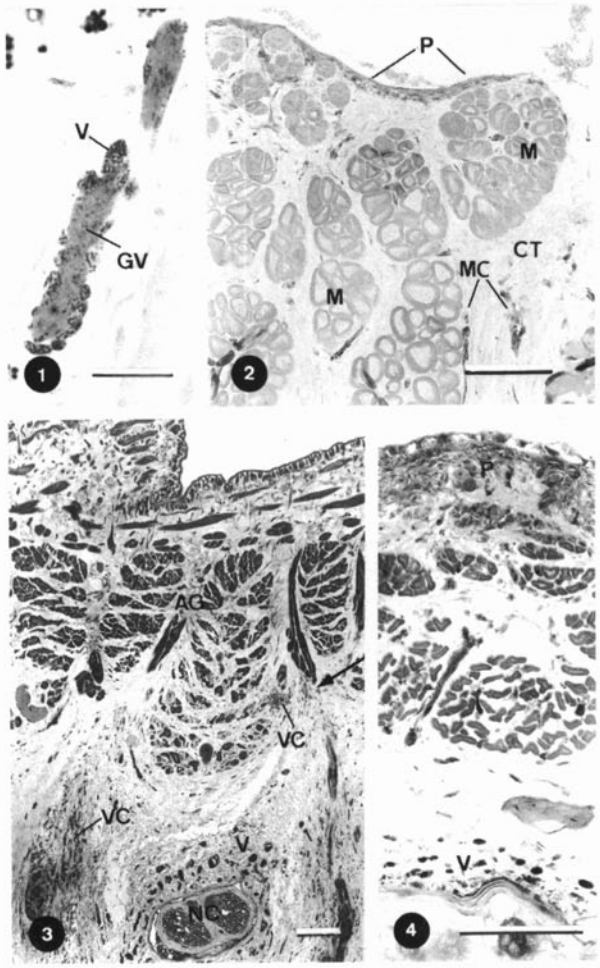
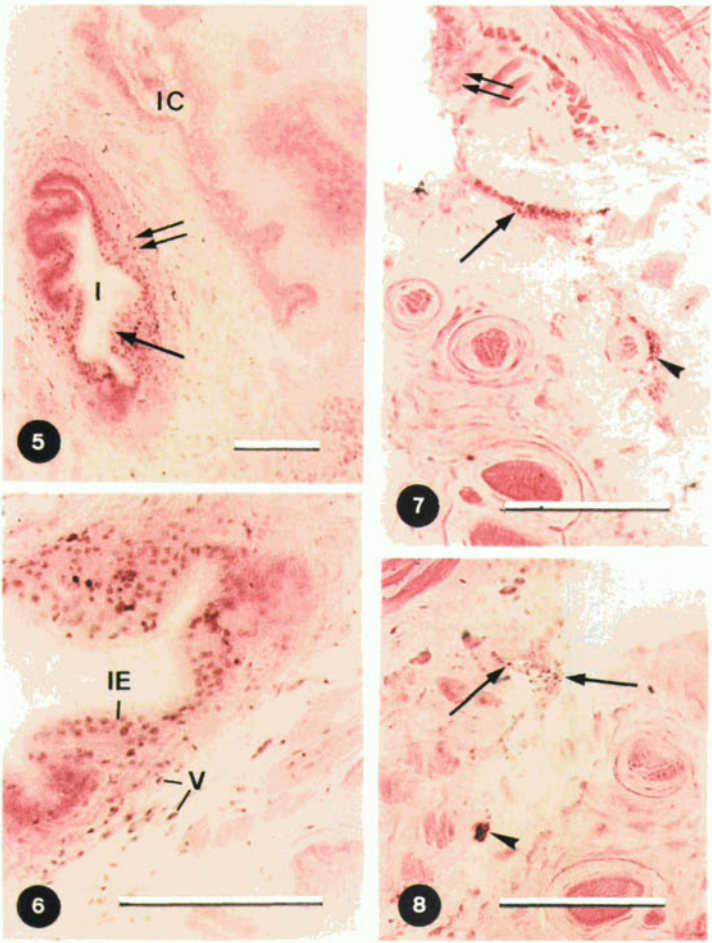


Fig. 1. Cluster of vasocentral cells surrounded by vasofibrous cell projections. Hematoxylin/eosin stained. GV, group of vasocentral cells; V, vasofibrous cell. Scale bar, 10 μm.

Fig. 2. Incision wound about 4 h post-injury. Vasocentral cells have reached the wound edges and the pseudoblastema is formed. Hematoxylin/eosin stained. CT, connective tissue; M, musculature; MC, migratory vasocentral cells; P, pseudoblastema. Scale bar, 100 μm.

Fig. 3. $N0_3Ag$ treatment 24 h post-injury. Argentic precipitates are seen in the body wall and vasocentral cells accumulate just below the impregnated zone (arrow). Hematoxylin/eosin stained. AG, argentic precipitates; NC, nerve cord. VC, vasocentral cell; V, vasofobrous tissue. Scale bar, 100 μm.

Fig. 4. 6 days post-injury the impregnated tissues had been spliced and the pseudoblastema recovers the external surface. Hematoxylin/eosin stained. P, pseudoblastema; V, vasofibrous tissue. Scale bar, 100 μm.



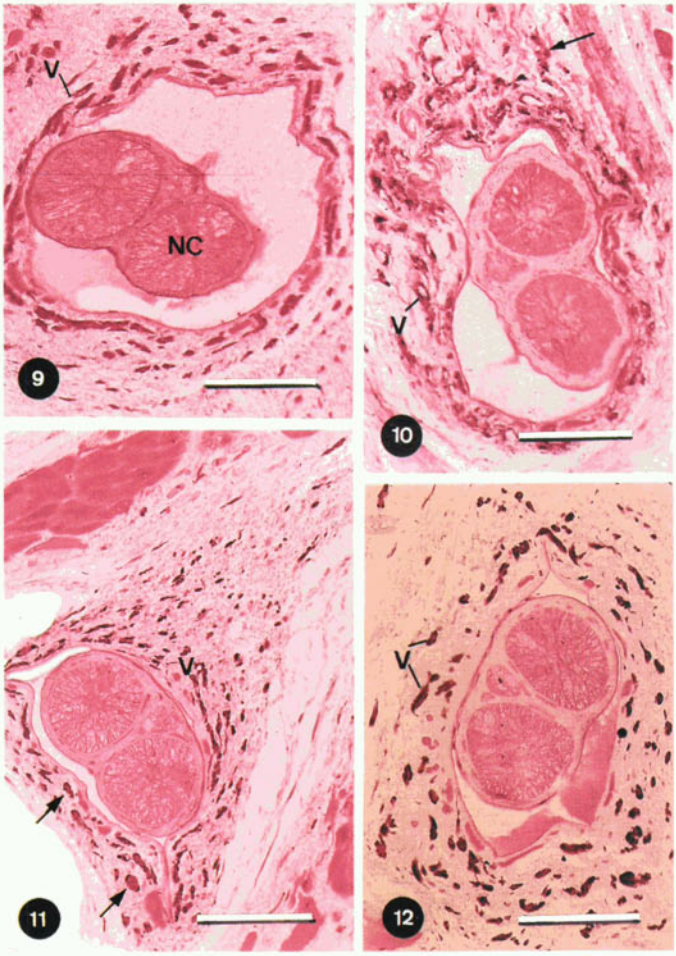
Figs. 5 to 8. Specimen with a incision wound 5 days post-injury incubated with BrdU. The presence of labeled nuclei is detected by immunohistochemistry with the presence of a brown precipitate.

Fig. 5. Peridigestive area. Nuclei of intestinal epithelium (arrow) and vasofibrous tissue of the intestinal vicinity (double arrow) are labeled. Hematoxylin/eosin stained. I, intestine; IC, intestinal caecum. Scale bar, 100 μm.

Fig. 6. Detail of the previous image. Hematoxylin/eosin stained. IE, intestinal epithelium; V, vasofibrous tissue. Scale bar, 100 μm.

Fig. 7. Pseudoblastema 5 days post-injury. Some pseudoblastema cells are labeled (arrow), mainly in the basal zone. In the upper zone of the pseudoblastema no labeling is observed (double arrow). Some labeled vasocentral cells are in the pseudoblastema vicinity (arrow point). Hematoxylin/eosin stained. Scale bar, 100 μm.

Fig. 8. Pseudoblastema 5 days post-injury. Pseudoblastema labeled cells (arrow) and labeled vasocentral cells in the pseudoblastema vicinity (arrow point). Hematoxylin/eosin stained. Scale bar, 100 μm.



Figs. 9 to 12. Vasofibrous tissue located around the nerve cord in different specimens during N0$_3$Ag treatments.

Fig. 9. Uninjured exemplar with normal vasofibrous tissue. Hematoxylin/eosin stained. NC, nerve cord; V, vasofibrous tissue. Scale bar, 100 μm.

Fig. 10. 4 h post-injury. Vasocentral cells detach from the vasofibrous tissue and initiate migration (arrow). Vasofibrous tissue appears vacuolated due to the looseness of vasocentral cells. Hematoxylin/eosin stained. V, vasofibrous tissue. Scale bar, 100 μm.

Fig. 11. 15 days post-injury. An increment of vasocentral cells in the vasofibrous tissue can be observed. Vasocentral cells form outstanding clusters in the interior of the vasofibrous fibers (arrows). Hematoxylin/eosin stained. V, vasofibrous tissue. Scale bar, 100 μm.

Fig. 12. Two months post-injury, when the pseudoblastema has been disintegrated, the vasofibrous tissue shows a normal aspect. Hematoxylin/eosin stained. V, vasofibrous tissue. Scale bar, 100 μm.

TABLE I. Cell proliferation tests using BrdU incorporation

BrdU INCORPORATION					HISTOLOGICAL PROCED.		IMMUNOHISTOCHEMICAL DETECTION			
	[BrdU] 100g corporal weigth	stock sol BrdU/ringer	BrdU/ml jar water	BrdU incubation	fixation	inclusion	H_2O_2	C1H	primary antibody	secondary antibody
Test nº 1	6 mg	4%	–	1h 20'	formol 10% PBS 0,1 M 3h	paraffin pF 56º	–	–	1h 30'	30'
							30'	–	1h 30'	30'
					formol 10% Sörensen phosphate buffer 3h	paraffin pF 56º	–	–	1h 30'	30'
							30'	–	1h 30'	30'
Test nº 2	12 mg	8%	–	18 h	formol 10% Sörensen phosphate buffer 4h	Medium plast pF 52º	1 h	–	16h	1h
								+	2h 30'	1h
								+	20h	1h
Test nº 3	67,5 mg	30%	0,6 mg	24 h	formol 10% Sörensen phosphate buffer 4h	Medium plast pF 52º	1 h	–	21h	1h
								+	3h	1h
								+	20h	1h
Test nº 4	225 mg	100%	0,6 mg	20 h	formol 10% Sörensen ph. buf. 4h	Medium plast pF 52º	1 h	+	20h	1h

(Continues)

TABLE I. Cell proliferation tests using BrdU incorporation (continuation)

	BrdU INCORPORATION				HISTOLOGICAL PROCED.		IMMUNOHISTOCHEMICAL DETECTION			
	[BrdU] 100g pes corporal	stock sol BrdU/ringer	BrdU/ml jar water	BrdU incubation	fixation	inclusion	H_2O_2	C1H	primary antibody	secondary antibody
Test nº 5	225 mg	100%	0,6 mg	48 h	acetone 20' acid ethanol 30'	criotomy	1h	+	20 h	1 h
Test nº 6	225 mg	100%	0,6 mg	48 h	acetone 20' acid ethanol 30'	criotomy	1h	+	20 h	1 h
Test nº 7	–	–	0,6 mg	48 h	acetone 20' acid ethanol 30'	criotomy	1h	+	20 h	1 h
Test nº 8	225 mg	100%	0,6 mg	48 h	acetone 20' acid ethanol 30'	criotomy	–	+	20 h	1 h
Test nº 9	225 mg	100%	0,6 mg	48 h	acetone 20' acid ethanol 30'	criotomy	–	+	20 h	1 h
mouse	6 mg	100%	–	24 h	formol 10% Sörensen ph. buf. 4h	Medium plast pF 52º	1h	–	3 h	1 h

In tests 1 to 7 and *Mus musculus* controls Amersham kit was used, and in tests 8 and 9 Boheringer kit was used.