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Planarian cell culture: a comparative review of methods and an improved protocol for primary cultures of neoblasts

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ABSTRACT. To develop an improved method for preparing and cultivating planarian cells, several protocols published previously were compared with each other. Cells, and in particular neoblasts, proved remarkably resistant to hyposmotic conditions. However, survival periods depended critically on the content in nutrients and on osmotic conditions. Starting from an optimized method to disintegrate planarian tissues and prepare purified neoblast fractions, different media and additives were tried. Hyposmotic media and layers of extracellular matrix components enhanced the adhesion of neoblasts and favoured the formation of transient processes. Proteins in the medium supported long-term survival of neoblasts that retained a spherical shape. Eventually, an isosmotic medium was devised that supported the survival of neoblasts with a viability of 46% on day 31 of primary cultures. With light microscopical techniques, no signs of differentiation were observed in these cultures. Mitoses were detected until the second day of cultivation. In contrast, cultures of total cells still displayed mitoses after 7 days of cultivation. Some guidelines are proposed for future research directed towards establishing permanent neoblast lines.

KEY WORDS: cell culture, culture media, extracellular matrix, neoblasts, osmotic effects, planarians, primary cultures, *Schmidtea (Dugesia) polychroa*, Platyhelminthes.

INTRODUCTION

The first attempt at planarian cell culture to be published originated during an initial boom in modern regeneration science and was undertaken in Ch. M. Child´s laboratory in Chicago (MURRAY, 1927). No report of any permanent platyhelminth cell line has been published up till now. The importance of the only proliferative cell type, the neoblast, in regeneration of turbellarians – especially in connection with the molecular data reported in recent times (for reviews, see BAGUÑÀ, 1998; BAGUÑÀ et al., 1994) and the experimental potential a permanent neoblast line would offer – should stimulate intensification of the efforts to establish such a cell line.

When devising planarian cell culture media, authors started from solutions used for amphibians (BETCHAKU, 1967), embryonic chick tissue (MURRAY, 1927), mammals (BETCHAKU, 1967; FRANQUINET, 1973) or a combination of recipes for vertebrate and snail cells (TESHIROGI & TOHYA, 1988). The importance of nutrients and osmotic conditions was realized early (MURRAY, 1927). Relatively later, analytical data from planarians have been used as a basis for deriving culture media. TESHIROGI & TOHYA (1988) added amino acids to the medium in the concentrations determined from planarian extracts. To guarantee isosmotic conditions, osmotic values of planarian tissues were taken into consideration by SCHÜRMANN & PETER (1993). A survey of the great variety of media is given in Table 1. The exact composition of the extracellular environment is, however, still unknown. Preparing an extract of the extracellular matrix that is free from any contamination originating from damaged cells is extremely difficult because of the parenchymal structure of triclad turbellarians. This fact contrasts sharply with the ease of obtaining and analyzing body fluids from vertebrates resulting, in the end, in an elaborate inventory of culture media and conditions.

The techniques to isolate cells from planarians may be grouped into a few categories. As it is not possible to dissect a triclad completely into its organs and neoblasts are widely scattered throughout the parenchyma, whole animals or defined body regions of them have to be the source

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for any cell preparation. Tiny fragments were obtained by simple cutting and kept either in tissue culture medium (FRANQUINET, 1973; SEILERN-ASPANG, 1960, among others) or cultivated under conditions leading to the accumulation of certain cell types, primarily neoblasts (BETCHAKU, 1967, 1970). To dissociate cells from planarian tissue, BETCHAKU (1967) chose moderately hyposmotic conditions (80 mOsmol/l, compared to 125-128 mOsmol/l for planarian tissues). Selective adhesion to glass and resistance to the osmotic conditions led to the accumulation of neoblasts within 1-2 days in this case. FRANQUINET (1981) introduced the dissociation of planarian tissues into single cells by disintegration in a Dounce homogenizer. To study DNA and RNA synthesis and other processes, cell suspensions were cultivated for up to 7 days without separating or enriching any cell type. In order to prepare large quantities of neoblasts within a reasonable time, we have combined the disintegration in a Dounce homogenizer first with preferential adhesion to glass and plastic dishes and prolonged survival of neoblasts under hyposmotic conditions (SCHÜRMANN & PETER, 1988) and later with a fractionation of cells by centrifugation in a Percoll density-gradient (SCHÜRMANN & PETER, 1993; SCHÜRMANN et al., 1998).

MATERIAL AND METHODS

Cells were prepared from *Schmidtea (Dugesia) polychroa* (Schmidt 1862). The laboratory cultures were originally derived from a population living in the river Amper (Bavaria, Germany) and kept in the dark at 19 ºC. The karyotype corresponded to the triplohexaploid "biotype B" (BENAZZI & BENAZZI LENTATI, 1976). The planarians were fed with tubificid worms at intervals of 7 days, starved a week and kept in 0.02% neomycin sulfate in culture water for at least 24 h prior to disintegration. Artificial pond water (MC CONNELL, 1967) was used for the planarian cultures. The reagents were purchased from Sigma, with a few exceptions cited in the following text. Cell culture grade was chosen, where available. To test their reproducibility and gain experience for developing an improved method, several protocols of previous publications were tried: those of BETCHAKU (1967), FRANQUINET (1981) in the original and in a modified form (SCHÜRMANN et al., 1998), FRANQUINET et al. (1985) and TESHIROGI & TOHYA (1988).

Osmolarities were determined by freezing point depression of media or planarian extracts with an Advanced Laboratory wide-range osmometer, model 3W2. For this purpose, planarians were disintegrated in distilled water by ultrasound (Branson Sonifier, model 250) and the dilution was taken into account in the calculations.

The disintegration method used to obtain cell suspensions was the same as described previously for the isolation and fractionation of neoblasts (SCHÜRMANN et al., 1998). A modification of this method is presented in this volume (BEHENSKY et al., 2001). For the primary cell cultures, however, any enzyme addition was omitted. To reduce viscosity caused by mucus, only "Digest-Eur" from Eurobio, Paris, was added to give a final dilution of 1% of the "10X" stock solution. This corresponded to a concentration of 0.00063% 2,3-dihydroxy-1,4-dithiolbutane with a little physiological saline. The confusion that arose in this context from different publications originating from Franquinet´s laboratory will be discussed later. Digest-Eur was present during all purification, washing and centrifugation steps, but was not contained in any of the culture media. All manipulations, beginning from disintegration, were done under sterile precautions. A laminar flow bench was used where appropriate.

For total cell cultures, the initial cell density was $6x10^5$ cells per ml. Neoblasts with a purity of roughly 90% were collected from a discontinuous four-step gradient (SCHÜRMANN, 1993; SCHÜRMANN et al., 1998) at the density boundary 1.05/1.07 and cultivated under sterile conditions in different media at 18 °C, in the dark except during the short observation and handling periods. Each Petri dish made of polystyrene (Greiner, Kremsmünster), with a diameter of 35 mm, was filled with 1 ml of a suspension containing $8x10^5$ cells. After 1 h, the cells had sedimented and the cultures were filled up with 1 ml of fresh medium. Media were changed every third day. A cooled incubator (Heraeus Cytoperm 8088, with 95% air + 5% CO_2) was used only for the cultures with the medium devised by TESHIROGI & TOHYA (1988). The cultures were viewed and photographed through an inverted microscope with phase contrast optics (Reichert Biovert). Control preparations stained with azure A – eosin B (PEDERSEN, 1959) confirmed the diagnosis of neoblasts based on their nucleocytoplasmic ratio observed with phase contrast illumination. To test viability, the stain exclusion method with nigrosin (50 µg/ml) was used (KALTENBACH et al., 1958). In several cases, the fluorescent LIVE/DEAD viability/cytotoxicity kit (L-7013) from Molecular Probes was applied (BELETSKY & UMANSKY, 1990; POOT et al., 1997). To determine mitotic indices, colcemide (100 or 60 µg/ml culture medium) was added and the arrested metaphases were stained with DAPI (Hoechst) and counted after 5 or 4 h.

Eventually, an isosmotic medium (Table 1) was developed, starting from the recipe given by TESHIROGI & TOHYA (1988). The main modifications consisted in adding the biological buffer Hepes and its sodium salt instead of $NaHCO_3$ and CO_2, and in the replacement of the amino acid mix by commercially available stock solutions. The calcium concentration was chosen to meet the requirement of 1 mM for maintaining RNA and DNA synthesis (MARTELLY, 1984). This medium had an osmolarity of 126 mOsmol/l and a pH of 7.40. One litre was prepared from 7,208.8 mg Hepes (free acid), 3,514.1 mg Hepes (sodium salt), 985.4 mg NaCl, 800.1 mg $NaHCO_3$, 26.4 mg KCl, 150.0 mg $CaCl_2.2H_2O$, 90.1 mg $MgSO_4.7H_2O$, 0.3 mg $MnCl_2.4H_2O$, 68.5 mg KH_2PO_4, 150.0 mg sodium pyruvate, 300.0 mg D-glucose, 50.0 mg D-trehalose, 49.0 mg L-glutamine, 0.3 mg d-biotin, 100.0 mg neomycin sulfate, 2.5 mg tricine, 2.0 mg phenol red, 10.00 ml BMS (Biochrom KG, Berlin), 2.00 ml MEM essential amino

acid solution (50x), 5.00 ml MEM non-essential amino acid solution (100x) and 3.00 ml MEM vitamine solution (100x). These components were dissolved in highly pure sterile water (double distilled quality) from a Millipore plant to give a final volume of 1 l. BMS is a standardized supplement containing compounds from fetal calf serum and bovine serum albumin; 1 l contains 15.00 g of protein. The MEM additives were obtained from GIBCO-BRL.

RESULTS

Reproducibility of methods published previously

BETCHAKU´S (1967) original method resulted in the accumulation of neoblasts he described. Both glass and polystyrene dishes were applicable. Cells adhered better to glass. For a successful isolation of neoblasts, it was essential that the tiny tissue fragments adhered to the bottom of the dish. Frequent gentle shaking increased the yield of neoblasts. When the method described by FRANQUINET (1981) was reproduced in strict compliance with the published protocol, cells could not be cultivated successfully and showed fragmented nuclei after one day. This was evidently due to the presence of the cationic detergent benzalkonium chloride. Viability tests (LIVE / DEAD test) with cells filtered through meshes down to 15 µm gauze showed no viable cells upon exposure to 1% benzalkonium for 1.5 h, and far less than 1% viable cells when the concentration was 0.033%. Cilia of epithelial cells ceased to beat at the latter concentration (SCHÜRMANN, 1993). As discussed below, Digest-Eur has evidently been taken erroneously for benzalkonium, resulting in a misleading description of the method. With benzalkonium chloride in the medium, neoblasts adhered readily to the culture dish and were firmly attached after 20 min. However, this might at best serve for enriching these cells immediately before fixation. Also Digest-Eur favours adhesion of neoblasts, although to a lesser extent. After 45 min, this cell type adheres preferentially to glass and plastic surfaces.

Influence of different culture media and matrices on the behaviour of neoblasts

Neoblasts isolated and purified by the new combination of methods mentioned above (BEHENSKY et al., 2001; SCHÜRMANN et al., 1998) adopted a spherical shape within a few hours from isolation (Fig. 1). Cells varied in size, depending on the osmotic values, with diameters from 11.0-13.2 µm (mean: 12.2 µm) in a hypotonic medium (TESHIROGI & TOHYA, 1988), from 10.3-13.0 µm (mean: 11.7 µm) in the newly devised isosmotic medium with 126 mOsmol/l (see above under materials and methods) and from 9.4-12.0 µm (mean: 10.7 µm) under strongly hyperosmotic conditions of 345 mOsmol/l (FRANQUINET, 1981). The cytoplasmic rim around the nucleus was, on the average, from 0.65-0.85µm wide, depending on the osmotic conditions. Cells mostly died on the first day in the hyperosmotic medium, but could be cultivated for weeks in the other media. Neoblasts adhered to glass or polystyrene dishes to a percentage of 80-90% two hours after seeding. Adhesion was far less firm than for mammalian cells and than for the neoblasts isolated after BETCHAKU (1967). The primary and secondary processes described by this author (Fig. 2) were observed within

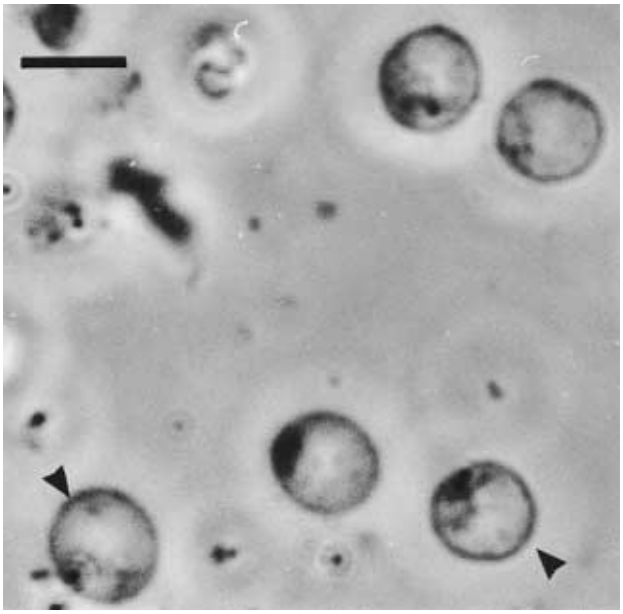

Fig. 1. – Neoblasts in hyposmotic saline (5/8-concentrated Holtfreter´s solution, 80 mOsmol/l, after BETCHAKU, 1967), cultivated for 7 h. Phase contrast optics, inverted microscope. The dark cytoplasmic rims (arrowheads) are clearly visible. Bar: 10µm.

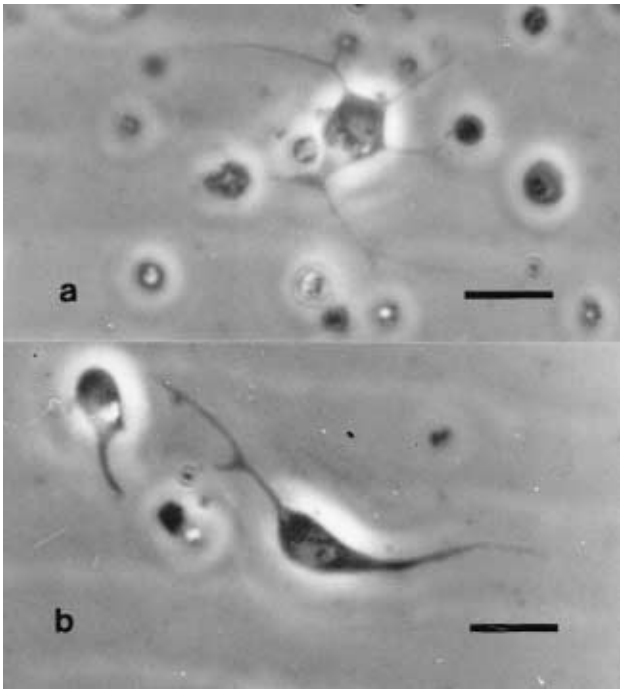

Fig. 2. – Neoblasts with processes formed in hypotonic medium (the same as for Fig. 1) in 46 h old culture: a) without protein, b) with 3% BMS in medium. Optics as for Fig.1. Bars: 20 µm.

24 h in roughly 10% of the cells under hypotonic conditions and in about 5% in the isosmotic medium. Multiple processes were observed. Lowering the osmotic value resulted in longer processes. All these were not permanent, but degenerated gradually from 48-72 h from the start of cultivation. Layers of collagen I from the rat favoured the formation of processes in the isosmotic medium (Fig. 3). In the hypotonic medium "DHM" with 80 mOsmol/l (see Table 1 and BETCHAKU, 1967), processes from 40-70 µm in length could be found on collagen layers.

The majority of cells retained the spherical shape. Attachment was enhanced up to 95% of the cells when the surface of the Petri dish was coated with extracellular

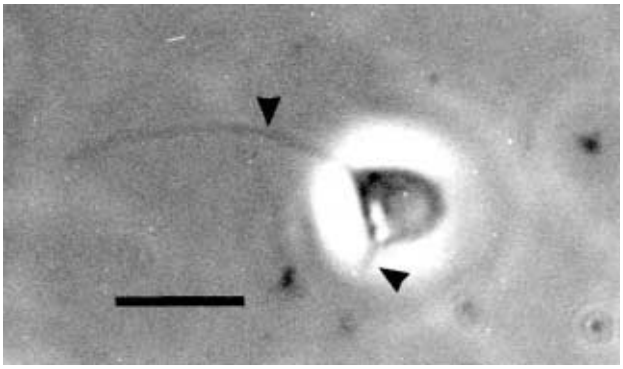

Fig. 3. – Neoblast cultivated in the new isosmotic medium on a layer of collagen I from rat tail for 26 h, with processes (arrowheads). Optics as for Fig. 1. Bar: 20 µm.

TABLE 1

Comparison of selected planarian cell culture media, with concentrations of major constituents

Medium:	**MPM**	**DHM**	**BPM**	**FMM**	**FM73**	**FM81**	**FM85**	**SPM**	**TTP**	**IPM**
mOsmol/l	30-150*)	80	69*)	160	175*)	345	155	139	85	126
pH	–	7.3	7.3	8.0	7.2-7.3	7.25	7.25	7.25	7.4-7.6	7.40
Inorganic ions [mM/l]										
Na^+	11.6-15.5	38.93	32.50	65.90	14.00	140.44	53.37	61.23	41.34	42.39
K^+	.22 -.30	.42	.34	2.13	6.71	10.50	7.40	7.22	.35	.89
Mg^{++}	+	–	–	1.07	.65	1.59	.70	1.05	.37	.38
Ca^{++}	.15-.20	.57	.45	.47	2.72	4.66	1.00	.42	.48	1.03
HCO_3^-	.20-.26	1.49	1.19	–	2.02	18.84	7.20	17.89	9.52	9.63
$H_2PO_4^- + HPO_4^{--}$	–	–	–	.62	–	.97	.48	.62	–	.51
SO_4^{--}	–	–	–	.67	.65	1.59	.70	1.05	.37	.37
Cl^-	12-16	39.00	31.20	67.85	24.13	139.96	55.00	50.33	33.13	20.34
Buffers [mM/l]:										
Hepes, anion + free acid	–	–	–	–	–	8.34	20.00	8.34	–	43.75
Tricine	–	–	–	30.00	–	–	–	–	.014	–
CO_2: 5% in atmosphere	–	–	–	–	–	–	–	–	+	–
Nutrients [mM/l]:										
Acetate	–	–	–	–	–	.34	–	.34	–	–
Pyruvate	–	–	1.36	.40	–	–	–	–	–	1.36
L-Glutamine	–	–	–	–	–	1.33	–	.34	.33	.34
D- Glucose	5.5-55.5	–	1.66	1.40	110.99	5.55	10.00	1.85	1.66	1.83
D- Trehalose	–	–	–	–	–	–	–	–	.15	.15
Amino acids	–	–	+	+	–	+	+	+	+	+
Vitamins	–	–	+	+	–	+	+	+	+	+
Antibiotics	–	–	N	PS	P	PS	PS	PS	–	N
Mammalian serum	0-25%	–	.3%	+/-	5%	–	–	–	1%	–
BMS	–	–	–	–	–	–	–	–	–	1%

Comments and references:

Solute compositions of culture media have been calculated from the original recipes. The constituents (ions, nutrients, vitamins etc.) added with the sera are not included in the concentrations listed. Osmolarities and pH values were taken from the original descriptions or measured, with three exceptions (*) that were estimated by calculations from the solute concentrations. The pH indicator phenol red is contained in all media except MPM, DHM and BPM. All media except MPM are prepared with distilled water.

+ indicates the presence, - the absence of a constituent.

N: neomycin sulfate, P: penicillin, S: streptomycin.

MPM: Murray´s planarian medium (MURRAY, 1927). Pretreated well water served as the solvent, with minor components and trace elements (Al^{+++}, Fe^{+++}, Mg^{++}, Mn^{++} and NO_3^-, among others). A wide range of concentrations and different variants, including the addition of some amino acids and peptone (1g/l), were tried. Dilutions without sheep serum had estimated osmolarities up to 88 mOsmol/l.

DHM: Dilute Holtfreter´s medium (5/8 of the original concentration for amphibian tissues), introduced by BETCHAKU (1967).

BPM: Betchaku´s planarian medium (BETCHAKU, 1967). Minor constituents: individual mixture of amino acids, vitamins and choline.

FMM: Fukushima´s and Matsuda´s medium (FUKUSHIMA & MATSUDA, 1991). Different modifications were tried. The composition optimized for maximal leucine incorporation is given in the table. This medium is prepared from the mammalian F–12K medium, that contains a cocktail of vitamins and amino acids, nucleosides, nucleotides, lipoic acid and minor amounts of Cu^{++}, Fe^{++} and Zn^{++}. Various additives, among them vertebrate sera and planarian extracts, were tested.

FM73: FRANQUINET´s (1973) medium. In addition to the constituents listed above, this medium contains hydrolyzed lactalbumin (500 mg/l) and yeast extract (250 mg/l) as sources for amino acids and vitamins.

FM81: FRANQUINET´s (1981) medium (see also FRANQUINET & MARTELLY, 1981) prepared from Gibco´s medium 199 with Hank´s salts (1/3) and Eagle´s MEM (2/3), including a combination of amino acids and vitamins. See text for the problems with this medium. In addition to the components listed above, a trace amount of Fe^{++}, polyvinylpyrrolidone (500 mg/l) and Tween 80 (66.67 mg/l) are present.

FM85: Franquinet´s medium 85 (FRANQUINET et al., 1985) contains polyvinylpyrrolidone (12 µM/l) in addition to the compounds listed above. Alternatively, calcium concentrations of 1 nM, 10 µM and 1mM per l were applied.

SPM: Schürmann´s planarian medium (SCHÜRMANN et al., 1998), prepared from Gibco´s medium 199 and Eagle´s MEM similar to FM81, but only with the vitamins and amino acids and without the salts contained in the MEM. See text for details. Also polyvinylpyrrolidone (500 mg/l) and Tween 80 are contained in this medium.

TTP: Tohya´s and Teshirogi´s planarian medium (TOHYA & TESHIROGI, 1988). This is the only medium requiring a CO_2-incubator. An amino acid mixture resembling the amino acid composition of planarian extracts (but without arginine), a vitamin combination adapted from other media, choline chloride and Mn^{++} (1.5 µM/l) are contained in addition to the components listed in the table. The osmolarity was measured after the addition of fetal calf serum. The original paper states an osmolality of 80 mOsm.

IPM: Isotonic planarian medium is the new culture medium presented in this paper. In addition to the components given above, it contains Mn^{++} (1.5 µM/l), d-biotin (.30 mg/l) and vitamins plus amino acids from the concentrated solutions for Eagle´s MEM (Gibco). Basal medium supplement (BMS), a standardized serum derivative, is used instead of other sera. Based on the analysis provided by the supplier, its ionic and other major components have been included in the above concentration listing. Details for preparing this medium are given in the text.

matrix components: collagen I from the rat acted similarly to a homologous planarian matrix preparation (PASCOLINI et al., 1992) and to other matrix layers (SCHÜRMANN, 1993). Protein in solution enhanced survival and reduced the attachment of neoblasts. In media with 5-10% fetal calf serum, cells did not adhere, retained their typical neoblast appearance to a considerable extent and still formed monolayers even after 30 days (Fig. 4). Aggregates formed in Petri dishes coated with fibronectin from the rat and even more in cultures without matrix coatings when the calcium concentration was elevated from 1 mM to 2 mM. Such aggregates tended to detach from the substratum with increasing cultivation periods.

Viability and longevity of cells in different culture media

Osmolarities of 125-128 mOsmol/l were found for *Schmidtea polychroa*. PRUSCH (1976) reported 126 mOsmol/l for *Dugesia dorotocephala*. For devising the isosmotic medium, isotonicity was assumed between the cells and the extracellular space. 345 mOsmol/l were measured for FRANQUINET´s (1981) medium.

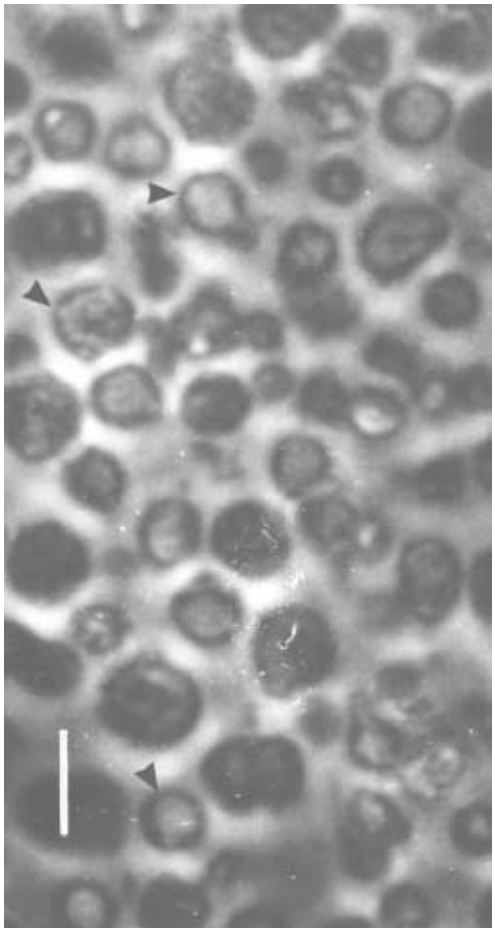

Fig. 4. – Neoblasts cultivated in the new isosmotic medium after 30 days in a Petri dish without matrix coating and with 3% BMS in the medium. Several cells show the characteristic narrow cytoplasmic rim (arrow heads), while others appear to be degenerating. Optics as for Fig. 1. Bar: 10 µm.

The initial viability of neoblasts differed little between the media tested in long term cultures. During extended cultivation periods, however, the new isosmotic medium proved superior and resulted in 46% of viable neoblasts after 31 days (Fig. 5). The cells cultivated in this medium

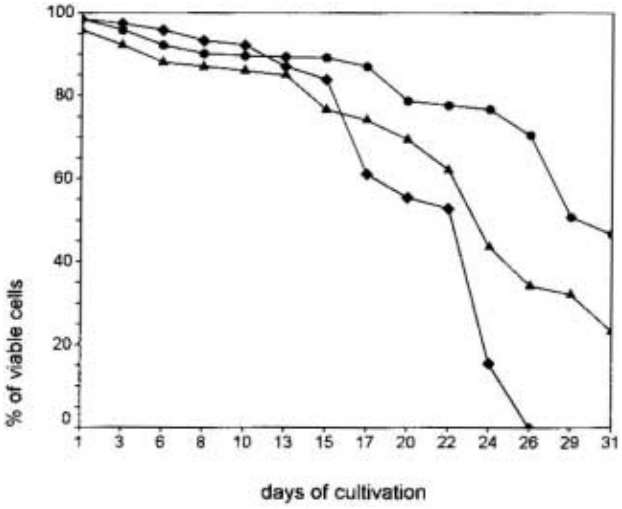

Fig. 5. – Viability of neoblast cultures from *Schmidtea polychroa* in different media, screened with the nigrosin stain exclusion test.
- ● Isosmotic medium (126 mOsmol/l) described in this paper
- ◆ Hyperosmotic medium: 154 mOsmol/l (FRANQUINET et al., 1985)
- ▲ Hyposmotic medium: 85 mOsmol/l (TESHIROGI & TOHYA, 1988)

showed no signs of differentiation when viewed through a light microscope. The same medium also supported the formation and survival of restitution bodies (PETER, 1995).The initially similar viabilities in media differing in composition were confirmed in a pilot count using the LIVE / DEAD kit to test neoblasts having been exposed for 1.5 h to the various media. The same percentage – 78% – of neoblasts was viable in a medium with 85 mOsmol/l (TESHIROGI & TOHYA, 1988) and in the new medium with 126 mOsmol/l. In contrast, hypertonic media as well as strongly hypotonic solutions heavily reduced viability: 10% Locke´s medium (30 mOsmol/l), recommended by MURRAY (1927) as an optimal medium, resulted in only 57% of viable cells. After one day, the majority of these cells had disintegrated, the culture dishes being covered with fragments and debris. The medium with 345 mOsmol/l (FRANQUINET, 1981) lowered viability only to 70%, but cells degenerated rapidly in this medium and could not be observed for more than two days. As will be discussed below, the cited osmotic value seems to result from an erroneous description in the published protocol.

In neoblast cultures with the isosmotic medium, mitoses could be observed during the first day of cultivation, with a fraction of 1.0% of the cells in metaphase. A slight increase to 1.2% was observed upon addition of 0.1 mM serotonin. When total cell suspensions were cultivated in a nearly isosmotic medium (139 mOsmol/l; see SCHÜRMANN et al., 1998) derived from the composition given by FRANQUINET (1981), metaphases were found at a rather constant rate slightly above or below 1% from the beginning of primary cultures until 7 days. This result confirms the original findings.

DISCUSSION

Planarian cells seem to be relatively resistant against hypotonic conditions. Survival seems, however, reduced in the respective media. The amitoses reported by MURRAY (1927) were obviously observed in dying cells. On the other hand, the same author reports good survival when nutrients such as 25% of horse serum were added. When estimating osmolarities, the lowest value is roughly 30 mOsmol/l for 1/10 LOCKE´s solution and 105-150 mOsmol/l for the medium containing serum. One difficulty in evaluating conditions and results in this and other older publications is that the establishment of hanging drop cultures, with a relatively high cell or tissue density in the media and generally without changes of the medium, implied restricted resources as well as poorly defined and hardly reproducible media compositions due to compounds diffusing into the solution from damaged tissues or cells.

Some irritation has arisen from the papers published by FRANQUINET and co-authors. The strongly hyperosmotic medium probably resulted from confusion of complete Eagle´s minimum essential medium (MEM) with the amino acids and vitamins contained in this medium. Eurobio Laboratories, Paris, have been the source for the culture media. Whereas MEM is listed in the catalogue in several variants, all with complete salts, amino acid and vitamin stock solutions for MEM are also offered separately. FRANQUINET (1981) states that the medium used previously (FRANQUINET, 1973, 1976) had been modified to yield the new recipe. This medium had an estimated osmolarity of about 175 mOsmol/l. If Hank´s medium 199 was diluted threefold and the amino acids and vitamins of MEM plus the additional salts listed separately by FRANQUINET (1981) were added, 139 mOsmol/l were measured (Table 1; see also SCHÜRMANN et al., 1998). The mitoses reported for 7 days in Franquinet´s paper and the related composition of a recipe published later (FRANQUINET et al., 1985), with an osmolarity of 155 mOsmol/l, favour the interpretation given above.

Digest-Eur has apparently been mistaken for benzalkonium chloride. In the catalogue issued by Eurobio, Digest-Eur is introduced as a mucolytic agent for clinical use in filtrating sputum, setting up bacterial cultures and, instead of trypsin, to detach cultivated cells from surfaces. It is described as a "concentrate 10X of 2,3-dihydroxy – 1,4-dithiolbutane for a rapid digestion and the fluidification of expectoration products" and delivered in 10 ml flasks. The chemical composition refers to dithiothreitol and / or

dithioerythritol, and the action is evidently based on the reduction of disulfide bridges formed by cysteine residues in glycoproteins. The kit offered contains, in addition, a solution of "Benzalkonium 1p. 3000" (=0.033%) in quantities of 100 or 500 ml to establish cultures of acid-resistant bacilles. In the pertinent papers, the term "benzalkonium" (FRANQUINET, 1981) changes to "benzalkonium (Digest-Eur, Eurobio)" in FRANQUINET & MARTELLY (1981) and later to "Digest-Eur" (FRANQUINET et al., 1985). The interpretation presented here is confirmed by the fact that the senior author discussed the discrepancies with Dr. Martelly, one of Franquinet´s co-workers, during a meeting in Graz in 1989. She remembered small vials as source for the "Digest-Eur" and somewhat later kindly sent a package of exactly those 10 ml vials with "10X Digest-Eur" that had been left over from the former experiments. FUKUSHIMA & MATSUDA (1991) report, indeed, correctly on the highly toxic effect of benzalkonium chloride. But apparently neither benzalkonium chloride nor strongly hyperosmotic conditions were really applied by FRANQUINET´s group.

With the exception of BETCHAKU (1967, 1970), previous attempts to cultivate planarian cells have used total cell suspensions. Under these conditions, the following survival periods were found: 3-15 days (MURRAY, 1927), 14 days (FRANQUINET, 1976), 2-3 weeks (TESHIROGI & TOHYA, 1988) and 4 days (FUKUSHIMA & MATSUDA, 1991). Purified neoblast fractions were cultivated only by BETCHAKU (1967), who arrived at a survival of 4 days at maximum. In no case has a standardized viability test been applied. The 14 weeks reported by SCHÜRMANN & PETER (1988) for primary cultures of neoblasts in the hypotonic (80 mOsmol/l) diluted Holtfreter´s medium (see BETCHAKU, 1967) refer to aggregates for which neither viability nor cell structure has been tested. Therefore, the 31 days reported here for primary cultures of purified neoblast fractions with a final viability of 46% in the isosmotic medium is the longest survival period found for neoblasts up till now, without differentiated cells and without microscopically visible signs of differentiation.

Further experiments with homologous matrix coatings should offer the possibility to simulate *in vivo* conditions on a less complex level by combining selected growth factors, tissue extracts and mitotically inactive cells with primary cultures of neoblasts, with the objective to study differentiation or stimulate proliferation *in vitro*. To promote mitoses, additional factors and / or the presence of differentiated planarian cells seem necessary (cf. BAGUÑÀ et al., 1989). The addition of X-irradiated planarian cells, possibly separated from the cultivated neoblasts by filters, and the use of homologous extracellular matrix components could bring improvements in this respect. Neoblasts form a heterogeneous pool (BAGUÑÀ, 1998). Selecting the true stem or progenitor cells among them should enhance the fraction of cells proliferating *in vitro*. For this purpose, additional criteria such as ultrastructural characters (RIEGER et al., 1999) and cytochemical data (BEHENSKY et al., 2001) may provide valuable tools to enrich special proliferative cells by appropriate separation techniques. Mere isotonicity might not be sufficient for optimal conditions. The available ion analyses refer solely to whole animals (PRUSCH, 1976). Determining the extracellular concentrations of inorganic ions and other components, for example by fluorimetric measurements following injections of appropriate dyes, may well supply essential data for a further improvement of culture conditions that might eventually result in an established neoblast line.

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REFERENCES

BAGUÑÀ, J. (1998). Planarians. In: *Cellular and Molecular Basis of Regeneration: From Invertebrates to Humans*. FERRETTI, P. & J. GÉRAUDIE (Ed.). J. Wiley & Sons, Chichester, New York etc.: 135-165.

BAGUÑÀ, J., E. SALÓ & R. ROMERO (1989). Effects of activators and antagonists of the neuropeptides substance P and substance K on cell proliferation in planarians. *Int. J. Dev. Biol.*, 33: 261-264.

BAGUÑÀ, J., E. SALÓ, R. ROMERO, J. GARCIA-FERNÀNDEZ, D. BUENO, A.M. MUÑOZ-MARMOL, J.R. BAYASCAS-RAMIREZ & A. CASALI (1994). Regeneration and pattern formation in planarians: cells, molecules and genes. *Zool. Sci.*, 11: 781-795.

BEHENSKY, Chr., W. SCHÜRMANN & R. PETER (2001). Quantitative analysis of turbellarian cell suspensions by fluorescent staining with acridine orange, and video microscopy. *Belg. J. Zool.*, 131 (Suppl. 1): 131-136.

BELETSKY, I.P. & S.R. UMANSKY (1990). A new assay for cell death. *J. Immunol. Meth.*, 134: 201-205.

BENAZZI, M. & G. BENAZZI LENTATI (1976). *Platyhelminthes. Vol.1 in Animal Cytogenetics*. JOHN, B. (Ed.). Gebr. Borntraeger, Berlin, Stuttgart (V + 182 pp.).

BETCHAKU, T. (1967). Isolation of planarian neoblasts and their behavior *in vitro* with some aspects of the mechanism of the formation of regeneration blastema. *J. Exp. Zool.*, 164: 407-434.

BETCHAKU, T. (1970). The cellular mechanism of the formation of a regeneration blastema of fresh-water planaria, *Dugesia dorotocephala*. I. The behavior of cells in a tiny body fragment isolated in vitro. *J. Exp. Zool.*, 174: 253-280.

FRANQUINET, R. (1973). Cultures *in vitro* de cellules de la Planaire d´eau douce *Polycelis tenuis* (Iijima). *C. R. Acad. Sc. Paris, Sér. D*, 276: 1733-1736.

FRANQUINET, R. (1976). Etude comparative de l´évolution des cellules de planaires d´eau douce *Polycelis tenuis* (Iijima)

dans des fragments dissociées en culture *in vitro*. Aspects ultrastructuraux, incorporation de leucin et d´uridine tritiée. *J. Embryol. exp. Morph*., 36 : 41-54.

Franquinet, R. (1981). Synthèse d´ADN dans les cellules de Planaires cultivées *in vitro*. Rôle de la sérotonine. *Biol. Cell.*, 40 : 41-46.

Franquinet, R. & I. Martelly (1981). Effects of serotonin and catecholamines on RNA synthesis in planarians ; *in vitro* and *in vivo* studies. *Cell Differentiation*, 10 : 201-209.

Franquinet, R., J. Moraczewski & A. Le Moigne (1985). Phosphorylation of endogeneous proteins, including histones, during initiation of planarian regeneration. *Comp. Biochem. Physiol.*, 80B : 661-669.

Fukushima, T. & R. Matsuda (1991). Experiments with culture media for planarian cells. *Hydrobiologia*, 227 : 187-192.

Kaltenbach, J.P., M.H. Kaltenbach & W.B. Lyons (1958). Nigrosin as a dye for differentiating live and dead ascites cells. *Exp. Cell Res.*, 15 : 112-117.

Martelly, I. (1984). Calcium thresholds in the activation of DNA and RNA synthesis in cultured planarian cells : relationship with hormonal and DB-cAMP effects. *Cell Differentiation*, 15 : 25-36.

Mc Connell, J.V. (Ed.) (1967). *A manual of physiological experimentation on planarians (2nd ed.)*. Planarian Press, Ann Arbor, Michigan : ix + 128 pp.

Murray, M.R. (1927). The cultivation of planarian tissues in vitro. *J. Exp. Zool.*, 47 : 467-505.

Pascolini, R., F. Panara, I. Di Rosa, A. Fagotti & S. Lorvik (1992). Characterization and fine-structural localization of actin- and fibronectin-like proteins in planarians (*Dugesia lugubris s.l.*). *Cell Tissue Res.*, 267 : 499-506.

Pedersen, K.J. (1959). Cytological studies on planarian neoblasts. *Z. Zellforsch.*, 50 : 799-817.

Peter, R. (1995). Restitution bodies from planarian cell suspensions : formation and survival in an isotonic culture medium. *Hydrobiologia*, 305 : 265.

Poot, M., L.L. Gibson & V.L. Singer (1997). Detection of apoptosis in live cells by Mito Tracker Red CMXRos and SYTO dye flow cytometry. *Cytometry,* 27 : 358-364.

Prusch, R.T. (1976). Osmotic and ionic relationships in the freshwater flatworm, *Dugesia dorotocephala*. *Comp. Biochem. Physiol.,* 54A : 287-290.

Rieger, R.M., A. Legniti, P. Ladurner, D. Reiter, E. Asch, W. Salvenmoser, W. Schürmann & R. Peter (1999). Ultrastructure of neoblasts in microturbellaria : significance for understanding stem cells in free-living Platyhelminthes. *Invertebr. Reprod. Dev.*, 35 : 127-140.

Schürmann, W. (1993). *Isolierung von Neoblasten aus Planarien*. Doctoral thesis submitted to the Faculty of Sciences at the University of Salzburg : vii + 169 pp.

Schürmann, W. & R. Peter (1988). Isolation and cultivation of planarian neoblasts by a novel combination of methods. *Fortschr. Zool.*, 36 : 111-114.

Schürmann, W. & R. Peter (1993). Primärkulturen von Neoblasten (Stammzellen) aus *Dugesia polychroa* (Turbellarien : Tricladida paludicola). *Verh. Dtsch. Zool. Ges*., 86.1 : 208.

Schürmann, W., S. Betz & R. Peter (1998). Separation and subtyping of planarian neoblasts by density-gradient centrifugation and staining. *Hydrobiologia*, 383 : 117-124.

Seilern-Aspang, F. (1960). Beobachtungen über Zellwanderungen bei Trikladengewebe in der Gewebekultur. *Roux´ Arch. Entwicklungsmech. Org*., 152 : 35-42.

Teshirogi, W. & K. Tohya (1988). Primary tissue culture of freshwater planarians in a newly devised medium. *Fortschr. Zool*., 36 : 91-96.