ACTES de COLLOQUES - n° 18 - 1995

European Workshop in Brest (France), April 28-30 1994

BIOLOGY of PROTOZOA INVERTEBRATES and FISHES

in vitro experimental models and applications

BIOLOGY of PROTOZOA, INVERTEBRATES and FISHES : *in vitro* experimental models and applications

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La BIOLOGIE des PROTOZOAIRES, INVERTÉBRÉS et POISSONS : modèles expérimentaux in vitro et applications

EUROPEAN WORKSHOP BREST
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PROTOZOA
PROTOZOAIRES

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COMPARISON OF PROTOZOA AND MAMMALIAN CELLS FOR IN VITRO TOXICOLOGICAL STUDY OF INORGANIC AND ORGANIC SUBSTANCES

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Abstract - *Nowadays, alternative methods are required for the evaluation of risks, as well in toxicology as in ecotoxicology. So, various models have been proposed and established cell lines are frequently used for the determination of the acute toxicity of xenobiotics, such as pharmaceutical drugs, cosmetic substances, food additives or environmental micropollutants. Furthermore, some other models have been proposed and among them, protozoa hold the attention specially in screening studies.*

Since 20 years, the ciliated protozoa Tetrahymena pyriformis have been increasingly used for toxicological evaluations. Moreover few studies have compared the sensitivity and the feature of this model to those of mammalian cells. After a brief presentation of the model (morphology, culture conditions, growth characteristics, previous works), the potential of Tetrahymena pyriformis strain GL as toxicological tool is discussed and compared to the potential of a mammalian cell line (L-929 murine fibroblasts), applied for the acute cytotoxicological study of 30 mineral and organic substances. A comparative study allows the advantages and the disadvantages of both models presented through specific examples to be specified. As general rule, the acute level of toxicity of all tested substances detected on Tetrahymena pyriformis GL with the simple generation time assay correlated significantly to the results obtained on L-929 with the more complex RNA synthesis rate assay, Neutral Red Incorporation assay and MTT Reduction assay. Moreover a improvement of Tetrahymena pyriformis GL culture in microtiter allowed a standardization and an automation of tests to be performed and a large number of substances rapidly to be tested. According to these results, Tetrahymena pyriformis GL can be considered as a sensitive, rapid, simple handling and cost-moderate model for in vitro toxicological screening studies.

INTRODUCTION

Nowadays, alternative methods are required for the evaluation of risks, in both toxicology and ecotoxicology. Simple handling assays performed on the most sensitive model would be the two main guidelines for the definition of a biological model. Established cell lines are frequently used for the determination of the acute toxicity of xenobiotics. Furthermore, some other models have been proposed and among them, protozoa have hold the attention specially in screening and

ecotoxicological studies. Over the last 20 years, ciliated protozoa *Tetrahymena pyriformis* have been increasingly used for toxicological evaluations. Moreover few studies have compared the sensitivity and the features of this model to those of mammalian cells, such as L-929 murine fibroblasts, both applied for the acute cytotoxicological study of inorganic and organic substances.

TETRAHYMENA PYRIFORMIS GL MODEL

The *Tetrahymena* species appears to be the most cosmopolitan freshwater ciliate. So, *Tetrahymena pyriformis* GL is an organism capable of independent life, but it is also a real pear-shaped eukaryotic cell (40-60 mm). It has absolute nutritional requirements of amino-acids, B-complex vitamins, purine, pyrimidine, and elements supplied in inorganic salts. *T. pyriformis* GL was the first organism grown axenically *in vitro* on defined media by Lwoff in 1923. *T. pyriformis* GL maintains its number by asexual reproduction which is dependent on various environmental conditions. At pH ranking from 6.5 to 7.0, the cell doubling time of *Tetrahymena pyriformis* GL is approximately 3 hours, but it can be easily modified in presence of xenobiotics. Among the ciliated protozoa, the *Tetrahymena* species is the best known group and have come to be used in fundamental research in genetics, cytogenetics, biochemistry and physiology (Elliott, 1973). Since 1980, *T. pyriformis* has also been an important tool for toxicological and ecotoxicological studies, adopted by some authors in various fields of investigations : acute cytotoxicity screening (Nilsson, 1989 - Yoshioka *et al.*, 1985), toxic effect determinations and metabolism reactions (Kergomard *et al.*, 1986), ultrastructural and biochemical impact of xenobiotics (Piccini *et al.*, 1987), definition of structure-toxicity relationships between the IC_{50} index of toxicity and the molecular descriptor of lipophilicity of tested organic substances (Schultz *et al.*, 1990). Moreover, these various studies were applied to all kinds of xenobiotics: pharmaceutical drugs, metal ions and organic substances.

TETRAHYMENA PYRIFORMIS GL OR L-929 MURINE FIBROBLASTS FOR *IN VITRO* TOXICOLOGICAL SCREENING STUDY OF INORGANIC AND ORGANIC SUBSTANCES ?

MODELS AND ASSAYS

The L-929 murine fibroblasts were grown in the Eagle's Minimum Essential Medium (M.E.M.) supplemented with 5% foetal calf serum, in a 5% carbon dioxide humidified incubator at 37°C. The assays performed for cytotoxicity evaluation were the determination of the cellular growth rate (CGR), the Neutral Red Incorporation Assay (NRI), the MTT Reduction Assay (MTT), and the RNA synthesis rate (RNA). *Tetrahymena pyriformis* GL were grown axenically at 28°C in capped flasks containing Plessner's PPYS medium, which is a proteose-peptone and yeast extract-base, supplemented with inorganic salts (Plessner *et al.*, 1964). The impact of tested substances was evaluated on the cell growth rate and the population doubling time.

TESTED INORGANIC AND ORGANIC SUBSTANCES

Both models were used to determine the acute cytotoxicity of 16 inorganic substances and 13 organic substances (Tab. 1). At to, tested substances were added directly to the culture media of cell models. For each substance, whatever the assay used, an index of cytotoxicity was determined. It was the IC_{50}, which was the concentration of tested substance required to induce a 50% inhibitory response of treated culture comparatively to the control culture. These values were secondly used for the comparison of both models.

RESULTS OBTAINED WITH THE L-929 CELLS

Whatever the assay used, the cytotoxic effects on L-929 were evaluated by end-point determination after a 24 hour incubation period, which is the doubling time of control culture in our experimental conditions. The acute cytotoxicity of tested substances was expressed by the value of their IC_{50}, which are summarized in the Table 1 according to the bioassays (CGR, NRI, MTT and RNA). Great differences of cytotoxicity intensity exist between these substances. The L-929 cells appeared to be more sensitive to the effects of inorganic substances than to organic ones. The most sensitive assays were ranking from the RNA, to the MTT, NRI and CGR assays.

RESULTS OBTAINED WITH *TETRAHYMENA PYRIFORMIS* GL

By end-point determination after 3-hour incubation period :

As for the L-929 cells, the cytotoxic effects were firstly sought after an incubation period with chemicals, which was the normal doubling time of the control population. In the experimental conditions used, this doubling time was approximately 3 hours; that is eight time less than with the L-929 cells. The results present in the Table 1 show that the cytotoxic effects of tested substances on the cell proliferation rate of L-929 and *T. pyriformis* GL are greatly and quantitatively different. With both models, no significant effect was detected for ethanol, chloroethanol, ethylene glycol and di-ethylene glycol. For other substances, a dose-dependent inhibitory effect on *T. pyriformis* GL was noted. The results showed that L-929 cells were more sensitive to the inorganic substances, and *T. pyriformis* GL were more sensitive to most organic substances.

By end-point determination after 3-hour, 6-hour and 9-hour incubation period:

The rapidity of *T. pyriformis* GL growth allowed easily the experiment to be extended and the effects of xenobiotics on several generations of cells to be evaluated. So, IC_{50} of tested substances were calculated by end-point determination after 3-hour (T 3H), but also after 6 hour (T 6H) and 9-hour (T 9H) incubation periods, which were the times required to obtain respectively 1, 2 and 3 generations of cells in the control culture.

This IC_{50} values showed, that most of the substances tested on *T. pyriformis* have dose-dependent, but also time-dependent inhibitory effects on the CGR, which were more marked for inorganic substances than organic ones. Firstly, whatever the incubation period may be, no cytotoxic effect was detected in our experimental conditions for ethanol, chloroethanol, ethylene glycol and diethylene glycol. A progressive decrease in IC_{50} values determined at T 3H, T 6H and T 9H was noted with inorganic substances (Ba, Co, Cu, Fe, Ge, Nb, Pb, Sb, Sn, V) as well as with organic substances (DEHP, terephthalic acid, glycolic acid and chloroacetic acid).

On the other hand, the inhibitory effects of some other substances were at their maximum levels as early as T 3H (for Cr, Zn, dichloroethane) or at T 6H (for Cd, Hg, Mn, Ti, VCM, acetaldehyde, chloroacetaldehyde, thioglycolic acid) and remained similar at T 9H.

Curves of dynamic growth of *T. pyriformis* GL :A 9 hour follow-up of *T. pyriformis* GL cultures by cell enumerations was performed and allowed the curves of the dynamic growth to be plotted. Two different kinds of curve models were observed :- on the first one, time-dependent, dose-dependent and linearly inhibition of CGR are observed; such curves are obtained for Ba, Hg, Nb, Ti, dichloroethane, chloroacetic acid and terephthalic acid;- on the second one, for some hours, a tempory inhibition of CGR followed by resumed growth at decreased rate and definitive inhibition after exposure to the highest concentration of tested substances are observed.; these responses are obtained for other substances (Cd, Co, Cr, Cu, Fe, Ge, Mn, Pb, Sb, Sn, V, Zn, MVC, DEHP, glycolic acid, thioglycolic acid, acetaldehyde and chloroacetaldehyde). Such behaviour has been previously mentioned for cadmium. The proliferation of surviving cells can start after a lag period, which could be explained by the cell selection, by the induction synthesis of metallothionein proteins or after a long exposure to metals, by a new proliferation rate which was related to a decreased concentration of metal in the culture medium. For *T. pyriformis* GL exposed to organic substances, the same phenomenon could be explained by metabolism and detoxification process. Even though the IC_{50} determined at T 3H, T 6H and T 9H gave some end-point information about the state of the *T. pyriformis* GL populations at a given time like a "photograph", the curves of the dynamic proliferation of cells showed better all the events which occured at different times and the natural evolution of the *T. pyriformis* GL populations, like a "movie". These observations oriented our research to the definition of a new parameter of cytotoxicity evaluation : the "Dynamic IC_{50}", which is calculated from the real doubling time of treated populations of *T. pyriformis* GL. A decrease of the cellular proliferation rate induced an increase of the population doubling time. This time is determined graphically on the curves of dynamic growth proliferation rate for each concentration of tested substance. Secondly, the concentration, which induced an 50% increase in the doubling time of *Tetrahymena* population is calculated by linear regression analysis.This new "dynamic IC_{50} index" was calculated for all tested susbtances and must be considered as the best index for the cytotoxicity evaluation of xenobiotics with *T. pyriformis* GL model. The comparison of this dynamic IC_{50} to the end-point IC_{50} calculated by the NRI, MTT and RNA assays on the L-929 cells proved the higher sensitivity of *T. pyriformis* GL to all organic substances and to numerous inorganic substances. Moreover, all results obtained with *T. pyriformis* GL correlated significantly to those obtained with the L-929 and the RNA, MTT, NRI and CGR assays.

	L-929 MURINE FIBROBLASTS				*TETRAHYMENA PYRIFORMIS* GL			
	CGR	NRI	MTT	RNA	" ENDPOINT " T 3H	T 6H	T 9H	" DY NAMI C "
Baryum	1.8	4.5	4.9	1.7	4.3	3.0	2.4	2.6
Cadmium	0.04	0.02	0.01	0.02	0.09	0.03	0.03	0.03
Cobalt	0.30	0.31	0.32	0.02	1.7	1.3	1.0	0.85
Chromium	7.6	7.1	5.4	0.67	1.2	1.0	1.0	0.77
Copper	0.30	0.35	0.32	0.002	1.7	1.3	0.9	0.47
Iron	1.6	0.63	0.77	0.17	7.6	7.4	4.7	1.9
Germanium	0.34	0.44	0.39	0.28	0.16	0.12	0.08	0.06
Mercury	0.02	0.02	0.02	0.005	0.16	0.02	0.01	0.01
Manganese	1.1	0.51	0.40	0.36	2.8	2.1	1.9	0.8
Niobium	0.19	0.11	0.19	0.06	0.09	0.08	0.06	0.02
Lead	1.4	2.8	0.47	0.68	1.4	0.9	0.7	0.87
Antimony	0.18	0.08	0.07	0.10	0.5	0.3	0.15	0.13
Tin	0.25	0.17	0.07	0.14	1.1	0.8	0.7	0.45
Titanium	0.30	0.29	0.38	0.09	0.7	0.4	0.4	0.42
Vanadium	0.68	0.31	0.37	0.002	2.4	0.9	0.4	0.35
Zinc	0.08	0.03	0.03	0.02	1.2	1.1	1.1	0.66
VCM*	35.2	7.9	6.8	7.7	12.9	6.9	6.5	8.6
Dichloroetane	2.2	2.7	2.7	3.2	4.5	3.7	3.5	2.0
Ethanol	>110	>110	>110	>110	>110	>110	>110	>110
Chloroethanol	>60	>60	>60	>60	>60	>60	>60	>60
Ethylene glycol	>80	>80	>80	>80	>80	>80	>80	>80
Diethylene glycol	>50	>50	>50	>50	>50	>50	>50	>50
Acetaldehyde	29.5	27.2	32.9	0.05	14.2	1.0	1.0	2.3
Chloroacetaldehyde	0.06	0.09	0.15	0.01	1.3	0.15	0.15	0.13
Chloroacetic acid	5.1	1.9	2.4	1.0	6.6	5.4	1.1	0.80
Glycolic acid	65.8	12.1	12.4	9.0	9.3	8.4	7.9	5.3
Thioglycolic acid	38.0	5.2	4.9	2.0	8.3	1.2	0.9	1.1
Terephthalic acid	3.5	3.1	2.9	1.5	3.9	2.8	2.1	1.7
DEHP **	3.3	2.0	2.3	0.13	0.6	0.25	0.15	0.08

* VCM = Vinyl Chloride Monomer/ ** DEHP = Di-Ethyl-Hexyl-Phthalate

Table 1 : IC_{50} VALUES (mmol/l)

CONCLUSION

As a general rule, the acute toxicity of all tested substances detected on *Tetrahymena pyriformis* GL with the simple generation time assay correlated significantly to the results obtained on L-929 with the more complex RNA synthesis rate assay, Neutral Red Incorporation assay and MTT Reduction assay.

According to these results, *Tetrahymena pyriformis* GL can be considered as both a sensitive and rapid model, which only requires simple handling and whose cost is moderate. To sum up, it is a helpful tool for *in vitro* toxicological and ecotoxicological screening studies.

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EFFECTS OF CADMIUM ON TETRAHYMENA

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Protozoa are useful as models for studying the toxicity of metals in more complex organisms and the biological mechanisms involved in detoxification. From this point of view, *Tetrahymena* has many advantages because it may be cultured in controlled conditions.

The data presented here concern three species of the *Tetrahymena* complex, *T.pyriformis, T.pigmentosa* and *T. thermophila*. All three species were grown axenically in 2% proteose peptone and 0.1 % Bacto yeast extract (Difco), enriched with Plesner (1964) inorganic salt solution and treated with various doses of cadmium (Cd) (as $CdCl_2.2.5\ H_2O$).

The effect of Cd on the growth of *T.pyriformis* is reported in Fig.1. No toxic effect is evident up to a dose of 8 μg ml^{-1} at 28°C. Some growth inhibition appears at higher doses, 9 or 10 μg ml^{-1} Cd. In any case, when a dose does turn out to be toxic, recovery takes place over a period of three or four days, which means that a process of adaptation is operating. *T.pyriformis* accumulates Cd like many other organisms (Tab. 1). An accumulation of 1500 μg ml^{-1} is not toxic, and the ultrastructure does not appear to be altered. In fact, in our experimental conditions, the morphology of both cytoplasm and nucleus of cells treated with 5 μg Cd ml^{-1} for 3 days was similar to that of controls (Piccinni et al., 1987).

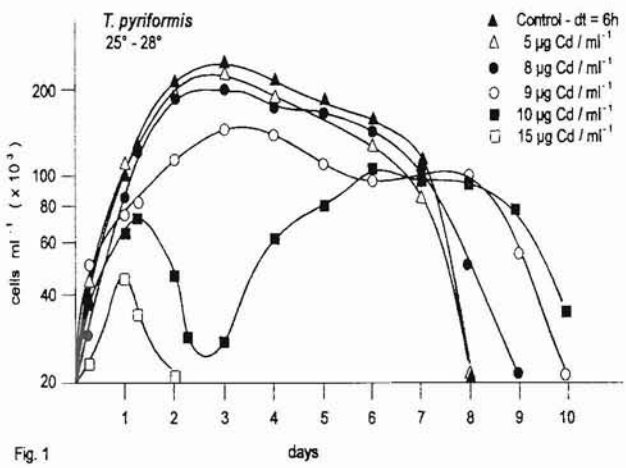
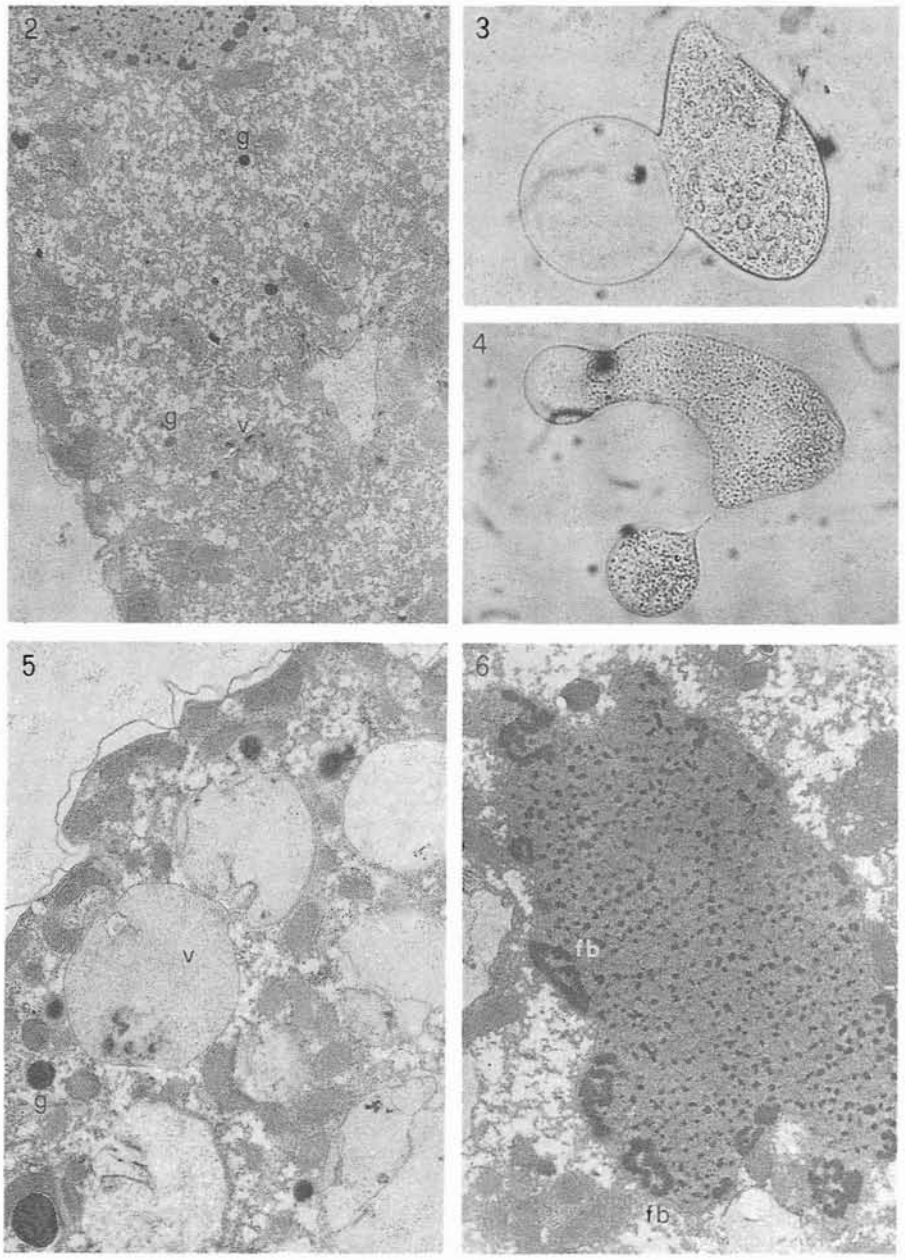


Fig. 1

These data are in contrast with those reported by other authors (e.g. Pyne et al., 1983) who found that growth is inhibited and morphology affected at lower doses. Because our observations are typical of the stationary phase of growth, some alterations induced by Cd may be masked. Short-term experiments were performed to overcome this condition: after 6h of treatment with 5 μg Cd ml^{-1}, no differences were observed between the nuclei of control and treated cells, but we did note enhancement of cytoplasm vacuolization. Membrane-bound vesicles with electron-dense material, cytolysomes and granules are found in



Figs.2-6. *Tetrahymena* Cd-treated cells. **2**. 5 μg Cd ml^{-1} after 6h. x 10500; **3**. 10 μg Cd ml^{-1} at zero time. x 575; **4**. 10 μg Cd ml^{-1} after 10 days. x 510; **5**. 15 μg Cd ml^{-1} after 6h. x 10500; **6**. 10 μg Cd ml^{-1} after 6h. x 9000. v, vesicles; g, granules; fb, nucleolar fusion bodies.

Cd-treated cultures (Fig.2). Many membranous structures representing material in decomposition could be seen in the nutrition vacuoles .

Toxic doses (10 or 15 μg ml^{-1}) produced great alterations visible by optical microscopy. Many cells, showing bubbles on the pellicle, were damaged even at zero time (Fig.3) ; after 10 days there were many vacuoles, and many monsters or cells showing altered shapes (Fig.4). After 30 days, the surviving cells showed normal shape. Electron microscopy observations after 6 h revealed dense granules and vesicles in greater amounts than in controls. Some mitochondria were degenerating, but morphological alterations were mainly found in the nucleus, which became an unusual shape . The nucleolar fusion bodies are also altered (Figs.5,6). All these changes were linked to the high accumulation of Cd in the cells (Tab.1).

It should be noted that granules may be regarded as structures involved in detoxification or metal ion regulation, as metals containing granules have been described in all invertebrate groups (Piccinni, 1989). In protists this phenomenon is well documented in *Tetrahymena* (Nilsson, 1981).

T. pigmentosa may be cultured at 29°C without damage, at least up to 5 μg Cd ml^{-1}. A dose of 8 μg ml^{-1} reduces the growth rate, and reduplication time is increased up to 10 h, as opposed to 7 h in controls (Fig.7). *T.thermophila* is much more sensitive to Cd, since a dose of as low as 2 μg ml^{-1} induces some inhibition of the growth rate at 32°C (Fig.8).

These data demonstrate that, in our experimental conditions, *T. pyriformis* is the most tolerant strain and *T. thermophila* the least tolerant. This behaviour may be explained by the faster growth of this species, which has a reduplication time of 3 h 36 min. Fast-growing cells are in fact usually more sensitive to external factors.

Tab. 1 Metal content in Cd treated cells (μg/g dry wt).

	Treatments μgCd/ml	2nd day Cd	2nd day Zn	5th day Cd	5th day Zn
T. pyriformis	–	–	100	–	nd
	5	1560	130	1700	190
	8	1500	270	1300	250
	15	2300	680	3500	570
T. pigmentosa	–	–	140		
	2	600	170		
	5	1500	180		
T. thermophila	–		70		
	2	460	200		

Tab. 2 - Distribution of metals between supernatant and pellet in controls and treated cells after two days of culture.- *T. pigmentosa* -

	Cd (%)		Zn (%)	
	Supernatant	Pellet	Supernatant	Pellet
Controls	–	–	40	60
2 μg Cd / ml	95	5	80	20
5 μg Cd / ml	95	5	85	15

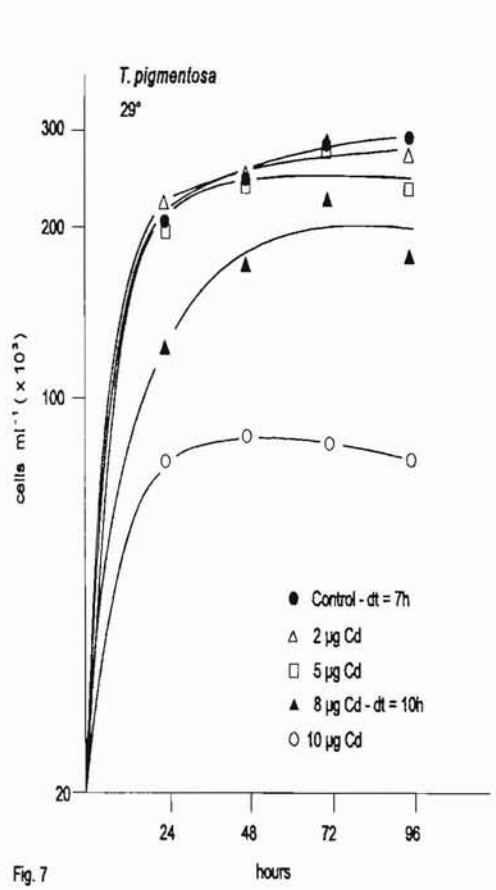


Fig. 7

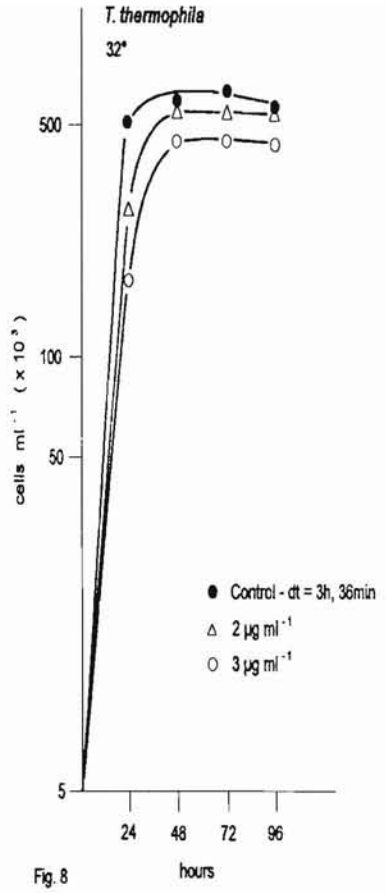


Fig. 8

All the species accumulate Cd, and the treatment also induces the coaccumulation of Zn (Tab. 1). This synergic effect is explained by enhancement of binding sites in the cells. Cd and Zn mostly accumulate in cell-free extracts. Increases in Zn contents in this fraction (when compared to controls) are to some extent due to the displacement of Zn from the particulate fraction of controls to the soluble fraction of Cd-treated cells (Tab. 2). In fact, in all three species, Cd treatment induces the formation of Cd-Zn-soluble chelating proteins similar to Metallothioneins (MTs) - low-molecular-mass, cysteine-rich, metal-binding proteins. This class of protein has been shown to be widely distributed in all kingdoms - fungi, plants, animals and prokaryotes.

Studies on the function of MTs have established that they play a primary role in the regulation of the essential metals Zn and Cu, and that they perform detoxification both of these metals when present in excess, and of non-essential metals like Cd. Many studies, especially on Cd which is one of the major inducers,

have established that MTs provide the cells with a mechanism to attenuate Cd toxicity.

In the animal kingdom, MTs have been reported in various phyla. Very few reports exist for protists. For some years now, we have been carrying out research on the inducibility of MTs by Cd in single-cell organisms, and have demonstrated that, in *Tetrahymena*, this metal induces chelating proteins with characteristics similar to those of invertebrate and vertebrate MTs: amino acid composition with high cysteine content, absence of aromatic amino acids, and spectroscopic features characteristic of metal-thiolate clusters (Piccinni et al., 1990). In all three species, we isolated two isothioneins with a very similar amino acid composition: MT-1 and MT-2. Several kinds of isothioneins are common in multicellular organisms. The functional significance of multiple forms of MTs still has to be clearly defined. Some of them are tissue-specific and may be related to differences in metal requirements during the life cycle and to different biological functions, but the factors that induce different isoprotein patterns are still unknown (Roesjiady, 1992).

Our finding of two isothioneins in *Tetrahymena* demonstrates that some polymorphism is also characteristic of single-cell organisms. The similarities of these isothioneins with classical mammalian MTs were studied by immunoblotting. A polyclonal antibody was produced against *T.pyriformis* in rabbit. Cross-reactivity was evident with MT-1 and MT-2 from *T.pyriformis, T. thermophila*, and *T.pigmentosa*, but not with MTs from mammals. Preliminary data on partial sequences of *Tetrahymena* MTs show some similarities whith pluricellular organisms. However, quite noteworthy is the presence of MTs in many phyla. Similar Cd-binding proteins are maintained through protists (Ciliophora), yeast (*Neurospora*), fungi (*Agaricus bisporus*), and animals, from invertebrates to vertebrates.

Tetrahymena MTs have the same function, and are thus physiologically analogous, to the MTs of other kingdoms, and are at the base of intracellular mechanisms involved in response to metals.

As previously mentioned, the other compartment used by organisms for detoxification of heavy metals is sequestration in cytoplasmic granules or membrane-bound vesicles. Thus, both types of compartmentalization in soluble and particulate fractions contribute to metal homeostasis, preventing the effects of toxic ions in both pluricellular and unicellular organisms.

In conclusion, the data presented here indicate that *in vitro* cultures of protists, in particular of *Tetrahymena*, are a good tool for studying the effects of heavy metals on biological systems, and may also be referred to environmental pollution.

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CHAPTER II

MARINE INVERTEBRATES
INVERTEBRES MARINS

European Workshop in Brest (France) April 28-30, 1994
ACTES DE COLLOQUES - 18 - ÉDITIONS DE L'IFREMER - 1995

MARINE BIVALVE CELL CULTURE: OPTIMISATION OF ISOLATION AND CULTURING

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Abstract - *Experiments were carried out for optimizing both cell isolation techniques and culture conditions, in order to obtain functional primary cultures of various tissues from bivalves (scallop and oyster). Different dissociation protocols were applied to oyster embryos and to mature organs from scallop, mainly gills and heart. Comparative studies led us to the conclusion that pronase alone is the most efficient, with cell viability ranging from 85 to 95 % for gills and heart. Suspensions from embryos and mature tissues contained single cells of different sizes and aggregates. These cell suspensions were seeded in plastic culture dishes. A sea-water-based medium allowed isolated cells to attach in 48 hours. The number of spreaded cells from gills and heart increased with time of culture, resulting either of proliferation or of migration. Terminal differenciation was obtained with heart cells: after eight days of culture, at cell confluency, formations similar to myotubes could be seen and, in parallel, beatings at regular intervals were observed.*

INTRODUCTION

The establishment of a suitable cell culture system from marine molluscs is important for applications especially in pathology and toxicology. The objective of this study was to optimize both the cell isolation techniques and the culture conditions in order to obtain short or long term primary cultures from Bivalves, the scallop *Pecten maximus* and the oyster *Crassostrea gigas*. Attempts have been performed from *Crassostrea gigas* embryos and *Pecten maximus* gills and hearts.

MATERIAL AND METHODS

Oysters *Crassostrea gigas*, collected from Aber Benoît and Brest Bay (France), were conditioned in hatchery during one month before experiments. After brushing the shells, the ripe animals were briefly washed in sterile sea water and rinsed with 70° ethanol. The oysters were then opened carefully and the tissues were rinsed with sterile sea water (S.S.W.) and with betadine in S.S.W. (1.1). The male and female gametes were taken from the gonad by aspiration with a syringe. The oocytes were transferred in S.S.W. supplemented with antibiotics and fertilized by adding a mixed spermatozoa. The embryos at 4-32 cell stages were sieved at 25 µm. The same treatment was applied to decontaminate scallops, *Pecten maximus*, collected from Brest Bay. Heart and gills were dissected and treated with antibiotics in decreasing concentrations. In the case of gills, a pretreatment by a mucolytic chemical was applied before the antibiotic treatment. Embryos and minced gills and

hearts were treated with a pronase solution. Then the isolated cells were filtered through a 60 µm nylon and centrifuged. The pellet was resuspended in S.S.W. and washed twice with S.S.W. to stop digestion. The final cell supensions were resuspended in a S.S.W.-based culture medium. Cell viability was assessed by the Trypan blue exclusion test adapted to the marine environment. Cells were seeded at a density of $1x10^6$ cells into 24-well dishes in a final volume of 0.5ml of culture medium and incubated. Daily microscopic observations were performed. Only one-half of the medium was changed within 2 or 3 days, depending whether the cells adhered to commercial plastic tissue culture. The medium was then replaced twice weekly. For gill and heart explants, small pieces of tissues were cultured in the same experimental conditions. After seven days of culture, adherent heart cells were fixed with glutaraldehyde fixative, postfixed with Osmium tetroxyde and ultrathin sections were prepared using conventional methods for electron microscopy (T.E.M.).

RESULTS

The experiments carried out with a view to dissociating oyster embryos and scallop gills and hearts with pronase allowed to obtain a cell viability of about 90% (Fig. 1).

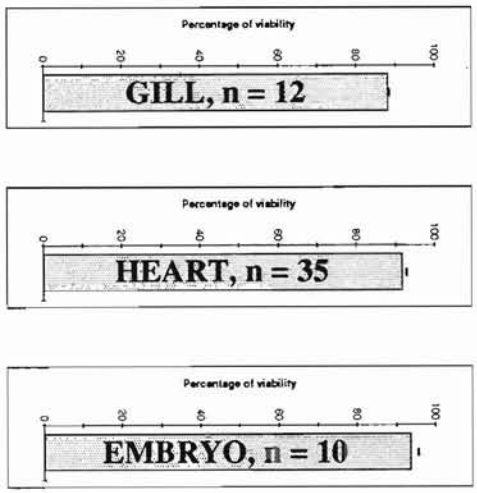

Figure1 - Average cell viability evaluated by the Trypan blue test

Quickly after seeding, isolated cells formed small clusters, most of which adhered to the plastic. After 2 days in culture spread cells were observed and their number increased with time (Fig. 2-6). It seems that most of the attached cells spreaded out from the cell clusters (Fig. 2, 3, 5). For embryos and gills (Fig. 2, 5, 6), adherent cells are fibroblastic-like cells. Some cell aggregates remained suspended and a ciliary activity could be observed during about one week.

For the heart (Fig. 3-4), 2 types of adherent cells were observed after 48 hours in culture, the epithelial cell type and the fibroblastic cell type, which is the dominant. Terminal differentiation was obtained after 8 days in culture: at cell

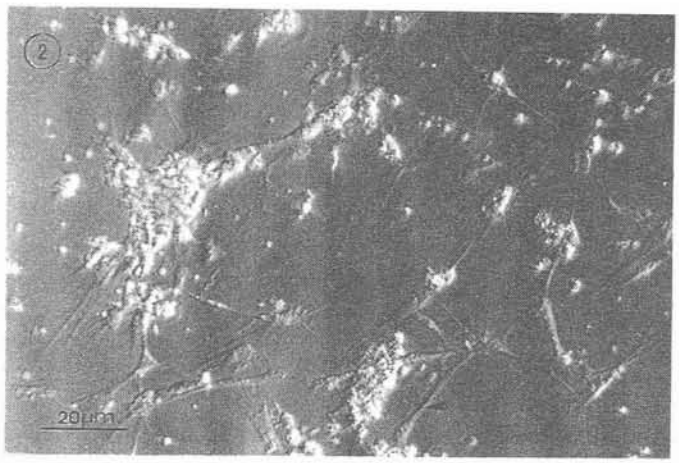

Fig. 2: Primary culture of oyster embryonic cells after 7 days in culture - (➞) fibroblast-like cells (phase contrast photograph).

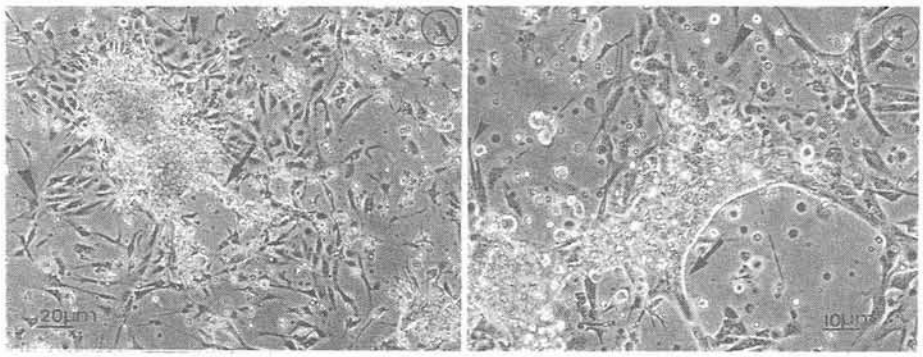

Fig. 3-4: Primary culture of scallop heart cells at 7 days: fibroblast-like cells (➞), epithelial-like cells (➞), formations similar to myotube (⟶) - (phase contrast photograph).

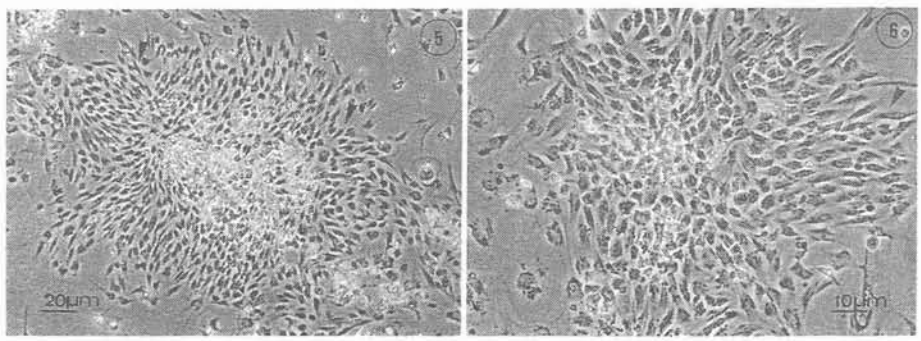

Fig. 5-6: Cluster of fibroblast-like cells (➞) from scallop gills - 7 days in culture - (phase contrast photograph).

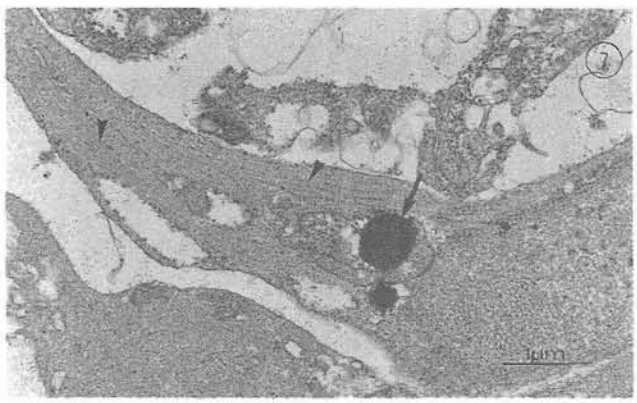

Fig. 7: Ultrathin section of spread heart cells after 8 days in culture: cardiomyocytes with myofilaments (➸) and lipid-like inclusions (➸).

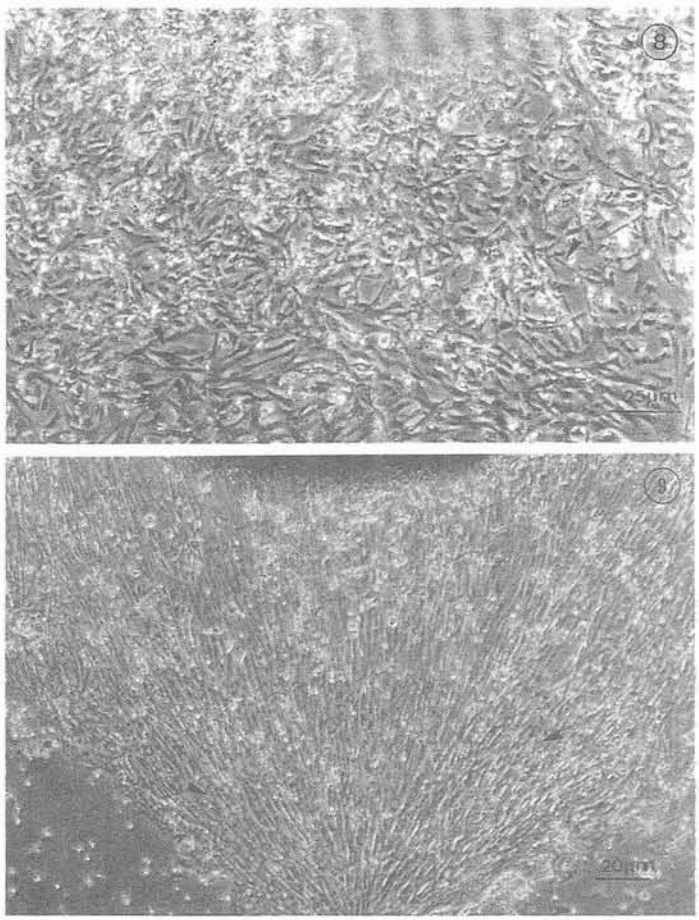

Fig. 8-9: Confluent monolayer of fibroblast-like cells (➸) from scallop heart explant on Day 5 of culture (Fig. 8), on Day 15 of culture (Fig. 9) - (phase contrast photograph).

confluency, formations similar to myotubes could be seen (Fig. 4) and in addition beatings at regular intervals were observed. Most of the adherent heart cells, examinated by T.E.M. after 8 days of culture (Fig. 7), are muscular cells characterized by the presence of myofilaments. Numerous cytoplasmic lipid-like inclusions were observed (Fig. 7). After about one month of culture, cells detached progressively, remained suspended and died.

By the explant technique, fibroblast-like cells migrating from heart and gill explants were also observed one day after explantation. The adherent cells formed a cell monolayer after 5-7 days in culture (Fig. 8-9).

DISCUSSION

Dissociation with pronase of oyster embryos and scallop gills and hearts allowed to obtain a good cell viability percentage. This enzyme had been chosen after having tested several dissociation protocols, performed either with trypsine, collagenase, hyaluronidase only or with a mixture of EDTA-trypsine, pronase-collagenase..., in accordance with the results of Wen *et al.* (1993 - a, b). For embryos, at 2-32 cell stages, pronase was the only enzyme which permitted cell dissociation. At this stage, the embryos are surrounded by a thick enveloppe which was resistant to other tested enzymes. This result differs from the observations of Odintsova and Khomenko (1991), who used collagenase but for trocophore larvae.

The dissociation protocol using pronase has been validated by the results of the cell culture. Indeed, adherent cells were systematically observed either for embryos or gills and hearts. A pretreatment of culture surface with adhesive proteins was not necessary for cell adhesion contrary to Odintsova and Khomenko (1991).

In culture, the fibroblast-like cells were predominant. However, in heart cell cultures some epithelial-like cells were also observed according to the results of Wen *et al.* (1993 - a, b) for hard clam. Transmission electron microscopy on scallop heart cells revealed that most of the adherent cells are muscular cells as seen also by Wen *et al.* (1993 - a). The presence of numerous cytoplasmic lipid-like inclusions observed in heart cells after 7 days in culture remains to be explained. Indeed, in heart muscular cells before or just after dissociation there were not so many lipidic inclusions.

Thanks to the antibiotic treatment (and to the pretreatment with the mucolytic chemical for gill cells), there was no real problem of contaminations of cell cultures.

The culture medium used in this study was sufficient to enable maintenance of cells during at least one month. Beatings of heart cells observed in culture according to Wen *et al.* (1993 - a) showed that the cardiomyocytes were functional. It remains to be explained how the number of spread cell increased during culture. It might be due to cell proliferation or migration from adherent cell clusters. That is the reason why some experiments of ^{3}H thymidine incorporation are undertaken.

With the establishment of these primary cultures of scallop heart and gill cells and oyster embryonic cells, it is now possible to focus on the improvement of the culture medium. The effect of lipids and growth factors from marine organisms will be assessed by the measurement of protein and D.N.A. synthesis.

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GROWTH FACTORS

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Abstract - The study of neuroendocrine controls of reproduction growth and digestion in Bivalve Molluscs is performed using bioassays and dissociated cell suspensions as biological material. According to first experiments (Lenoir et Mathieu, 1986), enzymatic dissociation with pronase 0,2 % and nutritic liquid medium (Hanks 199/Leibovitz) are regularly used.

Our researchs are concerning :
- a) regulation of somatic growth i.e. activation of protein and nucleic acid synthesis in somatic cells
- b) regulation of carbohydrate storage and mobilization
- c) regulation of digestive enzymes secretion
- d) regulation of gonial mitosis.

Concerning items a and d, identifyed neuropeptides are actually on the way of purification and can be considered as potential growth factors for Bivalve cell cultures. Two alternative ways of investigation (immunology and molecular biology) are completing this approach.

PHYSIOLOGICAL PROCESS	TARGET CELLS	SPECIES	BIOASSAY	NEURO-PEPTIDE	
Somatic growth	Mantle edge	*Pecten maximus* *Mytilus edulis*	^{14}C Amino Acid ^{3}H Thymidine ^{14}C Uridine incorporation	**P.S.A.F**	M. MATHIEU K. KELLNER J.Y. TOULLEC
Carbohydrate metabolism	Glycogen cells (purified)	*Mytilus edulis*	^{14}C Glucose ^{14}C O methyl incorporation	**G.M.H** **G.S.S.F**	M. MATHIEU I. ROBBINS F. LENOIR
Digestive enzyme activity	Digestive cells	*Pecten maximus*	α amylase activity	**FMRFa**	E. BOUCAUD W. GIARD
Gonial mitosis	Mantle (gonad)	*Mytilus edulis*	^{3}H Thymidine	**G.M.S.F**	M. MATHIEU

P.S.A.F	Protein Synthesis Activiting Factor	1.5 KD	Hydrophilic
G.M.H	Glycogen Mobilizing Hormone	20 à 30 KD	Hydrophobic
G.S.S.F	Glycogen Synthesis Stimulating Factor	1.5 KD	Hydrophobic
G.M.D.F	Gonial Mitosis Stimulating Factor	> 5KD	Hydrophobic

also provide useful non-vertebrate experimental systems for studies of basic cell processes, and could constitute a source of potentially valuable bioactive agents for therapeutic or diagnostic use. In addition, blood cell cultures from marine invertebrates may prove important for the assessment of sub-acute toxicity of environmental pollutants, and are needed to further our understanding and, hence control, of disease pathogenesis in commercial shellfish culture.

As yet there are no commercially available immortal blood cell lines from marine invertebrates, and while some success has been achieved with the maintenance of viable, and largely non-proliferative, cells *in vitro*, these have usually been for only very short periods (typically 1-6 h) and often under non-physiological conditions. Particular problems of culturing blood cells from invertebrates are associated with their biological functions. For instance, the cells are, by necessity, highly sensitive to non-self materials, especially endotoxin or other microbially derived carbohydrates, and tend to clot or transform upon exposure to these agents. They also constitute a heterogenous collection of cells which differ in their physiological requirements and behaviour *in vitro*. Some may adhere strongly to foreign surfaces, while others may undergo degranulation or lysis in culture. Several types are fully mature and lack proliferative capability. Successful culture of invertebrate blood cells therefore depends upon controlling these processes, whilst at the same time maintaining high cell viability and permiting normal cell activity.

REQUIREMENTS FOR INVERTEBRATE BLOOD CELL CULTURE

Successful culture of the blood cells from marine invertebrates entails a number of steps, including the collection of cells, their isolation or enrichment, maintenance *in vitro* and the induction of mitogenesis. The first step, removal from the host, crucially depends upon the prevention of coagulation and/or cell degranulation. A range of anticoagulants, of varying efficiency, have been reported in the literature. One, which has been found to be appropriate for marine crustaceans, is EDTA-citrate buffer at low pH (Söderhäll and Smith, 1983). A modified version at neutral pH effectively maintains blood cell integrity for ascidians, molluscs and echinoderms (Smith and Peddie, 1992; Smith, unpubl.). Use of an appropriate anticoagulant is particularly important for the subsequent separation or enrichment of the cells to obtain pure populations. For some groups single-step separation by density gradient centrifugation on 60% Percoll (Smith and Söderhäll, 1991) may be sufficient to yield functionally distinct populations of cells. In other cases second-step purifications, by cell affinity chromatography or panning, may be necessary. The development of suitable second step purifications rests largely with identification of distinct biochemical, surface protein or functional cell markers. In crustaceans, phenoloxidase activity is a convenient marker for the granular and semigranular cells (Söderhäll and Smith, 1983), while in molluscs, great strides have been made with monoclonal antibodies (Morvan *et al.*, 1991; Noël *et al.*, 1994). With the solitary ascidian, *Ciona intestinalis*, we have found that the phagocytic amoebocytes and the non-phagocytic, lymphocyte-like cells (LLC), which tend to settle out as two closely adjacent bands on Percoll, may be further enriched by differential nylon wool or glass bead adherence (Peddie and Smith, unpubl.). An alternative approach for the culture of blood cells from marine

invertebrates is to obtain cells from the haemopoietic tissue. This approach has been used successfully by Raftos et al (1990) to culture pharyngeal cells from the solitary ascidian, *Styela clava*. The use of pharyngeal explants avoids the problem of working with fully mature cells, but, with *C. intestinalis*, we have found that the explants may be inherently contaminated with protozoan parasites and bacteria, thus necessitating great scrupulousness in procedure and treatment of the tissue with broad spectrum antibiotics (Peddie and Smith, unpubl.).

As far as culture media are concerned, there are very few defined types suitable for marine animals that are available commercially. For short term culture, most workers have used either simple salines, constituted to mimick the ionic composition of the blood or body fluids, or artificial seawater (see for example, Smith and Ratcliffe, 1978; Smith and Peddie, 1992). While high cell viabilities (ie >95%) have been obtained with such salines, the culture period is often limited to 6-10 h at 15°C. With echinoderm and ascidian blood cells, inclusion of, variously, RPMI salts, Eagles Minimum Essential Medium (MEM), 199 medium, HEPES, glucose and/or peptone has been found to prolong cell viability for ca 10 d (Betheussen and Seljelid, 1978; Raftos *et al.*, 1990). For more extended periods of culture, however, the medium usually needs to be supplemented with fetal calf serum or host plasma (ca 20% vol/vol) and antibiotics (Raftos *et al.*, 1990; Rinkevitch and Rabinowitz, 1993). Recently we have cultured lymphocyte-like cells (LLCs) from *C. intestinalis* using a modification of the culture method described by Raftos *et al.* (1990). Briefly the medium contains RPMI 1640 powder (4.5 mg ml-1), streptomycin (500 æg ml-1), penicillin (1.0 unit ml-1), amphotericin B (2.5 æg ml-1), commercial sea salts (34 mg ml-1) and 20% vol/vol homologous plasma. After collection and separation, the cells are washed in sterile medium and then incubated in sterile flat bottomed 96-well culture plates (tissue culture grade) at 15°C in an atmosphere of 5% $C0_2$ in air. Although we have obtained good survival of the cells in this medium, there may be problems associated with the use of homologous plasma to support cell viability. One is the presence of naturally occurring cytokine-like molecules within the plasma (Raftos et al., 1991a) which may 'spontaneously activate' the cells *in vitro*. Another is subtle variations in the biochemical composition of the plasma due to seasonal effects or physiological changes within the host animals.

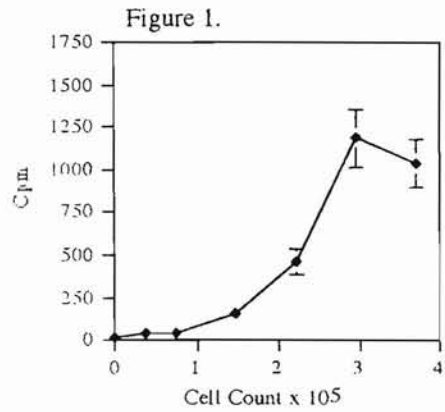
EXAMPLES OF SHORT TERM CULTURE

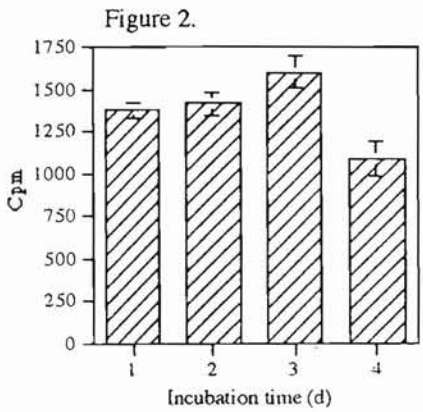
There are numerous reports of short term (< 10 h) culture of the blood cells from different marine invertebrate species. The majority have been concerned with crustacean or molluscan cells and have been used primarily to investigate aspects of phagocytosis or cell recognition. Early studies focussed on determining rates of uptake of various test particles *in vitro* and in attempting to detect opsonins in the serum, plasma or blood cells (see review by Bayne, 1990). More recently, effort has been directed at investigating the metabolic events underlying phagocytosis, particularly the generation of free oxygen radicals during the respiratory burst (Pipe, 1992; Bell and Smith, 1993). Other studies have used short term cell cultures to elucidate the nature of cell communication pathways in cellular defence (Johansson and Söderhäll, 1989). A few have examined cytotoxicity by invertebrate blood cells (Bertheussen, 1979; Peddie and Smith, 1993). Despite limitations in the culture

systems used, these studies have yielded important information about the biochemical events associated with cellular defence and are enabling us to learn not only how invertebrates respond to foreign entities but also how cellular reactivity might be regulated *in vivo*.

LONGER TERM CULTURE

Longer term culture of marine invertebrate blood cells has been used primarily to address fundamental questions about blood cell development, proliferation and the phylogenetic origin of immunological memory. It has been applied mainly to ascidian cells and, so far, has entailed culture of pharyngeal explants rather than of circulating blood cells. Long term cell culture is particularly useful for the study of cell proliferation, as it enables measurements to be made by ^{3}H-thymidine (^{3}H-TdR) incorporation, rather than by direct observation of mitotic figures in tissue sections. Raftos *et al.* (1991b) have employed ^{3}H-TdR incorporation to study cell proliferation in pharyngeal explants from *S. clava*. They found that proliferation was stimulated by recombinant human interleukin (IL-2) and the T cell mitogen, phytohaemagglutinin (PHA-P), but not by human IL-1 or the B cell stimulators, concanavalin A or pokeweed mitogen. The effect of IL-2 on the ascidian cells was dose dependent and affected mainly the LLCs, demonstrating that certain mammalian cytokines or mitogens may be used to stimulate invertebrate blood cells *in vitro*. As yet, few other mitogens have been tested for their ability to stimulate cell division in marine invertebrates, so the full range of agents which may induce mitosis in marine invertebrate cells is unknown. Likewise, little is known about the proliferative capability of the circulating cells *in vitro*, although recently, we have noticed that cytospin preparations of enriched LLCs from *C. intestinalis* contain a small proportion of mitotic figures (Peddie and Smith, unpubl). Autoradiographic examination of the cells, flash pulsed with ^{3}H-TdR, has further revealed that active DNA synthesis occurs in ca 30% of the LLCs in the circulation (Peddie and Smith, unpubl.). A more detailed analysis by ^{3}H-TdR incorporation has established that proliferation of circulatory LLCs is maximal after 3 d *in vitro* (Fig. 1) and is optimal at a cell concentration of ca 3 x 105 per well (Fig. 2).





Figures 1 and 2 - *Incorporation of ^{3}H-thymidine (^{3}H-TdR) into LLCs isolated and pooled from the circulation of twelve C. intestinalis.*

The cells were cultured in flat bottomed 96-well sterile culture plates at different concentrations at 15°C in a humid atmosphere of 5% CO2 in air. The cells (200 æl) were pulsed with 25 æl of culture medium containing 7.5 KBq ^{3}H-TdR for 16 h, harvested onto filter discs, dried and subjected to liquid scintillation counting. Results are expressed as the mean counts per minute (Cpm) ñ SEM. Figure 1 shows the rate of incorporation of 3H-TdR over time. Figure 2 shows the rate of uptake by LLCs at concentations ranging from ca 0.37 x 10^{5} to 3.7 x 10^{5}.

FUTURE PERSPECTIVES

In conclusion, there is clearly a great need for the development of long term culture methods for the blood cells of marine invertebrates, particularly for commercially important groups, such as crustaceans and molluscs. At present, the establishment of cell lines from these animals is limited by a number of factors, some inherent in the biological character of the cells themselves. Certainly, a wider range of cell specific markers and second step purification procedures need to be determined if pure cultures are to be set up *in vitro*. Monoclonal antibodies represent an especially useful category of markers, but they have yet to be raised for invertebrates other than molluscs. Most importantly, effort needs to be directed towards developing defined media capable of supporting the growth of the cells for prolonged periods *in vitro*. With suitable media and pure cell populations, it should then be possible to use cultured blood cells from invertebrates to obtain information about mitogenesis, cell maturation and cell to cell interactions during host defence. Perhaps also, as a way of overcoming the inability of some invertebrate blood cells to divide in the circulation, there may be merit in attempting to culture explants from haempoietic tissues of molluscs, crustaceans or echinoderms, perhaps along the lines already established for ascidians. In the meantime, short term culture of invertebrate blood cells is likely to continue providing us with baseline data on recognition events and

to offer a range of sensitive bioassay systems to evaluate some of the subtle physiological effects of environmental change on homeostatic integrity in marine invertebrates.

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CYTOSKELETAL ELEMENTS IN PRIMARY CULTURES OF ECHINODERMS

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Abstract - By comparison of cytoskeletal elements such as centrosomal proteins, actin-filaments and Ca^{2+} transporting membranes the distribution of these structures in cultured amebocytes and fibroblast-like cells, sperms and embryos of the sea urchin *Paracentrotus lividus* was studied. Amebocytes and fibroblast-like cells were obtained from the body cavity of adult animals. Amebocytes were cultured directly on polylysine-treated coverslips (1 mg/ml). Fibroblast-like cells were liberated by incubating small pieces from gut tissue in collagenase/Ca-PBS for 30 min at 15° C, separating the cells from the tissue by low-speed centrifugation and planting them in Petri-dishes on cover-slips, with and without polylysine. Both amebocytes and fibroblast-like cells were kept in sterile sea water, pH 7.4, supplemented with 15% horse serum and antibiotics (100 UI penicillin, 100 µg streptomycine, 0.25 µg amphotericin per ml). Cells stayed alive for several weeks in this medium. The distribution of the cytoskeletal elements was analysed using the appropriate antibodies, respectively phalloïdin in the case of the actin-filaments. The cells were fixed in cold ethanol followed by a 30 min treatment with cold acetone and viewed in an AXIOVERT 405 (ZEISS).

The results show that the centrosomal antigen is present in all cell types tested, actin filaments can be seen only in somatic cells, and Ca^{2+}-transporting membranes are continuously present in somatic cells, are absent in sperms and appear specifically at mitosis at the mitotic poles during the first rounds of divisions of early embryogenesis.

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CRUSTACEAN CELL CULTURES : STATE OF THE ART

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Abstract - *Crustacean constitute a class which includes animal species of biological interest and/or high commercial values. Crustacean cell culture has therefore gained recent attention as a potent model to assist in the development of diagnostic reagents and probes for the shrimp, crayfish and lobster industries. The avaibility of such cellular tools is especially important to developing industries which experience disease problems that are exaggerated by intensive culture methods. In addition such probes are of significant value to increase our knowledge on development and maturation processes or the endocrine metabolism of Crustaceans. Since the first paper describing the establishment of a continuous cell line of insect by Grace in 1962, several hundred cell lines have been established from approximately 50 species of invertebrates. Although a number of attempts have been made, no established cell culture of marine crustacean has been reported to date. However, primary cultures obtained from various organ sources are reported with increasing frequency. After a short review of various attempts in the establishment of continuous cell lines, we will discuss various applications of primary cell cultures and results obtained.*

INTRODUCTION

Crustacean cell culture has gained recent attention as a potent tool to assist in the development of diagnostic reagents and probes for the shrimp, crayfish and lobster industries. The availability of such cellular tools is especially important to developing industries which experience disease problems that are exaggerated by intensive culture methods. But, the interest of such a tool is not limited to the pathology. Crustacean constitute a class which includes animal species of high commercial value but also with biological interest. Such tools could be of significant value to improve our knowledge on development and maturation processes, or the endocrine metabolism of Crustaceans. It is therefore becoming an evidence that cell cultures are essential in Crustaceans as they are in Insects since thirty years.

Since the first paper describing the establishment of a continuous cell line of insect by Grace in 1962, several hundreds of cell lines have been established from approximatively 50 species of invertebrates. Although a number of attempts have been made, no established cell line of marine crustacean has been reported to date. For the last decade, most of attempts in the establishment of cell lines have been done on decapods with commercial value, with a special interest in the peneid shrimps (*Penaeus vannamei, stylirostris, japonicus, monodon, semisulcatus*). Most of the recent results presented in this short review have been obtained with these species (Table 1). All these experiments give us numerous informations about the different parameters which are involved in the survival but also, perhaps in the multiplication capacity of the cells in culture as the medium composition, the serum, the temperature, the osmolality etc. Some parameters seem to be accepted by the authors as a whole but others are discussed yet.

Cell cultures from different crustacean tissues

Reference	Testis	Ovary	Hepato & Gut	Lymp. o Hemat. t.	Nerve	Epiderm	Y organs	Embryos	Heart
Brody and Chang,1989	*H.american* 11 months			*H.american* 3 months.					
Chen *et al.*,**1986, 1989**	*P.monodon*	*P.monodon*	*P.monodon*	*P.monodon*					
	P.monodon 2 m.+ 2 Sc.								
Crozat and Patrois,1994 *(personnal communication)*								*P. indicus* 1 week.	
Graf and Cooke,1990					*H.american*				
Ellender *et al.*,**1988,92**			*P. vannamei* 2 m.+ 3 Sc		*P. setiferus*				
Fadool *et al.*,**1991**					*Panul. argus* > 23 days				
HuKe *et al.*,**1990**			*P. orientalis* 5 m+28 Sc						
Itami *et al.*,**1989**				*P. japonicus* 54 days					
Krenz *et al.*,**1990**					*P. clarkii* > 24 days				
Nadala *et al.*,**1993**	*P.vannamei* *P.stylirostris*	*P.vannamei*		*P.stylirostris*	*P.vannamei* > 3 weeks	*P.vannamei*	3 months		
Luedman/Lightner,1992	*P.vannamei* *P.stylirostris*								
Rosenthal/Diamant,1990	*P. semisul.* 6 days	*P. semisul.* 6 days		*P. semisul.* 3 weeks	*P. semisul.*				
Toullec, 1994 *(unpublished results)*	*P.vannamei* >2 months	*P.vannamei*		15 days		*P.vannamei*	*C. maenas*	4 months	> 5 days

Table 1: Main results obtained by differents authors. The names of the species are written in italics. They are followed by the survival time of the cultures.. m. = month. Sc. = Subculture.

CULTURE CONDITIONS

MEDIA

The choice of the medium for example is controversial. Most of the media were modified from commercially prepared media (M 199, MEM, RPMI 1640 and L-15). Two media were mainly used : the M 199 and the Leibovitz L15 often at double strength. It is suprising to note that two media which are quite different in proportion and number of components could have similar effects in close species and sometimes in the same species. Indeed, the L15 contains about 10 times more amino-acids and 100 times more vitamins than M 199. The fact that the difference between the both media is not obvious at the level of the survival or the multiplication of the cells, arise the question of the real importance of these components in comparison with supplements as serum or tissue extracts.

OSMOLALITY, PH, TEMPERATURE

Others parameters seem to be accepted by most authors as the osmolality of the medium from 750 to 770 mOsm excepted for *P. semisulcatus* which lives in a sea water with a higher salinity. Modifications generally reflected inclusion of physiological concentrations of inorganic ions, which increased medium osmolality to physiological levels. The pH used is generally the one which has been measured for the haemolymph of the animal. It ranges between 6.8 and 7.5. Temperature is maintained at 25-28 °C and corresponds to optimal temperature for intact animal.

SUPPLEMENTS

If the use of a supplement is general in all published studies where cells are maintened in culture for at least 2 weeks, its nature and its concentration are depending of the authors. Generally, foetal calf serum (FCS) is used at the concentration of 10 to 20%. Results obtained using Y-organ dispersed cells of crab (*Carcinus maenas*) have demonstrated that the secretory capacity of these endocrine cells was improved by addition of FCS. 10% of FCS seemed to be the optimal concentration, 5% leading to a weak effect and 20% giving no further amelioration (Toullec and Dauphin-Villemant, 1994). Although all the added FCS, some of them used haemolymph and/or tissue extracts. For instance, Chen *et al.* (1986) added up to 30% of muscle extract and 10% of haemolymph. Rosenthal and Diamant (1990) reported that cell proliferation was significantly enhanced by a combination of 5% heat inactived haemolymph of shrimp and more than 15% FCS. Moreover, the batch of serum used might affect the seeding efficiency of cells placed in the medium. Often, only one serum produced for high initial seeding efficiency and the choice of the serum supplement seems to be crucial to the establishment of a suitable confluent cell monolayer. Ellender *et al.* (1992) have made similar observations in the primary cell cultures of ovarian cells and hemocytes of Peneids.

ANTIBIOTICS

The use of antibiotics is also general in all papers. Indeed, antibiotics are necessary for long-term cell survival because bacteria and fungi are commonly found as part of the haemolymph cell mixture. Ellender *et al.* (1992) have demonstrated that the haemolymph samples collected and placed in antibiotic-free medium became contaminated during the first 48 hours of incubation, whereas, flasks which became contaminated using antibiotic-treated media did not show any visible contamination for 5-7 days. Apparently, the antibiotic mixture used in this investigation was not totally effective against all haemolymph contaminants. Broad spectrum antibiotics should be tested on isolated bacterial strains from shrimp haemolymph to evaluate for sensitivity. This approach has been used by Rosenthal and Diamant (1990). Streptomycin seemed to be the most effective antibiotic against

the bacterial strains isolated from hemolymph of *P. semisulcatus*. Then, they added penicillin and amphotericin B to eliminate any possible airborne bacterial and fungal contamination. Thus, the antibiotic cocktail they developped experimentaly is similar to the one generally used by most authors. That confirms that antibiotic composition, penicillin and streptomycin, waiting more important studies on hemolymph contaminants, should be the most efficient cocktail to avoid systematic contaminations. On the other hand, the various concentrations used set the problem of a possible impact of the antibiotics on the cultured cells.

ADHESION FACTORS

Tissue fragments of crustacean embryos, heart, stomach, hematopoietic tissue, ovary, testes and midgut gland often spontaneously attach to plastic culture flasks. Although coating of tissue culture flasks with adhesion factors (collagen, poly-lysine) can improve the percentage of attachment of tissue and cells, they were not often used. The characteristic of the explants to attach could be linked to the strong capacity of hemocytes to adhere. Indeed, all tissues contain haemolymph and haemocytes. Hemocyte-like cells could constitute a natural attachment factor. Hemocyte-like cells are the first cells to leave an explants. When this explant is sticked out, a layer of hemocyte-like cells remains attached to the flask. Most often when dispersed cells were seeded, this capacity to adhere was no longer observed.

TISSUES

The tissular origin of the cells used in culture varied greatly. But only few tissues retained more attention especially for virus studies : for example, hepatopancreas, ovary, and hematopoietic tissue from peneids. Although cell lines could never established, primary cells cultures were often obtained. For other applications than pathology studies, as cellular biology or endocrinology, various tissues of different species have been checked : testis, epidermis, Y-organs, neurons.

It is interesting to mention that most experiments used tissues taken from adults or juveniles. The use of differentiated tissues could partly explain the lack of established cell lines. Various attempts, particularly on regeneration buds and embryos gave encouraging results but unfortunately severe problems of contamination were encountered. If these problems could be solved, this would represent a valuable alternative to obtain established cell lines of crustaceans, despite the fact that further characterization of cell populations would require important experimental investments.

CONCLUSION

In conclusion, studies in Crustacean biology need the developpement of *in vitro* systems which offer a high experimental potential. Currently, the culture conditions generally used allow the establishement of primary cell cultures. So, crustacean cells can be kept in culture and used for various experiments. But in order to obtain established cell lines, the improvement of tissue culture media for growth and maintenance of cells should be the next step. It can be expected that due to the current multidisciplinary interest in the biology and aquaculture of Crustaceans, there will be major advances in crustacean cell culture in the near future.

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CULTURE STATES OF HEPATOPANCREATIC CELL SUSPENSIONS FROM SHRIMP *PALAEMON SERRATUS*

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Development of cell cultures and cell lines has provided essential tools for studying cellular metabolism *in vitro*. In marine invertebrates, various cell cultures from different organs have been studied, but to date, no subcultures have become available.

It is well established that the crustacean hepatopancreas consists of four different cell types of cells were classified by Hirsch and Jacobs (1930) from histological data as E (Embryonalenzellen), R (Resorptionzellen), F (Fibrillenzellen) and B (Blasenzellen) cells. Direct measurements of the absorption capacities of R and F cells of *Homarus americanus* and *Palaemon serratus* were achieved by Ahearn et al. (1983) and Toullec et al. (1992) using cells suspensions previously separated by density gradient centrifugation.

In vitro assay based on cell suspensions from the hepatopancreas of *P. serratus* can be used for studying the effects of various factors on protein synthesis. To be fully informative this test must use cells that have retained their integrity and their biological and chemical functions which be disrupted for a short period as a consequence of the separation stress. In this hypothesis, a culture in a survival medium is essential so that cells can recover their reactivity potential rapidly.

Survival of hepatopancreatic cells was tested in different culture media. Medium 199 (adapted for marine invertebrates) with 20 % low protein serum replacement (containing hormones, vitamins, growth factors and other growth promoting agents) was the best for obtaining a good survival of cells (Fig. 1). After a period of 25 hours in this medium, cell viability was maintained at 48 %. In contrast, incubation in saline solution resulted in low survival.

The present report describes a survival system to search for growth factors active on the hepatopancreatic cells of *P. serratus*. The effect of various culture conditions on cell survival and on the incorporation of radioactive aminoacid was studied as well as the response of the hepatopancreatic cells to treatment by dibutyryladenosine 3'-5' cyclic monophosphate (dcAMP), epidermal growth factor (from mouse) and extracts of eyestalks and of hepatopancreas (from shrimp *P. serratus*). Cell were incubated for 16 hours in culture medium (medium 199+20 % low protein serum replacement ± extract) and they were incubated in saline solution for 3 hours with [^{3}H] leucine. Eyestalks and hepatopancreas were homogenized by grinding in ammonium acetate buffer (pH = 7.4). The results (Fig. 2) show that substances tested stimulate protein synthesis in comparison with control. The presence of dcAMP and hepatopancreas extract increases significantly protein synthesis into dissociated cell suspensions. These first results show that the presence of endogenous growth factors in eyestalks extract and in hepatopancreas extract is able to stimulate protein synthesis. These factors are not available however they are fundamental for establishing an optimal culture medium.

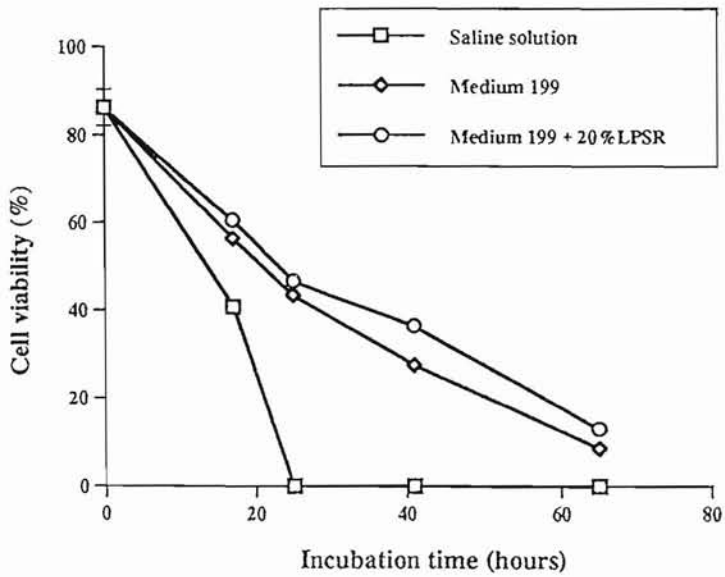

Figure 1 - Viability of hepatopancreatic cell suspensions. Each value represents the mean of triplicate samples and bars the S.D.

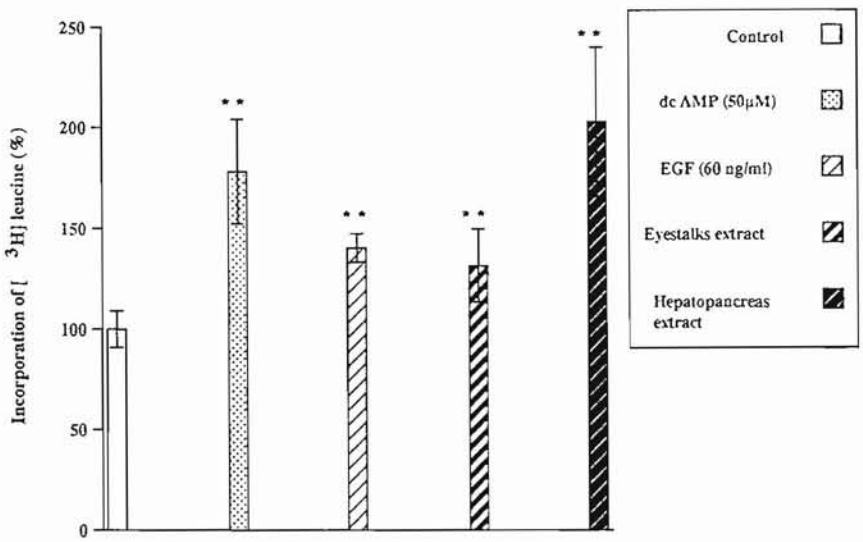

Figure 2 - Effects of dibutyryladenosine 3'-5' cyclic monophosphate (dcAMP), epidermal growth factor, extract of eyestalks and hepatopancreas on the incorporation of [^{3}H] leucine into dissociated hepatopancreatic cells of *P. serratus*. Each value represents the mean of triplicate samples and bars the S.D. (** : P>0.01 Student-t test).

Cell viability (Fig. 1) is practilly the same when cells were incubated in medium 199 with or without serum replacement (LPSR) strongly suggesting that cells do not seem to be sensitive to vertebrate growth factors. In marine invertebrates, there is a lack of informations concerning the fundamental growth factors. Little effort has been directed towards isolating and identifying growth factors in invertebrates. The identification of these factors is useful for increasing cell viability and cell growth. During the past decade, genetic and molecular analyses of genes that effect developmental processes in invertebrates have greatly expanded our understanding of the molecules that participate in developmental regulation. Studies of a number of invertebrate development regulatory genes have to led to some possible identifications. Some of these loci in flies, nematodes and sea-urchins encode proteins homologous to factors known or believed to regulate cell growth and development in vertebrates. These include epidermal growth factor (Muskavitch and Hoffman, 1990). The prospects for further advances in our understanding of the mechanisms by which invertebrate homologues of vertebrate growth factor function during development seem bright.

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CELL CULTURE FROM ADULT AND EMBRYONIC TISSUES OF THE PENAEID SHRIMPS

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Abstract - *In order to provide a useful tool to study fundamental and applied aspects of biology and pathology we derive cell cultures from adult and embryonic tissues of penaeid shrimp (Penaeus stylirostris, P. vannamei, P. monodon, P. indicus). Two different media were tested: Grace's medium and Eagle's Minimum Essential medium supplemented with fetal bovine serum (FBS) and antibiotics.*

Cells from ovary, hepatopancreas and embryos were routinely subcultured over six months. In primary culture, cells with various morphology and adhering to the flasks were observed. In the course of subcultures, aggregates of spherical cells containing refringent droplets become more and more abundant. We apply the cell cultures to the study of virulence factors responsible for the pathogenicity of Vibrio strains isolated during mass mortality events in penaeid shrimp hatcheries. We have also shown that the cultured cells can be cryopreserved thereby providing a constant standardized source of shrimp cells.

INTRODUCTION

During the last ten years, several attempts were performed to establish cell lines from penaeid shrimps. The authors obtained primary cell cultures from different tissues of the adult penaeid. Several morphological cell types were described, depending on the tissue origin, and on the medium used (reviewed in Najafabadi *et al.*, 1992). But until now, no permanent cell lines have been reported. The aim of this study was to attempt establishment of cell culture originating from different shrimp tissues, and therefore to gain useful tools for microbiology.

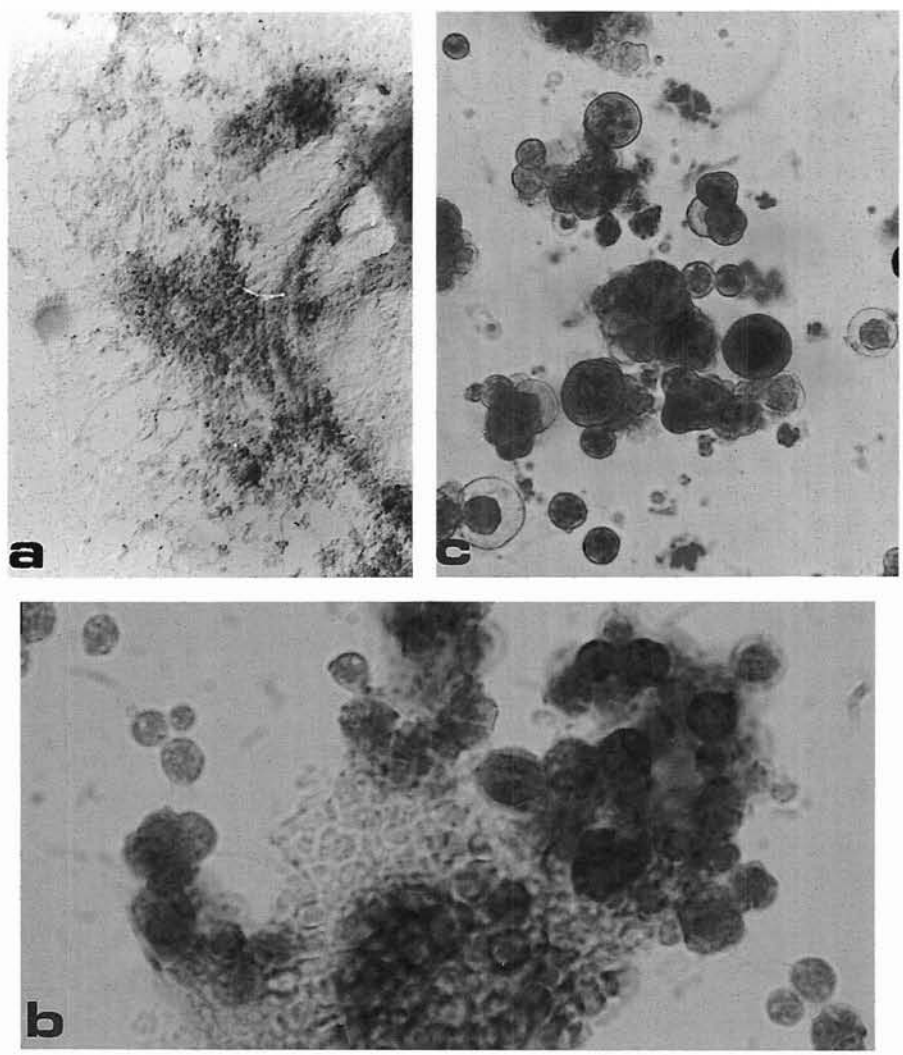

Figure 1 - a) Cells expanding from an explant of lymphoïd (Oka) organ of *Penaeus stylirostris* after 12 days in MEM supplemented with 10% FBS (X 100). b) and c) Cells from ovary of *Penaeus vannamei* in MEM. b) After six passages, cells have been assayed for bacterial attachment and stained by Giemsa (X 500). Both epithelioïd and spherical cells can be observed. c) Staining by Soudan black B and nuclear red attests for the lipidic nature of cytoplasmic inclusions in cell subcultured over one year (X 320).

PRIMARY CELL CULTURES AND SUBCULTURES FROM ADULT TISSUES

A variety of tissues removed from adult *Penaeus stylirostris*, *P. monodon*, *P. vannamei* were put in flasks using MEM or Grace medium supplemented with FBS and antibiotics after mechanical dissociation. Cells rapidly outgrew from heart, lymphoid (Oka) organ (Fig. 1a), and muscle explants forming a monolayer limited to tissue periphery. Adhering cells were mostly of epithelioïd type, and some of them were fibroblastic. A completely different situation occurred when digestive gland and ovary tissues were assayed. During the first two weeks of primary cultures, cells extended from the explant as previously described, but small groups of cells adhering to the flask were also noticed disseminated on the whole plastic surface. In the course of subcultures, two types of cells were noticed : epithelioïd cells, and cells with a special morphology, spherical in shape (Fig. 1b). Spherical cells had a large nucleus, and became more and more abundant, as reported previously by Hu (1990) with explants of the digestive gland. These cells provided free and attached aggregates, and also layers of cells on the bottom of the container (Fig. 1b). Nadala *et al.* (1993) also reported that attached Oka cells floated off, and reattached in other areas of the dish. Spherical cells from ovary have been routinely subcultured over a year, and have been recovered after freezing and thawing operations. Trypan blue test assessed for cell viability showed that over 90 % of these cells are viable. The spherical cells appeared to contain refringent droplets. After black Soudan B staining and nuclear red understaining the cells exhibited a rounded nucleus and a few large black inclusions attesting of the lipidic nature of the cytoplasmic droplets (Fig. 1c).

PRIMARY CELL CULTURES AND SUBCULTURES FROM EMBRYONIC AND LARVAE TISSUES

It was apparent from the experiments described before that cells originating from tissues with high storing ability could be more easily subcultured. This prompted us to try to culture embryonic and larvae cells from *Penaeus indicus*.

EMBRYONIC TISSUES IN CULTURE

Fertilized eggs were harvested just after spawning in aminotriazole solution, in order to weaken the hatching envelope. These eggs were transferred in culture. First eggs segmentation occurred, and after it attached to the dish. Cells migrated out from the attached embryos. These cells were of epithelioïd type, containing mainly large, refringent droplets. Repeated subcultures have been performed over a period of ten months.

LARVAE TISSUES IN CULTURE

Cells from Nauplii were isolated using Potter dissociation and gave rise to small aggregates of cells. These cells have also been subcultured, but less extensively than embryonic cells, only four subcultures were obtained.

CONCLUSION

Shrimp primary cell cultures have been obtained and were used in pathogenic assays for bacterial adhesion and cytopathic effects of bacterial extracellular products (Le Groumellec *et al.*, 1993). During subcultures, rounded cells with refringent droplets became more and more abundant. These cells resisted to freezing and thawing treatment, and resume their growing ability in small cells clusters, exhibiting low adhesive affinity for culture dishes. We are currently performing investigations to further characterise these cells. Are they a permanent cell line of penaeid shrimp origin ?

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UNIVERSAL CELL CYCLE CONTROL IN EUKARYOTES

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Abstract - *One of the most significant advances in our understanding of the eukaryotic cell cycle has been the elucidation of the role of the serine/threonine protein kinase p34 ^{cdc}2. The cdc2 gene in the fission yeast Schizosaccharomyces pombe, and its homologue the CDC28 gene in the budding yeast Saccharomyces cerevisiae, is essential at a control point in G1 phase called 'START' when the cell becomes committed to replicate its DNA, and also for the transition from G2 phase into mitosis.*

Define by their ability to complement a defective cdc2 or CDC28 gene when introduced into yeast, homologues of cdc2 have been found in all species. Although in yeast only one gene has been described. In higher eukaryotes several cdc2 related genes exist and compose the CDK (Cyclin Dependent Kinase) family. Each CDK comprises a cyclin regulatory subunit and a CDC2-family kinase subunit.

In bugging yeast, cell-cycle events are triggered by a single protein kinase subunit, Cdc28, which associates with a succession of different cyclins (Clns and Clbs). The programme is initiated in G1 phase by Cln3, which promotes accumulation of Cln1 and Cln2, leading to the cell-cycle commitment point known, as Start. In S phase, Clb5 and Clb6 replace Cln1 and Cln2, and, at M phase, Cdc28's partners change to Clb1 and Clb2. The abundance of cyclins rises and fails as cells progress through the cycle. In mammals, more than one kinase subunit is implicated in cell-cycle control. Thus progression from G1 to S phase involves Cdk4/cyclin D and Cdk2/cyclin E; S phase Cdk2/cyclin A; and M phase Cdc2/cyclin B. Cyclins D and E seem to be equivalent to Cln3 and Cln1/2 respectively, cyclin A to Clb5/6, and cyclin B to Clb1/2. Cln3 and cyclin D stand out, because their levels do not oscillate. They may act as 'initiator' cyclins that help to coordinate entry into the cell cycle with cell growth.

Cyclin oscillations are determined partly by transcriptional control of messenger RNA production and partly by specific proteolysis mediated by special ubiquitin ligases that turn on and off at particular times in the cell cycle.

Recently several inhibitors of the cyclin dependent kinases (CKI) have been isolated from different eukaryotes including yeast and mammals.

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CHAPTER III

FISHES
POISSONS

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METABOLISM OF FISH CELLS: OXYGEN CONSUMPTION, PROTEIN AND RNA SYNTHESIS

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Abstract - One of the fundamental properties of cells, protein turnover, is seen increasingly as playing an important role in cell energetics. In fish it has been estimated that protein synthesis can account for up to 40% of the total oxygen consumption but one of the determining factors in such calculations is the quantification of the energy cost of the synthesis of proteins. In this paper, data will be reported from experiments where protein synthesis and oxygen consumption have been measured simultaneously in a variety of fish cells. Energy costs of protein synthesis have been estimated from the slopes of the lines relating protein synthesis and oxygen consumption and from the effects of protein synthesis inhibitors. Generally speaking protein synthesis costs are lowest and approach theoretical values when the cells are synthesizing proteins rapidly.

There also seems to be an positive relationship between protein synthesis costs and the efficiency of RNA translation. In order to study RNA synthesis, experiments will be reported where cell proliferation rates, cellular RNA concentrations and uridine uptake in cultured fish cells have been studied. Proliferation rates (expressed as culture doubling times) were calculated from DNA concentrations. High rates of cell growth were associated with increased salvage of exogenous uridine and nucleotide recycling and also rapid sequential phosphorylation. It is possible that at low rates of protein synthesis, RNA synthesis represent a proportionally high cost associated with the synthesis of proteins (what could be called fixed costs) and that with increased rates of protein synthesis these fixed costs decline in importance relative to the synthesis of proteins.

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FISH CELL LINES : DEVELOPMENT AND APPLICATIONS IN FISH PATHOLOGY

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Abstract - Since the first continuous fish cell line (RTG2) was obtained in 1962, more than one hundred of cell lines have been established from freshwater as well as seawater fish species.

It is mainly the observation of viruses as causes of mortality among fish farmed, and the lack of host specific cell lines for viral detection that led to develop cell lines for isolation of the viral agents. The ease with which those cells can be handled makes them convenient for different laboratory procedures.

Most of those cell lines have been initiated from pieces of organs (skin, heart, fins, spleen, kidney, gonads...) or from larvae, dispersed by treatment with 0,25% trypsin in salt solution. The harvested cells are incubated in classical culture media: Eagle's minimal essential medium, Leibovitz medium, medium 199, buffered at pH 7.4 to 7.8 and supplemented with fetal bovine serum. Fish cells grow over a temperature range of 10° C to 35° C and most of the time do not require renewal of culture medium between subcultivations.

Cell culture represents the main tool in fish virology because it is the best and most useful approach for diagnostics as well as for research work. For diagnostic purposes they are used both for isolation and identification (by seroneutralisation or immunofluorescent tests) of viral and rickettsial agents. Cell lines are also commonly used in epidemiological studies to detect the presence of antibodies against the most important pathogenic viruses of fish. Research on fish viral diseases requires continuous cell lines for propagation and titration of the viruses in studies on their biological and biochemical properties. Titrations of the viral neutralizing activity of sera, and of the interferon production after experimental infection are commonly carried on in fish cell cultures. In order to get enough antigens to prepare a vaccine, continuous suspension culture of fish cells and microcarriers beads for cell cultivation have been developped for mass virus production.

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MYOSATELLITE CELLS OF *ONCORHYNCHUS MYKISS* : CULTURE AND MYOGENESIS ON LAMININ SUBSTRATES

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Abstract - *The critical role of extracellular matrix components in adhesion, growth and differentiation of many cells is wellknown to date. An immunoreactivity for mammalian basement membrane components observed around teleostean muscle fibers suggested to us to cultivate fish myosatellite cells on laminin rich substrates. As expected, myosatellite cells isolated from trout white muscle adhered, proliferated and formed large multinucleated myotubes on laminin or entactin/laminin substrates, whereas myogenesis was not promoted on surfaces devoided of laminin. The expression of a teleostean myogenic factor very related to MyoD, and the intracellular immunolocalisation of desmin and myosin demonstrated furthermore the muscular phenotype of the multinucleated myotubes generated in our in vitro myogenesis system. Interestingly, these myotubes reconstituated at their surface an extracellular matrix similar to those detected in vivo. We observed moreover, that the use of laminin substrates do not prevent the modulation of the morphogenetic events by other molecules such IGF I and II. Taken together, these results showed that mammalian extracellular matrix can be used for the optimization of fish cell culture.*

INTRODUCTION

Myosatellite cells located between the sarcolemma and the basal laminina of skeletal muscle fibers play an important role in muscle growth: they supply additional myonuclei in hypertrophy and after trauma new fibers arise from these cells. Interestingly, in contrast to mammals and birds where proliferation of satellite cells stops normally after birth, fish muscle development implies, beside hypertrophy (outgrowth of existing fibers), a continuous proliferation and differentiation of satellite cells.

The critical role of extracellular matrix components in adhesion, growth and differentiation of many cells is well known to date. It has been reported by example that laminin enhanced the adhesion rate of mammalian skeletal myoblasts on surface coated with type IV collagen when compared to fibronectin (Kühl et al., 1986). Furthermore, the formation of myotube is greatly enhanced *in vitro* with laminin (Foster *et al.*, 1987). In this study, we evidenced with mammalian antibodies, an immunoreactivity for laminin, type IV collagen and fibronectin at the surface of teleostean muscle fibers. Theses observations which demonstrated the phylogenetic conservation of matricial components antigens, especially those of basement membrane, lead to seed trout myosatellite cells on mammalian laminin-rich substrates.

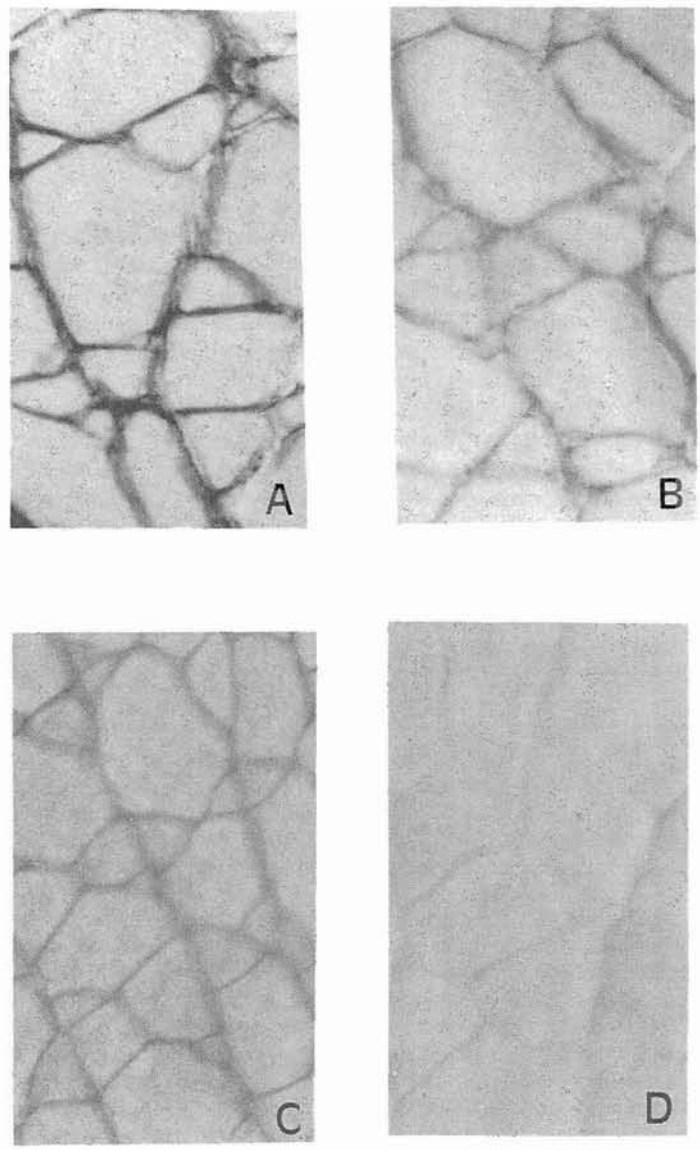

Figure 1 - Light microscopy, immunolocalization of fibronectin (A), laminin (B) and type IV collagen (C) in trout white muscle, control tissue section incubated with normal rabbit immunoglobulin (D). Cryostat sections : x 200

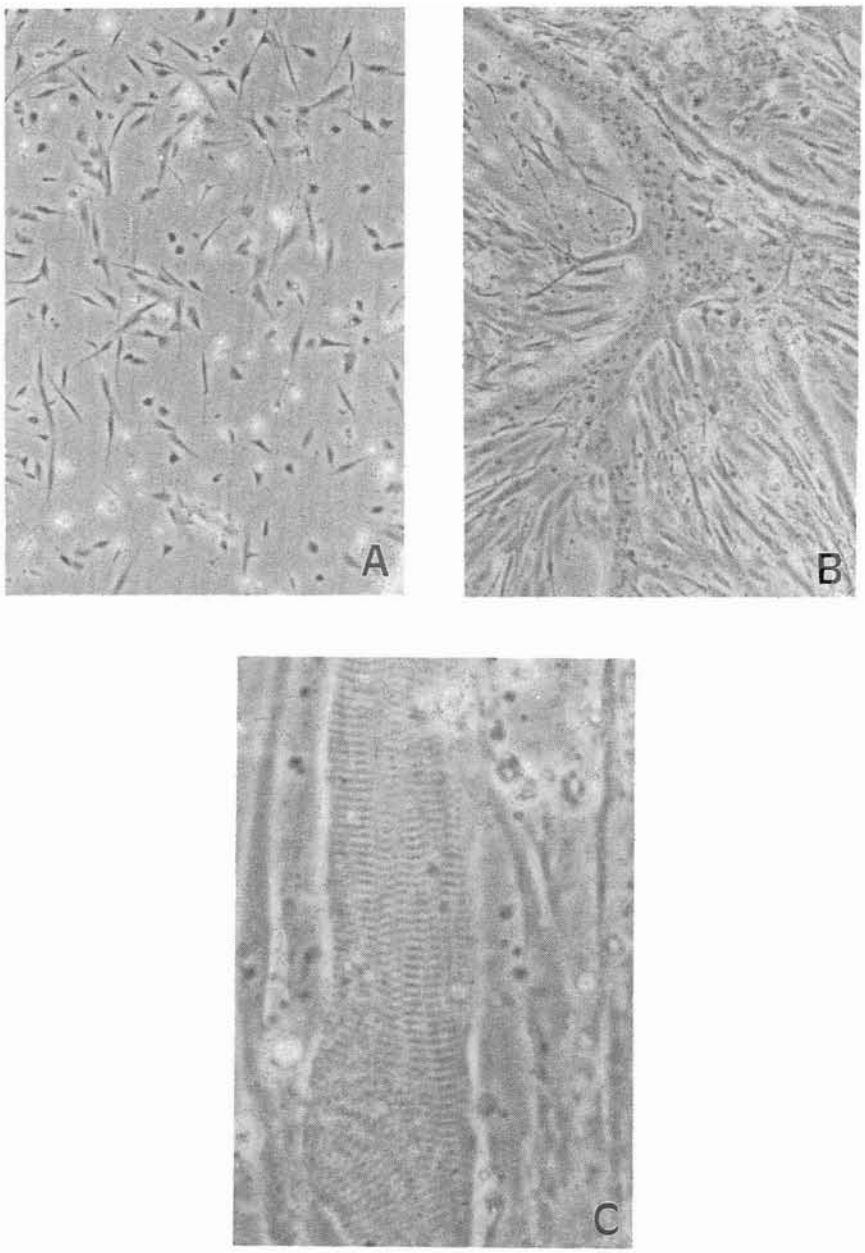

Figure 2 - Morphology of cultivated cells 24 hours after plating (A). Morphology of satellite cells derived multinucleated myotubes formed 8 days following plating (B), cross striation may be visible in some myotubes (C): x 200

RESULTS

MATRICIAL COMPONENTS RELATED TO FIBRONECTIN, LAMININ AND TYPE IV COLLAGEN SURROUNDED THE MYOFIBERS OF TELEOSTEANS

After fixation of trout muscle in a 4 % paraformaldehyde solution during 4 hours, cryostat sections were prepared and incubated with specific antibodies for laminin, fibronectin and type IV collagen. Specific immunoreactivity for these three matricial components was observed around muscle fibers (Fig. 1), whereas control sections incubated with normal immunoglobulins were devoid of labeling.Thus, the presence of basal membrane antigenic determinants at the surface of the trout myofibers suggested to cultivate myosatellite cells on laminin-rich substrates.

SATELLITE CELLS SEEDED ON LAMININ SUBSTRATES FORMED LARGE MULTINUCLEATED MYOTUBES

For isolation and cultivation of myosatellite cells, we took advantage of a protocol designed by Koumans *et al.* (1990). Briefly, white epiaxial muscle from 5 cm lenght trout was excised, minced, centrifuged and treated sequentially with a 0,2 % collagenase and a 0,1% trypsine solutions. Then fragments were triturated and the resulting suspension was filtered through a 50 µm filter gaze. Collected cells were then seeded and cultured in DMEM medium supplemented with 10 % calf serum and antibiotics. Isolated satellite cells adhered both on laminin and laminin/entactin substrates (Fig. 2, A), they proliferated, and formed large multinucleated myotubes (Fig. 2, B) which sometimes exhibited a typical cross striation (Fig. 2, C). Interestingly, such myogenesis was not promoted on surface devoid of laminin. This observation emphasized the significance of basement membrane components as substrates for the culture of myosatellite cells.

THE MYOTUBES FORMED *IN VITRO* EXPRESSED MANY MUSCULAR MARKERS

To validate our model, we have further studied the expression of many muscular markers. First of all, we analysed the expression of a teleostean cDNA isolated in our laboratory that appeared very related to the mammalian muscle regulatory factor MyoD. By northern blotting we observed a slight amount of the corresponding transcript at the beginning of the culture (4 hours after seeding), its level is dramatically increased in growing myosatellites cell cultures (48 hours after seeding) and remains elevated in myotubes until at least 11 days (Rescan *et a.l*, 1994). Thus, a major muscle regulatory factor homologue of MyoD is expressed in our cultures, indicating the muscular commitment of the cells. Furthermore, by immunocytochemical methods two proteins which expression is controlled by MyoD were also clearly detected in our system: desmin, a muscle-specific intermediate filament, and the heavy chain of myosin (Fig. 3, A). Altogether, these data indicated clearly the muscular phenotype of our cultivated cells.

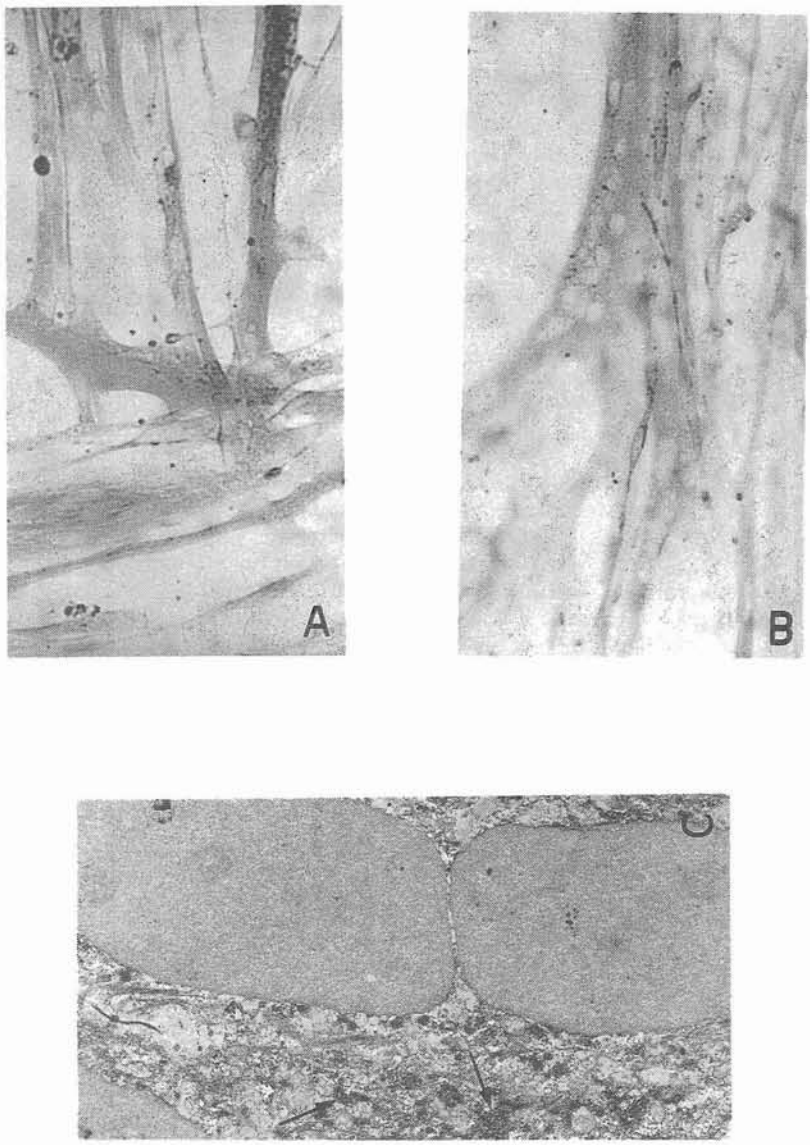

Figure 3 - Light microscopy, immunolocalization of the heavy chain of myosin (A) and type IV collagen (B) in satellite cells derived myotubes: x 200. Electron microscopy, immunolocalization of fibronectin in satellite cells derived myotube, intracellular labelling is restricted to the golgi apparatus and the endoplasmic reticulum (arrow): x 11000

THE MYOTUBES GENERATED *IN VITRO* RECONSTITUTED AN EXTRACELLULAR MATRIX SIMILAR TO THOSE DETECTED *IN VIVO*

Using laminin, fibronectin and type IV collagen antibodies, we evidenced an immunoreactivity for these three extracellular components at the surface of the neoformed myotubes (Fig. 3, B). Moreover an intracellular immunolabelling restricted to the golgi apparatus and the endoplasmic reticulum demonstrated the participation of the myotubes themselves to the synthesis of matricial components (Fig. 3, C). From these observations we can assert that *in vitro* the myotubes reconstitute at their surface an extracellular matrix similar to those detected *in vivo*. However, further studies are needed to demonstrate whether the synthesis of laminin chains by the teleostean satellite cells derived myotubes is associated, as in mammals (Kroll, 1994), with the expression of the myogenic factor Myod and the myogenic differentiation program.

CONCLUSION

Taken together, these results showed that the morphogenetic processes leading myosatellite cells to differentiated myotubes can be sum up *in vitro* when laminin-enriched substrates are used. Moreover, preliminary datas, showed that the use of laminin substrates do not prevent the modulation of morphogenetic events by other molecules such growth factors. Therefore, further studies on the regulation of satellite cells proliferation and differentiation may be carried out in our system. At last, we think that the use of mammalian basement membrane components as substrates may be intended to other fish and invertebrate cell culture models, especially laminin that has been shown to be closely conservated from drosophila to mammals and therefore may exhibited similar functional domains from one species to another (Fessler, 1987).

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ACKNOWLEDGEMENTS : *We would like to thank F. Levasseur and B. Clément (INSERM U49, RENNES) for the generous gift of antibodies and entactin/laminin substrate.*

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CULTURE,CRYOPRESERVATION AND IMMOBILIZATION OF *CALLIONYMUS LYRA* HEPATOCYTES

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Abstract - *Callionymus lyra* is a fish used as a sea water pollution bio-indicator. An IFREMER laboratory has shown that a hepatic enzyme was altered when fishes were captured in some sea areas. *Callionymus* hepatocyte cultures could represent a suitable tool to obtain information about the nature of the xenobiotics involved in these effects. A cell isolation method has been designed. Viability of the hepatocytes measured by trypan blue exclusion test was higher than 90 % and more than $30 . 10^6$ cells were obtained per liver. However, erratic availibility of these fishes, the high dissociation failure rate due to the stress of the fishes during capture and shipping, and the variable physiological state of the animals from different batches prompted us to store isolated cells obtained in excess. A cell bank should be set up with best cell suspensions. The cells were suspended in a medium containing 12 % dimethylsulfoxyde and the freezing rate was 5° C/min. Viability was not significantly decreased by the freezing process. However, both fresh and thawed cells poorly attached. To overcome this problem in getting good cultures, hepatocytes were entrapped in alginate beads. They remained viable during a 48 hour culture period at 4° C. Cultured or immobilised cells, either fresh or after thawing will be used to evaluate effects of potential sea pollutants.

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DEVELOPMENT OF SPECIFIC MARKERS FOR FISH BLOOD CELLS

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Abstract - *The usual characterisation of fish cells is performed by morphologic analysis in combination with cytochemistry. Recently, the identification of fish cells has been improved considerably due to the availability of conventional or monoclonal antibodies (MAbs) specific for cytoplasmic and/or cell surface molecules. The MAbs in particular form a dependable source of specific antibodies which can be used for detection, quantification and purification of defined molecules.*

It is not surprising that the panel of MAbs specific for fish cell markers is increasing (Secombes et al., 1983; Koumans-van Diepen, 1993). Different types of immunoglobulin (Ig) positive B lymphocytes or plasma cells can be identified or separated by using MAbs against serum or mucus Ig. In the meantime, MoAbs have become available for the detection of other blood cells, such as nonspecific cytotoxic cells, granulocytes or thrombocytes. However, MoAbs specific for fish T cells could not be produced till now. The major problem in producing fish leucocyte-specific MoAbs appeared to be the presence of a very immunodominant layer of carbohydrates (glycocalyx) at the surface of these cells. Tolerisation of young or adult mice against these common fish determinants or the use of highly purified antigens seems to be a promising new approach.

An other alternative may be the generation of "monoclonal" antibodies from Ig-gene libraries (McCafferty et al., 1990). In this novel technique the polymerase chain reaction is used to amplify cDNA encoding antibody heavy and light chain Fab fragments from B lymphocytes. Subsequently, a combinatorial library is formed which coexpresses, on the filomatous phage fd, Fab light and Fab heavy chains or a fusion protein wherein the variable domains of both chains are linked by a flexible 15 amino acid residue. This procedure generates an enormous diversity of antibody combinations. Positive phages can be rapidly selected by different panning procedures (e.g. against a particular fish cell marker). This new approach is faster than the usual procedure for MAb production and may make the immunization of large numbers of experimental animals superfluous in the future.

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DEVELOPMENT OF EMBRYONIC STEM (ES) CELLS IN HIGHER VERTEBRATES

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Abstract - Murine embryonic stem (ES) cells were first characterised by their ability to contribute to both germ line and somatic tissue development of the recipient host with high efficiency. Totipotency of ES cells was also demonstrated *in vitro* after their ability to differentiate into a wide variety of different cell types, including endoderm, mesoderm and ectoderm derivatives.

The ability to maintain ES cells *in vitro* as totipotent stem cells allowed genetic modification and subsequently production of new phenotypes in recipient mice. Presently, introduction of a transgene by DNA tranfection or retroviral infection, is able to produce stable and selected cells lines with defined criteria. Once reintroduced into recipient host, the genetically modified cells are able to transmit the mutation to their progeny and allow functional analysis of the transgene. This method appears to be very efficient and allows production of transgenic mice in a relative controlled way. New phenotypes such as resistance to diseases, or specific expression of proteins of interest can be generated by this method. Knocking out endogenous gene by homologous recombination is also widely used to investigate the role of any gene during development and adult life.

Based on the murine approach, an increasing number of laboratories is presently trying to identify embryonic stem cells in other mammalian or higher vertebrates species, including rabbit, pig, cow, sheep and chicken. As a first step towards this goal, one first has to define the *in vitro* culture conditions necessary to maintain the growth of undifferentiated stem cells. Concomitantly, one has to identify these putative stem cells using different criteria such as expression of specific antigens and specific enzymatic properties such as the alkaline phosphatase activity. The ability of such *in vitro* cultured cells to differentiate, to be induced in different lineages and to contribute to embryonic development when engrafted into a recipient host further demonstrate the totipotent nature of the isolated cells. The second part of the work will consist to define the culture condition necessary for indefinite culture of the previously identified embryonic stem cells. Established embryonic stem cells will be necessary to perform any genetic modification (gain-of-function or loss-of-function) as already done in the mouse.

CHAPTER IV

INDUSTRIAL CHALLENGES
PERSPECTIVES INDUSTRIELLES

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EVOLUTION OF ECOTOXICOLOGY IN THE RHONE-POULENC AGRO RESEARCH CENTER OF SOPHIA ANTIPOLIS

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Abstract - Pesticides are biologically active compounds deliberately released to the Environment. It is very important to predict and quantify any consequences of use.

Rhône-Poulenc Agro is concerned with the concentrations of chemicals that can be expected to occur in the Environment and to predict any effects that these concentrations may have on avian species - terrestrial wildlife and aquatic organisms. Ecotoxicity facilities have been developed at Sophia Antipolis in 1991 with for primary objective, to realise basic aquatic acute toxicity screening studies (Daphnids 48 h EC 50 - Trout 96 h LC 50) in order to support Research & Development of new compounds.

Since 1992, regulatory studies (acute and chronic toxicity studies) performed according to Good Laboratory Practice requirements have been developed for homologation purposes (Daphnids - Trout - freshwater green Algaes). The ecotoxicology approach is now being extended to other species such as Bluegills - Carps - Fathead minnows - Earthworms - Insects with the participation of the laboratory to international ring-tests.

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CARP (*CYPRINUS CARPIO*) HEPATOCYTES IN PRIMARY CULTURE: MORPHOLOGY AND METABOLISM[1]

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Abstract - *Teleosts show a high degree of diversity with respect to their ecology and biology. The present work describes aspects of the behaviour of primarily cultured hepatocytes isolated from two teleost species with contrasting physiology, the carnivorous trout Oncorhynchus mykiss and the omnivorous carp Cyprinus carpio.*

Isolation and culture: *Hepatocytes from trout liver are prepared by collagenase perfusion through the portal vein. Carp liver lacks a common portal vein, therefore, cell isolation is accomplished by perfusion through a branch of the dorsal aorta. Trout hepatocytes are delicate with respect to their seeding efficiency in plastic dishes, whereas carp liver cells readily attach to the culture substratum. Cell monolayers are maintained as monoloyers under air atmosphere in serum- and hormone-free media at 14°C (trout) or 20°C (carp). Viability (estimated as lactate dehydrogenase release) of hepatocytes from both species remains higher than 90 % during a three-day-incubation period, but declines thereafter.*

Morphology: *As evident from both qualitative and quantitative cytological examinations, isolated liver cells from trout and carp conserve an in-vivo-like ultrastructure during culture. Trout hepatocytes are much smaller than carp parenchymal cells (1200 μm^3 compared to 5500 μm^3). More than 20 % of the cell volume of trout hepatocytes is occupied by cisternae of the endoplasmic reticulum, whereas in carp liver cells the volume fraction of this organelle is less than 10 %. Within the first 12-24 hours of culture, the initially single cells start to aggregate and to form tubuli-like cell strands. Hepatocytes from both species establish cell junctions, however, only trout liver cells develop morphological structures identical to bile canaliculi. The absence of canalicular structures in carp hepatocyte cultures is related to the fact that carp liver in vivo possesses only intra-but not intercellular bile capillaries.*

Intermediary metabolism: *In vivo, trout liver is characterized by a high gluconeogenetic capacity but low glycogen levels. Carp liver, on the other hand, has a low gluconeogenetic capacity but high glycogen contents. These differences exist also in vitro. In cultured trout hepatocytes, lactate gluconeogenesis proceeds at a rate of 4.8 µmoles lactate incorporated /g protein/hour. The corresponding value in carp liver cells is 1.2 µmoles lactate incorporated/g protein/hour. Glycogen concentrations of carp hepatocytes are as high as 1000 mg glucosyl units/g protein, whereas trout cells usually contain not more than 200-300 mg glucosyl units/g protein.*

Xenobiotic metabolism: *Activities of ethoxyresorufin-O-deethylase (EROD) are approximately 10 times higher in trout than in carp hepatocytes, both in vivo and in vitro. This finding is in agreement with the ultrastructural*

[1] Supported by DFG (Se 466/2-1) and the State of Saxonia (7541.83 UFZ/305)

observations on the relative representation of the endoplasmic reticulum. Trout liver cells cultured for six to eight days display stable or even slightly increasing EROD activities. With carp hepatocytes, the initially low EROD activities decline further during culture. Preliminary studies indicate that the decrease of EROD activities in carp liver cells can be partly reduced by means of coculture with a fibrocytic fish cell line.

INTRODUCTION

Cultured hepatocytes are a promising tool to study liver physiology and toxicology in teleost fishes. However, since cellular structures and functions may change in acclimation to the *in vitro* environment, performance of the cultured cells needs sufficient characterization.

The vast majority of *in vitro* studies on teleost hepatocytes use freshly isolated cells (for reviews see Moon *et al.*, 1985; Baksi and Frazier, 1990). Such short-lived systems preclude the investigation of cellular responses which are detectable only after periods of many hours or days, e.g. the longterm effects of hormones or the chronic impact of toxicants. Techniques for prolonged culture of piscine liver cells *in vitro*, however, became available only recently (*Anguilla japonica*: Hayashi and Ooshiro, 1985; *Ictalurus punctatus*: Koban, 1986, *Oncorhynchus mykiss*: Maitre *et al.*, 1986; Mommsen and Lazier, 1986; Blair *et al.*, 1990). In this communication, we present few, selected data on the morphological and metabolic performance of carp, *Cyprinus carpio*, hepatocytes cultured as monolayer in chemically defined, serum-free media.

MATERIAL AND METHODS

Hepatocytes were isolated from 2-year-old carps by *in situ*-collagenase perfusion through the A. coeliaca. The isolated cells were collected and washed by centrifugation at 80 x g, resuspended in culture medium and seeded to Petri dishes (Falcon, Primaria) at a density of 1.5-2.0 x 10^6 cells/ml. The medium was composed of a modified Hank's buffer, MEM amino acids, basal media Eagle's vitamin mixture, glutamine, and antibiotics (for details see Segner *et al.*, 1993). The cells were maintained in a humified air atmosphere at 20°C.

Preparation of cell monolayers for electron microscopical investigations was done as described by Vogt *et al.* (1993). The stereological procedures are given in Segner and Braunbeck (1990). Enzymatic and protein analyses were performed according to Böhm *et al.* (submitted). 7-Ethoxyresorufin-*O*-deethylase (EROD) activity was measured by a direct fluorometric technique on a Perkin Elmer LS50B. Rates of lipogenesis were estimated from the incorporation of U-^{14}C-acetate into the cellular lipid fraction, rates of protein synthesis were estimated from the incorporation of U ^{14}-leucine into the TCA-precipitable cell fraction, and cellular glucose release was analysed by the glucose oxidase method (cf. Segner *et al.*, 1993).

RESULTS AND DISCUSSION

Stereological studies demonstrate that approximately 80 % of the volume of carp liver *in vivo* is occupied by hepatocytes, the remaining volume belonging to biliary epithelial cells, sinusoidal endothelial cells, fatstoring cells of Ito, (resident) macrophages as well as bile and blood spaces. In addition, carp liver tissue is intimately intermingled with parts of the exocrine pancreas. After collagenase perfusion of carp liver, the resulting lowspeed cell pellet is composed of approximately 90 % hepatocytes and 10 % pancreocytes, whereas the other cell types quantitatively remain in the supernatant (see also Bouche *et al.*, 1979).

When freshly isolated carp hepatocytes are added to culture plates, the preparation generally appears as a single-cell population, with some doublets. More than 85 % of the seeded carp liver cells firmly attach to plastic dishes within the initial six hours of culture. On the contrary, the adhesion of trout hepatocytes to uncoated dishes has been found difficult (cf. Blair *et al.*, 1990). During the first 12-24 hours of culture, carp liver cells start to aggregate and to form large, tubulilike cell strands. Between adjacent cells, tight junction-like plasma membrane specializations develop, indicating the existence of specific cell-to-cell interactions. However, contrary to trout hepatocytes (Blair *et al.*, 1990; Braunbeck and Storch, 1992), carp liver cells in culture do not re-establish bile canaliculi. Most likely, this is due to a particular organization of the canalicular system in carp liver *in vivo* (see Langer, 1979).

The intracellular composition of carp hepatocytes experiences no significant alterations during a 72-hour-culture period (Fig. 1).

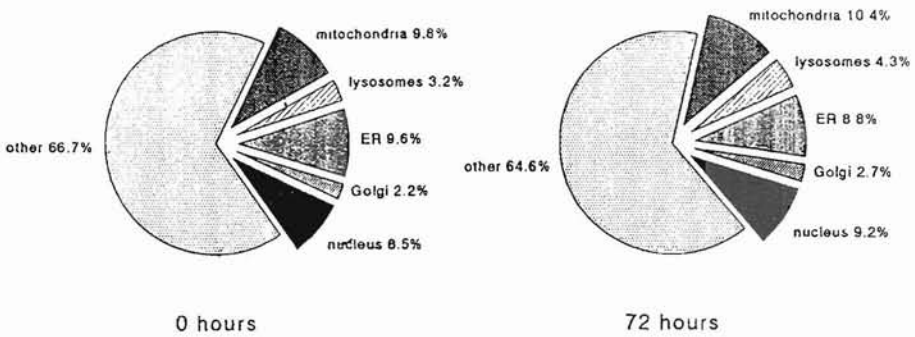

Figure 1 - Stereological evaluation of the subcellular composition of freshly isolated and 72 hours cultured carp hepatocytes. The data are mean values ± SEM from three independent incubations. The quantitative representation of the organelles within the cells remains constant during the *in vitro*-incubation. The endoplasmic reticulum (ER) consists almost exclusively of rough ER. The main component of the fraction "others" is glycogen. As confirmed in biochemical studies, cultured carp hepatocytes contain remarkably high and stable glycogen concentrations (up to 1200 mg glucosyl units/g protein).

In order to reveal whether the observed constancy of cellular structures indicates a conservation of metabolic functions as well, we measured 15 different marker enzymes from various metabolic pathways and/or cell organelles. An example from these studies is given in Figure 2. The enzymes glucose-6-phosphatase (G6Pase) and 7-Ethoxyresorufin-*O*-deethylase (EROD) are associated with the ER (microsomal fraction) of carp hepatocytes, as we proved in previous cell fractionation studies. The activity of G6Pase in carp hepatocytes remains stable during a 72-hour-incubation period (Fig. 2) what correlates with the stability of the ER volume (Fig. 1). The EROD activity, on the other hand, significantly decreases *in vitro*. Such a loss of hepatocellular drug-metabolizing capacity takes also place in cultured rat hepatocytes (Guguen-Guillouzo *et al.*, 1988) but does not occur in trout hepatocytes which display stable or even increasing EROD activities during culture (Pesonen and Anderson, 1991; Masfaraud *et al.*, 1992, Scholz, unpublished). Preliminary experiments on coculture of carp hepatocytes with a fibrocytic fish cell line failed to stabilize hepatocellular EROD activities (data not shown).

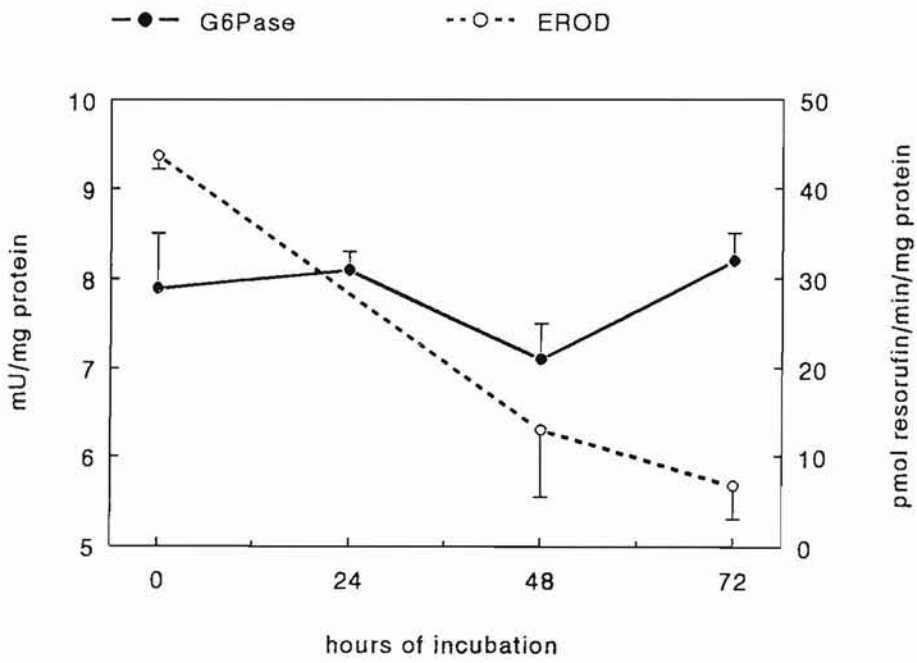

Figure 2 - Time-dependent changes of the activities of glucose-6-phosphatase (G6Pase, measured as µmol inorganic phosphate produced/min/mg protein) and 7-Ethoxyresorufin-*O*-deethylase (EROD, measured as pmol resorufin produced (min/mg protein) in cultured carp hepatocytes (0-72 hours). The data represent mean values ± SEM from three independent incubations.

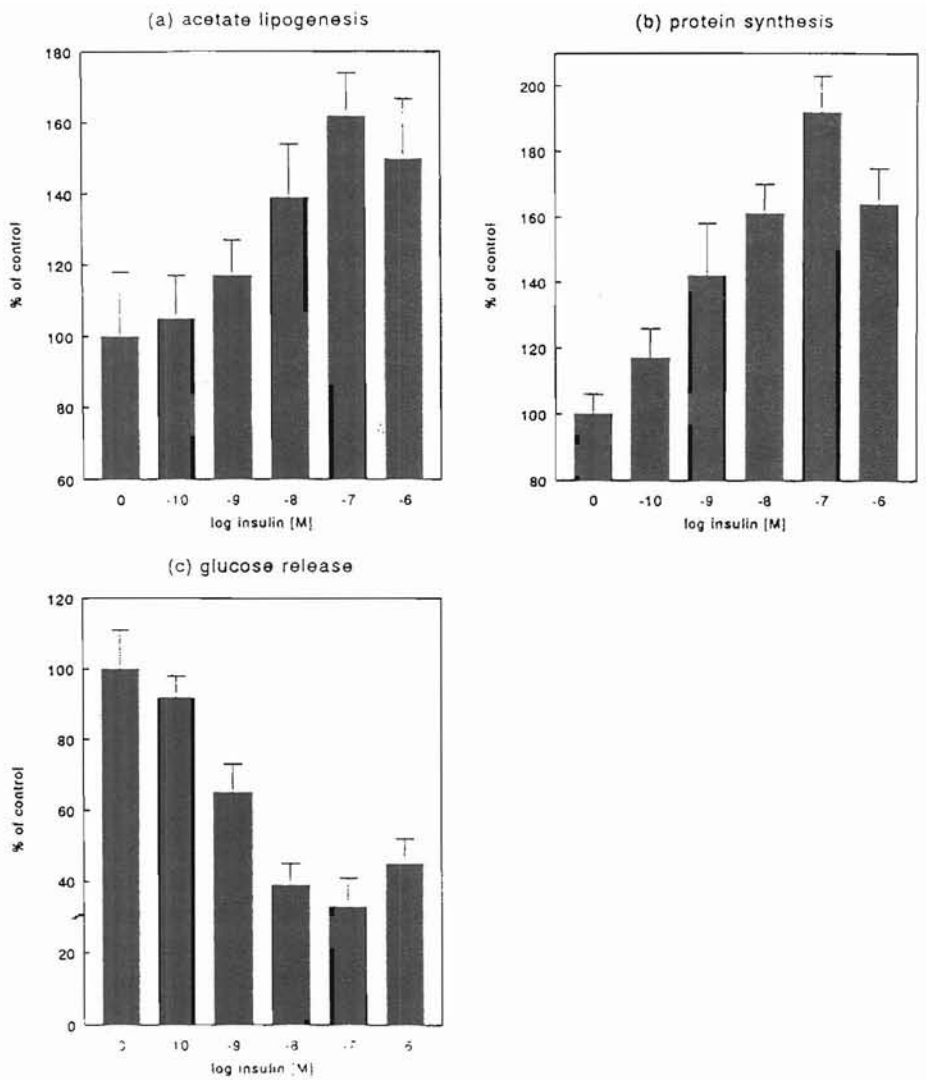

Figure 3 - Dose-dependent response of (a) lipogenesis (incorporation of ^{14}C-acetate into lipid), (b) protein synthesis (incorporation of ^{14}C-leucine into protein) and (c) glucose release into the medium to (bovine) insulin. The cells were pre-incubated for 12 hours to allow for recovery from the isolation procedure. Thereafter the cells were incubated with the hormone for another 12 (lipogenesis, protein synthesis) or 24 hours. Compared to results obtained after 1 or 4 hours of hormone exposure, insulin effects were more clearly expressed after the 12- or 24-hour-incubation period.

In vivo, liver metabolism is under strict endocrine control. Therefore, it is important to verify that the hepatic cells *in vitro* remain sensitive to hormonal regulation. In Figure 3, the dose-dependent action of insulin on the *de novo* synthesis of fatty acids, the protein synthesis and the glucose release of cultured carp hepatocytes is shown. The results demonstrate the conservation of hormone responsiveness in cultured carp liver cells. This agrees with previous findings on the presence of insulin receptors in cultured carp liver cells (Segner et al. 1993).

CONCLUSION

Only few data could be shown in the present communication, but they may illustrate that isolated carp hepatocytes maintain many differentiated hepatic functions during monolayer culture in defined media. The drug-metabolizing capacity, however, seems to decrease *in vitro*, as indicated by the findings on EROD activity. Since this represents a limitation, particularly with respect to toxicological studies, future work has to focus on the development of improved culture techniques supporting the stable expression of cytochrom-P-450-dependent enzyme activities in cultured carp liver cells.

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MARINE CELL CULTURE FOR TOXICOLOGICAL STUDIES

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Abstract - Marine animal cell cultures are becoming attractive *in vitro* models. Preliminary works have been performed with isolated gill and heart *Crassostrea gigas* cells exposed to mercury or cadmium. Neutral red incorporation, tetrazolium salt reduction, fluorescein diacetate hydrolysis and oxygen consumption have been selected to assess cell viability. Mercury was found to be more cytotoxic than cadmium. Identification of biochemical targets is in progress, using experimental approaches presently used for mammalian cells.

The chemiluminescence assay performed on whole cells in the presence of zymozan, now automated, will allow to evaluate effects of xenobiotics on the functions of the hemolymph cells. Other functional assays will be used as endpoints, simultaneously for the optimization of cell culture conditions and characterisation of cell types present in the cultures.

The cell models combined with appropriate test parameters should allow to better predict and understand ecotoxicological phenomena.

MONITORING THE BIOCHEMICAL EFFECTS OF POLLUTANTS IN MARINE ORGANISMS

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Abstract - Different approaches have been used for monitoring the biochemical effects of pollutants: benthic communities studies, ecotoxicological tests and molecular approaches. The use of biomarkers has been shown to be a valuable tool fitting the needs for setting up a network. Our work describe the strategy adopted for monitoring the biochemical effects of pollutants in fishes along the European coasts. Three main constraints were identified: the choice of the sites to be monitored, the choice of suitable target species and the selection of adequate biomarkers. Induction of Ethoxyresorufin-O-deethylase, a cytochrome P450 dependant monooxygenase, and inhibition of acetylcholinesterase were measured in fishes as a response to the presence of respectively aromatic (polychlorobiphenyls, polyaromatic hydrocarbons and dioxins) and both organophosphorus and carbamate compounds in the marine environment.

Present and future of monitoring are presented together with the development of new tools enabling the evaluation of biological responses of marine organisms to the presence of pollutans at an oceanographic scale.

European Workshop in Brest (France) April 28-30, 1994
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INSECT CELL CULTURE : INDUSTRIAL APPLICATIONS

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Abstract - Insect cells compete with other expression systems, among which the most widely used are bacteria (*Escherichia coli*), yeasts, mammalian cells. Many complex proteins do not meet biological activity criteria when produced in non-animal hosts. Conversely, mammalian cells generally express proteins correctly but at a high cost and with little flexibility. In insect cells, complex proteins are produced cost-effectively, under a biologically active form. These cells grow in low-cost culture media and secrete high levels of proteins.

The insect-cell system is a flexible process. There is no need for a lengthy generation of a specific cell line for each genetic construction : any desired change in the sequence of the gene may be implemented quickly through a modification of the Baculovector, while cell cultures are maintained independently and left unchanged. This system allows the fast and easy expression of large genes as well as co-expression of two or more genes from a single vector ; hence it is suitable for the synthesis of dimers, heterodimers, and oligomeric proteins (i.e. IgGs, IgAs, receptors, pseudoviruses, hormones) in a time-effective manner.

Efficient cultivation methods are a key issue for the industrial exploitation of insect cells system. The most promising approach involves cell culture in suspension using bioreactor. As infection of cells with baculovirus results after a few days in the death of the culture the process is a batch one. The maximal cell density currently obtained in a batch is on the range of 1 to 4.10^6 cells/ml. To improve recombinant protein production new culture processes are under development in order to increase cell density using perfused cultures with internal or external cell filtration.

From a regulatory standpoint, we believe that insect cells will be acknowledged as the safest animal-cell host. Indeed the likelihood of transmission of any known or unknown pathogen to man is minimal thanks to :

- the species barrier between insects and mammals : only few vertebrate virus, like arbovirus can replicate in both insect and vertebrates cells and fortunately these viruses are unable to replicate in lepidopteran cells used in the baculovirus-insect cell system
- the ability to establish continuous cell lines without using any viruses or genes, with culture conditions (26-28°C) that may not allow the growth of many vertebrate viruses
- the possibility to grow in protein-free media
- baculovirus does not transform vertebrate cells and are unable to replicate or persist in mammalian cells.

CHAPTER V

INSECTS
INSECTES

European Workshop in Brest (France) April 28-30, 1994
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THE EPITHELIAL CELL LINE FROM *CHIRONOMUS TENTANS* : HORMONAL REGULATION OF TISSUE DIFFERENTIATION AND CUTICLE-FORMATION

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Abstract - *The epithelial cell line from Chironomus tentans, established in 1982 by Wyss, is different from most other cell lines, since it grows exclusively as multicellular, monolayered vesicles. The vesicles can reach sizes in the mm range. Vesicles are not formed by aggregation of cells, but by cell division from a single cell. First an intracellular vacuole is formed and than cell proliferation starts. The cells in a vesicle are connected by all types of cell contacts, specific for insect epithelia.*

Moulting hormones (ecdysteroids) exert two different types of morphogenetic actions in this cell line: 1) they inhibit in a dose dependent and hormone specific way cell proliferation and thus also the formation of vesicles and 2) already formed vesicles change their structure: the originally squameous epitheliar cell monolayer forms a columnar and stratified epithelium. Concomitant with this process there is an increase in microtubules which are often arranged preferentially along the main axis of the cells. Tubulin and actin content increases. The increased expression of non-neuronal acetylcholinesterase and a transient increase in the muscarinic acetylcholine receptor accompany these morphogenetic events.

In addition to the effects on differentiation, moulting hormones also evoke regulations in metabolic processes which are typical for insect epidermis, like chitin metabolism and sclerotization. Chitin synthesis is inhibited by moulting hormones, whereas the expression and secretion of chitin degrading enzymes increase. Dopa decarboxylase -a key enzyme of sclerotization - is transiently increased, but depressed in the permanent presence of hormone.

The effectiveness of various moulting hormones and synthetic moulting hormone agonists corresponds quite well with their affinity to the intracellular ecdysteroid receptor from the cells.

INTRODUCTION

The suitability of permanent embryonic insect cell lines for studying ecdysteroid regulated processes has already been stressed severalfold (Dinan *et al.*, 1990; Porcheron, 1991). In contrast to other insect cell cultures, the epithelial cell line from *Chironomus tentans* (Wyss, 1982) grows exclusively as multicellular vesicles. Since each cell is connected to neighbouring cells by cell contacts, which are typical for insect epithelia, this cell line has basic characteristics of a permanent tissue culture (Spindler-Barth *et al.*, 1992).

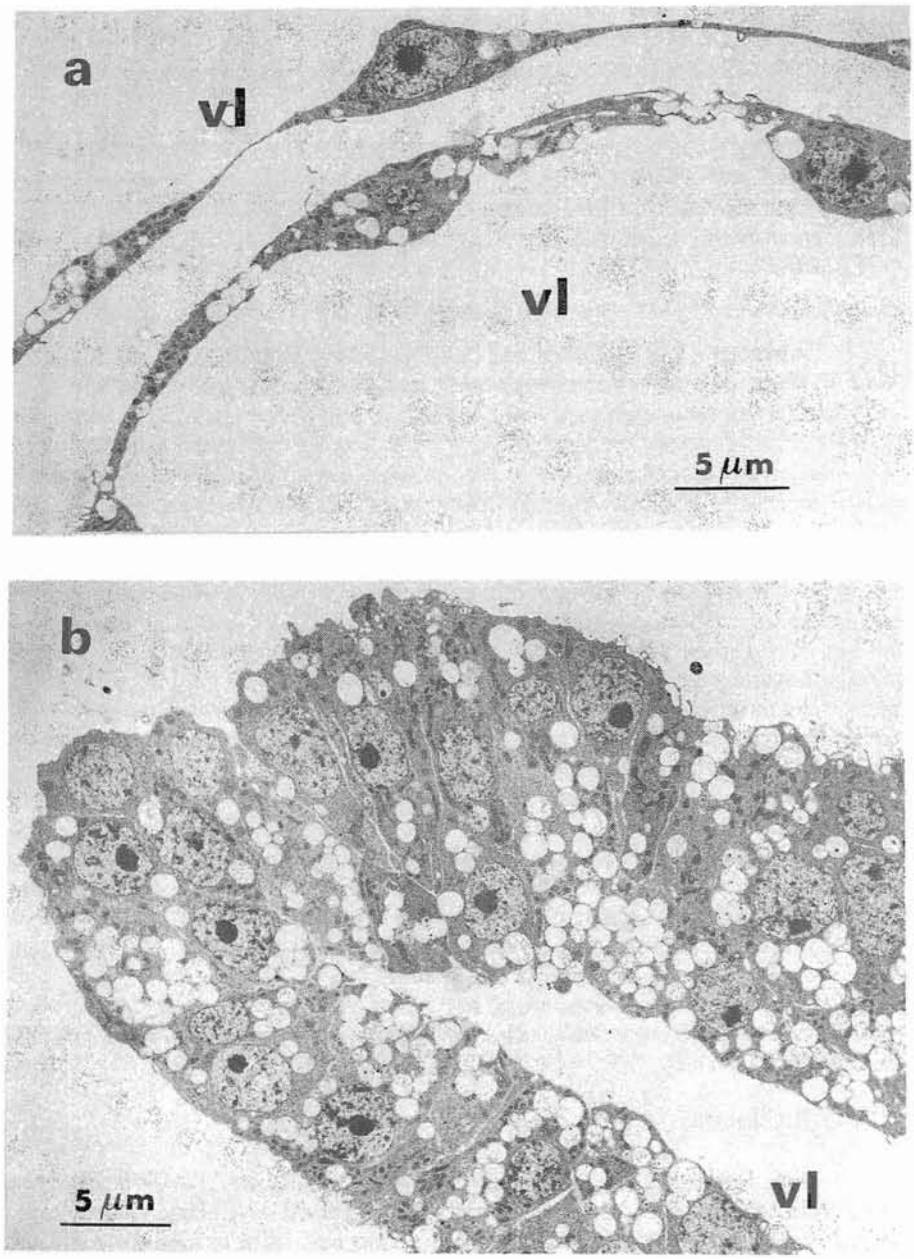
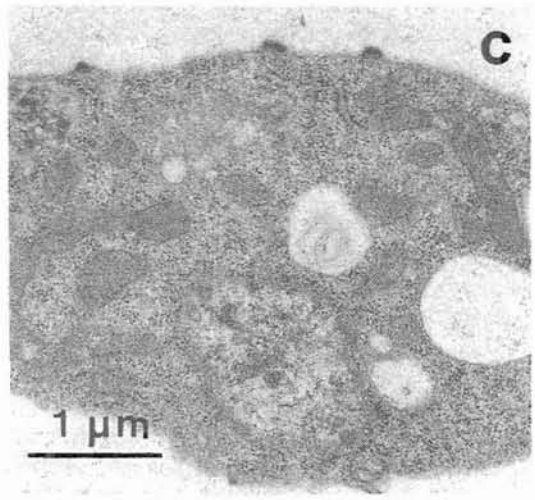


Figure 1 - a) Section profiles of thin platelike epithelial cells of two adjacent vesicles from *Chironomus tentans. b)* Rearrangement of the squamous epithelium into a stratified columnar epithelium after treatment with 1 μM 20-OH-ecdysone (4 days).



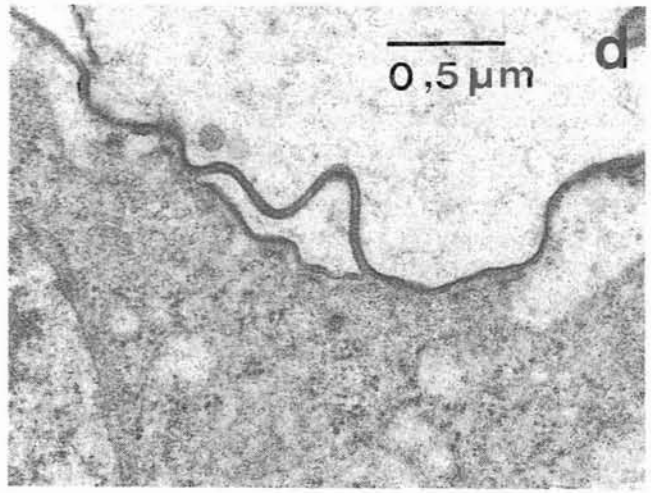


Figure 1 - c) small patches (control) and d) sheets of cuticulin deposits of the apical surface of vesicles (hormone treated).

An intracellular ecdysteroid receptor, which acts as ligand dependent transcription factor, with similar binding characteristics (Turberg *et al.,* 1988) and a high sequence homology of the corresponding gene to the *Drosophila melanogaster* ecdysteroid receptor is present (Imhoff *et al.,* 1993). Since metabolism of ecdysteroids added to the medium is rather low in the *Chironomus* cell line, longterm incubations with hormone under controlled conditions are possible (Spindler and Spindler-Barth, 1991). Therefore this cell line is especially suited to study hormonally regulated late events.

RESULTS AND DISCUSSION

The various ecdysteroid regulated effects observed in the epithelial cell line from *Chironomus tentans* can be divided in two groups: tissue differentiation and cuticle formation and degradation.

TISSUE DIFFERENTIATION

In the presence of ecdysteroids the squamous monolayer, which forms the multicellular vesicles of the *Chironomus* cell line, changes into a columnar, partially stratified epithelium (Fig. 1a, b; Spindler-Barth *et al.,* 1992) and is accompanied by a decrease in cell proliferation as measured by thymidine-incorporation (unpublished observation). This process resembles the ecdysteroid-induced changes observed during evagination of imaginal discs in Drosophila (Fristrom and Fristrom, 1993). The morphological changes in the cell line are accompanied by an altered pattern of newly synthesized proteins; most prominent is the increase in tubulin content (Fretz, unpublished observations), which can also be demonstrated morphologically. Simultaneously acetylcholinesterase activity increases up to 20fold (Spindler-Barth, *et al.,* 1988), which is considered as marker for differentiating cells. Quantitative evaluation of Western-blots revealed a transient increase of muscarinic acetylcholine receptors (Fig. 2) which is a central part of an embryonic muscarinic system involved in tissue differentiation both in vertebrates and invertebrates (Fig. 3). Immunohistochemically, an increased number of muscarinic acetylcholine receptors can also be demonstrated especially in the same regions, which are engaged in the morphogenetic process (Lammerding-Köppel *et al,.* in press). G_o-proteins, which are often associated with muscarinic receptors in vertebrates, are present in *Chironomus* cells (Wichelhaus and Eckels, personal communication), although their participation in signal transduction *via* the muscarinic receptor and the type of second messengers involved is not examined. According to preliminary results an increase in cGMP seems possible (Wegener, 1991). In differentiating chicken limb buds inositolphosphates and Ca^{2+} are involved in changes in cell shape 2 (Lohmann *et al.,* 1991). Whether this is also the case in *Chironomus* cells is not yet known.

CUTICLE FORMATION AND DEGRADATION

The main action of ecdysteroids in arthropods is the regulation of the molting cycle, which requires the periodic degradation of the old cuticle before molt and the subsequent formation of a new one. In *Chironomus* cells cuticle like structures can be seen at the apical side, which faces the culture medium, where numerous microvilli are present. Initially small spots are visible which combine lateron to sheets which cover the outside of the vesicles (Fig. 1c, d). Both, chitinases

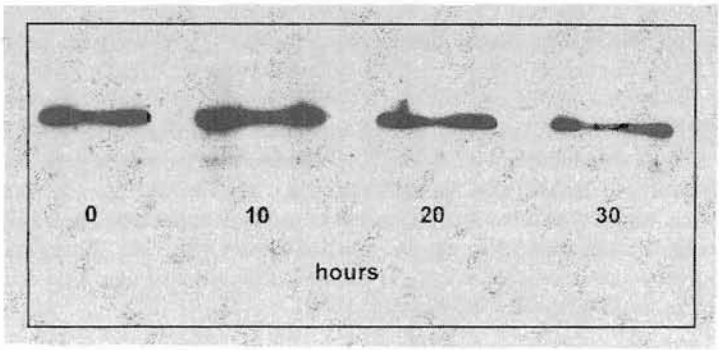


Figure 2 - Influence of 1 µM 20-OH-ecdysone on the muscarinic acetylcholine receptor in the *Chironomus tentans* cell line. Antibody M35 (directed against muscarinic receptor from calf brain; Lammerding-Köppel *et al.*, in press) was used for Western blots.

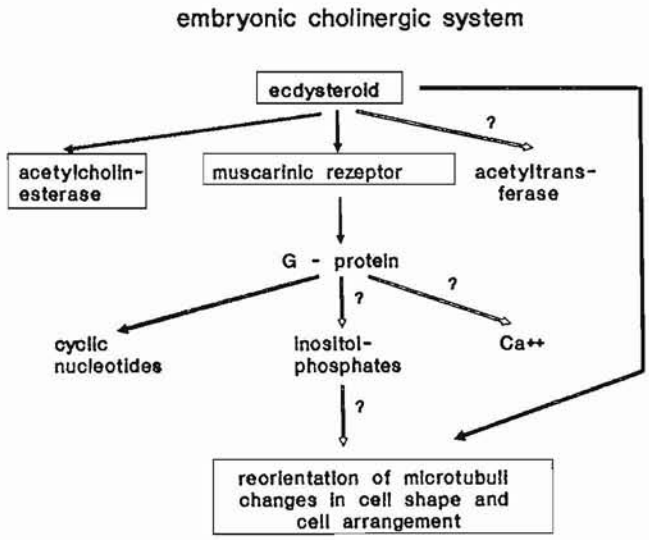


Figure 3 - Scheme of the "embryonic cholinergic system" in the cell line from *Chironomus tentans*. Dark arrows = experimentally verified in the *Chironomus* cell line.

and N-acetlyl-ß-D-glucosaminidases, are present in *Chironomus* cells and are secreted mainly into the culture medium (Spindler-Barth, 1993). After incubation with 20-OH-ecdysone the activity of both types of enzymes increases (own, unpublished results). In contrast, chitin synthesis is inhibited in the presence of ecdysteroids (Baumeister *et al.,* 1992). Dopadecarboxylase, a key enzyme for sclerotization of the cuticle, exhibits a dual regulation. In the presence of ecdysteroids, enzyme activity is diminished compared to controls, but after removal of hormone activity increases above control levels (Fig. 4). This type of dual regulation of genes involved in cuticle formation is already decribed for imaginal discs of *Drosophila* (Apple and Fristrom, 1991).

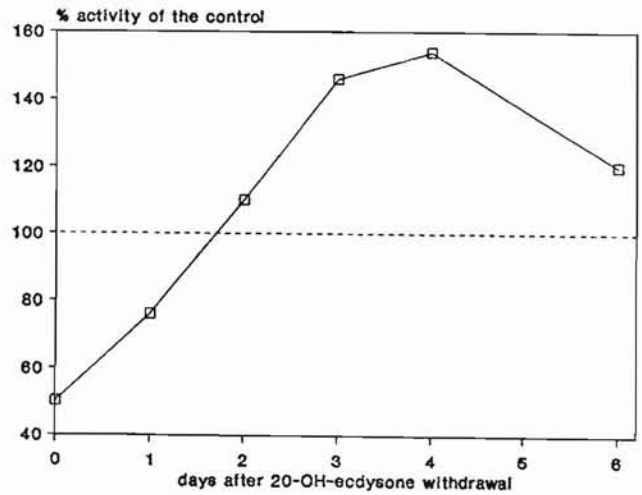


Figure 4 - Dual regulation of dopa decarboxylase activity in the epithelial cell line from *Chironomus tentans*. Cells were treated for 1 d with 1 µM 20-OH-ecdysone and then kept hormone free for the time periods indicated (determined according to Baumeister *et al.,* 1992; n = 3, S. D.< 5 %).

CONCLUSION

The various ecdysteroid regulated effects in the epithelial cell line from *Chironomus tentans* can be divided in two groups, one involved in tissue differentiation, the other in cuticle formation and degradation. The influence of molting hormones is quite different for various genes. Increase (acetylcholinesterase, chitinase, N-acetyl-glucosaminidase), inhibition (chitin synthase, dopadecarboxylase), transient changes (muscarinic receptors) and induction by a fall in ecdysteroid titer (dopadecarboxyl-ase) are observed. Time course and extent of induction vary also in a gene specific mode: About 20 fold stimulation in the case of acetylcholinesterase and 2 fold stimulation of chitinase activity. Since these different modes of hormonal regulations are caused by the same ecdysteroid receptor, additional factors must be involved in the regulation of these late ecdysteroid dependent genes.

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PHYSIOLOGICAL DIFFERENTIATION OF EMBRYONIC INSECT CELLS IN CULTURE

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Abstract - Early embryonic stages of some insect species can be dissociated and the cells cultured *in vitro* for several weeks. Such embryonic primary cell cultures offer a number of new possibilities to investigate problems of developmental biology and insect physiology. *In vitro*, the cells morphologically and functionally differentiate into various larval cell types, the best investigated being muscle-, fat body-, and imaginal disc cells. It is a major advantage of the system that no endocrine cells seem to assemble into functional endocrine glands and that no ecdysteroids are synthesized. Therefore, the cells remain hormonally naïve and constitute ideal experimental material for the investigation of ecdysteroid metabolism.

Using dissociated embryos of *Drosophila melanogaster*, we could show that cultured cells can express enzymatic activity for the biological activation and inactivation of ecdysteroid hormones, and that these processes are under a highly sensitive feedback control. The system also allowed us to determine the hormonal requirements for metamorphic development better than we could ever do by working with whole animals.

Some cell types, among them probably the stem cells of the haemocytes, are mitotically active in culture and give rise to large colonies, out of which permanent cell lines can be established. Presently, such cell lines are gaining importance for the investigation of new ways of biological insect pest control.

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USEFULNESS OF INSECT CELL CULTURE

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Almost seventy-five years ago insect tissue culture was a tool for pathfinders entomologists, embryologists and physiologists, asking questions about organs, embryos or imaginal discs development, endocrinological conditions for growth and differentiation (Day and Grace, 1959; Lenoir-Rousseaux and Lender, 1975; Landureau, 1976; Reddy, 1977). After *in vitro* explantation of ovaries, testis, brains, neuro-hemal organs, imaginal discs, epidermis, whole embryos, regenerats, primary cell culture appeared after mechanical dissociation from embryos at the gastrula stage or imaginal discs (Marks, 1980).

An important step was accomplished with the establishment of the first cell line from *Antherea* and *Drosophila*, the elaboration of defined media and the availability of the two developmental insect hormones: ecdysteroïds and juvenoïds as synthetic molecules (Oberlander, 1980; O' Connor and Chang, 1981; Mitsuhashi, 1982; Marks *et al.*, 1983; Riddiford, 1984; Oberlander and Miller, 1987; Yund, 1989; Dinan *et al.*, 1990) and the availability of growth factors (Porcheron, 1991).

Enzymatic dissociation was also performed from organs. Different cell types were cultivated such as hemocyte, visceral and striated muscles, fat body, neurones and epidermis.

The mealworm, *Tenebrio molitor*, a convenient model for developmental biology and endocrinology illustrates this short historical reminder. *In vitro* experiments started thirty years ago with organ explantation, genital apparatus, epidermis, regenerats. Isolated cell culture is available since ten years in Landureau's S20 medium and now in Insect X-Press medium (Bio-Whittaker) with or not fetal calf serum. Mesodermal male accessory glands obtained from newly ecdysed pupae produce after trypsinisation adherent cells that differentiate within a week in epithelial and muscle cells. Ultrastructural observations reveal that the spontaneous contracting visceral muscle presents contracting material in mononucleated cells. Epithelial cells offer granules that resemble to the several types that constitute the specific secretions of the spermatophora. Epidermal cells from young pupae wings aggregate and surround the production within a week of concentric sheets of cuticular layers. After six weeks of culture, cells dissociate from the cuticular nodule and produce either empty vesicle or flatten colonies growing in size up to more than 20 weeks by renewing the medium half part each week. Cuticular nodules present sheets of cuticle arranged in an helicoïdal way. This cuticle produces *in vitro* proteins that are recognized with a specific antibody built against the pre-post exuvial cuticle of *Tenebrio*. This cuticle is able to incorporate D-[6 $^{-3}$H]-glucosamine that is partly specific to chitin, increasing for 36 hours and then reaches a plateau in the presence or not of fetal calf serum. Epidermal cells from empty floating vessicle show a reverse polarisation from nodules with microvilli outside under a loose network of secreted material. Cells full of ribosomes and presenting vacuolae are tightly attached with overlaping convolutions of the cytoplasmic membrane.

Cells either mesodermal or epidermal are picked up while they are dividing in the early pupa. During the normal course of development in the pupa, the

Tab.

Fish, Insect and Mollusc Lines Held at the European Collection of Animal Cell Cultures			
SPECIES	**NAME**	**ECACC NO**	**DESCRIPTION**
Fish			
	BB	87101201	Fish Brown Bullhead posterior trunk tissue
	BF-2	87032603	Fish Bluegill fry caudal trunk
	CAR	89072611	Goldfish fin tissue
	CHH-1	92110412	Fish Chum heart
	CHSE-214	91041114	*Salmon* embryo
	EPC	93120820	Fish epithelioma
	FHM	88102401	Fish Fat Head minnow
	Grunt Fin GF	88010601	Fish Grunt fin tissue blue striped
	RTG	90102529	Rainbow trout gonad tissue
Insect			
	311	90070547	*Drosophila melanogaster* embryo
	AVL/CTVM 17	94022811	Tick *Amblyomma variegatum* larvae
	Aedes aegypti	87091801	Mosquito larvae
	Aedes albopictus	90100401	Mosquito larvae
	Antheraea cells	90111908	Moth (Lepidoptera) ovary
	BDE/CTVM 14	94022808	Blue Tick *Boophilus decoloratus* eggs
	BME/CTVM 4	94022804	Tick *(Boophilus microplus)* eggs
	BME/CTVM 6	94022803	Tick *(Boophilus microplus)* eggs
	Clone C6/36	89051705	Mosquito larvae, *Aedes albopictus*
	D1	90070552	Drosophila embryo
	D2	90070546	Drosophila embryo
	DH14	90070551	Drosophila embryo
	DH15	90070549	Drosophila embryo

SPECIES	NAME	ECACC NO	DESCRIPTION
	DH33	90070559	Drosophila embryo
	DM1	90070555	Drosophila embryo
	DM3	90070548	Drosophila embryo
	HAE/CTVM 11	94022801	Tick *Hyalomma anatolicum anatolicum* embryo (eggs)
	HAE/CTVM 15	94022810	Tick *Hyalomma anatolicum anatolicum* embryo
	HAE/CTVM 8	94022802	Tick *Hyalomma anatolicum anatolicum* embryo
	HAE/CTVM 9	94022809	Tick *Hyalomma anatolicum anatolicum* embryo (eggs)
	IZD-MB-0503	93090714	Lepidoptera *Mamestra brassicae*
	KC	90070550	Drosophila embryo
	MEL 9	90070553	Drosophila embryo
	Mshi	90070560	Drosophila embryo
	Neopu	90070561	Drosophila embryo
	P1	90070556	Drosophila embryo
	P2	90070558	Drosophila embryo
	P4	90070557	Drosophila embryo
	RAE/CTVM 1	94022805	Tick Brown Ear *Rhipicepahlus appendiculatus* eggs
	RAN/CTVM 3	94022806	Tick Brown Ear moulting nymph
	SL2	90070554	Drosophila embryo
	Sf9	89070101	*Spodoptera frugiperda*
	TRA-171	90120514	Mosquita larvae
	Tn 5B1-4 (High)	94020901	*Trichoplusia ni*
	ZIZ0LD-1	93022523	Colarado potato beetle, *Leptinotarsa decemlineata*
Mollusc			
	Bge	90120506	Mollusc embryo, *Biomphalaria glabrata*

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Le premier colloque européen traitant des cultures de cellules d'animaux aquatiques et terrestres s'est déroulé à Brest, du 28 au 30 avril 1994, sous le patronage de la Société Européenne de Culture de Tissus (SECT) de l'Université de Bretagne occidentale et de l'IFREMER.

Des spécialistes, venus d'Allemagne, de Belgique, de Grande-Bretagne, d'Italie, des Pays-Bas, de Suisse ainsi que de Polynésie Française et d'un certain nombre de villes françaises, se sont rencontrés pour faire le point sur des modèles expérimentaux nouveaux développés à partir d'organismes aussi divers que les insectes, les bivalves marins (coquilles Saint-Jacques, huîtres, moules), les échinodermes (oursins), les crustacés (crevettes) et les poissons.

Cette réunion scientifique a aussi été l'occasion d'échanges entre chercheurs et industriels. Une session a été organisée conjointement par une société rennaise BIOPREDIC, spécialisée dans la culture de cellules animales et le CRITT CBB Développement de Rennes. Les communications présentées par des industriels ont illustré l'intérêt des cultures cellulaires comme biotests dans des domaines divers, notamment ceux de l'environnement et de la toxicologie.....

The first European meeting entirely devoted to invertebrates and fishes organised by the European Tissue Culture Society in association with IFREMER and the University of Bretagne Occidentale, held in April 1994 in Brest.

This workshop described new in vitro cell model systems recently set up with all aquatic and terrestrial inferior eukaryotic species, focused on technical aspects of the models as well as general problems in cell biology, and dealed with various applications, including those related to problems in development and reproduction, environment and toxicology...

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