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Effects of Organic and Inorganic Substances on the Cell Proliferation of *L-929 Fibroblasts* and *Tetrahymena pyriformis GL* Protozoa Used for Toxicological Bioassays

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For *in vitro* toxicity testing, sensitive cells and simple handling assays have been sought by experimenters. A review of the literature showed that for some years, established cell lines have been frequently used to study the acute toxicity of xenobiotics. More recently, ciliated protozoa have been cited and among them, Tetrahymena pyriformis GL would be considered with great attention because of its short generation time with a doubling rate of less than 3 hr in defined medium (Carter and Cameron 1973; Ekwall 1983; Yoshioka et al. 1985; Nilsson 1989; Huber et al. 1990; Schultz et al. 1990; Grolière et al. 1992; Bamdad et al. 1993).

Whatever the model used, the cellular indicator of cytotoxicity must be adapted to the detection of small concentrations of substances and must be as easy as possible to perform. So, the cell proliferation rate (CPR) which resulted from all the toxic events occurring in the cell after contact with the xenobiotic, would be selected. The aim of the present study was firstly to specify the acute effects of 13 organic and 14 inorganic substances on the cell proliferation rate of two models L-929 murine fibroblasts (ECACC n°85011425) and Tetrahymena pyriformis GL protozoa by end-point determinations and secondly to compare the relative sensitivity of both models in such experimental conditions.

MATERIAL AND METHODS

The established cell line of L-929 murine fibroblasts was cultured in suspension in Eagle's Minimum Essential Medium (MEM) supplemented with 5% fetal calf serum, 1% L-glutamine, 1% non-essential amino-acids, 1% vitamins and 0.1 g gentamicine/L (Gibco BRL, France). 10-mL cultures in exponential phase growth were treated with sterile solutions of chemical substances and incubated at 37°C

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in a 5%-CO_2 humidified atmosphere. After a 24-hr exposure, corresponding to the normal doubling time of the population, the viability was evaluated by Trypan Blue Dye exclusion (Jauregui et al. 1981) and the impact on the viable cell proliferation was evaluated by counting achieved in Malassez hematocytometer under contrast phase microscope (Nikon). Tetrahymena pyriformis GL (TP) were grown axenically at 28°C in an autoclaved proteose peptone/yeast extract medium enriched with inorganic salts (PPYS) and elaborated according to procedure of Plessner et al. (1964). Chemical substances were added to the medium of exponential 100-mL growth culture (10^4 TP/mL) in a constant 1%-volume. For the determination of effects on the cell proliferation rate (CPR), some 1-mL aliquots were withdrawn from the cultures just after treatment with tested substances (time zero), then every hour for 9 hr. The viability and mobility of Tetrahymena were monitored by examination with a photonic microscope (Nikon). After fixation of the 1-mL cell suspension with 1 mL 4% formaldehyde in Isoton® buffer, the cell density of the culture was determined using an electronic particles Coulter Counter ZM® (Coultronics).

These two models were applied to the determination of the acute cytotoxicity of 13 organic and 14 inorganic substances, which were used for the synthesis of polyvinyl chloride (PVC) and polyethylene terephthalate (PET) or which could be generated simultaneously at the normal thermic transformation of these two plastic materials (Table 1). They have been selected from the substances which were known to be potentially toxic and which could be leached from the plasticwares into foodstuffs in contact with them. The inorganic substances were in salt chloride form, but the IC_{50} values were expressed in mg/L or mmol/L of inorganic part. All chemical substances were of analytical grade quality (purity > 98%) and were purchased from Merck (Darmstadt, Germany) except for germanium and niobium salts from Alfa Products (Danvers, Maine, USA). Previous to the experiments, concentrated stock solutions were prepared in deionized water (except for terephthalic acid and diethylhexylphthalate (DEHP) in ethanol and for monomer vinyl chloride (MVC) in N, N'-dimethylacetamide). In treated cultures, the percentages of solvants (0.1%) did not affect the CPR.

The acute toxicity of each substance was expressed by a biological index, which is the 50% growth inhibitory concentration (IC_{50}) of treated cultures compared to the control culture. With the L-929 cells, the IC_{50} values were determined after a 24-hr incubation period, which normaly corresponded to the doubling time of the cellular population. With the Tetrahymena, the IC_{50} values were

determined after 3, 6 and 9-hr incubation periods, which was the time required to obtain respectively 1, 2 and 3 generations of TP in the control culture. Concurrently, a 9-hr follow-up of the TP cultures allowed the dynamic proliferation of TP populations to be determined for each substance. Cell enumerations allowed the results to be expressed as ratios (Dx/Do) of the growth of each treated culture (Do= cell enumeration at the beginning of the experiment, i.e., time zero and Dx= cell enumeration at time zero + x hours). IC_{50} values were calculated with the linear regression model. The comparison of biological models was performed by regression analysis computed with Statview® (version II) on a Macintosh IIx.

RESULTS AND DISCUSSION

The acute toxicity of tested organic and inorganic substances was evaluated concurrently on two biological models, with two different methodologies adapted to each model. The acute toxicity is expressed by the index IC_{50}. All results are summarized in Table 1. A dose-dependent inhibitory effect on L-929 CPR was noted for the tested substances. The results showed a higher sensitivity of L-929 to inorganic substances than to organic ones (except for chloroacetaldehyde).

Similarly, most of the substances tested on TP have dose-dependent and time-dependent inhibitory effects on CPR, which were more marked for inorganic than organic substances. Previous studies have proved that the composition of the culture medium and the culture conditions of TP may greatly affect the CPR (Yoshioka & al. 1985; Liu et al. 1986; Huber et al. 1990). Moreover the concentration of proteose peptone in the medium could modify concentration of free metal ion tested in it (Larsen 1989). In this study, the IC_{50} values were calculated at various times, required to obtain one, two and three generations of TP in the control culture. So, these values showed that the IC_{50} of one substance could also be dependent on the time of exposure to tested chemicals. A progressive decrease of the IC_{50} values determined at 3 hr, 6 hr and 9 hr was noted with inorganic substances (Ba, Co, Cu, Fe, Ge, Pb, Sb, Sn) as well as with organic substances (DEHP, terephthalic acid, glycolic acid, chloroacetic acid, ethanol, ethylene glycol, diethylene glycol). The inhibitory effects of some other substances were at their maximum levels as early as 3 hr (Cr, Zn, dichloroethane) or 6 hr (Cd, Hg, Mn, Ti, MVC, acetaldehyde, chloroacetaldehyde, chloroethanol, thioglycolic acid) and remained similar at 9 hr.

Table 1 : IC_{50} of organic and inorganic substances

Times	Tetrahymena pyriformis GL						L-929	
	3 hr		6 hr		9 hr		24 hr	
Chemical Substances	mg/L	*mmol/L*	mg/L	*mmol/L*	mg/L	*mmol/L*	mg/L	*mmol/L*
Chloroacetic acid	626	*6.6*	510	*5.4*	106	*1.1*	480	*5.1*
Terephthalic acid	648	*3.9*	458	*2.8*	356	*2.1*	580	*3.5*
Glycolic acid	710	*9.3*	640	*8.4*	600	*7.9*	5000	*65.8*
Thioglycolic acid	765	*8.3*	114	*1.2*	83	*0.9*	3500	*38.0*
Acetaldehyde	625	*14.2*	44	*1.0*	44	*1.0*	1300	*29.5*
Chloroacetaldehyde	100	*1.3*	12	*0.15*	12	*0.15*	5	*0.06*
Dichloroethane	515	*4.5*	425	*3.7*	400	*3.5*	250	*2.2*
Chloroethanol	10110	*126*	9370	*116*	9000	*112*	4480	*56*
Ethanol	20900	*453*	17700	*384*	13100	*284*	11130	*242*
Ethylene glycol	15000	*242*	9820	*158*	9300	*150*	5050	*81*
Diethyleneglycol	91150	*859*	41000	*386*	31500	*297*	5220	*49*
DEHP*	228	*0.6*	92	*0.25*	60	*0.15*	1280	*3.3*
MCV**	806	*12.9*	430	*6.9*	405	*6.5*	2200	*35.2*
Barium (Ba)	585	*4.3*	415	*3.0*	330	*2.4*	250	*1.8*
Cadmium(Cd)	10	*0.09*	3	*0.03*	3	*0.03*	5	*0.04*
Cobalt (Co)	100	*1.7*	74	*1.3*	56	*1.0*	16	*0.3*
Chromium (Cr)	62	*1.2*	54	*1.0*	50	*1.0*	395	*7.6*
Copper(Cu)	105	*1.7*	80	*1.3*	60	*0.9*	21	*0.3*
Iron (Fe)	422	*7.6*	410	*7.4*	260	*4.7*	90	*1.6*
Germanium(Ge)	12	*0.16*	9	*0.12*	6	*0.08*	25	*0.34*
Mercury (Hg)	32	*0.16*	4	*0.02*	2	*0.01*	4	*0.02*
Manganese(Mn)	152	*2.8*	117	*2.1*	106	*1.9*	62	*1.1*
Lead (Pb)	286	*1.4*	190	*0.9*	148	*0.7*	290	*1.4*
Antimony (Sb)	60	*0.5*	38	*0.3*	20	*0.15*	22	*0.18*
Tin (Sn)	132	*1.1*	80	*0.8*	90	*0.7*	28	*0.25*
Titanium (Ti)	35	*0.7*	20	*0.4*	20	*0.4*	15	*0.3*
Zinc (Zn)	78	*1.2*	70	*1.1*	70	*1.1*	5	*0.08*

* DEHP = Diethylhexyl phthalate, ** MVC = Monomer Vinyl Chloride

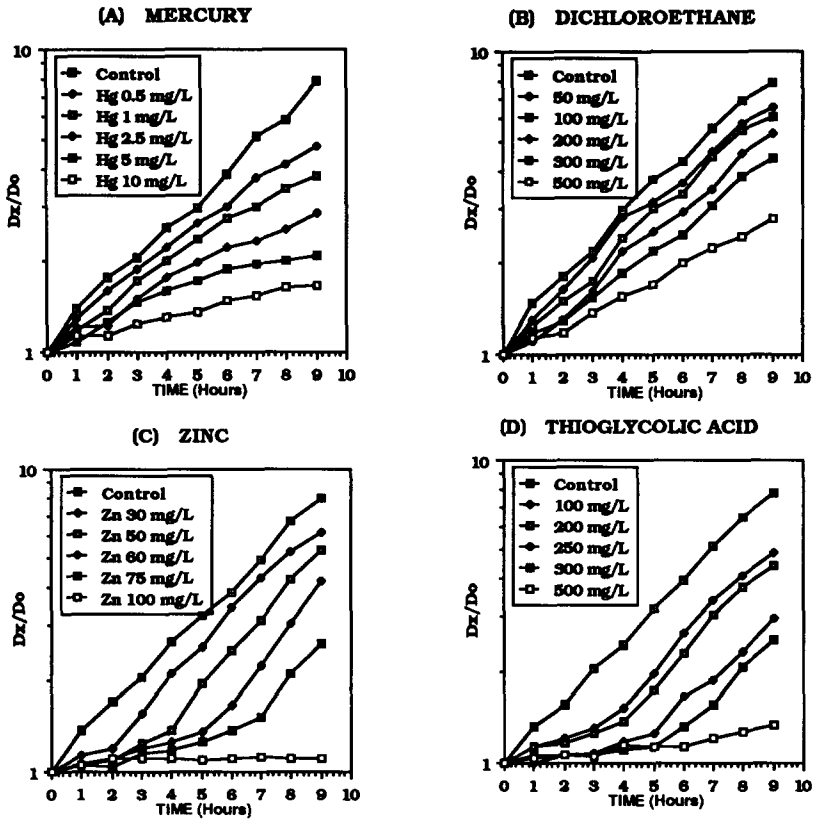

Figure 1 : Effects of inorganic and organic substances on the cell proliferation rate of Tetrahymena pyriformis GL (Do= cell enumeration at the beginning of the experiment, i.e., To and Dx= cell enumeration at To + x hours)
- Time-dependent, dose-dependent (i.e., *Mercury, Dichloroethane)*/
- Tempory inhibition of CPR followed by resumed growth and definitive inhibition after exposure to the highest concentrations of tested substances (i.e., *Zinc - Thioglycolic Acid)*

Concurrently, the TP proliferation rate was measured every hour in cultures exposed to at least five concentrations of each tested substance. These determinations allowed the dynamic of TP populations to be determined and the growth curves to be plotted. Two different kinds of curves were obtained with organic substances, as well as with inorganic ones. Typical examples of both models were presented in Figure 1. During the 9-hr exposure, on the first model (Figure 1A = Mercury and 1B = Dichloroethane), the CPR was dose-dependent and diminished progressively and regularly; such effects were also observed for barium, titanium, chloroacetic acid and terephthalic acid. For these substances, the slopes of the CPR curves of each tested concentrations differed significantly

from each other and from the slope of the control TP population. These features translate a real different proliferation rate of treated populations, directly related to the tested concentration and the toxic effects on TP. Moreover, another model of response was observed with both inorganic substances (Cd, Co, Cr, Cu, Fe, Ge, Mn, Pb, Sb, Sn, Zn) and organic substances (MVC, DEHP, glycolic acid, thioglycolic acid, acetaldehyde, chloroacetaldehyde, ethanol, chloroethanol, ethylene glycol, diethylene glycol). After exposure to these chemicals, a lag period followed by a marked reduction of CPR was observed for some hours; typical curve examples are presented with an inorganic substance (Figure 1C = Zinc) and an organic substance (Figure 1D = Thioglycolic Acid). For only the highest concentrations of these substances, a definitive inhibition of TP proliferation was noted. In other cases, the length of the lag period was in inverse ratio to the concentration tested, but it was followed by a resumed growth of TP populations, with a CPR similar to that of control population and visualized on the curves by the parallelism of the slopes of the tested concentrations. But, in fact, the same phenomenon of a lag period observed must be related to the specific detoxification mechanism of each tested substances. In a review about TP used as a cytotoxicological model, Nilsson (1989) underlined that cells, which proliferated in the presence of metal ions, had an increased generation time or had a proliferation starting only after a lag period. This last event could be explained by the cell selection, by the induced synthesis of methallothionein proteins or after a long exposure to metals, by a new proliferation rate which was related to a decreased concentration of the metal in the culture medium (Larsen 1989; Piccinni et al. 1987, 1990). For TP exposed to organic substances, the same phenomenon could be explained by the metabolism occurring concurrently to a detoxification process (Bamdad et al. 1993). The IC_{50} values of tested substances evolved with the time exposure. This could be explained by the relative sensitivity of the cells to them, by their speed and aptitude for going across the cellular membrane, for acting on the cellular target and lastly for generating specific toxic effects. Moreover, for organic substances, these effects are to be related to the lipophilicity behavior of the molecules. Even though the IC_{50} values determined at 3 hr, 6 hr and 9 hr gave some end-point information about the state of the TP populations at a given time like a "photograph", the curves of the dynamic proliferation of the TP populations showed all the events which occurred at different times and the natural evolution of the TP population, like a "movie". So, these two approaches complemented one another.

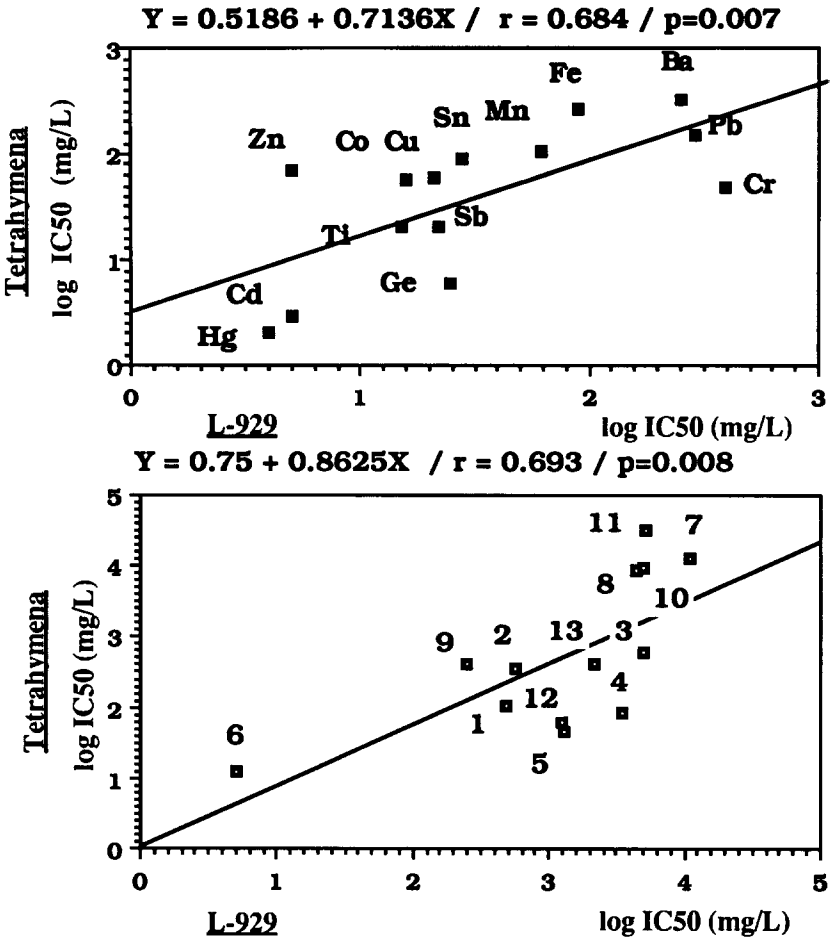

Figure 2. Comparison of the IC_{50} of organic and inorganic substances obtained with L-929 Fibroblasts and Tetrahymena pyriformis GL models.
Ba=Barium, Cd=Cadmium, Co=Cobalt, Cr=Chromium, Cu=Copper, Fe=Iron, Ge=Germanium, Hg=Mercury, Mn=Manganese, Pb=Lead, Sb=Antimony, Sn=Tin, Ti=Titanium, Zn=Zinc, 1=Chloroacetic acid, 2=Terephthalic acid, 3=Glycolic acid, 4=Thioglycolic acid, 5=Acetaldehyde, 6=Chloroacetaldehyde, 7=Dichloroethane, 8=Chloroethanol, 9=Ethanol, 10=Ethylene glycol, 11=Diethylene glycol, 12=DEHP, 13=MVC

The comparison of the IC_{50} values showed a similar level of sensitivity for both models, indicated by regression analysis, for the inorganic substances and for organic substances (Figure 2). One model must be considered to be more sensitive than the other, only for some substances for which the ratio (R or R') of IC_{50} values calculated on both models was arbitrarily fixed to be greater than four. So, TP were more sensitive than L-929 to chloroacetic acid (R=4.5), glycolic acid (R=8.3), thioglycolic acid (R=42.1), acetaldehyde (R=29.5), DEHP

(R=21.3), MVC (R=5.4), Cr (R=7.9) and Ge (R=4.2). Inversely, the L-929 were more sensitive than TP to diethylene glycol (R'=6) and zinc (R'=14).

The results of this present study indicated that both models, L-929 and Tetrahymena pyriformis GL, were adapted to the toxicological evaluation of inorganic and organic substances. Some avantages of each model must be underlined. Firstly, the past uses of established cell lines, such as L-929 fibroblasts, had contributed to the validation of such models for *in vitro* toxicological studies. TP cultures must also be considered with great attention as an inexpensive tool for the screening study of chemical substances especially as their handling is rapid and simple. TP appeared to be more sensitive to some inorganic and organic substances. Moreover, TP is a real eukaryotic organism and therefore allowed the metabolism of organic substances to be studied.

REFERENCES

Bamdad M, Groliere CA, Dupy-Blanc J, David L, Gendraud M (1993) Change in intracellular pH in Tetrahymena pyriformis GL in relation to the toxicity level of the ionophorous antibiotic Nigericin. Europ J Protistol 29:230-237

Carter JW, Cameron IL (1973) Toxicity bioassay of heavy metals in water using Tetrahymena pyriformis. Wat Res 7:951-961

Ekwall B (1983) Screening of toxic compounds in mammalian cell cultures. Ann NY Acad Sci 407:64-77

Groliere CA, Sefrioui S, Dupy-Blanc J (1992) Bioconversion of Thiram (TMTD) by Tetrahymena pyriformis. Arch Protistenk 141:135-140

Huber HC, Huber W, Ritter U (1990) Einfache in vitro-Prüfsysteme zur Töxizitätstimmung von Umweltchemikalien : Mikroculturen menschlicher Lymphozyten und monoxenische Ciliatenkulturen. Zbl Hyg 189:511-526

Jauregui HO, Hayner NT, Driscoll JL, Williams-Holland R, Lipsky MH, Galetti PM (1981) Trypan blue dye uptake and lactate dehydrogenase in adult rat hepatocytes. *In vitro* 17:1100-1110

Larsen J (1989) The influence of growth phase and culture conditions of Tetrahymena on effects of cadmium. Toxicology 58:211-223

Liu YF, Tang RH, Zhang QX, Shi JY, Li XM, Liu ZQ, Zhao W (1986) Stimulation of cell growth of Tetrahymena pyriformis and Chlamydomonas reinhardtii by trace elements. Biol Tr Elem Res 9:89-99

Nilsson JR (1989) Tetrahymena in cytotoxicology : with special reference to effects of heavy metals and selected drugs. Europ J Protistol 25:2-25

Piccini E, Irato P, Coppellotti O, Guidolin I (1987) Biochemical and ultrastructural data on Tetrahymena pyriformis treated with copper and cadmium. J Cell Sci 88:283-293

Piccini E, Irato P, Guidolin I (1990) Cadmium-thionein in Tetrahymena thermophila and Tetrahymena pyriformis. Europ J Protistol 26:176-181

Plessner P, Rasmussen L, Zeuthen E (1964) Techniques used in the study of synchroneous Tetrahymena.*In*: Zeuthen E (ed), Synchrony in cell division and growth, Intersciences Publ, New York, p534-565

Schultz TW, Lin DT, Wilke TS, Arnold LM (1990) Quantitative structure-activity relationship for the Tetrahymena pyriformis population growth endpoint : a mechanism of action approach. *In*: Karcher W and Devillers J (ed), Practical applications of quantitative structure-activity relationships (QSAR) in environmental chemistry and toxicology, p241-262

Yoshioka Y, Ose Y, Sato T (1985) Testing for the toxicity of chemicals with Tetrahymena pyriformis. Sci Tot Environ 43:149-157