Genetic basis of susceptibility for development of neoplasms following treatment with N-methyl-N-nitrosourea (MNU) or X-rays in the platyfish/swordtail system[1,2]

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Summary. Specific genotypes of the xiphophorine fish develop neoplasms following treatment with N-methyl-N-nitrosourea or X-rays. Several of these neoplasms can be related to the presence of specific chromosomes. The implications of these findings are discussed.

The xiphophorine fish, including platyfish and swordtails, carry an information for neoplastic transformation[4]; it was designated tumor gene (Tu)[5,6]. Tu may be present in the different genotypes in multiple form, distributed over different chromosomes[7]. Normally it is controlled through repressing genes (R-genes), which are arranged in a specific way: in certain genotypes Tu is mainly repressed by linked R-genes, in others mainly by nonlinked R-genes. In case R-genes are eliminated by crossing conditioned chromosome substitution, or are impaired by mutation, Tu may become expressed[6,7].

So far this principle could convincingly be shown to operate in the development of melanomas. The same principle may also apply for the development of thyroid tumors, and ocular tumors, which occur sporadically in the fish colonies and which have long been known to develop in certain genotypes of the xiphophorine fish[8,9]. Furthermore, there are indications that this principle may also apply for a variety of other neoplasms, which have recently been induced in the fish in our laboratory.

In order to find out to what extent genetic factors might be involved in the additional types of neoplasms, we have analyzed the susceptibility of a large number of defined genotypes including nonhybrids, F_1 hybrids and backcross hybrids, for development of neoplasms following treatment with mutagenic agents. We have chosen N-methyl-N-nitrosourea (MNU), which is a direct-acting carcinogen, not requiring metabolic activation[10], and X-rays, both exerting their carcinogenic effect, most likely, via mutation[11-13].

Materials and methods. Animals (table 1): 65 defined genotypes from *Platypoecilus maculatus, P. xiphidium, P. variatus, Xiphophorus montezumae cortezi, X. helleri guentheri,* as well as F_1 hybrids, and backcross hybrids were employed for the study. All genotypes carry 2 n = 48 chromosomes[14]. The genotypes exhibit, or lack, specific melanophore spot patterns, which are due to the expression of specific genes, of which each is located on a different chromosome. This makes it possible to recognize the presence, or the absence, of specific chromosomes in a given genotype. From each of the genotypes 2 age groups were used, one being about 6 weeks old, the other being about 6 months old; from each age group at least 50 individuals including both sexes were treated.

MNU-treatment. Animals were exposed in tanks to aqueous solution (10^{-3}M; pH 6.0–6.5) of N-methyl-N-nitrosourea (MNU; FERAK, Berlin). 4 treatments were given in 2-week intervals, of 1 h duration each.

X-irradiation. Animals were whole body irradiated with 1000 R for 45 min (dose rate 22 R/min; 150 kV, 12 mA)[15] 3 times at 6 week intervals. For the MNU-treatment and the X-irradiation each, about 3500 6-week-old, and about 3500 6-month-old animals were employed.

Histology. Neoplasms were promptly preserved in Bouin's fixative, embedded in paraffin, and cut into 5 µm thick sections, which were stained with hematoxylin-eosin.

Results. a) Age dependency of the susceptibility. From the 2 age groups treated, in the older one almost no neoplasms were observed; only the animals of the younger group

Table 1. Genotypes treated with MNU and X-rays

Species, populations, and hybrids	Melanophore spot pattern marker for different chromosomes*
Non-hybrids (highly inbred)	
Platypoecilus maculatus (Rio Jamapa)	Sd, Sd', Sp, Sr, Sr', Dt, Cr, Sh, Os, Sd^{del}, Sr^{del}
P. maculatus (Rio St. Pedro, Usumacinta-system)	Sh, Os; without markers
P. xiphidium (Rio Purification, Rio Soto la Marina)	Fl, Ct; Fl^{del}
P. variatus (Rio Panuco)	Li, Pu
Xiphophorus montezumae cortezi (Rio Axtla, Rio Panuco-system)	Sc
X. helleri guentheri (Belize River)	Db_1; without marker
X. helleri guentheri (Rio Lancetilla)	Db_2; without marker
Hybrids	
P. maculatus (Rio Jamapa) × *X. helleri* (Rio Lancetilla) without marker	
F_1	Sd, Sd', Sp, Sr, Sr', Dt, Cr, Sh, Os; Sd^{del}, Sr^{del}
BC_n (n = 1,3,5,7); BC-parent *X. helleri*	50% segregants with marker as in F_1, 50% without marker
P. maculatus (Rio Jamapa) × *X. montezumae cortezi*	
F_1	Sd, Sr, Sc
BC_n (n = 2,3,5); BC-parent *X. montezumae*	50% segregants with marker as in F_1, 50% without marker
P. maculatus (Rio Jamapa) × *P. xiphidium* Fl^{del}	
F_1	Sr, Sd, Sp, Sd, Sd^{del}
P. variatus × *X. helleri* (Rio Lancetilla) without marker	
F_1	Li
BC_n (n = 1,4,15); BC-parent *X. helleri*	50% segregants with marker as in F_1, 50% without marker
BC_n (n = 1,4,15); BC-parent *X. helleri* albino	50% segregants with marker as in F_1, 50% without marker

* Abbreviations: Cr, crescent; Ct, cut crescent; Db, dabbed; Dt, dot; Fl, flecked; Fl^{del}, deletion of flecked; Li, lineatus; Os, one spot; Pu, punctatus; Sc, spotted caudal; Sd, spotted dorsal; Sd^{del}, deletion of spotted dorsal; Sd', spotted dorsal mutation; Sh, shoulder spot; Sp, spotted; Sr, striped; Sr', striped mutation. For more detailed information see Wolf and Anders[17].

developed a respectable number of tumors, showing that the susceptibility is highly dependent on the age of the treated animal. The following section deals exclusively with the younger age group.

b) Susceptibility of the different cell types, tissues or organs. Following a latent period of about 3 months after the last treatment, a wide spectrum of neoplasms started occurring both following MNU-treatment and X-irradiation. So far 14 different types of neoplasms were classified (according to Neuhaus and Halver[16], Ribelin and Migaki[16], Ashley, Halver and Wellings[16]), including malignant melanoma, benign melanoma, neuroblastoma, squamous cell carcinoma, epithelioma, carcinoma (high differentiated), carcinoma (low differentiated), adenocarcinoma (kidney), adenocarcinoma (thyroid), papilloma, hepatoma, fibrosarcoma, rhabdomyosarcoma, and lymphosarcoma (table 2).

The proportions between the different types of neoplasms following MNU-treatment and X-irradiation are similar (table 2). However, the total incidences are apparently higher in the MNU-treated animals than in the X-irradiated ones (312 neoplasms for MNU; 163 for X-rays). The highest incidences were observed for the benign and the malignant melanoma, for the neuroblastoma and the fibrosarcoma. This remarkable variety of neoplasms suggests that all the different cell types, tissues or organs of the fish are susceptible.

c) Genotype-specific susceptibility. Within the genotypes tested, the neoplasms developed almost exclusively in backcross hybrids, and rarely in F_1 hybrids; only in 1 case was a neoplasm observed in a nonhybrid (1 lymphosarcoma). We have analyzed in more detail whether a relation exists between a neoplasm and a specific chromosome. Such a relation can be most easily recognized in backcrosses, which were selectively bred for a specific phenotypic marker, and thereby for a specific chromosome; such backcrosses segregate into 50% animals carrying the marker chromosome, and 50% lacking this chromosome. A susceptibility was observed in animals resulting from backcrosses of *P. variatus*/*X. helleri*-hybrids, or *P. maculatus*/*X. helleri*-hybrids using *X. helleri* as the recurrent parent (table 1). Within these animals, the benign and the malignant melanoma developed in those segregants exhibiting anyone of the following phenotypic marker: Li or Pu (derived from *P. variatus*), or Sr or Sd^{del} (derived from *P. maculatus*; for abbreviations see table 1); both earlier and later backcross generations are affected in the same way.

The neuroblastoma, as well as the epithelioma, developed exclusively in backcross segregants carrying the phenotypic marker Li; both earlier and later backcross generations were affected.

The fibrosarcoma is observed in both the Li-carrying and the Li-lacking segregants; in contrast to the above neoplasms, the susceptibility appears to exist only in earlier backcross generations, and is lost in later generations[18].

From the above observations it appears that the susceptibility to benign and malignant melanoma is associated with any one of the chromosomes marked by the Li, Pu, Sr, or Sd^{del}; the susceptibility for the neuroblastoma and for the epithelioma apparently depends on the Li-marked chromosome. The susceptibility for fibrosarcoma appears to be related to another chromosome, for which we have not found a phenotypic marker so far. It should be pointed out that these chromosomes apparently confer the susceptibility for the neoplasms both to MNU and to X-rays. For the other types of neoplasms, a similar genotype specific susceptibility is apparent, although additional data are required in order to establish such a relation.

Discussion. Small aquarium fishes have been widely used in chemical carcinogenesis studies[19,20], and the advantage of using these fishes instead of other animals in the analysis of potential mutagens-carcinogens has recently been emphasized[20,21]. However, to the best of our knowledge, so far no systematic studies on a broad scale, in which defined genotypes were tested for their susceptibility for tumor development following treatment with mutagens-carcinogens, have been carried out.

Table 2. Neoplasms induced in the xiphophorine fish by N-methyl-N-nitrosourea (MNU) and by X-rays

Type of neoplasm	No. of neoplasms		Incidence (%) based on total No. of animals treated*	
	MNU	X-rays	MNU	X-rays
Melanoma (benign)	112	93	3.2	2.7
Melanoma (malignant)	81	34	2.3	1.0
Neuroblastoma	43	7	1.2	0.2
Squamous cell carcinoma	2	0	0.05	0
Epithelioma	6	6	0.17	0.17
Carcinoma (low differentiated)	3	4	0.08	0.11
Carcinoma (high differentiated)	2	5	0.05	0.14
Adenocarcinoma (kidney)	3	2	0.08	0.05
Adenocarcinoma (thyroid)	2	3	0.05	0.08
Papilloma	4	0	0.11	0
Hepatoma	3	1	0.08	0.02
Fibrosarcoma	46	6	1.3	0.17
Rhabdomyosarcoma	4	2	0.11	0.05
Lymphosarcoma	1	0	0.03	0
Total	312	163		

* 3500.

The present investigation has revealed that in the xiphophorine fish the susceptibility for development of neoplasms following MNU-treatment and X-irradiation is not identical for the different genotypes. It could however be shown that certain genotypes carrying specific chromosomes are susceptible for neoplasms, while others lacking these chromosomes are non-susceptible. This suggests that the susceptibility for these neoplasms has a genetic basis.

To date we have no evidence in favour of a particular mechanism responsible for the preferential susceptibility of certain genotypes, and there might be various factors involved in sensitizing the genotypes for the susceptibility to tumor development. However, in this study, in our opinion, the most likely mechanism which is consistent with the present knowledge about how mutagens-carcinogens act in the transformation of normal cells into neoplastic ones, is a genetic change of a somatic cell, i.e. a somatic mutation.

Such a mutation could consist in a change in chromosome structure and/or chromosome number, which has been observed in cells of various origins following exposure to mutagens-carcinogens, including MNU[22]. These changes may lead to an imbalance between specific chromosomes containing information for either the expression or suppression of genes for neoplastic transformation, as could be shown by several authors[4-7,23].

On the other hand, such a mutation could be a point mutation leading to an impairment of genes involved in the suppression of other genes responsible for neoplastic transformation. Bouck and DiMayorca[11] presented evidence that this is the most likely primary event by which chemicals act in the neoplastic transformation of BHK cells; Huberman et al.[12] suggest that neoplastic transformation of cells by chemicals is due to mutation, which may occur in a single gene.

In the view that the xiphophorine fish contain a tumor gene Tu[5,6] responsible for the neoplastic transformation, it might be that the neoplasms result from an impairment or a deletion of genes involved in the regulation of the Tu[24]. Further experiments, including chromosome analyses as

well as breeding experiments, may help to answer this question. A more detailed presentation of the histology of the various types of neoplasms will be published subsequently.

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Effect of vagotomy upon the neurohistochemical and ultrastructural integrity of the inbuilt intrinsic nervous apparatus of the choledocho-duodenal junction

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Summary. The neurons of the choledocho-duodenal junction of the cat were shown to be neurohistochemically and morphologically independent of their extrinsic vagal connections. The effect of vagotomies upon the intrinsic nerve nets was also quite negligible.

There is convincing evidence that the integrity of the inbuilt intrinsic nervous apparatus of the choledocho-duodenal junction is of crucial importance for the maintenance of normal biliary dynamics[1-5]. The sympathetic influence upon the choledocho-duodenal junction is mediated through alpha receptors responsible for contraction of the sphincter following sympathetic nerve stimulation, and through beta receptors responsible for relaxation of the sphincter following stimulation by circulating catecholamines[3,4,6,7]. Recently, the presence of contraction-mediating atropine-sensitive cholinoceptors in the sphincter of Oddi of the cat has been confirmed[3]. In addition, it is generally believed that there is, in the regulation of the functioning of the extra-hepatic biliary duct system, a neurohumoral link between the vagal innervation and the effect of cholecystokinin, gastrin and secretin, all these humoral agents being released from the mucosal neuroendocrine cells by vagal nerve stimulation[8]. Finally, it has been shown that the physiological influence of cholecystokinin upon the biliary smooth muscle necessitates functioning of a reflex arch containing at least 1 synapse and procaine-sensitive receptors in relation to the cholecystokinin-releasing cells[5]. This further emphasizes the importance of the intrinsic innervation in the maintenance of normal biliary dynamics. However, until now, surprizingly little is known about the neurohistochemical nature and ultrastructural characteristics of the intrinsic neurons of the choledocho-duodenal junction, and this is true also concerning the neurohistochemical and ultrastructural consequences of vagotomies, although vagal denervation of the upper abdominal viscera has, during the last few years, become a common surgical procedure, and the role of the vagus nerves in the biliary dynamics has received special attention.

The nature of the intrinsic neurons of the choledocho-duodenal junction of the cat was studied: 1. by fluorescence microscopy (formaldehyde-induced and glyoxylic acid-induced fluorescence), 2. by light- and electron microscopical demonstration of the acetylcholinesterase enzyme [a) Gomori's modification of the Koelle-Friedenwald technique, b) the method of Karnovsky and Roots, c) the method of Lewis and Shute], 3. by examining the glutaraldehyde-osmiumtetroxide-fixed ultrastructure, and 4. by studying the effect of unilateral and bilateral subdiaphragmatic abdominal or cervical vagotomies performed 5 days - 3 months before sacrifice. A total of 30 adult cats was used for the present study.

In light microscopy, most of the neurons showed moderate to intense AChE activity (figure 1). At electron microscopy, intra-cellular distribution of the reaction product was