Genetic Basis of Susceptibility for Neuroblastoma following Treatment with N-Methyl-N-nitrosourea and X-Rays in Xiphophorus

Manfred Schwab, Gerhard Kollinger, Joachim Haas, Mulkh R. Ahuja, Safia Abdo, Annerose Anders, and Fritz Anders

ABSTRACT

In 65 genotypes of Xiphophorus (nonhybrids and interpopulational and interspecific F1 and backcross hybrids), the susceptibility to neuroblastoma following treatment with carcinogenic-mutagenic agents was tested. The fish were exposed to an aqueous solution of N-methyl-N-nitrosourea (MNU) or exposed to X-rays. Neuroblastoma was induced by MNU in 64 of about 3500 treated fish and by X-rays in 4 of about 5500 treated fish; in the control fish, as well as in the vast number of fish routinely bred in our fish laboratory during the last 20 years, neuroblastoma was not observed. Sixty of the 64 MNU-induced neuroblastomas and all 4 X-ray-induced neuroblastomas developed in the 605 and 859 treated fish, respectively, of a group of backcross genotypes carrying the "lineatus" chromosome. These fish are derived from Xiphophorus variatus × Xiphophorus helleri hybrids with X. helleri as the recurrent parent. The remaining four cases of neuroblastoma induced by MNU were distributed at random throughout fish of backcross genotypes derived from other species; within these, a relationship to the genotype is not detectable. In both X. variatus and the F1 (X. variatus × X. helleri), although carrying the same lineatus chromosome, as well as in X. helleri lacking this chromosome and in the lineatus-lacking lineatus backcross segregants, from each of which a comparable number of fish was treated, neuroblastoma could not be induced. The results are interpreted to imply that the neuroblastoma is triggered by somatic mutation of regulating genes suppressing another gene that favors neoplastic transformation and that is located on the lineatus chromosome. The differential susceptibility of the fish carrying this chromosome (X. variatus, F1, and backcross hybrids) might depend on the number of regulating genes. Accordingly, insusceptibility, as in the case of X. variatus, would be due to a polygenic regulating system, which, in practice, cannot be impaired in one cell. Susceptibility, as in the case of the lineatus backcross segregants, would be due to a monogenic regulating system, which can easily be impaired by one single mutation, and this monogenic state of regulation may be achieved by elimination of regulating genes in the course of selective backcrossing.

INTRODUCTION

Certain interpopulational and interspecific hybrids between the platyfish (Xiphophorus maculatus) and the swordtail (Xiphophorus helleri) spontaneously develop melanomas (20, 21, 24, 36), thyroid tumors (41), or ocular tumors (22). Subsequent analysis of melanomas as well as other neoplasms of Xiphophorus has led to the suggestion that development of a neoplasm may result from a misguided expression of an endogenous entity capable of transforming cells from the normal to the neoplastic state (4-6). This entity was called Tu* (7). Tu appears to be present in all populations and species of Xiphophorus and may exist in an individual in multiple form distributed over several chromosomes. Although the nature and the biological function of Tu are still largely obscure, some speculations on these aspects of Tu have been presented recently (2-4).

The present investigation is part of a broad-scale experiment in which a large number of defined genotypes were tested for their susceptibility to develop neoplasms following treatment with mutagenic-carcinogenic agents (1, 9, 10, 23, 35, 47-51). We have tested the susceptibility with 2 basically different types of agents, namely, a chemical agent, MNU, which is a direct-acting mutagen-carcinogen not requiring metabolic activation (37, 40, 42), and a physical agent, X-rays. Both exert their carcinogenic effect most likely via mutation (for MNU, see Refs. 15 and 28; for X-rays, see Refs. 13 and 17). This experiment has shown thus far, that, following exposure to both agents, various neoplasms can be induced, including melanoma, neuroblastoma, squamous cell carcinoma, epithelioma, carcinoma, adeno carcinoma, papilloma, hepatoma, fibrosarcoma, rhabdomyosarcoma, and lymphosarcoma; the susceptibility to several of these neoplasms appears to be confined to particular chromosomes (49, 51). In a previous paper, we have presented data on the susceptibility to both the fibrosarcoma and rhabdomyosarcoma (49). This report deals with the susceptibility to neuroblastoma.

MATERIALS AND METHODS

Fish Genotypes

Sixty-five different genotypes from X. maculatus, Xiphophorus xiphidium, Xiphophorus variatus, Xiphophorus

* This work was supported by Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 103 "Zellenergetik und Zeldifferenzierung" (Projects C 11 and C 12), Marburg; and by Land Hessen through Justus-Liebig-Universitaet Giessen. This work is dedicated to Peter Karlson on the occasion of his 60th birthday. This paper contains parts of the dissertations of Gerhard Kollinger, Joachim Haas, and Safia Abdo.

1 For whom requests for reprints should be addressed.

2 On leave from the University of Alexandria; supported by the Egyptian Ministry of Education.

Received August 11, 1978; accepted October 23, 1978.

FEBRUARY 1979

519
montezumae cortezi, and Xiphophorus helleri guentheri, as well as F1 and BC hybrids, were used in this study (Table 1). The species and subspecies were originally obtained from Myron Gordon in 1959 and from Curt Kosswig in 1960. Gordon had collected these fish between 1939 and 1951. The fish have been maintained since their collection in closed stocks. The approximate number of generations since then is between 40 and 60 for the different species (for further descriptions of these fish and for the localities where the original material was collected, see Refs. 29, 30, 58, and 59).

The different genotypes exhibit, or lack, specific melanophore spot patterns, which are due to the expression of specific genes. Each is located on a particular chromosome. All these genes are in any genotype coexpressed (codominant); this makes it possible to recognize the presence or absence of specific chromosomes in any genotype.

Treatment

From each of the genotypes, at least 50 individuals (for some genotypes, up to 700) were treated, all being between 6 weeks and 6 months old. About 3500 fish were treated with MNU, and about 5500 fish were treated with X-rays. The same number of animals served as controls; they were handled like the treated ones.

MNU Treatment. Animals were exposed in aquariums to freshly prepared aqueous solutions of MNU (1 mM; pH 6.0 to 6.5; 27°). Four 1-hr treatments were given in 2-week intervals.

X-irradiation. Animals were whole-body-irradiated with 1000 r for 45 min (dose rate, 22 R/min; 150 kV, 12 ma); this dose was administered 3 times at 6-week intervals (the total dose was 3000 R/fish; for details, see Ref. 46).

Examination

Light Microscopy Examination. Whole fish were fixed in Bouin’s solution, dehydrated, and then embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin and were selectively stained with periodic acid-Schiff, Van Gieson’s stain, and Gomori’s stain.

Electron Microscopy Examination. Tissue samples were fixed in 2.5% glutaraldehyde in PBS. All specimens were postfixed for 2 hr in 1% osmium tetroxide. After dehydration, they were embedded in ERL-4206 (Serva). Ultrathin sections were cut with a diamond knife using a Reichert Om U2 ultramicrotome. Sections were stained with uranyl acetate and lead citrate and were examined in a Zeiss EM 10 electron microscope.

RESULTS

Description of the Neuroblastoma

Morphology. Following a latent period of 3 to 7 months after the last treatment, the first clinically observable change in the area of the eye occurs. It consists of a slight protrusion of the eye, apparently due to proliferation of the underlying tissue. During the following 2 to 3 weeks, the tumor grows rapidly and may reach up to 7 mm in diameter within this period. In most cases, only one eye is affected (Fig. 1, a and b) and, exceptionally, both eyes show the protrusion.

The eye itself apparently remains functional in most of the fish as judged from their response to stimuli.

Histology. Sagittal sections revealed that the neuroblastoma is in most cases located exterior to the sclera in the retrobulbar space within the area of the orbita (Fig. 1 b). Only in advanced stages are the tumor cells also found in the subcutaneous tissue.
Neuroblastoma Induced in Xiphophorus

Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treated</th>
<th>Survived</th>
<th>Neoplasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MNU</td>
<td>X-rays</td>
<td>MNU</td>
</tr>
<tr>
<td>X. variatus Li/Li</td>
<td>286</td>
<td>698</td>
<td>271</td>
</tr>
<tr>
<td>X. helleri —/—</td>
<td>198</td>
<td>68</td>
<td>182</td>
</tr>
<tr>
<td>F, Li/—</td>
<td>103</td>
<td>476</td>
<td>89</td>
</tr>
<tr>
<td>BC, Li/—</td>
<td>226</td>
<td>684</td>
<td>212</td>
</tr>
<tr>
<td>BC, —/—</td>
<td>201</td>
<td>352</td>
<td>198</td>
</tr>
<tr>
<td>BC,5 Li/—</td>
<td>181</td>
<td>104</td>
<td>170</td>
</tr>
<tr>
<td>BC,5 —/—</td>
<td>153</td>
<td>88</td>
<td>146</td>
</tr>
<tr>
<td>BC,6 Li/—</td>
<td>198</td>
<td>111</td>
<td>193</td>
</tr>
<tr>
<td>BC,6 —/—</td>
<td>165</td>
<td>91</td>
<td>151</td>
</tr>
</tbody>
</table>

* Percentage of incidence based on number of survivors.

* The susceptibility of the Li segregants differs significantly from the insusceptibility of the group of other genotypes, including the Li-lacking segregants, the F, X. variatus, and X. helleri; p < 0.001.

* The susceptibilities of the BC, and BC, are not significantly different; p < 0.001.

* The incidences at BC, and BC, to those at BC, fit a 1:2 ratio; p < 0.05. p calculated by the χ² method.

within the eye, invading the sclera and the retina as well as the vitreous body and the area around the iris; the optic nerve is invaded in few cases. These findings would suggest that the nidus of the neuroblastoma is outside the eye. The tumor often shows extensive necrotic and hemorrhagic areas.

The major part of the neuroblastoma is packed densely with round to ovoid, closely aggregated, regular hyperchromatic cells, measuring between 3 and 7 μm in diameter. The cells possess a scanty, ill-defined cytoplasm (Fig. 1c). The nuclei usually occupy an abnormally large portion of the cell; mitotic figures are abundant in some areas of the neuroblastoma. The tumor cells are frequently arranged in rosette-like structures (Fig. 1c, arrows).

Ultrastructure. The nuclei are polymorphic, with pockets and projections (Fig. 1d), and the nuclear mass appears frequently bipartite, depending on the direction in which the nucleus had been sectioned. This nuclear appearance in neuroblastoma is similar to that found in earlier investigations in the melanoma of Xiphophorus (54, 55). Cytoplasmic organelles are scarce in most of the cases; they show a few abnormally large mitochondria and small vesicles, few endoplasmic reticula, and varying numbers of ribosomes. In other cases, however, large areas of distinct endoplasmic reticula were observed. In many cells, characteristic solitary cilia were detectable which, without exception, consisted of 9 pairs of peripheral doublets of microtubules with no central axis (Fig. 1d, inset). In addition, in some of the neoplasms, particles were detectable that resemble in size and structure C-type viruses (see Ref. 35).

Dependence of the Susceptibility on the Genotype. Within the 3500 fish of the 65 genotypes treated with MNU, the neuroblastoma developed in 64 fish, and within the 5500 treated with X-rays, neuroblastoma was found in 4. In the untreated controls, which were handled the same as the treated fish, neuroblastoma did not develop. In addition, in the vast number of fish of the same genotypes used in this study that were bred and maintained over the last 20 years in our fish laboratory, neuroblastoma has not been observed. This would imply that all of the neuroblastomas observed following treatment with MNU and X-rays in fish of these genotypes are due to the action of these agents.

In the case of the MNU-induced neuroblastoma, all but 4 fish developed neuroblastomas in one group of backcross segregants carrying the Li chromosome (derived from X. variatus; its presence can be recognized by the Li spot pattern) (Fig. 2); the remaining 4 neuroblastomas were distributed at random throughout backcross genotypes derived from other species, and within these a relationship of the susceptibility to the genotype cannot be drawn. In the case of X-ray-induced neuroblastoma, all fish developed neuroblastomas also in the group of BC segregants carrying the Li chromosome.

The Li backcross segregants, in which the high susceptibility for neuroblastoma was observed, were obtained according to the crossing principles shown in Fig. 2. In this study, according to availability, 2 earlier backcrosses, BC, and BC, were used (Table 1). MNU and X-rays induced the neuroblastoma in BC, in 17 of 212 fish (8.0%) versus 2 of 661 fish (0.3%); in BC, in 15 of 170 fish (8.8%) versus 1 of 102 fish (1.0%); and in BC, in 28 of 193 fish (14.5%) versus 1 of 96 fish (1.0%; Table 2). For MNU, the incidences between BC, and BC, are not significantly different (p < 0.001), and the mean value of the incidences at BC, and BC, fit a 1:2 ratio; p < 0.05. p calculated by the χ² method.

As a result, only the Li segregants of the backcrosses are susceptible to neuroblastoma, both following MNU treatment and X-irradiation. In contrast, the F, and the parental species X. variatus, although carrying the same Li chromosome, are insusceptible, and the BC segregants lacking the Li chromosome, as well as the parental species X. helleri, which also lacks this chromosome, are also insusceptible (p < 0.001).

DISCUSSION

The Relation of the Induced Neuroblastoma to Ocular Diseases in other Vertebrates. Protrusion of the eye.
called exophthalmus or "pop-eye," is a disease that is found frequently in Xiphophorus. It may be caused, for instance, by infection with a variety of organisms (trematodes, bacteria, fungi, viruses, etc.), by hormonal influences, or by mechanical injury (see Ref. 19). The exophthalmus described in the present article could be identified by histological examination as due to a neoplasm of the retrobulbar space of the eye (35). It has never been observed in our laboratory to occur spontaneously. The histological picture is that of a neuroblastoma which consists of imperfectly differentiated cells of neural origin. This neuroblastoma is rather similar to the eye tumor of apparently neural origin described earlier in Xiphophorus (39). It is, however, different from the neoplasms originating from pigment cell precursors also induced by MNU and by X-rays in various tissues, including the eye, in Xiphophorus.* This neoplasm, furthermore, with respect to its histological structure, is similar to the neoplasms induced by nitrosamines and nitrosamides in the nasal cavity and in the olfactory bulb of the rat (11) and also resembles the extraocular orbital tumors of apparently neural origin induced by human adenovirus type 12 in the hamster (45). The histological picture of fish neuroblastoma is also similar to that of the intraocular retinoblastoma-like tumors induced by human adenovirus in the rat (34). The neuroblastoma, however, to the best of our knowledge was not induced thus far in fish by treatment with mutagens-carcinogens (for review on carcinogenesis in aquarium fish, see Ref. 44).

**Genetic Basis of the Susceptibility to Neuroblastoma.**

By far, most of the neuroblastomas in this study were induced in fish of the *Li* genotype. This would suggest that the susceptibility to neuroblastoma following MNU treatment and X-irradiation is determined by the genotype in Xiphophorus.

The agents used in this susceptibility test, both the "radiomimetic" MNU (43) and the X-rays, are known to be potent mutagens (for a comparison between MNU and X-rays, see Ref. 38). It has been proposed (12) that some type of mutation is involved in the initiation step of the process leading to neoplasia (for more recent analyses, see Refs. 25 and 26). Such a mutation could be a change in either chromosome number and/or chromosome structure leading to an imbalance between genes for "expression" or "suppression" of neoplastic transformation (14, 27, 52), or it could be a single gene mutation (15), involving a single base missense (28) leading to the impairment of genes that are involved in the suppression of genes responsible for neoplastic transformation.

Speculating that the neuroblastoma induced in the present investigation might be due to somatic mutation, one has to assume that the susceptibility in the *Li* segregants depends on a single gene. If 2 or more genes would be involved, the incidence should be extremely low (for relation between mutation rate, tumor incidence, and number of genes controlling the susceptibility, see Ref. 4). On this basis, one might formally interpret the result that the parental species *X. variatus* and *X. helleri*, their *F*₁ and the *Li*-lacking segregants from backcrosses are insensitive, whereas the *Li* segregants are susceptible to neuroblasto-
nonhybrids of Xiphophorus, which stem from natural populations, are apparently insusceptible, certain backcross genotypes develop neoplasms following the treatment of various organs or tissues (see "Introduction"); see also Refs. 47 to 51). On the basis of the findings in Xiphophorus, one might speculate on a more general mechanism for the differential susceptibility of wild-type and domesticated animals. According to these findings, insusceptibility of the wild-type animals might be due to a polygenically controlled balance between genes favoring neoplastic transformation and genes suppressing neoplastic transformation, and this balance might have been built up during the evolution of the populations in genetic isolates. In the course of crossing and subsequent backcrossing, as in the case of Xiphophorus in the breeding of the Li segregants, or in the case of other animals during domestication, genes suppressing neoplastic transformation may be eliminated. If all such genes are eliminated, neoplasms develop spontaneously without any further treatment. The most well analyzed example is the spontaneously developing melanoma of certain breeds of Xiphophorus (4-8). If all of these genes are not eliminated, depending on the importance of the remaining genes, a more or less susceptible animal is obtained that may develop neoplasia following a further genetic event induced by mutagenic-carcinogenic agents; a typical example is the neuroblastoma described in this paper (for further neoplasms, see Refs. 49 to 51). These apparent parallels in the etiology of cancer between Xiphophorus and higher vertebrates imply that this model might be suitable for analyzing further the genetic basis for neoplasia in general (for broader discussion, see Refs. 2 to 4).

ACKNOWLEDGMENTS

We are indebted to Dr. H. D. Menzel, Pathologisches Institut der Universität Freiburg im Breisgau, for his help in the identification of the neoplasm. We thank Kaete Klinke for her help in the breeding of the fish, and Jutta Siegers for excellent technical assistance.

REFERENCES


Fig. 1. a, Li-carrying segregant of the BC, (X. variatus × X. helleri) × X. helleri exhibiting an MNU-induced neuroblastoma in the area of the left eye. x 3. b, sagittal section showing the tumor mass causing protrusion of the eye. H & E, x 6. c, neuroblastoma cells. Note rosette-like arrangement (arrows). H & E, x 1400. d, electron micrograph of the neuroblastoma cells. Note nuclear pockets (P), bipartite nuclear mass (N), abnormally large mitochondria (M), and small vesicles (V). x 14,000. Inset, cross-section of a cilium exhibiting the 9 + 0 pattern of microtubules. x 44,000.
Fig. 2. *X. variatus* (Li/Li), *X. helleri* (−/−), their F₁, (Li/−), and segregants of the BC₁, (Li/−, and −/−) resulting from backcrossing of the F₁ to *X. helleri*. Further backcrosses were obtained by selective backcrossing of the Li segregants to *X. helleri*. Only the Li segregants were susceptible to neuroblastoma following MNU treatment and X-irradiation.
Genetic Basis of Susceptibility for Neuroblastoma following Treatment with \textit{N}-Methyl-\textit{N}-nitrosourea and X-Rays in \textit{Xiphophorus}

Manfred Schwab, Gerhard Kollinger, Joachim Haas, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/39/2_Part_1/519

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.