Environmental Genotoxicity Evaluation Using Cytogenetic End Points in Wild Rodents

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We analyzed cytogenetic end points in three populations of two species of wild rodents—*Akodon montensis* and *Oryzomys nigripes*—living in an industrial, an agricultural, and a preservation area at the Itajaí Valley, state of Santa Catarina, Brazil. Our purpose was to evaluate the performance of the following end points in the establishment of a genotoxic profile of each area: the polychromatic/normochromatic cell ratio; the mitotic index; the frequency of micronucleated cells both in the bone marrow and peripheral blood; and the frequency of cells with chromosome aberrations in the bone marrow. Preparations were obtained using conventional cytogenetic techniques. The results showed *a*) the role of the end points used as biomarkers in the early detection of genotoxic agents and in the identification of species and populations at higher risk; *b*) the difference in sensitivity of the species selected as bioindicators in relation to the cytogenetic end points analyzed; *c*) the need to use at least two sympatric species to detect the presence of genotoxins in each locality; and *d*) the need to use several end points when trying to establish a genotoxic profile of an area. *Key words*: cytotoxicity, genotoxicity, *in situ* biomonitoring, wild rodent populations. *Environ Health Perspect* 108:1165–1169 (2000). [Online 13 November 2000]
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The analysis of cytogenetic end points in organisms exposed to chemicals in their natural environment may help in the early detection of genotoxic damage. This methodology was used to evaluate the impact of environmental compounds occurring in the Itajaí Valley, state of Santa Catarina, Southern Brazil. The lumber, textiles, paper, cigarette, and pesticide industries, besides intense rice cultivation, are the main economic sources of the region. Three areas were chosen for this study: an industrial, an agricultural, and a permanent preservation area. Two species of wild rodents naturally occurring in the three areas, *Akodon montensis* and *Oryzomys nigripes*, were analyzed.

Cytogenetic preparations were obtained using conventional methods. The end points under study were the polychromatic/normochromatic cell ratio (PCE/NCE); the mitotic index (MI); the frequency of cells with micronuclei in the bone marrow and peripheral blood; and the frequency of cells with chromosome aberrations in the bone marrow.

The purpose of the study was to determine the performance of the species and cytogenetic end points as indicators of genotoxic activity in the study areas. The results show *a*) the role of the end points used as biomarkers in the early detection of genotoxic agents and in the identification of species and populations at higher risk; *b*) the difference in sensitivity of the species selected as bioindicators in relation to the cytogenetic end points analyzed; *c*) the need to use several end points when trying to establish a genotoxic profile of an area; and *d*) the need to use at least two sympatric species to detect the presence of genotoxins in each locality.

Materials and Methods

Collecting areas. The area of study is located at the Medium Valley of the Itajaí River, state of Santa Catarina, Brazil. We selected three collecting points: a preservation area, to monitor animals not directly exposed to environmental pollution and for comparison with the other two points; a rice field, to evaluate the genotoxic effects of pesticides used in the rice culture; and an industrial area, to assess the effect of industrial, domestic, and agricultural waste.

Animals. Because there were no previous records on the diversity and density of wild rodents in the region, we chose the species for this study after the captures. Three criteria were established: range of distribution, including the three selected locals; population density (enough to ensure a sample of approximately 10 animals of each species at each collecting point during a 1-year period); and sympatry.

Based on these criteria and on the results of the captures, two species of wild rodents were chosen: *Akodon montensis* ($2n = 24$) and *Oryzomys nigripes* ($2n = 62$), both well-known cytogenetically. Individuals were live-trapped at the three collecting areas.

Cytogenetic preparations and analysis. Once in the laboratory, the animals were weighed, sexed, and marked. One hour after being injected with a 0.01% colchicine solution (1 mL/100 g/body weight), they were killed by ether inhalation. We used each individual for estimating all cytogenetic end points. Micronuclei and metaphase preparations were obtained using published techniques (*1,2*), with the substitution of sodium citrate by KCl 0.0075M, and the staining of the slides with Giemsa.

We prepared five slides per animal for assessing micronucleated cells in the bone marrow. Five smears prepared from blood, drawn by heart puncture from each specimen, were used for scoring peripheral-blood micronucleated erythrocytes. The smears were air dried for 24 hr, fixed in methanol P.A. for 10 min, and stained in Feulgen/Fast-Green.

We selected 100 well-spread metaphases per animal from 5 slides for scoring the frequencies of cells with gaps and breaks, cells with at least one chromosome showing early separation of the centromere (early segregation), polyploid, and extremely damaged (i.e., with nuclear material visibly different from normal patterns of cell division stages).

All cytologic analyses were carried out in a blind test, using an optical microscope at a 10×100 resolution.

The following parameters were analyzed in each specimen: *a*) the frequency of PCE among the first 100 NCE observed in each slide (PCE/NCE ratio); *b*) the frequency of micronucleated polychromatic erythrocytes (MNPCE) within 2,000 polychromatic erythrocytes counted; *c*) the frequency of micronucleated normochromatic erythrocytes (MNNCE) within 2,000 normochromatic erythrocytes counted; *d*) the frequency of dead cells (DC; erythrocytes with > 3 micronuclei in the bone marrow) within 2,000 cells; *e*) the frequency of micronucleated peripheral blood erythrocytes (MNPBE) within 4,000 erythrocytes; *f*) the frequency of metaphases among 2,000 cells (MI); and *g*) the frequencies of cells with chromosome aberrations among 100 metaphases.

The frequencies of micronucleated cells from the bone marrow and peripheral blood in both species allowed three types of analyses to be made: the intraspecific sympatric comparisons, where the different types of cells were compared in each species, at the three localities; the intraspecific allopatric comparisons, where the same end point in each species was compared among the three localities; and the interspecific sympatric comparisons for each end point at each locality. Only the last two types of comparisons were done for the frequencies of chromosomal aberrations.

Statistical analysis. To choose appropriate statistical methods to be used in our data analysis, we took into consideration the following facts: the counts for the end point PCE/NCE are observations of a negative binomial variable; recall that the frequency of PCE cells was counted to obtain the 100th NCE cell. The counts for all other parameters were considered as observations of Poisson variables. Note that they are counts of rare events.

Comparisons are of six groups (two species in three areas) for each of the end points, where the observations are either a negative binomial variable sample or counts of rare events (Poisson variable sample).

For the case of the negative binomial model, we used the regular chi-square test that compares the observed frequency with the expected frequencies under the null hypotheses. For the Poisson case we used the conditional likelihood (*3*). Whenever low frequencies were obtained, we used an exact conditional test, and for larger frequencies we used the chi-square test. For details on conditional tests that compare counts of rare events, see Pereira (*4*).

In calculating the figures to perform the test, we added the individual frequencies of each group. In both cases, the negative binomial and Poisson, the total frequency is a sufficient statistic, and any decision rule can be focused in its value replacing the set of individual values. This procedure considers each group as a random sample of a common distribution, negative binomial, or Poisson. To test differences among groups, we computed the expected frequencies by weighing each group by its sample size (the number of units in the group).

To compare the frequencies of MN in different groups of cells (PCE and NCE, for instance) within the same group, we considered the square root of the frequencies—a transformation used to achieve constant variance—and used the *t*-test for paired samples. For discussion about the transformation, see Bishop et al. (*5*).

To simplify the notation, we represented the two species, *Akodon montensis* and *Oryzomys nigripes*, by *Am* and *On*, respectively. The three areas, preservation area, rice field, and industrial area, were denoted by PA, RF, and IA, respectively. The six independent sample groups were then represented by *Am* PA, *Am* RF, *Am* IA, *On* PA, *On* RF, and *On* IA.

Results and Discussion

Tables 1, 2, and 3 show all of the observed values for each animal. The number of animals in each sample varied depending on the capture and because some animals did not yield good preparations for the analyses of all end points.

To evaluate the genotoxicity of environmental compounds, we compared indices of genotoxicity in animals from an "exposed" and a "less exposed" area. Our "less exposed" area was the wildlife preservation. We assumed that exposure to environmental compounds occurred to a lower degree in the preservation compared to the other two study areas.

Intraspecific Comparisons

The visualization of micronuclei and chromosome aberrations depends on the proliferation capacity of the cells subjected to the initial damage. Analyses of the PCE/NCE ratio and the MI are therefore indispensable for the interpretation of the results obtained for those end points. Besides revealing possible cytotoxic effects of environmental contaminants, these indices may also reflect the

Table 1. Individual frequencies for each cytogenetic end point: preservation area.

Species/sample	Sex	PCE per 100 NCE	MNPCE per 2,000	MNNCE per 2,000	DC per 2,000	MNPBE per 4,000	Metaphases per 2,000	Cells with breaks[a]	Extremely damaged[a]	Polyploid[a]	Early segregation[a]
Akodon montensis (*n* = 12)											
PA1	M	19	7	15	5	34	31	6	0	0	4
PA2	M	52	9	3	0	20	19	10	0	0	0
PA3	M	50	1	2	0	35	26	2	0	2	0
PA4	F	31	8	7	0	24	54	3	0	3	1
PA5	F	68	6	6	0	33	22	1	0	1	0
PA6	F	53	6	4	0	39	35	5	0	4	4
PA7	M	45	12	16	0	26	30	1	0	1	0
PA8	M	28	3	4	0	25	46	6	0	0	0
PA9	M	99	6	3	12	32	21	2	0	0	1
PA10	M	27	5	2	0	28	27	2	1	1	2
PA11	M	31	8	11	0	34	45	2	0	0	0
PA12	M	57	14	9	0	12	32	3	0	6	0
Total		560	85	82	17	342	388	43	1	18	12
Mean		46.7	7.1	6.8	1.4	28.5	32.3	3.6	0.1	1.5	1.0
Oryzomys nigripes (*n* = 11)											
PO1	M	21	29	23	15	34	45	3	0	0	4
PO2	M	69	44	51	0	11	55	4	0	0	0
PO3	M	26	15	7	1	15	84	2	0	0	0
PO4	M	80	24	21	1	32	26	2	0	0	0
PO5	F	40	8	9	0	29	49	9	0	0	0
PO6	F	59	16	9	14	18	60	5	0	0	0
PO7	M	41	25	20	0	83	95	9	0	0	0
PO8	F	137	6	7	1	24	61	0	0	2	3
PO9	M	216	12	24	0	55	62	2	0	1	0
PO10	F	135	4	15	16	25	72	3	0	1	3
PO11	M	84	11	24	42	21	29	3	0	0	1
Total		908	194	210	90	347	638	42	0	4	11
Mean		82.5	17.6	19.1	8.2	31.5	58.0	3.8	0.0	0.4	1.0

[a]Per 100 cells.

influence of confounding factors such as animal age, health, nutritional status, presence of pathogens, and intra- and interpopulational genetic variability. Although these factors may be present in the animals from all areas of study, there is no reason to suspect that they prevail in any of them.

The analysis of the PCE/NCE ratio at the three areas showed that the majority of the individuals in both species had a ratio within or very close to the values reported for mice in the literature—that is, between 0.5 (*6*) and 1.0 (*7*). We assume, therefore, that the end points analyzed in this cell-line are not compromised.

Table 2. Individual frequencies for each cytogenetic end point: rice field.

Species/sample	Sex	PCE per 100 NCE	MNPCE per 2,000	MNNCE per 2,000	DC per 2,000	MNPBE per 4,000	Metaphases per 2,000	Cells with breaks[a]	Extremely damaged[a]	Polyploid[a]	Early segregation[a]
Akodon montensis (n = 13)											
RA1	M	86	23	13	23	21	45	3	0	0	0
RA2	M	31	8	19	2	35	112	2	0	1	1
RA3	M	83	13	3	0	17	80	1	0	0	0
RA4	M	58	17	2	0	12	53	10	3	0	2
RA5	M	31	6	4	0	17	58	3	0	3	0
RA6	F	90	14	9	0	16	69	3	0	2	2
RA7	M	175	12	15	1	12	17	3	0	3	0
RA8	M	124	24	7	6	29	44	3	0	0	2
RA9	M	64	5	4	0	12	39	0	0	0	0
RA10	M	105	7	10	2	30	98	2	0	1	5
RA11	F	120	12	8	6	13	71	1	0	3	0
RA12	F	39	14	7	0	55	84	12	2	2	0
RA13	F	27	9	2	1	40	80	5	0	1	0
Total		1,033	164	103	41	309	850	48	5	16	12
Mean		79.5	12.6	7.9	3.2	23.8	65.4	3.7	0.4	1.2	0.9
Oryzomys nigripes (n = 8)											
RO1[b]	M	81	19	17	10	-	48	10	0	0	0
RO2[b]	M	44	15	14	24	-	54	9	0	0	6
RO3[b]	M	51	22	31	3	-	102	18	0	1	1
RO4[b]	M	48	4	6	0	-	23	5	8	0	1
RO5	F	43	18	10	0	59	18	4	0	0	2
RO6	M	85	11	13	75	36	32	1	1	0	1
RO7	M	59	33	20	10	24	58	8	0	1	7
RO8	M	67	21	10	18	4	63	1	1	1	9
Total		478	143	121	140	123	398	56	10	3	27
Mean		59.8	17.9	15.1	17.5	30.8	49.8	7.0	1.3	0.4	3.4

[a]Per 100 cells. [b]Animals that did not yield good MNPBE preparations.

Table 3. Individual frequencies for each cytogenetic end point: industrial area.

Species/sample	Sex	PCE per 100 NCE	MNPCE per 2,000	MNNCE per 2,000	DC per 2,000	MNPBE per 4,000	Metaphases per 2,000	Cells with breaks[a]	Extremely damaged[a]	Polyploid[a]	Early segregation[a]
Akodon montensis (n = 13)											
IA1	F	37	6	7	22	19	75	6	0	0	0
IA2	M	131	10	6	0	23	44	2	3	0	0
IA3	F	100	11	4	0	15	82	6	0	0	0
IA4	M	90	10	8	0	20	40	6	0	2	1
IA5	F	56	9	8	0	31	74	9	1	1	2
IA6	F	45	13	7	0	14	63	2	0	1	4
IA7	M	63	9	4	0	41	42	1	0	0	1
IA8	M	46	6	5	0	28	56	4	0	1	1
IA9	M	27	1	2	0	29	57	1	0	1	0
IA10	M	45	2	6	0	30	51	7	0	0	1
IA11	M	37	24	18	0	37	54	6	0	0	0
IA12	F	98	17	17	0	37	68	2	0	0	0
IA13	M	65	33	17	0	44	52	1	0	2	0
Total		840	151	109	22	368	758	53	4	8	10
Mean		64.6	11.6	8.4	1.7	28.3	58.3	4.1	0.3	0.6	0.8
Oryzomys nigripes (n = 10)											
IO1[b]	F	18	23	9	0	-	67	8	0	2	0
IO2	F	18	24	16	2	24	94	1	0	1	0
IO3	M	66	20	18	10	51	46	0	0	0	0
IO4	F	36	30	10	1	20	77	2	0	0	5
IO5	M	20	14	17	35	89	48	1	0	1	0
IO6	F	37	19	21	0	64	49	1	1	3	0
IO7	F	52	10	13	9	26	60	5	0	1	5
IO8	M	25	20	6	32	60	89	2	0	7	3
IO9	F	86	11	4	0	44	50	4	0	0	0
IO10	F	62	10	7	1	32	77	5	0	0	6
Total		420	181	121	90	410	657	29	1	15	19
Mean		42.0	18.1	12.1	9.0	45.6	65.7	2.9	0.1	1.5	1.9

[a]Per 100 cells. [b]Animals that did not yield good MNPBE preparations.

The significantly ($p = 0.000\%$) higher values found for MI in the exposed areas for *Am* suggest the presence of cytotoxic agents that reached the bone marrow, inducing either a regenerative effect or a mitotic delay. For *On*, although the same explanation could account for the high values in IA ($p = 0.01\%$), only a strong cytotoxic effect could account for the significantly ($p = 1.62\%$) lower MI in the RF.

For the group *Am* PA, there was no significant difference between the frequencies of young MNPCE and mature MNNCE, but significantly ($p = 0.12\%$) higher values were found in the frequency of MNPBE. The *Am* individuals of PA had the lowest ($p = 0.001\%$) value of MNPCE when compared to the ones of other areas, as expected.

The fact that the frequencies of MNNCE cells did not differ significantly among the three collecting areas may seem paradoxical because we could expect higher values at the exposed areas. But taking into account a higher mortality of the younger cells in the exposed areas, these results could be explained. The RF animals exhibited the lowest ($p = 0.89\%$) frequencies of MNPBE. Because these originate from live micronucleated cells in the bone marrow, we might expect a high mortality of polychromatic injured cells at RF, suggesting an important cytotoxic effect.

At the IA, the higher values of MNPCE in relation to PA are indicative of a strong genotoxic effect.

The higher MI in exposed areas and of micronucleated cells in bone marrow, however, are not accompanied by an increase of the frequency of extremely damaged cells or by cells with chromosome breaks. The presence of spindle poisons at the contaminated areas seems to be an acceptable explanation. The situation for *Am* reinforces the possibility that erythrocytes and other cell lines in the bone marrow may have different susceptibilities to the same agent (*8–10*). It also emphasizes the need for using several end points in distinct cell lines of the individuals in a population to establish the genotoxic environmental profile of an area.

In the group *On*IA, the frequency of MNPCE was higher ($p = 1.74\%$) than that of MNNCE, indicating an apparent intense and continuous genotoxic effect in the animals from this locality. [The effects observed in the target cells of animals living in the wild may be classified depending on the combination of two factors: the intensity and the frequency of the animal's exposure to environmental genotoxins. The first may be intense, moderate, or weak, and the second may be accidental, intermittent, continuous (for a long period), or chronic (lifelong).] However, because the frequency of MNPBE is higher ($p = 0.000\%$) than that of the bone marrow (MNPCE + MNNCE), a moderate and chronic effect may be the actual situation. There may be a contaminant that exerts a cytotoxic effect in IA, which would account for the decrease in the frequency of micronucleated cells in the bone marrow, as compared to the peripheral blood. The higher MI observed in this region may support this hypothesis.

In relation to MNPCE, nonsignificant differences among the three areas were detected for *On*. This result could be explained by a high mortality of these cells at the exposed areas, not allowing a differentiation from the PA, where we expected lower frequencies. The higher ($p = 0.02\%$) values of MNNCE frequency at PA and of dead cells ($p = 0.000\%$) at RF corroborate this hypothesis and also suggest a strong genotoxic and cytotoxic effect at RF.

Based on what was found for the above mentioned end points, we would expect for MNPBE frequencies a higher value at the PA, where the frequency of MNNCE was higher and a lower value at the RF because of the higher frequency of dead cells. This was not observed: the frequency of MNPBE was significantly ($p = 0.00\%$) higher at IA and did not differ at the other two. This seems to indicate a strong genotoxic effect for *On* at IA as compared to PA. In other words, if only the bone marrow parameters were considered for *On*, we would predict a stronger genotoxic and cytotoxic action at RF, whereas the analysis of the peripheral blood could point to a stronger effect at IA.

The *On*RF population showed significantly higher frequencies of cells with breaks ($p = 0.003\%$), extremely damaged ($p = 0.002\%$), and cells with early segregation of one or more chromosomes in relation to the other areas ($p = 0.13\%$).

The presence of one or more chromosomes with early or asynchronic separation of the centromere may be characteristic of a species or may result from the action of environmental agents (*11–14*). In *On*, this phenomenon was more frequent at RF, suggesting that even if genetic factors are involved, the interpopulational variation observed must be influenced by outside factors (i.e., environmental agents). It is possible that the early segregation induced by extrinsic factors results from agents altering the cell cycle.

Some carbamates are known to induce aneugenic (*15*), clastogenic (*16*), and asynchronic centromeric separation (*14*); others have no cytogenetic effect either in mitosis or meiosis (*17*). It is also known that the effects of a mixture of pesticides are often different from the individual effects of each drug (*18*).

At RF, at least two types of carbamates are used: Furadan 5G and Satanil E, besides the organophosphate Roundup, which was proved nonmutagenic in several test systems.

Considering the pesticide mixture used at the RF, the higher frequency of cells with chromosome breaks and asynchronic centromere segregation in animals from this area could result from the action of both clastogenic and aneugenic (spindle poison) agents. The cytogenetic effects would probably be a consequence of the mixture of pesticides used at the area rather than of the individual effects of each one of them.

The frequency of polyploid cells was significantly higher at IA, suggesting the presence of aneugenic substances also at this locality. It is interesting that the chromosome number exhibited by these cells was never an exact multiple of the haploid number for the species, a characteristic of spindle poison-induced polyploidy (*19*).

Results obtained with the micronucleus test in the bone marrow and peripheral blood of the same individuals of *On* also pointed to a strong genotoxic effect at the RF and IA. The discrimination between a clastogenic and an aneugenic origin of the micronuclei was not attempted.

Interspecific Comparisons

The comparisons of the frequencies of micronucleated cells, including dead cells at all areas suggest a higher sensitivity of *On* to the genotoxic environmental agents; the exception was the frequency of MNPBE at the PA, where no statistical difference was found between the species.

The fact that *On* PA had higher values for MI than *Am* PA may indicate that this end point is naturally higher for *On*. If this were true, the results obtained for the exposed areas might be revealing a stronger cytotoxic effect in RF for *Am*.

The nonsignificant result in the comparison between the frequencies of DC in animals from the PA and IA may suggest a cytotoxic and genotoxic effect for *On* from PA. This might explain the nonsignificant difference in the frequencies of MNPBE between the two species in this area. It would also account for the results of MNPCE frequency.

The analysis of the end points that may disclose a difference in susceptibility to clastogenic agents between these two species (cells with breaks) showed that the values at the RF were significantly higher in *On*. Apparently, this species is more sensitive than *Am* to clastogens present at this locality.

The end points that may show a species difference to the effects of agents interfering with the mitotic spindle are also informative. *On* and *Am* did not differ significantly in the frequency of cells with early segregation at the PA, but values were higher for *On* at the exposed areas. The frequency of polyploid

cells was higher at RF and PA in *Am*, and higher (*p* = 0.09%) at the IA in *On*.

These results suggest the presence of at least two types of spindle poisons at the exposed areas—one inducing aneuploidy and another polyploidy. The occurrence of at least a polyploidy inducer is possible at PA. It is noteworthy, however, that the same substance (benomyl, a carbamate fungicide) can be responsible for both effects (*20*).

Summing up, our results suggest that *a)* *On* is more sensitive than *Am* to spindle poisons, and *b)* *Am* is more susceptible to polyploidy inducers at the PA and RF, and *On* at the IA. This effect could result from the different nature of these substances at the three areas, different interactions of the same agent at different areas, or both.

Conclusions

This study showed that the use of only one cytogenetic end point is not sufficient to understand the range of the effects of the environmental agents on the genetic material in an *in situ* study. However, when other end points are included, the method can be very promising.

Because of differences in sensitivities, it is necessary to have at least two sympatric species for comparison to establish a genotoxic profile of each area. The idea that different responses to genotoxic agents may reflect intrinsic differences rather than differences related to exposure is corroborated (*21*). The presence or absence of certain species in an environment may often be related to severe effects of environmental stressors, which lead to the elimination of one or more species from certain localities. The main purpose of monitoring the environmental health of populations is the early detection of the effects of stressors. These effects must be reflected in the whole population, or they will not be relevant either ecologically or evolutionally (*22*).

The genotoxic effects here identified, in the three populations of both species, showed that micronucleated cells are a relevant biomarker for the early detection of environmental stressors and give an indication of which intraspecific and interspecific populations are at higher risk. An environmental genotoxic profile may thus be drawn for a certain locale, for a certain period, based on a specific biomarker, which is also an important indicator of possible future alterations in the fauna of local communities.

We cannot say that the analysis of the frequencies of cells with chromosome aberrations of any species, at different localities, is sufficient to detect environmental genotoxic effects. However, when results obtained with another sympatric species are considered, and a joint analysis of several cytogenetic end points and proliferative indices (including micronucleated cells, chromosome aberrations, MI, and poly/normochromatic erythrocyte ratio) from both species are made, they can be used either in the early detection or in the discrimination of genotoxins in the environment.

The use of terrestrial and aquatic organisms for *in situ* environmental assessment is becoming a widely accepted method for identifying risks to ecosystems and human health (*23*). More research is needed on the development of methods. The choice of suitable biological indicators and end points will help decision makers to take corrective measures against environmental contamination.

Results obtained using the *in situ* cytogenetic biomonitoring, although relevant, cannot by themselves serve as an environmental diagnosis or prognosis. They may, however, contribute as indicators in a wider approach.

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