

Item ID Number 03100 **Not Scanned**

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Report/Article Title Typescript: Histological Evaluation of Brain Sections
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2,4-D in a 2-Year Chronic Oral Toxicity Study

Journal/Book Title

Year 0000

Month/Day

Color

Number of Images 15

Description Notes

HISTOLOGICAL EVALUATION OF BRAIN SECTIONS OBTAINED FROM F344
RATS EXPOSED TO VARIOUS DOSES OF 2,4-D IN A
2-YEAR CHRONIC ORAL TOXICITY STUDY

Adalbert Koestner

My name is Adalbert Koestner. My curriculum vitae is attached to this report which reviews brain sections obtained from F344 rats exposed to various doses of 2,4-D in a 2-year chronic oral toxicity study.

Objectives of this consultation

- A. To review brain tumor sections of affected animals and reconfirm diagnoses rendered by project assigned pathologists.
- B. To determine whether a prospective causal relationship exists between the presence of the gliomas and the feeding of the 2,4-D herbicide.

Results:

1. Brain Tumor Review

	<u>Male</u>	<u>Female</u>
C	1/60	0/60
1 mg/kg	0/60	1/60
5 mg/kg	0/60	2/60
15 mg/kg	2/58	1/60
45 mg/kg	5/60	1/60
TOTAL	8/298 (2.6%)	5/300 (1.6%)

As Table I indicates there was a noticeable greater number of brain tumors in the male high dose group than in the control group, namely 5 to 1. A sixth tumor was added to the high dose group after additional brain tissue was embedded and sectioned (case #23473, high dose male). This small lesion is most likely not neoplastic. It consists of a mixed glial and mesenchymal cell population and has, therefore, not been included in the table. In our experience, early astrocytomas are remarkably monomorphic and elicit no tissue reaction. A typical lesion of that sort is illustrated in a 2 mm astrocytoma of the olfactory bulb (case #23505, high dose male). In contrast, the lesion in case #23473 consists of a pleomorphic perivascular and dispersed cell population including granulocytes and lymphocytes in addition to glial cells. Special stains in this case reveal reticulin and collagen formation which is a function of specific mesenchymal cells but not of astrocytoma cells.

All but one of the brain tumors in the high dose group male rats were discovered at termination of the experiment (105 weeks); the one was incidentally found (small lesion in olfactory bulb, 1-2 mm, case #23505) in a rat which died for other reasons at 93 weeks of age. There were no brain tumors in male rats in the 1 and 5 mg/kg dose groups. Two tumors were recognized in the 15 mg/kg dose group at 94 and 105 weeks. The one tumor in a control rat (case #23025) was a large neoplasm which was identified by the dissecting pathologist as the cause of death of this rat at 21 weeks of age. None of the 2,4-D exposed rats died as a consequence of astrocytoma development. There is no brain tumor increase in females in the high dose group. The 5 brain tumors in female rats were all detected at the termination of the experiment (105 weeks) and were, like those in the males, between 2-5 mm in their longest diameter.

Histologic characterization of these brain tumors:

All tumors are of glial origin and were diagnosed as astrocytomas. They are generally well differentiated with little reaction by the surrounding brain. In some there are areas of necrosis with subsequent repair responses. One astrocytoma (case #23500) is a mixed glioma (oligoastrocytoma), mostly astrocytic. All gliomas in these rats are morphologically similar and they are identical to glial tumors routinely recognized in aged rats.

B. Determination of evidence or lack of evidence of a causal relationship between tumors and feeding of the 2,4-D herbicide:

Background: At the age of 2 years and beyond, a variety of neoplasms occur spontaneously in various organs of rodents in a random distribution among experimental and control groups. A higher tumor in control rats over those exposed to a test compound is easily discarded as a chance incidence. However, the opposite usually creates severe problems in interpretation. Statistical analysis in these cases is often equivocal at best, and sometimes meaningless in assessing the carcinogenic potential of the test substance. It is, therefore, advisable to precede statistical evaluation with a careful biologic analysis permitting distinction between spontaneous and induced tumor incidences. While this distinction may not be completely achievable in every case, separation in most instances with a high degree of certainty is possible (Koestner 1984).

Biological Criteria for Evaluation of Neurocarcinogens:

1. Increased Incidence Beyond Expected Control Levels
2. Shift of Tumor Appearance to a Younger Age (decreased survival time)

3. Demonstration of Dose-Effect Relationship
4. Higher Tumor Incidence after Transplacental Exposure
5. Trend Toward Anaplasia
6. Presence of Preneoplastic Lesions
7. Multiplicity of Tumors in Individual Animals
8. Tumor Occurrence in Both Sexes
9. Tumor Occurrence also in Peripheral Nervous System
10. Tumor Induction Outside the Nervous System
11. Genotoxicity, Mutagenicity, Chromosomal Aberrations

It will depend upon the experimental design, the carcinogenic potential of the test substance and the availability of tissue samples at various stages during the course of the experiment whether all of these criteria apply to or are testable in any single case. Some, however, will always be present and will permit a distinction to be made between experimentally induced and naturally occurring brain tumors (Koestner, 1986).

Let us follow these criteria one by one and compare the outcome of the 2,4-D study with that of known neurocarcinogens such as methyl- and ethylnitrosoureas (MNU and ENU).

1. Increased incidence beyond expected control level.

In order to assess this criterion it is necessary to review critically the natural brain-tumor incidence in rats. Brain tumors in various rat strains were recorded by commercial breeding companies, individual researchers and governmental agencies. However, many surveys of naturally occurring brain

tumors in rats either were done in animals that were too young or were restricted to grossly visible tumors. Therefore, a great variation in the natural brain tumor incidence in rats is reported in the literature. Recent reports are more reliable because of the awareness of the unexpected high natural incidence of gliomas in rats as compared to other animal species and man. Table II presents incidences of gliomas in control male Sprague-Dawley rats encountered by commercial testing laboratories. The range was from 0-10%.

Table II

VARIABILITY IN BRAIN GLIOMA INCIDENCE
IN CONTROL MALE SPRAGUE-DAWLEY RATS 1 YR. AND OLDER
(SELECTED FROM SWENBERG, J.A. 1986)

NUMBER	COLOR	LABORATORY	CONTROL 1(%)	CONTROL 2(%)	CONTROL 3(%)
1-5	Diff. Colors	IRDC	0/292 (0)	2/287 (0.7)*	2/137 (1.4)
6	Red No. 33	IRDC	3/57 (5.2)	0/59 (0)	2/58 (3.4)
7	Green No. 3	Biodynamics	0/52 (0)	5/55 (9)**	--
8	Blue No. 2	Biodynamics	0/59 (0)***	2/59 (3.4)	--
9-13	Diff. Colors	Biodynamics	2/290 (0.7)	2/289 (0.7)	4/231 (1.7)
14	Red No. 9	Litton	4/58 (6.9)****	6/60 (10)	2/57 (3.5)
15	Red No. 27	Litton	2/54 (3.7)	0/55 (0)	--
16	Red No. 36	Litton	2/57 (3.5)	1/59 (1.7)	0/53 (0)
17	Red No. 30	Hazleton	3/59 (5.1)	1/55 (1.8)	--

* One of the rats died on day 350 with a glioma

** Additional sections resulted in 6/55 (10.9%)

*** Additional sections resulted in 2/59 (3.4)%

**** One glioma diagnosed at 12 mos. interim sacrifice

There is less experience with the F344 rat strain, however, in the NCI chronic carcinogenicity studies the incidence of gliomas in 4700 male & female F344 rats ranged from 0-3.3% (Ward & Rice, 1982). Soleveld et al (1984) reported a 2.8% incidence of gliomas in the male and 1.6% in the female F344 control rats in a life-time toxicity study. These incidences are generally based on only 3 sections per brain. It should be emphasized that most of these gliomas in 2-yr. old rats are "microtumors", meaning that they are small enough and unobtrusive enough so that they can only be recognized upon microscopic examination.

If we consider that nothing had been done to the 600 rats in the 2,4-D study, the incidence of expected brain tumors would have been about the same as it actually occurred. Table I indicates that 8 gliomas were identified in 298 male rats for a total of 2.6% and 5 gliomas were recognized in 300 female rats amounting to 1.6%. This incidence is well within the reported incidence of gliomas in the F344 rat. It should be emphasized that this incidence was achieved on the basis of sections from 7 blocks as compared to 3 in standard tests. Two of the astrocytomas, for instance, were tiny lesions (1-2mm in diameter) found after additional embedding and sectioning in the olfactory bulb which is routinely never embedded and examined.

The first criterion for neurocarcinogens has not been met in the 2,4-D study since the substance has not been able to raise the brain tumor incidence beyond the expected spontaneous tumor level.

2. Shift of tumor appearance to a younger age.

This is a general principle in experimental carcinogenesis but applies particularly to brain tumors since space-occupying lesions within a restricted cranial cavity, whether benign or malignant, inevitably shorten survival. When methylnitrosourea (MNU), a potent neurocarcinogen, was administered by repeated injections to young adult rats, 97% of the exposed animals developed tumors of the nervous system, with an average survival time of 339 days (Koestner, 1978; Swenberg, Koestner & Wechsler, 1972). The tumors killed the great majority of the animals within the first year of life. In contrast, all tumor-bearing experimental animals in the 2,4-D study survived the 2 years (except for 2 which died for other reasons at 93 and 94 weeks respectively and the control rat which died as a consequence of the large astrocytoma at 21 weeks of age) and the tumors were mostly undetectable by gross observation. Tomatis (1979) lists 21 chemical substances capable of producing neurogenic tumors. There was a decreased latency and/or survival time for brain-tumor-bearing rats with all the compounds for which such data were recorded. Even a very weak carcinogenic compound such as methyl methanesulphonate (MMS), producing only a 6% gliomas incidence (out of a 12-20% total neuroectodermal tumor burden) reduced the survival of animals with brain tumors to an average of 292 days (Kleihues, Mende & Reucher, 1972). Spatz & Laqueur (1967) reported a 7% glioma incidence in Sprague-Dawley rats following exposure to crude cycad material. The range of survival of brain-tumor-bearing rats in this experiment was between 6.5 and 15 months, with a mean of 12 months. Decreased latency seems to be a fairly consistent characteristic of neuro-oncogenic substances. It was not observed in the 2,4-D exposed animals.

3. Demonstration of dose-effect relationship

Using the potent carcinogen ethylnitrosourea (ENU) as an example, one can demonstrate a clear dose-effect relationship; as the dose is increased the tumor incidence increases and the survival time is shortened (Table III). If we compare these results with those obtained with the weak carcinogen MMS, we find a similar dose-effect relationship (Table IV).

TABLE III: TRANSPLACENTAL TUMOR INDUCTION WITH ENU IN SPF SPRAGUE-DAWLEY RATS ON THE 20TH DAY OF GESTATION (KOESTNER, 1971; 1978)

Single Dose I.V. mg/kg	Number of days	Number of Offspring	Number of Rats with Tumors	Average Survival Time (Days)
1	4	46	13	487
5	3	24	14 (58%)	368
20	2	17	16 (94%)	269
50	2	25	25 (100%)	211

TABLE IV: NEUROGENIC TUMORS IN BD RATS FOLLOWING PRENATAL ADMINISTRATION OF METHYL METHANESULPHONATE

Dose (mg/kg)	No. of Offspring*		
	Total	With tumors	With malignancies
20	19	2(10.5%)	1
40	18	3(16.6%)	2
68	10	2(20%)	2

* Calculated from results reported by Kleihues et al. (1972).

No comparable dose-effect relationship exists in the 2,4-D study. The expected tumor incidence shows an unequal distribution with a cluster in the high dose group and no shortening of survival time.

4. Higher tumor incidence after transplacental exposure

The fourth criterion of higher tumor incidence after transplacental exposure can not be evaluated because it was not tested.

5. Trend toward anaplasia

The tumor spectrum in the 2,4-D study was comparable to that found in surveys of the spontaneous brain tumors in rats. Histological examination showed the latter to consist primarily of mature and differentiated tumors of the astrocytic cell population. In contrast 53% of the brain tumors produced with MNU were either unspecified gliomas (14%), anaplastic gliomas (14%) or gliosarcomas (7%). Table IV also indicates that the weak carcinogen methylmethanesulphonate also produced primarily malignancies; 5 of the 7 tumor-bearing animals had malignant neurogenic neoplasms (most rats had several tumors).

6. Presence of preneoplastic lesions

Toxic lesions and preneoplastic or early neoplastic proliferations preceding micro- and macrotumors have been reported in many studies dealing with chemically induced neoplasms, including studies with neurocarcinogens. Such lesions are particularly apparent in experiments in which sequential killing of the animals was performed during the latency period as part of the

experimental design. Among offspring of rats exposed to ENU during gestation, 50% have been shown to contain early neoplastic proliferations in their trigeminal nerves at 3 weeks of age and over 90% had these lesions at 3 months of age, although the peak of neurinomas is at 7 months (Koestner, 1983; Swenberg, Clendenon, Denlinger & Gordon, 1975). Early neoplastic (preneoplastic) glial proliferations were recognized at 4 months of age, while glial tumors appeared much later (Swenberg et al. 1972a). These early neoplastic glial proliferations are always a good indicator of chemical tumor induction in contrast to the occurrence of spontaneous neoplasms in aged rats, when such early proliferations rarely occur. There were no preneoplastic lesions in the 2,4-D study in neither rats killed prematurely or those killed at termination of the experiment.

7. Multiplicity of tumors in individual animals

Multiplicity of neurogenic tumors induced with ENU and with MNU is the rule rather than the exception (Kleihues et al. 1972; Koestner et al. 1971; Swenberg et al. 1972). In our studies with N-nitrosoureas (Koestner et al. 1971; Swenberg et al. 1972) up to 3 and 4 tumors of the nervous system occurred in the individual rats, most of them malignant. In contrast, no rat in the 2,4-D study had more than one neurogenic neoplasm.

8. Tumor occurrence in both sexes

In the nitrosourea studies (Koestner et al. 1971; Swenberg et al. 1972) a high incidence of neurogenic neoplasms occurred in both sexes and no statistically significant difference was discernable. In the 2,4-D study the overall

incidence in the male rats is slightly higher than in the female (2.6 to 1.6) as is the case under natural conditions. The female rats in the 2,4-D study show by chance an almost equal tumor distribution among the groups.

9. Tumor occurrence in both the central and peripheral nervous system

In the nitrosourea studies almost all rats which lived long enough (peripheral nervous system tumors have a longer latency period) developed neurinomas. Kleihues (1972) with the weak neurocarcinogen methylmethanesulfonate produced more tumors of the peripheral nervous system than of the central nervous system.

No tumors of the peripheral nervous system were found in the 2,4-D study.

10. Tumor induction outside the nervous system

Most systemic neurocarcinogens produce in addition to neurogenic tumors extraneural neoplasms such as leukemia, lymphoma, carcinomas of various organs and sarcomas.

There was no significantly higher extraneural tumor incidence in 2,4-D treated rats.

11. Genotoxicity, mutagenicity, chromosomal aberrations

Most known carcinogenic agents are also genotoxic. The herbicide 2,4-D (Kociba, 1983) proved negative in the most commonly used mutagenicity tests such as the Ames test, other bacterial tests, the erythrocyte micronucleus test in mice, the dominant lethal study for mutagenesis in mice and on the

genetic material of human lymphocytes. Only in vitro mutagenicity tests in Saccharomyces cerevisiae showed a positive response and mixed results were obtained in tests with fruit flies (Drosophila melanogaster). Overall these tests indicate little or no mutagenic potential for man.

CONCLUSION

Evaluation of the neurocarcinogenicity of chemical substances in chronic (2-year) toxicity studies should not be accomplished by statistics alone without a thorough biological analysis of the results. The variation of incidences of naturally occurring brain tumors in rats and the high chances of a haphazard distribution among the randomly grouped animals should be expected. In case of unequal tumor distribution favoring high dose groups the above listed criteria (page 3) should be tested. If all or some of these criteria are met, a statistical analysis for significance is most appropriate and essential. None of these criteria (criterion 4 not tested) were met in the case of the 2,4-D study and the substance should not be considered to be a neurocarcinogenic agent. The uneven distribution of the gliomas, clustering within the high dose group is most likely by chance. There are many examples where the highest tumor incidence occurred in the control group. As in all areas of biology, we must expect to deal with a wide spectrum of biologic variations. Meticulous analysis of consistently detectable characteristics of experimentally induced neoplasms, as compared to spontaneous tumors, is the only way to reduce existing uncertainties and establish sharper borderlines between the two.

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