Grant Application to

International Lead Zinc Research Organization, Inc.

Title of proposal: Lead induced encephalopathy and glioma in rats

Total direct cost requested: 35,170

Principal investigator and his organization:

Ryoichi Oyasu, M. D., Assistant Professor of Pathology, Department of Pathology, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, Illinois 60611. Ryoichi Oyasu M.D.

Summary of the proposal:

The proposed investigation is an experimental study on the effect of administration of lead carbonate on the rat brain. The specific aims are 1) to elucidate the mechanism of development of hemorrhagic lead encephalopathy in neonatal and young rats, and 2) to study the etiologic relationship between chronic lead administration and the development of cerebral gliomas. To study aim #1, lead encephalopathy will be induced in sucklings by feeding their lactating mothers with a 4% lead carbonate diet. Our investigations will include a) electron microscopic study of the brains of these sucklings to observe the earliest and the subsequent structural changes, especially changes developing in the cerebral blood vessels, b) determination of the distribution of lead in terms of blood brain barrier after i.p. administration of radioactive lead to sucklings, c) observation of the effect of delayed initiation of lead administration to sucklings. After the above studies have been completed we intend to investigate the prevention of the development of cerebral hemorrhage and e) the possible reversibility of lead effects through simultaneous administration of vitamin C or the antioxidant BHT (2,6 DL-tert. butyl-4 methylphenol). Aim #2, the relationship of lead to gliomas will be investigated in weanling rats fed lead chronically for as long as 18 months. The brain will be studied by electron microscopy to determine if viral particles are present during the early stage of lead treatment. Also planned is the quantitative study of lead distribution in brain tissue of patients with brain tumors, gliomas in particular. Autopsy brain tissue (both neoplastic and non-neoplastic portions) of such patients and age and sex-matched control patients will be analyzed for lead by atomic absorption spectrometry.

Detailed Budget for first 12-month period

1. Personnel	Amount Requested Salary	Fringe Benefits	To
R. Oyasu, M.D., Principal Investigator	None		
H.A.Battifora,M.D., Associate Investigator	None		
J.E.Leestma,M.D., Consultant	None		
Electron Microscope technician (½ time)	4,000	404	4,
Histology technician (1/3 time)	2,500	253	2,
Chemistry-Animal Care technician (½ time)	4,000	404	4,
	Total		11,

2. Supplies

Initial cost and maintenance for 60 pregnant rats for 3 weeks	
Maintenance for approximately 150 rats (7¢/day) for a year	3,
Rockland ground chow, lead carbonate and radioactive lead	
Lab. glassware, reagents and EM supplies	1,

3. Other expenses

Radiation safety control service charge (see remark)	
Publication costs (film, preparation with reprints)	
Total direct cost	17,

Indirect cost: Northwestern University requests an indirect cost according to your policy.

Budget for the second year

1.	Personnel	12,139
2.	Supplies	5,210
3.	Other expenses	450
	Total direct cost	17,799

Remarks

1. Fringe benefit rate is 10.1% of S. & W.
2. Personnel salaries normally increase at 4 to 5% per year. Here annual increase of 5% is requested. For supplies, annual increase of 3% is requested.
3. A 2 year support is requested because of the nature of experiment; the glioma study requires at least 18 months to complete the scheduled experiment.
4. Radiation Safety Department of Northwestern University Medical School surveys the laboratories using radioactive materials at least once a month. Their charge for such service for radioactive lead is $450 annually.

BIOGRAPHICAL SKETCH

NAME	TITLE	BIRTHDATE (Mo.,Day,Yr.)
Ryoichi Oyasu	Assistant Professor of Pathology	12/4/29

PLACE OF BIRTH (City,State,Country)	PRESENT NATIONALITY	SEX
Nagano, Japan	U. S. A.	Male

EDUCATION

INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED	SCIENTIFIC FIELD
Kyoto Univ. Liberal Arts Course		1951	Chemistry
Kyoto Univ. Faculty of Medicine	M. D.	1955	Medicine
Interne, Michael Reese Hospital,Chicago		1958	Medicine
Resident in Path.,Presby-St.Luke's Hosp. Chicago		1962	Medicine
Research Fellow in Pathology, as above		1967	Medicine

HONORS

Doctor Medical Science Awarded from Kyoto Univ., 1963

Travel grant from Nat. Academy of Sciences, Nat. Res. Council to attend and present a paper to the 9th Internat. Cancer Congress, Tokyo, 1966.

MAJOR RESEARCH INTEREST

Chemical carcinogenesis

Toxicology of lead

ROLE IN PROPOSED PROJECT

Principal investigator

RESEARCH SUPPORT

NIH CA-11036 Urinary bladder carcinogenesis in animals. Percent effort on this project 40%. Support for current year 23,702. Total funds for the entire project period [illegible],038

PROFESSIONAL EXPERIENCE

Assistant Professor of Pathology, Northwestern University Medical School, Attending pathologi Passavant Memorial Hospital, Chicago, Illinois (1967 to present), Attending pathologist, Veterans Administration Research Hospital, Chicago, Illinois (1968 to present), Research fellow, Presbyterian-St.Luke's Hospital (1964 to 1967), Instructor in Pathology, University of Illinois College of Medicine, Chicago, Illinois (1960 to 1962, 1964 to 1967). USPHS postdoctoral research trainee in Pathology (2G-129) (1964 to 1967).

Personal Publications

Lead Studies

1. Mass, G.M., McDonald, J.H., Oyasu, R., Battifora, H. and Paloucek, J.T.: Renal neoplasia

induced by combinations of dietary lead subacetate and N-2-fluorenylacetamide. In: First International Symposium on Renal Neoplasia, Stanton King,Jr. Ed. Little Brown and Co. Boston, 1967. pp. 377-412.

2. Oyasu, R., Battifora, H.A., Clasen, R.A., McDonald, J.H., and Hass, G.M.: Induction of cerebral gliomas in rats with dietary lead subacetate and 2-acetylaminofluoren Cancer Res. 30:1248-1261, 1970.

3. Shakerin, M., Paloucek, J., Oyasu, R., and Hass, G.M.: Carcinogenesis in rats due to dietary 2-AAF and lead subacetate. Fed. Proc. 24:684, 1965.

Other Personal Publications

1. Oyasu, R., Miller, D., McDonald, J. and Hass, G.: Neoplasms of the urinary bladder and liver in rats fed 2-acetylaminofluorene and indole. Fed. Proc., 20:290, 1961.

2. McDonald, J.H., Oyasu, R. and Hass, G.M.: The relation of liver neoplasms to bladder tumors produced by 2-acetylaminofluorene. Trans. Am. Assoc. Genito-Urinary Surgeons, 53:19-28, 1961.

3. Oyasu, R., Miller, D.A., McDonald, J.H. and Hass,G.M.: Neoplasms of rat urinary bladder and liver. A.M.A. Arch. Path., 75:184-190, 1963.

4. Oyasu, R.: Effect of pretreatment with hepatotoxic substances on 2-acetylaminofluore and indole tumorigenesis in rats. Effect of carbon tetrachloride and dl-ethionine. Gann, 54:339-351, 1963.

5. Battifora, H., Clasen, R.A. and Oyasu, R.: The effect of local tissue injury on the incidence of gliomas in rats ingesting 2-AAF. Fed. Proc., 22:317, 1963.

6. Oyasu, R.: C.P.C. Enshu (How to discuss the C.P.C. cases). Kimpodo Publisher, Kyoto (234 pages), 1964.

7. Shakerin, M., Paloucek, J., Oyasu, R. and Hass, G.M.: Carcinogenesis in rats due to dietary 2-AAF and lead subacetate. Fed. Proc., 24:684, 1965.

8. Oyasu, R., Battifora, H.A. and McDonald, J.H.: Enhanced urinary bladder tumorigenes in rats by 2-AAF treatment in the neonatal period. Fed. Proc., 25:291, 1966.

9. Oyasu, R., Battifora, H.A., Eisenstein, R., McDonald, J.H. and Hass, G.M.: Enhancement of tumorigenesis in the urinary bladder of rats by neonatal administration of 2-acetylaminofluorene. J. Nat. Cancer Inst. 40:377-388, 1968.

10. Oyasu, R. and Sumie, H.: Neoplasms of the hamster urinary bladder induced by 2-acetylaminofluorene (AAF) and 3-methylcholanthrene (MC). Proc. Am. Assoc. Cancer Res. 11:62, 1970.

11. Hsu, G., Oyasu, R., McDonald, J., Malikova, S. and Hass, G.: Relations of renal excretion of N-hydroxy-acetylaminofluorene to occurrence of urothelial tumors in animals ingesting 2-acetylaminofluorene. Lab. Invest. 22:501, 1970.

12. Oyasu, R., Sumie, H. and Burg, H.E.: Neoplasms of urinary bladder of hamsters treated with 2-acetylaminofluorene and indole. J. Nat. Cancer Inst. 45:853-860, 1970.

Brief resume of specific aims and methods

Specific Aim #1

Hemorrhagic lead encephalopathy in neonatal rats.

The most serious clinical manifestation of plumbism is acute encephalopathy. Although the incidence of acute encephalopathy appears to be decreasing in major cities where case-detecting program is under way, approximately 40% of the survivors sustain significant permanent brain damage. Possible effect of continuous chronic exposure to lead of a lower level on the mental development is not known. But it is quite likely that such lower level exposure would likewise cause significant damage to the brain.

Experimental study of lead encephalopathy was greatly stimulated when Pentschew first reported a successful development of hemorrhagic encephalopathy in neonatal rats. Paraplegia developed in about 90% of the sucklings sometime between 23 and 29 days after delivery when their mothers were fed continuously postpartum with a 4% lead carbonate diet. Postmortem examination showed hemorrhage and brown discoloration of the cerebellum in all affected animals.

We have been interested in the problem of plumbism and have conducted several experiments on the effect of chronic lead feeding of young adult rats. These animals developed a large number of renal tumors and a small number of gliomas. However, none of these developed paraplegia or microscopic changes suggestive of lead encephalopathy. The marked difference in susceptibility to lead encephalopathy between the adult and suckling rats was striking. We repeated Pentschew's experiment and confirmed his findings; a large number of sucklings develop marked hemorrhagic encephalopathy 3 weeks after birth. However, the cerebellar changes were too advanced and destructive to evaluate the pathogenesis of lead encephalopathy.

In the experiments conducted over the past 2 years, we have developed a model of neonatal encephalopathy that develops following ingestion of lead in suckling rats. These preliminary experiments which are being prepared for publication were done with the following in mind; to develop an adequate experimental model for the study of the earliest morphological changes of lead encephalopathy and second, to determine the reason why hemorrhagic encephalopathy occurred at such an early time as well as why hemorrhage was restricted to certain portions of the brain.

Briefly, feeding the lactating mothers with 4% lead carbonate diet following delivery results in hemorrhagic encephalopathy in half of their sucklings as early as 1 week of age. Hemorrhage observed in the cerebellum (and also at other sites when cerebellar hemorrhage is marked) is milder than the lesions observed in the similarly treated but older weanling rats. Therefore, we have an excellent model for the study of the earliest changes.

It has been generally agreed on that the primary target of lead in the brain is the vessels. The above experimental results as well as many other reports seem to support this hypothesis. Light-microscopic evidence of severe vascular injury antidating the terminal episode (symptomatic encephalopathy) has been shown in human cerebellum. To our knowledge there has been no study by electron microscopy made on the early changes antidating the clinical manifestation of lead encephalopathy in either experimental animals or humans. Our working hypothesis is that lead exerts its effect selectively on the actively proliferating endothelial cells. Such vessels are particularly abundantly found during the first 2 weeks of life in the rat brain (Stage 2 of McIlwain) and probably also in human brain up to 36 months of age. Therefore our understanding of lead encephalopathy would be enhanced if we had adequate information on the morphological and biochemical changes which occur in the early (preclinical) stage of plumbism.

We will focus our attention on the earliest ultrastructural changes in the vasculature, particularly in the endothelial cells and tight junctions. Our specific objectives are:

a) What are the earliest changes in the cytoplasmic organelles of the endothelium, neuroglia etc. in treated vs. non treated rats?
b) Does lead affect only those capillaries which are open by the time of birth because they are easily accesible to lead?
c) Or does lead affect those immature vessels (vascular strands observed by Caley) as well as those patent from the beginning? Or, does lead affect the immature vessels selectively at the time of rapid vascular growth around the 10th postnatal day? If so, are these the capillaries which rupture when blood begins to flow through them?
d) Why are the cerebellar vessels affected selectively? Is this because these vessels are different in the structure, or staging in maturation?
e) Will the tight junctions be disrupted some time before massive pericapillary hemorrhage occurs?
f) What is the significance of incomplete support of neonatal vessels by astrocytic foot processes to their rupture?

Several studies including ours have shown that the brain of the lead-treated animals contains a fair amount of lead. We want to know:

g) Is vascular injury by lead a pre-requisite for lead itself to enter the brain substance?
h) If that is the case, does the entrance of lead precede the destruction of the blood brain barrier as tested by horseradish peroxidase and Evans blue?

To answer the above questions Evans blue, horseradish peroxidase and radioactive lead (Pb^{210}) will be used to observe their distribution in the brains of sucklings of various ages which have varying degrees of previous exposure to lead.

i) After the above proposed experiments have been completed, we plant to perform a series of studies in which initiation of administration of lead is delayed. This study is very important because the human lead encephalopathy occurs most commonly in children between 12 and 36 months of age, probably due to increased oral activity. This age group will correspond to stage 4 of brain growth and development in the rat by McIlwain's classification. Therefore, in order to conduct a study of experimental lead encephalopathy at the stage which corresponds to the stage where the human disease commonly occurs, one must use the rats which are approximately three weeks old at the time of initial exposure to lead. As an initial step of this type of experiment, we will install a group in which lead treatment will be delayed until the sixteenth day of life.

Eventually we hope to study therapeutic approaches to plumbism including:

j) Study of the prophylactic use of drugs to prevent or suppress the passage of lead to the brain substance or to prevent its binding to intracellular organelles. Since lead binds with sulfhydryl groups of enzymes in particular, its effects might be mitigated by administration of reducing agents such as vitamin C or BHT (2,6 DL-tert. butyl-4 methylphenol) (Aldrich Chem.). If they are effective, their usage may have clinical significance in prevention of lead encephalopathy as well as potential reversal of lead effects on cerebration. Their possible effect can be monitored by determining ALA dehydratase activity in blood. One study has shown that ALA dehydratase activity in blood of mentally retarded children is decreased even when the blood lead level is considere to be in the upper range of normal (20-40 micrograms per 100 ml).

Specific Aim #2
Experimental glioma in lead treated rats

In a study of neoplasms induced by 2-acetylaminofluorene (AAF) and lead subacetate, we found 25 gliomas and 3 extracerebral intracranial tumors in 988 Wistar and C.D. Sprague-Dawley rats of which 663 were in experimental and 325 in control groups. The tumors usually developed after 52 weeks of age. Most tumors were classified as poorly differentiated malignant gliomas. The highest incidence (8.6%) of gliomas was in animals ingesting lead subacetate and the difference compared with the incidence (0.3%) among control was statistically significant ($p < 0.05$). Addition of AAF to the diet of rats ingesting lead subacetate did not increase the incidence of gliomas (8.3%). We believe that all these tumors except 2 which occurred in control animals, were not spontaneous but were produced by diets containing AAF and lead. Furthermore, we believe that administration of lead may be etiologically related to the induction of gliomas. Since the naturally occurring and experimentally induced gliomas are morphologically similar, it seems likely that many arise through a common mechanism perhaps involving the virus-like particles Zimmerman's group has demonstrated in hydrocarbon-induced glioma studies. Any investigations of the mechanism of induction of experimental tumors are greatly accelerated by a model with higher yield of tumors arising as quickly as possible. Thus based on our previous experience with lead encephalopathy in young adult rats we intend to determine if earlier initiation of lead treatment will accelerate and enhance the development of gliomas. Electron microscopic study will be added with particular attention to demonstrate virus particles throughout the period of study. Also included in our studies is an analysis of lead content of human brain tissue. Since we believe that in the above described experiment, administration of lead may be etiologically related to the induction of gliomas, we wonder if this might also be the case in the human. Exposure to an excessive amount of lead during the childhood might cause the development of brain tumors at an early age as well as at a later age.

Recently we have had an opportunity to review an unusual autopsy case by courtesy of Dr. Sion of Jackson Park Hospital, Chicago. The child was a 3 year old Negro child from one of the high risk areas of lead poisoning in Chicago. He was brought into the hospital because of convulsion and coma. At autopsy (70A51, Jackson Park Hospital), he was found to have a small ependymoma in the third ventricle causing hydrocephalus. The analysis of his brain tissue for lead by the Cook County Coroner's Laboratory revealed 250 micrograms/100 gm wet tissue (normal for this age group, 2-3 micrograms/100 gm wet tissue). This unusual case prompted us to investigate the possible role of lead on the development of gliomas in humans.

For analysis, autopsy material will be used. Using clean technique, 5 to 10 grams of cerebral tissue will be obtained at the time of autopsy examination. The tissue will be taken from the right frontal lobe known to be free of tumor. If the tumor is easily accessible without distorting the structure, a small amount of tumor tissue will also be submitted for analysis. The resected samples will be immediately placed in a previously acid-washed plastic bag and will be stored frozen until analyzed. The material for analysis is available from the teaching hospitals affiliated with the Medical School. Control samples matched for age and sex, will be similarly collected and analyzed.

Expected Results and Interpretation of the Results

Expected results are as follows:

The following are our working hypotheses:

1. Damaging effect of lead on the endothelial cells are greatest in endothelial cells in the proliferative state.

2. Active proliferation of cerebral capillaries according to Caley occur between days 10 and 15 of life. Therefore, if the sucklings are exposed to lead during and shortly before this period, the vascular damage will be more severe. As a result hemorrhage follows several days after initial exposure to lead.

3. We hypothesize that the blood brain barrier which has been established by birth for protein-bound dye and probably also for lead, will be destroyed by continued lead treatment and that hemorrhage is a manifestation of severe vascular injury and destruction of the barrier. Therefore, Pb^{210} and dye should be demonstrated in the brain substance even as early as day 6 in the group of sucklings in which lead treatment was initiated at birth.

4. In the group where lead treatment will be delayed until day 16, the vascular changes will be milder and blood brain barrier will be less severely involved than in the first group because, by day 16 a great majority of the capillaries have been opened and the rate of vascular proliferation has been slowed. Hence these vessels should be less susceptible to the injurious effect of lead.

5. We believe that maturation of vasculature varies from place to place in the brain. We believe that more capillaries are actively growing in the cerebellum postnatally than in the cerebrum in the rat. In other words, delayed growth in the vessels until postnatal period and administration of lead at the time of active growth are the major cause of massive hemorrhage which occurs preferentially in the cerebellum.

6. We believe that administration of lead (lead subacetate or lead carbonate) is causally related to the development of gliomas in the rat. Therefore, initiation of lead treatment in an earlier period such as 10 days and 3 weeks after birth, would enhance the incidence of gliomas because of increased concentration of lead in the brain substance at the time of active growth of glial cells. Although in the previous study of induction of gliomas, we observed only one case of glioma occurring in the cerebellum, the present experiment might result in induction of more cerebellar gliomas.

7. The result of the studies of lead distribution in human brains are not predictable. No such study has been made and an elevation in lead levels in brain tissues of glioma patients would be of interest and potential significance.

Significance

The results of the proposed studies will give information on the following points of interest:

a) They should give good data suggesting whether the capillaries are the primary target of lead in the brain.
b) They should give data as to why lead encephalopathy occurs selectively in young children.
c) The relationship among the lead content in the brain, mental retardation, encephalopathy and vascular injury is not clear. The present investigation will shed a light on the relation between capillary injury and amount of lead in the brain and between capillary injury and lead encephalopathy.
d) Our future research will be directed to the investigation of possible reversibility of lead effect following withdrawal, combined treatment of lead and vitamin C, lead and antioxidant such as vitamin E or BHT. Vitamin C deficiency in guinea pig (scurvy) has been shown to cause decrease in thickness of capillary basement membrane and partial separation of tight junction in the capillaries of lead-treated rats. Additio of vitamin E or BHT may restore decreased ALA-dehydratase activity in the brain.
e) The cause of gliomas in humans is totally unknown. If we can demonstrate in the current investigation that lead is causally related to the development of gliomas in rats, this would have a great clinical significance. Some gliomas in adults as well as in children might be a late effect of childhood plumbism (either clinical or subclinical). If this is the case, these gliomas would come in the realm of "preventable" cancer. The data from man also will be of interest in relating Pb to cerebral tumors.

Facilities Available

The animal care department of Northwestern University Medical School has a large animal quarter occupying the entire spaces of the 14th and 15th floors of the Medical School buildings. The entire floors are air-conditioned. A room with adequate number of cages for study is available for this investigation. There is a special animal room which meets every requirement of radiation safety standard. This room has enough capacity to keep our radioactive animals. The room is regularly surveyed by Radiation Safety Department personnel.

The electronmicroscope to be used is a Philips model 300 and is located in the Department of Pathology, Chicago Wesley Hospital. My collaborator, Hector A. Battifora, M.D., who is a full time member of the faculty of Northwestern University Medical School (Associate Professor of Pathology), is in charge of the microscope. It is fully available for the proposed investigation and we expect no difficulty in operation if a half-time technician in charge of preparation of the material is available.

The atomic absorption spectrometer to be used is a model 82-500 of Jarrel-Ash Co. which is owned by the Department of Pathology and is currently being used for a variety of trace metal analysis. Jan E. Leestma, M.D., our consultant, is a neuropathologist who is currently serving in U. S. Air Force Medical Corps stationed at the Neuropathology Branch, Armed Forces Institute of Pathology, Washington, D.C. He will join the faculty of Northwestern University Medical School on June 1, 1971.