Japan. J. Med. Sci. Biol., 38, 67-72, 1985.

Short Communication

PATHOLOGY OF TOXIC DEATH IN MICE FOLLOWING INTRAVENOUS INJECTION OF *RICKETTSIA TSUTSUGAMUSHI* STRAIN GILLIAM: EXAMINATION BY LIGHT AND SCANNING ELECTRON MICROSCOPY

Stanley C. OAKS, Jr.¹, Francis K. P. NG², Michael R. ELWELL³, Michael G. GROVES⁴ and George E. LEWIS, Jr.

United States Army Medical Research Unit, Institute for Medical Research, Jalan Pahang, Kuala Lumpur 02-14, Malaysia and ³U.S. Army Medical Component, Armed Forces Research Institute of Medical Sciences, Rajvithi Road, Bangkok 4, Thailand

(Received January 11, 1985. Accepted June 4, 1985)

SUMMARY: The pathologic manifestations of the toxic death elicited by the iv injection of mice with *Rickettsia tsutsugamushi* strain Gilliam were examined by light and scanning electron microscopic techniques to ascertain the cause of death. Light microscopic examinations of hematoxylin and eosin-stained sections of organs suggested the presence of fluid in the lungs; this was confirmed by an increase in the weight of the lungs of moribund mice. Scanning electron microscopy of blood cells demonstrated a marked crenation of erythrocytes from these mice. Death appears to be the result of shock brought on by vascular collapse secondary to plasma leakage.

A lethal, toxic reaction to the iv injection of mice with *Rickettsia tsutsugamushi* strain Gilliam-infected yolk sac suspension was first reported by Smadel et al. (1). They noted that the "toxin" was neutralized by homologous antiserum and that several other *R. tsutsugamushi* strains did not elicit the toxic reaction in mice. Kitaoka and Tanaka (2) demonstrated that two additional strains, Karp and Kato, were toxic. They also reported that the lethal "toxin" was removed by centrifugation at 1,500 xg for 15 min, heating at 50 C for 10 min, or treatment with 0.05% formalin at 4 C for 15 min. The pathologic manifestations of this toxic reaction have not been previously examined.

This research was supported by Research Grant No.DAMD17-82-G-9502 from the U.S. Army Medical Research and Development Command, Fort Detrick, Maryland 21701-5012.

"In conducting the research described in this report, the investigators adhered to the 'Guide for the Care and Use of Laboratory Animals', prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No.(NIH) 80-23, Revised 1978-reprinted 1980)."

Address reprint requests to: Commander, U.S. Army Medical Research Unit, Kuala Lumpur, Department of State, Washington, D.C. 20520.

Present addresses: ¹Department of Pathology and Area Lab Services, William Beaumont Army Medical Center, El Paso, Texas 79920, ²P.O. Box 124, St. Lucia, Brisbane, Queensland, Australia 4067 and ⁴Military Disease Hazards Research Program, U.S. Army Medical Research and Development Command, Fort Detrick, Maryland 21701-5012.

In the present study, we determined the most likely cause of death by observing the pathologic changes resulting from the iv injection of *R. tsutsugamushi* strain Gilliam into BALB/c mice. Vital organs were examined by light and scanning electron microscopic methods.

Rickettsiae: The Gilliam strain of *R. tsutsugamushi* was propagated in the yolk sacs of embryonated chicken eggs (SPAFAS, Inc., Norwich, CT) as previously described (3) and in gamma-irradiated L-929 cells by the method of Nacy and Oaks (4). The infected yolk sac (166th passage) and infected L-929 cell (1st passage) homogenates were stored as 20% suspensions in Snyder I (5) in liquid nitrogen.

Mice: BALB/c mice (Jackson Laboratories, Bar Harbor, ME), 20-25 g and either sex, were used throughout the study.

Titration of rickettsial stocks: The 50% mouse lethal dose (MLD_{50}) was determined by the method of Reed and Muench (6). The titer of the yolk sac suspension was 5.2×10^8 $MLD_{50}/0.2$ ml and that of the L-929 cell suspension 2.7×10^8 $MLD_{50}/0.2$ ml.

Mouse inoculation: Mice were injected intravenously, in the tail, with 0.2 ml each of rickettsial stock diluted in Snyder I. The time of inoculation was recorded and the mice were observed periodically for signs of toxicity. Control mice received either Snyder I, normal (uninfected) yolk sac suspension, heat-killed Gilliam rickettsiae, or normal L-929 cell suspension injected in the same manner as the rickettsial stock.

Removal of organs for weighing and fixation: Moribund and control mice were sacrificed by cervical dislocation and the kidneys, spleen, heart, and liver of each mouse were removed, weighed, and fixed. Prior to removal of the lungs, the jugular vein was severed and 10 ml of sterile physiological saline solution was infused into the posterior vena cava to flush blood from the vessels. The lungs were then removed and fixed.

Ten percent formalin for 16-18 hr (24 C) was used for fixation of organs to be sectioned for staining with hematoxylin and eosin (H & E), while 2.5% glutaraldehyde (Polaron Instruments, Inc., Doylestown, PA) for 3 hr (4 C) was used for those organs to be examined by scanning electron microscopy.

Processing of fixed organs and blood for scanning electron microscopy: Fixed organs were cut into 1 to 2-mm pieces, washed 3 times with 0.1 M phosphate buffer (PB, pH 7.4), and post-fixed in 1% osmium tetroxide (in PB) (Polaron) for 30 min at 4 C. The specimens were then dehydrated in a graded ethanol series (30, 50, 70, 95, 100, 100%) and dried at the critical point of CO_2 in a Hitachi HCP-2 Critical Point Drier (Nissei Sangyo Co., Ltd., Tokyo, Japan). Samples of blood were prepared for scanning electron microscopy by placing a drop on a glass coverslip, spreading it to cover the entire surface, and then air drying. Fixation and processing were done as for organs. After critical point drying, specimens were mounted on stalks with silver paint (Ernest F. Fullam, Inc., Schenectady, NY) and coated with carbon on a Hitachi

Hus-5GB High Vacuum Evaporator and with gold on an Eiko IB-2 Ion Coater (Eiko Engineering, Ibaragi, Japan). The specimens were examined under a Hitachi S430 Scanning Electron Microscope.

H & E staining and sectioning: Formalin-fixed organs were each cut into several pieces and then processed through a Shandon 2L Tissue Processor MKII (Shandon Southern Products Ltd., Cheshire, England) prior to embedding in paraffin blocks. Thin sections (5-8 μ m) were cut on an A/O Spencer 820 Microtome (American Optical Corp., Buffalo, NY).

Effect of dilution on toxic effect

Toxic death occurred, usually within 1.5-2 hr, after injection of either undiluted or 1:2 dilution of original stock suspension yolk-sac propagated rickettsiae. Occasional deaths were observed at a 1:4 dilution; however, most of these mice developed signs of toxicity and then returned to a normal appearance overnight. Injection of cell culture-propagated Gilliam, at dilutions of undilute to 1:8 of original stock suspension, resulted in a very sporadic death pattern. Some mice died as rapidly as in one minute post-injection and others required up to 8 hr or did not die until 8-10 days later; toxic death being dilution independent. Toxic death was defined as death occurring within 5 hr post-injection. Prior to death, mice developed a bluish-purple skin coloration, their coat became roughened, and they became very lethargic. Control BALB/c mice, observed for 10 days post-infection, did not die or exhibit any signs of toxicity.

Effect of iv injection on organ weight

Organs were removed from control mice and mice moribund as a result of injection of a toxic dose of yolk-sac-propagated Gilliam strain. The organs were weighed and their average weights calculated. Table I contains the results of these calculations. When compared to the controls, the most important change observed was in the weight of the lungs. The change in the average weight of the kidneys and spleen are small in comparison and are not important with such a small sample size.

Scanning electron microscopy of organs and blood cells

Examinations of lung, spleen, kidney, heart, and liver tissues from moribund mice by scanning electron microscopy did not detect any significantly altered pathology, when compared to control mice. However, similar examinations of blood smears showed that a high percentage of erythrocytes from the moribund mice were crenated (Figs. 1 and 2). This could be due to a severe loss of plasma volume. This phenomenon was seen on repeated examinations and correlates well with the difficulty experienced when attempting to collect blood from the moribund mice; the blood was very viscous when compared to blood of control mice.

Table I. *Effect of Rickettsia tsutsugamushi strain Gilliam on iv injection of the weight of selected BALB/c mouse organs*

Organs	Weight (grams)*		% change
	Normal	Infected	
Lungs	0.113 $\pm$ 0.009	0.187 $\pm$ 0.017	(+) 65**
Kidneys	0.207 $\pm$ 0.041	0.247 $\pm$ 0.049	(+) 19
Spleen	0.095 $\pm$ 0.014	0.082 $\pm$ 0.034	(-) 14
Liver	1.338 $\pm$ 0.175	1.449 $\pm$ 0.203	(+) 8
Heart	0.116 $\pm$ 0.012	0.108 $\pm$ 0.018	(-) 7

*Weights are averages from 10 mice $\pm$ one standard deviation.

**p < 0.05, Student's t test.

Fig. 1. Scanning electron micrograph of erythrocytes from mice approximately 3 hr post-injection (iv) with *Rickettsia tsutsugamushi* strain Gilliam (5×10^7 MLD₅₀). Magnification = 14,500 (bar = 1 μ m).

Fig. 2. Scanning electron micrograph of erythrocytes from mice approximately 3 hr post-injection (iv) with heat-killed *Rickettsia tsutsugamushi* strain Gilliam (5×10^7 MLD₅₀; prior to heating at -50 C for 10 min). Magnification = 14,500 (bar = 1 μ m).

H & E staining of organ sections

Examination of H & E- stained organ sections demonstrated changes in the lungs of moribund mice consistent with pneumonitis and either hypovolemic or septic (endotoxic) shock. The alveolar walls were thickened, with congested (erythrocyte filled) capillaries and an increased number of leukocytes in the capillary space itself. Close examinations revealed a considerable amount of eosinophilic material within the alveolar walls and in some cases in the alveolar spaces. The overall picture was that of a "shock lung". Significant changes in the pathology of the other organs examined were not observed.

The findings of this study suggest that the rapid toxic manifestations experienced with BALB/c mice after the iv injection of *R. tsutsugamushi* strain Gilliam are primarily due to plasma loss, with the resulting death caused by shock and pulmonary edema.

Wattenberg et al. (7) noted that the iv injection of sufficient murine typhus rickettsiae (*R. typhi*) into mice resulted in acute plasma loss, hemoconcentration, and eventual death from vascular collapse. A similar, if not identical, phenomenon appears to occur with *R. tsutsugamushi* strain Gilliam-induced toxic death. The complete cessation of blood flow in the vascular system of the mouse, as seen in the previously noted study (7), coincides with the great difficulty we experienced in collecting blood from the moribund mice.

The collection of fluid in the lungs of mice in the present study was considered a significant finding. It was suggested by the findings of the microscopic examinations of H & E-stained sections and confirmed by the large increase in weight of the lungs from moribund mice when compared to control mice. This is in contrast to the reported reaction in mice to the *R. typhi* "toxin" (7). They found that plasma transudation of the lungs was no more significant than in any of the other tissues or organs.

While it appears that the iv injection of mice with large numbers (approximately 5×10^8 MLD₅₀) of *R. tsutsugamushi* strain Gilliam causes an increase in the vascular permeability with resulting plasma leakage and death, the mechanism of this phenomenon remains unknown.

ACKNOWLEDGEMENT

The authors wish to thank Dr. George de Witt, Director, Institute for Medical Research, for his support of this study; Drs. Daryl J. Kelly and David L. LaBarre for their expert assistance; Lee Eng Kee, Chan Teik Chye, and Victor Sankaran for their excellent technical help; and the technical staffs of the United States Army Medical Research Unit and the Institute for Medical Research for their valuable assistance.

REFERENCES

1. Smadel, J. E., Jackson, E. B., Bennett, B. L. and Rights, F. L. (1946): *Proc. Soc. Exptl. Biol. Med.*, 62, 138-140.
2. Kitaoka, M. and Tanaka, Y. (1973): *Acta Viro.*, 17, 426-434.
3. Catanzaro, P. J., Shirai, A., Hildebrand, P. K. and Osterman, J. V. (1976): *Infect. Immun.*, 13, 861-875.
4. Nacy, C. A. and Oaks, S. C., Jr. (1981): In D. O. Adams, P. J. Edelson and H. Koren [eds.], *Methods for studying mononuclear phagocytes*. p.725-743. Academic Press, Inc., New York.
5. Jackson, E. B. and Smadel, J. E. (1951): *Amer. J. Hyg.*, 53, 326-331.
6. Reed, L. J. and Muench, H. (1938): *Amer. J. Hyg.*, 27, 493-497.
7. Wattenberg, L. W., Elisberg, B. L., Wisseman, C. L. and Smadel, J. E. (1955): *J. Immunol.*, 74, 147-157.