Encephalitogenic Properties of Crude and Protamine Treated Rabbit and Mouse Brain Suspensions. (20333)

PHILIP Y. PATERSON, WILLIAM L. POND, JOEL WARREN, AND MARVIN L. WEIL.

From the Department of Virus and Rickettsial Diseases and Department of Bacteriology, Army Medical Service Graduate School, Washington, D. C.

Man occasionally develops paralytic complications following the repeated injection of rabbit spinal cord during the course of the Pasteur treatment for rabies(1). Encephalomyelitis has been produced in several experimental animals following inoculation of homologous or heterologous brain tissue with(2-9) or without(10) the adjuvant mixture devised by Freund(11). These facts suggest that the vaccination of man with any preparation containing crude brain components may be hazardous.

The elimination of encephalitogenic activity from infected central nervous system tissue used for the preparation of several neurotropic virus vaccines would be of considerable merit. Such a method of preparing rabies vaccine has been reported by one group of workers(12,13). Previous reports from this laboratory(14,15) indicated that protamine treatment of infected crude brain material is useful for purifying a number of neurotropic viral agents. Furthermore, 2 mouse brain vaccines, Japanese and Russian Spring Summer encephalitis, prepared by this method gave initial immunologic responses comparable to crude preparations when tested in mice and human volunteers(16). Therefore, since this technic may have possible future application in the preparation of neurotropic viral vaccines of the mouse brain type, studies were carried out to determine the effect of protamine sulfate precipitation on the encephalitogenic activity of crude suspensions of rabbit and mouse brain tissue.

Materials and methods. Animals used for studies. All brain tissue was obtained from adult Swiss mice of the Bagg strain or adult, New Zealand White rabbits. The guinea pigs were bred at Walter Reed Army Medical Center and weighed 250 to 450 g each. The rhesus monkeys had been used several months previously for titrations of poliomyelitis virus and had shown no neurologic signs prior to use in these studies. *Preparation of brain tissue suspensions.* Freshly harvested mouse or rabbit brain tissue was prepared as a 20% suspension in buffered (pH 7.4) physiologic saline by use of a mortar and pestle or a Waring blendor. An aliquot of this preparation was used as the "crude uncentrifuged" brain suspension. A second aliquot was treated with powdered protamine sulfate (Lot No. 16693 or 1097397, Squibb & Co.) for 30 minutes at 4-6°C as previously described(14). A third untreated aliquot was held for a similar period of time at room temperature and subsequently centrifuged along with the protamine treated brain material in a mean gravitational field of 1200 g for 10 minutes. The resultant supernates were designated as the "crude supernate" and "protamine supernate" suspensions respectively. The sediments after resuspension in buffered saline to approximately their original volume served as the "crude sediment" and "protamine sediment" suspensions respectively. In 2 experiments designed to test the effects of freezing and thawing freshly harvested brain was stored at —20°C for 24 or 48 hours prior to being processed as described above. All brain-adjuvant emulsions were prepared in a mortar by mixing equal volumes of a given brain suspension and adjuvant mixture, the latter prepared according to the method of Freund(11). Each milliliter of final emulsion contained 0.5 ml brain suspension, 0.45 ml of liquid petrolatum, 0.05 ml of mannide mono-oleate (Arlacel-A, Atlas Powder Co.) and 2 mg of heat killed, dried *Mycobacterium tuberculosis,* strain H37Rv. *Inoculation of animals.* Each guinea pig received 3 subcutaneous inoculations of 1.5 ml of brain-adjuvant mixture along the dorsum at weekly intervals, except in Exp. 1 and 2, where only a single inoculum was administered. These animals were observed daily for neurologic signs for at least 4 and up to 8 weeks following the last inocula-

TABLE I. Incidence of Encephalomyelitis in Guinea Pigs Following Injection of Crude and Protamine Treated Rabbit and Mouse Brain Suspensions.

Brain tissue	Suspension injected: Crude Uncent.	Crude Supe.	Crude Sed.	Protamine Supe.	Protamine Sed.
Rabbit	10/15†	ND‡	ND	0/15	14/15
"	13/18	ND	ND	0/18	11/17
"	8/14	2/15	10/11	0/15	10/15
" *	11/13	5/12	7/12	0/12	8/12
" *	ND	6/15	ND	0/15	ND
Total incidence	42/60	13/42	17/23	0/75	43/59
Mouse	5/15	0/14	ND	ND	ND
"	8/12	1/12	6/12	0/12	4/12
"	6/12	2/12	6/12	0/12	4/12
Total incidence	19/39	3/38	12/24	0/24	8/24

* Frozen-thawed brain tissue employed.

† Numerator = No. of guinea pigs showing neurologic signs. Denominator = No. of guinea pigs inj.

‡ ND = Not done.

tion of brain material. The monkeys received 1.5 to 2.0 ml of a given brain-adjuvant suspension intramuscularly in each thigh on 2 occasions during a 3- to 6-week period and were observed daily for central nervous system signs for up to 3 months following the second inoculation.* *Criteria for evaluating encephalitogenic properties of inocula.* Encephalitogenic activity in a preparation was manifested by the development of paralysis and other neurologic signs in a group of inoculated guinea pigs. The absence of such clinical abnormalities in all animals of a group was taken as presumptive evidence that the suspension inoculated was devoid of encephalitogenic properties. In certain of these groups without neurologic signs histopathologic studies were carried out to detect subclinical encephalopathy. Brains and upper segments of spinal cord were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin for microscopic examination. Thus, the complete absence of both clinical signs and histopathologic lesions, as described by Lumsden(17), served to indicate that a given inoculum possessed little if any encephalitogenic activity. Microscopic study was not performed on the monkeys employed in this study and the sole criteria were the presence or absence of clinical neurologic signs.

Results. The results of 8 experiments in which 5 different types of rabbit or mouse brain suspensions were used to inoculate groups of guinea pigs are summarized in Table I. It will be noted that all crude brain preparations contained encephalitogenic activity although the actual incidence of neurologic signs varied greatly from experiment to experiment and from group to group. The highest incidence followed the inoculation of either crude uncentrifuged or crude sedimented material. Both these preparations produced signs in 50% or more of the animals in each group except in Exp. 6, where neurologic signs appeared in only 5 of 15 guinea pigs receiving crude mouse brain material. The crude suspensions following centrifugation, on the other hand, were less encephalitogenic and produced clinical signs of encephalopathy in less than 50% of every group. This decrease of encephalitogenic activity following centrifugation was most marked in Exp. 6, 7, and 8, where only 3 of a total of 38 guinea pigs developed neurologic signs after the inoculation of crude supernatant mouse brain material.

The effect of treating 7 of these crude suspensions with protamine sulfate will be noted in Table I. The protamine supernatant suspensions produced no paralysis or other neurologic signs in the 7 groups totaling 99 guinea pigs. Fifty-one of these animals, approximately half of which had received protamine supernatant material, prepared from rabbits and the remainder prepared from mice, were sacrificed for histopathologic studies. No microscopic encephalopathy was evident in any of these sections. Considerable encephalitogenic activity was demonstrable in the protamine sedimented inocula, all 6 of which elicited clinical signs when inoculated into animals.

The effect of protamine treatment was further demonstrated in an experiment employing 3 groups of monkeys consisting of 8 animals each. Crude uncentrifuged and protamine sedimented suspensions prepared from rabbit brain produced marked neurologic signs in 4 and 2 monkeys, respectively. None of

* The technical assistance of Dr. J. A. Morris in the monkey studies is gratefully acknowledged.

the 8 monkeys in the third group which received protamine supernatant material developed clinical signs. It should be noted that, in an additional experiment, 10 monkeys received crude uncentrifuged mouse brain material and failed to develop clinical signs of encephalopathy during a 3-month observation period.

Discussion. The results of these studies indicate that protamine sulfate can precipitate most, if not all, of the encephalitogenic activity associated with crude rabbit and mouse brain tissue. The question might well be raised as to whether the single cycle of low speed centrifugation, used to sediment the protamine precipitated material, was responsible for this marked decrease in encephalitogenic activity. Centrifugation, as employed in these studies, did decrease the encephalitogenic character of the initial crude brain tissue. However, a small but significant amount of encephalitogenic activity remained in the majority of resulting supernatant fluids and thus, the effect of protamine treatment cannot be attributed to centrifugation *per se*.

These experiments were not designed to compare the difference in the encephalitogenic activity of crude rabbit and crude mouse brain tissue. However, it is worthy of comment that mouse brain, in general, appeared to be less encephalitogenic than rabbit brain tissue. This fact was suggested by the lower incidence of neurologic signs observed in guinea pigs receiving crude or centrifuged mouse brain suspensions and the failure of the former suspension to produce any abnormal signs in 10 monkeys. Neurotropic viral vaccines prepared from infected mouse brain tissue and clarified by low speed centrifugation have been employed for vaccination of man. Sabin has reported that this type of Japanese encephalitis vaccine resulted in no apparent paralytic complications when used during an outbreak of Japanese encephalitis on Okinawa during World War II(18). In this connection, it is of interest to mention that 2 vaccines prepared by the method of Sabin(19) from mouse brain infected with Japanese encephalitis virus were tested for encephalitogenic properties in this laboratory in 2 groups of 15 guinea pigs each. Neither vaccine, even when combined with adjuvants, produced any clinical signs of encephalopathy in this susceptible host. Such observations might be cause for minimizing the potential hazards associated with vaccination procedures employing this species of brain tissue. However, this study indicates that centrifugation alone cannot be relied upon to remove regularly encephalitogenic activity of both mouse and rabbit brain material. For this reason, the protamine technic may be of advantageous use in the preparation of certain neurotropic vaccines of the mouse brain type. Such vaccines would be expected to possess little, if any, encephalitogenic properties.

Conclusions. 1. Crude uncentrifuged rabbit or mouse brain tissue, combined with adjuvant, elicited acute encephalomyelitis in more than 50% of 99 guinea pigs. 2. The encephalitogenic activity of both types of brain tissue was decreased but not eliminated by low speed centrifugation. 3. Protamine sulfate appeared to sediment most, if not all, of the encephalitogenic activity of both rabbit and mouse brain material.

1. Sellers, T. F., *J. Med. Assn.*, Georgia, 1947, v36, 30.

2. Morrison, L. R., *Arch. Neur. and Psych.*, 1947, v58, 391.

3. Olitsky, P. K., and Yager, R. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 600.

4. Koprowski, H., and Jervis, G. A., PROC. SOC. EXP. BIOL. AND MED., 1948, v69, 472.

5. Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, v85, 117.

6. Morgan, I. M., *J. Exp. Med.*, 1947, v85, 131.

7. Good, R. A., Campbell, B., and Good, T. A., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 341.

8. Thomas, L., Paterson, P. Y., and Smithwick, B., *J. Exp. Med.*, 1950, v92, 133.

9. Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, v57, 179.

10. Rivers, T. M., and Schwentker, F. F., *J. Exp. Med.*, 1935, v61, 689.

11. Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, v49, 548.

12. Hottle, G. A., Nedzel, G. A., Wright, J. T., and Bell, J. F., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 289.

13. Bell, J. F., Wright, J. T., and Habel, K., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 457.

14. Warren, J., *Bact. Rev.*, 1950, v14, 200.

15. Weil, M. L., Warren, J., Breese, S. S., Russ, S. B., and Jefferies, H., *J. Bact.*, 1952, v63, 99.
16. Warren, J., and Russ, S. B., unpublished data.
17. Lumsden, C. E., *Brain*, 1949, v72, 198.
18. Sabin, A. B., *J. Am. Med. Assn.*, 1947, v133, 281.
19. ———, PROC. SOC. EXP. BIOL. AND MED., 1947, v65, 127.

Received May 13, 1953. P.S.E.B.M., 1953, v83.

Effect of Toluidine Blue on Metastatic Calcification.* (20334)

ROGER BAKER, GERALD REAVEN, AND JOHN SAWYER. (Introduced by L. R. Dragstedt.)
From the Department of Surgery, Division of Urology, University of Chicago.

Hyperparathyroidism is the most common cause of metastatic calcification. Calcified lesions due to hyperparathyroidism have been reported in the connective tissue of the stomach, pulmonary alveoli and kidney(1). Within the kidney the peritubular basement membrane is involved. Gersh(2) considers the basement membrane as a plastic, well-organized ground substance that is highly labile in certain physiologic and pathologic states. Heller-Steinberg(3) and also Engel (4) have demonstrated that the histological changes seen in bone after administration of parathyroid extract, can be explained on the basis of depolymerization of connective tissue ground substance. Gersh and Catchpole(5) believe that calcification is most likely to occur in depolymerized matrix. Rubin and Howard(6) have confirmed this work and also observed that there is a relationship between the state of the connective tissue and the ability of the tissue to become calcified. They contend that more reactive groups are available to bind with calcium when the matrix is being depolymerized. The basic dye toluidine blue is known to combine with mucopolysaccharides of the ground substance. Sylven(7) and others believe this is due to the presence of chondroitin sulfate. Toluidine blue binds on reactive groups of the ground substance coinciding with calcified portions of the matrix. Furthermore, in three week-old rats in which calcification has not yet appeared, toluidine blue binds intensely with the matrix in precisely the geographical locations eventually to become calcified. Miller, *et al.*(8) have shown that *in vitro* calcification of rachitic rat cartilage is blocked by toluidine blue. These authors have demonstrated that this inhibition of calcification is directly proportional to the amount of toluidine blue present in the system. Marc and Wenk(9) and France(10) have reported that crystallization of certain inorganic salts from a supersaturated solution are markedly inhibited by dyes which stain the mother crystal.

Based on the above data, the object of this paper is to experimentally study the *in vivo* effect of dye binding on metastatic calcification produced in rats by the administration of parathyroid extract.

Methods. Young rats of the Sprague-Dawley strain, weighing 200-250 g and of the same sex, were used in the experiment. They were maintained on a routine laboratory diet and given water *ad libitum*. Fifteen rats each received a total dose of 1625 units of parathyroid extract (Lilly) administered over a 2 week period as described by Chown(11). Five rats each received 1625 units of parathyroid extract and 3 cc (intraperitoneally) of a 0.9 % saline solution each injection day of the experiment. Another group of 15 rats received the same dosage of parathyroid extract and in addition an intraperitoneal injection of 3 cc of a 0.04% solution of toluidine blue each injection day of the 2 week period. At the end of this time all animals were sacrificed by a blow to the head. Blocks of tissue obtained from the kidney, stomach, spleen, blood vessels and heart of each animal were fixed in absolute alcohol. Von Kossa and hematoxylin and eosin stains were obtained. To quantitate the extent of calcification in the

* This study was aided by Fred and Harvey Goldberg.