degeneration and necrosis may proceed along different pathways. The role played by the adrenal medulla in the development of this degenerative response is not defined. Nonetheless, the rather striking shifts in the pattern of response merit further study.

Summary. Immunological sympathectomy does not protect rats against the hepatotoxic properties of CCl_4. This substantiates the idea that sympathetic discharge in and of itself is not responsible for development of the lesion. However, adrenal demedullation seems to alter the pattern of the lesion, in that hydropic degeneration becomes more extensive after demedullation.

1. Calvert, D. N., Brody, T. M., *Am. J. Physiol.,* 1960, v198, 669.

2. Brody, T. M., Calvert, D. N., Schneider, A. F., *J. Pharm. Exp. Ther.,* 1961, v131, 341.

3. Stern, P. H., Brody, T. M., *ibid.,* 1963, v141, 65.

4. Larson, R. E., Plaa, G. L., Crews, L. M., *Toxicol. Appl. Pharm.,* 1964, v6, 154.

5. Larson, R. E., Plaa, G. L., *Experientia,* 1963, v19, 604.

6. Levi-Montalcini, R., *Science,* 1964, v143, 105.

7. Brody, M. J., *Circulation Res.,* in press.

8. Brody, M. J., PROC. SOC. EXP. BIOL. AND MED., 1963, v114, 565.

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Cytochemistry of Inclusion Bodies in Tissue Culture Cells Infected with Rabies Virus.* (29306)

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Contradictory reports have been published regarding the nature of the inclusion bodies in cells infected with rabies virus. Wolman and Behar(1) found Feulgen-positive material in some inclusion bodies in the hippocampal cells of mice infected with street virus and Moulton(2) made similar observations regarding the Negri bodies in the brain of rabid skunks. These observers were unable to detect any ribonucleic acid in the inclusions. Sourander's careful study(3) of Negri bodies in dogs failed to reveal any nucleic acid. More recently, Sokolov and Vanag(4) claimed to have demonstrated presence of granules containing DNA and RNA in Negri bodies. Lépine and Atanasiu(5) found that Negri bodies in the brain were stained red by concentrated solutions of acridine orange in distilled water or in buffer at pH 7.0.

Inclusions produced in cells *in vitro* after infection with rabies virus could not, however, be readily distinguished from the intensely red-staining cytoplasm(5). Finally, Kissling and Reese(6) found round to irregular-shaped, dense reddish-orange masses in the cytoplasm of hamster kidney cells inoculated with a tissue culture-adapted strain of rabies virus, standard challenge virus (CVS) 11, and stained with acridine orange at pH 4. Fernandes *et al*(7) have adapted the CVS strain of rabies to several tissue culture systems, observing the formation of cytoplasmic inclusion bodies closely resembling Negri bodies which have provided good material for cytochemical study. The two rabies-infected tissue culture systems chosen for this study were the neonatal hamster kidney fibroblast line, BHK-21, clone 13 (BHK-21) where the number of cells showing the presence of cytoplasmic inclusions and size of the inclusions vary from cell generation to generation, and the rabbit endothelium (RE) line in which a true carrier state has been established with all cells containing inclusions of uniform size.

Materials and methods. Virus-infected cells. Monolayer tissue cultures of RE and BHK-21 cells(8) were propagated in milk dilution bottles and passed twice weekly. The medium consisted of modified Eagle's basal medium (9) supplemented with 10% millipore-filtered calf serum and containing 25 ml 5.6% sodium

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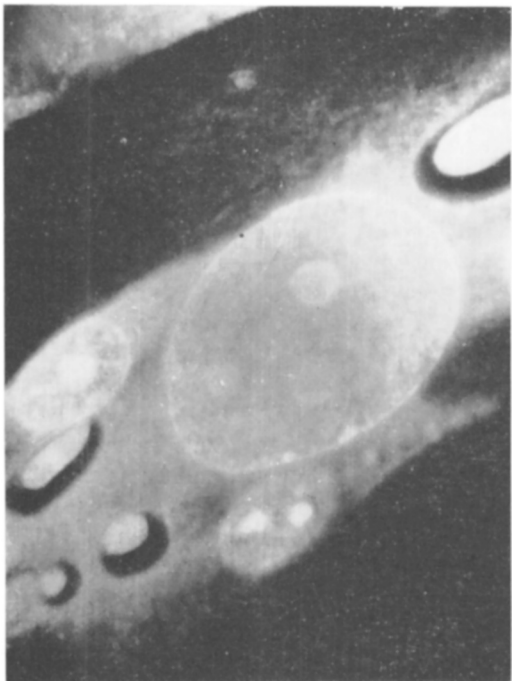

FIG. 1. Cell of a 7th subculture of a CVS-infected stable line of neonatal hamster kidney fibroblast (BHK-21). Acridine orange staining technique. Black and white copy of Kodachrome A Professional Original. Magnification × 1500.

bicarbonate, 10^5 μg streptomycin and 10^5 units of penicillin or 5×10^4 μg of Aureomycin per liter. For cytochemical studies, the cells were grown on coverslips in Petri dishes in the same medium and incubated in a humidified CO_2 incubator. The CVS,† a fixed rabies virus propagated in mouse brain, was originally used to infect the cultures and to establish the chronically infected lines(7).

Cytochemistry (a) Nucleic acids. The specificity of the staining methods was checked by digestion with ribonuclease (RNase) or deoxyribonuclease (DNase), or both, in that order or in the reverse order (10), with or without treatment with proteolytic enzymes, as indicated in the protocols. Nucleic acids were also removed by hot trichloroacetic acid (TCA)(11). The following methods were used:

(1) Toluidine blue-molybdate (TBM), (methods A, B, C, D, and E)(12);

(2) Dilute toluidine blue (TB) (0.0004% in McIlvaine's buffer, pH 3.0) for 18 hours after nitrosation(10);

(3) Acridine orange (10 minutes in 0.01% in McIlvaine's buffer, pH 3.5), after fixation for 6 minutes in Bouin's solution and then 15 minutes in Carnoy's solution. (These fixatives were chosen because they caused the inclusions to shrink away from the rest of the cytoplasm, leaving them surrounded by an unstained halo (Fig. 1). This enabled a clear distinction between staining of the inclusion and that of the surrounding cytoplasm.) Since the inclusions were stained green by this method after digestion with RNase and DNase, the possibility of the presence of double-stranded RNA that was resistant to digestion by RNase was investigated by digesting the inclusions for 5 minutes in 0.002% pepsin in 10% acetic acid at 20-25%, before and after RNase, as described by Gomatos *et al*(13). Pepsin was also used before and after extraction with hot TCA. Due to the fact that ribonuclease effectively prevented staining of the inclusions by the TB and TBM methods involving nitrosation, the preparations were treated with HNO_2 as for TBM, digested with all the enzymes, separately or sequentially, and stained with acridine orange;

(4) Acridine orange in phosphate buffer, pH 7.0, as described by Lépine and Atanasiu (5). Since pre-digestion in RNase had no effect upon the red staining of the inclusions, preparations were digested in trypsin, and then by RNase(5); and

(5) Methyl green pyronin.

(b) Lipids. Sudan black B(14).

(c) Mucopolysaccharides. Alcian blue and the periodic acid-Schiff method (PAS)(14).

(d) Protein-bound groups.

(1) Lillie's modification of the Morel-Sisley reaction(15);

(2) A modification of Pauly's method for histidine(16);

(3) The dinitrofluorobenzene reaction(17);

(4) The ninhydrin-Schiff method(17);

(5) The alkaline fast green method for histones(18).

Results. At pH 3.5 all inclusions were stained green by acridine orange, very pale

† Supplied by Nat. Inst. Health, Bethesda, Md.

purple by TBM, method C, and very pale green by dilute TB. Whereas staining with TB and TBM was prevented when the inclusions were pretreated with either RNase or hot TCA, it was unaffected by DNase or pepsin. The following procedures did not interfere with the green staining by acridine orange: DNase; RNase; DNase followed by RNase, and *vice versa*; or extraction with hot TCA with or without treatment with pepsin. After nitrosation, acridine orange stained the inclusions yellowish-green at pH 3.5 and red (like the rest of the cell) at pH 7.0. The extraction procedure did not affect the yellowish-green staining and the red stain was not influenced by digestion with RNase or trypsin followed by RNase(5). Red staining of the inclusions by methyl green pyronin was also unaffected by digestion with ribonuclease. TBM methods A, B, D, and E did not stain the inclusions and they were Feulgen-negative. No material staining with Sudan black, alcian blue or PAS was detected in the inclusions, but they were stained by all the methods for protein-bound groups, except for the alkaline fast green method. The inclusions, therefore, contained protein and small amounts of RNA.

Discussion. The green coloration of the inclusions in the pH 3.5 acridine orange preparations was not due to binding of dye by nucleic acids. Hydrolysis of nucleic acids prevented staining by TB and TBM but had no effect on the fluorescent green staining of the inclusions by acridine orange. Similarly, digestion with proteolytic enzymes and nucleases did not affect the red staining by pyronin-methyl green or by acridine orange at pH 7.0. In the paper by Kissling and Reese(6), there was no mention of digestion by nucleases and, therefore, no proof that nucleic acids were being colored red by acridine orange. Since there was no staining by alcian blue, toluidine blue after ribonuclease digestion, or by the PAS method, it is highly unlikely that there are mucopolysaccharides in the inclusions. On the other hand, the inclusions were readily stained by all methods for protein-bound groups (except the alkaline fast green method), so that it seems probable that the binding of acridine orange and pyronin was caused by the anionic carboxyl groups. This would be most likely when the dye was used at the higher pH where more carboxyl groups would be charged.

The inclusions have been shown to contain antigen(7). Several attempts to demonstrate virus in the inclusions of cells infected with rabies have been unsuccessful(19). Matsumoto(20) has recently published a very clear demonstration of the virus and its absence in the inclusions. It would seem most probable, therefore, that the inclusions contain viral antigenic protein and a small amount of RNA.

Summary. The cytoplasmic inclusions present in rabbit endothelial and hamster kidney fibroblast cells, chronically infected with CVS rabies virus, can be stained green or red by acridine orange and are colored red by methyl green pyronin. These staining reactions do not appear to be caused by the presence of nucleic acids in the inclusions. RNA, which is sensitive to digestion with RNase, is stained by toluidine blue and toluidine blue-molybdate (Method C) in the inclusions. The inclusions appear to contain viral antigenic protein and small amounts of RNA.

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1. Wolman, M., Behar, A., *J. Infect. Dis.*, 1952, v91, 69.
2. Moulton, J. E., *Am. J. Path.*, 1954, v30, 533.
3. Sourander, P., *J. Path. Bact.*, 1956, v72, 257.
4. Sokolov, N. N., Vanag, K. A., *Acta Virology*, 1962, v6, 452.
5. Lépine, P., Atanasiu, P., *C. R. Acad. Sci.*, 1963, v256, 4783.
6. Kissling, R. E., Reese, D. R., *J. Immunol.*, 1963, v91, 362.
7. Fernandes, M. V., Wiktor, T. J., Koprowski, H., *Virology*, 1963, v21, 128.
8. Macpherson, I., Stoker, M.G.P., *ibid.*, 1962, v16, 147.
9. Eagle, H., *J. Exp. Med.*, 1955, v102, 595.
10. Love, R., Rabotti, G., *J. Histochem. Cytochem.*, 1963, v11, 603.
11. Schneider, W. C., *J. Biol. Chem.*, 1945, v161, 293.
12. Love, R., Walsh, R. J., *J. Histochem. Cytochem.*, 1963, v11, 188.

13. Gomatos, P. J., Tamm, I., Dales, S., Franklin, R. M., *Virology,* 1962, v17, 441.

14. Pearse, A. G. E., *Histochemistry, Theoretical and Applied,* Little, Brown & Co., 1960.

15. Lillie, R. D., *J. Histochem. Cytochem.,* 1957, v5, 528.

16. Reavan, E. P., Cox, A. J., *ibid.,* 1963, v11, 782.

17. Burstone, M. S., *ibid.,* 1955, v3, 32.

18. Alfert, M., Geschwind, I. I., *Proc. Nat. Acad. Sci.,* 1953, v39, 991.

19. Atanasiu, P., Lépine, P., Dragonas, P., *Ann. Inst. Past.,* 1963, v105, 813.

20. Matsumoto, S., *J. Cell. Biol.,* 1963, v19, 565.

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Characteristics of Human Adenovirus Type 12 Induced Hamster Tumor Cells in Tissue Culture.* (29307)

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Previous reports(1-4) have described tumor induction in Syrian hamsters with human adenoviruses. In addition, adenovirus type 12 (A-12) has been found to induce tumors in rats(5) and mice(6) but with a lower incidence. In contrast to the findings with other viruses oncogenic for newborn hamsters, *i.e.,* polyoma and Simian virus 40 (SV-40)(7-9), all attempts to isolate the virus from A-12 induced hamster tumors have been negative. The findings that some animals with primary tumors develop neutralizing antibodies(10) and that most animals with primary or transplant tumors develop A-12 type specific complement-fixing antibodies(5) suggest the presence of the virus or viral antigen in some form.

In an effort to detect A-12, a hamster tumor was serially propagated in tissue culture and attempts were made, by a variety of methods, to demonstrate the presence of the virus. Other characteristics studied were the response to superinfection with A-12, morphology and growth characteristics, and transplantability. Detailed karyotype analysis will be presented separately.

Materials and methods. Establishment of tumor cell line. A male hamster which had been inoculated with A-12 within 24 hours after birth developed intraperitoneal tumors. The tumor tissue was removed 42 days after inoculation, minced into small fragments about 0.5-1.0 cm and trypsinized overnight at 4°C with a magnetic stirrer. The suspension was centrifuged at 1200 rpm for 5 minutes, the pellet resuspended in growth medium, the cell suspension inoculated in 60 mm glass petri dishes and incubated at 37°C in a CO_2 incubator. The tumor cell line will subsequently be referred to as hamster tumor, line 1 (H.T.-1).

Growth medium. Eagle's medium (MEM) containing 10% horse serum was used as the initial growth medium and for the first 10 subcultivations; subsequently the serum content was changed to 10% calf serum unless otherwise indicated. All sera were heat inactivated at 56°C for 30 minutes. The medium contained 200 units of penicillin, 200 μg of streptomycin, and 20 units of Mycostatin per ml. All washing was done in phosphate buffered saline (PBS).

Superinfection studies. The MEM plus 10% calf serum was decanted from 2 oz bottles containing approximately 10^6 hamster tumor cells of the 25th passage. A-12 was added, and after incubating for one hour (37°C), the bottles were drained, washed 3 times with 2 ml of PBS, refed with 4 ml of MEM plus 10% horse serum, and incubated at 37°C for the 10 days of the experiment. Cytological observations were made from cover slips. Supernatant fluid and cells were titrated for virus separately, the latter after

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