the esterase or the epoxide hydrase responsible for JH metabolism, we have evidence (*9*) that other JHA may act through epoxide hydrase activation. The initial response of insects treated with each type of material is of course different though the end result, disruption of development, is apparently the same.

Our data may indeed provide the basis for a more rational approach to the design and development of a variety of compounds with potential JHA activity.

MICHAEL SLADE
C. F. WILKINSON
Department of Entomology,
Cornell University,
Ithaca, New York 14850

References and Notes

1. J. J. Menn and M. Beroza, Eds., *Insect Juvenile Hormones: Chemistry and Action* (Academic Press, New York, 1972).
2. H. Röller and K. H. Dahm, *Recent Progr. Hormone Res.* **24**, 651 (1968).
3. W. S. Bowers, *Naturally Occurring Insecticides* (Dekker, New York, 1971), pp. 307–332.
4. K. Slama, *Annu. Rev. Biochem.* **40**, 1079 (1971).
5. W. S. Bowers, *Science* **164**, 323 (1969).
6. P. A. Cruickshank, *Bull. WHO* **44**, 395 (1971).
7. W. S. Bowers, *Science* **161**, 895 (1968).
8. M. Slade and C. H. Zibitt, in "Chemical releasers in insects," *International IUPAC Congress 2nd, Pesticide Chemistry: Proceedings*, A. S. Tahori, Ed. (Gordon & Breach, New York, 1971), vol. 3, pp. 45–58.
9. M. Slade and C. F. Wilkinson, in preparation.
10. A. S. Meyer and H. A. Ax, *J. Insect Physiol.* **11**, 695 (1965).
11. R. I. Krieger and C. F. Wilkinson, *Biochem. Pharmacol.* **18**, 1403 (1969); R. S. H. Yang and C. F. Wilkinson, *Biochem. J.* **130**, 487 (1972).
12. W. Hafferl, R. Zurfluh, L. Dunham, *J. Labelled Comp.* **7**, 331 (1971).
13. C. E. Dyte, *Proc. Brit. Insec. Fungic. Conf. 5th* **1**, 393 (1969).
14. G. T. Brooks, in *Agricultural Research Council Annual Report for 1970–71* (Her Majesty's Stationery Office, London, 1972), p. 36.
15. T. Ohtaki, K. Kiguchi, H. Akai, K. Mori, *Appl. Entomol. Zool.* **7**, 161 (1972).
16. We thank H. K. Hetnarski for technical assistance, Dr. W. S. Bowers of the New York State Agricultural Experiment Station, Geneva, for compound **2**, and Dr. P. A. Cruickshank of FMC Corporation, Princeton, N.J., for compounds **3** and **6** (NIA 23509 and NIA 16388). Supported in part by PHS training grant ES 00098, PHS grant ES 00400, and Rockefeller Foundation grant RF 69073.

16 March 1973; revised 24 April 1973

Human Papovavirus (JC): Induction of Brain Tumors in Hamsters

Abstract. *Eighty-three percent of hamsters inoculated at birth with JC virus, a human papovavirus isolated from brain tissue of a case of progressive multifocal leukoencephalopathy, developed malignant gliomas within 6 months. Three brain tumors have been serially transplanted as subcutaneous tumors. JC virus was isolated from five of seven tumors tested. Cells from four tumors were cultivated in vitro. These cells contained an intranuclear antigen with the characteristics of a T antigen, and this antigen was antigenically related to SV40 T antigen. Although virus was not recovered from extracts of serially cultured tumor cells, JC virus was rescued when one tumor cell line was fused with permissive cells.*

A new human papovavirus, JC virus, was recently isolated (*1*) from brain tissue of a case of progressive multifocal leukoencephalopathy (PML). Although PML is a degenerative disease of the brain of man, the scattered giant astrocytes usually present in the lesions cannot be distinguished from the malignant astrocytes of pleomorphic glioblastomas (*2*). This observation in itself could raise a question concerning the oncogenic potential of this virus, but, in addition, the following points suggest that the tumor-inducing capacity of JC virus should be examined: (i) JC virus belongs in that subgroup of papovaviruses (polyoma, SV40) that have a strong capacity for transforming cells in vitro and for inducing tumors in laboratory animals; (ii) JC virus is found in deep tissues of man (in contrast to the human papilloma virus); and (iii) approximately 75 percent of adults have serum antibody against JC virus, which is evidence of infection by this virus (*3*). The data presented here are part of such an examination and indicate that JC virus is strongly oncogenic in the central nervous system of hamsters.

JC virus is distinct from polyoma, SV40, and other papovaviruses in several respects (*1*); however, we have unpublished evidence of a weak relationship between JC and SV40 virion antigens. Because of this relationship and because polyoma virus and SV40 are known to be highly oncogenic in newborn hamsters, care was taken to reduce the possibility that the observed effects could be due to adventitious SV40 or polyoma viruses.

Golden Syrian hamsters (*Mesocricetus auratus* from Lakeview Hamster Colony, Newfield, New Jersey) less than 24 hours old were inoculated both intracerebrally and subcutaneously with JC virus or with control material. Inoculation was into the right cerebral hemisphere and under the skin over the shoulders. The virus dose in each site was 10^6 $TCID_{50}$ (tissue culture infective dose, 50 percent effective) (2048 hemagglutinating units) in 0.02 ml.

The virus inoculum was from the third passage in primary human fetal glial (PHFG) cell cultures (*1*) and was concentrated and partially purified by differential centrifugation. The control inoculum was a concentrated and partially purified extract of uninfected PHFG cells. The inocula were cultured for mycoplasma (*4*) with negative results. That the virus inoculum had the characteristics typical of JC virus was checked by inoculation onto PHFG cells and two cells derived from African green monkey kidney cultures (Vero and CV-1). JC virus does not produce cytopathic effects in Vero or CV-1 cells, but SV40 virus does. During a 21-day observation period, cytopathic effects were seen only in the PHFG cultures, and only JC viral antigen was detected (in the PHFG cultures) when inoculated cultures of all three cell types were stained by the indirect immunofluorescent technique with anti-JC, anti-SV40, and anti-polyoma serums.

There was no evidence of acute disease caused by the inocula. Sixty-three of 100 hamsters inoculated with JC virus and 39 of 69 inoculated with control material survived the initial manipulations and were placed under long-term observation.

Three months after inoculation, four animals from each group were killed, and their tissues were examined for gross and microscopic lesions. Although no signs of disease had been seen in these animals, one of the four inoculated with JC virus had a definite microscopic tumor deep in one cerebral hemisphere. The other three had collections of abnormal cells in the brain that suggested early tumor foci. The control hamsters showed no evidence of disease.

Approximately 4 months (123 days) after inoculation overt signs of central nervous system disease were recognized in a JC virus–inoculated hamster. Additional animals with signs of central nervous system disease appeared at a fairly steady rate of five or six per week. Central nervous system disease usually was signaled by decreased activity, somnolence, unsteady gait, and circling. In order to obtain fresh tissues, most animals were killed within 5 days after onset of signs of disease; otherwise they usually died within 10 days.

Six months after inoculation the nine remaining JC virus–inoculated hamsters and the control animals were

killed. A complete autopsy was performed on every animal in this experiment, and the tissues were fixed in 10 percent formalin. Blood was collected at death whenever possible. All of the serums (51 from JC virus–inoculated hamsters and 37 from control animals) were tested for antibody against JC virion coat protein by using the hemagglutination-inhibition technique (*3*). Twelve serums, all from JC virus–inoculated animals, had detectable antibody.

The only significant pathologic findings were related to brain tumors in the JC virus–inoculated group. Table 1 provides a summary of the results. Tumors were found in all the major areas of the brain with some preference for the thalami and the cerebellar cortex. In gross appearance, the tumors were gray in color and ranged from 2 to 7 mm in diameter. On microscopy, the tumors were found to be malignant and of glial origin. Many contained bizarre giant cells. Most of the tumors were glioblastomas (Fig. 1), medulloblastomas, or unclassified primitive tumors. Papillary ependymomas were found in two animals only. Most brains contained multiple tumors, and it was not uncommon for tumors of different histologic appearance to be located in the same brain. No metastases were found in other organs, and no tumors were observed at the site of the subcutaneous inoculation.

Transplantability of the tumors was tested with tissue from seven animals. Excised tumor tissue was broken up by repeated aspiration and ejection from a pipette and by forcing through a 20-gauge hypodermic needle. Each tumor suspension was injected subcutaneously (0.2 ml) into two weanling hamsters. Within 1 to 2 months, six tumor cell suspensions had developed into progressively growing subcutaneous tumors. One tumor suspension caused small (< 1 cm) nodules within 2 months, but these did not increase in size. Three of the progressively growing tumors were selected for serial transplantation and are now in the fifth subcutaneous passage.

Attempts were made to isolate JC virus from tumor tissue of seven animals. Each tumor was disrupted by forcing it through a 20-gauge hypodermic needle and was suspended in culture medium as a 10 percent suspension. It was frozen and thawed once and sonicated for 3 minutes at 20 kc/sec. Each suspension was then layered over four cultures of PHFG cells in Leighton tubes. The PHFG cell cultures were maintained with twice-weekly changes of Eagle's minimum essential medium containing 3 percent fetal calf serum. After 3 weeks of incubation, the cultures were examined for cytopathic effects. The cells were then disrupted, a sample was tested for hemagglutinin (*3*), and the remainder was used to inoculate four new PHFG cultures. These cultures similarly were examined for cytopathic effects and hemagglutinin production at the end of 3 weeks.

A virus was recovered from five tumors. The virus was identified as JC virus in each instance by demonstration of a hemagglutinin characteristic of JC virus (*3*) and by use of the indirect immunofluorescent technique to test for viral antigen. Cells in infected cultures contained antigen that reacted with antiserum against JC virus but did not react with antiserums against SV40 or polyoma virus. No virus was isolated from two tumors.

An effort was made to establish cells in culture from five tumors and from brain tissue of two control animals. Tissue was minced and expressed through a 20-gauge hypodermic needle. It was planted in plastic flasks in Eagle's minimum essential medium containing 10 percent fetal calf serum. Cells from four of the tumors were successfully grown and are in the 10th to 31st subculture. Brain cells from control animals could not be carried beyond the first culture.

The serially cultured tumor cells were tested for the presence of JC virus coat protein by the indirect immunofluorescent test with the use of a rabbit anti-JC virus serum known to be potent in labeling JC antigen in infected PHFG cells. All four tumor cell lines were negative. They were also negative in tests using antiserums against SV40 and polyoma coat protein.

Most cultured tumor cells contained an intranuclear antigen that reacted with serum collected from certain of the tumor-bearing hamsters. This antigen was similar to the T antigens described for other papovaviruses (*5*) in that it filled the nucleus but spared the nucleoli and was heat labile (56°C for 30 minutes). Thirty of the 51 serums collected from JC virus–inoculated hamsters had antibody against this antigen, as detected by indirect immunfluorescent tests. Serums from 21 JC virus–inoculated animals and all 37 serums available from control animals lacked such antibody. When tumor cells were reacted with antiserums against SV40 and polyoma T antigens in indirect immunofluorescent tests, clear-cut nuclear staining was observed with the SV40 antiserum.

Table 1. Brain tumors appearing within 6 months in hamsters inoculated at birth with JC virus.

Inoculum	Brain tumors*	
	No. with tumors/ No. inoculated	Percent with tumors
JC virus†	52‡/63	83
Control extract§	0/39	0

*Tumors confirmed by histologic examination. † 10^6 $TCID_{50}$ (2048 hemagglutinating units) both intracerebrally and subcutaneously less than 24 hours after birth. ‡Three additional hamsters, not counted as positive, had only foci of abnormal cells suggestive of early tumor formation. §Concentrated extract of normal primary human fetal glial cells.

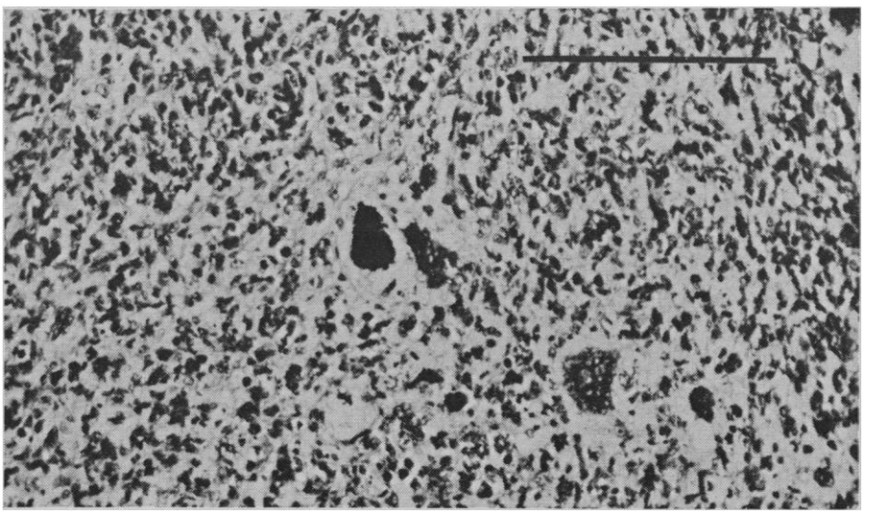

Fig. 1. An extremely cellular, pleomorphic tumor (glioblastoma multiforme) from thalamic region of the brain. Paraffin section, hematoxylin and eosin stain (× 340). Bar represents 0.1 mm.

Cross-reactions among the T antigens of papovaviruses have not been reported previously. Therefore, it was necessary to investigate the cause of the observed reaction between the cultured hamster tumor cells and SV40 T antiserum. The relationship between JC and SV40 T antigens was investigated in PHFG cultures, and it was established that the T antigens of these two viruses are related. Cultures of PHFG were inoculated with a mixture of JC virus and a potent SV40 neutralizing antiserum. Twenty days later, many cells in the culture contained a nuclear antigen that reacted with the SV40 T antiserum in immunofluorescent tests (*6*).

One tumor cell line, HJC-15, started from a mixed malignant glioma of astrocytic and ependymal derivations, was selected for further study. No infectious virus was recovered from this cell line when a sonicated suspension of 5×10^6 cells at the 12th passage level was inoculated into PHFG cell cultures (42-day observation period).

JC virus was rescued from the 14th subculture of HJC-15 cells after fusion with PHFG cells with the use of Sendai virus inactivated with β-propiolactone. Suspensions of 2×10^6 HJC-15 cells were fused with 3×10^6 PHFG cells or 2×10^6 Vero cells with the use of 4000 hemagglutinating units of Sendai virus in 2 ml (*7*). Each fused cell suspension was planted in two plastic flasks (250 ml) in maintenance medium. After 16 days of incubation at 37°C the cells were disrupted by freezing and thawing followed by sonication. A thick suspension of cell debris from the HJC-15 : PHFG fused cultures was layered on PHFG cells in Leighton tubes, while that from the HJC-15 : Vero fused culture was layered on CV-1 cells. These cultures were fixed 21 days later and tested for viral coat protein antigens with immunofluorescent techniques using antiserums against both JC and SV40 viruses. Many cells in the PHFG cultures contained antigen that reacted with JC virus antiserum only, whereas the CV-1 cells were negative with both antiserums.

This initial experiment demonstrates that a human papovavirus (JC), which is associated with a demyelinating disease of the brain, is capable of inducing malignant gliomas in a high percentage of hamsters inoculated intracerebrally as newborns. The majority of these gliomas are of types not previously reported to be induced by papovaviruses in in vivo experiments. In hamsters, SV40 virus induces glial tumors of the ventricular lining only (ependymomas, papillary ependymomas, or plexus papillomas), while polyoma virus induces mesenchymal tumors in the brain (*8*). The rescue of JC virus from hamster tumor cells by fusion with permissive cells is evidence for the continued presence of the JC virus genome in the cultured tumor cells.

DUARD L. WALKER
BILLIE L. PADGETT
GABRIELE M. ZURHEIN
ALBERTINA E. ALBERT
Departments of Medical Microbiology and Pathology, University of Wisconsin Medical School, Madison 53706
RICHARD F. MARSH
Department of Veterinary Science, University of Wisconsin Agricultural and Life Sciences, Madison 53706

References and Notes

1. B. L. Padgett, D. L. Walker, G. M. ZuRhein, R. J. Eckroade, B. H. Dessel, *Lancet* **1971-I**, 1257 (1971).
2. K. E. Åström, E. L. Mancall, E. P. Richardson, Jr., *Brain* **81**, 93 (1958); G. M. ZuRhein, *Progr. Med. Virol.* **11**, 185 (1969).
3. B. L. Padgett and D. L. Walker, *J. Infec. Dis.* **127**, 467 (1973).
4. V. Allen, S. Sueltmann, C. Lawson, *Health Lab. Sci.* **4**, 90 (1967).
5. J. Pope and W. A. Rowe, *J. Exp. Med.* **120**, 121 1964); P. H. Black, *In Vitro* **2**, 61 (1966).
6. Details of this experiment are in preparation.
7. P. Gerber, *Virology* **28**, 501 (1966); H. Koprowski, F. C. Jensen, Z. Steplewski, *Proc. Nat. Acad. Sci. U.S.A.* **58**, 127 (1967); J. M. Neff and J. F. Enders, *Proc. Soc. Exp. Biol. Med.* **127**, 260 (1968).
8. R. L. Kirschstein and P. Gerber, *Nature* **195**, 299 (1962); D. Duffell, R. Hinz, E. Nelson, *Amer. J. Pathol.* **45**, 59 (1964); F. Unterharnscheidt, O. Bonin, K. Schmidt, I. Schmidt, *Acta Neuropathol.* **3**, 362 (1964); A. S. Rabson and R. L. Kirschstein, *Arch. Pathol.* **69**, 663 (1960).
9. We thank Martha Blakis and Doris Metcalf for expert technical assistance. This work was supported by grant AI-09023 from the National Institute of Allergy and Infectious Diseases.

14 May 1973

Stimulus-Coupled Secretion of γ-Aminobutyric Acid from Rat Brain Synaptosomes

Abstract. *Synaptosomes treated with radioactive γ-aminobutyric acid can be stimulated to release this substance. The release is maximal within 40 seconds after stimulation and is dependent on calcium. Magnesium and manganese ions, known to block stimulus-secretion coupling processes, depress calcium-dependent release. This release is specific to synaptosomes because microsomal or myelin fractions do not release accumulated γ-aminobutyric acid. The data illustrate a simple in vitro system suitable for analysis of secretion of γ-aminobutyric acid in brain and in addition describe several new aspects of uptake and secretion of this compound at brain nerve endings.*

Understanding the mechanism of synaptic transmission is essential for understanding brain function. Although synaptic transmission is chemical in nature, few chemical techniques are available to directly study the process. In particular we need to understand (i) the nature of the secretory process; (ii) the nature of the transmitters secreted; and (iii) the action of drugs which affect neuronal transmission. Ideally in order to study function at chemical level, it is essential to study the synapse directly. Centrifugation techniques have made it possible to isolate presynaptic endings (synaptosomes) in abundance and in fairly pure fractions from the central nervous system. It has now been demonstrated that the function of synaptic endings can be studied in vitro under a wide range of controlled conditions.

Studies from several laboratories indicate that putative transmitters are released from synaptosomal fractions (*1*, *2*). However, specific criteria required for demonstration of transmitter release have not yet been met. Investigations on neurosecretory activity of isolated synaptosomes require that a number of criteria must be met in order to assume that the observed chemical changes are due to stimulus-induced secretion: (i) The appropriate stimulus should trigger a very rapid efflux of neurotransmitter; (ii) the secretion should be Ca-dependent; (iii) the secretion should be blocked by Mg and other antagonist divalent ions; (iv) the secretion should be specific to synaptosomes; and (v) the Ca-dependent secretion should be distinguished from possible artifacts of the system such as blockage of reuptake and surface exchange. All of these criteria are general properties of stimulus-secretion coupling processes found in various systems in vivo (*3*).

We have characterized a functional synaptosomal system for the release of γ-aminobutyric acid (GABA) which meets all of the criteria listed above. To study transmitter release, synaptosomes