(1963); D. H. Adams, *Pathol.-Biol.* **18**, 559 (1970); R. L. Chandler, *Res. Vet. Sci.* **4**, 276 (1963).
6. G. D. Hunter and G. C. Millson, *J. Gen. Microbiol.* **37**, 251 (1964b); D. L. Mould and A. MCL. Dawson, *J. Comp. Pathol.* **80**, 595 (1970).
7. C. J. Gibbs, *Curr. Top. Microbiol. Immunol.* **40**, 44 (1967); ———, D. C. Gajdusek, J. A. Morris, in *Slow, Latent and Temperate Virus Infections*, National Institute of Neurological Diseases and Blindness Monograph No. 2, D. C. Gajdusek, C. J. Gibbs, Jr., M. Alpers, Eds. (PHS Publ. 1378) (Government Printing Office, Washington, D.C., 1965), p. 195.
8. I. Zlotnik and J. C. Rennie, *Br. J. Exp. Pathol.* **48**, 171 (1967); I. H. Pattison and G. C. Millson, *J. Comp. Pathol.* **71**, 350 (1961).
9. S. B. Prusiner *et al.*, *Ann. Neurol.* **11**, 353 (1982).
10. R. F. Marsh and R. H. Kimberlin, *J. Infect. Dis.* **131**, 104 (1975); R. H. Kimberlin and C. Walker, *J. Gen. Virol.* **34**, 295 (1977).
11. Thermal inactivation experiments are complicated by the asymptotic approach to thermal equilibrium. In these experiments the equilibration period was minimized and all samples were brought to the test temperature in 60 seconds or less by means of a brief thermal pulse in a 156°C oil bath. The temperature of the vigorously agitated samples was monitored with a recording thermometer (Fig. 1) and the samples were transferred at an empirically determined point to the test bath in such a way that the temperature continued to rise quickly to the test temperature and then stopped.
12. S. S. Block, Ed., *Disinfection, Sterilization and Preservation* (Lea and Febiger, Philadelphia, 1977); J. E. Salle and J. B. Gori, *Ann. N.Y. Acad. Sci.* **83**, 609 (1960).
13. W. Ginoza, in *Methods in Virology*, K. Maramorosch and H. Koprowski, Eds. (Academic Press, New York, 1968), p. 139; S. Gard and O. Maaloe, in *The Viruses*, F. M. Burnet and W. M. Stanley, Eds. (Academic Press, New York, 1959), vol. 1, p. 359.
14. R. G. Rohwer, in *Virus Non Conventionnels et Affections du System Nerveux Central*, L. A. Court and F. Cathala, Eds. (Masson, Paris, 1983), p. 84.
15. C. M. Eklund, W. J. Hadlow, R. C. Kennedy, C. C. Boyle, T. A. Jackson, *J. Infect. Dis.* **118**, 510 (1968); D. Burger, J. R. Gorham, R. W. Leader, in *Slow, Latent and Temperate Virus Infections*, National Institute of Neurological Diseases and Blindness Monograph No. 2, D.C. Gajdsuek, C. J. Gibbs, Jr., M. Alpers, Eds. (PHS Publ. 1378) (Government Printing Office, Washington, D. C., 1965), p. 307; D. P. Gray, thesis, University of Toronto (1964).
16. G. Siegle, C. Hallauer, A. Novak, G. Kronauer, *Arch. Gesamte Virusforsch.* **35**, 91 (1971); C. Wallis, J. L. Melnick, F. Rapp, *Virology* **26**, 694 (1965); P. Pohjanpelto, *ibid.* **15**, 225, 231 (1961); G. Fassell and S. G. Wildman, *Biochim. Biophys. Acta* **72**, 230 (1963).
17. K. O. Stetter, *Nature (London)* **300**, 258 (1982).
18. J. Baross and J. Deming, *ibid.* **303**, 423 (1983); J. Baross, M. D. Lilley, L. I. Gordon, *ibid.* **298**, 366 (1982).
19. Phage fd was inactivated by the violent homogenization procedure applied to the scrapie samples. Therefore, phage fd–containing samples had to be homogenized by gentler non-inactivating means 10 seconds at 70 percent of full power with a Tekmar SDT Tissumizer (Tekmar Co., Cincinnati, OH). If phage fd aggregates with itself or with tissue coagulated during exposure to heat, the lower relative titers seen for phage fd in 10 percent brain homogenate may reflect inadequate homogenization of this virus.
20. R. G. Rohwer, in preparation.
21. In thermistor-controlled tests of our own autoclaves we have observed long delays in reaching 121°C, depending on the mass and type of sample. Some surfaces of the glassware placed in a kill pan full of empty glassware can take over 1 hour to reach 121°C in an ambient environment of 121°C (*14*). This suggests that some of the survival after autoclaving may be due to incomplete exposure to 121°C. Because the approach to thermal equilibrium is asymptotic, 121°C is attained much more readily by operating the autoclave at 132°C (*14*). Therefore, it may be advisable to operate the autoclave at that temperature to ensure that all materials contaminated with scrapie or other subacute spongiform virus encephalopathies spend as much time as possible at 121°C or greater.
22. The output of Yellow Springs Instruments No. 421 thermistor was monitored by a Yellow Springs Instruments Model 42 Tele Thermometer and recorder. The measured time constant of the thermistor configured in this way was 5.3 seconds.
23. When exposed to 100° or 121°C, brain homogenate coagulates into soft gray curds. At 80°C the homogenate gradually turns gray and grainy over an hour's time. At 60°C samples remain homogeneous and retain their pinkish color for almost an hour.
24. Previous tests have shown scrapie infectivity titers to be stable to this treatment (R. G. Rohwer, unpublished data).
25. J. Goudsmit, R. G. Rohwer, E. K. Silbergeld, D. C. Gajdusek, *Brain Res.* **220**, 372 (1981).
26. C. Masters *et al.*, in preparation.
27. L. J. Reed and J. Muench, *Am. J. Hyg.* **27**, 493 (1938).
28. I thank G. Sokol for technical assistance, E. Green for supervision of the animal colony, and D. C. Gajdusek for support, encouragement, and critical reading of the manuscript.

23 June 1983; accepted 19 December 1983

A New Type D Retrovirus Isolated from Macaques with an Immunodeficiency Syndrome

Abstract. *Macaque monkeys with the recently described acquired immunodeficiency syndrome show a marked defect in T-lymphocyte function and die with opportunistic infections and lymphoproliferative abnormalities. In the study described here a new type D retrovirus was isolated from two* Macaca cyclopis *with this syndrome. This virus is related to, but distinct from, Mason-Pfizer monkey virus, a type D retrovirus previously isolated from a mammary tumor of a rhesus monkey* (Macaca mulatta).

Endemic immunodeficiency diseases in colonies of macaque monkeys at the New England Regional Primate Research Center (NERPRC) and the California Primate Research Center at Davis have many similarities to the acquired immunodeficiency syndrome (AIDS) in humans (*1, 2*). Consistent immunologic abnormalities have been noted in affected macaques of the NERPRC colony. Proliferative responses of their peripheral blood lymphocytes to antigens and lectins are dramatically diminished (*1*). Furthermore, ratios of the T4 (helper/inducer) to T8 (suppressor/cytotoxic) circulating T-lymphocytes of the macaques in the colony at greatest risk for developing this syndrome (*Macaca cyclopis*) are considerably less than those in other macaques (*1*). Peripheral blood smears from affected monkeys reveal an immature circulating mononuclear cell with vacuolated cytoplasm and prominent nucleoli (*1*).

We have transmitted spontaneously occurring lymphomas in rhesus monkeys to healthy monkeys by means of tumor cell suspensions (*3*). The recipient animals developed undifferentiated or poorly differentiated lymphomas or parenchymal lymphoproliferative abnormalities suggestive of early lesions of lymphoma. They also developed opportunistic infections with agents such as cytomegalovirus (CMV) and *Cryptosporidium* and showed evidence of an abnormal peripheral blood mononuclear cell morphologically similar to that seen in macaques with the immunodeficiency syndrome. These findings suggested a link between the transmissible lymphomas and the immunodeficiency syndrome in macaques. In fact, by inoculating previously healthy macaques with either tissue or a cell-free filtrate from a macaque lymphoma we were able to transmit the immunodeficiency syndrome (*4*). The recipients developed evidence of profound lymphocyte dysfunction or died with infections by opportunistic agents, including *Candida albicans*, *Cryptosporidium*, and CMV.

These studies implicated an infectious agent in the syndrome. Attempts to isolate a virus from macaques with this syndrome were made by using explant cultures of minced tissues (lymph nodes, spleens, and parenchymal tissues) and by cocultivation of cell suspensions, minced tissues, and throat and rectal swabs with a battery of indicator cell lines. Viruses identified by these approaches included adenoviruses, simian virus 40 (SV40), CMV, paramyxoviruses including measles virus, and foamy viruses. Viruses were identified on the basis of reactivity in indirect immunofluorescence tests with specific antisera and by electron microscopy. None of these viruses, however, seemed likely to be etiologic in the macaque immunodeficiency syndrome.

We reasoned that since the presumed etiologic virus was causing immunosuppression, we might be able to isolate it by cocultivation of tissue from an infected animal with lymphoid cell lines. We used tissue from two 3-year-old *M. cyclopis* (Mc) in late stages of the immunodeficiency disease. One, Mc 184-80, was dehydrated and had lymphadenopathy (*5*). Peripheral blood mononuclear cells (PBM) from this animal showed no significant in vitro proliferative responses to pokeweed mitogen, concanavalin A, xenogeneic cells, or *Candida albicans* antigen. Lymph node biopsies 1 month

before the animal's death revealed an effacement of nodal architecture with an absence of follicles and a depletion of lymphocytes. At necropsy, paramyxovirus inclusions were noted in periportal hepatocytes and CMV inclusions were seen in lymph nodes. Nodular aggregates of mature lymphocytes were seen in the bone marrow.

The second macaque, Mc 398-80, was dehydrated, cachetic, and had generalized lymphadenopathy (*5*). A lymph node biopsy 7 months before the animal's death revealed paracortical hyperplasia and sinus histiocytosis with erythrophagocytosis. CMV was isolated from oral swabs. Although PBM from this animal showed normal in vitro proliferative responses to lectins and antigens, a dramatic depletion of lymphocytes from lymph nodes was noted at necropsy. Furthermore, nodular infiltrates consisting of small, well-differentiated lymphocytes were noted in the kidneys. These unusual findings and the progression of morphological changes in lymph nodes are characteristic pathologic features of the macaque immunodeficiency syndrome (*6*).

Peripheral blood lymphocytes from Mc 398-80 and Mc 184-80 were cocultivated with Raji cells, a lymphoblastoid B cell line of human origin, and examined microscopically daily with the cell cultures being split at 3- to 4-day intervals. After 7 days, large unusual cells began to appear in the cultures (Fig. 1A). These cells varied in size, with some being as much as 20 times larger than normal Raji cells. Examination of these cells after fixing and staining with hematoxylin and eosin revealed that the smaller cells had one or sometimes two nuclei, whereas the large cells had multiple, closely packed nuclei, often two to four times larger than the nuclei of normal Raji cells (Fig. 1B). The cytoplasm was scant, granular, and usually vacuolated. No inclusion bodies were seen. Electron microscopic examination revealed numerous type D retrovirus particles in the large vacuolated cells (Fig. 1, C to E). Infected cells contained intracytoplasmic A particles that were budding with a complete nucleoid from the cell membrane. Mature particles had a central nucleoid and lacked surface spike projections on their envelope. These findings are characteristic of type D retroviruses.

The original selection of Raji cells for use in these cocultivations was fortuitous since 12 other B and T cell lines did not display the unusual cytopathic changes when infected with this agent. Several control experiments showed that these results were not due to artifact. Virus was passed from infected cultures to uninfected Raji cells by means of supernatants from the cocultivations passed through a 0.2-μm filter; secondary infections again resulted in the large, bizarre cells. Mason-Pfizer monkey virus (MPMV), a type D retrovirus originally isolated from a mammary neoplasm in a rhesus monkey (*Macaca mulatta*), and squirrel monkey type D retrovirus (SMRV-D) produced the same type of cytopathic effect on Raji cells. Raji cell cultures from four separate laboratories have behaved similarly upon infection with type D retroviruses. Control Raji cell cultures examined by electron microscopy on three separate occasions did not show these virus particles, and the large cells shown in Fig. 1A were never seen in control cultures. The unusual cytopathic effect of this virus on Raji cells has allowed us to titer the virus by limiting dilution; virus titers in the supernatants of infected Raji cell cultures have been shown to exceed 10^6 infectious particles per milliliter.

The Raji cocultivation procedure thus provides an efficient, convenient means for isolating and growing type D retroviruses and does not appear to permit the growth of potential contaminating viruses such as foamy viruses, CMV, and SV40. Cocultivation of infected Raji cell supernatants with indicator monolayer

Table 1. Number of restriction endonuclease cleavage sites in replicative intermediate DNA from infected cells.

Restriction endonuclease	Source of replicative intermediate DNA			
	D398	D184	MPMV	SMRV-D*
Eco RI	1	1	0	1
Bam HI	1	1	2	3
Sst I	0	0	1	3
Kpn I	1	1	1	0

*Data from Chiu *et al.* (*8*) and Chiu and Aaronson (*12*).

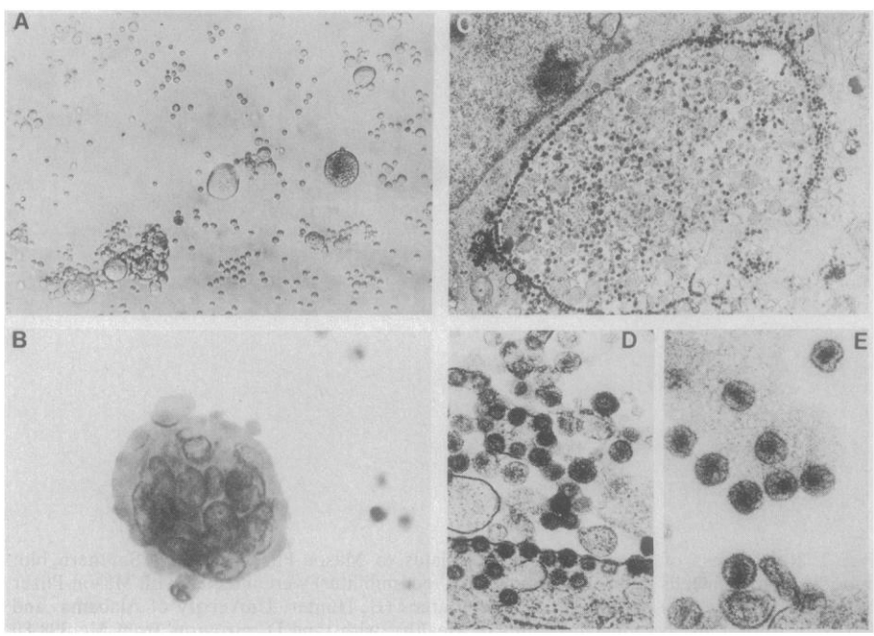

Fig. 1. Raji cells infected with retrovirus isolate from animal Mc 398-80. Approximately 5×10^5 peripheral mononuclear cells from Mc 398-80 were cocultivated with 10^7 Raji cells in 10 ml of RPMI 1640 medium containing 10 percent fetal calf serum. Peripheral mononuclear cells were prepared by Ficoll-Hypaque density gradient centrifugation. Cell cultures were split at 3- to 4-day intervals. For electron microscopy, cell pellets were fixed with 1 percent glutaraldehyde followed by osmium tetroxide and embedded in Epon 812. Thin sections were prepared with an ultramicrotome and stained with uranyl acetate and Sato's lead stain. (A) Large, unusual Raji cells 9 days after cocultivation (Leitz Diavert inverted light microscope, ×340). (B) Large multinucleated Raji cell after fixation and staining with hematoxylin and eosin (×1600). (C) Electron micrograph of a portion of one of the large Raji cells in which there are numerous viral particles aligned around and budding into a cytoplasmic vacuole (×12,000). (D) Higher magnification electron micrograph of a retrovirus-filled vacuole. Numerous cytoplasmic, 90-nm type A retrovirus particles are in the process of budding through the membrane of the vacuole to become enveloped 120-nm type D particles. The enveloped particles are devoid of surface spikes and have a centrally located nucleoid (×60,000). (E) Electron micrograph of a group of extracellular, enveloped viral particles with barrel-shaped nucleoids and blunt knobby surface projections on the envelope, morphologic features characteristic of mature type D retroviruses (×75,000).

cultures has not revealed the outgrowth of other virus types. We have also isolated type D retrovirus from macaque pharyngeal secretions and from supernatant fluids of lymph node and spleen monolayer cultures using the Raji cocultivation procedure.

The relation of these new virus isolates to MPMV was examined by cutting replicative intermediate DNA (Hirt supernatant DNA) from infected cells with restriction endonucleases and analyzing it by Southern blot hybridization (Fig. 2). Cells infected with D398 (the isolate from Mc 398-80) and D184 (the isolate from Mc 184-80) contained replicative intermediate DNA of about the same size as MPMV (approximately 8.2 kilobase pairs). Approximately ten times more Hirt supernatant DNA from D398 and D184 infected cells than from MPMV infected cells was required to give approximately equal intensity in the autoradiogram when a ^{32}P-labeled MPMV DNA fragment was used as the hybridization probe. That D398 and D184 are indeed distinct from MPMV is reflected in differences in restriction endonuclease fragmentation patterns (Table 1). For example, while Eco RI and Bam HI cut D398 and D184 DNA once each, Bam HI cut MPMV DNA twice and Eco RI did not cut MPMV DNA at all (Fig. 2). When MPMV DNA was compared to D398 and D184 DNA with Pst I and Hpa II being used as the restriction enzymes, very different fragmentation patterns were observed. We have recently cloned the full length D398 Bam HI fragment into pBR322 and derived detailed restriction endonuclease maps. These maps were compared to MPMV maps (*7*). Although there were some similarities, especially with Hpa I and Hind III, most (> 50 percent) restriction endonuclease sites were not conserved. Thus these results demonstrate that D398 and D184 are distinct from but related to MPMV. Furthermore, the restriction endonucleases that have been used to date did not show any differences between the D398 and D184 isolates. Restriction endonuclease maps for cloned squirrel monkey type D retrovirus (SMRV-D) DNA (*8*) also clearly distinguish SMRV-D from D398 and D184 (Table 1).

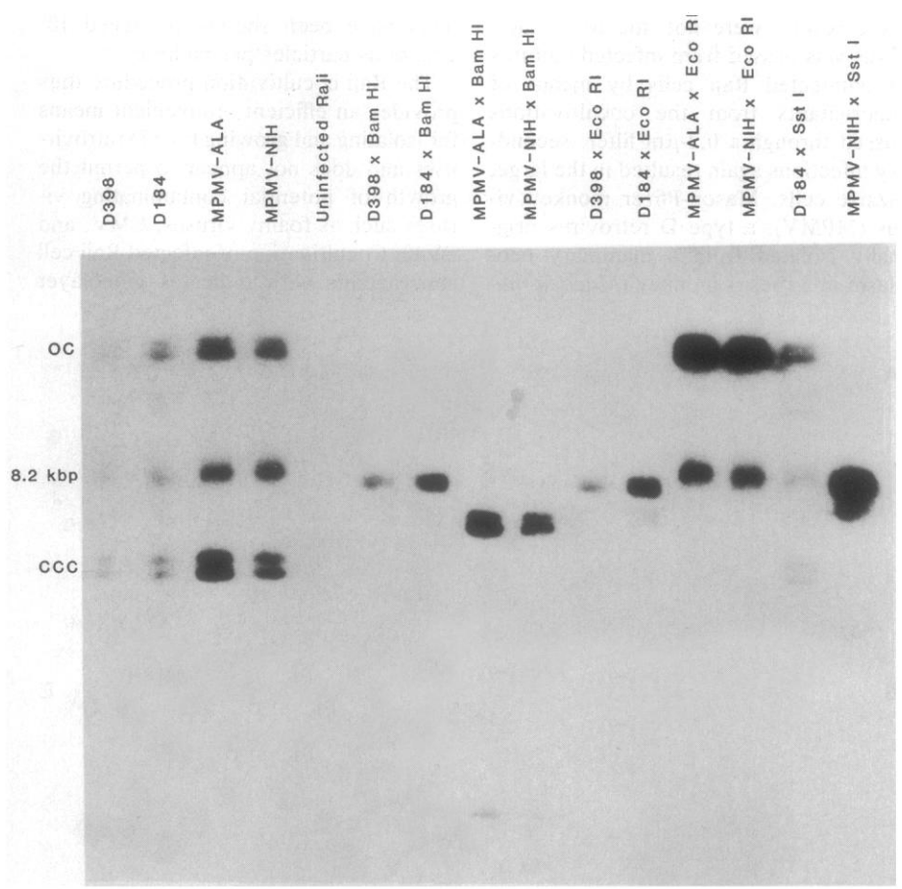

Fig. 2. Relatedness of type D retrovirus isolates to Mason-Pfizer virus by Southern blot hybridization. Raji cells (approximately 3×10^5 per milliliter) were infected with Mason-Pfizer monkey virus (*MPMV*) obtained from two sources (E. Hunter, University of Alabama, and Research Resources, National Institutes of Health), with type D retrovirus from Mc 398-80 (*D398*), and from Mc 184-80 (*D184*) at a multiplicity of infection of approximately 0.1. Virus titers were determined by limiting dilution on Raji cells. Seventy-two hours after infection, the cells were harvested and Hirt supernatant DNA was prepared (*13*). Portions of DNA, uncut or cut with the indicated restriction endonuclease, were subjected to electrophoresis through a 1.0 percent agarose gel and blotted to nitrocellulose by the procedure of Southern (*14*). Eight times more Hirt supernatant DNA from D398 and D184 than from MPMV infected cells was applied to the gel to give similar intensity in the autoradiogram. When equal volumes of the Hirt supernatant DNA's were subjected to electrophoresis in parallel slots, the ethidium bromide stain intensity of the mitochondrial DNA revealed equivalent yields (within 10 percent) from the four infected cultures as well as the uninfected control culture. A ^{32}P-labeled 2.5-kb fragment of MPMV DNA obtained from the cloned DNA pMP-6 (*7*) was hybridized with transferred DNA at 67°C in 4 × SSC (0.6*M* sodium chloride plus 0.06*M* sodium citrate) and 0.1 percent sodium dodecyl sulfate, and the filter was rinsed extensively and placed onto film (*15*). The fragment was purified by electroelution from an agarose gel following digestion of pMP-6 DNA with Hind III. When equal amounts of Hirt supernatant DNA were applied to the gel, lanes containing MPMV DNA gave a much stronger signal (not shown). *CCC* and *OC* correspond, respectively, to the expected locations of covalently closed circular and open circular forms of retroviral DNA; the doublets at these positions probably represent molecules with one and two long terminal repeats.

We obtained additional type D retrovirus isolates from four of seven *M. cyclopis* with lymphadenopathy and neutropenia. Peripheral blood lymphocytes from 34 apparently healthy *M. cyclopis* yielded no type D retrovirus when cocultivated with Raji cells.

Since new type D retrovirus isolates were obtained from *M. cyclopis* with this immunodeficiency syndrome but not from apparently healthy *M. cyclopis*, one may speculate as to whether this virus is responsible for the endemic disease in our macaque colony. When Fine *et al.* (*9*) inoculated 68 newborn *M. mulatta* with MPMV, 41 of the monkeys died during the 35 weeks following inoculation with a disease spectrum that included lymphadenopathy, anemia, neutropenia, opportunistic infections, and failure to thrive. These clinical findings are strikingly similar to those in macaques with the immunodeficiency syndrome at NERPRC. Long-term study of experimentally infected juvenile macaques will be needed to determine definitively whether type D retroviruses are indeed the cause of the macaque immunodeficiency syndrome or whether they are opportunistic agents.

It is conceivable that the type D retroviruses of macaques represent an extended family of viruses. In this regard, we have found that endogenous DNA sequences from healthy macaque tissues contain large amounts of DNA homologous to MPMV DNA (*10*); similar results

have been obtained in another laboratory (*11*). A family of related type D retroviruses could conceivably cause a spectrum of immunodeficiency diseases in nonhuman primates.

MUTHIAH D. DANIEL
NORVAL W. KING
NORMAN L. LETVIN
RONALD D. HUNT
PRABHAT K. SEHGAL
RONALD C. DESROSIERS*
New England Regional Primate Research Center, Harvard Medical School, Southborough, Massachusetts 01772

References and Notes

1. N. L. Letvin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2718 (1983).
2. R. V. Henrickson *et al.*, *Lancet* **1983-I**, 388 (1983).
3. R. D. Hunt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5085 (1983).
4. N. L. Letvin *et al.*, *Lancet* **1983-II**, 599 (1983).
5. Hematologic data for Mc 184-80 were as follows: hemoglobin, 7.4 g/dl with microcytic hypochromic indices; a leukocyte count of $1.5 \times 10^3/mm^3$ with a differential count of 46 percent neutrophils, 6 percent band forms, 46 percent lymphocytes, and 2 percent monocytes. Hypoproteinemia was detected with total protein 3.3 g/dl, albumin 1.6 g/dl, and globulin 1.7 g/dl. The data for Mc 398-80 were: hemoglobin, 9.6 g/dl with microcytic hypochromic indices; a leukocyte count of $4.8 \times 10^3/mm^3$ with a differential count of 21 percent neutrophils, 5 percent band forms, 67 percent lymphocytes, 3 percent eosinophils, 2 percent monocytes, and 2 percent blast forms. Liver function tests were normal.
6. N. W. King, R. D. Hunt, N. L. Letvin, *Am. J. Pathol.* **113**, 382 (1983).
7. C. Barker, J. Wills, E. Hunter, in preparation.
8. I.-M. Chiu, P. R. Andersen, S. A. Aaronson, S. R. Tronick, *J. Virol.* **47**, 434 (1983).
9. D. L. Fine *et al.*, *J. Natl. Cancer Inst.* **54**, 651 (1975).
10. R. Desrosiers, in preparation.
11. E. Hunter, personal communication.
12. I.-M. Chiu and S. Aaronson, personal communication.
13. B. Hirt, *J. Mol. Biol.* **26**, 365 (1967).
14. E. M. Southern, *ibid.* **98**, 503 (1975).
15. R. C. Desrosiers, *J. Virol.* **39**, 497 (1981).
16. We thank E. Hunter and S. Aaronson for providing materials and information prior to publication and for helpful discussion, E. Gelmann for interest in this work, and A. Bakker, B. Price, W. Aldrich, and J. MacKey for technical assistance. This work was supported by grants RR00168 from NIH Division of Research Resources and R01-A1 20729 from USPHS.
* To whom correspondence should be addressed.

1 November 1983; accepted 20 December 1983

Epinephrine Enables Pavlovian Fear Conditioning Under Anesthesia

Abstract. *Rats underwent Pavlovian defensive conditioning (noise paired with shock) while under general anesthesia. Peripheral administration of epinephrine (0.01 to 1.0 milligram per kilogram of body weight) during training resulted in the acquisition of conditioned fear, as shown 10 days later by conditioned suppression of water drinking. Analysis of heart rate and measurement of reflexes during training indicated that epinephrine did not lighten the state of anesthesia. These results indicate that epinephrine enables the learning of conditioned fear in the anesthetized brain.*

Sensory systems process stimuli during states of deep anesthesia. Most knowledge of sensory neurophysiology is based on recordings obtained from anesthetized animals. Yet the results of such sensory processing apparently are not remembered, or at least not retained in a form that is later expressed in behavior. For example, anecdotal reports and clinical studies of learning in humans under general anesthesia have failed to provide unequivocal evidence for learning. Apparent learning occurs only if the (usually auditory) stimuli have "high emotional content, coinciding with lightening of anesthesia" (*1*). Animal studies have yielded similar findings (*2*). Learning and memory can be facilitated by pharmacological agents and hormones (*3*). We asked whether a facilitatory treatment would enable learning to take place and be detectable behaviorally later, during a state of general anesthesia. We now report that epinephrine administered to deeply anesthetized rats enables Pavlovian fear conditioning. The effect is not due to a lessening of the depth of anesthesia by epinephrine.

Male Sprague-Dawley rats ($N = 44$) 80 to 120 days of age at the time of testing were maintained in individual cages in a vivarium with a 12-hour light cycle. Some subjects had not been anesthetized previously, in which case the electroencephalogram (EEG) was recorded from needle electrodes (Grass) inserted bilaterally in the scalp after animals were anesthetized. Other subjects had screw electrodes implanted bilaterally over the cerebral cortex (7 mm posterior and 2 mm lateral to bregma) under sodium pentobarbital anesthesia (48 mg per kilogram of body weight, injected intraperitoneally) from 14 to 21 days before training (*4*).

On the day of training, animals were anesthetized with sodium pentobarbital (48 mg/kg, intraperitoneal) supplemented with chloral hydrate (30 to 60 mg/kg, intraperitoneal) (*5*). The animal was placed on a heating pad within an acoustic chamber and held lightly by the mouth plate and snout bar of a rat stereotaxic apparatus. An earphone was placed within 1 cm of the right ear. The EEG leads were connected and bilateral needle electrodes were inserted subcutaneously into the thorax to record the electrocardiogram (EKG). Needles were inserted into the right posterior flank and attached to a constant current stimulator by way of a stimulus isolation unit (*6*). Reflexes were then tested; if present to any degree, the animal received a supplemental injection of chloral hydrate and was retested within 5 minutes. Training was not initiated until all reflexes were absent (*7*).

The conditioned stimulus (CS) was 15 seconds of white noise (90 dB); the unconditioned stimulus (US) was a 50-msec train of 50-Hz, 5.0-msec pulses (4 to 6 mA) delivered to the right hindlimb at the offset of the CS on paired trials (*8*). Responsiveness to the US was tested three times (average intertrial interval, 1.3 minutes) 4 minutes before and again 4 minutes after a subcutaneous injection of either saline or epinephrine (*9*). Ten paired trials of the CS and US were presented, with an average intertrial interval of 1 minute (range, 30 to 90 seconds on an irregular schedule). After trial 10, reflexes were tested, and the animal was allowed to recover from anesthesia in a warm environment.

To determine the effect of training, animals were tested for conditioned suppression in the presence of the white noise 10 days after training. Either 2 or 3 days after recovery from anesthesia, water bottles were removed, and the animals were given the opportunity to drink their entire supply during 5 minutes (at approximately 5:00 p.m.). Animals quickly learned to drink almost continuously while water was available. They lost not more than 15 percent of their weight (*10*). All animals were drinking continuously before the test for conditioned suppression, held on day 10. During testing, the animal's cage was placed near a loudspeaker. During the first minute of water availability, the speaker was off. During minutes 2 through 5, the speaker provided white noise (85 dB within the cage); during minutes 6 and 7, the noise was off. For each minute of testing, the experimenter recorded the cumulative number of seconds of drinking on electronic timers (*11*). The effects of training were assessed by determining a suppression ratio: duration of drinking during minute 2 divided by that during minute 1. Conditioned suppression to a CS after aversive training in waking animals is regarded as evidence of Pavlovian fear conditioning (*12*).