ministered in stimulating or depressing dosage. It was not possible to test the therapeutic efficacy of reserpine after serotonin administration, as the neurological symptoms induced by intra-arterially injected serotonin were of too short duration.

Summary. 1) Serotonin, administered into brain-tissue via an extravascular route, induces edema, possibly due to its musculotropic property. 2) Serotonin was confirmed to have a neurotropic action which appeared to be responsible for the reversible hemiplegic symptoms. 3) The neurotropic effect of serotonin is biphasic, *i.e.* when administered in low dosage via internal-carotid artery, it causes transient spastic paralyses. In high dosage, it causes flaccid paralyses without inducing cerebral edema. 4) Chlorpromazine and reserpine both block the edema-inducing action of serotonin. Reserpine is, however, effective in a much lower dosage. 5) Chlorpromazine potentiates the neurotropic depressant effect of serotonin, but apparently does not alter its stimulating action. 6) Reserpine inhibits the neurotropic effect of serotonin. This holds true for the spastic as well as for the flaccid symptoms. 7) Therapeutic considerations suggest that reserpine may be specifically indicated in prophylactic treatment of apoplexy.

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Japanese B Encephalitis Virus in Tissue Culture.* (23011)

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The virus of Japanese B encephalitis (JBE) grows readily in a variety of tissue culture systems. Kawakita(1) successfully propagated JBE virus serially in Maitland-type cultures of minced chick embryo brain, and in minced whole chick embryo(2). Scherer and Syverton(3) reported 13 serial passages of JBE virus in HeLa cell cultures. Cytopathogenic effect (CPE) was noted only with the first 4 passages although continued virus proliferation was evidenced by mouse pathogenicity of subsequent tissue culture passages. Mason reported growth of 2 strains of JBE virus in HeLa cell cultures with CPE noted only in 13th and 41st passages of one strain and only in 32nd passage of the other. In general, however, consistent and reproducible CPE has not been observed. The present report is concerned with the propagation of JBE virus in several tissue culture systems, primarily in the cell line currently known as Detroit-6 (originally isolated by Berman, Stulberg and Ruddle(4) from human bone marrow), the only type of cells which has shown consistent CPE in our experience.

Materials and methods. Viruses: *a) Japanese B. encephalitis.* Strain M_1311 was received as the seventh mouse brain passage of a virus isolated from a pool of *Culex tritaeniorrhynchus* mosquitoes. These were trapped near Tokyo, Japan in 1951 by members of the 406th Medical General Laboratory (U.S. Army). Strain M_32204 B was received as the third mouse brain passage of a similar isolation made in 1953. Both were kindly supplied by Major Edward L. Buescher, Walter Reed Army Institute for Research. Both strains were passed one time in suckling Swiss albino mice for stock suspensions, used to initiate tissue culture passages. *b) West Nile.* Strain EV 101 was supplied as third mouse brain passage by Dr. Joseph L.

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Melnick. *Detroit-6 cell cultures*: The original supply was kindly furnished by Drs. I. W. McLean and W. A. Rightsel of Parke, Davis and Co., Detroit, Mich. Stock cultures in 3 oz. pharmacy bottles were maintained in 15% pooled human serum in Eagle's synthetic medium(5). When a confluent sheet had formed (usually 5 to 7 days after seeding and one medium change) the cells were scraped off with a rubber policeman into 20 ml of fresh medium. (For some cultures 40% pooled human serum in Hanks' balanced salt solution was used.) A dispersion consisting predominantly of single cells was obtained by passing this suspension repeatedly through a 22 gauge needle attached to 2 ml automatic Cornwall syringe. The suspension was diluted with growth medium to a concentration of 200,000 cells/ml. Tubes receiving 0.5 ml of this suspension were incubated at slight slant at 36-37°C. They were fed the next day with 0.5 ml of fresh growth medium. Solid sheets of cells were present after 3 to 5 days. Media were usually changed 24 hours prior to inoculation. The cultures were washed 2 to 3 times with Hanks' BSS immediately prior to introduction of 0.9 or 1.8 ml of maintenance solution (10% normal horse serum, 10% tryptose phosphate solution, and 80% mixture 199) and an inoculum of 0.1 or 0.2 ml of a virus or control suspension in the same medium. Cultures were observed daily for 8 to 12 days and fluids were changed approximately every 4 days. *Other tissue culture systems*: 1) *Chick embryo fibroblast cultures* were prepared by 2 methods: a) implantation in chicken plasma clot of skin-muscle fragments from 9-day-old chick embryos; b) direct growth on glass of trypsinized cells from minced torsos of 9-day-old chick embryos. 2) HeLa cells were obtained from Microbiological Associates ready for inoculation. 3) Monkey kidney epithelial cells were prepared by the method of Rappaport(6). *Assay of tissue culture fluids for virus content*: The same method was used for all cell lines. Eight to 10 g, weanling Swiss albino mice were inoculated by intracerebral route with 0.03 ml of serial 10-fold dilutions of tissue culture fluids (diluted in same medium as that used for maintenance of the cell line). Specificity of animal deaths was ascertained at various tissue culture passage levels by use of hyperimmune guinea pig serum. *Tissue culture neutralization tests*: Virus (tissue culture fluids) and serum dilutions were prepared in ice bath (2-3°C) so that in the final mixture a volume of 0.1 or 0.2 ml contained approximately 100 or 1000 TCD_{50} of virus, and serum in a final dilution of 1:10 or 1:100. These mixtures were then incubated at 37°C for one hour prior to inoculation of the cultures. Simultaneous virus titrations and serum controls were included. *Preservation of tissue culture passage fluids*: Aliquots of fluid pools from each passage level were shell frozen in glass sealed ampoules in a CO_2-alcohol bath and stored at −70°C in a CO_2 chest.

Results. Evidence of continued virus proliferation was obtained with 10 or more serial passages of both strains ($M_1$311 and $M_3$2204 B) of JBE virus in each of the cell lines studied. Maximal virus titers in the range of $10^{4.0}$ to $10^{7.0}$ mouse LD_{50}/ml were observed in chick embryo fibroblast, HeLa cell and monkey kidney epithelium cultures, but no specific, consistent or reproducible CPE was observed. Such an effect was noted in the first 15 continuous passages of strain $M_3$2204 B in the plasma-clot cultures of chick

TABLE I. Comparison of Serum Neutralization Tests in Mice and Tissue Cultures (Dt-6 Cells).

Serum (1:10 final dilution when mixed with virus dilutions)		JBE virus—strain $M_1$311	
		8th passage mouse brain* LNI‡	12th passage T.C. fluid† LNI‡
Guinea pig	Normal pool	.0	.0
	Immune "	3.2	2.8
Human A	Pre-vacc.	.0	.3
	Post "	1.8	1.8
B§	Pre "	1.6	1.3
	Post "	3.4	2.7

* Titer in 8-10 g mice (intracer.) $= 10^{-7.4}$.

† Representing a total accumulative dilution of 10^{-27} of 8th mouse brain passage. Titer in Dt-6 cells (by CPE) $= 10^{-6.3}$.

‡ LNI = Log Neutralization Index calculated on the basis of 5 mice or 3-4 tube cultures inoculated per virus dilution-serum mixture.

§ Prior to vaccination this individual had acquired antibodies through inapparent infection during Army duty in Japan and Korea.

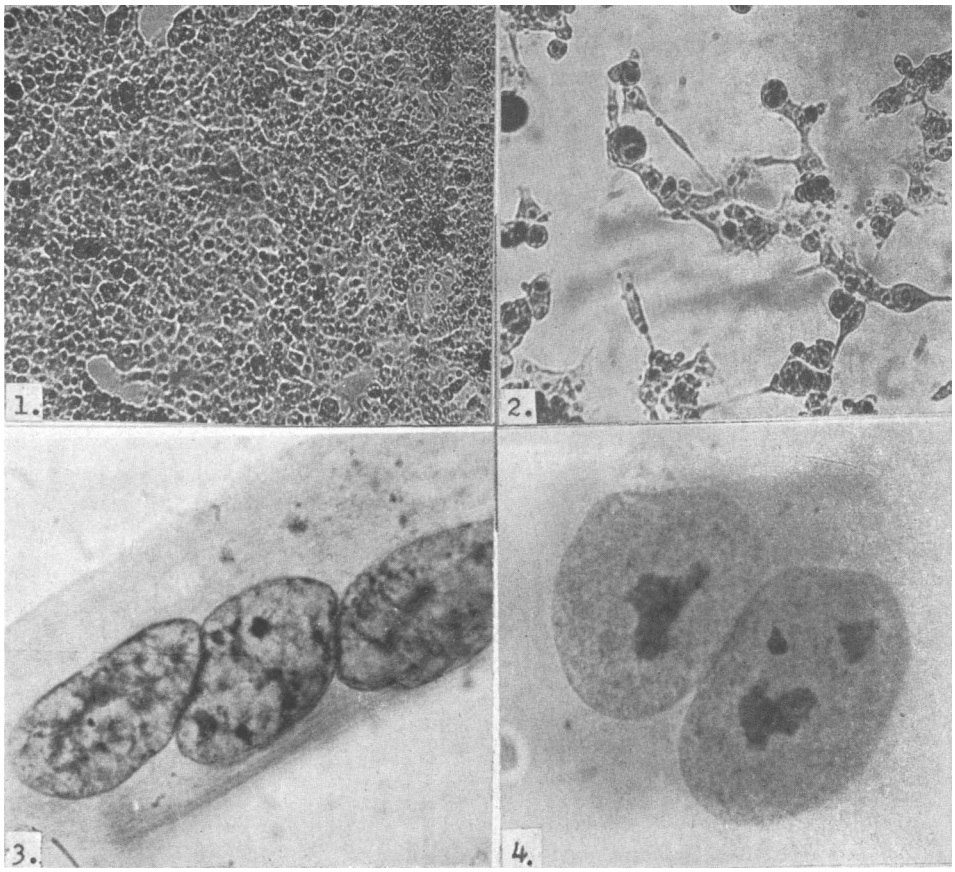

FIG. 1. Dt-6 cell culture showing appearance of uninfected cells 10 days after inoculation of undiluted control fluid pool from previous passage. Unstained preparation, approx. X 80.

FIG. 2. Dt-6 cell culture showing advance CPE 10 days after inoculation of 32,000 TCD_{50} of 9th passage JBE virus, strain M_1311. Onset of CPE was on 8th day. Unstained preparation, approx. X 80.

FIG. 3. Dt-6 cells 6 days after inoculation (10,000 TCD_{50} JBE virus, strain M_1311) showing cytoplasmic contraction, thickening of nuclear membrane, clumping of chromatin and fragmentation of nucleoli. Such changes are most obvious in the larger multinucleated cells which are scattered throughout the cultures. H and E stained coverslip preparation, approx. X 800.

FIG. 4. Dt-6 cells 6 days after inoculation of control fluid from previous passage. Cytoplasmic boundaries are not seen. Chromatin is finely granular. Nucleoli are irregular but intact. H and E stained coverslip preparation, approx. X 800.

embryo fibroblasts. However, it could not be reproduced with the frozen tissue culture passage materials and its disappearance remains unexplained.

A cytopathogenic effect was observed, however, with each passage of both strains in Detroit-6 cells and this effect has been reproduced with frozen materials from representative passages at all levels. Through 16 passages of strain M_1311 the cumulative total dilution of the original mouse brain inoculum was 10^{-43}. The titer of the 16th passage fluid was $10^{-6.0}$/ml in mice and $10^{-5.5}$/ml in tissue culture (based on CPE). In general, comparative titrations in mice and tissue cultures have given essentially equal results. Through 7 passages of strain M_32204 B the virus titers, as determined by CPE or mouse infectivity, have been similar. Neutralization of the CPE produced by JBE (M_1311) virus in Detroit-6 cells has been demonstrated (Table I) by use of hyperimmune guinea pig serum and human immune serum (both post-infection and post-vaccination).

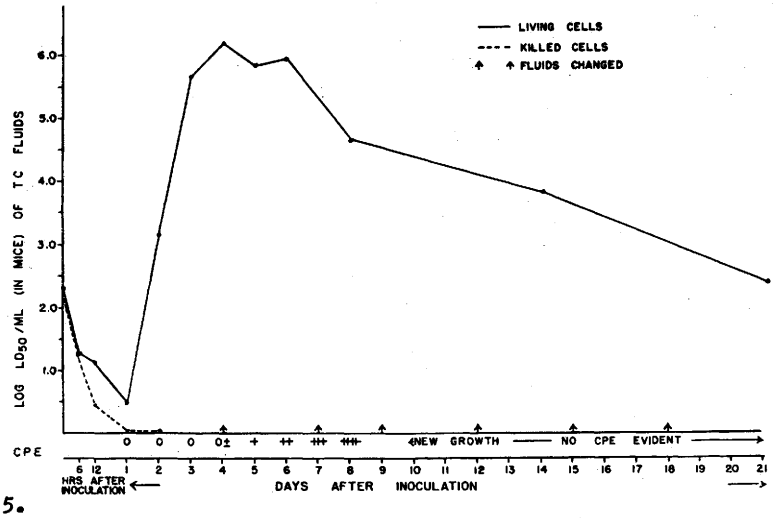

FIG. 5. Growth curve of JBE virus, strain M_1311, 12th Dt-6 tissue culture passage. Titers are expressed as mouse LD_{50}/ml of pooled fluids from 4 tissue culture tubes. Degenerated cell sheet had completely recovered by 14th day. No further CPE was observed even though virus proliferation continued.

Cytopathogenicity usually becomes apparent on 5th day after inoculation, but may appear as early as the fourth day or as late as the eighth day, depending to some extent on dilution of the inoculum. The earliest evidence of CPE consists of small focal areas of heaped-up, rounded cells. Shortly thereafter the cellular sheet in these areas begins to retract and scattered, large, swollen cells may be noted. Within several hours retraction has increased so that the sheets exhibit a "Swiss cheese" appearance. During the next 24 to 48 hours large numbers of cells become detached. Degeneration proceeds rapidly but is never 100% complete. Fig. 1 and 2 illustrate respectively a control culture and an infected culture in an advanced state of degeneration.

Hematoxylin and eosin stains of JBE infected Detroit-6 cells show changes confined primarily to the nuclei though cytoplasmic contraction is apparent. Nuclear changes consist of washing out of the fine chromatin material, clumping and thickening of chromatin toward periphery of the nucleus, thickening of the nuclear membrane, fragmentation of the nucleoli, and the appearance of variable sized eosinophilic inclusions. There is rather marked variation in the size of cells and nuclei within a given culture and these changes are most striking in the larger cells which are often multinucleated. Fig. 3 shows such nuclei demonstrating these changes as compared, in Fig. 4, with the appearance of nuclei of similar size in an uninfected control culture of the same age.

Limited growth studies (Fig. 5), determined by measurements of extracellular virus, indicate that there is an initial drop in titer during the first 24 hours. For the first 6 hours this seems to parallel the drop in titer observed in virus inoculated cultures of cells killed by repeated freezing and thawing. Virus proliferation (or release) is maximal on the fourth day, one day prior to the first definite evidence of CPE. A relative plateau is maintained for 2 days after which the titer falls gradually. Cultures which have exhibited maximal (but not 100%) degeneration show continued cellular proliferation if the fluids are changed frequently. One such group of cultures, kept for 21 days (with fluids changed 6 times), yielded fluid with a virus titer of $10^{2.3}$ mouse LD50/ml, an amount equivalent to the original inoculum.

Limited attempts to utilize the fluids from various tissue culture systems as complement-fixing and hemagglutinating antigens have been essentially unsuccessful.

West Nile (WN) virus (Egypt 101)

through 6 serial passages in Detroit-6 cells gave a picture identical to that observed with JBE virus. In general, the onset of CPE was noted one to 2 days later than with JBE virus, but degeneration progressed in the same manner.

Discussion. Although these studies confirm previous observations(1-5) that JBE virus grows readily in a variety of tissue culture cell lines, CPE has previously been a rare indicator of virus production and its appearance has been irregular. Detroit-6 cells appear to be unique among cell systems used thus far, in that CPE has served as a consistent indicator of virus proliferation and infectivity. The fact that degeneration has never been total and cellular and viral proliferation will continue for prolonged periods under proper conditions suggests the possibilities that: 1) some cells undergo a mutation or change rendering them unsusceptible to CPE while leaving them susceptible to infection; or 2) there are actually 2 (or more) different cell types in the cultures capable of supporting virus multiplication, but only one subject to degeneration in the process. In fact, careful microscopic examination of our Detroit-6 cell cultures has revealed the presence of what appear to be several cell types. The cells which degenerate, however, would appear to be more efficient virus producers since virus titers are highest prior to their gross degeneration (see growth curve). Fluids harvested on 3rd to 6th day (0-2+ degeneration) have consistently yielded higher titers than those tested at later times even when renewed growth has produced an almost complete sheet of cells.

The failure to obtain higher virus infectivity titers in Detroit-6 cells as well as in other cell systems used, is probably due to the marked thermolability of JBE virus. Tissue culture fluids containing over 5 logs of virus/ml (as measured by mouse infectivity immediately after harvesting) had dropped to less than 1 log/ml after 12 hours incubation at 37°C, and no detectable mouse or tissue culture infectivity after 24 hours. Since the cultures are maintained at 36-37°C, there would appear to be a high ratio of inactive to active virus in the fluids at any time beyond the first few hours after beginning of release of virus from the cells. It has been observed repeatedly that CPE becomes apparent from one to 2 days earlier in cultures receiving 100 to 1000 TCD_{50} than in cultures inoculated with larger doses. That this delay is probably due to interference was demonstrated by inoculating cultures with large amounts of inactive virus (kept at 37° for 24 hours) 6 hours prior to the addition of active virus. The CPE in these tubes appeared from 2 to 3 days later than in controls inoculated with the active virus only.

The fact that West Nile virus, another member of the serologically related B group of the arthropod-borne viruses, produces a CPE in Detroit-6 cells similar to that produced by JBE virus suggests the possibility that this cell line might be susceptible to other members of the same group.

Summary. Two strains of Japanese B encephalitis virus have been serially propagated in tissue cultures of chick embryo fibroblasts (both plasma-clot explants and trypsinized cells), monkey kidney epithelium, HeLa cells, and Detroit-6 cells. Cytopathogenicity, which was consistent and reproducible, was observed only with the Detroit-6 cells. Maximal virus growth and release was observed 2 days before CPE became pronounced. This effect was readily neutralized by JBE immune serum. A similar CPE has been observed in Detroit-6 cells infected with the Egypt 101 strain of West Nile virus.

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