7. Dunn, T. B., *in* "The Physiopathology of Cancer" (F. Homburger, ed.), 2nd ed., Chap. 3. Harper, New York (1959).
8. Mühlbock, O. and Boot, L. M., Biochem. Pharmacol. **16**, 627 (1967).
9. Dux, A. and Mühlbock, O., Intern. J. Cancer **1**, 5 (1966).
10. Pullinger, B. D., Brit. J. Cancer **6**, 78 (1952).
11. Prehn, R. T., Main, J. M., and Schneiderman, M., J. Natl. Cancer Inst. **14**, 895 (1954).

Received Sept. 25, 1968. P.S.E.B.M., 1969, Vol. 130.

The Effect of Hyperthermia on Dengue Virus Infection of Mice* (33555)

GERALD A. COLE[1] AND CHARLES L. WISSEMAN, JR.
Department of Microbiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

[1] Trainee in Virology and Rickettsiology under NIH Training Grant 2 T 1 A1 16. Present addreess: Department of epidemiology, School of Hygiene, The Johns Hopkins University, Baltimore, Maryland 21205.

The relationship between the expression of viral virulence and an optimal environmental temperature was demonstrated in cell culture systems (1) and *in ovo* (2). Equally convincing studies showed that the temperature of the intact experimental animal plays an important role in determining the degree of its susceptibility to viral infection. Walker and Boring (3) found that when mice inoculated with a lethal dose of Coxsackie virus were held at 36°, the resulting 2–3° increase in their body temperature prevented virus replication and no deaths occurred. In similar experiments by other workers, passively induced hyperthermia could prevent or delay the lethal effects in mice of several different viruses (4–6).

The present work deals with the effect of hyperthermia on the course of experimental central nervous system (CNS) infections of weanling mice produced by a low (MP-33) and high mouse brain-passaged line (MP-125) of type 1 dengue virus of the Hawaiian strain. Both of these lines were uniformly lethal for weanling mice after intracerebral (i.c.) inoculation but differences in their neurovirulence could be shown in adult animals which, although highly susceptible to MP-125, were relatively resistant to MP-33. Since virulence of other viruses has been correlated with an ability to replicate at elevated temperatures (4), it was of interest to determine for each of the two dengue lines: (i) the possible relationship between temperature sensitivity *in vivo* and the differences in their virulence; and (ii) whether any demonstrable protection by elevated temperature was due to its direct effect on virus multiplication in the CNS or was mediated by an enhancement of the immune or interferon response of the host.

Materials and Methods. Virus. Both lines of type 1 dengue (Hawaiian strain) were prepared as clarified 20% brain suspensions from infected moribund suckling mice. Low passaged MP-33 was identical to the MD-1 human attenuated vaccine strain developed by Wisseman and co-workers (7) and had initially undergone 18 i.c. passages in young weanling mice followed by 15 additional passages in 3–5 day-old sucklings. Highly neurovirulent MP-125 had a history of 125 serial brain passages. Initially adapted to 10–12-day-old suckling mice, weanlings were employed through passage 92 and 3–4 day-old animals were used for remaining passages.

The mean i.c. LD_{50} in 4-week-old weanling mice of the low and high passaged lines were, respectively, $10^{7.5}$ and $10^{8.40}$/g of brain.

* Carried out under the auspices of the Commission on Rickettsial Diseases of the Armed Forces epidemiological Board and Partially supported by contract DA-193-49-MD-2277 with the U. S. Army Medical Research and Development Command.

Induction of hyperthermia. Animals were housed in metal cages equipped with a continuous food and water supply and placed in one of two large self-regulating forced draft incubators maintained at 35°. The desired hyperthermic state (39–40°), determined rectally with a thermister probe, was achieved in animals within 3 hr after incubation.

Virus titrations. All titrations were performed in 4-week-old Swiss white mice, I.C.R. strain, obtained from a single commercial breeder. Serial tenfold dilutions of brain suspensions were inoculated i.c. into groups of 6–8 animals and LD_{50} values calculated by the method of Karber (8).

Antibody assays. Intracerebral neutralizing (NT) antibody assays were carried out in weanling mice using equal volumes of undiluted serum fortified with accessory factor and tenfold dilutions of virus (9). Neutralization indices represented the differences in LD_{50} between normal and test serum-virus mixtures. Hemagglutination-inhibiting (HI) antibody was measured using the basic method of Clark and Casals (10) modified for microtitration plates (11).

Interferon assay. Twenty percent brain homogenates were acidified to pH 2.0 with 5 *N* hydrochloric acid, stored at 4° for 72 hr, neutralized with 5 *N* sodium hydroxide and clarified by centrifugation. Interferon levels in twofold dilutions of test samples were measured by vesicular stomatitis virus plaque reduction in primary mouse embryo cell cultures (12). Representative samples showing antiviral activity were further tested for the basic properties of interferon (13).

Results. Effect of hyperthermia on LD_{50}. To explore the relationship between mouse neurovirulence and resistance to elevated temperature, both virus lines were titrated in mice held at 35° and in similar control animals housed at 22°. The mean body temperatures of these animals were 39 and 37°, respectively. As shown in Table I, MP-33 was markedly temperature sensitive showing a reduction in titer of greater than 2.5 logs in hyperthermic animals. By comparison, MP-125 was much less affected. Although the onset of disease produced by MP-125 was delayed by 1–2 days in the incubated animals, their mortality patterns were essentially identical to the controls.

TABLE I. Effect of Hyperthermia on Virus Titers of Two Lines of Type L Dengue Virus.

Virus line	Expt. no.	Titer[a] after 21 days at: 22°	35°	Reduction
MP-33	1	7.0	≦4.0	≧3.0
	2	7.5	4.9	2.6
MP-125	1	8.5	7.6	0.9
	2	8.6	8.0	0.6

[a] Log_{10} LD_{50}/g of brain.

Effect of restoration to normal temperature. Hyperthermic mice, surviving the initial titrations of MP-33 and MP-125, were removed from the incubator after 21 days, placed at 22° with survivors of the control titrations and observed for an additional 14 days. During this period many of the previously asymptomatic hyperthermic animals which had been infected with MP-33 developed signs of neurologic disease and died in patterns very similar to those which had occurred earlier in the controls housed at 22°. This temperature-mediated delay in mortality is illustrated in Fig. 1 for animals infected with 100 LD_{50} of MP-33. The LD_{50},

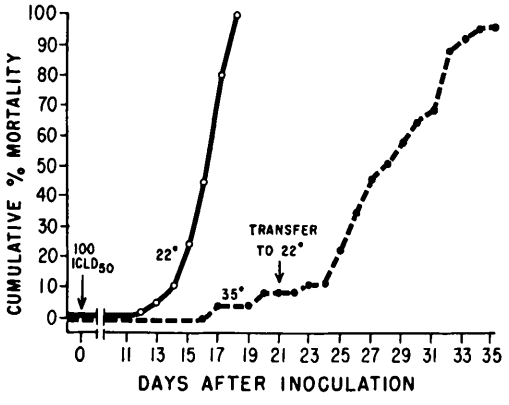

FIG. 1. Cumulative mortality following intracerebral inoculation of normal (22°) and hyperthermic (35°) weanling mice with 10^2 LD_{50} of the MP-33 line of type 1 dengue virus. On day 21, hyperthermic animals were transferred to 22°.

TABLE II. Virus Titers of MP-33 and MP-125 after Restoration of Normal Body Temperature.

Virus line	Virus titers[a] After 21 days 35°	14 Days after transfer to 22°	Increase
MP-33	≦4.0	6.7	≧2.7
	4.9	7.0	2.1
MP-125	7.6	7.7	0.1
	8.0	8.3	0.3

[a] Log_{10}/g of brain.

when recalculated on postinoculation (PI) day 35, had increased by 2 or more logs (Table II) and closely approached that usually obtained in normal animals (Table I). In the analogous MP-125 infected mice, only an insignificantly few virus-specific deaths occurred during this same period. When, on PI day 30, animals surviving titrations of either virus line were challenged with 100 weanling mice LD_{50} of MP-125, all died within a predictable time, suggesting that none had experienced an inapparent immunizing infection regardless of the temperature(s) at which they originally had been housed.

Effect of prolonged hyperthermia. The MP-33 infected mice were maintained at 35° for periods up to 35 days. Under these conditions, up to 50% survived i.c. infection with 1000 LD_{50} of virus although many developed nonfatal neurological disease. No additional deaths occurred when these animals were returned to room temperature and after 14 days they were solidly immune to i.c. back-challenge with 100 LD_{50} of MP-125.

Comparative virus, interferon, and antibody levels. Quantitative studies were performed on the relative abilities of MP-33 and MP-125 to multiply and to induce interferon and antibody production in hyperthermic and normal mice. Groups of 80 mice were inoculated i.c. with 10,000 suckling mouse LD_{50} of each virus. Half of the mice in each group were held at 35° and half at 22°. At selected intervals during the course of infection with each virus, 5–6 animals in each temperature group were sacrificed. Serum pools were tested for HI and NT antibodies while brain homogenates were titered for virus and interferon.

As shown in Fig. 2, temperature-sensitive MP-33 multiplied in brains of hyperthermic mice at a reduced rate in comparison to the control animals whereas MP-125 was much less affected. Interferon levels in the brains of mice infected with both viruses were, irrespective of temperature, directly related to the degree of viral replication and usually were not detectable until virus titers reached about $10^{5.0}$/g of brain. Thus, in hyperthermic animals infected with MP-33, interferon production was minimal and delayed by about a week as compared with normal animals. In MP-125 infected hyperthermic mice, interferon production was neither delayed nor significantly reduced.

In MP-33 infected hyperthermic mice an immune response, present on PI day 24, coincided with a fall in virus titer and was detected about a week later than in normal animals

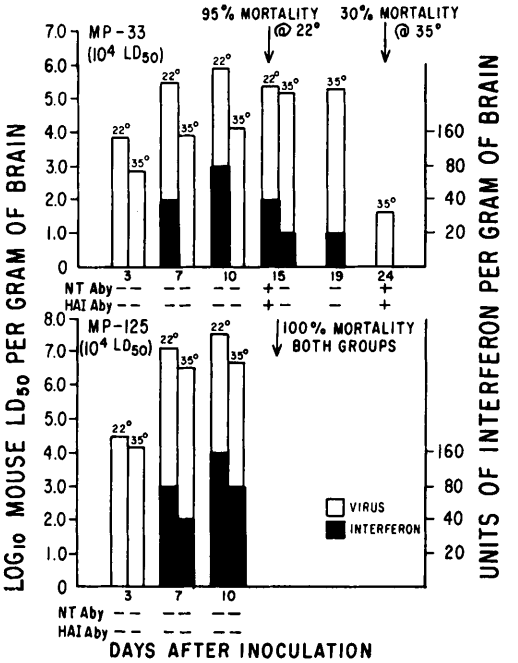

FIG. 2. Brain virus, interferon, and serum neutralizing (NT) and hemagglutination inhibiting (HI) antibodies in normal (22°) and hyperthermic (35°) weanling mice infected with 10^4 LD_{50} of the MP-33 and MP-125 lines of type 1 dengue virus.

in which circulating antibodies were usually present by the end of the second week of infection. The differences in percentage mortality between normal and hyperthermic animals infected with MP-33 is also indicated in Fig. 2. By PI day 15, 95% of the normal animals were dead whereas at 24 days only 30% of those with hyperthermia had died. In contrast, MP-125 killed all animals within 11–13 days regardless of temperature and before circulating antibodies became detectable.

Discussion. These data support previous observations that elevated body temperature can protect mice against a lethal i.c. virus challenge (4–6, 17). However, the protection afforded to mice against dengue CNS infection by passively induced hyperthermia is dependent on the brain passage history of the virus. Thus, temperature sensitivity of different lines of dengue virus may be related to their degree of neuroadaptation. In contrast to the more or less absolute resistance conferred on cell cultures by incubating them at elevated temperature after infection with temperature-sensitive strains of other animal viruses (14, 15), the hyperthermic state imposed for 21 days on MP-33 infected animals merely reduced the rate of virus multiplication in the brain (Fig. 2). The appearance of overt disease followed by mortality after restoration of normal temperature (Fig. 1) and the attainment of LD_{50} values similar to those observed in normal animals (Tables I and II) also support this conclusion.

It has been suggested that some viruses produce more interferon when cultivated at temperatures above the optimum (16). Since the quantitative relationships between virus and interferon production seen in normal animals also held true for those with hyperthermia (Fig. 2), temperature per se had no measurable effect on interferon induction by either MP-33 or MP-125. Rather, the delay in its appearance and the decreased levels observed particularly during MP-33 infection seemed to be a reflection of the reduced rate of virus multiplication. These observations are similar to those of Kern *et al.* for Sindbis virus (17) and indicate that the inhibiting effect on the replicative cycle of MP-33 was not mediated by interferon.

Our results suggest that the immune response played an important role in the outcome of infection, as evidenced by the decrease in infectious virus soon after the appearance of circulating antibodies, as well as the permanent survival of infected animals whose normal body temperature was reestablished after prolonged periods of hyperthermia. In both situations, hyperthermia probably created a host-favored relationship between the rate and extent of viral replication and the development of the immune response, resulting in delayed disease or abortive infection. The concept that the efficacy of the immune response may play a major role in the outcome of primary viral infections of the CNS is supported by other observations. The disappearance of virus from brains of adult mice during inapparent CNS infection with incompletely neuroadapted type 1 dengue virus is correlated with the appearance of the immune response (18). Such inapparent infections can be converted to uniformly lethal infections in animals which are immunosuppressed (19).

Summary. Passively induced hyperthermia significantly delayed or prevented lethal CNS infections of mice produced by a line of type 1 dengue virus with a history of 33 serial mouse brain passages (MP-33) but not one with a history of 125 passages (MP-125). This effect was not due to any enhancement of interferon production in the brain but was the result of a reduced rate of viral multiplication. Permanent recovery from MP-33 infection in hyperthermic animals was correlated with the appearance of the immune response.

1. Baron, S., Advan. Virus Res. **10**, 39 (1963).

2. Bedson, H. S. and Dumbell, K. R., J. Hyg. Comb. **59**, 457 (1961).

3. Walker, D. L. and Boring, W. D., J. Immunol. **80**, 39 (1957).

4. Lwoff, A., Bacteriol. Rev. **23**, 109 (1959).

5. Baron, S. and Buckler, C. E., J. Immunol. **93**, 45 (1964).

6. Schmidt, J. R. and Rasmussen, A. F. Jr., J. Infect. Diseases **107**, 356 (1960).

7. Wisseman, C. L. Jr., Sweet, B. H., Rosenzweig,

E. C., and Eylar, O. R., Am. J. Trop. Med. Hyg. **12**, 620 (1963).

8. Karber, G., Arch. Exptl. Pathol. Pharmakol. **162**, 480 (1931).

9. Wisseman, C. L. Jr., Kitaoka, M. and Tamiya, T., Am. J. Trop. Med. Hyg. **15**, 588 (1966).

10. Clarke, D. H. and Casals, J., Am. J. Trop. Med. Hyg. **7**, 561 (1958).

11. Sever, J. L., J. Immunol. **88**, 320 (1962).

12. Baron, S. and Buckler, C. E., Science **141**, 1061 (1963).

13. Isaacs, A., Lindenmann, J., and Valentine, R. C., Proc. Roy. Soc. (London), Ser. B. **147**, 268 (1957).

14. Lwoff, A., Cold Spring Harbor Symp. Quant. Biol. **27**, 159 (1962).

15. Burge, B. W. and Pfefferkorn, E. R., Virology **24**, 126 (1964).

16. Ruiz-Gomez, J. and Isaacs, A., Virology **19**, 8 (1963).

17. Kern, A., Schieffer, A., and Tinland, R., Nature **215**, 86 (1967).

18. Cole, G. A., Ph.D. thesis submitted to University of Maryland Graduate School, 1966.

19. Cole, G. A. and Wisseman, C. L. Jr., Federation Proc. **27**, 561 (1968).

Received July 10, 1968. P.S.E.B.M., 1969, Vol. 130.

Changes in Surface Antigens of SV40-Virus Transformed Cells (33556)

H. Thomas Robertson and Paul H. Black
(Introduced by P. S. Russell)

Harvard Medical School, Department of Medicine, Massachusetts General Hospital, Boston 02114

The changes that the surface components of mammalian cells undergo in the process of virus transformation are of particular interest because of their possible role in the loss of contact inhibition that these cells exhibit. Of the antigenic changes that have been detected, both virus specific and nonspecific changes were noted. The virus specific surface antigenic changes have been best characterized by transplantation resistance studies in syngeneic hosts (1, 2). More recently, fluorescent antibody (3-5) and colony inhibition techniques (6) with unfixed cells were used to demonstrate virus specific surface antigens that appear to be identical to those antigens detected by transplantation resistance. The antibody utilized in these tests to demonstrate virus specific changes was obtained from animals exposed to virus and/or homologous or isologous transformed cells. The nonspecific antigenic changes in virus transformed cells, however, were best delineated by use of heterologous antisera. Utilizing heterologous antisera formed against extracts of virus transformed cells, Fogel and Sachs (7) and O'Neill (8) failed to demonstrate the virus specific surface antigen, but did report the presence of an antigen having the properties of the Forssman antigen in polyoma virus transformed cells.

The present studies were undertaken to study the antigenic changes on the surfaces of virus transformed cells. Utilizing heterologous antisera prepared in rabbits and immunofluorescent techniques, a new antigenic reactivity was detected on the surfaces of SV40 and polyoma transformed hamster cells which was characterized as that of the Forssman antigen. Adenovirus transformed hamster cells, however, did not contain such Forssman reactivity.

Materials and Methods. Cell lines. The cell lines utilized in the present studies are listed in Table I. The passage history of the BHK-21 and 3T3 cell lines and the details of the transformation studies carried out with SV40 DNA (BHK) (10) and SV40 virus (3T3) (14) resulting in the A-8 and 3T3-SV40 cell lines, respectively, have been described. These studies were carried out with clonal populations (the BHK-21 C/13 and 3T3 M clones) derived from these cell