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The Effect of Host Age, Virus Dose and Route of Inoculation on Inapparent Infection in Mice with Japanese Encephalitis Virus.* (31418)

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The pathogenesis of Japanese encephalitis (JE) and other arbovirus encephalitides is poorly understood. The relationships between pathogenicity and infectivity of the encephalitic arboviruses are not clear; and it is unknown, for example, why man is rarely diseased though often infected by JE virus (1,2). The following factors may be important considerations: 1) the route of inoculation of virions by vector mosquitoes, *i.e.*, into the lumina of blood vessels for immediate transport to the central nervous system or into subcutaneous tissues where virus proliferates before entering the circulatory system; 2) the quantities of infectious virus inoculated by different mosquitoes; 3) variations in host resistance mechanisms, *e.g.*, related to age, that might affect interferon or antibody production; and 4) differences in virulence among JE virus strains in nature.

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Previous studies have described how the route of inoculation and the dose of virus may be related to the *lethal effects* of JE and other arboviruses(3-6), but the relationship of *infectivity* to pathogenicity has not been defined. This study was undertaken to evaluate the possible effects of route of inoculation and dose of virus on the ability of JE virus to produce disease as opposed to inapparent infection. A single strain of JE virus in low suckling mouse passage was used to avoid variation among strains and to minimize the possible effects of passage on pathogenicity and infectivity of naturally occurring virus. Mice were chosen as hosts because of their known susceptibility to JE virus and because of the ease of making observations upon large numbers of animals.

Materials and methods. Virus. The JE virus strain M5/596 used for infection as well as antibody tests had been recovered from a suspension of mosquitoes collected in Japan in 1956(7) and passaged 6-8 times intracranially in suckling mice. Virus suspensions were kept in sealed glass ampoules on dry ice as 10% suckling mouse brain suspensions in Hanks' balanced salt solution (H) containing 40% rabbit serum, and penicillin, 100 units,

and streptomycin, 100 μg, per ml. Dilutions of virus were made in 10% normal rabbit serum in H and kept in an ice bath until inoculations were completed.

Mice. Swiss albino mice were obtained from Taconic Farms, Germantown, N. Y., or from a colony of these mice maintained in this laboratory. Suckling mice were used at 1-4 days of age, newly weaned mice at 4 weeks, and adult mice, approximately 6-to-7 months.

Inoculation of mice. Intracranial, subcutaneous, intraperitoneal, and *intradermal* inoculations were done without anesthesia. *Duodenal* inoculation was accomplished during laparotomy upon mice anesthetized by intraperitoneal injection (0.01 ml/g of body weight) of a mixture containing glycerin 20 cc, absolute ethyl alcohol 10 cc, distilled water 70 cc and veterinary nembutal 10 cc containing 60 mg/cc. After iodine preparation of the shaved skin, the abdominal wall was incised, and the duodenum brought anteriorly and held gently with forceps for inoculation with a no. 26 needle through the wall into the lumen. The needle was changed between the inoculation of each mouse in an attempt to avoid contamination of the peritoneum with virus. Mice were lightly etherized for *nasopharyngeal* administration of virus. *Intravenous* injection was performed through a tail vein. All mice were allowed free access to food and water; those which were to undergo laparotomy were deprived of food the preceding night.

Tests for antibody. Mice were bled from the retro-ocular venous plexus into Pasteur pipettes 21 to 50 days after inoculation. Hemagglutination-inhibition (HI) tests were performed either on heparinized plasma or the supernatant fluid of blood diluted immediately in saline and reconstituted after acetone precipitation to yield approximately a 1:5 dilution of plasma, based on the usual hematocrits of 50% observed in mice (see *Results*). The initial concentration of heparin in blood was estimated to be 10-100 mg %. The procedures for the HI, complement fixation, and neutralization (N) tests have been described (8). The 3 concentrations of virus used in mouse N tests were 5, 50, and 500 weanling mouse intracranial LD_{50} doses.

Results. Pathogenicity vs *infectivity of JE virus.* To relate the pathogenicity to the infectivity of JE virus, adult mice 6 months of age were inoculated concurrently by various routes, and mortality and hemagglutination-inhibiting (HI) antibody responses were measured. As anticipated, when virions were put directly into the brains of mice, mortality corresponded to infectivity (Fig. 1). However, after intraperitoneal (IP), intravenous (IV), subcutaneous (SC) or intradermal (ID) inoculation of virions, deaths occurred most often with large doses of virus; and there was a great discrepancy between the dilutions of virus which produced death and those which caused non-lethal infection. Infectivity end-points achieved after peripheral injection were 10^{-1}, although curiously a few mice died after receiving 10^{-5} or 10^{-6} dilutions of virus IV or ID (Fig. 1).

Since these peripheral routes of inoculation sometimes caused death, although irregularly, other portals of viral entry were sought that would consistently permit survival with infection and antibody production. Inoculation of virus either onto the pharynx or into the duodenum (during laparotomy) allowed survival with antibody production, whereas intranasal inoculation caused death (Fig. 2). It must be recognized that duodenal infection could have occurred not only from the lumen of the duodenum but also by intramural placement of virus in the needle puncture wound. However, since IP injection produced some mortality, it is unlikely that duodenal inoculation through the intestinal wall by needle merely resulted in IP infection, unless anesthesia (used for duodenal but not for IP inoculations) protected mice against lethal disease. Certainly pharyngeal inoculation appeared innocuous in contrast to nasal injection where virions probably reached the brain by way of the olfactory nerves through the cribriform plate.

Influence of age. The age of mice was a factor in resisting the lethal effects of JE virus. In intracranial (IC) titrations performed simultaneously, the negative $\log_{10}$ LD_{50}/0.03 ml were 10.0 for mice at 4 days, 9.4 at 4 weeks, 8.8 at 2 months, and 8.4 at

6 months of age. This finding documents previous observations that younger mice are more susceptible than older mice to the lethal effects of JE virus given IC(3,4).

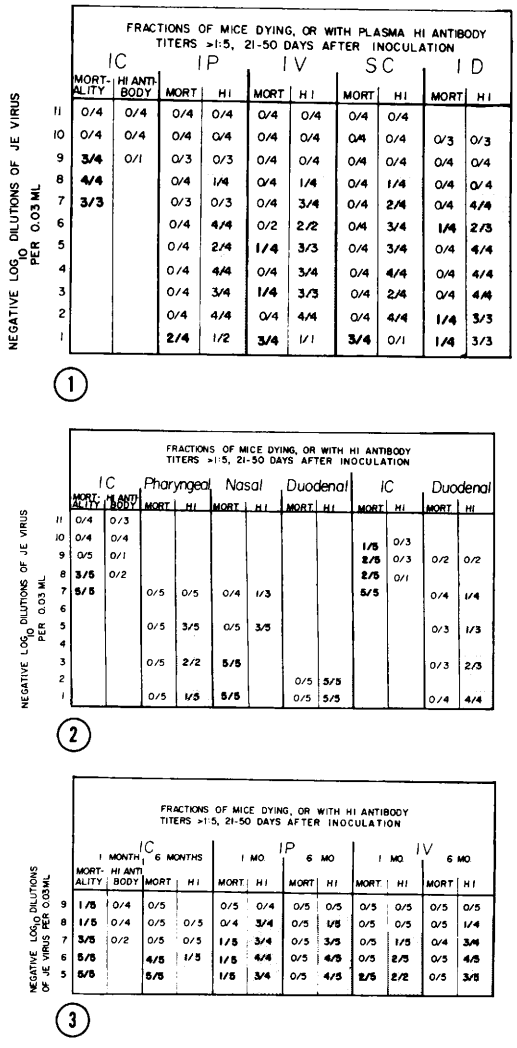

FIG. 1. Relationship between mortality and infectivity following inoculation of Japanese encephalitis virus by intracranial (IC), intraperitoneal (IP), intravenous (IV), subcutaneous (SC), and intradermal (ID) routes. Fractions shown in bold type indicate mortality, and stippled areas infectivity.

FIG. 2. Relationship between intracranial (IC) inoculation to pharyngeal, nasal, and duodenal injections. Bold face indicates mortality, and stippled areas infectivity.

FIG. 3. Comparison of young (1-month-old) and old (6-month-old) mice following inoculations of selected dilutions of Japanese encephalitis virus by intracranial (IC), intraperitoneal (IP), and intravenous (IV) routes. Bold face indicates mortality, and stippled areas infectivity.

In an experiment where one-month-old mice and 6-month-old mice were inoculated IC, IP, and IV with graded dilutions of virus, greater mortality was always observed in the younger group (Fig. 3). Deaths occurred sporadically after IP or IV inoculation only in the younger mice, but antibody was produced up to dilutions of 10^{-7} or 10^{-8} in both age groups.

Magnitude and nature of antibody responses. In the experiments described above, the easily performed and inexpensive hemagglutination-inhibition test was used to detect antibody. However, to validate its use, it was necessary to determine whether this test would detect all inapparent infections of mice or whether neutralizing and/or complement fixation tests also had to be employed. Because sufficient blood could not be obtained by venipuncture, and serial cardiac puncture was often fatal, mice were bled from the retro-orbital venous plexus. However, even this method did not always provide sufficient plasma; therefore, blood had to be diluted in saline, centrifuged to remove erythrocytes, and the diluted plasma used in HI tests. To test the validity of this procedure, 20 mice were bled 23 or 24 days after inoculation, and supernatant fluid from blood (diluted 1:5 in normal saline) was obtained for HI antibody test: the same mice were sacrificed and bled by cardiac puncture to obtain heparinized plasma 36 or 37 days after inoculation. Both specimens yielded HI antibody titers of $<1:5$ for 11 mice and $\geqq 1:5$ for one mouse; however, the supernatant of diluted blood was $<1:5$ and the plasma $\geqq 1:5$ for 8 mice. Therefore, an HI antibody titer $<1:5$ on diluted supernatant fluid could not be interpreted as negative or positive, but a titer $\geqq 1:5$ could be considered positive since in this experiment there were no falsely positive results on diluted supernatant fluid.

With HI titers $\geqq 1:5$ on diluted blood or plasma considered as positive and $<1:5$ on plasmas as negative, the results of HI tests for JE virus antibody correlated well with the results of neutralizing (N) antibody tests done in mice (Table I). There were no positive-HI, negative-N antibody test results, and only 3 plasmas were negative by HI and positive for N antibody, indicating that the

TABLE I. Correlations of Japanese Encephalitis Viral Neutralizing (N), Hemagglutination-Inhibiting (HI) and Complement-Fixation (CF) Antibody Titers of 1-6-Month-Old Mice, 23-27 Days After Inoculation of Virus.

Numbers of mice with antibody titers indicated as reciprocals of serum dilutions or log neutralization indices (LNI)				
	HI*		CF	
	≧5	<5	≧4	<4
N >1.7	8	3†	7	1‡
1.0-1.6	1	0	1	
<1.0	0	9	3§	5
CF ≧4	27	2‖		
<4	4¶	5		

* HI titers ≧5 indicate results of tests of plasma or supernatant of diluted blood; titers <5 represent tests of plasma.

† LNI = 2.2, 1.8, 2.3; CF titers = 4, <4, anticomplementary.

‡ LNI = 1.8; HI titer = 20.

§ CF titers ≧4, ≧4, 8; HI titers <5, not tested, not tested.

‖ CF titers ≧4, 4; LNI = 1.0, 1.8.

¶ HI titers 20, 5, ≧40, 5; LNI = 1.8, not tested, not tested, not tested.

HI tests failed occasionally to detect antibody 23-37 days after inoculation. The results of the CF tests correlated less well with those of the N-antibody tests than did the results of the HI tests. There were 3 positive-CF, negative-N antibody test results as well as one negative-CF, positive-N antibody result (Table I). The positive-CF, negative-N antibody test results are interpreted as probably false-positive CF tests for antibody. The correlation between HI and CF test results was good except for 2 positive-CF, negative-HI results, (one of which was also positive by N antibody test), and 4 negative-CF, positive-HI antibody test results (one of which was positive by N antibody test). Because (a) the CF test yielded some false positive results, (b) the N antibody test in mice required undiluted plasma, and (c) the HI antibody test could be done on supernatant fluid of diluted blood, the HI test was chosen for detection of antibody even though some false negatives could be expected to occur.

The magnitudes of HI antibody responses in 6-month-old mice were not related to concentration of virus injected or route of inoculation. Very low concentrations of virus could produce titers ≧1:40, and high concentrations of virus sometimes caused titers of only 1:5 (Table II). Since no dose-response relationship was observed, it seemed that a) mice were *infected* by peripheral inoculation of virus instead of responding to a *nonreplicating* antigenic stimulus, and b) the titer of HI antibody following infection was a function of the individual animal and not related to dose of *replicating* antigen administered.

HI antibody was long-lasting in some mice as evidenced by its detection 149 days after inoculation. IP inoculation of dilutions 10^{-2} (2 mice), 10^{-6} (2 mice), or 10^{-8} (1 mouse) of JE virus provoked long-lasting titers ≧1:40. Titers of ≧1:40 were also found after ID inoculation of 10^{-7} dilution of JE virus (1 mouse), IV injection of 10^{-2} (2 mice), and 10^{-3} dilution (1 mouse).

Virus challenge after a single inapparent infection. The relationship between resistance to homologous virus and the presence of HI antibody from a single previous inapparent infection was studied with mice available from the above experiments. It was thought im-

TABLE II. Hemagglutination-Inhibiting (HI) Antibody Responses in Individual 6-Month-Old Mice Inoculated with Graded Doses of JE Virus by Different Routes.

	Numbers of mice with indicated JE viral HI antibody titers in plasma or diluted blood 23-27 days after inoculation by the indicated routes											
$-\log_{10}$ dilution JE virus inoculated/0.03 ml	≧1:40				1:10–1:20				1:5			
	IP	IV	SC	ID	IP	IV	SC	ID	IP	IV	SC	ID
1		1		1			1	1	1			1
2	2	4	1		1		3	2	1			1
3		1			2		1	1	1	2	1	3
4		1	1		3	2	2	1	1		1	3
5			1		1	2	2	3	1	1		
6	1	1		1	1	1			2			1
7		1				1	1	2		1		
8	1	1	2	2			1	1	1			

portant to use a peripheral route of virus challenge to approximate the natural mode of infection by mosquitoes; intracranial challenge is neither a satisfactory nor necessarily valid test for immunity since virus inoculum is placed directly in contact with highly susceptible brain cells not protected by antibody. Challenge experiments were therefore undertaken using the intravenous route since it repeatedly produced the highest mortality rates of all the peripheral routes of inoculation tested (approximately 75%) at 10^{-1} dilution of virus suspension. Mice surviving the first injection of virus were inoculated intravenously with 0.05 ml of the supernatant (clarified by centrifugation 400 $\times$ *g* for 10 minutes) of 10% suckling mouse brain suspension containing 10^8 weanling mouse IC LD_{50}. The results can be summarized as follows: 1) 74% of 13 mice that survived intracranial inoculation and that were without detectable HI antibody died following intravenous virus challenge, 2) all of 9 mice that had received duodenal inoculation of virus and that had developed HI antibody resisted intravenous challenge 46-54 days after the first inoculation and 19-21 days after antibody test, and 3) there was a positive correlation in 13 other mice between protection and the presence of HI antibody. These results suggest that a single previous inapparent infection of mice with JE virus confers immunity to peripheral challenge with homologous virus. Further studies are needed with more animals and with strains of challenge virus more virulent by peripheral inoculation than the one employed here to establish these points conclusively.

Discussion. The experiments presented herein relate inapparent infection as well as disease and death to the routes of inoculation and doses of JE virions in young and old mice. Whereas intracranial inoculation predictably produced death at selected dilutions of virus with no (or a very rare) mouse surviving to develop antibody, injections given intraperitoneally, subcutaneously, intravenously, or intradermally produced deaths very irregularly. Mice died after receiving large doses of virus (10^{-1} or 10^{-2} dilutions), and deaths occurred occasionally with intermediate doses (10^{-5} or 10^{-6} dilutions). In contrast very small inocula (10^{-7} and 10^{-8} dilutions) provoked antibody responses without disease.

The mechanism is obscure by which large doses of virus apparently can invade the central nervous system to produce encephalitis and death, whereas smaller inocula only multiply peripherally to provoke an antibody response. The deaths observed with large peripheral inocula of virus may be due to early penetration of nervous tissue before mechanisms of host resistance can operate adequately. Interferon, for example, has been shown to be induced rapidly (within hours) after intravenous administration of large doses of arboviruses as well as other viruses(9). If interferon were operative in the limitation of infection to an inapparent process, it would have to be continually produced during the critical events determining entry and spread of JE virus in brain. The relatively high mortality seen in these experiments after intravenous injection of 10^{-1} dilution of JE virus, which should have produced large quantities of interferon, may be evidence against the protection afforded by interferon as a primary mechanism; however, interferon was not measured in these studies.

It might be postulated that the reticuloendothelial system is temporarily overwhelmed with large inocula. Tissue macrophages have been thought to play a role in the blockade of the spread of herpes virus to the brain in older mice inoculated peripherally because they would trap, but not release, virus(10). Other somatic cells might also participate during aging in the physiological differentiation that has been thought to occur in macrophages in their response to viral infection. It is of course possible that the mice which died following peripheral inoculation of virus did so because they failed to produce antibody, but this possibility could not be checked in these studies.

Physiological maturity and resistance to viral infection are often correlated. Children in Japan are the ones who usually suffer Japanese encephalitis, but the explanation usually given is that the adult population is protected by antibody as a result of widespread

inapparent infection. However, decreasing susceptibility due to unknown causes but correlated with increased age may also be a factor since this phenomenon has been noted in animal studies with various neurotropic viruses, for example, rabies, vesicular stomatitis, eastern and western encephalitis viruses, as well as JE virus(3-5). In mice the resistance to peripheral infection as observed by the failure to induce a lethal infection with JE virus increases rapidly between 10 and 21 days of age(3,4).

No answer is available for questions as to whether human JE epidemics are caused by viruses of greater virulence than those which cause endemic, inapparent infections or whether disease is solely a function of the individual host and his resistance mechanisms when small virus doses are received either intradermally, subcutaneously, or intravenously from mosquitoes. Certainly in these experiments individual variations in host response seemed to be as important as dosage or route of peripheral inoculation in determining the outcome of infection with JE virus, especially in younger animals.

If the observations made here in mice have any relevancy to human infection with JE virus, the results may be helpful in evaluating strains of JE virus for possible use as live virus vaccines. For example, the strain of JE virus used here, with relatively low peripheral invasiveness and relatively high antigenic potency, could be a candidate for further study. These results also raise the question whether live virus immunization against arboviruses by gastrointestinal administration might not be profitably investigated. It has yet to be shown that JE virus can infect through *intact* duodenal mucosa without producing disease since in the present studies the duodenum was inoculated transmurally during laparotomy. The oral administration of live, unencapsulated virus would be potentially hazardous in man inasmuch as JE virus invaded (presumably *via* olfactory nerves) the brains of mice that had been given virus intranasally. If a capsule containing live virus can be satisfactorily coated so that it will dissolve in the duodenum, but not in the stomach where gastric acid inactivates virus(11), oral immunization against Japanese encephalitis might become feasible.

Summary. The occurrence of inapparent infection or death due to Japanese encephalitis virus has been related to the dose and route of inoculation of virus in young and old mice. The virus strain employed, which had been isolated from naturally infected mosquitoes and used in low passage, predictably produced death on intracranial inoculation at high dilutions with only a rare mouse surviving to develop antibody. In contrast, virus inoculated intraperitoneally, subcutaneously, intravenously, or intradermally produced only occasional deaths and usually at low virus dilutions; yet very small inocula provoked hemagglutination-inhibiting antibody responses which correlated well with neutralizing and complement-fixing antibodies. Intravenous administration of 10^8 weanling mouse IC LD_{50} of virus consistently produced about 75% mortality; intranasal injection of virus killed mice, whereas duodenal inoculation (during laparotomy) or pharyngeal administration did not. A single previous inapparent infection of mice inoculated into the duodenum conferred immunity to intravenous virus challenge 46-54 days later. Six-month-old mice were more resistant than young mice to peripheral as well as intracranial inoculation although infectivity was about the same in both groups. The mechanisms involved in the determination of apparency of infection which can be correlated with host age, virus dose, and route of inoculation have yet to be learned.

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Isolation of Toxohormone from Tumor Tissues of Germfree Mice.* (31419)

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Investigators in cancer research are generally careful about the state of the tumor tissues they use for chemical studies. Adherent normal tissues and necrotic areas in the tumor are carefully trimmed away, and it is tacitly understood that any grossly infected tumors are not to be used at all. On the other hand there is always a possibility that some microbial contamination could escape notice, and so introduce extraneous substances into tumor tissue components. A confusing development became apparent in studies on toxohormone, the tumor cell product which is responsible for the depression of liver catalase in tumor-bearing animals(1), when Kampschmidt(2) claimed that tumor toxohormone comes not from the tumor tissue itself but from contaminating bacteria. The tumor used by Kampschmidt was very heavily infected with *Salmonella typhimurium,* and *Salmonella* endotoxin was found to be highly active in depressing liver catalase activity when injected into normal animals.

In making this sweeping statement Kampschmidt ignored all the relevant experimental data to the contrary(1), and our recent study on the relation of bacterial contamination to tumor toxohormone showed anew that Kampschmidt's allegation is unfounded(3). The experiment presented in this paper should clear away any possible doubt in the matter. We have found that a tumor, produced and maintained in germfree mice, is as productive of toxohormone as other tumors derived from conventional, non-germfree mice.

Material. Germfree tumor tissue was collected from 7 germfree Lobund C3H mice carrying transplants of fibrosarcoma tissue which was originally induced by injections of a sterile solution of 20-methylcholanthrene in olive oil(4). The germfree mice and their tumors were free of bacterial flora, as determined by test procedures described by Wagner(5). Individual tumors weighed 5 to 10 g, wet weight. A pool of 41.4 g of tumor tissue was stored in the freezer until dried at 70°C in the vacuum oven. The desiccated tumor tissue was shipped from Notre Dame to Tokyo for further examination.

Methods. Procedures for extraction of toxohormone and its bioassay were carried out at the National Cancer Center Research Institute. 7.04 g of desiccated tumor was reconstituted in 200 ml of water, and homogenized with a Waring blendor. The homogenate was centrifuged and the supernatant fluid was boiled for 2 hours in a water bath. It was condensed to about 1/10 original volume, and centrifuged again to remove precipitated material. Two volumes of absolute ethanol were then added to the clear supernatant fluid. The precipitate thereby formed was collected by centrifugation, washed with ethanol and then with ether. This is the original toxohormone fraction of Nakahara and Fukuoka (1). The yield of the toxohormone fraction was 650 mg (9.25% of the original dried material), which is approximately the yield to be expected from any other tumor tissue.

The toxohormone activity of the preparation from germfree tumor tissues was tested by injecting it intraperitoneally into adult mice of ddN strain, about 20 g in body weight, and determining the liver catalase activity of the mice at 24 hours after the

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