PATHOGENESIS OF H-1 VIRUS INFECTION OF EMBRYONIC HAMSTER BONE IN ORGAN CULTURE*

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(Received for publication 27 October 1970)

The picodna viruses, pico referring to their small size and dna to the nature of their nucleic acid, were originally recovered from certain rat and human tumors and have been recently reviewed by Toolan (1). Inoculation of hamsters during the suckling period with the H-1 strain of picodna virus produces various abnormalities among which are runting, microcephaly, cranial lacunae, dysplastic teeth, and unusually docile behavior (2-5). Transplacentally induced infection with H-1 virus is highly embryocidal in the hamster and surviving fetuses exhibit a wide spectrum of congenital malformations which include skeletal defects (6). Particularly affected are tissues of mesodermal origin. Infection of adult animals, although easily produced, is usually not apparent. However, infection of adult hamsters with the H-3 strain of picodna virus following dental extractions or fractures of long bones results in a marked delay in the healing of these osseous wounds (7).

Since invasion of bone is a seldom considered and potentially important aspect of viral infections of man, the pathogenesis of the damage to bone which occurs in this animal model of viral osseous infection was studied. The effects of H-1 virus infection observed in the hamster by other investigators (2-7) strongly suggest that the defects observed in teeth and bone are the direct result of infection of bone by the virus. However, the possibility that these effects on bone are attributable to indirect mechanisms must be considered, since retarded growth, especially in the very immature animal, is a phenomenon which is often associated with various chronic infections. Also, because H-1 virus infects many tissues, it must be recognized that an apparent isolation of virus from bone may represent contamination of the bone by infected blood, muscle, or other tissues rather than infection of bone by the virus. In this regard, another concern relates to the unusual resistance of H-1 virus to physical and chemical inactivation, an attribute which probably enables the virus to persist in the environment for long periods of time (1). Thus, the presence of H-1 virus in bone must

* These studies were supported by the Rainbow Hospital Research Fund for Children, Cleveland, Ohio, and by National Institutes of Health Grants HD-04110 and FR-5410.

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be shown to result from infection and replication in osseous tissue and not from persistence of the original inoculum. In view of these and other unknown or uncontrollable variables encountered in vivo, it was decided to study the effects of H-1 virus infection on embryonic hamster bone in organ culture and thus make possible direct observation of the reaction of bone to virus in a regulated environment.

Materials and Methods

Animals.—Pregnant Syrian golden hamsters and Sprague-Dawley rats were obtained from commercial sources.

Preparation of Rat Embryo Cell Cultures.—Rat embryos of 14–16 days gestation were finely minced with scissors and suspended in Hanks' balanced salt solution containing 0.25% trypsin. After 90–120 min of mechanical stirring at room temperature, undigested pieces of tissue were removed by decanting through sterile gauze. The resulting cell suspension was then centrifuged at 3000 rpm for 5 min, resuspended in Eagle's minimal essential medium containing 10% fetal calf serum, and seeded in a concentration of 50,000 cells/ml into tubes or flasks as required. After formation of confluent cell monolayers (usually 5 days after seeding), fluids were changed to Eagle's minimal essential medium containing 2% fetal calf serum for maintenance, and the cultures were used for propagation or titration of H-1 virus.

Virus.—The H-1 strain of picornavirus was kindly provided by Lawrence Kilham, M.D., Hanover, N. H. Samples of H-1 virus were inoculated into rat embryo cell cultures. When viral cytopathic effects were evident in 50–75% of the cells in culture, the supernatant fluids were removed and centrifuged at 2000 rpm for 10 min. The sediment was discarded and the supernatant fluids were sealed in glass ampules and stored at −60°C until needed for inoculation of organ cultures of bone. Control fluids for inoculation were prepared from the same batch of rat embryo cell cultures in an identical manner except for the omission of virus. The virus content of the stock preparations was calculated by the Reed-Muench method (8) using a cytopathic effect in rat embryo cell cultures as an indicator system. Because of the frequency of latent infection of rats by rat virus, another member of the picornavirus group (9), control cultures from each batch of rat embryo cells were observed until senescence for the cytopathic effects of rat virus. Whenever such cytopathic effects were suspected, the stocks of H-1 virus and control fluid prepared from that batch of cells were discarded.

Preparation and Inoculation of Organ Cultures of Hamster Tibiae.—Pairs of tibiae were dissected from hamster embryos obtained by hysterotomy on the 13th–15th days of pregnancy. One bone from each pair was placed in a test group of 5–10 bones and its mate was assigned to a control group of the same size. The bones were rinsed with Hanks' balanced salt solution and were transferred to plastic Petri dishes to which was added Eagle's minimal essential medium containing 20% fetal calf serum and added glucose to produce a final concentration of 5.0 mg/ml. Cultures were incubated at 37°C in an atmosphere of 5% carbon dioxide. Test cultures were inoculated with H-1 virus prepared in rat embryo cell cultures and control cultures were inoculated simultaneously with fluids from uninoculated rat embryo cultures. The use of control fluids from uninoculated rat embryo cultures was felt to be essential because of the possibility of undetected contamination of the stocks of H-1 virus by rat virus from the rat embryo cell cultures. If, despite the precautions described in the section on virus preparation, undetected rat virus was present in the stocks of H-1 virus, it was also present in the controls. The concentration of virus in the fluids of inoculated cultures at the beginning of each experiment was approximately $10^8$ tissue culture-infective doses (TCID$_{50}$) per 0.1 ml. 24 hr after inoculation (or 2 hr after inoculation in the case of

1 Abbreviation used in this paper: TCID$_{50}$, tissue culture-infective doses $50$.
determinations of the viral growth curve in bone) the virus inoculum was removed and the bones were rinsed five times in phosphate-buffered saline to dilute any residual virus and were placed in new culture dishes containing fresh medium.

Testing for H-1 Virus Infection of Bone.---To determine whether viral infection of bone had occurred, tibiae were harvested in pairs at various times after inoculation. Each bone sample was pulverized using a mortar and pestle and then suspended in 1.0 ml of Eagle's minimal essential medium containing 2% fetal calf serum. The suspension was centrifuged at 2000 rpm for 10 min and the supernatant fluid, now an extract of bone, was tested for H-1 virus by inoculation into rat embryo cell cultures which were then observed for viral cytopathic effect. Portions of supernatant fluids from bone cultures were also harvested and tested for virus.

Measurements of Bone Weights and Lengths.---Changes in wet weights of bone were detected by removing the bones from the culture dish, blotting on sterile filter paper, and weighing on an analytical balance. The bones were then returned to the culture dish for further observation or were harvested for other studies. For determination of dry weight, bones were desiccated in an oven at 100°C for 24 hr, allowed to cool to room temperature, and weighed on an analytical balance. Changes in bone length were measured with a microscope eyepiece micrometer.

Histologic Examination of Bones.---For histologic study, bones were fixed in Bouin's solution, washed, dehydrated, and embedded in paraffin. Decalcification was found to be unnecessary. Sections were cut 6 μ in thickness and were stained with hematoxylin and eosin, toluidine blue, periodic acid Schiff-Alcian blue, and trichrome stains.

RESULTS

During a period of 14 days after inoculation, H-1 virus was isolated repeatedly from bones and supernatant fluids from test cultures, but not from control cultures. The curve of viral replication in bone is shown in Fig. 1. The time at which the inoculum was removed and the bones were placed in fresh medium is indicated as 0. Virus was not detected in the washed bones or fresh medium at that time. During the following 72 hr, the titer of virus in bone extract increased to $10^4$ TCID_50/0.1 ml. Between 24 and 30 hr after inoculation, virus was released from bone into the culture fluids, and by 72 hr after inoculation, the virus content of culture fluids and bone extract were approximately equal. However, since bone extract represented a 1:100 to 1:1000 dilution of bone in Eagle's medium, the concentration of H-1 virus was proportionately greater in bone than in culture fluids.

As viral replication progressed, infected bones became translucent and slender, while bones in cultures inoculated with control fluids became more opaque and developed bulbous epiphyses. This change in appearance is demonstrated in Figs. 2 and 3 in which infected and control bones from embryos of 15 days gestation were photographed in culture 7 and 17 days after inoculation. In addition to these morphologic differences, control bones developed an envelope of cells and tended to become attached by this envelope to the bottom of the culture dish. Once attached, the uninfected bone became a nidus for cellular outgrowth. Cellular envelopes and outgrowths were not produced by infected bones. This cellular proliferation was more marked in uninfected bones from embryos of 13 days gestation than in bones harvested from 15-day em-
bryos. Also, after a few days in culture 13-day control bones became quite distorted, presumably from stresses applied by the envelope of cells, while 15-day bones had sufficient structural rigidity to resist distortion. Fig. 4 demonstrates an uninfected bone from a 13 day embryo distorted by its cellular envelope, and an infected bone, undistorted because of the absence of a cellular envelope. In Fig. 5 an uninfected control bone with its cellular outgrowth is shown next to a free-floating infected bone. These bones were placed together in the same dish only for photographic purposes. The photographic technique, which in this figure is directed toward showing cellular detail, has produced silhouettes of the straight infected bone and the distorted uninfected bone surrounded by its cellular outgrowth.

A regular increase in the wet weights of embryonic tibiae in culture was observed. After 7–9 days in culture, control bones had usually tripled in weight. In contrast, the wet weights of bones infected with H-1 virus usually increased during this period by a factor of not more and often less than two. The increase in weights of infected and control bones in one experiment are compared in Fig. 6. As is apparent from the figure, uninfected control bones gained weight more rapidly and attained a greater final wet weight than did infected bones, despite the fact that the mean wet weights of both groups of bones were approximately equal at the beginning of the experiment. Evidence that impaired weight gain was consistently observed in tibiae infected with H-1 virus is presented in Fig. 7.

The mean dry weights of infected and control tibiae were found to be directly proportional to their mean wet weights. From their wet and dry weights the water contents of the bones were calculated according to the formula: Per cent water = (mean wet weight – mean dry weight) × 100/mean wet weight. As is demonstrated in Fig. 8, no differences were found between the water contents of infected and control tibiae.

In contrast to their greater weights, control bones tended to be shorter than
Fig. 2. Tibiae from hamster embryos of 15 days gestation after 7 days in organ culture. × 20. Upper bone is an uninfected control. Lower bone was infected with H-1 virus on the 1st day in organ culture.

Fig. 3. Tibiae from hamster embryos of 15 days gestation after 17 days in organ culture. × 20. Upper bone is an uninfected control. Lower bone was infected with H-1 virus on the 1st day in organ culture.
FIG. 4. Tibiae from hamster embryos of 13 days gestation after 4 days in organ culture. X 25. Photographic technique was directed toward showing bone detail. Left: An uninfected control tibia, attached to the surface of the culture dish, has been distorted into a horseshoe configuration by its cellular envelope. Right: A tibia infected with H-1 virus on the 1st day in organ culture is undistorted and floats free in the culture fluids.

FIG. 5. Tibiae from hamster embryos of 13 days gestation after 4 days in organ culture. X 25. Photographic technique was directed toward showing cellular detail. Left: A cellular outgrowth emanates from an uninfected control tibia which has become attached to the culture dish. Right: A tibia which was infected with H-1 virus on the 1st day in organ culture is undistorted and floats free in the culture fluids.
Fig. 6. The effect of H-1 virus infection on the increase in wet weight of organ cultures of tibiae from hamster embryos of 15 days gestation. Each point on the graph represents the mean wet weight of 12 tibiae.

Fig. 7. The effect of H-1 virus infection on the mean wet weights attained by tibiae from hamster embryos of 15 days gestation in organ culture. The mean wet weights of infected and control bones determined between the 7th and 16th day after inoculation with H-1 virus or control fluids are plotted as coordinates. The line with a slope of 1.0 is the line of no difference. All points but one fall below this line, indicating that at the end of each experiment the mean wet weight of the uninfected tibiae was greater than that of the infected bones.
infected bones at the end of the period in organ culture. The mean lengths of infected and control tibiae are compared in Fig. 9.

Longitudinal sections cut at the widest diameter of infected and control bones confirmed the morphologic differences observed in organ culture and provided evidence of histologic abnormalities in infected bones. Whole bone sections are compared in Fig. 10. The uninfected control bone seen on the 7th day in culture exhibits broad epiphyses and is rimmed by a mantle of periosteum and perichondrium. In the mid-shaft area, deposition of subperiosteal bone has taken place. In contrast, the epiphyses of the infected bone, also examined on the 7th day in culture, appear more narrow and angular and the rim of periosteum and perichondrium is indistinct. Subperiosteal bone formation is much less than in the control. Examination of fixed sections of the mid-shaft region of bone at higher magnification (Fig. 11) shows that the rim of periosteum around the infected bone is almost acellular and consists largely of amorphous debris. Subperiosteal bone formation again is less than in the control bone, and several multinucleated giant cells, presumably osteoclasts, are present in the diaphysis.
No viral inclusion bodies are identified. In comparison the uninfected bone possesses an intact cellular periosteum and giant cells are absent.

**DISCUSSION**

These observations show that the H-1 strain of picodna virus can directly infect and damage embryonic hamster bone. Detection of increasing quantities of virus in the bone after inoculation attests to the ability of the virus to replicate in this tissue. Thus indirect mechanisms need not be postulated to explain the osseous defects produced by this infection in embryonic and suckling hamsters, although the possible operation of such mechanisms as contributing causes cannot be excluded by this study.

Histologically the abnormalities seen in the infected bones in organ culture were degeneration of the periosteum and perichondrium and diminution of subperiosteal bone deposits. Multinucleated giant cells, presumed to be osteoclasts in function, were noted in apposition to subperiosteal bone. Therefore the decreased amount of subperiosteal bone was probably the result of a combination of diminished deposition by the impaired periosteum and resorption of
FIG. 10. Longitudinal sections of tibiae from hamster embryos of 15 days gestation after 7 days in organ culture. X 15. Hematoxylin and eosin stain. Left: An uninfected control bone. Right: A bone infected with H-1 virus on the 1st day in organ culture.

FIG. 11. Longitudinal sections from the mid-shaft regions of the embryonic tibiae shown in Fig. 10. X 430. Hematoxylin and eosin stain. Left: Uninfected bone. Right: Infected bone. P, periosteum; SPB, subperiosteal bone; OC, osteoclast.
bone by osteoclasts. This defect of the periosteum probably accounts for the
slender, delicate appearance of the infected tibiae illustrated in Figs. 3 and 10,
since in the long bones subperiosteal bone deposition is essential to normal
circumferential growth. Impaired periosteal function may also explain the
presence of the cranial lacunae and "mongoloid" deformities of the flat bones
of the skull described in young hamsters infected with H-1 virus. These ab-
normalities presumably result from defects in the formation of the bony tables
of the cranial vault and failure of the skull to undergo normal growth and re-
modeling during maturation. Both of the latter processes normally occur at the
periosteal surfaces of the membranous bones of the skull and require an intact
periosteum.

Viral inclusion bodies were not identified in cartilage or periosteum in this
study, but infectivity tests demonstrated the presence of virus. Ferm and
Kilham (6) reported the finding of intranuclear inclusion bodies in the cartilage
and notochord of hamster embryos infected with H-1 virus, but they are not
mentioned in a description by Cohen and Shklar (10) of the skull and teeth of
hamsters with deformities induced by H-1 virus. In a study of infection of
periodontal tissues by rat virus, a member of the picorna virus group closely
related to H-1 virus, Baer and Kilham describe the presence of inclusion bodies
in the periodontal membrane in one report (11) and their absence in another (5).
The observation by Moore (12) that H-1 virus had to be passed 20 times in
hamster embryo cells before it produced cytopathic changes in tissue culture
suggests that production of cellular inclusions is a variable property of the virus.

In addition to its effects on the histologic structure of the tibia, H-1 virus
interfered with growth of bone as measured by increase in wet weight. The
greater weight gain of the control bones may be related in part to the unim-
paired growth of periosteal tissue resulting in a greater cell mass and in deposi-
tion of greater amounts of subperiosteal bone in the uninfected tibiae. Other
differences such as changes in bone composition may be involved, however. In
this study no difference was found between the water contents of infected and
control bones after 7-16 days in organ culture. This determination was carried
out because of the finding of Biggers (13) that as a normal bone ages in organ
culture, it imbibes water. In contrast, dead bone or bone which has been dam-
aged by exposure to certain chemicals does not show this phase of hydration.
Apparently infection of bone by H-1 virus does not interfere with this incorpora-
tion of water. Staining of sections of bones with toluidine blue, periodic acid
Schiff-Alcian blue, and trichrome stain failed to show histochemical differences
in the matrix of infected and control bones. Further studies of bone composition
are in progress.

The mean lengths of infected tibiae were found to be somewhat longer than
controls. This difference in lengths, although consistently observed, was not as
marked as the difference in weights. Since the bones studied were from embryos
in late gestation, only a moderate increase in length was expected during the
culture period, in contrast to the change in wet weight which usually tripled during the same interval of time. Thus the opportunity for development of marked differences in length was less than for weight differences. Greater growth in length occurs in bone rudiments cultured from embryos in earlier stages of gestation, but the distorted shapes often assumed by these younger bones in culture make accurate measurement of their lengths difficult.

To explain the greater length of infected tibiae, the possibility of stimulation of this aspect of growth by the virus must be considered. Although runting and impaired dentition are the striking features seen in the infected hamster, occasionally overgrowth of the incisor teeth occurs to the extent that clipping of the teeth becomes necessary to allow the animal to masticate (1, 10). The structure of these overgrown teeth is abnormal, however. In view of the disruptive effects of H-1 virus on bone in organ culture, it seems unlikely that the greater length of infected bones in this study is due to stimulation of growth by the virus. Instead, the difference in length between infected and control bones may be related to encasement of the latter in an intact periosteal and perichondral membrane. Although this membrane is probably necessary for normal bone growth in vivo, it may restrict elongation of the uninfected bone in organ culture. In this regard it must be appreciated that bone in organ culture behaves differently than in the intact animal, and that the damaged infected bone which elongated under the conditions of organ culture might have become a shortened, deformed bone if subjected to the stresses encountered in vivo.

Three viruses have been reported to affect human bone. In certain smallpox epidemics viral osteitis and arthritis, often associated with residual disability, have been reported to occur in 2–5% of affected children (14). Viral invasion of bone is also encountered as a rare but serious complication of vaccinia virus infection (15, 16), and osteolytic lesions from which virus can be recovered are seen in infants with congenitally acquired rubella (17, 18). In addition, transient arthritis is a common complication of rubella in women and is also found as a minor concomitant of other viral illnesses (19). Picorna virus infection of hamster bone in organ culture provides a useful model for the study of the pathogenesis of viral osseous infection which may have important implications for human disease. Modification of the system described in this report to the study of the effects of viruses on organ cultures of human embryonic bone, often available from therapeutic abortions, is suggested as a promising approach for investigating this aspect of viral infections in man. Also, if viruses are associated with conditions such as the osteochondroses, bone neoplasms, and other disorders of bone and cartilage in man, it is attractive to consider that they might be detected by inoculation of affected tissues into organ cultures of bone.

SUMMARY

H-1 virus infection of hamsters has been shown to produce runting, microcephaly, cranial lacunae, and deformed teeth in animals inoculated during the
suckling period and to cause various abnormalities, including skeletal defects, in embryos infected transplacentally. To explore the pathogenesis of these effects of viral infection on bone, the response of embryonic hamster tibiae in organ culture to inoculation with the H-1 strain of picornavirus was studied. This system made possible the direct observation of the reaction of bone to virus in a regulated environment. During a period of 7–17 days after inoculation the following observations were made: (a) H-1 virus was found to infect and replicate in bone. (b) Infected bones became more translucent, slender, and elongated than control bones. (c) Bone growth as measured by increase in wet weight was reduced in infected tibiae. (d) Infected bones showed periosteal and perichondral degeneration and diminished deposits of subperiosteal bone. It was concluded that the skeletal abnormalities which develop in embryonic and suckling hamsters after H-1 virus inoculation are the direct result of viral replication in bone, and that indirect phenomena such as those associated with chronic infection need not be postulated to explain the deformities seen in these animals.

The author expresses his thanks to Mr. James D. McCallum for his excellent technical assistance, to Miss Patricia Bene for the preparation of bone sections, to Dr. Robert P. Bolande for his aid in interpretation of the histologic changes in bone, and to Mr. Donald N. Schad for his photographic services.

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