Comparative Susceptibility of Neonatal Rabbit Skin to Herpes Simplex Virus *in vitro*.* (32426)

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The mammalian neonate is remarkably susceptible to infection by herpes simplex virus (HSV)(1,2). In contrast with more mature members of each species, HSV may disseminate widely and in great quantity in the newborn host, often causing death(3,4).

The factors surrounding resistance of the host to viral infection, particularly infection of the neonate, are complex. They involve the relative importance of immunologic responses, such as circulating antibody and delayed hypersensitivity, *versus* the intrinsic resistance of the cells and tissues of the host. One might suggest that the host cell is the most crucial parameter of innate resistance to viral infection. The infected cells and tissues allow for the dimension of viral multiplication which may characterize either mild or major disease and perhaps define the heightened susceptibility of the newborn host.

The quantitative and relative importance of the various components of resistance to viral infection has been difficult to resolve with experiments *in vivo*. A simple system was developed in which HSV and rabbit skin might be interacted *in vitro* in the absence of antibody or the expression of delayed hypersensitivity. Pursuing the question of comparative resistance to viral infection, as related to age, subsequent experiments revealed that immature skin synthesized considerably more virus than mature skin, apparently consequent to the biochemical attributes of newborn tissue.

Materials and methods. Tissue. Multiple 4 mm punch biopsies of skin were obtained simultaneously from New Zealand white rabbits of varied age. None of the rabbits possessed neutralizing antibody to HSV. Specimens of skin weighed before experimental preparation and use were unsatisfactory for subsequent optimal synthesis of HSV. On separate occasions multiple biopsies obtained from each of several newborn and older rabbits were weighed on a Mettler microbalance. Attempts to disperse suspensions of cells from rabbit skin, using both trypsin and collagenase, were unsuccessful. Biopsies were individually minced with crossed scalpel blades and carefully rinsed free of potential specific or nonspecific viral neutralizing substances. Each tissue mince, representing the component parts of a single biopsy, was placed in a 13 × 100 mm screw-cap glass tube, containing 1 to 2 ml of maintenance medium (5 volumes calf serum, 95 volumes Eagle's basal supplement in Hanks' balanced salt solution), and incubated upright in a gyrotary shaker bath at 35°C.

Virus. Approximately 10^4 — 10^5 plaque forming units (PFU) of HSV, prepared as stock in HeLa cells, were added in 0.1-0.2 ml volumes per suspension culture. At appropriate intervals a freeze-thaw lysate of the minced tissue and medium contained in each culture was harvested for subsequent assay of virus and interferon. Virus was assayed in replicate 16 × 125 mm slanted tube cultures of stable rabbit (RK-13)‡ and human (LL-B) heteroploid cells by serial 10-fold dilution of test fluids, addition of 0.1 ml volumes of each dilution in duplicate to cell sheets, adsorption for 1 hour at 37°C, and subsequent liquid overlay. Cytopathic primary plaque foci of HSV were enumerated after 48 hours of cultivation by microscopic examination (50×) of either a stained (0.01% crystal violet) or unstained cell sheet.

Interferon-like-substance. Interferon-like-substance (ILS) was assayed by overnight treatment of tube cultures of primary rabbit

* Supported by Grant AI 04702 01A1 from Nat. Inst. of Allergy & Infect. Dis. (NIH).

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renal cells with heat-inactivated (56°C × 2 hours) test fluid and subsequent challenge with approximately 50 PFU of vaccinia virus prepared as stock in LL-B cells. Titers of ILS were estimated as the reciprocal of the dilution of test fluid which effected a 50% reduction in the number of vaccinia virus plaques. Heat-inactivated fresh medium and spent medium controls (medium harvested in similar fashion from cultures containing minced tissue without HSV) were included in assays of ILS. Fluids were considered to contain ILS because inadequate volume remained to characterize the interfering principle as classical interferon.

Attachment of virus. The attachment of HSV to minced rabbit skin *in vitro* was studied in the same system by the recovery and assay of residual unattached virus from the supernatant fluid at hourly intervals over a period of 3 hours following initial virus-tissue interaction at 35°C.

Nucleic acid. The kinetics of nucleic acid metabolism in newborn and adult rabbit skin were studied by the addition of 1 microcurie (specific activity, 30 millicuries/millimole) of uridine-2-C^{14} and thymidine 2-C^{14} to duplicate suspension cultures of uninfected minced rabbit skin as a 2-hour pulse. In the cold, the minced tissue was removed, rinsed carefully with buffered balanced salt solution, triturated with mortar and pestle following treatment with 88% formic acid, and precipitated with 14% trichloroacetic acid. The precipitate was transferred to a millipore filter, placed on a metal planchette, allowed to dry overnight at 4°C, and then counted in a windowless geiger counter (Nuclear-Chicago Corporation).

Results. The weights of skin biopsies ranged from 6 to 10 mg. For example, 4 biopsies from 1-day-old-rabbit weighed 6, 8, 8, and 9 mg; 4 biopsies from a 49-day-old-rabbit weighed 6, 7, 8, and 10 mg.

The results of a representative experiment in which replicate skin biopsies, obtained from immature and mature rabbits, were infected with HSV and viral multiplacation characterized throughout an extended period of time are summarized in Fig. 1. Cutaneous tissue from a 5-day old rabbit synthesized 10-fold more virus than tissue from a 28-day old rabbit. Five additional experiments were performed, comparing tissue from newborn and other rabbits, in which, although the comparative difference in the peaks of viral replication varied from 3 to 10-fold, the minced cutaneous tissue from the newborn rabbit consistently synthesized virus in greater and more persistent fashion than tissue from the older rabbit.

Skin biopsies were also obtained from a non-immune adult rabbit and a hyperimmune adult rabbit with a HSV-neutralizing antibody titer of 1:2048. The carefully rinsed tissues from both rabbits yielded indistinguishable patterns of viral multiplication.

To expand these experiments in a cross-sectional manner, single biopsies were obtained from 3 rabbits in each age-contrasted group. The infected tissue and fluids were harvested only once after 48 to 72 hours, to compare the mean of cumulative viral multiplication. After 72 hours, comparably greater multiplication of HSV was observed in cultivated cutaneous tissue of 1-day old rabbits (Fig. 2). Whereas in other experiments ILS was either undetectable or detected in only equivocal amounts, in this instance the yield of ILS by the minced skin from the 3 age-contrasted groups of rabbits was equal at titered units of 4. In a similar but shorter 48 hour experiment the cutaneous tissues of the newborn again elaborated almost 10-fold more virus than that of the adult (Fig. 3). In these experiments the intragroup variation in synthesis of virus was approximately 20%.

Figure 4 depicts the disappearance of HSV from the supernatant fluid in cultures containing age-contrasted tissue, in comparison with a thermolability control, and delineates the rate of attachment of virus to minced skin during the period of study. These studies suggest an equivalent rate of attachment of virus to cutaneous tissues of the newborn and adult rabbit.

Two-hour pulse incorporation of isotopically-labeled nucleoside by rabbit skin, as counts per minute (CPM) per approximately 8 mg of cultivated minced tissue, revealed enhanced uptake of thymidine (DNA) by newborn tissue *versus* that of the adult

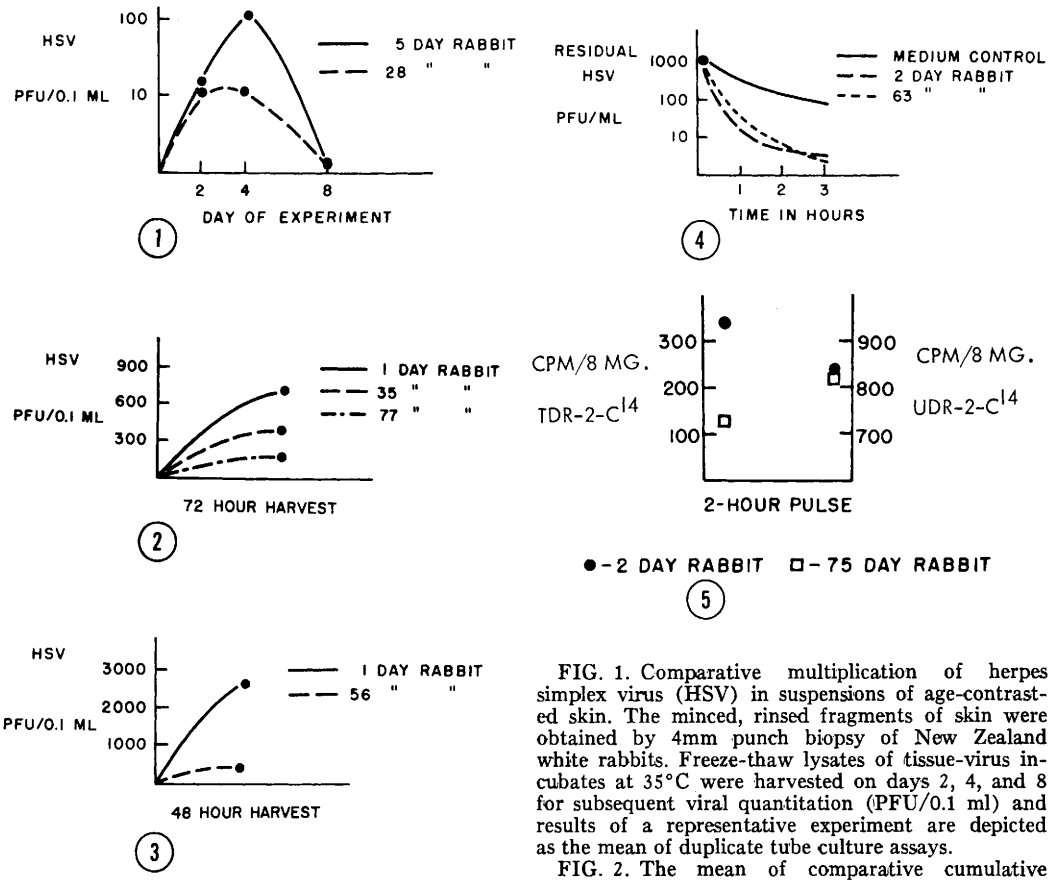

FIG. 1. Comparative multiplication of herpes simplex virus (HSV) in suspensions of age-contrasted skin. The minced, rinsed fragments of skin were obtained by 4mm punch biopsy of New Zealand white rabbits. Freeze-thaw lysates of tissue-virus incubates at 35°C were harvested on days 2, 4, and 8 for subsequent viral quantitation (PFU/0.1 ml) and results of a representative experiment are depicted as the mean of duplicate tube culture assays.

FIG. 2. The mean of comparative cumulative multiplication of herpes simplex virus (HSV) in triplicate suspensions of age-contrasted rabbit skin. Freeze-thaw lysates were harvested after 72 hr of incubation at 35°C and assayed in duplicate for virus (PFU/0.1 ml) and interferon-like-substance (ILS). The yield of ILS from the 3 age-contrasted experimental preparations was equal at 4 units/ml.

FIG. 3. The mean of comparative cumulative multiplication of herpes simplex virus (HSV) in triplicate suspensions of age-contrasted rabbit skin. Freeze-thaw lysates were harvested after 48 hr of incubation at 35°C and assayed in duplicate for virus (PFU/0.1 ml).

FIG. 4. Rate of attachment of herpes simplex virus (HSV) to suspensions of age-contrasted rabbit skin, compared with tissue-free thermolability control. Attachment was assessed by quantitation of residual unattached virus in the supernatant fluid of the virus-tissue incubate over a period of 3 hrs at 35°C.

FIG. 5. Incorporation of C^{14}-labeled thymidine (TDR) and uridine (UDR) by virus-free duplicate suspensions of age-contrasted rabbit skin and expressed as the mean count per minute (CPM) per 8 mg sample following a 2 hr pulse at 35°C.

(Fig. 5). On the other hand, the incorporation of uridine (RNA) was equivalent for cutaneous tissues from both age-contrasted hosts.

Electron microscopic study of rabbit skin failed to delineate definite lysosomal structures in epidermal cells.§ Ultrastructural cytochemical methods for the demonstration of lysosomal acid phosphatase did not detect specific deposits of reaction-product in these cells.

Light microscopic study of cutaneous biopsies from newborn and mature rabbits revealed no apparent difference in the cell population or morphology of the epidermis and corium.

Discussion. An obvious criticism of these experiments is the lack of quantitative information concerning the actual population of cells in each skin biopsy. More vigorous

§ These studies were performed kindly and expertly by Dr. James G. White, Dept. of Pediatrics, Univ. of Minnesota Med. School.

enzymatic treatment of the tissue might allow dispersion of single, selected cells. The same is true for plasma clot-explants of skin. However, while these techniques may provide eventual cell monolayers, one wonders whether such cell populations truly represent the original host tissue. The fact that the incorporation of uridine -2-C^{14} by minced rabbit skin from both age-contrasted hosts was similar suggests that the cell population of each original 4 mm punch biopsy was equivalent.

Several factors render cells either susceptible or resistant to infective virus. Viral receptors, localized on the cellular surface and intracellular membranes, allow for the attachment and perhaps penetration of viral particles into the cell(5,6). Kunin(7) has suggested that an abundance of receptors may explain the susceptibility of the newborn mouse to certain enteroviruses.

Lysosomal enzymes may effect intracellular uncoating of viral particles thus allowing viral genome to initiate synthesis of new virions (8). In other experimental circumstances lysosomes seem to augment the resistance of the cell. Prompt lysosomal activation in hepatic cells may destroy mouse cytomegalovirus, while concurrent persistence of virus in salivary glands may occur because of relatively low lysosomal activity(9). What role lysosomes play in age-dependent suceptibility to viral infection is unknown.

The host cell also possesses the ability to repress synthesis of new virus, at least that destined to occur in neighboring and perhaps distant cells, by the elaboration of interferon(10). Heineberg and her colleagues(11) have suggested that scant synthesis of interferon by the tissues of the suckling mouse accounts for the enhanced multiplication of enterovirus in these tissues and subsequent death. However, the ultimate and uniform importance of interferon in resistance to viral infection is somewhat obscured by studies of arbovirus infection in genetically susceptible and resistant mice. Susceptible mice synthesized not only more virus but also more interferon than resistant animals(12). So, as with receptors, interferon may explain comparative resistance or susceptibility of the host to some viruses but not all. Indeed, HSV appears less facile in induction of interferon than other viruses(13,14). It is also conceivable that the lower temperature of these experiments mitigated against optimal synthesis of interferon or interferon-like-substance(15).

The equivalent synthesis of HSV by non-immune and hyperimmune rabbit skin excludes from this experimental system the classic immunologic responses of antibody and delayed hypersensitivity. Johnson(16) has demonstrated that resistance to HSV in the maturing mouse may result from viral containment in the infected macrophage. We have assumed, nonetheless, that the essential host cell in this experimental milieu is the epidermal cell. Thus, the apparent susceptibility of the newborn rabbit to the lethality of HSV infection may be explained by the capacity of newborn tissue itself to synthesize a greater magnitude of virus for a protracted period of time. Campbell(17) has made similar comparative observations with foot-and-mouth disease virus infection of mouse renal cells and tissue *in vitro*.

Receptor density and the capacity of tissues to elaborate inteferon do not explain the comparative differences in multiplication of HSV in this system. The same seems to be true for the lysosome. The potential for DNA synthesis and turnover in newborn rabbit skin may elucidate not only the enhanced replication of DNA-containing-HSV in this and perhaps other immature tissues but also the subsequent fulminant nature of HSV infection in the intact newborn host.

The biochemical attributes of neonatal tissue fail to explain susceptibility to RNA-viruses, such as the enteroviruses, since these viruses may multiply in the absence of functional cell DNA(18). In these instances, perhaps cellular receptors and interferons do indeed play the dominant role(s) in the virus-cell conflict.

Summary. The remarkable susceptibility of the mammalian neonate to some viral infections raises the issue of the relative importance of humoral immunity, delayed hypersensitivity, and the intrinsic resistance of cells and tissues of the host. The present

studies focused on the tissue of the host and involved the interaction *in vitro* of herpes simplex virus with preparations of skin fragments from newborn and more mature rabbits. The cutaneous tissue from 1-2-day-old rabbits consistently synthesized 3-10-fold more virus than tissue from 1-2-month-old rabbits. Studies indicated no apparent difference in the attachment of virus to immature and mature rabbit skin. Interferon-like-substance was elaborated in equivalent, low titers by cutaneous tissue from newborn and adult rabbits. Light and electron microscopic study of rabbit skin failed to discern apparent differences in the population, morphology, or lysosomal content of cutaneous tissue. Virus-free preparations of 2-day-old rabbit skin incorporated 100% more thymidine-2-C^{14} than skin from 2-month-old rabbits but the incorporation of uridine-2-C^{14} by these tissues was essentially equal. Thus, the cutaneous tissue of the newborn rabbit appears particularly well suited biochemically for the synthesis of DNA-containing-herpes simplex virus.

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Received June 19, 1967. P.S.E.B.M., 1967, v126.

Neoplastic Conversion *in vitro* of Newborn Mouse Thymic Cells.* (32427)

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Newborn mouse thymic cells have been cultured *in vitro* for over two years(1). In the present study their possible malignant conversion has been tested by injecting the cultured cells into newborn and adult syngeneic hosts.

Material and methods. The thymic cells were derived from minute thymus fragments of newborn MA mice. The procedure used for establishing the cell line, as well as its morphological characteristics, has been previously described(2). The thymic cultures were started in August 1963 and maintained in continuous culture until March 1966, when they were stored at −70°C. Since December 1964, subcultures from the original thymus culture were started by transferring a minute amount of cells obtained by mechanical scraping at the edges of the solid sheet of epithelial cells. Occasionally subcultures were obtained by trypsinization.

In the present experiment only subcultures obtained by mechanical scraping were used. The cultures were maintained in Leighton tubes, T-flasks or Milk bottles at 37°C in an incubator without CO_2. The culture medium

* This work was supported in part by Grant CA-6162 from Nat. Cancer Inst., NIH, USPHS and in part by Contract PH 43-65-67.

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