"product 2" and 0.2 ml calcium chloride. After 20 minutes' incubation, 0.1 ml of the mixture was added to a mixture of 0.2 ml human fibrinogen and 0.1 ml of the inhibitor or normal control fractions and the clotting times recorded. The inhibitor fractions had no effect on clotting times.

*Discussion.* The findings that the inhibitors from both patients interfered with the reaction of the sedimented intrinsic blood thromboplastin ("product 2") with human but not with bovine prothrombin preparation suggest that the inhibitors are directed against human prothrombin. Another possible explanation is that the inhibitors interfere with some other activity in the prothrombin preparation necessary for prothrombin conversion. However, it is generally believed that "product 2" when prepared with factor V converts prothrombin to thrombin in the presence of calcium and that no other factors are needed for the conversion. If this is so, a new clotting component difficult to separate from prothrombin would have to be postulated. A third possibility is that the inhibitors require a species specific co-factor for the development of their activity; this seems unlikely as the addition of human serum to the bovine prothrombin preparation did not result in inhibition of thrombin formation by "product 2." The most logical explanation of our findings is that the inhibitors are species specific inhibitors of human prothrombin. The finding of a naturally occurring species specific inhibitor of prothrombin has not been previously reported to our knowledge. Fantl and Nance(8) claimed that one of their patients developed a species specific inhibitor against tissue thromboplastin but this finding was based on indirect evidence and their conclusions would be considered invalid in the light of more recent work(3).

*Summary.* Fractions were prepared by 0 to 33% saturation with ammonium sulfate from the sera of two patients with lupus erythematosus who had circulating anticoagulants. The fractions prolonged the prothrombin and partial thromboplastin times of human but not bovine or canine plasma and inhibited the conversion of human but not bovine prothrombin to thrombin by "product 2." The findings suggest that the circulating anticoagulants are species specific inhibitors of prothrombin.

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## Study of Virulence and Tumorigenicity of Variants of Herpes Simplex Virus.* (29248)

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The virulence of various strains of herpes simplex virus (HSV) in newborn and young adult mice has been studied in many laboratories. Efforts to test the potential tumori-

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genicity of HSV in newborn animals have failed(1) because introduction of the virus even in low concentration has resulted in a high fatality rate in inoculated animals. The isolation of genetically purified populations of HSV variants that were less virulent in mice and hamsters was therefore attempted. A previous study(2) has reported isolation and partial characterization of such strains, in which virulence was measured by ability to induce keratitis on the cornea of the rabbit and by the number of plaque-forming units (PFU) required to kill 50% of 3-week-old mice inoculated.

The present study compares the virulence of 3 selected variants of HSV in newborn mice and hamsters. Observations concerning transplacental passage and attempts to induce tumors in mice and hamsters by inoculation of large concentrations of virus into newborn animals will also be described.

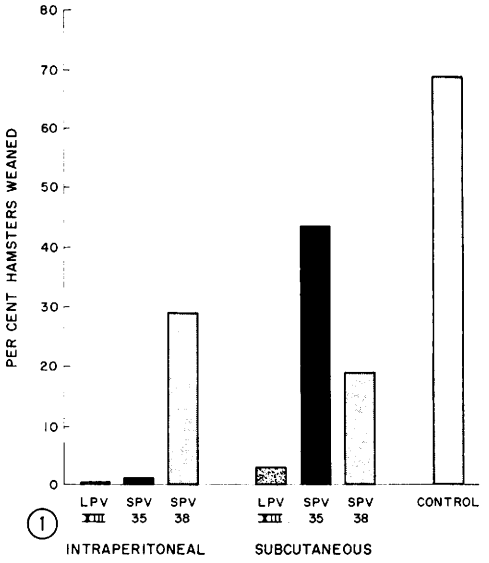
*Materials and methods. Viruses.* Three plaque-purified variants of the JES strain of HSV, previously designated(2) as large plaque-forming (LPV) XIII and small plaque-forming (SPV) 35 and 38 were used throughout this study. Virus stocks were prepared in cultures of primary young rabbit kidney fibroblasts, growing as monolayers in 16 oz bottles. The cells were maintained in a nutrient fluid composed of 98% Eagle's basal medium(3) with 2% calf serum; 100 units of penicillin and 100 $\mu$g of streptomycin/ml were added to all fluids. Cultures were incubated at 34°C until approximately 75% of the cells showed cytopathic effects (CPE). Cells were ruptured by alternate cycles of quick-freezing and thawing in the nutrient fluid. The virus suspensions were clarified by centrifugation at 1000 rpm at 4°C for 10 minutes and dispensed in aliquots in ampoules which were subsequently sealed. The virus was quick-frozen in an alcohol-dry ice bath and stored at —65°C. Virus stocks were titrated by the plaque assay technique in rabbit kidney cells under a methylcellulose overlay as previously described(2). Aliquots used to inoculate animals were retitered at time of inoculation. All stocks contained at least $10^7$ PFU/ml.

*Animals.* Newborn Syrian hamsters were inoculated within 24 hours after birth with 0.1 ml containing $10^5$ PFU of virus by either the intraperitoneal (IP) or subcutaneous (SC) route. To reduce cannibalism, litters were checked only once a week until 3 weeks post-inoculation, when the animals were weaned. Survivors were separated according to sex and were observed for a period of 6 months.

Pregnant hamsters, previously anesthetized with sodium nembutal, were inoculated intracardially with $10^5$ PFU of virus in 0.1 ml. Thirteen hamsters were inoculated with each variant. The hamsters were approximately half-way in their gestation cycle. Embryos from each group of animals were aseptically removed at daily intervals for 5 days following inoculation. A 20% suspension of the embryos was prepared in 95% Eagle's basal medium and 5% calf serum. Prior to removal of the embryos, blood was taken from the pregnant animals. In addition, newborn hamsters were sacrificed 24 and 48 hours after delivery from inoculated animals and 20% tissue suspensions were prepared as above. The preparations were assayed for HSV in rabbit kidney cells growing in plastic petri dishes by the plaque assay technique. Four cultures of rabbit kidney cells growing in constricted tubes(4) were also inoculated with each sample. The latter were observed daily for the development of CPE and harvested when cytopathic changes became evident. All isolates of the virus were identified with anti-herpes simplex virus serum prepared in rabbits.

Newborn Swiss mice were inoculated IP or SC within 24 hours after birth with $10^5$ PFU of the virus variants. Litters were observed daily until the mice were weaned. Survivors were kept until 6 months post-inoculation. They were then autopsied for evidence of tumor induction.

Pregnant Swiss mice were inoculated into the tail vein with $10^4$ PFU/0.1 ml of virus approximately 3 days before term. Embryos were harvested 1 and 2 days post-inoculation and newborns were sacrificed within 24 and 48 hours after birth (between 3 and 4 days post-inoculation of the mother). Attempts to isolate virus were carried out in a manner



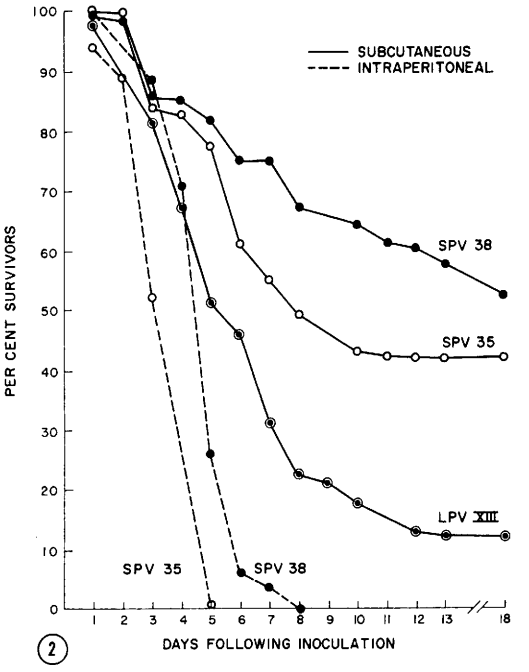




FIG. 1. Effect of variants of HSV cn newborn hamsters inoculated intraperitoneally or subcutaneously.

FIG. 2. Effect of variants of HSV on newborn mice inoculated intraperitoneally or subcutaneously. SPV 35 and 38—$10^5$ PFU were inoculated. LPV XIII—$10^4$ PFU were inoculated.

similar to that described for isolation of virus from hamster tissues.

*Results. Introduction of HSV into newborn hamsters.* A total of 393 newborn hamsters were inoculated by either the SC or IP route with either LPV XIII, SPV 35 or SPV 38; an additional 57 animals served as uninoculated controls. The results are summarized in Table I. Only 2% of all animals inoculated with $10^5$ PFU of LPV XIII survived. However, 17% and 24% of the hamsters inoculated with SPV 35 and SPV 38 respectively survived to weanling age. It is apparent from Table I and Fig. 1 that inoculation of SPV 35 by the IP route is more virulent than when this same variant is introduced *via* the SC route. The newborn hamsters tolerate SPV 38 equally well regardless of route of inoculation. The somewhat higher figures obtained for the virus-free controls probably reflect in part a lower tendency toward cannibalism on the part of mothers whose babies had not been disturbed.

TABLE I. Response of Newborn Hamsters to Inoculation with Variants of HSV.

| Variant | Route of inoculation | No. inoculated | No. weaned | % survivors |
|---|---|---|---|---|
| LPV XIII | SC | 63 | 2 | 2 |
| LPV XIII | IP | 43 | 0 | |
| SPV 35 | SC | 65 | 28 | 17 |
| SPV 35 | IP | 102 | 1 | |
| SPV 38 | SC | 78 | 15 | 24 |
| SPV 38 | IP | 42 | 12 | |
| None | None | 57 | 39 | 68 |

The 58 inoculated surviving hamsters were observed for 6 months. At that time, they were autopsied or checked at site of inoculation for evidence of neoplasia. In no instance was evidence of tumor induction obtained.

Thirty 3-week-old hamsters (not previously inoculated) were inoculated into the cheek pouches with $10^5$ PFU of the variants. The 10 animals inoculated with LPV XIII succumbed within a week after inoculation. Fifty percent (10 out of 20) of the hamsters survived inoculation of SPV 35 and 38. These animals were also carefully checked for tu-

TABLE II. Recovery of HSV from Blood and Embryos of Inoculated Hamsters.

| Hamster No. | Variant | Specimen | Day post-inoc | Titer of sample* (PFU/ml) |
|---|---|---|---|---|
| H-01 | LPV XIII | Embryos | 1 | $2.0 \times 10^1$ |
| H-04 | *Idem* | " | 3 | $7.1 \times 10^2$ |
| H-04 | " | Blood† | 3 | $1.0 \times 10^1$ |
| H-05 | " | Embryos | 4 | $2.5 \times 10^3$ |
| H-07 | " | " | 8 | $7.0 \times 10^1$ |

* 20% suspensions. † From pregnant hamster.

mor induction but none was observed.

*Introduction of HSV into pregnant hamsters.* Thirteen pregnant hamsters were each inoculated intracardially with $10^5$ PFU of the variants. Embryos were harvested from different animals at daily intervals; blood from the mothers was also collected at that time. A total of 54 assays were carried out for detection of HSV. The results are summarized in Table II. Only LPV XIII was reisolated. This variant was isolated from embryo homogenates derived from 4 litters. Virus was detected in the blood of one mother whose embryos also carried the virus. The titer of virus in the embryo was, however, higher than that found in the mother's blood. Other isolates were obtained from the embryos in the absence of virus in the blood of the mother. In each instance, virus was also recovered under fluid in rabbit kidney cells growing in tubes. This virus was identified with rabbit serum prepared against HSV by neutralization tests. SPV 35 and SPV 38 were not recovered from either embryo homogenates or from the blood of the inoculated adult hamsters.

*Introduction of HSV into newborn mice.* Inoculation of large numbers of newborn mice by various routes confirmed that LPV XIII was more virulent than SPV 35 or SPV 38 (Table III and Fig. 2). Only 11% of the animals survived $10^4$ PFU of LPV XIII administered SC while 42% and 57% survived SPV 35 and 38 respectively even though a log more virus of the latter 2 variants was introduced into the mice. However, introduction of $10^5$ PFU of SPV 35 or SPV 38 *via* the IP route caused the deaths of all animals inoculated (Table III and Fig. 2). Animals died more rapidly after IP inoculation than when the virus was administered SC (Fig. 2). The elapsed time required between inoculation and death was longer when $10^5$ PFU of SPV 35 or SPV 38 were inoculated SC than when $10^4$ PFU of LPV XIII was inoculated *via* the same route (Fig. 2). No animals died after the 13th day post-inoculation.

The 97 mice surviving to weanling age were observed for 6 months. No evidence of tumor induction by HSV was obtained.

*Introduction of HSV into pregnant mice.* Forty-five pregnant mice were inoculated into the tail vein with $10^4$ PFU of either LPV XIII, SPV 35 or SPV 38. A total of 16 attempts were made to isolate virus from either mother's blood, embryo homogenates or from newborn mice. Virus was not isolated from any of the specimens sampled. Approximately 80% of the newborn animals reached weanling age. Since these animals are highly susceptible to HSV, it seems probable that they were not exposed to HSV by inoculation of the mother.

*Discussion.* The observations that HSV variants SPV 35 and SPV 38 are less virulent for newborn mice and hamsters than is LPV XIII confirms differences in virulence previously established in weanling mice(2). Other studies have also reported similar differences among strains of HSV(5,6). A difference in neurovirulence for mice of variants in the JES strain of HSV (the same as used in this study) was also noted by Kolhage and Siegert(6). It appears from the data presented here that newborn hamsters respond to the HSV variants in a manner similar to that exhibited by newborn mice.

The ability of HSV to cause transplacental infection in the rabbit has been reported(7,

TABLE III. Response of Newborn Mice to Inoculation of Variants of HSV.

| Variant | Route of inoculation | No. inoculated | No. weaned | % survivors |
|---|---|---|---|---|
| LPV XIII* | SC | 101 | 11 | 11 |
| SPV 35† | SC | 88 | 37 | 42 |
| SPV 35† | IP | 109 | 0 | 0 |
| SPV 38† | SC | 87 | 49 | 57 |
| SPV 38† | IP | 82 | 0 | 0 |

* $1 \times 10^4$ PFU were inoculated.
† $1 \times 10^5$ PFU were inoculated.

8). While conflicting reports have appeared, it seems probable that intrauterine infection with HSV does occasionally occur in man(9). Our observations in hamsters demonstrate that at least some variants of HSV can cause transplacental infection. Further work is in progress to determine whether this ability is a unique property of LPV XIII. Failure to cause intrauterine infection in mice, reported here, has also been reported by Berry and Slavin(10) who failed to isolate virus from mouse embryos although they could detect virus in the blood of the adult infected mice.

Failure of HSV to induce tumors in either newborn mice or hamsters adds one more virus to those human viruses not found to cause induction of malignancy in these animals(1). It is of course possible that other strains of HSV or other routes of inoculation might yield different results. The techniques used, however, regularly result in tumors when adenovirus types 12(1,11) or 18(1,12) or the papovaviruses SV40(13,14) and polyoma (15) are introduced into newborn animals. It is of interest that the HSV variants employed in this study have been shown to cause selective aberrations of mammalian chromosomes in both diploid Chinese hamster and embryonic human cells(16). It therefore appears likely that ability to damage chromosomes does not necessarily confer oncogenic capability to viruses.

*Summary.* Variants of herpes simplex virus were shown to differ in virulence for both newborn mice and hamsters. Intraperitoneal introduction of all variants led to a higher death rate than subcutaneous inoculation. The virulent variant caused transplacental infection of hamster, but not of mouse, embryos. Inoculation of $10^5$ plaque-forming units of the attenuated variants into newborn hamsters and mice within 24 hours after birth failed to induce tumors within a period of 6 months.

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## Studies of Germfree Animals I. Response of Mice to Infection With Influenza A Virus.* (29249)

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There are few reports of the effects of viruses on germfree (GF) animals. Rous sar-

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