PATHOGENICITY FOR SUCKLING MICE OF COXSACKIE VIRUSES ADAPTED TO HUMAN AMNION CELLS*

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The observation that viruses may exhibit different biological properties when maintained in different hosts is well established. Because of its practical consequences the phenomenon of reduced virulence has been the subject of numerous studies. However, owing to the complexity of host-virus interactions, basic information is still scanty (1), and it is mainly for this reason that use of attenuated variants for active immunization has been greatly hampered, the possibility of reversion to virulence being of great concern. The more recent finding that polioviruses may become attenuated for their original primate host when passed in cells *in vitro* (2, 3) has once more stimulated attempts to obtain insight into the basic mechanisms governing the appearance and selection of attenuated variants.

In spite of great efforts, study on polioviruses has progressed slowly, mainly owing to the high cost and difficulty in maintaining a stock of monkeys large enough to permit work on a sound quantitative basis. In looking for a substitute, it was found that Coxsackie viruses, which are similar to polioviruses in their biological and physical properties, lost pathogenicity for suckling mice when passed in cells *in vitro* (4). Thus a model became available permitting the study of attenuation of enteroviruses much more economically than had been possible so far. Experiments designed to characterize this system will be presented.

Materials and Methods

Cell cultures.—Human amniotic membranes were treated according to a standard method recently developed in our laboratory (5). Growth medium consisted of Eagle's medium (6) supplemented with 0.5 per cent lactalbumin hydrolysate (LaH) and 10 per cent human serum (HuS). Cells from Roux bottles were seeded into cell culture tubes and square screw cap bottles (7). After 24 to 72 hours cultures were rinsed 3 times and used for assay or virus multiplication with maintenance medium containing yeast extract 0.05 per cent, LaH 0.25 per cent (prepared in water as 1 per cent and 5 per cent stock solutions, respectively), and calf serum (CaS) 10 per cent in Hanks's balanced salt solution (BSS) with additional $NaHCO_3$ to give

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a final concentration of 0.11 per cent. Penicillin, streptomycin, and tetracycline were added to give 100 units, 100 μg. and 25 μg. per ml., respectively. Monkey kidney (MoK) cell cultures were prepared as described by Rappaport (8). Growth medium consisted of 0.5 per cent LaH in BSS with 5 per cent CaS (9). After inoculation, cells were maintained under a similar medium with CaS reduced to 1 to 2 per cent and $NaHCO_3$ (added as a 1.4 per cent solution in water) increased to a final concentration of 0.14 per cent.

Suckling Mice.—Litters from Swiss albino mothers were used when 12 to 36 hours old.

Viruses.—Coxsackie group A viruses had had a history of passages in newborn mice only. We are indebted to Dr. G. Dalldorf for strains "Belgium 1" (A-11), "Flores" (A-13), "G 14" (A-14), "G 9" (A-15), and "G 13" (A-18). From Dr. J. L. Melnick we received "Wiederhold" (A-9) and "Alaska" (A-10). Pools of group A viruses were prepared in suckling mice. After intraperitoneal injection of 0.05 ml. of mouse suspension containing 100 to 1000 LD_{50} the animals were killed with ether when extensive paralysis had developed. They were skinned, eviscerated, and ground with alundum in a mortar. A 1:10 suspension (*w/v*) was prepared with BSS containing 10 per cent inactivated CaS and antibiotics. After centrifugation the supernate was stored in ampoules at −30°C. This virus source was considered 10^{-1} for further calculations. When this study was initiated only Coxsackie B viruses, which had previously been passed in HeLa and MoK cells, were available. "Connecticut-5" (B-1) had been passed 17 times in HeLa and once in MoK cells before a working pool from MoK cell cultures was prepared. "Ohio 1" (B-2) had had a history of 8 HeLa and 8 MoK passages, "Nancy" (B-3) of 12 HeLa passages, and "Powers" (B-4) of 14 HeLa and 6 MoK passages before a final MoK pool was prepared. "Faulkner" (B-5) had been maintained in HeLa and MoK (3 and 1 passages, respectively) prior to pool production. These viruses had come to our laboratory originally from Dr. A. B. Sabin (B-2 and B-4), Dr. J. L. Melnick (B-3), Dr. G. Dalldorf (B-5), and from the American Type Culture Collection (B-1). Pools from MoK cultures were prepared as follows: monolayer cultures in 200 ml. bottles were infected with high doses of virus. After almost complete cell destruction, cells and medium were frozen and thawed once, centrifuged, and stored in ampoules at −30°C. For calculations this material was considered to be undiluted (10°C.).

Through the kind help of Dr. J. L. Melnick, Dr. A. B. Sabin, and Dr. L. Rosen it later became possible to include the same Coxsackie group B strains without any cell culture history. Pools as 10 per cent mouse suspension were prepared as with group A viruses, except that the intracerebral route of inoculation (0.02 ml.) was employed.

Passages.—For high concentration passages, between 100 and 10,000 $TCID_{50}$ were inoculated into tube cultures. After extensive cytopathic effect (CPE) had developed, cells and medium were frozen and thawed once, pooled, and used for further passage under similar conditions. For high dilution passages, cultures were infected as for titration. All tubes of the highest dilution showing CPE were frozen and thawed once and the pooled contents were used for the next passage.

Back passages in mice were done at high concentration only. In the case of group A viruses 0.05 ml. of a 10 per cent virus suspension was inoculated intraperitoneally into each of 6 to 8 mice. Animals were killed when paralysis was extensive or by the 5th day if no symptoms developed. A 10 per cent suspension from at least 4 mice was prepared and used for further passage. The procedure for group B viruses was essentially the same except that 0.02 ml. were inoculated intracerebrally *and* 0.05 ml. were inoculated intraperitoneally.

Clonal Selection of Viruses.—Although most of the Coxsackie viruses used in this study produced a marked CPE on amnion cells under a liquid medium, A-10, A-11, A-13, A-14, A-15, and A-18 and B-2 and B-4 developed plaques under agar irregularly and of pin-point size only. Therefore, purification was attempted by limiting dilution procedure. 5 to 6 tubes per tenfold dilution step were inoculated. Cultures showing CPE were frozen at −20°C. At the end of the observation period of 8 days the content of a single tube from a dilution at

least 1 step above the calculated $TCID_{50}$ was used for further passages in the same manner. Since some of the Coxsackie viruses produce little CPE with low virus concentrations even after prolonged observation periods (10), end points are not always sharp and might well be underestimated. That this imposes serious limitations on purification procedures based on the limiting dilution principle is obvious. No other approach, however, was available. Types A-9 and B-3 were also plaque-purified. Virus diluted in 0.5 log steps was spread on cell monolayers in square screw cap bottles. After 2 hours' adsorption, cultures were rinsed 3 times with 5 ml. BSS and overlaid with a medium containing 0.75 per cent agar. After 36 to 48 hours a second overlay with neutral red 1:10,000 was added. Discrete plaques from bottles containing only 1 to 3 plaques were picked with a bent pipette, transferred to 1 ml. BSS, and frozen and thawed once. After filtration through a membrane filter (Millipore type HA) this suspension was again diluted and directly transferred onto new monolayers for further plaque passage.

Virus Titrations.—6 tube cell cultures per tenfold serial dilution of virus were used. Virus was diluted in maintenance medium and inoculated in 1 ml. amounts into cultures. All final readings were done on the 9th day. Titrations in suckling mice were performed by inoculating 0.05 ml. intraperitoneally or 0.02 ml. intracerebrally for group A and B virus respectively into at least 8 mice per tenfold dilution step. All mice dead between the 4th and 14th day were considered positive. The titers were calculated according to Behrens (11) and Reed and Muench (12), and expressed on a 1 ml., 0.05 ml., and 0.02 ml. basis for titrations done in cell cultures and mice, group A and group B, respectively. Passages in amnion cell cultures resulted in a marked attenuation of the viruses for mice, associated with a significant flattening of the dose-response curve. Accordingly attenuated viruses were titrated in duplicate or triplicate to compensate for loss of accuracy.

Virus Neutralization Tests.—Monkey immune sera, previously prepared in our laboratory and tested by Dr. R. L. Crowell of this department, diluted 1:20 in maintenance medium, were added to an equal volume of virus suspension diluted to contain approximately 200 $TCID_{50}$/ml. After 1 hour of incubation at 37°C., 1 ml. of this mixture was added to each of 6 tissue culture tubes. The final reading was done on the 9th day. Appropriate controls were included to ensure the correct amount of the challenging virus.

Definitions.—"Adaptation" is used to indicate increase in titer and degree of cell destruction and a decrease in time of appearance of CPE in the course of passage of the virus in cell cultures. "Attenuation" or "loss of virulence" denotes decrease of pathogenicity of the virus for mice when control titrations in cell cultures fail to show a corresponding drop of titer.

EXPERIMENTAL

Adaptation to Human Amnion Cells in Primary Culture.—From the 24 Coxsackie prototype viruses tested in human amnion cells *in vitro*, A-9, A-10, A-11, A-13, A-14, A-15, and A-18 and B-1 to B-5 could be passed with CPE (10). Continuous high concentration passage of these 7 group A and 5 group B strains resulted in increase in infectious titer and proportion of cells destroyed and a decrease in time for the appearance of CPE. Although these observations were unequivocal, definite conclusions as to the degree of adaptation could not be drawn from the results. Therefore an experiment was done in which 1st and 10th amnion passage viruses were compared under more controlled conditions.

Cultures were prepared from a single placenta. They were inoculated with the original mouse or monkey kidney virus and with the 9th amnion passage virus of types A-9, A-11,

A-13, A-15, A-18, B-1, B-2, B-3, and B-5. An attempt was made to use equal infectious doses for both passage levels. The 10th passage cultures showed cell destruction faster and more extensively than those of the 1st passage. This phenomenon was particularly striking in the case of the group A viruses which will be considered first. Comparative titrations of the 1st and 10th passage pools prepared as described were performed using tube cultures from 1 placenta.

Results are shown in Table I. It can be seen that in all cases the 10th passage titers exceeded those of the 1st passages. The differences were marked in most cases and left no doubt as to their significance. Since A-10 and A-14 showed no CPE with 1st amnion passage, they were not included in the comparative

TABLE I

Titers in Amnion Cell Cultures and in Suckling Mice of Coxsackie A Virus Strains, 1st and 10th Amnion Passages

Coxsackie type	Titer* of amnion virus in			
	mice		amnion	
Passage No.....	1	10	1	10
A-9	3.6	3.4	6.8	8.2
A-11	3.7	<1.0	2.5	5.7
A-13	4.9	1.0	5.5	7.0
A-15	5.4	<1.0	4.5	5.5
A-18	2.9	2.0	5.7	6.3

Cultures from a single placenta were inoculated with original mouse virus and 9th amnion passage virus. Comparative titrations of 1st and 10th passage pools thus obtained were performed in mice and in tube cultures from 1 amnion.

* Negative log LD_{50} per 0.05 ml. and $TCID_{50}$ per 1 ml., respectively.

assay just described. The extent of adaptation can, however, be assessed from Table II which shows significant increase of infectious titers with successive amnion passages.

The group B viruses showed little, if any, adaptation when 1st and 10th amnion passage of viruses previously maintained in cell cultures were compared by amnion cell assay. When reference is made to viruses without *in vitro* history, adaptation with continuous passages in cell cultures of primate origin appears to be very pronounced (Table III). Only the "Nancy" strain of B-3 virus exerted extensive cell destruction with high titers from the beginning and did not change this property with passages in cells.

Attenuation of Virulence for Newborn Mice.—When comparable virus preparations from 2 separate culture passages were titrated in mice it was seen that the gain in infectivity for cells *in vitro* was associated with a decreased pathogenicity for mice (Table I). Attenuation was most obvious with types A-11 and A-15. Here the inoculation of more than 10,000 $TCID_{50}$ caused the

TABLE II

Comparative Titrations in Amnion Cell Cultures and in Suckling Mice of Coxsackie A Viruses after Passages in Amnion Cells

Coxsackie type	Assayed in	Titers* after passages in amnion			
		0	1	10	15
A-9	Amnion	7.5	6.8	8.2	8.7
	Mice	4.6	3.6	3.4	3.9
A-10	Amnion	≦1.5	n.d.‡	5.4	5.2
	Mice	6.8	n.d.	4.5	3.2
A-14	Amnion	≦1.5	n.d.	5.0	6.6
	Mice	6.1	n.d.	1.0	1.4

The stored virus from amnion passages 1, 10, and 15 together with the original material was titrated in mice and in tube cultures from human amniotic membranes.

* Negative log LD_{50} per 0.05 ml. and $TCID_{50}$ per 1 ml., respectively.

‡ n.d., not done.

TABLE III

Comparative Titrations in Amnion Cell Cultures and in Suckling Mice of Coxsackie B Virus Strains after Passages in Cell Cultures

Coxsackie type	Assayed in	Titers*				
		Of original mouse passage virus	After passages in MoK and HeLa cell cultures	After passages in amnion cell cultures		
				1	10	15
B-1	Amnion	3.3	5.5	6.4	6.5	7.7
	Mice	6.4	5.5	1.5	<1.0	<1.0
B-2	Amnion	2.0	4.9	6.5	6.0	5.9
	Mice	5.4	<1.0	<1.0	<1.0	<1.0
B-3	Amnion	7.8	8.0	6.6	6.7	6.7
	Mice	6.4	5.8	4.8	4.4	4.8
B-4	Amnion	≦1.5	2.3	<1.0	3.5	5.3
	Mice	6.5	5.4	n.d.‡	<1.0	<1.0
B-5	Amnion	5.5	5.6	5.0	6.3	7.3
	Mice	4.0	1.4	<1.0	<1.0	<1.0

Monkey kidney prepared viruses after passages in HeLa and monkey kidney cells and amnion prepared viruses after 1, 10, and 15 passages in amnion cells together with original mouse passage virus were titrated in mice and in tube cultures from human amniotic membranes.

* Negative log LD_{50} per 0.02 ml. and $TCID_{50}$ per 1 ml.

‡ n.d., not done.

death of less than 50 per cent of the mice. The most conspicuous finding in the surviving animals was their retarded growth compared with the control litter mates. Only some of the mice appeared sick, and occasionally mild paralysis could be observed. Suckling mice infected with undiluted 10th amnion passage virus of Coxsackie A-11 and A-15 showed histologically isolated focal areas of muscle destruction as described by Gifford and Dalldorf (13). In order to gain some insight into the kinetics of this attenuation the following experiments were performed.

Five additional high concentration passages were carried out in amnion cells. The stored suspensions from amnion passages 0 to 6, 8, 10, and 15 of A-11, A-13, A-15, and A-18 were titrated in suckling mice and also in amnion cell cultures. For A-9, A-10, and A-14 only passage numbers 0, 1, 10, and 15 were similarly titrated in both host systems.

The results are presented in Fig. 1 and Table II. Kinetics of attenuation seem to be similar for all viruses tested. The speed, however, with which the lowest levels of virulence for mice were reached differed from virus to virus. With some strains attenuation progressed so fast that it became doubtful whether virus which was still virulent for mice had multiplied at all in amnion. Inactivation rates of these viruses at 37°C. in cell culture medium were determined (14). Calculated titers based on thermal inactivation and dilution are included in Fig. 1. A significant gap between titers thus expected and titers observed indicates that multiplication of mouse virulent viruses in amnion took place. Results with group B viruses treated in the same manner are given in Table III. For comparison, titers obtained with original mouse passage virus and viruses grown in MoK cells after passages in MoK and HeLa cell cultures have been included. The results correspond to those seen with group A. Again, B-3 behaved as an exception. No attenuation for mice with consecutive passages in human cells *in vitro* could be detected.

Stability of attenuation was checked by passing attenuated viruses in high concentration in mice. Coxsackie A-11 and A-15 after 10 amnion passages and all 12 strains after 15 amnion passages were thus tested. Results are presented in Figs. 2 to 4. While A-15, B-1, B-2, and B-5 revealed a certain stability in that they did not reach their original mouse titers after several passages in mice, most group A viruses and B-4 reverted to their original levels of infectivity within 1 or 2 mouse passages.

A-15, B-1, B-2, and B-5 were passed further in mice which led to an additional increase of virulence (Fig. 5). When these materials were also titrated in human amnion cell cultures it was found that, irrespective of low or increased virulence for mice, large amounts of amnion pathogenic viruses were produced *in vivo* at each passage level (Fig. 5). Extent of CPE and infectious titers for cells remained high except for B-2, for which titers in mice and in amnion cells varied inversely. Assay in MoK cells revealed that large amounts

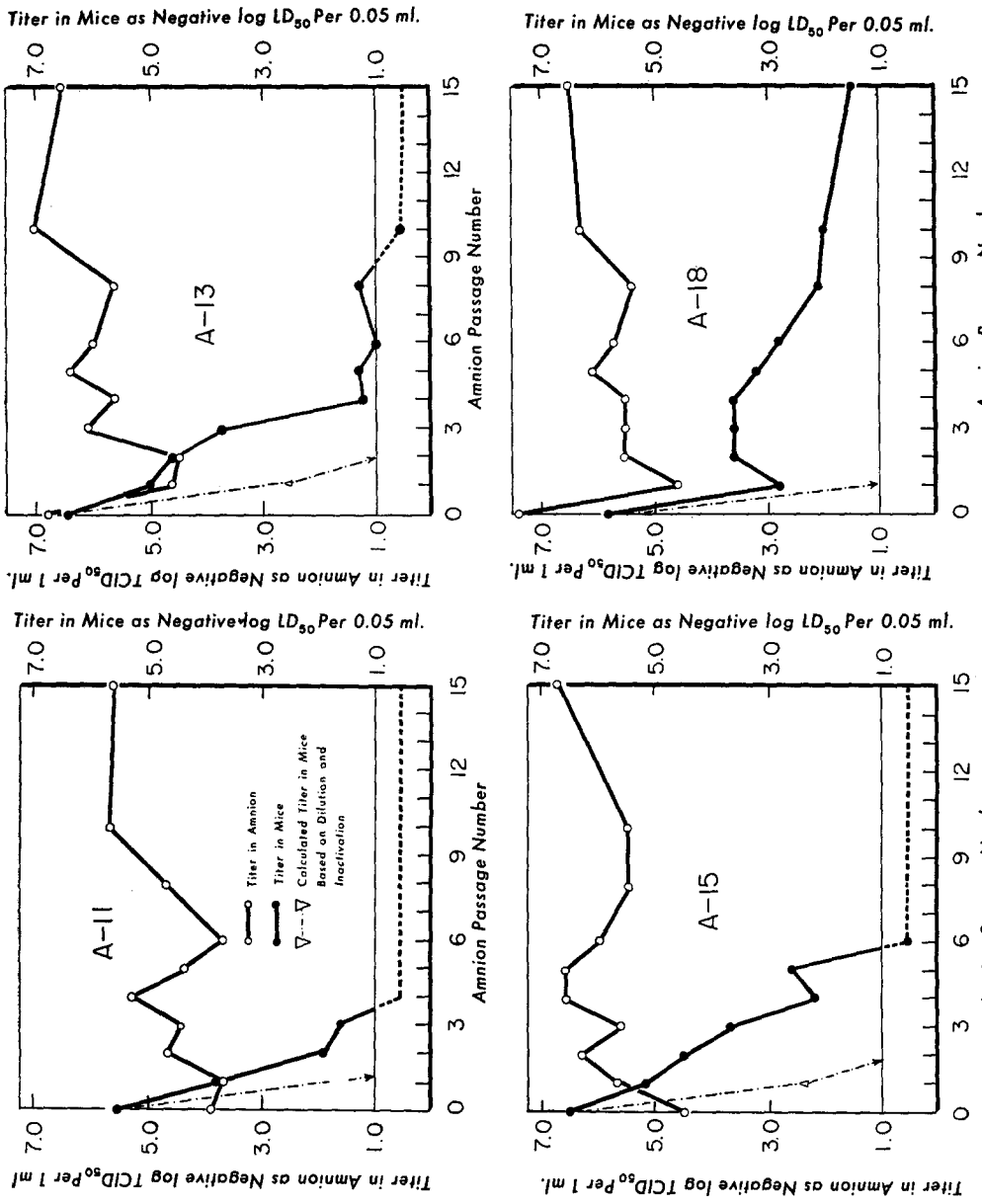

FIG. 1. Stored virus from amnion passages 1 to 6, 8, 10, and 15 together with original mouse passage virus were titrated in suckling mice (closed circles) and in amnion cell cultures (open circles). Interrupted lines indicate expected titers from dilution and heat inactivation with successive amnion passages.

of virus were produced in mice irrespective of the degree of pathogenicity for amnion cells or for mice.

These observations suggested that the virus preparations contained two types of particles, one of high, the other of low mouse virulence. If this assumption were true, clonal selection should lead to a purified preparation containing one or the other variant only.

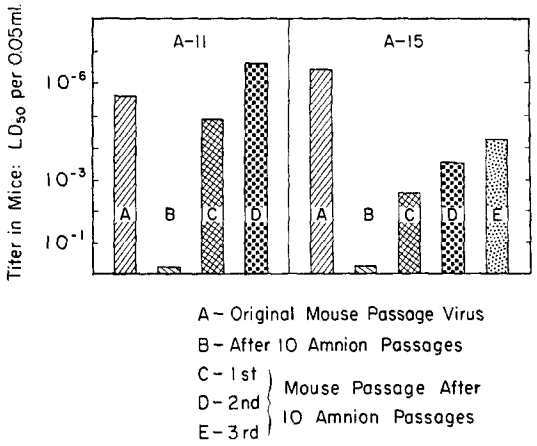

FIG. 2. After 10 high concentration passages in amnion cells viruses were passed in newborn mice again. Titers in mice as obtained with original material, after passages in human amnion cells, and back passages in mice are shown.

Clonal purification was attempted with all viruses showing little (A-15) or no stability (A-9, A-10, A-11, A-13, A-14, A-18, B-3, and B-4) of attenuation when passed back in mice. In addition to the 15 high concentration passages, 5 high dilution passages followed by 4 limiting dilution passages were performed as described under Methods. Production of final (25th amnion passage) pools was done by inoculating approximately 10 $TCID_{50}$ per ml. into bottle cultures. A-9 and B-3 were also 3 times plaque-purified after 15 passages at high concentration, final pools being prepared as for limiting dilution purification.

Comparative titrations of these purified viruses in amnion cells and in suckling mice indicated further attenuation of most viruses (see Figs. 6 and 7). After triple plaque purifications, as compared with limiting dilution procedure, A-9 appeared to be more attenuated. Neither limiting dilution nor picking of plaques significantly changed the original properties of B-3.

Stability of attenuation after purification was tested with A-9 (plaque-purified) and A-11, A-13, and A-15 viruses. In contrast to the results obtained after high concentration passages only (see Fig. 3) purified viruses shared a degree of stability (Fig. 8) similar to that seen before with B-1, B-2, and B-5. It is noteworthy that limiting dilution procedure markedly increased the stability of A-15 which had shown slow reversion to virulence after high con-

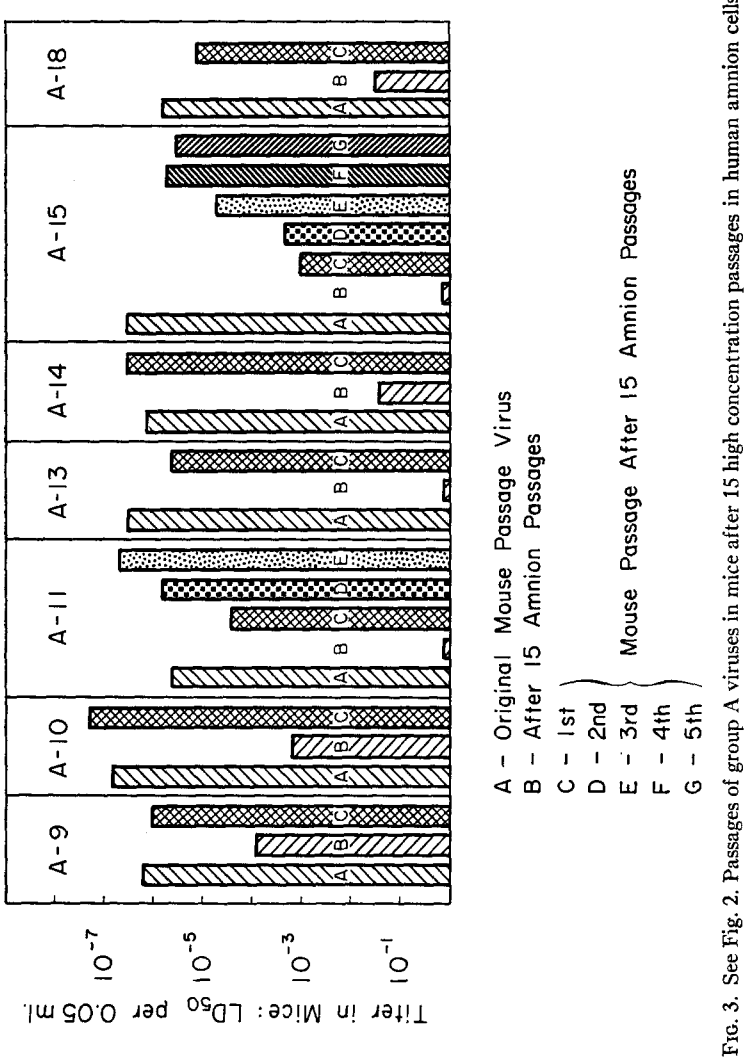

FIG. 3. See Fig. 2. Passages of group A viruses in mice after 15 high concentration passages in human amnion cells.

centration passages. Again, a large amount of amnion pathogenic virus was produced in the mice at each passage level, irrespective of whether virulence for mice slowly increased as seen with A-11, A-13, and A-15 or remained essentially low as with A-9.

Neutralization of viruses with homologous immune sera was done after 10, 15, and 25 amnion cell passages and with material from final back passages in mice. In all cases viruses were rendered non-infective after interaction with homologous antiserum. It should, however, be stressed that minor antigenic

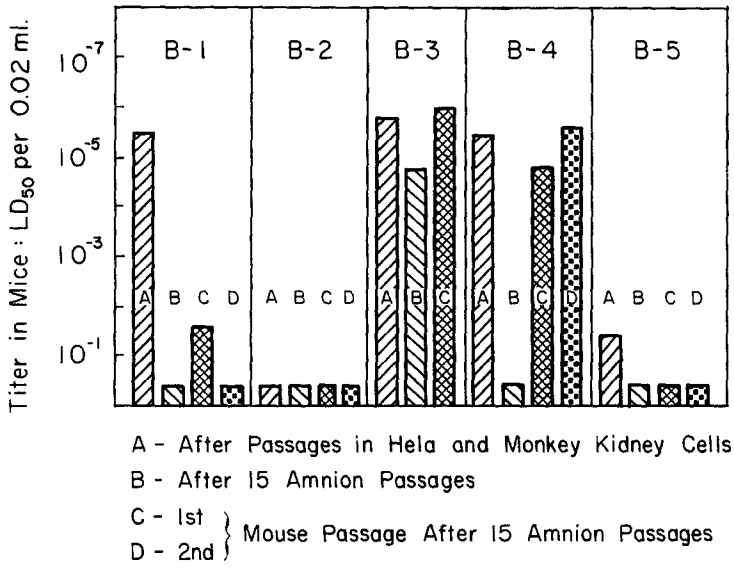

FIG. 4. See Fig. 2. Passages of group B viruses in newborn mice after 15 high concentration passages in human amnion cells.

changes would have remained undetected by the qualitative technique employed.

The dose-response curve in mice of most Coxsackie viruses is remarkably steep, extending usually over only 2 to 3 $\log_{10}$ dilution steps. Furthermore, recovery of mice from Coxsackie disease is exceptional. In contrast, after attenuation some strains did not induce any overt signs in mice when large amounts of virus had been inoculated (*e.g.*, A-11, A-13, A-15, and B-4); others (*e.g.*, B-1, B-2, and B-5) caused retarded growth, occasional paralysis, and some deaths even at high dilutions although undiluted cell culture fluids killed less than 50 per cent of the inoculated animals. The flattening of the dose-response curves became particularly pronounced at intermediate stages of reversion to virulence after some back passages in mice, with specific deaths extending over a range up to 10^6 $TCID_{50}$.

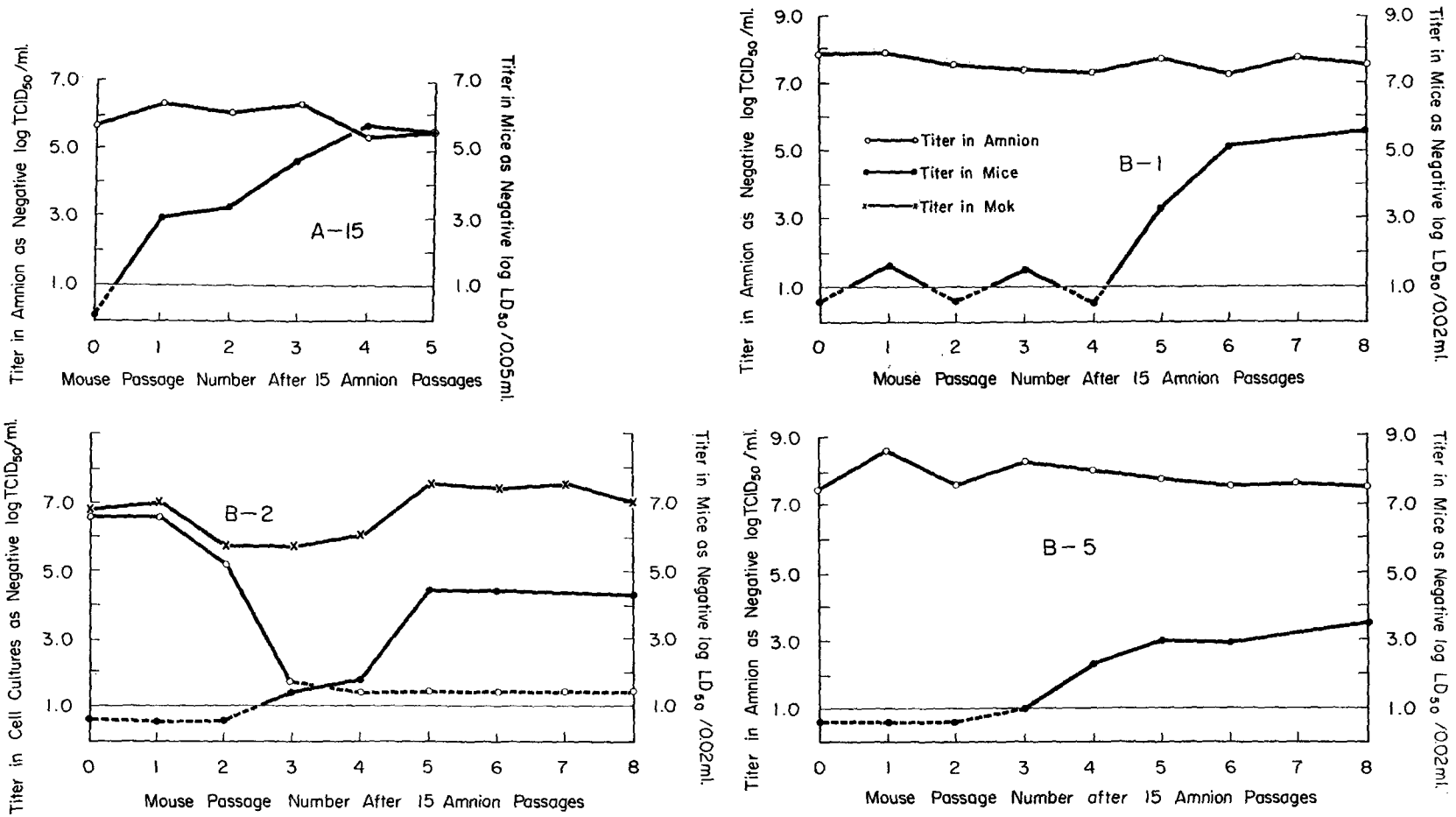

FIG. 5. After 15 high concentration passages in amnion cells viruses were again passed in newborn mice. Titers in mice (closed circles) of various back passages *in vivo* are shown in comparison with titers as obtained in human amnion cell cultures (open circles). Crosses (×) denote results from assays in monkey kidney cultures.

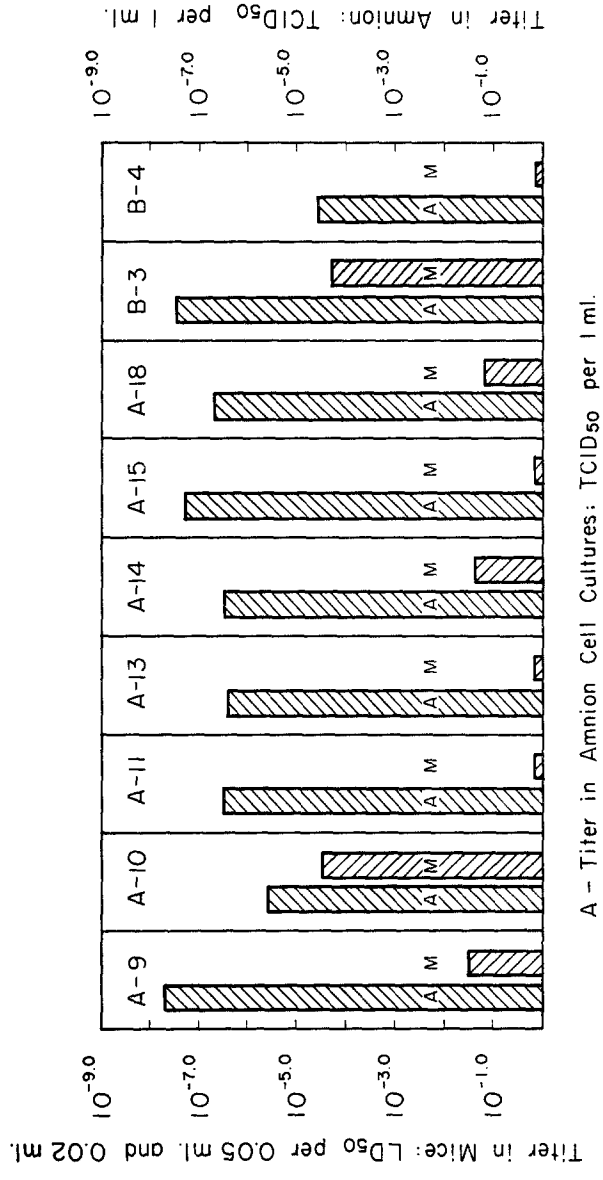

FIG. 6. Comparative titrations in amnion cell cultures and in newborn mice after 15 high concentration passages followed by 5 passages in high dilution, 4 in limiting dilution, and again 1 in high dilution.

DISCUSSION

Attenuation of animal viruses for their original host after propagation in cells *in vitro* seems to be the rule rather than the exception. After Theiler's classical work with yellow fever virus which led to the 17 D variant (15) a variety of different agents with decreased virulence for their previous animal hosts have been obtained by passages in tissue or cell cultures. The viruses of Venezuelan equine encephalomyelitis (16, 17), fowl plague (18), Newcastle

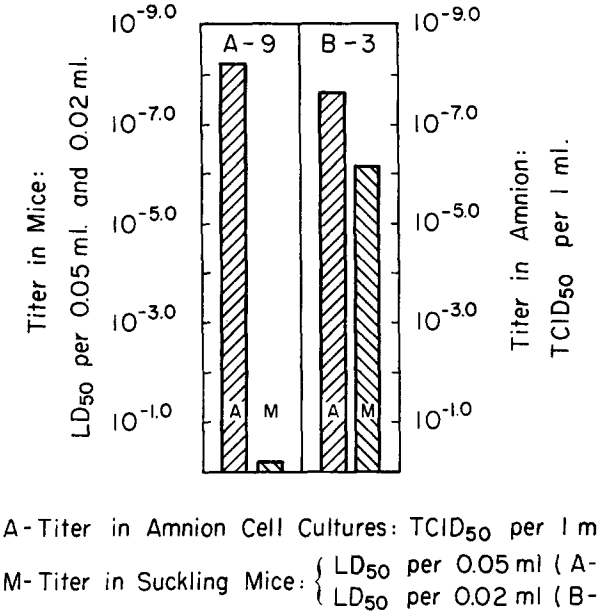

FIG. 7. Comparative titrations in amnion cell cultures and in newborn mice after 15 high concentration passages followed by 3 successive plaque purifications.

disease (19, 20), Teschen disease of swine (21), West Nile virus (22), Col SK strain of the encephalomyocarditis group (23), and the 3 types of polioviruses (24) are examples. Attenuated variants of certain Coxsackie strains have also been obtained (25–28). Continuous passages of Coxsackie prototype strains in human amnion cells resulted in increase in infectious titer and degree of cell destruction, and a decrease in time for the appearance of CPE in 11 out of 12 strains. This gain in infectivity was in all cases associated with a decreased pathogenicity for mice. The speed with which some strains became attenuated is remarkable (Fig. 1). Yet in most cases 15 passages employing large inocula did not lead to any degree of genetic stability. With the exception of A-15, B-1, B-2, and B-5, titers reached their original values after 1 or 2 back passages in mice. However, limiting dilution and plaque purification changed the

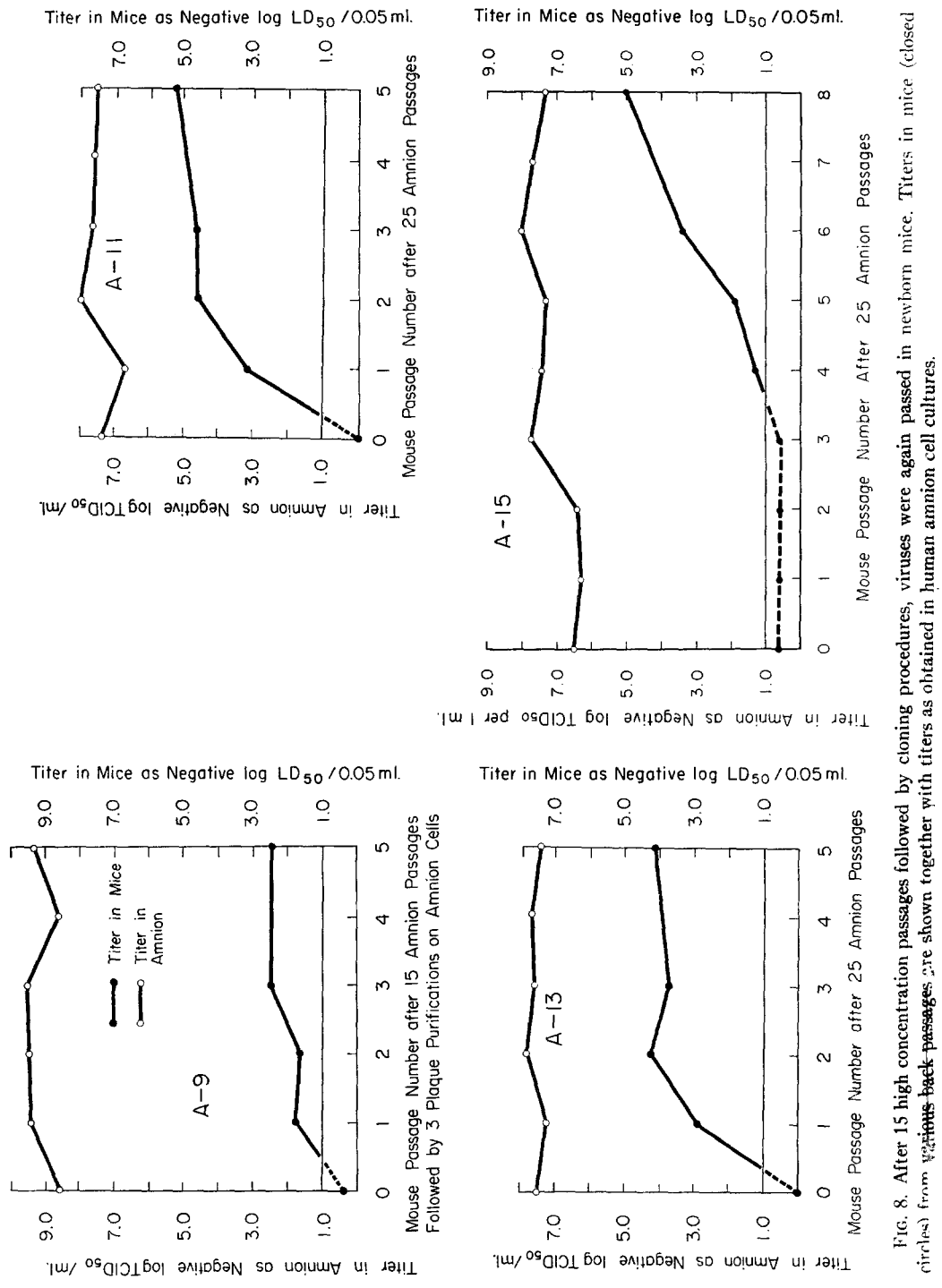

FIG. 8. After 15 high concentration passages followed by cloning procedures, viruses were again passed in newborn mice. Titers in mice (closed circles) from various back passages are shown together with titers as obtained in human amnion cell cultures.

pattern, and greater stability upon back passages became evident. Irrespective of virulence for mice as determined by infectivity titrations, large amounts of amnion pathogenic virus were produced *in vivo*.

Although the course of adaptation and attenuation of Coxsackie viruses after passages in cells of primate origin can readily be recognized from our results not much can be said about the underlying principles which are operative. It is likely that a genetic mechanism is involved. The only alternative, namely a host-induced change which has been shown to occur with bacterial viruses (29, 30) and possibly with animal viruses (31, 32) should lead to phenotypic changes only. The possibility of a host-induced change which is ultimately genotypic (33) cannot, however, be ruled out. It must also be assumed that adaptation of Coxsackie viruses to cells *in vitro* and attenuation to mice occur—with the possible exception of B-2—independently of each other. Reversion to mouse virulence did not as a rule lead to a corresponding drop of pathogenicity for cells *in vitro*. There can be little doubt that the change in virulence of Coxsackie viruses after passages in cells of primate origin is due to a qualitative alteration of the virus particles. The other possible explanation is that a large proportion of amnion infective virus is completely avirulent for mice and that the comparatively low titers *in vivo* are due to a small number of fully virulent mouse particles. The flattened dose-response curves as obtained with all attenuated variants strongly points towards a qualitative change (34). That interference due to some factor produced in amnion cells is operative in mice only is unlikely because attenuated variants maintained their properties over several back passages in mice. Furthermore, a limited number of experiments did not indicate any ability of attenuated virus produced in amnion to interfere with the fully virulent original agent in mice (14).

The following hypothesis seems to explain adequately most of the observations. The virus as it is passed and propagated in suckling mice is pathogenic for mice and to a much lesser degree for certain cells in culture. Mutations leading to a variant with decreased mouse pathogenicity, and increased effect on cells in culture, occur at a high rate. This variant which is at a competitive disadvantage in mice outgrows the original type in cells *in vitro*.

No explanation can be offered for the genetic instability of attenuated viruses which must be inferred from the gradual reversion to virulence—even after purifying procedures—upon back passage in mice. Burnet and Lind (35) found a similar phenomenon with influenza virus. Dulbecco and Vogt, when studying the stability of poliovirus mutants which had a low plating efficiency under acid agar, found likewise a stepwise reversion towards the original wild type (36). It is also well known that attenuated polioviruses, even after purification procedures, can revert to increased neurovirulence following multiplication in man (37, 38) or when passed in human cells in culture (39).

The reduced pathogenicity of the viruses for mice in spite of unaltered ability

to multiply is also unexplained as yet. It could be a change of tissue tropism with shift of multiplication to organs not essential for survival. It also could be due to a reduced speed of multiplication *in vivo*, thus permitting the mouse to reach a certain critical age beyond which even maximal amounts of virus do not cause overt paralysis, as was found by Eggers and Sabin with ECHO 9 after passage in monkey kidney cells in culture (40). Experiments are now planned to obtain more information about the action of attenuated Coxsackie viruses in mice.

In view of the recent discussion in the literature concerning classification of the group of enteroviruses, our findings are significant. It seems that any classification based solely or mainly on the host spectrum of the agent can lead to conclusions not always justified, if its previous history—in nature or in the laboratory—can be suspected to have altered the host spectrum. It is possible that some of the Coxsackie viruses, if tested in mice after isolation and passages in cell culture, would have been classified as ECHO types. A similar confusion might have resulted as it now exists with ECHO 9, whose unexpected mouse pathogenicity led to attempts to reclassify the Coxsackie and ECHO viruses (41, 42). Probably for such reasons, in the discussions on nomenclature at the Fifth International Congress of Microbiology held in 1950 at Rio de Janeiro, there was "unanimous" opinion that "symptoms produced in the host should, with emphasis, be placed at the bottom" of the list of criteria to be applied in classifying viruses (43).

SUMMARY

Continuous passages in primary human amnion cell cultures of Coxsackie prototype viruses A-9, A-10, A-11, A-13, A-14, A-15, A-18, B-1, B-2, B-4, and B-5 increased the titers, hastened and enhanced cytopathic effect, and in varying degrees caused loss of virulence for newborn mice. Only B-3 behaved differently in that neither adaptation to cells *in vitro* nor attenuation with respect to the original animal host could be observed. Types A-15, B-1, B-2, and B-5 slowly regained virulence when passed in mice after high concentration passages in amnion cells whereas all other viruses reverted to their original virulence after only 1 or 2 passages in the animal host. When these strains, however, were purified by cloning procedures, they too showed markedly increased stability. In all stages of attenuation, viruses multiplied extensively *in vivo* as could be shown by titration in amnion cell cultures. It is suggested that a genetic mechanism is operative leading to virus populations in which the majority of the particles is qualitatively changed.

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