RELATIONSHIP OF DONOR AGE TO IN VITRO PRODUCTION OF FOOT-AND-MOUTH DISEASE VIRUS BY MOUSE KIDNEY CELLS

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The development of age resistance by mice has been reported for a number of viral infections including infection wth foot-and-mouth disease virus (FMDV). Skinner (1) and Graves and Poppensiek (2) found that mice are rather uniformly susceptible to FMDV until they are approximately 2 weeks old, after which they progressively become resistant. At 5 to 6 weeks of age very few remain susceptible.

Although age resistance has been demonstrated many times, the mechanism of this resistance is not clear (3). A number of possibilities have been investigated or suggested including blocking of virus pathways (4–7), production of inhibitory substances (8), production of more effective immunological responses with maturation (9, 10), and hormonal effects (11). There is also the possibility that age resistance is related to the production during maturation of cells resistant to virus infection. The studies of Goodman and Koprowski (12) and Kantoch *et al.* (13) on the association of cell susceptibility to susceptibility of cell donors provide evidence for this hypothesis. Previous to these investigations, Sabin and Olitsky (5) found a relation between intramuscular multiplication of vesicular stomatitis virus and age of the animal, and recently Lerner *et al.* (14) reported a similar relationship with Coxsackie A4 virus. Also working with Coxsackie viruses, Kunin (15) obtained evidence which indicated that the susceptibility of animals at various ages was related to the relative amount of receptor-like substances in the tissues. The experiments presented here were performed, therefore, to investigate the possibility of a cellular mechanism for the development of age resistance to FMDV. Kidney cells from mice 1 through 5 weeks of age were exposed to virus, and virus growth curves were obtained by assay of samples removed at various intervals. These growth curves were then compared to determine if a relationship existed between virus production and age of the cell donors.

Materials and Methods

Virus.—Foot-and-mouth disease virus (FMDV), type A, strain 119, passaged 115 to 119 times in primary monolayer cultures of calf kidney cells was used throughout the experiments.

Culture fluid containing virus was centrifuged at 900 *g* to remove cellular debris, after which the infectious supernatant fluid was stored in glass ampoules at −40°C.

Virus Assay.—Virus assays were performed by the plaque method of Bachrach *et al.* (16) in primary cultures of calf kidney cells contained in 4-ounce prescription bottles (17). In brief, duplicate cultures were inoculated with serial 10-fold dilutions of the virus samples, each culture receiving an inoculum of 0.1 ml. The cultures then were incubated at 37°C for 60 minutes to permit virus adsorption, after which the nutrient fluid and agar overlay was added. Plaques were counted after incubation of the cultures in an inverted position at 37°C for 48 hours.

Mouse Kidney Cell Preparations.—Production of FMDV was studied in three types of cell preparations from the kidneys of 7-, 14-, 21-, 28-, and 35-day-old mice. Swiss mice of the Rockefeller H strain produced at this laboratory were used in all of the experiments. The cells in each preparation were representative of a pool of kidneys removed aseptically from at least 5 mice of the same age. After being washed in several changes of Hanks' balanced salt solution (BSS), the kidneys from the mice of the several age groups were processed simultaneously for culture preparation.

Monolayer cultures: Kidneys were minced finely with scissors after which the fragments were transferred to trypsinizing flasks containing 0.25 per cent trypsin in a ratio of 150 ml for each gm of tissue. Following incubation at 35°C for 20 minutes, the trypsin was decanted and replaced with an equal volume of fresh trypsin. The suspension was then agitated for 30 minutes on a magnetic stirrer, filtered through two layers of gauze and diluted with an equal volume of cold BSS. Dispersed cells were concentrated by centrifugation at 250 *g* for 10 minutes at 6°C, suspended at a concentration of one million cells/ml in BSS containing lactalbumin hydrolysate, 0.5 per cent, and bovine serum, 10 per cent and dispensed in 10-ml amounts into 4-ounce prescription bottles. Cultures were incubated at 37°C, during which time the growth medium was changed at approximately 3-day intervals. Confluent cell sheets were obtained after incubation for 5 to 7 days with cultures from younger animals becoming confluent earlier than those from older mice. When all cultures were confluent, the fluid was removed from 5 cultures from each age group and replaced with 15 ml of BSS containing lactalbumin hydrolysate, 0.5 per cent, and bovine serum, 2 per cent. An equal amount of FMDV calculated to give a final virus concentration of approximately 10^4 plaque-forming units (PFU) per ml, was added to each culture. During incubation at 37°C, the pH of the fluid was maintained at 7.6–7.8 by occasional addition of 2.8 per cent sodium carbonate solution. At times specified by the particular experimental design, 1 ml samples of the fluid were removed from each of the 5 bottles of each group, pooled according to group, and centrifuged at 900 *g* for 10 minutes at 6°C to remove cellular debris. The supernatant fluid was stored at −10°C until it was assayed for virus by the plaque method.

Suspensions of dispersed cells: Mouse kidney tissues were processed and cells were dispersed with trypsin by the method described for the monolayer cultures. After the cells were concentrated by centrifugation, a 20 ml suspension of each group of cells, each containing one million cells/ml, was prepared in BSS containing lactalbumin hydrolysate, 0.5 per cent, and bovine serum, 2 per cent. To each suspension, contained in a 50 ml silicone-coated serum bottle, an equal amount of FMDV calculated to give a final virus concentration of approximately 10^4 PFU/ml was immediately added. The preparations were incubated at 37°C without agitation unless stated otherwise, and the pH of the fluid was maintained at 7.6 to 7.8 with 2.8 per cent sodium carbonate. Samples for virus assay were removed after the preparations were thoroughly agitated by pipetting to produce a homogeneous suspension. Removal of the samples, therefore, did not change the cell-fluid ratio. The samples were then processed as described for the monolayer cultures.

Suspensions of minced tissue: Intact mouse kidneys were washed as described previously

and after being transferred to a dry, wide-mouthed vial they were minced finely with scissors. Suspensions of the tissue fragments in a ratio of 1 gm tissue to 100 ml medium were prepared in BSS containing lactalbumin hydrolysate, 0.5 per cent, and bovine serum, 2 per cent. The amount of minced tissue for each preparation was adjusted by weighing to be about 0.2 gm, so that after addition of medium, the final volume of each was approximately 20 ml. After being placed in 50-ml silicone-coated serum bottles, each suspension was inoculated with FMDV calculated to give a final virus concentration of approximately 10^4 PFU/ml. The preparations were incubated without agitation and samples for virus assay were removed and processed as described for the suspensions of dispersed cells.

Nuclei Counts.—After lysis of the cells with citric acid, the nuclei were stained with crystal violet and counted in a hemocytometer as described by Sanford *et al.* (18).

TABLE I

Response of Mice of Various Ages to FMDV

Mouse age	LD_{50} inoculum per mouse		
	10	100	1000
days			
7	10/10*	10/10	10/10
14	8/10	10/10	10/10
21	2/10	9/10	9/10
28	2/10	2/10	7/10
35	0/10	0/10	4/10

* Numerator, number of mice which died; denominator, number of mice which were inoculated.

RESULTS

Effect of Age on Response of Mice to FMDV.—Since age resistance of mice to FMDV was previously demonstrated, the experimental results recorded in Table I were obtained only to illustrate the response of mice of the particular age groups which were used in this study. Groups of 10 mice, 1 to 5 weeks of age, were inoculated intraperitoneally with 10, 100, and 1000 LD_{50} (determined in 7-day-old mice) and observed for 10 days after inoculation. The response to each quantity of virus administered demonstrates the increase in resistance which develops with age.

Multiplication of FMDV in Monolayer Cultures of Kidney Cells from 1- through 5-Week-Old Mice.—Primary monolayer cultures of cells from kidneys of 1- through 5-week-old mice were inoculated with virus when confluent and samples were removed at 4-hour intervals for virus assay. As shown in Fig. 1, the pattern of virus multiplication for all cultures was similar. There was a brief lag between 0 and 4 hours, but after that, multiplication occurred rapidly and to approximately the same extent in all cultures regardless of cell donor age. Although there is a marked difference in the susceptibility of 1- through

4-week-old mice, exactly the same peak titers of virus multiplication were obtained in cultures prepared from the kidneys of such mice. A decline in virus from the 5 week culture occurred between 16 and 20 hours, but likely this was due to smaller number of cells in this culture. However, the amount of virus

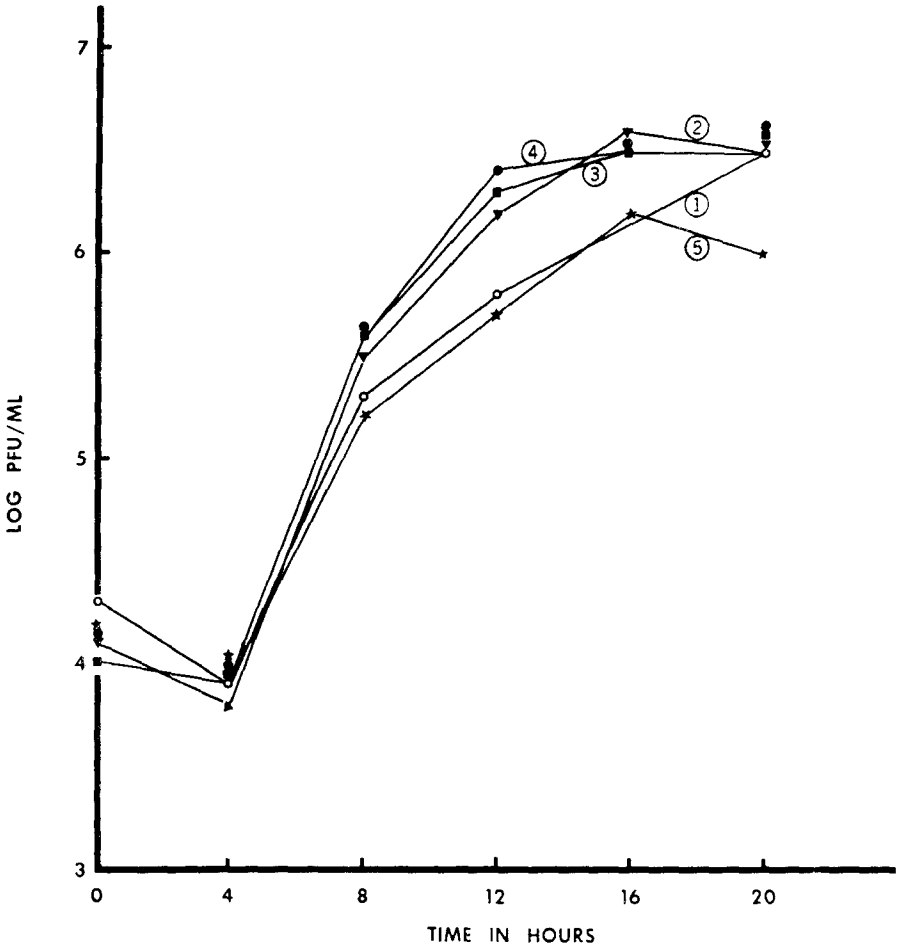

FIG. 1. Multiplication of FMDV A119 in primary monolayer cultures of kidney cells from mice of various ages: 0 to 20 hours after inoculation. Numerals refer to age of cell donors in weeks.

demonstrated in the 5 week culture at 20 hours was not appreciably different from that of the other cultures. The experiment demonstrated therefore, that cultured cells derived from the kidneys of 1- through 5-week-old mice did not differ significantly in their ability to be infected with and produce FMDV.

Multiplication of FMDV in Suspensions of Trypsin-Dispersed Kidney Cells

from 1- through 5-Week-Old Mice.—On the basis of the above results with monolayer cultures, one would assume that in cells from 1- through 5-week-old mice the ability to produce FMDV was not related to the various degrees of resistance of the cell donors. However, Fig. 2 represents the production of FMDV from 0 to 96 hours after inoculation of trypsin-dispersed cell suspensions prepared from kidneys of 1- through 5-week-old mice. This experiment, in contrast to the results with cells in monolayer cultures, demonstrated a striking difference in virus production by cells from the various aged mice. Virus multiplication began earlier in the cells from younger animals and more virus was produced by such cells. After the peak of virus multiplication, the decline was similar in all cases.

To obtain more information on virus adsorption and the beginning of virus multiplication in suspensions of cells from 1- through 5-week-old mice, an experiment was performed in which the amount of virus was determined every 2 hours for 20 hours after inoculation. The results emphasized that the commencement of virus multiplication was related to the age of the donors with cells from younger animals producing virus earlier (Fig. 3). Furthermore, the experiment demonstrated that there was essentially no difference in virus adsorption by the various cells, as evidenced by the amount of extracellular virus present 2 and 4 hours after inoculation.

While these experiments demonstrated that suspensions of kidney cells from younger mice produced virus more rapidly and in higher amounts than cells from older donors, the possibility existed that cellular multiplication, particularly of cells from the younger animals, might have influenced the results. Although culture bottles were coated with silicone to prevent cellular attachment, a deposit of cells was observed on the bottom of the bottles after a day or 2, particularly in those bottles containing cells from younger animals. To determine if cells were multiplying after settling to the bottom of the culture vessel, experiments were performed in which duplicate suspensions were prepared from kidneys of 1- and 5-week-old mice. One culture of each group was agitated by a mechanical shaker to prevent the cells from settling; while the other culture was incubated, as usual, without agitation. Nuclei counts were performed on the cell residue of each 4 hour sample after the fluid phase had been collected for virus assay.

The results of one of these experiments is illustrated in Fig. 4. There was a slight decrease in the number of nuclei from all cultures after incubation for 4 hours, but afterwards there was very little change in counts for the agitated cells from 1-week-old mice and in either of the suspensions from 5-week-old mice. However, the number of nuclei from the stationary suspension of 1-week-old mouse cells declined steadily over the 24 hour period. This probably was due to cells adhering to the glass after settling to the bottom. Whether such cells multiplied is not known, since no attempt to remove them was made,

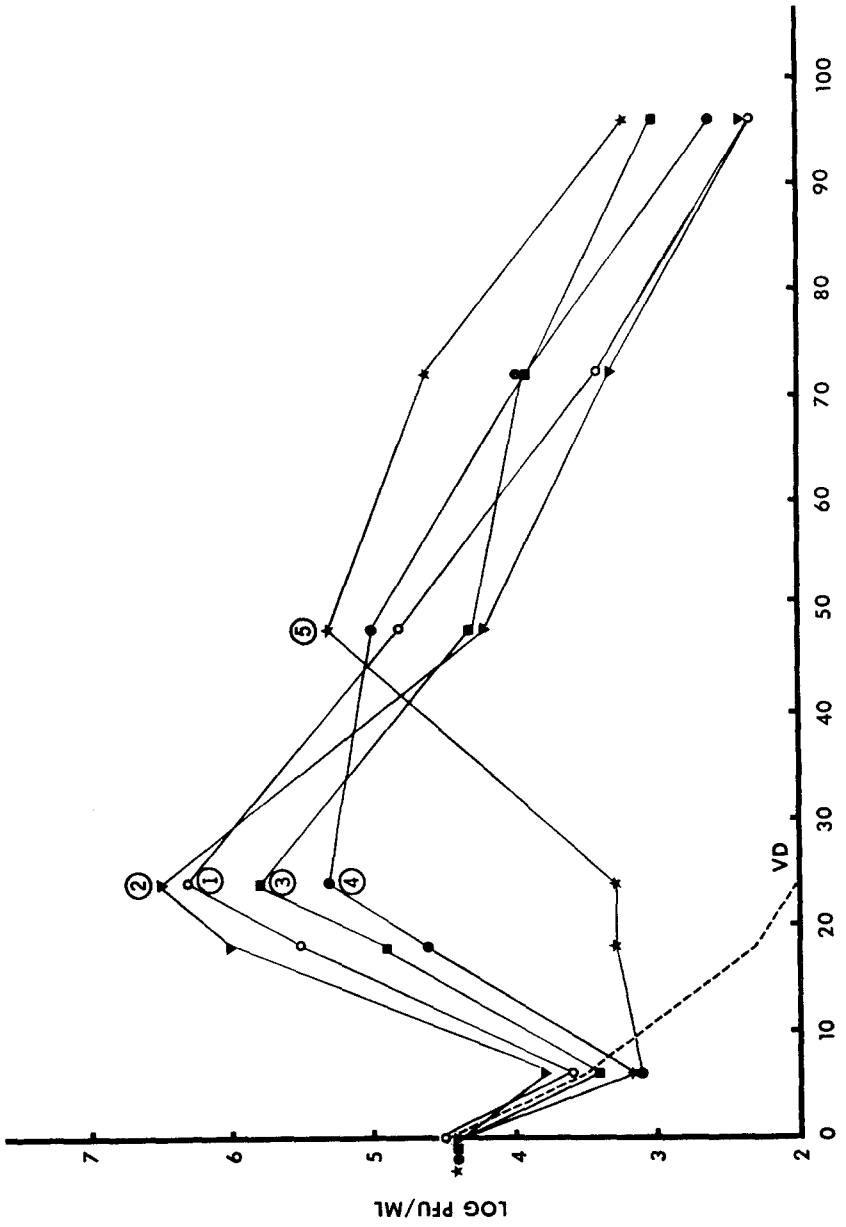

FIG. 2. Multiplication of FMDV A119 in suspensions of trypsin-dispersed kidney cells from mice of various ages: 0 to 96 hours after inoculation. Numerals refer to age of cell donors in weeks. VD, virus decay rate at 37°C.

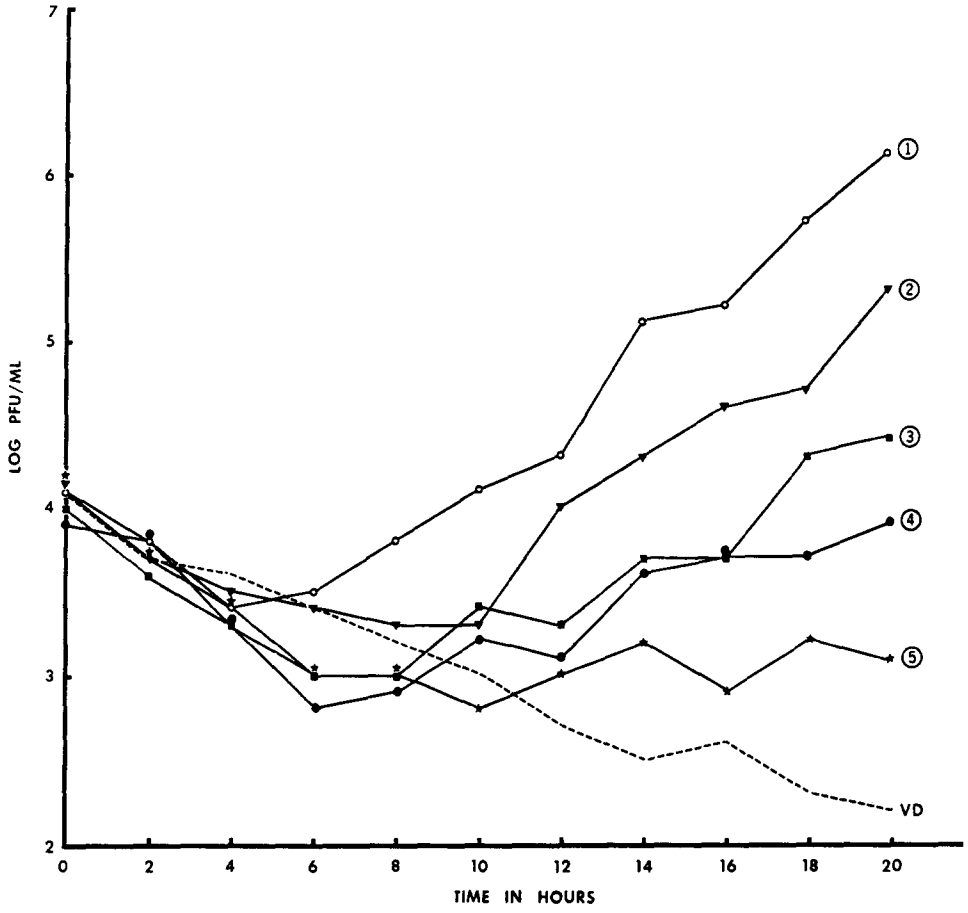

FIG. 3. Multiplication of FMDV A119 in suspensions of trypsin-dispersed kidney cells from mice of various ages: 0 to 20 hours after inoculation. Numerals refer to age of cell donors in weeks. VD, virus decay rate at 37°C.

but Fig. 4 demonstrates that this preparation produced no more virus than its counterpart which was agitated. In fact, the agitated suspensions of both groups of cells produced virus earlier than the stationary suspension. This probably was related to earlier contact between cells and virus under the agitated condition. In each case, however, peak virus titers of duplicate cultures occurred at about the same time, and approximately the same amount of virus was produced. The experiment again demonstrated that cells from 1-week-old mice produced 90 to 99 per cent more virus during the 24 hour period than did such cells from 5-week-old mice.

Multiplication of FMDV in Suspensions of Minced Kidney Tissue from 1-

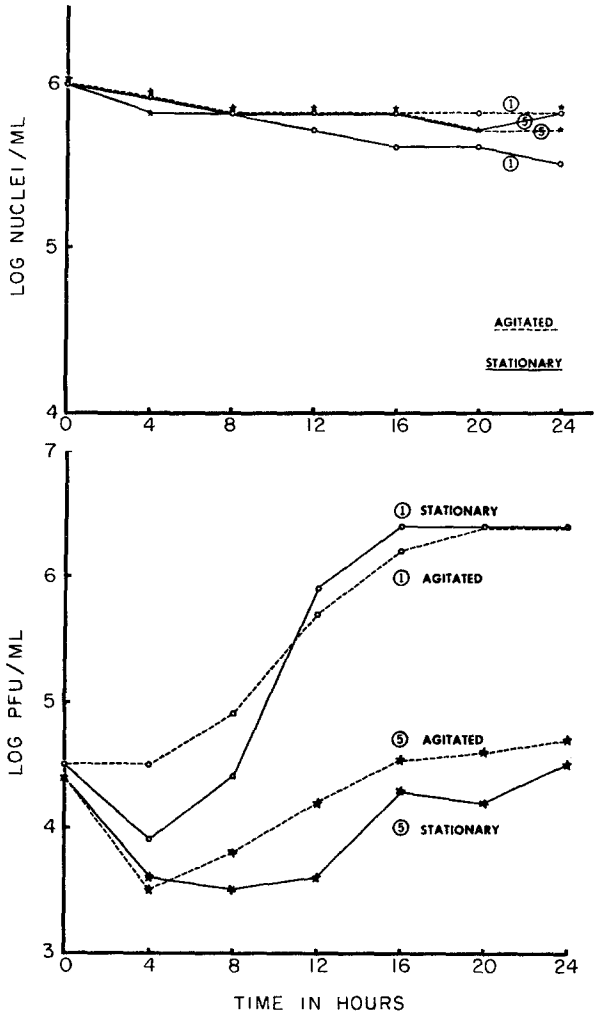

FIG. 4. Nuclei counts and multiplication of FMDV A119 in stationary and agitated suspensions of trypsin-dispersed kidney cells from 1- and 5-week-old mice. Numerals refer to age of cell donors in weeks.

through 5-Week-Old Mice.—While the above experimental results seemed to coincide with the working hypothesis that age resistance was related to a cellular mechanism, there was the possibility that treatment of kidney fragments with trypsin might affect and select cells, so that the observed reaction would not be representative of kidney cells not treated in this manner. To eliminate this objection, cultures were prepared by simply mincing kidneys and suspend-

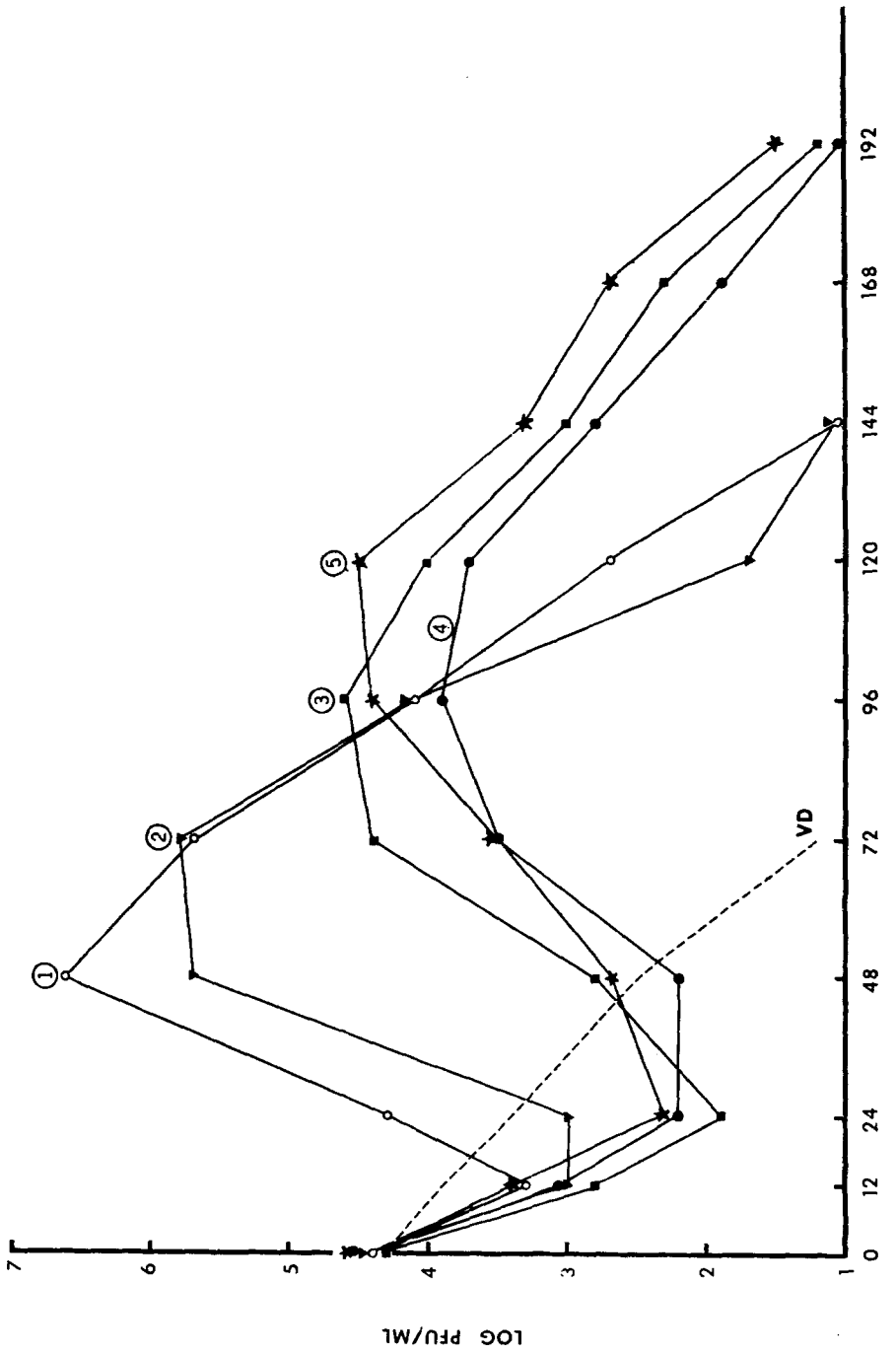

FIG. 5. Multiplication of FMDV A119 in suspensions of minced kidneys from mice of various ages: 0 to 192 hours after inoculation. Numerals refer to age of cell donors in weeks. VD, virus decay rate at 37°C.

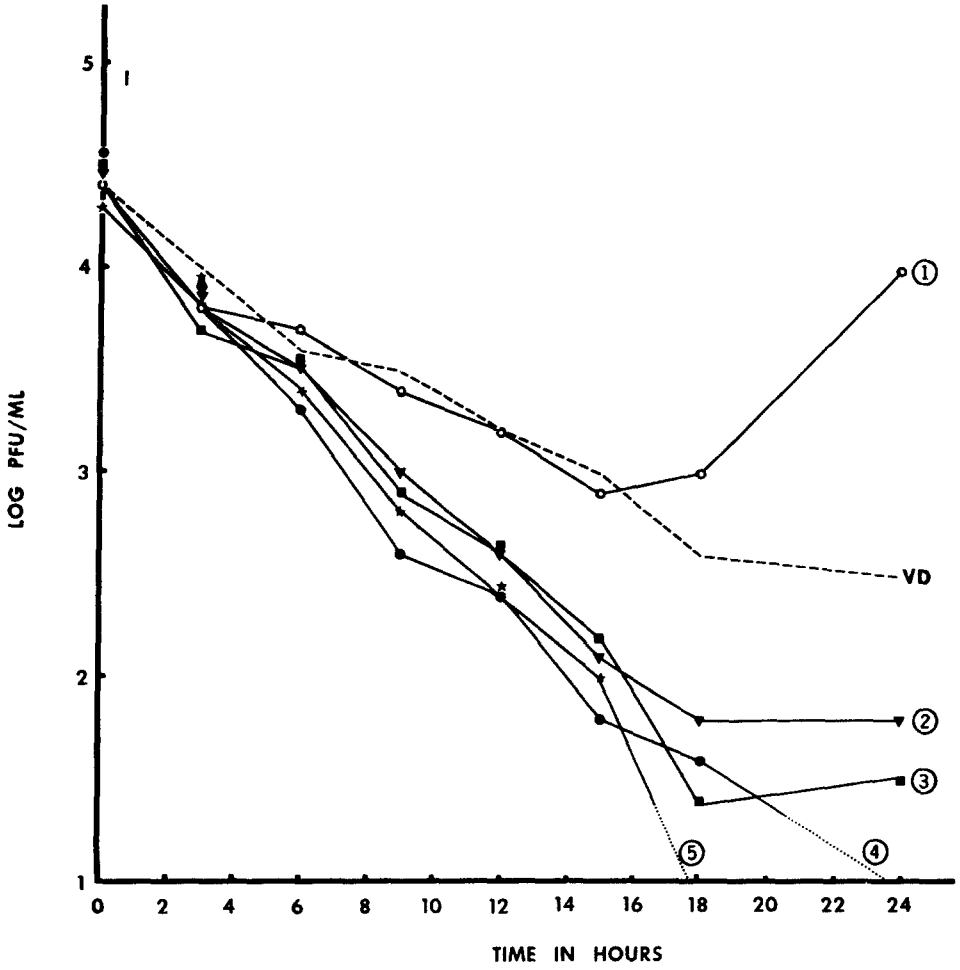

FIG. 6. Multiplication of FMDV A119 in suspensions of minced kidneys from mice of various ages: 0 to 24 hours after inoculation. Numerals refer to age of cell donors in weeks. Dotted lines show that no extracellular virus was detected at the indicated time. VD, virus decay rate at 37°C.

ing the fragments in maintenance fluid after which they were inoculated immediately with FMDV.

Fig. 5 illustrates the multiplication of FMDV from 0 to 192 hours in such suspensions prepared from the kidneys of 1- through 5-week-old mice. Except for finding that peak titers occurred later in these preparations than in suspensions of trypsin-dispersed cells, the results of the two methods are quite similar. Again virus began to multiply earlier in cells from younger mice and

higher peak titers were achieved in suspensions of these cells than in cells from older animals.

Another experiment (Fig. 6) emphasized the similarity of the two types of suspensions by demonstrating that the rate of virus adsorption was about the same in all 5 different donor age suspensions, but that the lag phase was shorter with cells from younger mice than with those from older animals. By 24 hours there was definite evidence of virus multiplication in tissue suspensions from

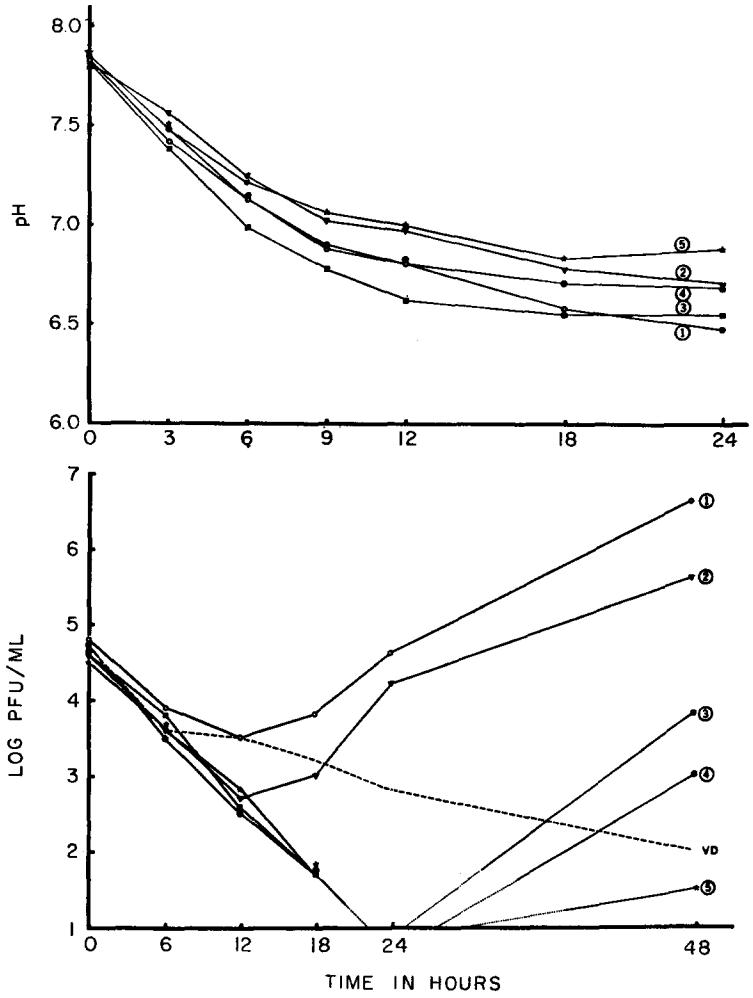

FIG. 7. Changes in pH and multiplication of FMDV A119 in suspensions of minced kidneys from mice of various ages. Numerals refer to age of cell donors in weeks. Dotted lines show that no extracellular virus was detected at the indicated time. VD, virus decay rate at 37°C.

younger mice but no virus could even be demonstrated in the preparations from the 4- and 5-week-old donors.

Since these experiments revealed the ability of younger mouse cells to produce more virus, consideration was given to the possibility that more favorable cultural conditions for these cells, rather than their origin in susceptible animals, might have been responsible for the results. In a test for metabolic activity, therefore, duplicate suspensions of minced kidneys from 1- through 5-week-old mice were prepared and inoculated with virus. Samples were taken from one of each suspension for pH determinations, while the duplicate suspensions in which the pH was maintained at 7.6–7.8 furnished samples for virus assay.

As shown by Fig. 7, there was a rather constant change in pH for all of the preparations. After incubation for 24 hours, there was some difference in pH among the various suspensions but the spread appeared to be random and not related to the age of the cell donors. Assay of the virus produced in the duplicate cultures, however, demonstrated the same pattern as observed previously. Although the pH changes were irregular in relation to the age of the donors, virus production was directly related to age with younger mouse cells producing virus earlier and in larger amounts than cells from older animals.

DISCUSSION

The review by Sigel (3) on the influence of age on susceptibility contains many examples of the development of resistance to viral infections as mice become older. While the development of resistance with age is well established, why animals become resistant as they mature is not clear. Probably the mechanism is not the same for every host-virus system, and it very well might vary from host to host, with various disease agents, and in relation to the passage history of the agents. It also seems likely that any factor which could retard or prevent virus contact with susceptible cells or multiplication within such cells would also afford a greater opportunity for the development of an effective immunological response. Therefore, to be fully effective, the mechanism of age resistance probably involves the interplay of several factors.

Considerable evidence indicates, however, that age resistance might be dependent on the production of cells during maturation which are not affected by a given virus or in which multiplication of the virus occurs slowly or not at all. For example, Sabin and Olitsky (5) found that following intramuscular injection vesicular stomatitis virus multiplied in the muscle of young susceptible mice and invaded the sciatic nerve, while in old resistant mice there was no evidence of either local multiplication or invasion. Similarly, Lerner *et al.* (14) observed that with Coxsackie A4 virus, high titers of virus with diffuse myositis were produced in young mice while low titers and focal myositis occurred in older animals. Illustrating strain differences, however, they found

that frank myocarditis and high titers of virus were produced by Coxsackie A9 virus in older mice in contrast to low titers and no demonstrable heart lesions in young mice. While not directly related to age resistance, the studies of Goodman and Koprowski (12) and Kantoch *et al.* (13) pointed to the possibility of a cellular mechanism for age resistance. Their experiments demonstrated that virus susceptibility of mouse macrophages was associated with the susceptibility or resistance of the strains of mice supplying the cells. Additional evidence was produced in a study of age susceptibility by Kunin (15), who detected in mouse brain a receptor-like substance, the distribution and abundance of which seemed to be related to age and tissue susceptibility.

In establishing an association between age of cell donors and *in vitro* production of FMDV by mouse kidney cells, the present work indicates that a cellular mechanism of age resistance exists in mice exposed to this virus. Examination of the peak virus titers produced in the various cell suspensions reveals a pattern very similar to the susceptibility pattern of mice 1 through 5 weeks of age (Table I). In these, and in a number of experiments not reported here, cells from 1- and 2-week-old mice, which are rather uniform in their response to FMDV, produced peak titers of about the same magnitude, after which the titers decreased progressively with age (Figs. 2 and 5). Although suspensions of such cells adsorbed virus at approximately the same rate for the first 4 to 6 hours regardless of the age of the cell donors (Figs. 3 and 6), cells from younger mice began producing virus earlier and higher titers occurred in these suspensions than in suspensions of cells from older animals (Figs. 2 and 5). The difference between cells from younger and older animals seems to be related, therefore, not to variations in ability to adsorb virus but to variations in ability to produce virus.

Although there was a relation between mouse age and production of virus by suspended cells, primary monolayer cultures which originated from kidney cells of 1- through 5-week-old mice did not vary appreciably in their pattern of virus production (Fig. 1). This observation was not surprising, since primary monolayer cultures are representative only of cells from the donor's tissue which are able to reproduce under the cultural conditions. This results in a selection of cells, and those which do survive and reproduce are not characteristic physiologically or morphologically of the original cell population. Since virus production was approximately the same in all of the monolayer cultures and since the cells from younger mice formed confluent sheets earlier, however, one might assume that the cells selected by the cultural conditions were susceptible ones and, therefore, that more of such cells were in the kidneys of the younger mice. Goodman and Koprowski (12) also found no difference in production of West Nile virus by monolayer cultures of kidney cells from resistant and susceptible mice, although virus production by mouse macrophages was related to the resistance or susceptibility of the donors. However, Hsiung (19)

observed that monolayer cultures from kidney cells of human babies supported the propagation of enteroviruses much better than cultures prepared from kidneys of adults.

As with the majority of work of this type, relating *in vitro* results to *in vivo* observations is difficult. Systems such as the present one for *in vitro* study lack the biological control of cell multiplication, the immunological response, and the interplay of hormonal and other systems which occur in the animals. Also, regardless of attempts such as those illustrated by Figs. 4 and 7 to determine if the experimental conditions are more favorable for one or more groups of cells than for others, there is always the possibility that slight but significant changes occur and go undetected. Therefore, while the demonstrated association of FMDV production by mouse kidney cells to the age of the cell donors supported the working hypothesis of a cellular mechanism for age resistance, further work is necessary before a definite relation of such virus production to the resistant status of the animal can be presumed.

SUMMARY

Multiplication of foot-and-mouth disease virus (FMDV) was compared in kidney cells from 7- to 35-day-old mice representing various degrees of age resistance to this virus. Three types of cell preparations were used: primary monolayer cultures, suspensions of dispersed cells, and suspensions of minced tissue.

Virus multiplication in the two types of cell suspensions was related to the age of the donors both in regard to time when multiplication first became evident and to the amount of virus produced. While adsorption rates were similar in the cells from all age groups, virus multiplication began earlier in cells from younger mice and more virus was produced by these cells than by cells from older animals. There was no significant difference in the virus growth rates in the primary monolayer cultures of cells.

The results indicate that kidney cells from mice 7 to 35 days old vary in their ability to produce virus in relation to the degree of susceptibility of the cell donors. After propagation of the cells in primary monolayer cultures, however, this difference no longer exists probably because of cell selection under the cultural conditions.

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