Mycoplasma Effects on SV40 Transformation of Human Amnion Cells[1] (34763)

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The reproducible SV40 transformation of human epithelial amnion cells *in vitro* and the characteristics of the strains of transformed cells during subcultivation, have been reported previously (1–4). Since we have established that mycoplasma infection may affect spontaneously transformed amnion cells of the FL line, causing changes in morphology and growth (5), in chromosome numbers and abnormalities (6–8), and in tumor-producing capacity (9), it seemed pertinent to investigate possible effects of mycoplasma on SV40 transformation of amnion cells.

The present data demonstrate that concurrent SV40 and mycoplasma infection modified the SV40 transformation. Effects were noticeable during the early events of transformation, and the characteristics of the transformed amnion strains were also affected.

Materials and Methods. Virus. The SV40 strain VA 45-54 GMK 4 used in the present experiments, has been previously described (3). Details of methods for determining the infectious unit, ID_{50}/ml of culture fluid, using African green monkey kidney cells, have also been reported (3). Since samples for virus titration contained mycoplasma which propagated to high titers in the test cultures, it was determined if the presence of mycoplasma had any effects on the virus titers. A series of SV40 and mycoplasma infected culture fluids were collected, and mycoplasma was eliminated from one aliquot of each by treatment with chlortetracycline HCl (Aureomycin Lederle), 1000 μg/ml for 5 hr at 37°. These and the untreated aliquots were serially diluted for comparative end-point titrations in cultures of the monkey kidney cells. In the range of virus titers tested ($10^{4.5}$–$10^{7.5}$), the end points remained the same regardless of the presence or absence of mycoplasma. However, the time of appearance of maxium cytopathic effect was delayed several days when mycoplasma was present, and the morphological changes differed from those observed in the absence of mycoplasma. Thus, the vacuolization of the cells was less pronounced, and the cell sheet was held together by a stranded matrix. These differences were taken into consideration in the following experimental virus titrations.

Mycoplasma. Mycoplasma, strain HT (5), was originally isolated as a tissue culture contaminant. It has been identified as a strain of mycoplasma fermentans (PG-18) by Dr. M. Barile, Division of Biologics Standards, National Institutes of Health, Dr. J. G. Tully, Laboratory of Bacterial Diseases, National Institutes of Allergy and Infectious Diseases, and Dr. Donald Armstrong, Sloan-Kettering Institute. The mycoplasma strain received many tissue culture passages as an experimental contaminant of FL cells and the derived, modified cell lines (5–9). For infection of amnion cultures in the present experiments, the mycoplasma was subcultured several times in BYE broth (10) to prevent the introduction of foreign cell types into the experimental cultures. The concentration expressed as colony-forming units (CFU) was determined by inoculating proper dilutions into 35-mm plastic petri dishes containing BYE agar (10) with 15% human serum. The plates were incubated at 37° in 5% CO_2 in nitrogen, and colony numbers were determined at days 3 to 5.

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The presence or absence of mycoplasma in experimental cultures was demonstrated by the direct microscopical mycoplasma technique (11), using cultures of FL cells as indicator cultures. After inoculation and incubation for 1 to 3 days, the FL cultures were exposed to hypotonic treatment, air-drying, and staining with orcein. Observation under the microscope, using phase optics, revealed the mycoplasma in association with the FL cells.

Mycoplasma elimination was accomplished by treating the infected cultures, after rinsing with medium, with Aureomycin at a concentration of 200 μg/ml for 1 hr at 37° in McCoy's medium 5a (12) with 10% agamma calf serum. After several rinses with the medium, the Aureomycin concentration was reduced to 100 μg/ml during the following week's incubation. The culture fluid was changed three times during this period.

Cells. Primary cultures of human amnion cells were prepared by trypsinization of amniotic membranes obtained from local hospitals. Routinely, the cultures were established in medium 512 (2), supplemented with 15% fetal bovine serum; penicillin, 100 units/ml; and streptomycin, 100 μg/ml. The cultures were infected while grown in McCoy's medium 5a with 10% agamma newborn calf serum, penicillin, and streptomycin, and were carried in this medium during all subsequent cultivation. pH was adjusted to be similar in cultures infected only with SV40 and in those infected with SV40 and mycoplasma. The transformed strains were transferred according to the CSK (cell seed constant) method (13), *i.e.*, 2×10^5 cells were seeded/ml of medium.

The complement fixation and immunofluorescent methods used to demonstrate the presence of T antigen have been reported (3, 14).

Results. In a great number of experiments, involving SV40 infection of cells in primary culture from many individual human amniotic membranes, SV40 transformation had occurred without exception. It was then observed, in a pilot experiment, that amnion cultures exposed to both SV40 and mycoplasma failed to develop into transformed strains. In spite of a high multiplicity of virus exposure (100 ID_{50}/cell) in McCoy's medium with 10% agamma calf serum, and subsequent cultivation in this medium, conditions which in the absence of mycoplasma promote early and pronounced transformation (13), the additional mycoplasma infection reduced the presence of SV40 transformed cells to the extent that subcultures could not be established 6 weeks after infection. The results were similar for primary cultures inoculated with SV40 and mycoplasma simultaneously or at 2-week intervals. In this experiment, observation of all cultures was discontinued approximately 2 months after SV40 infection.

In the following experiment (A222) cultures of primary amnion cells, 8 days after seeding, were also infected with SV40 and mycoplasma. The multiplicity of virus exposure was increased to 300 ID_{50}/cell; the mycoplasma inoculum was 10^6 CFU/ml of culture medium. The latter was prepared in primary amnion cell cultures in McCoy's medium with 10% agamma calf serum which were infected with mycoplasma from a broth culture. After proper fluid changes, the mycoplasma-infected tissue culture fluid was harvested, 1 month later, and was filtered through Millipore filter, pore size 0.45 μ. Virus and mycoplasma inoculation for different culture groups (a–e) are indicated in Table I, which also shows the days after virus infection at which SV40 transformed foci were first observed. Cultures infected with SV40 (group d) contained foci 4 weeks after infection. The two culture groups, a and b, infected with SV40 and mycoplasma (a was infected with mycoplasma 14 days after SV40 infection; b received SV40 2 weeks after mycoplasma infection) showed no transformed foci as late as 2 months after the SV40 infection. Cultures of group c, infected with SV40 and mycoplasma simultaneously, contained transformed foci in the ninth week after infection (in the first culture passage made 41 days after infection). The uninfected control culture (group e) did not show foci.

During the following 5 months the strain developed from group c cultures was subcultured 15 times. The split ratios, however,

TABLE I. Time Postinfection of First Observed Transformed Foci in Exp. A222 and A245 (+, foci observed; —, no foci observed).

Exp. no.	Infection with	Culture designation	Post virus infection (days)						
			Primary culture				Pass. 1		Pass. 2
			28	32	41	48	58	63	88
A222	SV40 + Mycoplasma	a[a]	—		—		—	—	
		b[b]	—		—		—	—	
		c	—		—		+		
	SV40	d	+		+				
	Uninfected	e	—		—		—		
A245	SV40 + Mycoplasma	A,B,C,D,		—		—		—	—
		E,F		—		—		+	+
	SV40	G,H		+		+			
	Mycoplasma	I,J		—		—		—	
	Uninfected	K,L		—		—		—	

[a] Infected with mycoplasma 2 weeks after SV40 infection.
[b] Infected with SV40 2 weeks after mycoplasma infection.

were considerably reduced (1:2 or 1:3) compared to SV40 transformed amnion strains in general (2, 4, 13). A most unusual SV40 production picture was observed. The virus titers during the primary culture period were high ($>10^{7.5}$ ID_{50}/ml) but they started to decrease in culture passage, and from passage 7 no virus could be detected in the culture supernatant. A subline, established from culture passage 12 of this strain, and from which mycoplasma was eliminated by treatment with Aureomycin for 1 week, was also negative for SV40 when tested 3 culture passages later. These strains were lost in an incubator accident.

In a more comprehensive experiment, (A245), a number of primary amnion cell cultures, 9 days after seeding, were infected with SV40, with mycoplasma, or with SV40 and mycoplasma simultaneously (Table I). SV40 multiplicity of exposure was 1000 ID_{50}/cell; the mycoplasma inoculum was 10^5 CFU/ml of culture fluid. Cultures inoculated only with SV40 (G and H) contained transformed foci in stained preparations when examined after 32 days. Of the 6 culture groups inoculated with SV40 and mycoplasma (A–F), transformation was observed in two groups (E and F), and not until 63 days after infection, in the first culture passage. The four other groups (A–D) were negative even at 88 days post infection. Groups I and J, inoculated only with mycoplasma, and the two uninfected control groups (K and L), showed no signs of transformation. Those groups which had not shown transformation in stained preparations during the first 3 months, were observed as living cultures for a total of 6 months after infection. No evidence of transformation was seen.

During serial cultivation (according to the CSK method) the transfer dilution factors were also higher in this experiment for strains transformed by SV40 than for those transformed by SV40 with mycoplasma present, as demonstrated in Fig. 1 for strains H and E.

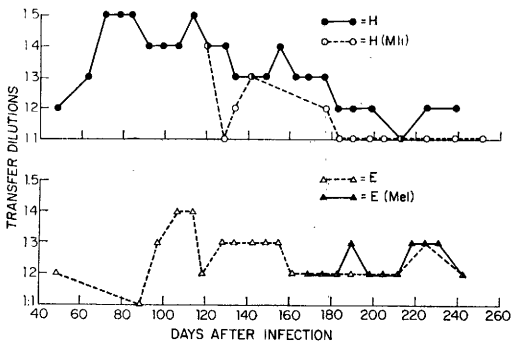


FIG. 1. Dilution factors as applied at weekly culture transfers for strains H, H (Mli), E, and E (Mel) of Expt. A245.

TABLE II. Longevity in Terms of Number of Culture Passages, Days of Cultivation Prior to Crisis, and Number of Cell Divisions for Different Culture Groups of Exp. A245.

Initial infection with	Culture designation	No. of passages	Crisis (day post-SV40 inf.)	No. of divisions[a]
SV40 + Mycoplasma	A,B,C,D	1	—	1
	E	20	237	25
	E (Mel)[b]	22	259	27
	F	20	259	23
SV40	G	Not passed[d]	—	—
	H	24	259	38
	H (Mli)[c]	21	217	19
Mycoplasma	I,J	1	—	1
Uninfected	K,L	1	—	1

[a] Based upon transfer dilutions.
[b] E (Mel), mycoplasma eliminated from day 170 post-SV40 infection.
[c] H (Mli), mycoplasma infected from day 105 post-SV40 infection.
[d] Subcultivation of transformed strain G not attempted beyond passage 1.

As shown in Table II, the concurrent SV40 and mycoplasma infection affected the longevity of the transformed strains when compared to SV40 infection in the absence of mycoplasma. The number of culture passages prior to "crisis" (15) was only slightly reduced after the concurrent infection (4 passages), but the number of cell divisions, based upon transfer dilutions (4), was greatly diminished (38 for strain H; 25 and 23 for strains E and F). However, the period of time between infection and occurrence of "crisis" was changed only for one strain (259 days for strain H; 237 or 259 days for strains E and F).

The virus production pattern during sub-

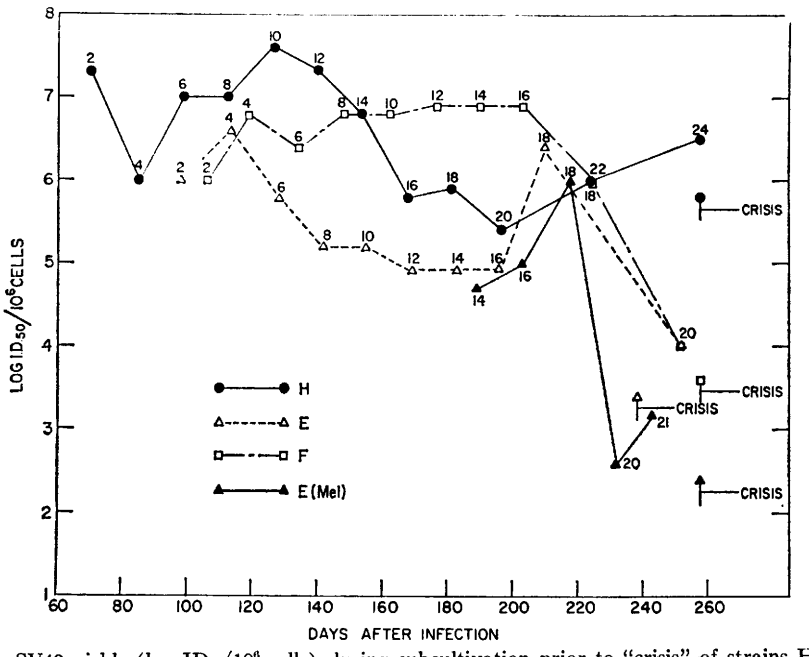


FIG. 2. SV40 yields (log $ID_{50}/10^6$ cells) during subcultivation prior to "crisis" of strains H, E, F, and E (Mel) of Expt. A245.

cultivation was also affected by the presence of mycoplasma in this experiment (Fig. 2). Strain H showed a pattern similar to that previously observed (13) in McCoy's medium with agamma calf serum. The virus titers were high initially, decreased during serial passage, but increased again in the passages prior to "crisis." During the earlier passages, both strains E and F produced less virus than did strain H. Strain E continued low virus production during many passages; strain F produced more virus than strain H after culture passage 8. Strains E and F both showed a decrease in virus production prior to "crisis."

Effect of elimination of mycoplasma from a strain transformed by SV40 and mycoplasma. Cultures of strain E of Expt. A245 in the 12th culture passage were exposed to Aureomycin in the period of day 161 to 168 after infection. Several subsequent tests demonstrated successful mycoplasma elimination. As a consequence, this strain, E (Mel), could be maintained for two additional culture passages before "crisis" occurred (Table II), the total number of population doublings increased from 25 to 27, and "crisis" was observed 20 days later than in strain E, still infected with mycoplasma. As compared to strain E, the amount of cell destruction and debris was considerably reduced. Some of the weekly transfer dilution factors were slightly increased (Fig. 1). The virus production was similar in pattern to strain E. However, strain E (Mel) showed an even greater decrease in virus titers prior to "crisis" (Fig. 2).

Strain E (Mel) was examined for the presence of T antigen in the 6th passage after mycoplasma elimination. The cells reacted to a titer of 1:16 in complement-fixation tests, and 100% positive cells were observed by immunofluorescence. A comparable passage level of strain H gave a CF titer of 1:8 and also 100% positive cells by immunofluorescence.

Mycoplasma infection of SV40 transformed strain in subculture. For comparison, the effects of "late" mycoplasma infection of an SV40 transformed and subcultured strain were examined by infecting cultures of Strain H in passage 8 with mycoplasma at day 105 after the initial SV40 infection [strain H (Mli)]. This late mycoplasma infection reduced the total period before "crisis" from 259 to 217 days (Table II). The culture passage number was reduced by 3, and there were 19 less population doublings than in strain H (Fig. 1). Cell destruction and the amount of culture debris were increased for strain H (Mli).

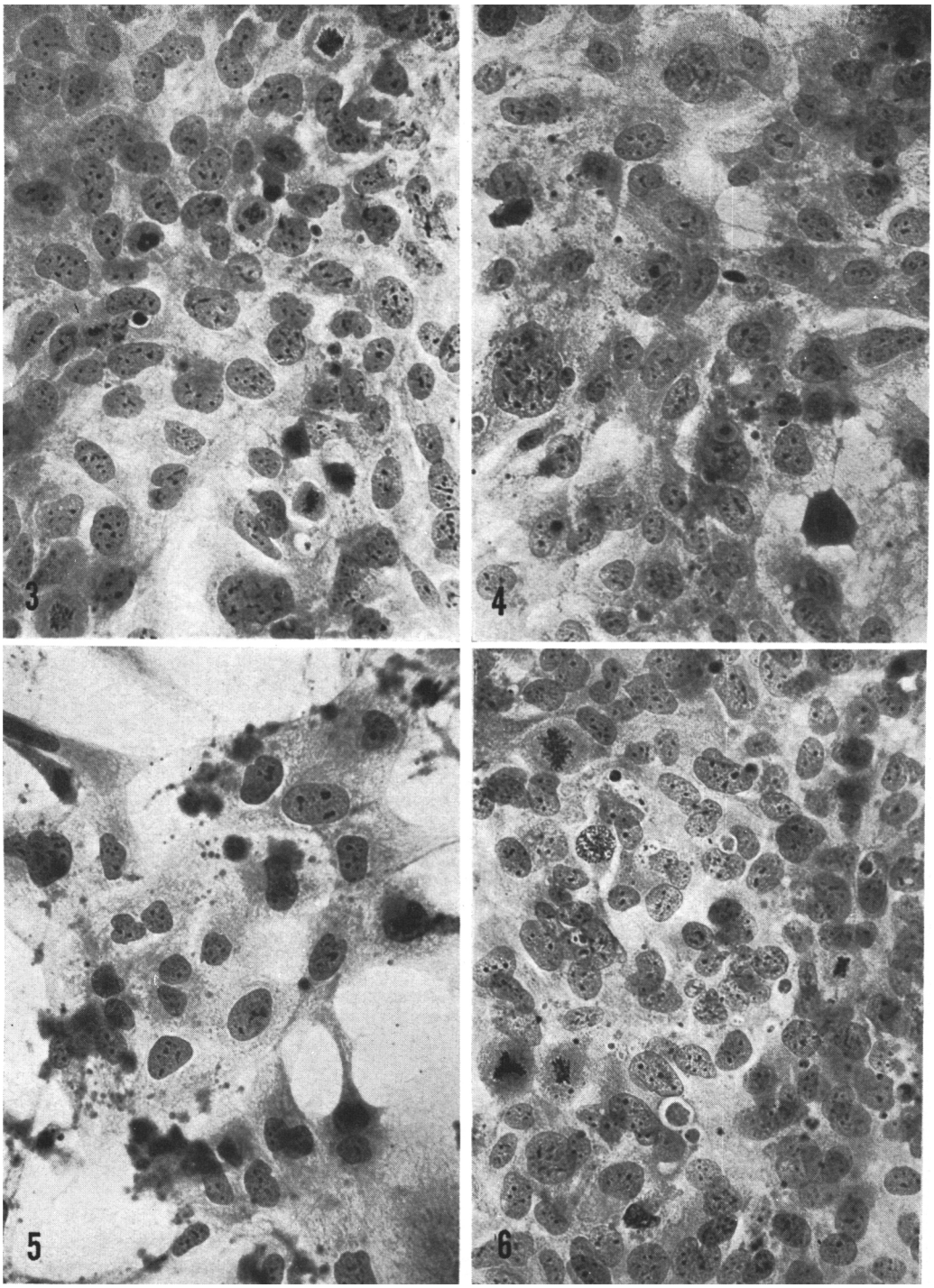
Recovery of post-"crisis" lines. Established lines were derived during recovery from "crisis" from three cultures of strain E (Mel) as follows:

Two cultures were in "crisis" in the 21st culture passage at day 259 after infection, and in the 10th culture passage after Aureomycin treatment [E $(Mel)_1$ and E $(Mel)_3$]. At day 350 after infection, there was sufficient recovery to make a transfer of both cultures at a dilution of 1:2. The following transfer of E $(Mel)_1$ could be made 7 weeks later, and presently (September 12, 1969) this line has received 30 culture passages since recovery. E $(Mel)_3$ was not transferred again until day 484 after infection, and from then has received 18 culture passages.

A culture, E $(Mel)_2$, from the same experiment also showed "crisis" at day 259, in the 19th culture passage postinfection, and in passage 8 post-Aureomycin treatment. It could also be transferred 100 days later at a dilution of 1:2, and is presently in 18th culture passage after recovery.

Throughout the whole period of "crisis," there was a substantial amount of cells present in each of the three cultures (>500,000 cells/T-30 flask containing 5 ml of medium). Culture transfer was attempted when the number of cells appeared to be increasing, and the cell morphology was more homogenous. Strain H, which was not exposed to mycoplasma, entered "crisis" at day 259 and was not recovered. The characteristics of these three post-"crisis" lines as compared to two other SV40 transformed amnion cell lines, recovered during "crisis," will be reported separately.

Cell morphology. The morphology of the transformed cells in subculture in the presence or absence of mycoplasma is illustrated by the four strains in Fig. 3 to 6.



FIGS. 3–6. Hematoxylin and eosin stained cultures at comparable passage level of strains H (Fig. 3); strain E (Fig. 4); strain H (Mli) (Fig. 5); and strain E (Mel) (Fig. 6) of Experiment A245; ×306.

Strains H, E, and E (Mel) contain cells of epithelial-like morphology (Figs. 3, 4, 6); the cells of strain H (Mli), (Fig. 5) are more fibroblastic in shape. In all strains there is variation in nuclear shape and size. This variation is most pronounced in the mycoplasma-infected strains E and H (Mli), which also show a more disorganized growth pattern and greater amounts of cell destruction and debris. There is more destruction of the cell sheet in strain H (Mli), and many intracellular bridges are seen between cells of this strain.

Discussion. Although the population in primary cultures of amnion cells appear uniform, it represents cells with individual characteristics and potentials, for example with regard to the relative proportion of cells which will undergo abortive infection, lytic infection resulting in virus production and cell destruction, or defective infection leading to transformation (13). There is also evidence that the original population of SV40 transformed cells may represent a distribution of cell growth potentials (13).

Some of the effects of concurrent SV40 and mycoplasma infection vary under identical conditions. Presumably, the results can best be interpreted as a combination of effects. The HT strain of mycoplasma is cytopathic for SV40 transformed amnion cells [pronounced destruction in strain H (Mli)], as for FL cells (5) and other spontaneously transformed cells, whereas primary amnion cells are relatively resistant. Lack of transformation, late appearance of transformed foci, and lesser growth potential of the strains resulting from concurrent infection with SV40 and mycoplasma may, therefore, in part, be explained by the mycoplasma's cell destructive effects, both initially and throughout subculture. The particular susceptibility to destruction may reduce the number of cells expressing transformation in the primary culture; hence the number of transformed foci simulate the results observed after a lesser multiplicity of virus exposure (4). Some cell destruction during subcultivation must be taken into consideration. Elimination of mycoplasma [strain E (Mel)] increased both the transfer dilutions and the cell strain longevity prior to "crisis."

Medium and serum during the period of transformation can affect the transformation incidence and the growth potential of transformed strains (13). Thus, a depletion of the culture medium by the mycoplasma, may similarly reduce both the transformation incidence and growth potential. It should be pointed out that the HT strain is not arginine dependent (5), as has been shown for many other mycoplasma strains. The relative resistance to the destructive mycoplasma effects observed for transformed strains E and F (A245), as compared to the low resistance of strain H (Mli), indicates a cell change related to the concurrent mycoplasma and SV40 exposure of the primary amnion culture. This change, which is correlated with an increased resistance to mycoplasma, may represent a selection of cells with changed metabolic pathways, induced by mycoplasma-depleted medium. However, whether this mechanism, an adaptation process, or direct mycoplasma-induced genetic changes, is the more likely interpretation, is pending the collection of more specific data, including chromosome analysis. The consistency of the appearance of new chromosome varieties after exposure of spontaneously transformed FL cells to the HT strain of mycoplasma, point to mycoplasma-induced genetic changes as the underlying mechanism in this system (6). Some of the major chromosome changes were irreversible and persisted after elimination of mycoplasma from the cultures.

In all previous experiments, utilizing the presently employed medium–serum combinations, the virus production pattern has been triphasic, with an increase in virus titers prior to "crisis." Different virus production patterns were observed for the three strains examined after concurrent SV40–mycoplasma infection. Virus production ceased in cultures of Expt. A222, both before and after mycoplasma elimination. With one exception (16), all previously reported SV40 transformed human cells have produced virus prior to "crisis". Virus-producing cells may have been eliminated by mycoplasma or it may be proposed that this strain, at a very early time of cultivation, entered an abortive

"crisis." Thus, the virus-free passages could represent post-"crisis" cells, as reported (15) for lines of SV40 transformed fibroblasts.

The consistent differences in virus production from week to week between strains E and F (Fig. 2), otherwise identical in environmental conditions, indicate that the virus production pattern is determined early after transformation, and may reflect the characteristics of the cell present in the original transformed focus. It is not likely that a much greater proportion of virus-producing cells is destroyed by mycoplasma in consecutive cultures of one strain. The decrease in virus titers prior to "crisis" for the two strains, and the even more pronounced decrease observed for strain E (Mel) is interesting. Indeed this may explain the recovery of post-"crisis" lines from strain E (Mel) from which mycoplasma was eliminated. Although strains E and F also showed low virus titers, the persistent mycoplasma infection of these strains conceivably might have destroyed the cell population with potential for "crisis" survival. In a great number of experiments involving SV40 transformation of amnion cells (without mycoplasma), attempts to recover cells from "crisis" have been negative. An increase in virus titers prior to "crisis," has always been observed, and it has been shown that "crisis" was correlated with a cellular change towards a decreased resistance to the virus after a certain number of cell divisions (17). Strain H of Expt. A245, transformed by SV40 alone, was not recovered from "crisis".

Although apparently not a transforming agent in the present system by itself, the HT strain of mycoplasma modified the virus transformed human amnion cells to the extent that long-lasting (perhaps permanent) changes in their characteristics occurred. Whether this is a general effect of mycoplasma is not known at this time.

Summary. Incidence of transformation of human amnion cells by SV40 was reduced and the appearance of transformed foci delayed by concurrent SV40–mycoplasma infection of primary cultures. The number of population doublings during serial cultivation was reduced, as was the number of culture passages and the time before "crisis" of the SV40 transformed cells. Mycoplasma infection of an SV40 transformed strain already in serial cultivation caused even more cell destruction and effects on the population doubling number. The virus production pattern changed, but differently for individual strains. After mycoplasma elimination, the number of passages and cell divisions, and the time before "crisis" increased, and several strains were recovered from "crisis" as permanent cell lines.

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1. Fogh, J., Proc. Amer. Ass. Cancer Res. **7**, 21 (1966).

2. Fogh, J., Ramos, L., and Fogh, H., *in* "Axenic Mammalian Cell Reactions" (G. L. Tritsch, ed.), p. 59. Dekker, New York (1969).

3. Gaffney, E., Ramos, L., and Fogh, J., Cancer Res., in press.

4. Fogh, J., and Gaffney, E., Proc. Amer. Assoc. Cancer Res. **10**, 26 (1969).

5. Fogh, J., Hahn, E., and Fogh, H., Exp. Cell Res. **39**, 554 (1965).

6. Fogh, J., and Fogh, H., Proc. Soc. Exp. Biol. Med. **119**, 233 (1965).

7. Fogh, J., and Fogh, H., Proc. Soc. Exp. Biol. Med. **126**, 67 (1967).

8. Fogh, J., and Fogh, H., Proc. Soc. Exp. Biol. Med. **129**, 944 (1968).

9. Fogh, J., Cancer Res. **29**, 1721 (1969).

10. Barile, M., Yaguchi, R., and Eveland, W., Amer. J. Clin. Pathol. **30**, 171 (1958).

11. Fogh, J., and Fogh, H., Proc. Soc. Exp. Biol. Med. **117**, 899 (1964).

12. McCoy, T., Maxwell, M., and Kruse, P., Proc. Soc. Exp. Biol. Med. **100**, 115 (1959).

13. Fogh, J., and Gaffney, E., J. Nat. Cancer Inst. in press.

14. Petursson, G., Fogh, J., de Harven, E., and Armstrong, D., Virology **28**, 303 (1966).

15. Girardi, A., Jensen, F., and Koprowski, H., J. Cell. Comp. Phys. **65**, 69 (1965).

16. Khoury, G., and Van der Noordaa, J., Proc. Soc. Exp. Biol. Med. **131**, 297 (1969).

17. Fogh, J., Loveless, J., and Gaffney, E., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **28**, 297 (1969).

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