and threonine than those of rats subsisting on heated sbf.

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Irreversibility of Major Chromosome Changes in a Mycoplasma-Modified Line of FL Human Amnion Cells.* (32369)

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Previously reported characteristic chromosome changes in cells of the FL human amnion line after mycoplasma (PPLO) infection (1) could be classified as 3 types: reduction in chromosome number; increase in chromosome aberrations; and the appearance of new chromosome varieties. The major changes developed very slowly. Thus chromosome numbers, predominantly between 70 and 76 for uninfected FL cells, continued to decrease in mycoplasma-infected cultures, and this was found to be true when counts were made even as late as the 8th and 10th months of cultivation (time of last report). Most chromosome aberrations in mycoplasma-infected FL cultures represented an accentuation of aberrations observed to a minor degree in uninfected FL cells. Three new chromosome varieties were seen: A very large teleocentric chromosome, which increased in frequency with time of culture and, after 10 months, was seen in 28.5% of metaphase plates in one of the mycoplasma-infected cell lines; in addition, a large metacentric chromosome and a large subteleocentric chromosome, both of which were seen with less frequency, although the latter in some preparations occurred in 20% of metaphase plates.

Studies have been continued to answer the two questions: 1) Would the mycoplasma-infected and -modified cells change further on continued cultivation with mycoplasma present? 2) What would be the effect on the modified chromosome picture if mycoplasma were eliminated from the culture? This report concerns these questions.

Materials and methods. Cell line F 138 has previously been described(1) as a line of FL human amnion cells exposed to myco-

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plasma (strain HT) and cultured as mycoplasma-infected for an extensive period. The composition of the media used in this study, LY medium(2) with 20% human serum and medium 512(3) containing 15% fetal bovine serum, has also been previously reported. For chromosome analysis cell cultures were prepared by a method based on the suspension technique developed by Moorhead *et al*(4) and with ignition of the fixative. The direct microscopical demonstration method(5), as well as various agar cultivation methods, were used for mycoplasma tests.

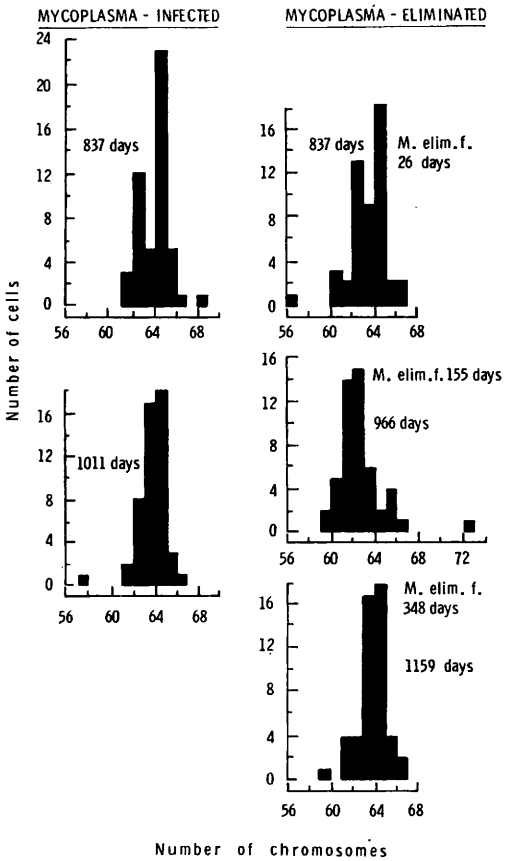

FIG. 1. *Left side*: Chromosome numbers of mycoplasma-modified FL cells of the F 138 line after 837 days and 1011 days of cultivation with mycoplasma present. *Right side*: Chromosome numbers of the F 138 line after elimination of mycoplasma (F 138cl). *Upper graph:* After 837 days of culture since initial mycoplasma infection; mycoplasma eliminated during the last 26 days. *Middle graph:* 966 days since initial infection; mycoplasma eliminated the last 155 days. *Lower graph*: 1159 days since initial infection; mycoplasma eliminated during the last 348 days.

Results. The F 138 line of mycoplasma-infected and -modified FL human amnion cells has now been cultured for more than 1000 days. Further reduction in chromosome number, an additional increase in frequency of some aberrations, particularly of dicentric chromosomes, and a very definite increase in frequency of metaphase plates containing the very large teleocentric chromosome, have occurred during the additional two years of cultivation since the time of the original report. At that time the F 138 line had been observed for 318 days.

Fig. 1 shows that 49 of 50 cells had chromosome numbers less than 68 at 837 days after the initial mycoplasma infection. At 1011 days all cells had less than 68 chromosomes. In these late analyses most cells had numbers between 62 and 64, and thus the number of chromosomes had decreased since the 318th day, at which time (as previously reported) the numbers were in the range of 63 to 68, with 78% of cells having numbers less than 68.

Data in Table I illustrate the percentage of metaphase plates with chromosome abnormalities, as observed in the mycoplasma-infected F 138 line 1011 days after infection, in comparison with uninfected FL cells. Most notable is the increased frequency of metaphase plates with dicentric chromosomes and the more frequent occurrence of translocational exchanges. Cells with chromatid breaks were also more frequent in the infected cultures, as was chromosome destruction. This analysis therefore confirms the observations made during the first 10 months of culture of the mycoplasma-infected line, although the absolute frequencies vary. Cells in endoreduplication, which in previous analysis was a predominant trait, were not seen in these late cultures.

Changes in the incidence of the 3 new chromosome varieties previously observed in the mycoplasma-infected F 138 line have occurred during the last 2 years, most conspicuously of the new large teleocentric chromosome which, at 1011 days, was present in practically all metaphase plates (98.3%). The large subteleocentric chromosome at this stage was present in 16.5% of metaphase

TABLE I. Percentage of Metaphase Plates with Chromosome Abnormalities and with New Chromosome Varieties in FL Cells (Control), in FL Cells Carrying Mycoplasma Infection for 1011 Days (F 138 Line), and in Cells of the F 138 Line at 155 and 348 Days After Mycoplasma Elimination (F 138cl).

	Type of cell			
	Control (FL)	Mycoplasma infected (F 138)	Mycoplasma infection eliminated (F 138cl)	
Days after mycoplasma inoculation	—	1011	966	1159
Days after mycoplasma elimination	—	—	155	348
No. metaphase plates	387	121	106	164
Metaphase plates with abnormalities	%			
Endoreduplication	.5	—	1.9	—
Dicentric chromosomes	1.0	9.1	.9	1.8
Acentric fragments	.8	.8	—	1.2
Minute chromosomes	1.8	1.7	.9	.6
Translocational exchanges	observed	more frequent	observed	observed
Ring chromosomes	—	.8	—	—
Secondary constrictions	observed	observed	more pronounced	more pronounced
Chromatid breaks	.8	1.7	—	1.8
Chromosome destruction	observed	more frequent	observed	not observed
2S*	3.3	6.0	5.2	4.7
Metaphase plates with new varieties				
Large teleocentric	—	98.3	99.1	95.7
Large metacentric	—	—	1.9	1.2
Large subteleocentric	—	16.5	—	.6

* Based on 300 metaphase plates.

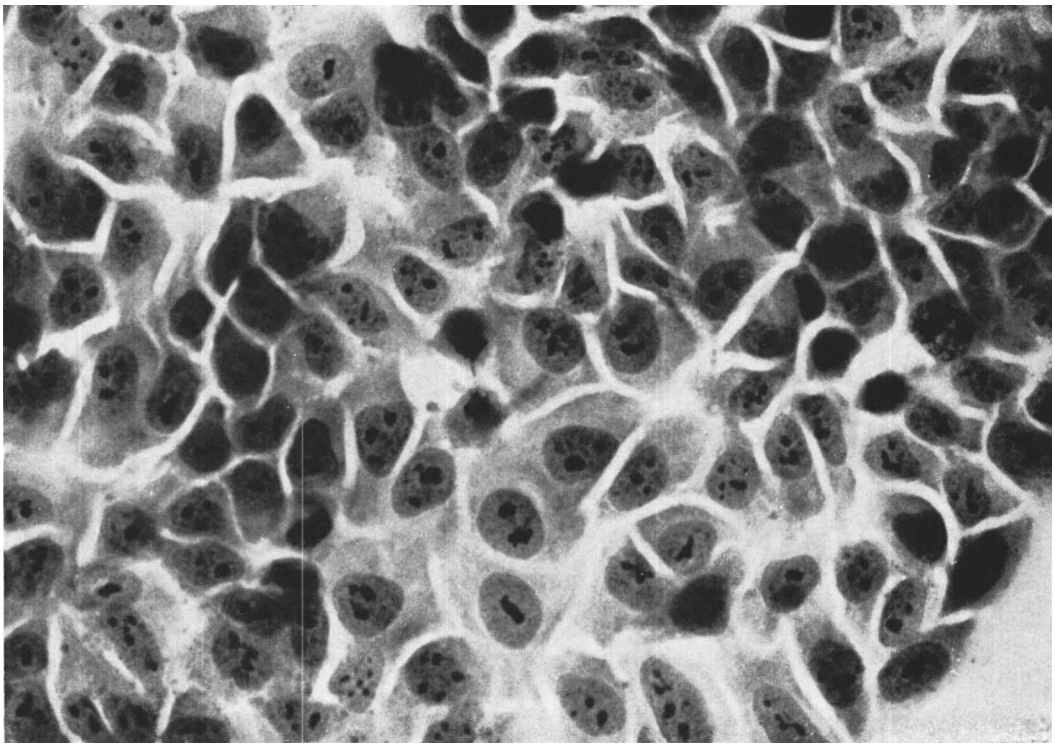

FIG. 2. Culture of the F 138cl line stained with hematoxylin and eosin. 653 ×.

plates, whereas the large metacentric chromosome was not seen.

The data presented here in table form represent only individual samples out of a large number of preparations examined during this long period of study. All other analyses have conformed with this picture. The constant presence of mycoplasma in the F 138 line has been ascertained by frequent mycoplasma testing during this long period.

Several attempts to eliminate mycoplasma from the infected F 138 cell line were unsuccessful, although kanamycin and Aureomycin were included in the culture fluid in concentrations and according to the procedure(6) which had previously eliminated contamination from many lines of other cells. Elimination, however, was accomplished after approximately 800 days of cultivation of the infected F 138 cells by the following procedure: The cells which had been continuously cultured in LY medium plus 20% human serum were transferred, by trypsinization, to medium 512 containing 15% fetal bovine serum. Six days later Aureomycin was added to the culture fluid to a concentration of 100 μg/ml. The inside of the culture container was bathed with the Aureomycin containing medium several times daily. The culture medium was renewed once daily, and at this time the silicone stopper, after thorough flaming of the culture container opening, was replaced with a new stopper. Aureomycin was omitted from the culture medium after 10 days, and the cells were further cultured in medium 512 with 15% fetal bovine serum. Tests for mycoplasma on culture fluid and cells were performed one week later and from there on repeatedly in the following weeks and months. All such tests showed absence of mycoplasma. The untreated F 138 cell cultures in LY medium with 20% human serum or in medium 512 with 15% fetal bovine serum continued to show presence of mycoplasma. Two weeks after termination of the Aureomycin treatment cells were transferred back to LY medium with 20% human serum and were subsequently carried in this medium, in which they also continued to show absence of mycoplasma. The F 138 line after elimination of mycoplasma (F 138c1) has presently been cultured for 348 days.

Previous attempts to treat F 138 cells with Aureomycin by a comparable procedure, but in LY medium with 20% human serum, had, as mentioned, failed to eliminate the microorganisms from the human cell cultures. Cells derived from such an attempt were subsequently cultured for a number of months in the presence of low Aureomycin concentrations (25 μg/ml), and several times during this period subcultures were tested for mycoplasma after discontinuation of the Aureomycin. At no time, however, was mycoplasma elimination accomplished. Cells of this line were now transferred to medium 512 containing 15% fetal bovine serum, and new attempts were made to eliminate the mycoplasma by treatment with Aureomycin, 100 μg/ml, in view of the successful results obtained with previously untreated F 138 cells in this medium. All efforts, however, were unsuccessful in eliminating the mycoplasma from the previously Aureomycin-treated cells, and it may therefore be concluded that Aureomycin resistance had developed during the longtime cultivation of the mycoplasma-infected cells in the presence of low Aureomycin concentrations.

Chromosome preparations of the F 138c1 line have been repeatedly examined. As shown in Fig. 1, the chromosome numbers of this line from which mycoplasma have been eliminated have not reverted to higher numbers. Counts made 26 days after elimination of mycoplasma, this date corresponding to 837 days after the initial mycoplasma infection, showed a great similarity to the counts of the infected F 138 cell line. Similar results were obtained 155 days and 348 days after mycoplasma elimination, corresponding to 966 days and 1159 days after initial infection of FL cells with mycoplasma. At this latest date, chromosome numbers in the F 138c1 line ranged between 59 and 67, with most cells containing 63 and 64 chromosomes. These numbers closely resemble the numbers counted in the mycoplasma-infected F 138 line 1011 days after mycoplasma infection.

Many chromosome aberrations decreased

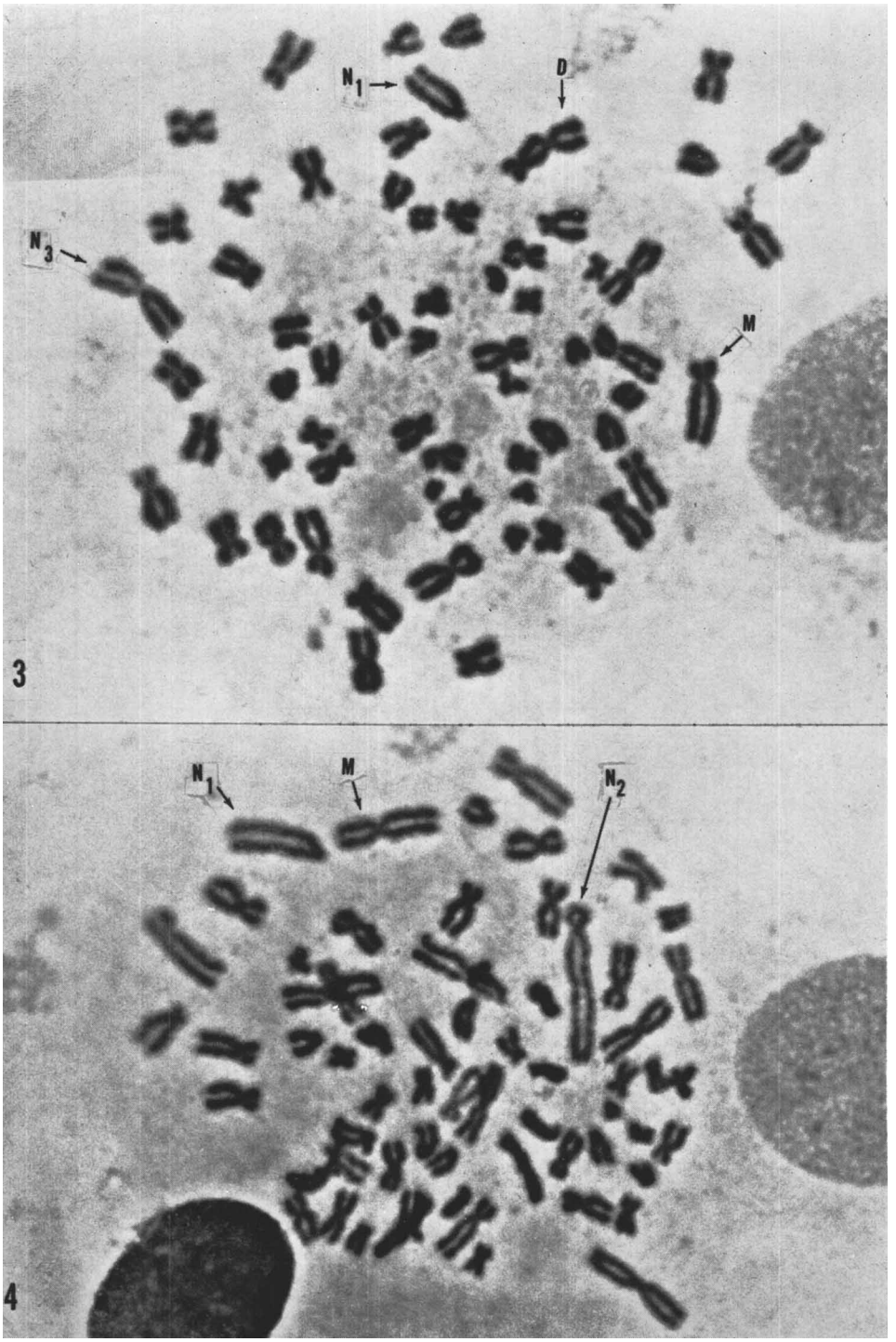

FIG. 3 to 5. Metaphase plates of cells of the F 138 line after 1011 days of cultivation with mycoplasma present.

FIG. 3. 63 chromosomes, including marker chromosome (M), dicentric chromosome (D), the new large teleocentric chromosome (N_1), and the new large metacentric chromosome (N_3).

FIG. 4. This metaphase plate shows marker chromosome (M), the new large teleocentric chromosome (N_1), and the new large subteleocentric chromosome (N_2).

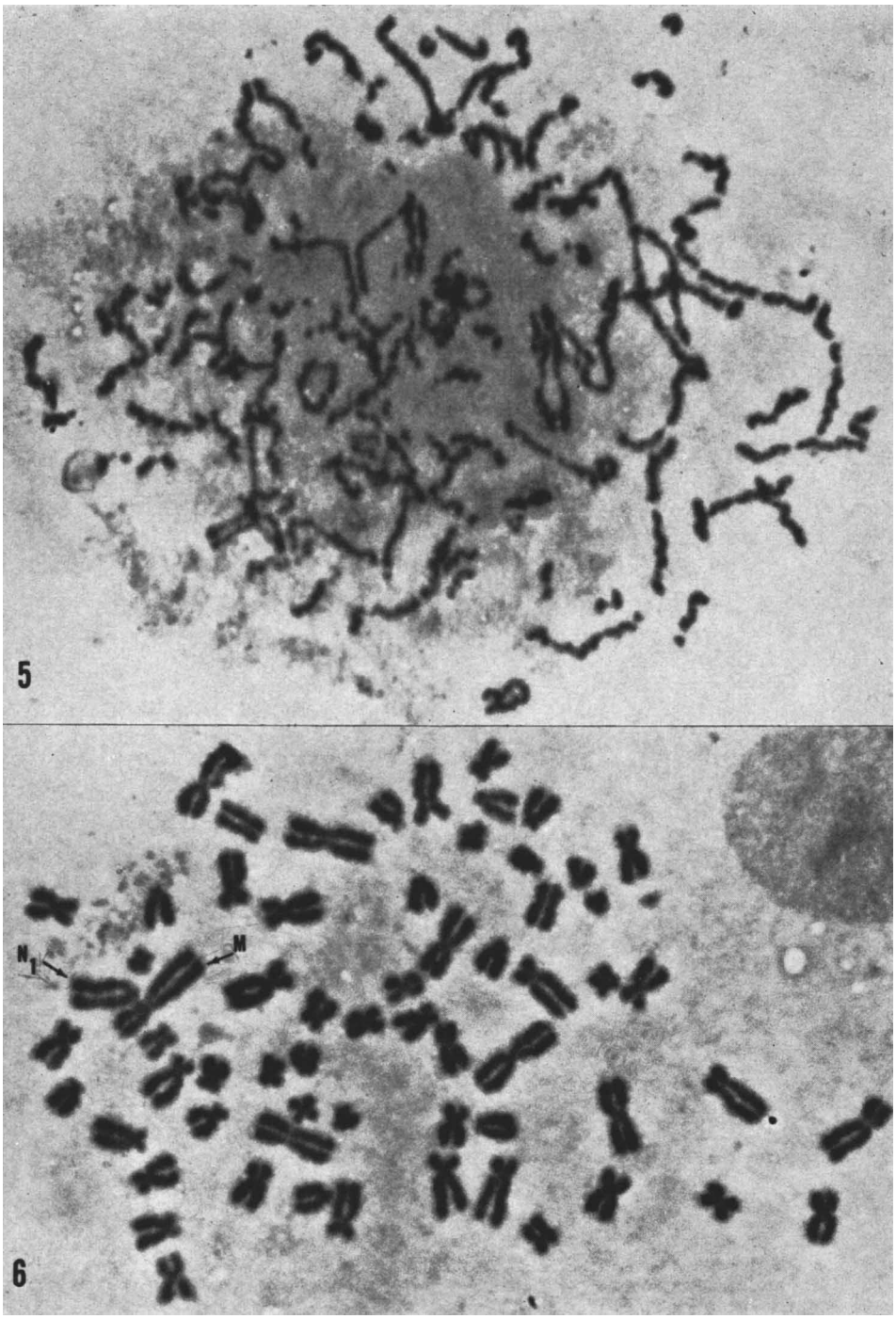

FIG. 5. Metaphase plate with high degree of chromosome destruction.

FIG. 6. Metaphase plate of F 138cl cell. At the time of preparation the line had been cultured 1159 days since initial infection; mycoplasma eliminated during the last 348 days. There are 63 chromosomes, among which the marker chromosome (M) and the new large teleocentric chromosome (N_1). 2041 ×.

considerably in frequency after mycoplasma elimination, as shown in Table I. Dicentrics were now observed only at low frequencies, and cells with minute chromosomes, translocational exchanges, ring chromosomes, and chromosome destruction were less frequently observed both at 155 and at 348 days after mycoplasma elimination; the aberration frequencies resembled those of uninfected FL cells.

Among the new chromosome varieties observed in the F 138 line, the large teleocentric chromosome persisted at very high frequencies in the F 138c1 line after mycoplasma elimination, and it was observed in 99.1% of metaphases at 155 days, in 95.7% of cells at 348 days after mycoplasma elimination. The large metacentric chromosome was observed at low frequencies, and the large subteleocentric chromosome, appearing frequently in the infected line, was very infrequent at 155 and at 348 days after mycoplasma elimination.

Discussion. In mycoplasma-infected cultures of FL cells, an early stage of cell destruction was followed by the outgrowth of a population of cells with reduced rate of division and increased variation in cell size and shape(7). This population, in addition to showing chromosomal changes(1), differed also from uninfected FL cells in cultivation characteristics, resistance to mycoplasma, and in tumor-producing capacity(8), which was reduced when mycoplasma-modified cells were injected into the cheek pouch of cortisonized weanling hamsters. It is conceivable that the changes in biological characteristics are related to some of the chromosome changes.

As shown in this report, additional changes in the chromosomes occurred even during the second and third year of cultivation of the mycoplasma-infected cells. The number of chromosomes decreased further, some aberrations increased in frequency, and the large teleocentric chromosome, never observed in uninfected FL cells, became present in essentially all cells. After elimination of mycoplasma the chromosome numbers did not revert from the low level, and the large teleocentric chromosome had persisted. The other new chromosomes are also still present at low frequencies. Many chromosome aberrations, however, decreased in frequencies to the level observed in uninfected FL cells. The tumor-producing capacity continued to be reduced after mycoplasma elimination from the modified cell line(8).

The similarity between the changes observed in this mycoplasma-human cell system and chromosome effects of various viruses has been previously emphasized(1). The mechanism resulting in these exceedingly slowly developing changes is still uncertain. For a possible interpretation of the phenomenon, as well as for practical purposes, the irreversibility of the changes in chromosome number and in the occurrence of new chromosome varieties appears to be of importance. When compared to virus-induced *in vitro* transformations the effected irreversible changes in mycoplasma-modified FL cells can quite appropriately be referred to as reverse transformation or, perhaps, retransformation. Other cell lines are being studied. Certain chromosome changes in a mycoplasma-infected human diploid cell strain have now been reported(9). The prospect that irreversible changes in the genetic constitution and in *in vitro* and *in vivo* behavior of mammalian cells may be a rather general effect of mycoplasma contamination calls for great caution in considering mycoplasma contamination an innocent phenomenon without consequences, even when mycoplasma elimination has been accomplished.

Summary. A line of mycoplasma-infected FL human amnion cells (F 138) has shown further chromosome changes from the first to the third year of cultivation, with mycoplasma present. Additional reduction in chromosome numbers to a present range between 61 and 66, with predominant numbers of 62 to 64 chromosomes per cell, an increase in some chromosome aberrations, in particular the frequency of dicentrics, and further increase in the number of metaphase plates with new chromosome varieties, most notably the large teleocentric chromosome, have occurred during this period. After elimination of mycoplasma from the cell line (F 138c1) with Aureomycin by a special method

which is described, the chromosome numbers did not revert to higher numbers, and the large teleocentric chromosome persisted in practically all cells during one year of cultivation (present time). Many chromosome aberrations decreased in frequency to the level observed in uninfected (control) FL cells. The irreversible changes, which were correlated with alterations of *in vitro* and *in vivo* behavior of the cells, are referred to as reverse transformation or retransformation.

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Variation in Adrenal Cortical Hormones Within Physiologic Ranges, Stress and Interferon Production in Mice.* (32370)

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The relationship of adrenal corticosteroid administration and of stress to the clinical course of virus infections and to interferon production has not been established(1,2,3). Corticosteroid treatment did not appear to affect the clinical course of poliomyelitis(4) or mumps(5) but did appear to predispose to relapses of infectious hepatitis(6). Stress or substantial doses of adrenal steroids rendered normally resistant adult mice susceptible to Coxsackie B virus(7,8,9). ACTH and the stress of overcrowding were both noted to reactivate rabies virus in guinea pigs (10,11). Under certain conditions, various stresses increased susceptibility of mice to vesicular stomatitis virus and herpes simplex virus(12,13,14). However, intact mice inoculated after a second 3-hour daily period of sound stress were more *resistant* to vesicular stomatitis virus, though adrenalectomized mice inoculated on the second day of stress showed increased mortality from encephalitis (14). *Decreased* susceptibility to poliomyelitis was found in monkeys subjected to 24 hours of avoidance stress(15). In humans, delayed recovery from infectious mononucleosis was correlated with deficient ego strength (16), and from influenza correlated with depression(17).

Several studies report suppression of interferon production *in vivo* and *in vitro* by adrenal cortical steroid hormones in high doses(18,19,20,21,22). Cortisone in doses of 0.5 to 10.0 mg/mouse administered 24 hours prior to Newcastle disease virus (NDV) injection reduced the interferon titer 6 hours after injection 2½ to 8-fold(23). The administration of 1 to 5 mg of hydrocortisone to 1 kg rabbits markedly suppressed interferon production by *E. coli* endotoxin, but single doses of hydrocortisone up to 250 mg did not decrease circulating interferon levels following inoculation with Sindbis virus or NDV(24). Adrenalectomy markedly poten-

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