Viral-induced Fusion of Human Cells

I. QUANTITATIVE STUDIES ON THE FUSION OF HUMAN DIPLOID FIBROBLASTS INDUCED BY SENDAI VIRUS [1,2]

ANTONIO VELÁZQUEZ,[3] FRANCIS E. PAYNE AND ROBERT S. KROOTH
Department of Human Genetics, Medical School, and Department of Epidemiology, School of Public Health, The University of Michigan, Ann Arbor, Michigan

ABSTRACT   Human diploid fibroblasts in monolayer cultures underwent cytoplasmic fusion in the presence of UV-inactivated Sendai virus. The proportion of nuclei in the cultures which were in polykaryocytes ("polykaryocytic index") under the conditions used in the present study, was about 0.50. When the cells were fused by the methods developed for the present study, the polykaryocytic index was substantially higher than when the conventional method was employed. The extent of fusion obtained was dependent, up to a certain degree, on the concentration of the virus and on the cell population density. The polykaryocytic index reached a maximum four to six hours after the cells were exposed to virus, but the frequency of cells with very high numbers of nuclei continued to increase for at least two days. Evidence is presented which suggests that the population of mononucleate cells is heterogeneous as regards their ability to fuse. The distribution of multinucleate cells with various numbers of nuclei roughly fits the Poisson distribution, although the frequency of cells with very large numbers of nuclei is substantially greater than the Poisson would predict.

The studies to be reported here were undertaken in order to measure the effect of several experimental variables on the frequency of cytoplasmic fusion of human diploid fibroblasts. The ultimate goal of these experiments was to develop conditions yielding a high frequency of polykaryocyte formation.

Most human cell lines, growing *in vitro*, can be placed into one of two classes, depending on the variation in chromosome complement between the cells of a single clone (Krooth, Darlington and Velazquez, '68). *Homonuclear* lines are characterized by the fact that there is very little variation from cell-to-cell; *heteronuclear* lines, on the other hand, show marked variation (we shall use the words "strain" and "line" interchangeably). Human diploid fibroblasts are one kind of homonuclear cells.

When using the conventional method, which involves the exposure of a cell suspension to Sendai virus (Okada, '62; *inter alia*), viral-induced cell fusion is readily achieved with most human, and other mammalian, *heteronuclear* cell lines. In contrast, cells recently adapted to grow in culture show low fusion capacities (cf. Okada and Tadokoro, '63). But it is the use of cell fusion between this latter kind of cells that is likely to be especially important for the study of interactions between human genes, since a great number of Mendelian mutations have been found to affect specific molecules in them (Krooth, '69). Although human homonuclear strains sometimes hybridize (Siniscalco et al., '69), the formation of hybrid cells in culture is

a rare event and to isolate somatic cell hybrids to study genetic interactions, one needs a selective system which will eliminate the two parental strains. Other difficulties encountered in the use of hybridization between human homonuclear cells include their low cloning efficiency and their finite life-span *in vitro*. Many of these limitations can be avoided by fusing the cytoplasm of cells in a mixed culture with the aid of inactivated viruses (Okado and Murayama, '65; Harris and Watkins, '65) and making the relevant observations directly on the heterokaryocytes. For this purpose, one would like to employ a procedure that would result in the collective fusion of as many cells as possible in a culture.

MATERIALS AND METHODS

Cell cultures

Three lines of human homonuclear cells were used in this study. *CR* was started from the foreskin of a normal male infant. *JDU* was developed from a skin biopsy of a female who was mutant homozygous for galactosemia (Krooth and Weinberg, '61) *AUC* was similarly obtained from a male infant homozygous for orotic aciduria (Krooth, '64). The human heteronuclear strain employed (D98/AH-2; Szybalski and Szybalska, '62) may in fact be a subline of HeLa cells (Gartler, '67). It was obtained from the American Type Culture Collection (CCL 18.3).

Virus

Parainfluenza virus type I, strain Sendai (Fukai and Suzuki, '55), was originally isolated from a mouse by Dr. Nakao I. Ishida (Tohoku University Medical School, Sendai, Japan). The virus had been passed several times through mice, through a rat, and again through mice, before being adapted to grow in the allantoic cavity of chick embryos.

Composition of culture medium and solutions

Balanced salt solution (BSS) was prepared according to the formula of Hanks ('48). Phosphate-buffered saline, pH 7.0, was prepared as described by Dulbecco and Vogt ('54). The cells were grown in modi-fied "nucleomedium" (Krooth, '64) composed of Eagle's Minimum Essential Medium (Eagle, '59), 5 volumes % NCTC-135 (Evans et al., '64), 12 volumes % fetal bovine serum, the "non-essential" amino acids, sodium pyruvate (1.0 mM), five nucleosides (adenosine, thymidine, cytidine, uridine and guanosine), each at 15 μg/ml, ascorbic acid (500 μg/100 ml), penicillin (100 U/100 ml), streptomycin (12 μg/ 100 ml) and tetracycline (250 μg/100 ml). The versene and trypsin solutions were prepared as described by Martin ('64).

Growth of the virus

Sendai virus was grown in ten or eleven-day-old embryonated chicken eggs. After the allantoic fluid was harvested, it was centrifuged at 680 g for 20 minutes. The supernatant fluid was again centrifuged at 34,850 g for 20 minutes, and the pellet was resuspended, by gentle grinding with the tip of a pipette, in 10 times less than the original volume. For experiments in which the effects of virus dose were examined, the virus was concentrated 100-fold (by using, instead, a volume of the suspending medium 100 times less than the original volume). The suspending medium was BSS with penicillin G (100 U/ml) and streptomycin (50 μg/ml). An aliquot of the suspension was used to measure its hemagglutination titer. The ten-fold concentrated virus suspension had a titer of about 40,000 hemagglutinating units (HAU) per milliliter. The virus was stored frozen, in 1.0 ml aliquots, at −60°C. The storage period varied from several hours to more than a year.

Virus inactivation

On the same day on which a fusion experiment was to be performed, the virus suspension was thawed and 1.0 to 1.5 ml of the suspension were placed in 60 mm diameter Petri dish (Falcon Plastics, Los Angeles, California). The dish was then exposed for three minutes to a General Electric germicidal lamp (no. 630T8) at a distance of 7.5 inches. Next, the suspension was stored at 4°C until it was added to the cell culture — two to four hours later. Following this procedure, virus suspensions having up to 10^6 infectious units per 0.1 ml contained no detectable infec-

tivity when inoculated into embryonated chick eggs.

Method of cell fusion

One milliliter of the suspension was placed in each of several 16 × 150 mm Leighton tubes. The cultures were incubated at 37°C for six to eight hours, by which time the cells had attached to the flat surface of the Leighton tubes to form a confluent monolayer, whose density was about 1 × 10⁵ cells per square centimeter. Without removing the 1.0 ml of medium present, 0.1 ml of the virus suspension was added to each tube. The final concentration of the virus was 4,000 to 5,000 HAU per milliliter of medium. Control cultures received 0.1 ml of BSS instead of virus suspension. In both cases, the pH of the medium was 7.4. The virus and the medium were mixed by agitation, and the cultures were incubated at 37°C for an additional 12 hours. Thereafter the cells were treated in the manner described below.

Scoring procedure

When human homonuclear cells are examined after they have grown to a high cell population density, it is almost impossible to determine the outer border of each cell. Hence, after exposure to virus the cells were subcultured from a dilute suspension into 35 mm dishes containing 22 mm² coverslips. Immediately after the cells were placed in these plates, they were observed through the microscope, and only those dishes containing a low concentration of monodispersed cells were used. The number of cells per dish was not fixed, but was low enough as to assure a very small probability of two cells of being close to one another. Plates containing too many cells, or significant numbers of aggregated cells,

were discarded. The cells were then allowed to attach to the coverslips at 37°C during a six- to eight-hour incubation period. Afterwards, the coverslips were washed once in warm BSS. They were then fixed in 95% ethanol for 30 minutes, stained with Giemsa, and mounted on glass slides.

RESULTS

A. Quantitation of cell fusion: the polykaryocytic index

The parameter used to quantitate cell fusion will be called the *polykaryocytic index*. It is defined as the proportion of all nuclei in a culture which are present in multinucleate cells. It is therefore estimated by dividing the number of nuclei in multinucleate cells by the number of nuclei in all cells counted in a given preparation. The binomial standard error has been used for this estimate. On the following assumptions, the polykaryocytic index represents the proportion of cells in the original population which underwent fusion: (1) all the multinucleate cells observed arise by fusion; (2) there is no cell growth between the time the virus is added and the time when the cells are fixed; and (3) no nuclear fusion takes place among nuclei present in polykaryocytes.

B. Cell culture variables

1. *Biology of the cell lines used.* Table 1 shows the results of an experiment in which Sendai virus, instead of being added to cell suspensions, was added to monolayers of human homonuclear (CR) and heteronuclear (D98/AH-2) cells. This experiment demonstrates that, under the present conditions, human homonuclear cells fuse as readily as heteronuclear ones.

TABLE 1

Polykaryocytic indices of human homonuclear (CR) and heteronuclear (D98/AH-2) cells in the absence and presence of UV-inactivated Sendai virus [1]

	Without virus		With virus	
	CR	D98/AH-2	CR	D98/AH-2
Polykaryocytic index	0.063 ± 0.012	0.086 ± 0.010	0.341 ± 0.017	0.322 ± 0.017
Number of nuclei counted	413	628	760	746

[1] The chi-square value, comparing the homonuclear and heteronuclear cell cultures exposed to virus, was 0.61, with 1 degree of freedom. $p = 0.30$–0.50.

Data based on comparing different experiments, in each of which a different line of human homonuclear cells was used, have revealed no significant differences in the fusion abilities of strains derived from different donors.

Although no rigidly controlled experiments were performed, the length of time during which a homonuclear strain had been propagated *in vitro* did not seem to affect its fusibility. Fusibility has not, however, been tested in the case of homonuclear lines that are clearly senescent.

2. *Method of cell fusion.* In fusion experiments previously reported, Sendai virus has been added to a cell suspension (Okada, '62a; *inter alia*). The cell suspension was kept at 4°C, which reportedly facilitates the agglutination of the cells by the virus. The suspension was then warmed to 37°C, at which temperature fusion takes place. When the maximum degree of fusion had been achieved, the cells were transferred to culture flasks to whose surface they attached. In the fusion technique we have used, the cells are already attached to the surface of the flasks and their fusion was induced simply by adding the virus to the medium, in sufficient amounts to obtain the desired final concentration.

In the following experiment the two procedures, *as they are usually carried out,* were compared.

10^7 cells (AUC strain) suspended in nucleomedium were placed in each of six centrifuge tubes. The medium was removed by low speed centrifugation and the cells were then resuspended with 0.5 ml of BSS at 4°C. 0.5 ml of viral suspension (also suspended in BSS) was added to each tube. The concentration of virus was adjusted so that final concentrations in the tubes were, respectively, 2×10^2, 2×10^3 and 2×10^4 HAU per milliliter. Two tubes received each concentration. The cells and virus were then mixed. The cells underwent agglutination almost immediately, and the tubes were left at 4°C for 15 minutes. Then the tubes were transferred to a water bath at 37°C, where they were agitated at a rate of approximately 100 strokes per minute for 45 minutes. Afterwards, the cells were diluted 1:100 with nucleomedium and plated into 35 mm diameter Petri dishes (Falcon Plastics) containing 22 mm² coverslips. The cells were allowed to attach at 37°C for six hours. Simultaneously, six replicate cultures of AUC cells, which had been plated six hours before, were fused in monolayer using the procedure described under MATERIALS AND METHODS. The final concentrations of virus in each Leighton tube were, respectively, 4×10^2, 4×10^3 and 4×10^4 HAU per milliliter. Two duplicate tubes received each concentration of virus.

Table 2 shows the results of this experiment. There was significantly higher fusion when the cell monolayer was exposed to the virus, than when the cells were fused by the conventional method. However, the two methods are not strictly comparable. The final concentrations of virus were not the same in the cultures fused in suspension as those fused in monolayer. Nonetheless, when cells in suspension were exposed to a concentration of virus as high as 2×10^4 HAU per milliliter, the polykaryocytic

TABLE 2

Comparison of results obtained using different methods for the fusion of human homonuclear cells [1] *in the presence of different concentrations of Sendai virus*

Method employed	Virus concentration (HAU/ml)	Polykaryocytic index	Number of nuclei counted
Suspension	2×10^2	0.098 ± 0.014	451
	2×10^3	0.137 ± 0.016	467
	2×10^4	0.188 ± 0.018	475
Monolayer	4×10^2	0.532 ± 0.028	324
	4×10^3	0.498 ± 0.025	412
	4×10^4	0.509 ± 0.027	340

[1] AUC cells were fused in monolayer using the technique described under Materials and Methods, and in suspension using the method of Okada ('62), and modified by Harris et al. ('66).

index was still substantially lower than that observed when a concentration of only 4×10^2 HAU per milliliter was used to fuse cells in monolayer. The time of exposure of the cells to virus was also different in cultures fused in suspension (45 minutes) and in monolayer (12 hours). Finally, there is no really rigorous way of controlling cell population density when comparing cells in monolayer with those in suspension. In monolayer cultures, cell population densities are expressed as number of cells per *square* centimeter of surface area available for growth, while in suspension culture the corresponding figure is number of cells per *cubic* centimeter of suspending medium.

In order to evaluate the effect of the duration of exposure to virus on the fusion of homonuclear cells fused in monolayer or in suspension, an experiment similar, but not identical, to the one just described was performed. In this instance, the cell monolayers of two replicate cultures were exposed to the virus for only six hours, and their average polykaryocytic index was compared to that of four suspension cultures in which the exposure to the virus at 37°C had lasted 45 minutes in the case of two, and six hours in the other two. After viral exposure, the cells were subcultured at low population density to dishes containing coverslips, which were then fixed after six hours of incubation at 37°C. The final concentration of virus used in the cultures in monolayer was 4×10^4 HAU per milliliter, and in those in suspension, 2×10^4 per milliliter. The cell strain used in this experiment was CR. The difference in virus concentration is due to differences, in this respect, in the protocol of the two methods. However, these differences in the final con-

centration of virus are most likely not the cause of the results obtained, since the polykaryocytic index does not vary at these different viral concentrations (cf. table 2, fig. 3).

As shown in table 3, although the polykarocytic index was low in the suspension cultures exposed to virus for only 45 minutes, when the exposure lasted six hours, the suspended cells had about the same index as those in monolayer exposed to virus for a similar length of time. For the reasons discussed above, these experiments do not, in any rigorous sense, compare the relative efficiency of suspension versus monolayer cultures for achieving cell fusion. They do, however, show that using the techniques described here, one can obtain similar polykaryocytic indices by these two methods. The monolayer method is much easier to use, and, in the case of human homonuclear lines which will not grow, and will eventually die, in suspension culture, perhaps less deleterious to the cells.

It is interesting that, although the spatial relationships of cells to one another in monolayer and suspension cultures are very different, after six hours of exposure to virus the distribution of cells with varying numbers of nuclei are remarkably similar. Figure 1 shows the distribution of multinucleate cells among the various classes following exposure to virus.

Because of their shape, these distributions are reminiscent of the Poisson. The complete Poisson is not applicable in this case, however, since the class zero (i.e., cells with zero nuclei) does not exist. It becomes necessary to generate a theoretical truncated Poisson distribution lacking the class zero, which can then be compared to

TABLE 3

Polykaryocytic indeces of human homonuclear cells exposed, in suspension and in monolayer, to Sendai virus for different lengths of time [1]

	Method and time of exposure of cells to virus		
	Suspension 45 minutes	Suspension 6 hours	Monolayer 6 hours
Polykaryocytic index	0.108 ± 0.010	0.424 ± 0.024	0.402 ± 0.019
Number of nuclei counted	1059	406	654

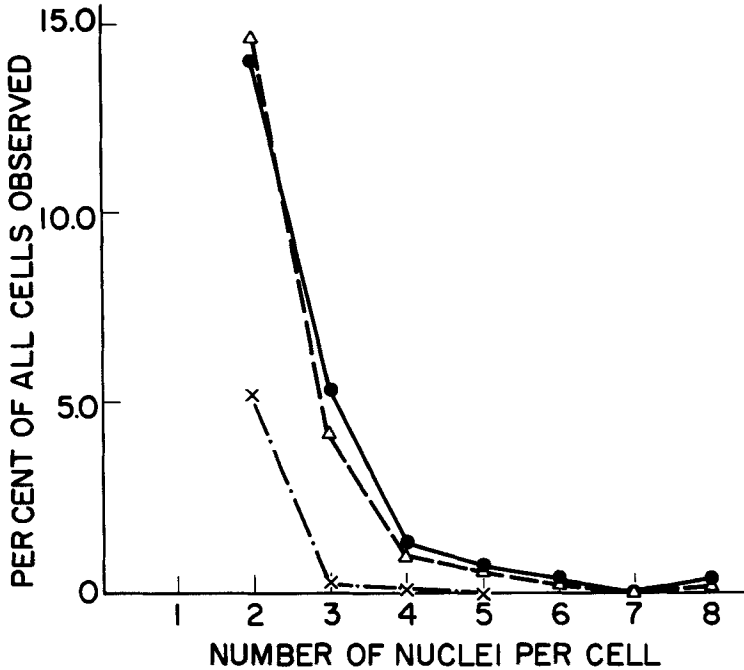[1] The results of chi-square tests of homogeneity are shown in table 5.

Fig. 1   Frequency distribution of *cells* with different numbers of nuclei after exposure to Sendai virus using two techniques for effecting cell fusion. Only the frequencies of poly-karyocytes are shown in this figure. CR cell strain was employed. (×—·—·—×): exposure in suspension to virus for 45 minutes; (·————·): exposure in suspension to virus for six hours; (△ – △ – △ – △): exposure in monolayer to virus for six hours.

the observed distribution. In addition to this comparison, an attempt was made to determine whether the mononucleate cells form a heterogeneous population with respect to their capacity to undergo fusion. For this purpose, the observed distributions were also truncated to eliminate the mono-nucleate class ("class one") and compared with a truncated Poisson lacking the classes zero and one. Because there was no significant difference between the distributions of cells exposed in suspension or in mono-layer to virus for six hours (see table 5), these data were pooled in the statistical tests for comparison with the truncated Poisson distributions. The procedures used for this analysis are described in the APPENDIX.

The results are shown in table 4. Table 5 summarizes the results of χ^2 tests of homogeneity, contrasting the observed and the expected distributions. Both in the case of cells exposed to virus for 45 minutes and of cells exposed for six hours, the observed

distribution of *all* cells does not fit the Poisson distribution, truncated to omit the zero class, even at a probability level of 0.001. However, when the distributions are truncated to eliminate mononucleate cells, and the observed and expected distributions of *only* the polykaryocytes are compared, there is a much better fit. The fit is improved both for the cultures exposed to virus for 45 minutes and for those exposed for six hours. In each instance, chi-square is insignificant.

Note, incidentally, that although the data satisfactorily fit (table 5) a Poisson distribution, truncated to eliminate classes zero and one, there tends to be an excess of cells with the highest number of nuclei (table 4). The interpretation of these findings will be discussed later.

3. *Cell population density.*   Cell-to-cell contact is obviously necessary if cell fusion is to occur. Therefore, it is to be expected that population density of the cultures would affect the frequency of fusion. How-

TABLE 4

Comparison of cell fusion in monolayer and in suspension. Observed and theoretical frequency distributions [1] of cells with different number of nuclei

Number of nuclei per cell	Exposure in suspension for 45 minutes				Exposure in suspension for 6 hours				Exposure in monolayer for 6 hours			
	All cells		Polykaryocytes only		All cells		Polykaryocytes only		All cells		Polykaryocytes only	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
1	94.50	80.44	—	—	78.00	66.04	—	—	79.20	66.04	—	—
2	5.20	16.91	94.25	80.52	14.00	25.74	63.64	54.34	14.60	25.74	70.19	54.34
3	0.20	2.37	3.64	16.57	5.33	6.69	24.24	29.03	4.20	6.69	20.19	29.03
4	0.10	0.25	1.82	2.56	1.33	1.30	6.06	11.63	1.00	1.30	4.81	11.63
5	0	0.02	0	0.32	0.67	0.20	3.03	3.73	0.60	0.20	2.88	3.73
6	0	0	0	0.03	0.33	0.02	1.52	1.00	0.20	0.02	0.96	1.00
7	0	0	0	0	0	0	0	0.23	0	0	0	0.23
8	0	0	0	0	0.33	0	1.52	0.04	0.20	0	0.96	0.04
Number of cells counted	1000	100	55	100	300	100	66	100	500	100	104	100

[1] The theoretical distributions were obtained using the procedures described in the appendix. The results of chi-square tests of homogeneity are shown in table 5.

ever, one cannot be certain how sensitive cell fusion might be to population density, since cultured homonuclear cells are usually highly motile (Abercrombie, '65; *inter alia*). The following experiment was intended to measure the effect of cell population density.

Triplicate cultures of CR cells, growing in monolayer at different population densities, were exposed to Sendai virus for 12 hours. As shown in figure 2, there is a progressive increase in the polykaryocytic index with higher cell population density. The function seems to reach a plateau at 2.5 to 5×10^4 cells per square centimeter. Human homonuclear cells form a confluent monolayer at a population density of 10^5 cells per cm².

4. *Effect of pH*. Cell fusion induced by some paramyxoviruses, such as SV-5, reportedly occurs at higher frequency at a pH of about 8.4 than at pH 7.4 (P. W. Choppin, personal communication). An experiment was performed in order to determine whether the polykaryocytic index of human homonuclear cells would be different if the cells were exposed to Sendai virus at pH 8.4 instead of 7.4. As described under MATERIALS AND METHODS, the pH of the viral suspension previously employed was 7.4. The medium in four replicate cultures of JDU cells was removed and replaced, in two of them, by nucleomedium at pH 7.4, and in the other two, by nucleomedium adjusted to pH 8.4 by the addition of sodium hydroxide. Following exposure of the cells to virus the cultures were again incubated in medium at pH 7.4. As shown in table 6, there is a significant but rather small difference between the cultures, the polykaryocytic index being slightly higher in those cultures exposed to virus at pH 8.4. This result was consistently found in two similar experiments performed on different occasions. However, when the cells are incubated at the higher pH, unlike those incubated at pH 7.4, they develop an abnormal morphology. Moreover, no mitoses are subsequently observed in these cultures.

C. Concentration of the virus

Experiments were performed in order to measure the effect of different concentrations of UV-inactivated Sendai virus on the

TABLE 5

*Results of tests of homogeneity comparing observed and theoretical distributions of cells
exposed in suspension and in monolayer to virus for different lengths of time* [1]

Distributions being compared	χ^2	Degrees of freedom	Probability
Exposure in suspension and in monolayer for 6 hours	1.07	5	0.95–0.98
Exposure in suspension for 45 minutes and for 6 hours	112.72	3	< 0.001
Exposure in suspension and monolayer for 6 hours, and truncated Poisson without class zero	42.72	4	< 0.001
Exposure in suspension and monolayer for 6 hours, and truncated Poisson without classes zero and one	9.12	4	0.05–0.10
Exposure in suspension for 45 minutes, and truncated Poisson without class zero	92.32	3	< 0.001
Exposure in suspension for 45 minutes, and truncated Poisson without classes zero and one	5.26	2	0.05–0.10

[1] Chi-square tests done on data from tables 3 and 4.

polykaryocytic index of human homonuclear cells. The virus suspension was harvested, concentrated and irradiated with ultraviolet light, as described under MATERIALS AND METHODS. The virus suspension was then serially diluted to the desired concentrations using BSS. Each Leighton tube containing a confluent cell monolayer received 0.1 ml of one of the viral dilutions. As before, the control cultures received BSS instead of virus. Each result is the average of those found in three replicate cultures.

In one of these experiments CR cells were employed. As shown in figure 3, there is a very large increase in the index after the cells were exposed to only 80 HAU per milliliter, followed by only a moderate additional increase when the virus was about 100-fold more concentrated. Over the range of viral concentrations used, there was no inhibition of cell fusion by the higher titers of virus. The shape of the distribution of cells with different numbers of nuclei did not change appreciably with varying viral concentrations.
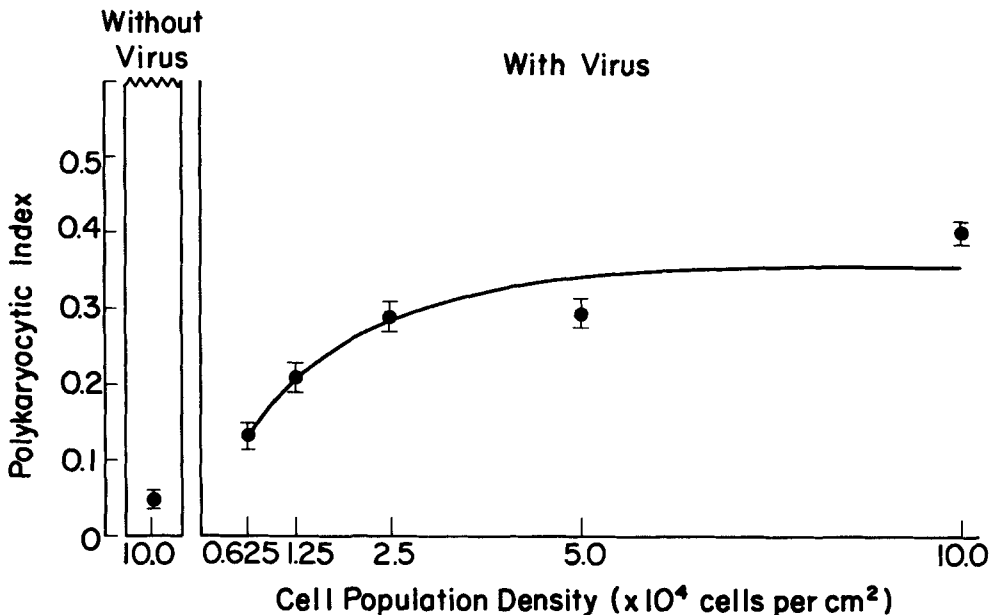

Fig. 2　Effect of varying cell population density on the polykaryocytic index of human homonuclear cells in monolayer exposed to Sendai virus. Human homonuclear cells form a confluent monolayer at a population density of 10^5 cells per cm².

TABLE 6

Effect of pH on the polykaryocytic index of human homonuclear cells [1]

pH	Polykaryocytic index	Number of nuclei counted
7.4	0.338 ± 0.021	503
8.4	0.425 ± 0.021	534

[1] JDU cells were employed. The chi-square value was 8.31, with 1 degree of freedom. p = 0.001–0.010.

D. Conditions of virus preparation

Guggenheim et al. ('68) studied the fusion in suspension of human fibroblasts with chick embryo erythrocytes. They reported that the frequency of heterokaryocytes was greater when the cells were exposed to fresh infected allantoic fluid, than when they were exposed to an equal volume of a ten-fold concentrate of the virus, suspended in BSS instead. They concluded that in this system, some of the fusing activity is lost in the discarded allantoic fluid. However, they did not consider the possibility that the higher virus concentration could inhibit to some degree the formation of the human-chicken heterokaryocytes. They also report that part of the fusing activity is lost if the virus suspension is stored for one week at either 4°C or −70°C. They do not explain, however, whether the virus was UV-inactivated before or after it was frozen and thawed.

The following experiments were performed in order to determine whether the removal of allantoic fluid, or the freezing and thawing of the virus suspension, lower the frequency of polykaryocytes in our system.

Infected allantoic fluid was harvested and pooled. Four aliquots were centrifuged. In one of the tubes, the pellet was resuspended in the same allantoic fluid, and in another the pellet was resuspended in BSS. In both tubes the volume of the suspending medium was the original one, i.e., the virus was not concentrated. The virus suspension was irradiated with ultraviolet light and, afterwards, 0.1 ml from each tube was added to a set of triplicate cultures of CR cells in the same way as in the previous

TABLE 7

Polykaryocytic indeces of cells exposed to Sendai virus suspended in different media [1]

Medium used for viral suspension	Polykaryocytic index	Number of nuclei counted
Fresh allantoic fluid	0.323 ± 0.017	752
Hanks' balanced salt solution	0.334 ± 0.017	760

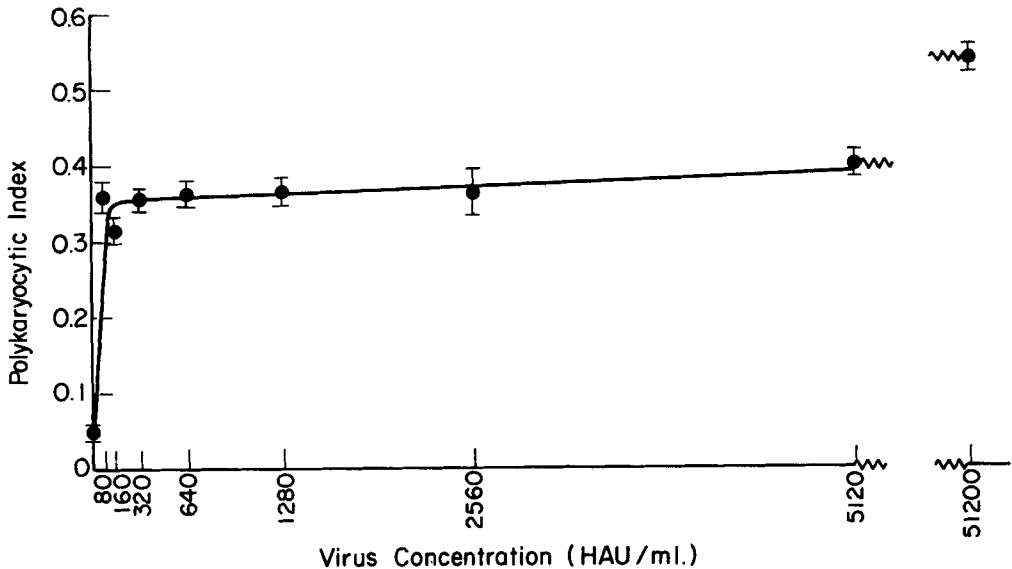[1] The chi-square value was 0.209, with 1 degree of freedom. p = 0.50–0.70.

Fig. 3 Effects of different concentrations of UV-inactivated Sendai virus on the polykaryocytic index of human homonuclear cells. HAU: hemagglutinating units.

experiments. The results are shown in table 7. There was no difference between the fusing activities of Sendai virus suspended in fresh allantoic fluid and in BSS. Therefore, in the case of human homonuclear cells, no fusion activity was lost when the supernatant allantoic fluid was discarded during the preparation of the virus.

The other two aliquots of the pooled viral suspension were concentrated ten-fold with BSS. One of these aliquots was kept at 4°C while the other was stored in a freezer at — 60°C. After five hours, the latter was quickly warmed up by submersion into a water bath at 37°C and was continuously agitated until the moment the solution was completely thawed, as determined visually. Both suspensions were then irradiated with ultraviolet light. Subsequently, 0.1 ml of each aliquot were added to triplicate monolayer cultures of CR cells. As shown in table 8, the polykaryocytic index was not diminished when the virus suspension was frozen and thawed.

The above experiment tested the effect of short-term freezing of the virus suspension on its fusion activity. Experiments comparing long-term effects of storage at 4°C and −60°C have not been performed. This is because a rigorous test of the effects of *prolonged* storage at freezing temperatures cannot be performed since it is not reasonable to use as a control an unfrozen virus suspension harvested at the same time as the frozen suspension. Therefore, the use of a freshly harvested suspension would necessarily introduce a second variable to the experiment, i.e., the lot of virus being used. However, data based on comparing different experiments suggest that virus suspensions kept at −60°C for more than one year induce as high polykaryocytic indices in human homonuclear cell cultures as suspensions frozen for only a few hours.

TABLE 8

Effect of freezing and thawing on the ability of Sendai virus to promote polykaryocyte formation [1]

Temperature at which the virus was stored after purification	Polykaryocytic index	Number of nuclei counted
4°C	0.305 ± 0.017	747
− 60°C	0.390 ± 0.017	792

[1] The chi-square value was 12.24, with 1 degree of freedom. p < 0.001.

E. Cell fusion kinetics

The following experiments were undertaken in order to study the time course of some of the events leading to viral-induced cell fusion. To design such experiments, one needs to employ assumptions concerning the mechanism of cell fusion. The present evidence suggests that fusion occurs as the result of several discrete events: The viral particles are adsorbed to the cell, the cell surface is interrupted and the interrupted sites are then either reconnected or fused with those of neighboring cells. Cytoplasmic bridges are thus formed between the cells leading to cell fusion (Okada, '62a,b; Okada et al., '66; Harris et al., '66).

When performing kinetic studies on cell fusion, it is desirable to separate the process of adsorption of virus to the cell surface from the processes leading to fusion itself. It is known that fusion does not occur at 4°C (Okada and Tadokoro, '63; Schneeberger and Harris, '66). However, adsorption of viral particles to the cells seems to take place rapidly at this temperature (Zhdanov and Burinskaya, '62).

In the experiment to be described, CR cells were first overlaid with the virus suspension at 4°C for one hour. The "control" cultures were not exposed to the virus, but were instead overlaid with BSS, also at 4°C for one hour. Time zero, as depicted in figures 4 and 5, was the moment when the cells were washed and fed with warm nucleomedium, and then transferred from 4° to 37°C. At each specified time, two or three replicate cultures were subcultured and plated at a very low population density into dishes containing coverslips. After six hours, the coverslips were fixed and the polykaryocytic indeces were determined. The cells that were subcultured during the first 10 or 15 minutes after time zero, tended to form tight clumps which were difficult to disperse, and it was impossible to obtain a perfect monodisperse cell suspension.

Figure 4 shows the kinetics of change of the polykaryocytic index in this experiment. At time zero, the polykaryocytic index is already substantially greater than the one observed in the control cultures. However, if the cells exposed to virus are
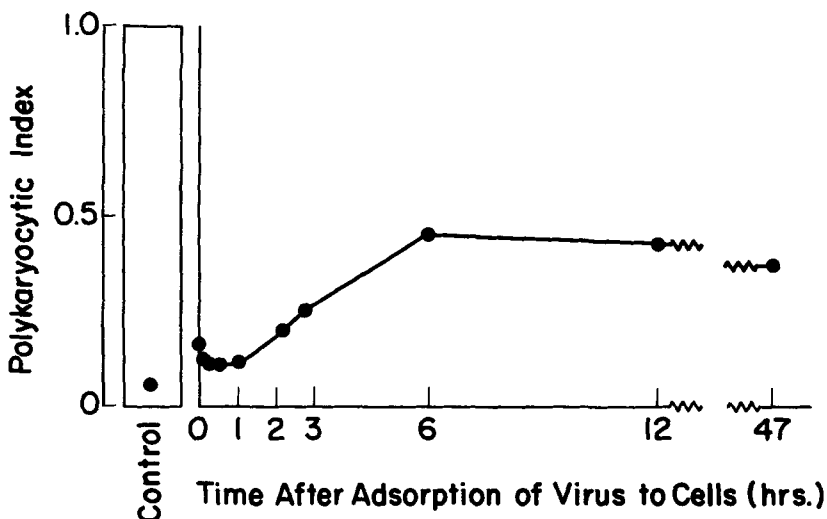
Fig. 4 Kinetics of change of the polykaryocytic index. Virus suspension was overlaid on CR cells at 4°C for one hour. Control cultures were overlaid with BSS. Time zero is the moment when the cultures were transferred to 37°C.

transferred from 4° to 37°C and are then incubated for 20 to 30 minutes before being subcultured, the polykaryocytic index is somewhat decreased. It is likely that the initial rise in the polykaryocytic index is due to the plating on the coverslips of clumps of cells, which then fuse in the following hours of incubation at 37°C. It must be remembered that cells are incubated for six additional hours in order to allow them completely to attach to the coverslips. When cells exposed to virus in the cold are incubated for 20 or 30 minutes at 37°C, before they are subcultured, it is easier to disperse them and few, if any, cell clumps are plated on the coverslips. Consequently no additional cell fusion will take place. Hence a lower polykaryocytic index is obtained for those cultures incubated at 37°C for 20 or 30 minutes, than for the cultures subcultured immediately after the incubation period at 4°C. Okada ('62a) has reported that when cells are fused in suspension, they are strongly agglutinated by the virus at 4°C, but when they are transferred to 37°C the cells in these aggregates are no longer so tightly agglutinated, and the large clumps of cells break apart into smaller ones. These clumps probably occur as a result of the large amounts of viral particles ad-

sorbed to the surface of the cells. The number of these particles probably decreases after the cells are incubated at 37°C. As was mentioned previously, Schneeberger and Harris ('66) observed that Sendai virus remains adsorbed to the cell surface so long as the temperature is kept at 4°C. However, after 20 minutes of incubation at 37°C, very few particles remain on the surface.

At about one hour, the index begins gradually to increase until it reaches a maximum between the fourth and the sixth hour. Over the next 42 hours, a slight decrease is observed, perphaps due to proliferation of the unfused cells. At the virus concentrations used in the present experiment, no nuclear division was observed in polykaryocytes. During the first 48 hours, no evidence of degeneration of polykaryocytes was detected.

The kinetics of fusion of human homonuclear cells contrast sharply with the time course followed by most heteronuclear cells fused in suspension. When a suspension of these latter cells is transferred to 37°C after having been exposed to Sendai virus at 4°C, they start fusing almost immediately, and 30 minutes later fusion is almost complete (Okada, '62a; Schneeberger and Harris, '66). It seems likely that the slow kinetics of increase in the poly-

karyocytic index which we have observed in our experiments is a characteristic of human homonuclear cells, rather than of the method employed to induce their fusion, because when human homonuclear cells in *suspension* are exposed to virus, there is also considerably higher fusion when the viral exposure lasts six hours than when it is limited to 45 minutes (table 3).

The proportion of nuclei at each point in time, occurring in cells with 1, 2, 3, ... n nuclei was also measured. These data are shown in figure 5 which describes the relative frequency with which nuclei were observed in each class at each point in time. If one assumes that all the polykaryocytes were formed by the fusion of mononucleate cells after the addition of virus, the slopes of these curves represent the rates of incorporation of the original members of the cell population into each class of polykaryocytes. After the increase observed at time zero, and the subsequent decline, there is a progressive incorporation of nuclei into polykaryocytes, paralleled by a decrease in mononucleate cells. The frequency of nuclei in binucleate cells first increases — probably as a result of recruitment of monokaryocytes. Afterwards, there is a decline of the frequency of nuclei in binucleate cells over the next 47 hours. This is probably due, at least in part, to the donation of nuclei from these cells to cells with a larger number of nuclei. As shown in figure 5, after 47 hours, the proportion of nuclei in cells with five or more nuclei has increased in spite of a small rise in mononucleate cells. Cells with a higher number of nuclei appear later than cells with a lower number. Cells with more than 11 nuclei were observed only after 47 hours. This suggests that the fusion of several cells into a single one is more likely to take place sequentially rather than simultaneously.

It might be argued that the late appearance of cells with a large number of nuclei is the result of amitotic divisions by polykaryocytes containing a lower number of nuclei, since in multinucleate cells nuclear mitosis is frequently not followed by division of the cytoplasm (Harris et al., '66). However, this is unlikely, since at the concentrations of virus used in the present study, no nuclear mitoses were observed in polykaryocytes. Furthermore, it is reported when two or more nuclei of a polykaryocyte undergo division synchronously, they usually fuse, giving rise to a single, giant nucleus (Harris et al., '66; Holmes and Choppin, '66). Rao and Johnson ('70) have observed a very high degree of mitotic synchrony among nuclei in fused HeLa cells. If these effects apply to human homonuclear cells, they would tend to decrease, rather than increase, the proportion of polykaryocytes having many nuclei with time. It is unlikely that the late appearance of large polykaryocytes is the result of multiplication of residual live virus surviving the ultraviolet treatment. The kinetics of appearance of these cells is the same whether the concentration of the virus is ten-fold greater or lesser than the concentration employed in the experiment summarized in figure 5.

F. Effect of multiple additions of the virus

As shown in the above experiments, the maximum polykaryocytic index obtained following a single exposure of human homonuclear cells to UV-inactivated Sendai virus was about 0.5. It was, therefore, of interest to determine whether additional exposures of the cells to the virus would result in an increase in the polykaryocytic index.

In one experiment, nine replicate cultures of CR cells were plated in Leighton tubes. Three of these cultures were used as controls, and were overlaid with 0.2 ml of BSS on three different occasions, for one hour each, at 24-hour intervals. Another set of triplicate cultures received only a single dose of virus. They were overlaid with 0.6 ml of the virus suspension for three hours, six hours after the cells had been plated. At 24 and 48 hours after this exposure, the cells were overlaid with 0.2 ml of BSS, for one hour. The last set of cultures were exposed to three doses of virus at 24-hour intervals. On each of these occasions, they were overlaid with 0.2 ml of virus suspension for one hour. All cultures were then subcultured, for evaluation of the polykaryocytic index, 72 hours after the first exposure to virus. The purpose of this experimental design was to prevent
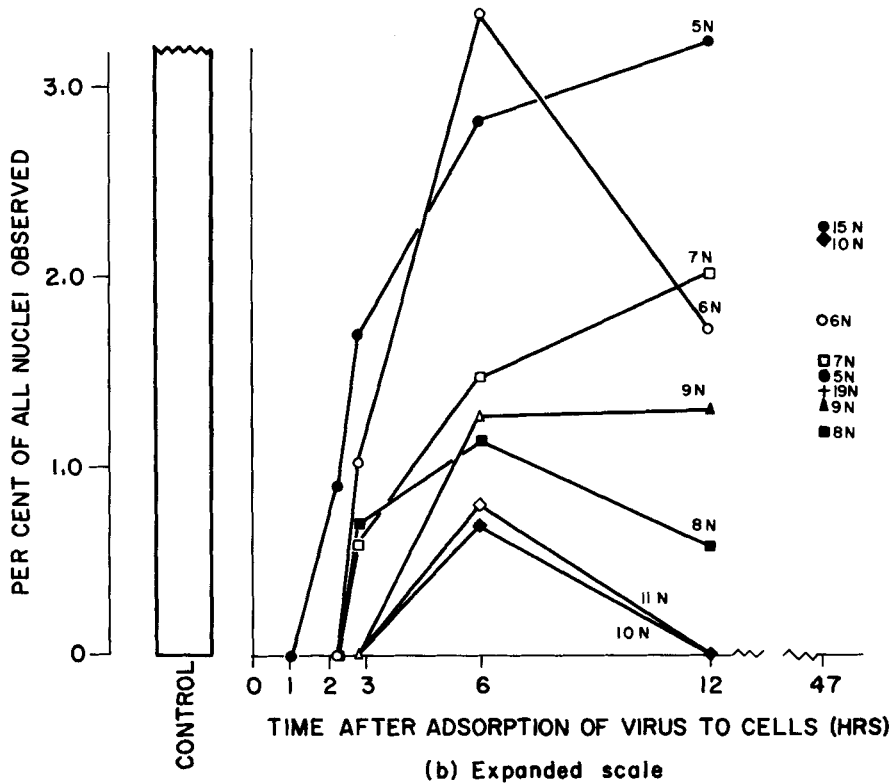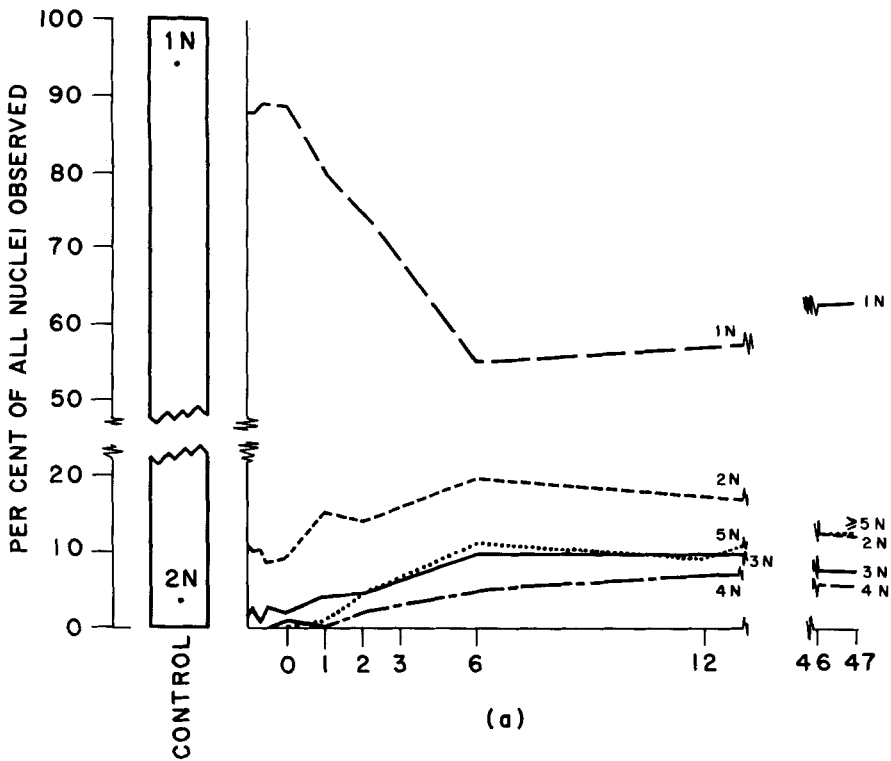
Fig. 5 (a) Kinetics of change of the frequencies of nuclei in the different cell classes. "N" refers to the number of nuclei per cell. In order to simplify the graph, the points at the forty-seventh hour have not been connected with the earlier points. (b) The same experiment on an expanded scale and representing only the frequencies of nuclei in cells with five or more nuclei. Note that cells with more than 11 nuclei were observed only at the forty-seventh hour.

the *amount* of exposure of cells to virus from confounding the *frequency* of exposure. All exposed cells were exposed to the same quantity of virus for the same total time, but in the case of one set of cultures, the virus suspension was divided into three aliquots. The cells were exposed to these aliquots 24 hours apart, rather than to all three aliquots at once. As shown in table 9, the polykaryocytic index of cultures exposed to the virus only once was almost identical to the one of the cultures that was exposed three times. Similar results were observed when the intervals between viral exposure lasted only six hours.

Therefore, additional doses of UV-inactivated Sendai virus added to human homonuclear cells at either 6 or 24 hours after a first exposure, do not seem to lead to higher polykaryocytic indices. Although other experiments, with perhaps more rigorous design, for inquiring into this matter are feasible, there is little reason to believe that they would yield valuable information. The results of the experiments just described make it very unlikely that intermittent exposure of the culture to virus promotes increased cell fusion.

DISCUSSION

The experiments reported here show that human homonuclear cells can be formed into polykaryocytes by the use of UV-inactivated Sendai virus. A very simple procedure which appears to work well is described. While the present work was in

progress, Siniscalco et al. ('69) reported results of experiments (whose main purpose was the formation of somatic cell hybrids) in which cells from two different human homonuclear strains were fused in suspension. To effect fusion, they exposed cells to Sendai virus which had been inactivated with beta-propiolactone. Their cultures showed 7 to 8% binucleate cells; less than 0.1% of cells had three or more nuclei. This would seem to correspond to a polykaryocytic index of 0.13 to 0.15.[4]

Under the conditions used in the experiments reported here, human homonuclear cells appear to undergo cytoplasmic fusion as readily as human heteronuclear cells. Okada and Tadokoro ('63), working with HeLa cells exposed in suspension to Sendai virus, obtained considerable fusion with relatively low concentrations of virus. However, higher viral concentrations seemed to have an inhibiting effect on the fusion of the cells. Beyond a certain titer, the degree of fusion obtained decreased with increasing viral concentration. This may perhaps explain why in the present experiments, where high concentrations of Sendai virus were employed, a line of human heteronuclear cells, suspected of being derived from the HeLa cells (Gartler, '67), fused to only a limited extent.

Although the fusion of cells in monolayer and in suspension is not strictly comparable, the polykaryocytic index of human homonuclear cells fused in monolayer is considerably higher than when cells of the same strain are fused by the conventional method in suspension. Fusion in monolayer has previously been produced in other cell-virus systems (Kohn, '65; Holmes and Choppin, '66; Davidson, '69; Klebe et al., '70). However, the technique that we have used is perhaps simpler since it requires only the addition of virus to the medium covering the cells. The results obtained when cells are exposed to virus in this manner are similar to those obtained when the medium is removed and the cell monolayer is overlaid for one hour with the virus suspension (cf. fig. 4). This latter procedure is more laborious, and probably in-

TABLE 9

Effect of multiple additions of the virus, at 24-hour intervals, on the polykaryocytic index of human homonuclear cells

Number of viral additions	Polykaryocytic index	Number of nuclei counted
0 [1]	0.039 ± 0.010	612
1 [2]	0.433 ± 0.017	824
3 [3]	0.436 ± 0.017	837

In all cases, the cell monolayers were subcultured to dishes with coverslips 78 hours after having been seeded.
[1] Monolayers were overlaid with 0.2 ml BSS three times, for one hour each, at 24 hour intervals.
[2] Monolayers were overlaid with 0.6 ml of virus suspension for three hours, on only one occasion. They were then overlaid with 0.2 ml BSS for one hour at 24 and 48 hours after exposure to virus.
[3] Monolayers were overlaid with 0.2 ml of virus suspension three times, for one hour each, at 24 hour intervals.
The value of chi-square comparing the results obtained after one and three viral additions is 0.017 with 1 degree of freedom. p = 0.80-0.90.

[4] If 7 to 8% of the cells are binucleate, then 14 to 16 nuclei of every 107 to 108 are, on the average, in polykaryocytes. Therefore, the proportion of nuclei in multinucleate cells (the polykaryocytic index) is 0.13 to 0.15.

creases slightly the risks of microbial contamination.

It is interesting that the cell-to-cell contact, among cells in a confluent monolayer, seems to be sufficiently intimate to allow as much fusion as when the cells are clumped together in compact agglutinates, as occurs when virus is added to a suspension culture. Nonetheless, despite the fact that cultured homonuclear cells are highly motile (Abercrombie, '65), the cell population density of a culture exposed to virus needs to be relatively high for the polykaryocytic index to reach a maximum (fig. 2).

The polykaryocytic index of human homonuclear cells fused in monolayer reaches near-maximum values at relatively low concentrations of Sendai virus. Afterwards, there is a very moderate, but persistent, increase of the index as the virus concentration is increased 100-fold more. This result contrasts markedly with the one obtained by Okada and Tadokoro ('63) for heteronuclear cells fused in suspension. These authors observed a gradual increase of the fusion index with higher virus concentrations and, as was mentioned before, after reaching a maximum there was a decline of the index with additional amounts of virus. It has been observed that aerobic conditions and an energy supply are essential when cells are fused in suspension by Sendai virus (Okada, '62b; Okada et al., '66). On the basis of these findings, Okada and Murayama ('68) have hypothesized that an excessive amount of virus inhibits fusion, because, under these conditions, very large cell aggregates would form and cells inside these large aggregates would not be adequately aerated. Whatever the inhibitory mechanism might be, it does not seem to be operative in fusion of homonuclear cells in monolayer.

As was shown in tables 4 and 5, the observed distribution of cells among the various nuclear classes, following exposure to virus, does not fit a Poisson distribution which has been truncated to eliminate the zero class. However, if the observed distribution is truncated to eliminate class one (mononuclear cells), the fit with a correspondingly Poisson is much better. These observations suggest that perhaps the population of mononuclear cells is heterogeneous, consisting of both cells capable and incapable of undergoing fusion.

There is a discrepancy, however, between the observed and the expected (on the basis of a Poisson distribution) frequencies of polykaryocytes with many nuclei. The Poisson distribution predicts the frequency of polykaryocytes with five or more nuclei to be virtually zero. However, as shown in table 4, this is not the case. Moreover, with higher polykaryocytic indices there is an even higher frequency of, say, cells with five or more nuclei. In some instances, cells with 20 to 30 nuclei have been observed. One reason for this may be that the stochastic properties of the fusion process alter during the period of observation. If the frequency of cell fusion is sufficiently high, the size and composition of the population at risk for cell tension could significantly change with time. As was shown in figure 5, the frequency of trinucleate and tetranucleate cells, probably recruited partly from the population of binucleates, eventually increases, and thus causes the population of binucleates to decrease. Such a process creates a problem in hypergeometric sampling, for which the Poisson distribution does not allow.

There are two other possible mechanisms which may also contribute to the observed excess of polykaryocytes with large numbers of nuclei. As noted above, the kinetic experiments suggest that these cells are frequently the result of fusion of polykaryocytes with lower number of nuclei. If this is indeed the case, the formation of a polykaryocyte with larger numbers of nuclei would not be an event independent of the formation of polykaryocytes with smaller numbers of nuclei. For example, a cell with eight nuclei could result from fusion of two tetranucleate cells. Furthermore, if the probability that a cell undergoes fusion is higher if it has fused before, then fusion between, say, two binucleate cells is apt to occur more frequently than between two mononucleate cells. Both these effects would tend to contradict elemental assumptions in the mathematical argument which generates the Poisson distribution.

There is currently not enough information to explain why no more than 50% of human homonuclear cells in a given cul-

ture undergo fusion in the presence of UV-inactivated Sendai virus. However, a polykaryocytic index of 0.5 is quite favorable for many kinds of experiments. Moreover, it may be possible, in the near future, to attempt to separate all the mononucleate cells, which did not fuse, from the polykaryocytes. Efficient methods have recently been developed for the fractionation of mammalian cell populations on the basis of relative cell size by sedimentation velocity techniques (Miller and Philips, '69).

APPENDIX

In order to determine whether an observed distribution from which one or several classes of events have been omitted ("truncated"), fits the Poisson distribution, from which the corresponding classes have been omitted, it is first necessary to estimate from the data the mean of a complete Poisson distribution. We computed these means by iteration, using the formulas that follow.

The mean of a distribution from which the first class (zero events) has been truncated, is given by

$$\bar{x}_t = \frac{\bar{x}}{1 - e^{-\bar{x}}} \tag{1}$$

$\bar{x}_t$ is the mean of the truncated distribution; $\bar{x}$ is the complete Poisson mean which is to be iterated. It is then used to give the truncated Poisson distribution

$$\sum_{i=1}^{\infty} p_i = \frac{1}{1 - e^{-\bar{x}}} e^{-\bar{x}} (\bar{x} + \frac{\bar{x}^2}{2!} + \cdots + \frac{\bar{x}^j}{j!} + \cdots) = 1 \tag{2}$$

The mean of a distribution from which the first *two* classes have been deleted can be computed as follows

$$\bar{x}_{tt} = \frac{\bar{x} - \bar{x}e^{-\bar{x}}}{1 - e^{-\bar{x}} - \bar{x}e^{-\bar{x}}} \tag{3}$$

and the truncated Poisson distribution without the first two classes is given by

$$\sum_{i=2}^{\infty} p_i = \frac{1}{1 - e^{-\bar{x}} - \bar{x}e^{-\bar{x}}} e^{-\bar{x}} (\frac{\bar{x}^2}{2!} + \frac{\bar{x}^3}{3!} + \cdots + \frac{\bar{x}^j}{j!} + \cdots) = 1 \tag{4}$$

LITERATURE CITED

Abercrombie, M. 1965 The locomotive behavior of cells. In: Cells and Tissues in Culture. E. N. Willmer, ed. Academic Press, New York, pp. 177–202.

Davidson, R. 1969 Regulation of melanin synthesis in mammalian cells as studied by somatic hybridization. III. A method of increasing the frequency of cell fusion. Exp. Cell Res., 55: 424–426.

Dulbecco, R., and M. Vogt 1954 Plaque formation and isolation of pure lines with polyomyelitis viruses. J. Exp. Med., 99: 167–182.

Eagle, H. 1959 Amino acid metabolism in mammalian cell cultures. Science, 130: 432–437.

Evans, V. J., J. C. Bryant, H. A. Kerr and E. L. Schilling 1964 Chemically defined media for cultivation of long-term cell strains from four mammalian species. Exp. Cell Res., 36: 439–474.

Fukai, K., and T. Suzuki 1955 On the characteristics of a newly-isolated hemagglutinating virus from mice. Med. J. Osaka Univ., 6: 1–15.

Gartler, S. M. 1967 Genetic markers as tracers in cell culture. Natl. Cancer Inst. Monograph, 26: 167–194.

Guggenheim, M. A., S. Tyrrell and A. S. Rabson 1968 Studies on Sendai virus cell fusion factor. Proc. Soc. Exp. Biol. Med., 129: 854–857.

Hanks, J. H. 1948 The longevity of chick tissue cultures without renewal of medium. J. Cell. and Comp. Physiol., 31: 235–260.

Harris, H., and J. F. Watkins 1965 Hybrid cells derived from mouse and man: artificial heterokaryons of mammalian cells from different species. Nature, 205: 640–646.

Harris, H., J. F. Watkins, C. E. Ford and G. I. Schoefl 1966 Artificial heterokaryons of animal cells from different species. J. Cell Sci., 1: 1–30.

Holmes, K. V., and P. W. Choppin 1966 On the role of the response of the cell membrane in determining virus virulence. J. Exp. Med., 124: 501–520.

Klebe, R. J., T. Chen and F. H. Ruddle 1970 Controlled production of proliferating somatic cell hybrids. J. Cell Biol., 45: 74–82.

Kohn, A. 1965 Polykaryocytosis induced by Newcastle Disease Virus in monolayers of animal cells. Virol., 26: 228–245.

Krooth, R. S., and A. N. Weinberg 1961 Studies on cell lines developed from the tissues of patients with galactosemia. J. Exp. Med., 113: 1155–1172.

Krooth, R. S. 1964 Properties of diploid cell strains developed from patients with an inherited abnormality of uridine biosynthesis. Cold Spring Harbor Symp. Quant. Biol., 29: 189–212.

——— 1969 Genetics of cultured somatic cells. Med. Clin. N. Amer., 53: 795–811.

Krooth, R. S., G. A. Darlington and A. Velazquez 1968 The genetics of cultured mammalian cells. Ann. Rev. Genet., 2: 141–164.

Martin, G. M. 1964 Use of tris (hydroxymethyl) aminomethane buffers in cultures of diploid human fibroblasts. Proc. Soc. Exp. Biol. Med., 116: 167–171.

Miller, R. G., and R. A. Philips 1969 Separation of cells by velocity sedimentation. J. Cell. Physiol., 73: 191–202.

Okada, Y. 1962a Analysis of giant poly-nuclear cell formation caused by HVJ virus from Ehrlich's ascitis tumor cells. I. Microscopic observation of giant poly-nuclear cell formation. Exp. Cell. Res., 26: 98–107.

—————— 1962b Analysis of poly-nuclear cell formation caused by HVJ virus from Ehrlich's ascitis tumor cells. III. Relationship between cell conditions and fusion reaction or cell degeneration reaction. Exp. Cell Res., 26: 119–128.

Okada, Y., and F. Murayama 1965a Multinucleated giant cell formation by fusion between cells of different strains. Biken. J., 8: 7–21.

—————— 1965b Multinucleated giant cell formation by fusion between cells of different strains. Exp. Cell Res., 40: 154–158.

—————— 1968 Fusion of cells by HVJ: requirement of concentration of virus particles at the site of contact of two cells for fusion. Exp. Cell Res., 52: 34–42.

Okada, Y., and J. Tadokoro 1963 The distribution of cell fusion capacity among several cell strains or cells caused by HVJ. Exp. Cell Res., 32: 417–430.

Okada, Y., F. Murayama and K. Yamada 1966 Requirement of energy for the cell fusion reaction of Ehrlich ascites tumor cells by HVJ. Virol., 28: 115–130.

Rao, P. N., and R. T. Johnson 1970 Mammalian cell fusion: Studies on the regulation of DNA synthesis and mitosis. Nature, 225: 159–164.

Schneeberger, E. E., and H. Harris 1966 An ultrastructural study of interspecific cell fusion induced by inactivated Sendai virus. J. Cell. Sci., 1: 401–406.

Siniscalco, M., H. P. Klinger, H. Eagle, H. Koprowski, W. J. Fujimoto and J. E. Seegmiller 1969 Evidence for intergenic complementation in hybrid cells derived from two human diploid strains each carrying an x-linked mutation. Proc. Nat. Acad. Sci., 62: 793–799.

Szybalski, W., and E. H. Szybalska 1962 Drug sensitivity as a genetic marker for human cell lines. Univ. Mich. Med. Bull., 28: 277–293.

Zhdanov, V. M., and A. G. Bukrinskaya 1962 Studies on the initial stage of virus-cell interaction. Acta Virol. (Prague), 6: 105–113.