Persistent Non-Cytocidal Infection of BHK 21 Cells by Human Parainfluenza Type 2 Virus

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(Received 9 November 1965)

SUMMARY

The BHK 21-C13 line of hamster fibroblasts was infected with human parainfluenza virus type 2. The infection persisted through 40 subcultures without affecting the plating efficiency of the cells and without degeneration of the monolayers. Although all cells contained fluorescent-staining virus antigen and most became specifically haemadsorbing and contained inclusion bodies, the titre of virus in 4-day-old cultures was only equivalent to 0.0001 plaque-forming unit/cell. Infection of cells continued when clones were grown in anti-parainfluenza serum. There was only very slight interference to infection with other viruses and superinfection with parainfluenza type 2 virus caused degeneration of the culture. Persistent infection of BHK 21 cells with human type 2 parainfluenza virus produced no loss of contact inhibition and no ability to grow in semi-solid media, changes which are characteristic of BHK 21 cells when transformed by polyoma virus or Rous sarcoma virus.

INTRODUCTION

There are now many descriptions of persistent infection by viruses in continuous cultures of animal cells. In many of these the balance between virus and host is controlled by the presence of specific antibody or of interferon in the system (Ginsberg, 1958; Isaacs, 1963), which limit infection to a minority of the cells in the culture. Continuously infected cultures have recently been described in which antibody is unnecessary and interferon not predominant in maintaining equilibrium, and the infecting viruses are frequently myxoviruses or other RNA-viruses which resemble myxoviruses (Rustigian, 1962; Walker & Hinze, 1962*a, b*). The host cells are usually epithelial. Exceptions to this type of host are found in chronic infection of BHK 21 cells with rabies virus (Fernandes, Wiktor & Koprowski, 1964) and in a fluctuating balance between polyoma virus, a DNA-virus, and mouse L cells (Henle, Hinze & Henle, 1963; Hare & Morgan, 1964).

The present account of persistent infection of BHK 21 cells is of interest because the cells are a line of diploid fibroblasts which, when transformed by polyoma virus (Macpherson & Stoker, 1962) or Rous sarcoma virus (Macpherson, 1965), show a characteristic loss of contact inhibition and a new ability to grow in semi-solid media, but which are largely unaffected in cultural behaviour when all cells in the culture are carrying parainfluenza virus type 2.

METHODS

Media. Growth medium (ETC) is a modified Eagle medium containing 10% (v/v) calf serum and 10% (v/v) tryptose phosphate broth. Occasionally the species or concentration of serum was changed, as mentioned in the text. Overlay medium consisted of 0.9% (w/v) Difco Bacto agar with 5% (v/v) inactivated calf serum in Eagle medium.

Cells. The continuous line of hamster kidney cells BHK21-C13 (Stoker & Macpherson, 1964) was grown in ETC at 37° in 4% (v/v) CO₂ in air. Subcultures of infected and non-infected cells were made every 3 or 4 days by inoculating about 2×10^6 cells into 10 ml. of ETC. When first used, the cells had been subcultured for at least 200 generations and will be referred to as C13 cells. BHK21-C13-404 and C13-405 were cell batches 3 or 4 passages distant from the stock clone C13 and recovered after preservation at -80° in ETC with 5% (v/v) glycerol. They will be designated C13-404 and C13-405 cells.

A line of polyoma-transformed virus-free cells—BHK21/13/PyY (Stoker & Macpherson, 1964)—was also used. Secondary cultures of rhesus-monkey kidney were used for preparing stocks of parainfluenza virus and a line of HEp2 cells was used for titrations of virus infectivity. The cells were grown in ETC and maintained during virus growth by replacing the calf serum with 2% (v/v) horse serum after inoculation of virus. Cells were removed from glass or plastic surfaces with 0.05% (w/v) bovine trypsin in 0.02% (w/v) EDTA.

Virus. Stocks were prepared from a strain of parainfluenza virus type 2, grown in rhesus-monkey kidney cells for 5 successive passages, and were stored at -60°. Titres varied from 10^6 to 10^7 plaque-forming units (p.f.u.)/ml. by the haemadsorption method given below. Viruses other than parainfluenza type 2 were assayed on BHK21 cells as described by Gharpure (1965). Assay of parainfluenza virus-infected C13 cells by immunofluorescence followed previous methods for polyoma virus infection (Fraser & Gharpure, 1962).

Titration. Parainfluenza virus was titrated on C13 monolayers by haemadsorption. Washed monolayers were inoculated with 0.1 ml. of virus dilution which was adsorbed for 30 min. at 37°, when the inoculum was removed and 5 ml. of overlay medium applied. After 3 days of incubation at 37° the agar was peeled off, the monolayer washed and covered with a 1% (v/v) suspension of guinea-pig red cells in saline. These were allowed to adhere for 20 min. at room temperature, then washed off gently and the monolayers examined with a low-power objective. Plaques were easily seen, very variable in size, irregular in shape, often branching to follow the lie of the cells, and gave reproducible counts in proportion to each dilution of inoculum. Overlapping of plaques caused a decrease in the apparent count at numbers above 150 per plate. Titres are given in p.f.u./ml.

Antiserum. Rabbit immune sera were inactivated at 56° for 30 min. and stored at -20°. Serum at 1/25 dilution neutralized 10^4 p.f.u. when equal volumes of serum and virus suspension were allowed to react for 30 min. at room temperature.

Fluorescent staining. The parainfluenza 2 antiserum was used in the indirect method of immunofluorescent staining with anti-rabbit goat globulin conjugated with fluorescein isothiocyanate as second layer. Infected cells, grown on coverslips, were fixed at suitable times in acetone for 10 min. at room temperature. Uninfected

cells and sera immune to influenza and polyoma viruses were used in appropriate controls of the specific staining. Photographs were taken in a Reichert fluorescence microscope on Ektachrome high-speed film.

Growth rates. Infected and uninfected cells were plated on 60 mm. dishes over circular coverslips under ETC. Cell counts were made from a fixed number of randomly chosen fields on coverslips removed from each culture at the 1st, 2nd and 3rd days of incubation and stained with Giemsa's stain.

Plating efficiency of cells. One ml. of cell suspension containing 300 or 100 cells was plated in each of 6 plates over feeder layers of X-irradiated mouse-embryo cells. Two plates were used for haemadsorption counts by the method given below. One week later colonies on the other four plates were fixed in 10% (v/v) formol saline, stained with Leishman's stain and counted.

Agar culture. Suspension culture for the selection of virus-transformed cells was described by Macpherson & Montagnier (1964); this technique was used here.

Examination of monolayers by haemadsorption. The density and firmness of haemadsorption on cultures of parainfluenza-infected cells varied with the cultures, in none of which it was possible to count individual haemadsorbing cells in the confluent monolayer. A modification was therefore made of the original method of Vogel & Shelokov (1957).

Cells from a monolayer were well dispersed in ETC, 5×10^4 cells in 60 mm. Petri dishes, and because freshly trypsinized C13 PF2 cells (see Results), unlike influenza-infected cells, are always non-haemadsorbing they were examined after overnight culture by washing the cells with normal saline, adding 2.0 ml. 0.25% (v/v) guinea-pig red cells and adsorbing these for 20 min. at room temperature. The Petri dish was then filled with normal saline containing 1% (v/v) inactivated calf serum, covered with a 90 mm. lid from a glass Petri dish and inverted. Five ml. of serum saline was then added to the lid. After 1 hr, when red cells had fallen into the lid, the dish was moved aside from the obscuring layer of red cells and the number of haemadsorbing and non-haemadsorbing C13 cells counted with low and high-power objectives. The number of cells counted was 200–400 on each plate, except when most cells seemed to be positive, when fewer sufficed. Cells to which two or more erythrocytes adhered were scored as positive. Cells tended to round up in serum saline, but did not come off the glass for several hours. Data on the validity of the method appear under Results. The specificity of the reaction was tested from time to time with a 1/5 dilution of specific antiserum which neutralized all haemadsorption. A 1/25 dilution of antiserum decreased the count of positive cells and the number of adherent red cells on each positive cell. Antisera to parainfluenza types 1 and 3 and various anti-influenzal sera had no neutralizing effect. Occasional preparations of red cells adhered non-specifically to glass or to plastic, but non-specific adhesion tended to disappear when the tests were left inverted for some hours or the plates tapped firmly from time to time. When non-specific adhesion persisted, tests were made with fresh red cells.

RESULTS

When 0.05 ml. of parainfluenza type 2 virus, titre 2×10^6 p.f.u./ml. was inoculated into about 5×10^6 C13 cells, the monolayer showed no cytopathic changes 2 days after infection, but testing with guinea-pig red cells gave confluent, firm

haemadsorption. Subcultures were made from these monolayers by trypsinizing the cells and inoculating about 2×10^6 washed cells into 10 ml. of ETC. Monolayers again grew that gave confluent haemadsorption at the third day of incubation and showed no cytopathic effect. Such cells were termed C13-PF2 cells and serial passages from the 4th pass onwards were examined in greater detail. They will be referred to as C13-PF2 cells except where exact designation is necessary.

Two further infected cultures were made for comparison, C13-404-PF2, at a virus-to-cell ratio of 1 and C13-405-PF2 at a ratio of 5. There was, this time, visible cytopathic change in both starting cultures, greater with the higher virus dose which delayed the formation of a complete monolayer for 6 days. No experiments were made to establish the factors which initially produced this non-cytopathic haemadsorption.

C13-404-PF2 and C13-405-PF2 thereafter behaved similarly and only the observations made on C13-PF2 and C13-404-PF2 are given in the paper.

Table 1. *Proportion of haemadsorbing cells and the titre of released parainfluenza type 2 virus in successive cultures of C13-PF2 cells*

Passage no.	Series I, C13-PF2		Series II, C13-404-PF2	
	% haem-adsorbing cells	Virus titre p.f.u./ml.	% haem-adsorbing cells	Virus titre p.f.u./ml.
1	—	—	92.0	7.5×10^6
2	—	—	93.4	2.75×10^5
3	95.6	1.62×10^5	—	—
4	84.3	—	18.0	—
5	85.4	—	31.1	—
6	81.6	—	32.4	—
7	66.8	—	—	—
11	13.8	1.68×10^3	—	—
12	31.6	9×10^3	—	—
13	67.0	—	—	—
15	20.0	—	—	—
17	41.8	—	—	—
18	39.6	—	—	—
19	53.8	—	—	—
21	79.6	1×10^3	—	—
22	79.4	1.2×10^3	—	—
23	91.3	0.6×10^3	—	—
31	99.0	1.25×10^3	—	—
32	99.9	1.75×10^3	—	—
33	—	—	76.0	—
34	98.5	0.1×10^3	40.0	—
36	—	—	30.0	0.4×10^3
37	94.5	—	60.0	—
38	—	—	76.5	0.35×10^3
39	—	—	81.9	0.5×10^3

Persistence of infection during continuous propagation of cells

All three infected cultures could be propagated as described in Methods without distinguishable alterations of the macroscopic or microscopic appearances of unfixed cultures from uninfected C18 cells. Infectious parainfluenza virus was recovered at intervals throughout the passages shown in Table 1 and the widespread nature of

the infection is shown by the fact that in all cultures haemadsorbing cells were present and usually predominated.

Virus yields. The titre of infectious virus in supernatant fluids of C13-PF2 cells and C13-404-PF2 cells, harvested at the time of subculture, fell rapidly from about 10^6 p.f.u./ml. in the beginning of the series to less than 100 p.f.u./ml. at the end. Since the number of cells in each culture was $10-16 \times 10^6$ in 10 ml. of fluid, this represented a ratio of about 1.0 p.f.u./cell at the beginning of the chronic infection and 0.0001 p.f.u./cell at the end. Similar titres were found when the virus was titrated on BHK21 cells, HEp2 cells and monkey kidney cells. No higher yields were found at intermediate times between subculturing and harvesting the cells, nor was the amount increased by freezing and thawing the cultures. Yields of haemagglutinin were too low, varying from less than 1 up to 8 agglutinating doses per ml., against 1% guinea-pig red cells, to correlate yield of haemagglutinin with the infectious titre of the virus or with the amount of haemadsorption.

The haemadsorption count and its significance. There was a tendency in both series for the proportion of haemadsorbing cells to diminish towards the middle, and to increase again towards the end of the series. There was no concomitant decrease and increase in the titre of infectious virus. Continued passage resulted in a less intense haemadsorption even when the proportion of haemadsorbing cells remained high. Fewer red cells stuck to each cell and they were more easily washed off, but strongly and weakly adsorbing C13-PF2 cells existed in the same culture. Different degrees of haemadsorption are illustrated in Pl. 1, figs. 1-3.

As explained in Methods, it was necessary to decide whether cells which had been removed from a monolayer and which became haemadsorbing during overnight culture represented cells already producing virus or cells invaded by virus during or following plating. The low p.f.u./cell ratio in the late passages made it virtually impossible for infection during plating to account for the large number of cells showing specific haemadsorption. Secondary infection was disproved by growing cells under an agar overlay. Equal numbers of cells from various passages of C13-PF2 cells were plated in ETC and one half the number of plates overlaid with nutrient agar after 5 hr incubation at 37° , when cells had stuck to the glass. After incubation for a further 18 hr, agar was removed and cells washed and tested by the inversion method of haemadsorption. There was neither significant difference between the proportion of haemadsorbing cells in ETC and the proportion under agar nor any great loss of cells on removing the overlay (Table 2).

Subsidiary tests showed in addition that harvesting and plating the C13-PF2 cells in 1/25 antiserum did not decrease the proportion of haemadsorbing cells. They also became positive after plating even when nutrient agar was replaced by serum saline or when the incubation temperature was decreased to 20° , conditions under which newly infected C13 cells did not produce specific haemadsorption. Haemadsorption as tested by the method used thus indicated a property of the cells that had been removed temporarily during subculture.

Studies were made to see whether haemadsorbing cultures contained any other evidence of virus infection.

Inclusion bodies. Staining of C13-PF2 cells with dilute Giemsa's stain for 20 hr showed many inclusion bodies that were not present in uninfected C13 cells. They varied from sharply defined, densely eosinophilic, oval bodies in the cytoplasm, to

irregular patches of faintly staining material (Pl. 1, figs. 6, 8). The number of cells containing such bodies was greater in the early passages and roughly related to the intensity of haemadsorption. No nuclear inclusions were seen. A small proportion of multinucleate cells was usually present.

Immunofluorescent staining. Plate 1, fig. 4 shows cytoplasmic masses of brightly staining specific antigen which was present as loose aggregates and also as brilliant oval bodies. These corresponded exactly to irregular eosinophilic masses (Pl. 1, figs. 5, 6) or to the oval inclusions (Pl. 1, figs. 7, 8). The amount of specifically stained material was roughly proportional to the amount of haemadsorption, but no cells which were non-haemadsorbing were quite free of virus antigen.

Table 2. *Comparison of the proportion of haemadsorbing cells in C13-PF2 cells, when plated in growth medium and under agar*

C13-PF2 passage no.	No. cells plated	Growth medium			Agar		
		No. cells counted	% haem- adsorbing cells	Average no. cells per field	No. cells counted	% haem- adsorbing cells	Average no. cells per field
7	10 ⁵	212	67.4	—	230	65.6	—
9	10 ⁵	169	19.8	—	590	22.0	—
18	10 ⁴	245	35.1	9.07	204	41.6	7.9
	10 ⁵	574	37.4	114.8	496	39.6	99.2

— = not done.

Infectious centres. The number of virus-yielding cells in a culture can normally be estimated from the proportion of infectious centres formed on plating a known number of cells. Four different subcultures of C13-PF2 cells and two of C13 cells, freshly infected at a ratio of about 3 p.f.u./cell, were tested by plating up to 10⁴ cells on monolayers of BHK21 or of monkey kidney cells. The proportion of infectious centres was always less than 1% of the cells plated. It was not therefore possible to decide how many C13-PF2 cells produced infectious virus.

Culture in antiserum. Cultures were also made in specific antiserum. Six successive passages, each of 2×10^6 cells, made in 1/5 and 1/10 rabbit immune antiserum did not reduce the proportion of haemadsorbing cells present in the final culture when the serum was removed.

The effect of cloning C13-PF2 cells

Continuous cultivation of mass cultures of C13-PF2 cells was similar to the passage of uninfected cells. Since each cell was infected, in that all contained virus antigen and most displayed specific haemadsorption when tested, it was possible to study persistent infection arising from single cells.

Plating efficiency. The proportion of viable cells in C13 and C13-PF2 cultures was studied by comparing the number of colonies appearing from a given inoculum of each type of cell (see Methods). The plating efficiency of the parainfluenza-infected cells is equal to that of uninfected cells; and although virus antigen must be present at the surface of haemadsorbing cells, plating efficiency was not diminished in virus-specific antiserum (Table 3).

Haemadsorption on single colonies. Cells were plated as in the plating efficiency

test, but no feeder layers were used. An inoculum sufficient to provide 3–30 colonies/plate was used, and at the end of the incubation period these were not fixed but examined by haemadsorption. They could be divided into haemadsorbing and non-haemadsorbing colonies. Some colonies showed only slight haemadsorption, a few hundred red cells being scattered over the surface of the colony, others showed dense, confluent haemadsorption through which the C13 cells could not be seen. The presence of non-haemadsorbing colonies at the end of incubation for 7 days and the fact that the proportion of haemadsorbing colonies roughly equalled the proportion of haemadsorbing cells at the start of the experiment (Table 4), suggests that spread of virus from colony to colony was infrequent.

Table 3. *Plating efficiency of C13–PF2 cells*

A. In ETC, C13–PF2 cells compared with C13 cells

	Number of colonies from 300 cells/plate				
	Plate				Average
	1	2	3	4	
C13 cells	134	165	133	129	140
C13–PF2 cells	127	139	141	134	135

B. C13-404–PF2 and C13–PF2 cells in ETC and in ETC plus anti-parainfluenza serum 1:10

Series and passage no.	Medium	Number of colonies from 300 cells/plate			
		Plate			Average
		1	2	3	
C13-404–PF2, 4th pass	ETC	174	163	158	165
	ETC + antiserum	181	178	159	173
C13–PF2, 30th pass	ETC	191	174	190	185
	ETC + antiserum	185	205	193	194

Table 4. *Comparison of the proportion of haemadsorbing C13–PF2 cells, after 24 hr incubation, with the proportion of haemadsorbing colonies, from the same culture, after 7 days incubation*

C13–PF2 passage no.	% haem-adsorbing cells at 24 hr	No. cells/plate	No. colonies counted on all plates	% haem-adsorbing
22	78	300	90	64.4
		100	44	65.9
34	98.5	300	54	100
		100	16	100

Subculture of single colonies. Cells negative to haemadsorption have been shown above to contain specific virus antigen. It is probably for this reason, and not because of spread of infectious virus from colony to colony, that non-haemadsorbing colonies have always given haemadsorption-positive cells when subcultured to make monolayers. Table 5 gives results of one series in which the progeny of all colonies was haemadsorbing when tested in the usual way for the proportion of haemad-

sorbing cells. It will be seen from Table 5 that the intensity of haemadsorption was less in subcultures derived from non-haemadsorbing colonies. No attempt was made to select completely uninfected cells by this method.

Single-cell cultures. The technique of picking single colonies could not exclude the chance of secondary infection of the colony during sampling or in the haemadsorption test. The passage of infection from parent cell to progeny was confirmed by culturing single C13-PF2 cells in anti-parainfluenza serum. Of 12 clones grown in droplets, 6 were cultured in ETC and 6 in 1/25 antiserum. All droplet cultures were grown into monolayers in antiserum and all gave haemadsorbing cells when the serum was removed and the cells tested by overnight culture. Six clones from the single-cell cultures in antibody were recloned in antiserum with the same haemadsorption result as before and two of these were passed a third time in single cell culture. These were haemadsorption-negative at the first pass, but became positive on further culture. Thus it was certain that infection could be passed intracellularly to daughter cells, but the question of eventual cure of the culture from parainfluenzal infection was not settled.

Table 5. *Haemadsorption in monolayers derived from single colony cultures of C13-PF2 cells*

Origin	1st passage, single colonies	2nd passage, monolayer from each colony
A. A colony with confluent haemadsorption	5 haemadsorbing	→ 5 haemadsorbing*
	6 non-haemadsorbing	→ 6 haemadsorbing†
B. A colony with moderate haemadsorption	4 haemadsorbing	→ 4 haemadsorbing*
	2 non-haemadsorbing	→ 2 haemadsorbing†

* Haemadsorption equal in density to colony A or colony B.

† Haemadsorption 80 % less dense than colony A or colony B.

Serial single cell cultures were also made in stronger antiserum, 1/9 in ETC, but cultures died out at or before the 4th single-cell subculture. Whether this was an effect of the anti-parainfluenzal globulin on the surface of the cell or whether the cultural conditions favoured the eventual lytic cycle of the virus is not known. No attempts were made to recover infectious virus from single cells because of the low yield of virus (0.0001 p.f.u./cell) in monolayer cultures.

Cultural characteristics of C13-PF2 cells

Since no obvious cytopathic effect had been seen during passage of mass cultures of C13-PF2 cells, the growth rate and plating efficiency in fluid medium and agar were tested, as described under methods.

Growth rate. No significant difference was detected between C13 cells and C13-PF2 cells (Table 6).

Growth in agar. In 3 out of 4 experiments using the agar technique, it was found that C13-PF2 cells, like C13 cells, did not proliferate when colony formation in agar was attempted (Table 7). In the same tests polyoma-transformed C13 cells produced too many colonies to be counted.

Interference and superinfection. Interference with plaque formation by other viruses is shown in Table 8, which compares the number of foci on C13-PF2 monolayers with the number on monolayers of C13 cells. There was a significant, but slight, interference with encephalomyocarditis virus, with the NWS strain of influenza A and with polyoma virus but not with vaccinia. Decrease of plaque diameter on C13-PF2 cells was also a sign of interference. The mean diameter of 20 plaques of EMC virus on C13 cells was 9.6 mm. (range 9.0–11.0) and on C13-PF2 cells the mean was 5.5 mm. (range 4.0–6.5).

Table 6. *Growth rate of C13-PF2 cells compared with that of C13 cells*

		Average no. of cells in 10 fields at:			Multiplication factor at 72 hr
		24 hr	48 hr	72 hr	
C13-PF2	1st slip	43.8	169.6	331.5	—
	2nd slip	37.6	161.2	346.5	—
	Average	40.07	165.4	339	× 8
C13	1st slip	40.2	154	414	—
	2nd slip	54.6	139.4	364	—
	Average	47.4	147	389	× 8

Table 7. *Comparative growth in agar of C13-PF2 cells, C13 cells and polyoma-transformed BHK21 cells (Py/Y)*

Expt. no.	C13-PF2		C13-404-PF2	
	1	2	3	4
Inoculum No. cells/plate	2×10^4	5×10^4	5×10^4	5×10^4
	No. of colonies/plate (5 plates)			
C13-PF2 or C13-404-PF2 cells	19	0	0.2	0.2
C13 cells	0	0	0.2	0.2
Py/Y cells	570	Very numerous	Very numerous	Very numerous

Table 8. *Number of foci produced by four viruses on monolayers of C13-PF2 cells compared with the number produced by an equal inoculum on C13 cells*

Virus	C13-PF2, monolayer	C13, monolayer	Ratio, % C13-PF2/C13
Encephalomyocarditis	13*	28.5	55 %
NWS, influenza A	134*	325	41 %
Vaccinia	47.5*	57.5	83 %
Polyoma	0.18†	0.45	41 %

* Average number of plaques on 4 plates.

† Percentage of fluorescent-stained nuclei in 40,000 cells.

Plaque titration of parainfluenza virus on C13-PF2 cells was not possible but, if there were any interference to superinfection with the same virus, it was not absolute. The inoculation of 0.2 ml. of undiluted stock parainfluenza type 2 virus caused confluent haemadsorption and destruction of the monolayer 4 days later.

Polyoma-transformed BHK21 cells, free of polyoma virus, were readily infected by parainfluenza type 2 virus and persistent infection followed.

Reaction to altered cultural conditions. Several attempts were made to upset the balance of synthesis between virus and cells. Replacement of ETC with a medium containing only 1 % calf serum resulted in destruction of the cells in 3 or 4 days, but the titre of infectious virus was not increased and the concentration of haem-agglutinin was only occasionally increased. Temperatures of incubation were varied from 37° to 32°, 35° and 39.5°. These did not alter the state of C13-PF2 cells or alter the titre of cell-free virus. Monolayers of C13-PF2 cells degenerated before similar monolayers of C13 cells when incubation was prolonged to 6 or 7 days in ETC. There was no increase in the yield of infectious virus but the yield of haem-agglutinin rose as high as 16 agglutinating doses per ml.

DISCUSSION

The principal features of this persistent association between parainfluenza type 2 virus and BHK21 cells were the continuous production of antigen, shown by haemadsorption and by fluorescent antibody, the low yield of infectious virus and the unaltered cultural characteristics of the infected C13-PF2 cells. The fall and rise in the proportion of haemadsorbing cells may have been caused by extraneous factors which were not tested, such as the presence of antibody or inhibitors to parainfluenza virus in different batches of calf serum. It may also be a rhythmical alteration similar to changes in the yield of infectious influenza virus that occur on passage of infected cultures (Tyrrell, 1959). This will only be apparent when the infected cell line is propagated further.

The large amount of antigen indicated by haemadsorption and also by immunofluorescent staining is, surprisingly, not accompanied by large yields of virus haem-agglutinin, but the rough correlation between haemadsorption and specific immune staining does suggest that haemadsorption is dependent on the amount of antigen being extruded from the cell. When cytoplasmic stores diminished haemadsorption was absent. There was no concentration of virus antigen at the cell margin of C13-PF2 cells as there is with influenza A antigen in the same species of cell (K. B. Fraser, personal observation). The loss of haemadsorbing properties in newly trypsinized cells and their recovery under conditions which did not permit virus synthesis also suggests that surface antigen was replaced from the cytoplasmic virus.

The small quantity of infectious virus in C13-PF2 cultures, which was not due to an attenuated infectivity for C13 cells alone, may well come from a minority of cells which produce large individual yields of virus and fail to divide. The higher yield of virus in the early passages of the series, in conjunction with undiminished plating efficiency of cells, suggests that, initially at least, most cells produced some infectious virus. It is clear that parainfluenza virus-infected cells divided, as was already known to Gresser & Enders (1961), who used non-continuous cell lines; it is not known whether dividing cells synthesized virus. The very poor plating efficiency of infectious centres and the very low yield of released virus per single cell make these questions difficult to decide.

There are many similarities between C13-PF2 cells and other systems in which

persistent infection of the majority of cells in culture has been described. Continuing production of measles antigen by infected HeLa cells with little production of infectious virus (Rustigian, 1962) is very similar to the C13-PF2 system. In chronic infection of human conjunctival cells with mumps virus (Walker & Hinze, 1962*a*) auto-interference was striking, in contrast to C13-PF2 cells; interference with unrelated viruses was negligible. In the mumps system, anti-virus serum decreased the amount of antigen in each cell, but all cells became haemadsorbing when antiserum was removed. (Walker & Hinze, 1962*b*). This again is similar to C13-PF2 cells.

The pathogenicity of mumps virus decreased in chronically infected cells, and rabies virus also has been reported to lose virulence in persistently infected rabbit and hamster cells (Fernandes *et al.* 1964). On the other hand, interference in rabies-infected cultures was not confined to auto-interference or even to viruses of the same presumed group.

Persistent infections by mumps, rabies and parainfluenza viruses show certain common features: no great effect on the cultural characteristics of the cells; a large percentage of infected cells present in the culture; diminished infectivity or virulence of the virus; inaccessibility of the virus to immune serum; the presence of cytoplasmic inclusion bodies and aggregates of virus antigen; rather poor, but variable, resistance to infection by other viruses. The type of association is distinct from steady-state cultures which are maintained by cytolytic infection of a minority population of cells amongst a resistant or protected majority.

The use of diploid BHK21-C13 cells in this study has a bearing on virus transformation not illustrated in previous studies. Alteration of the morphology of the colonies, and acquisition of ability to grow in agar, are manifestations of virus transformation by either polyoma virus (Macpherson & Stoker, 1962) or Rous sarcoma virus (Macpherson, 1965). They are readily demonstrable in BHK21 cells and are usually regarded as a result of close integration of the virus genome with the cell. It is thus of some interest that persistent infection of BHK21 cells with parainfluenza virus is not accompanied by any of the cellular changes that are characteristic of virus transformation. This shows that persistent infection alone is not the cause of virus transformation.

We are indebted to Mr James Stott and Professor N. R. Grist for strains of parainfluenza viruses and their type-specific sera, and to Mr Alan McIlroy for the photographic prints.

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EXPLANATION OF PLATE

Figs. 1–3. Different intensities of specific haemadsorption of guinea-pig erythrocytes in the same culture of C13–PF2 cells. Culture cells rounded by the action of non-buffered serum-saline. Fig. 1. Dense haemadsorption to a single cell; $\times 480$. Fig. 2. Moderate haemadsorption to 3 cells; $\times 480$. Fig. 3. Scanty haemadsorption to 4 cells, one on a lower plane; $\times 480$.

Fig. 4. Immunofluorescent staining of C13–PF2 cells, 85 % of which were haemadsorbing. Virus antigen (white) present in all cells as inclusion bodies and as diffuse antigen. $\times 480$.

Figs. 5–8. A comparison of fluorescent-stained virus antigen with inclusions in C13–PF2 cells. Fig. 5. A multinucleate cell showing irregular aggregates and discrete flecks of virus antigen; $\times 480$. Fig. 6. The same cell stained with dilute Giemsa's stain. Note the correspondence of cytoplasmic inclusions with fluorescent-staining of virus antigen. $\times 480$. Fig. 7. Three cells showing circular and oval cytoplasmic aggregates of virus antigen; $\times 480$. Fig. 8. The same cells, stained with dilute Giemsa's stain, to show correspondence of inclusions with fluorescent-staining; $\times 480$.