HYBRID CELLS DERIVED FROM MOUSE AND MAN: ARTIFICIAL HETEROKARYONS OF MAMMALIAN CELLS FROM DIFFERENT SPECIES

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THE ability of certain animal viruses to induce the formation of multinucleate cells by fusing together single cells suggested the possibility that these viruses might be used to amalgamate different cell types and thus produce artificial animal cell heterokaryons. In this article we describe some properties of heterokaryons which we have produced by fusing together cells of human and murine origin.

The two cell types used in the present experiments were HeLa cells (originally derived from a human carcinoma) and mouse Ehrlich ascites tumour cells. These cells were chosen because they could be obtained in quantity as suspensions of single cells: the HeLa cells were grown *in vitro* in suspension culture[1]; the Ehrlich tumour was maintained in the ascitic form in the peritoneal cavity of Swiss mice. Some experiments we have made with other cell types indicate, however, that the technique can be applied to a variety of cells, including differentiated somatic cells, provided that they are susceptible to the strain of virus used to produce fusion. Of the large number of viruses which are known to induce the formation of multinucleate cells, the para-influenza I group of myxoviruses seemed the most promising, because it had been shown by Okada[2] that one strain of these viruses (*HVJ*) could induce rapid fusion in suspensions of Ehrlich ascites cells *in vitro*. The virus used in the present experiments was a strain of Sendai virus supplied by Dr. H. G. Pereira of the National Institute for Medical Research, Mill Hill.

The virus was propagated in the following way. Infected allantoic fluid with a titre of 8,000 haemagglutinating units/ml. was diluted 1 in 10^4 with phosphate-buffered saline: 0·1 ml. of this preparation was injected into the allantoic cavity of 10- or 11-day-old fertile hens' eggs, which were incubated for 3 days at 37° C. The eggs were then maintained at 4° C overnight and the allantoic fluid collected. The pooled allantoic fluid was centrifuged at 400*g* for 10 min and the haemagglutination titre of the supernatant determined. The supernatant was then centrifuged at 30,000*g* for 30 min and the deposit re-suspended in one-tenth of the original volume in Hanks's solution[3] (used throughout the present experiments without glucose). The haemagglutination titre was again determined and the concentrated virus suspension stored in 1-ml. lots at −70° C. This suspension, suitably diluted in Hanks's solution, was used for the experiments. Haemagglutination titrations were performed in Salk-pattern haemagglutination trays. Doubling dilutions of virus were made in 0·5 ml. of phosphate-buffered saline, and approximately $2 \cdot 5 \times 10^7$ guinea-pig erythrocytes suspended in 0·05 ml. of this saline were added to each cup. The smallest amount of virus which produced complete haemagglutination after 2 h at room temperature was defined as one haemagglutination unit (HAU).

Since any investigation of the physiology of the heterokaryons would be greatly complicated if these cells were engaged in virus production, the virus used to produce cell fusion was inactivated by ultra-violet light. One ml. of the concentrated suspension of virus in a watch-glass was exposed for 3 min to ultra-violet light emanating from a Philips 15-W 18-in. germicidal tube, type 'T.U.V.'. The intensity of the radiation incident on the surface of the fluid was 3,000 ergs/cm²/sec. The suspension of virus was mixed by pipetting at the end of the first and second minutes. Infectivity titrations on the irradiated virus were carried out by a modification of Fulton's method[4] in which pieces of chorio-allantoic membrane were incubated with the virus in Medium 199 (ref. 5) in a haemagglutination tray. It was found that after 3-min exposure to ultra-violet light under the present conditions, the infectivity of the virus, as measured by the production of haemagglutinin, was reduced to levels at which the measurements were obscured by the haemagglutination produced by the initial viral inoculum. This represented a reduction in infectivity of at least 3 logs. Although the ability of the virus to multiply in the chorio-allantoic membrane had thus been drastically reduced, its ability to induce cell fusion *in vitro* remained unimpaired. Evidence will be presented to show that the virus inactivated by ultra-violet light was not reactivated in the vast majority of the heterokaryons.

The technique used for inducing cell fusion was essentially similar to that described by Okada[2]. HeLa cells from a suspension culture were spun down and re-suspended at a concentration of 2×10^7 cells/ml. in Hanks's solution. Ehrlich ascites cells, withdrawn from the peritoneal cavity, were washed once by centrifugation in this solution and then resuspended in it at a concentration of 2×10^7 cells/ml.: 0·5 ml. of each cell suspension was pipetted into a chilled inverted T-tube together with 1·0 ml. of the suspension of virus, diluted, if necessary, in Hanks's solution. The cells clumped immediately, and the size of the clumps was roughly proportional to the amount of virus added. The T-tube was kept at 4° C for 15 min and then shaken in a water bath at 37° C for 20 min at a rate of 100 excursions/min. During this time the cells in the clumps underwent varying degrees of fusion. Fig. 1 shows an electron micrograph of a section through a pellet of cells obtained by centrifuging a preparation at 200*g* for 10 min immediately after the period of shaking at 37° C. A trinucleate cell is shown, formed by the fusion of three discrete cells; and a fourth cell is seen in the process of fusing with this trinucleate cell.

One ml. of the cell suspension and 5 ml. of culture medium were pipetted into a 6-cm-diameter Petri dish containing 15 coverslips 1 cm in diameter. The culture medium consisted of 20 per cent calf serum and 1 per cent tryptose broth in Medium 199, to which penicillin at a concentration of 100 international units/ml. and streptomycin at a concentration of 100 μg/ml. were added. The dishes were incubated at 37° C in a gas mixture of 5 per cent carbon dioxide in air. The coverslips were transferred to fresh medium after 24 h and again after 4 days.

Four h after the cell suspension had been introduced into the Petri dishes multinucleate cells were found to have adhered to the coverslips; and within 24 h most of these had flattened out on the glass. From cell counts it could be calculated that the multinucleate cells adherent to the coverslips accounted for about 10 per cent of the single cells originally present in the suspension. Each of these multinucleate cells contained from 2 to about 20 nuclei. The nuclei were of two easily distinguishable morphological types. With May–Grünwald–Giemsa or Leishman stain one type of nucleus stained more deeply than the other. The more deeply staining nucleus contained numerous small nucleoli or coarse condensations of chromatin, while the less deeply staining nucleus con-

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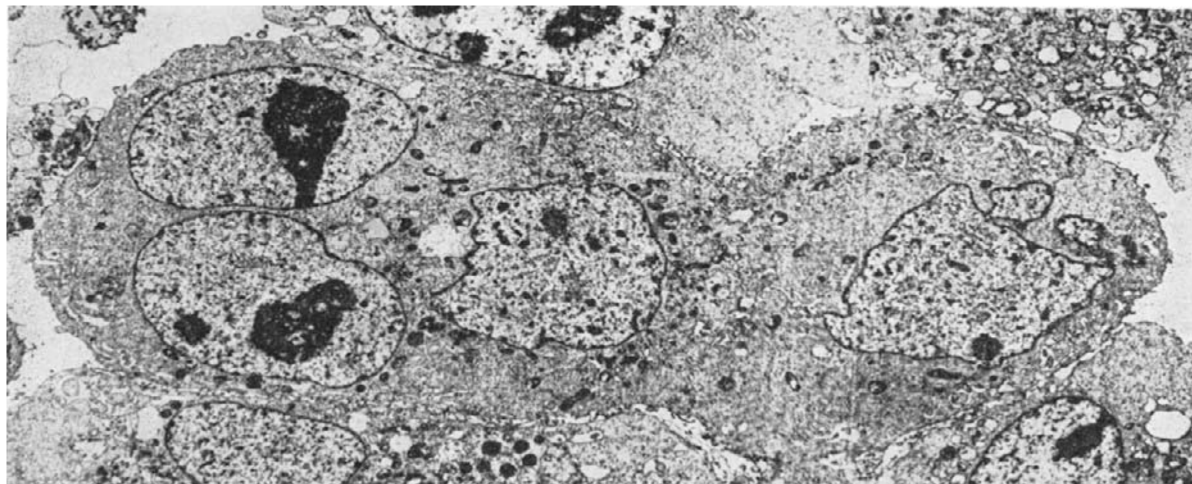

Fig. 1. An electron micrograph of a section through a preparation in which HeLa cells and Ehrlich ascites cells were fused together. A trinucleate cell is shown, formed by the fusion of three discrete cells. A fourth cell, on the right, is seen in the process of fusion with the trinucleate cell. (By courtesy of Dr. G. I. Schoefl)

tained one to three large nucleoli. The two types of nucleus are illustrated in Fig. 2, which shows a tetranucleate cell containing two nuclei of each type. Since the deeply staining pachychromatic nuclei clearly resembled the nuclei of Ehrlich ascites cells, and the less deeply staining nuclei resembled the nuclei of HeLa cells, it was difficult to avoid the conclusion that the multinucleate cells were formed by fusion of the two cell types. This was confirmed in the following way: 100 μc. of tritiated thymidine was added to a suspension culture of HeLa cells at 10 a.m., 2 p.m., 6 p.m. and 10 p.m. on one day, and again at 7 a.m. and 10 a.m. on the following day. Since the generation time of the cells under these conditions was about 20 h, it was thought that this procedure would succeed in labelling the nuclei of virtually all the cells in the culture. Autoradiographs of smears of the cells, made 2 h after the final addition of tritiated thymidine, confirmed that this was so. When a mixture of labelled HeLa cells and unlabelled Ehrlich ascites cells was treated with virus, autoradiographs of the resulting multinucleate cells revealed that the deeply staining pachychromatic nuclei were not labelled, whereas the less deeply staining nuclei with the large nucleoli were. This is illustrated in Figs. 3–5, which show a series of heterokaryons containing labelled and unlabelled nuclei in varying proportions. When the reciprocal experiment was made with labelled Ehrlich ascites cells and unlabelled HeLa cells, it was found that the deeply staining pachychromatic nuclei were labelled and the others were not. It is thus clear that the multinucleate cells were indeed formed by the fusion of HeLa cells with Ehrlich ascites cells. At least 98 per cent of the multinucleate cells were heterokaryons when virus concentrations of 8,000 HAU/ml. or more were used to induce the cell fusion (Table 1).

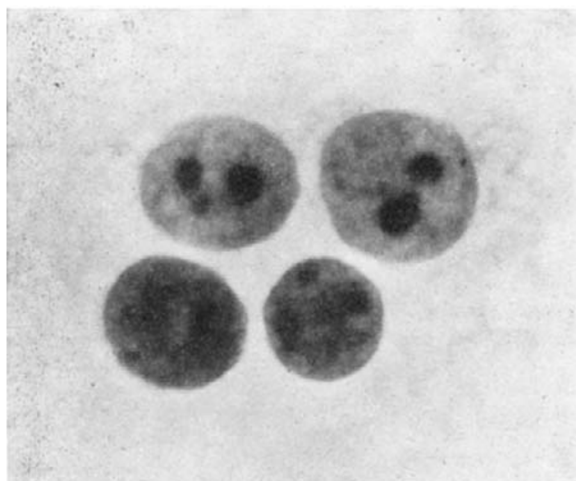

Fig. 2. A tetranucleate cell in which the two upper nuclei are derived from HeLa cells and the two lower ones from Ehrlich ascites cells (×1,300)

Table 1. EFFECT OF VIRUS CONCENTRATION ON THE CHARACTER OF THE HETEROKARYONS PRODUCED

Counts made 24 h after formation of heterokaryons	Concentration of virus used: Virus inactivated by ultra-violet light 800 HAU	8,000 HAU	80,000 HAU	Infective virus 8,000 HAU
Percentage of multinucleate cells which were heterokaryons	77	99	98	97
	84	98	97	99
	85	97		99
Mean	82	98	98	98
No. of nuclei per heterokaryon	4·0	(18·4)	10·0	6·3
	4·3	5·9	8·5	5·1
	4·4	9·4		7·4
Mean	4·2	(11·2)	9·3	6·3
Ratio of HeLa nuclei to Ehrlich ascites nuclei	2·8	1·9	3·2	2·3
	1·9	2·1	2·7	2·6
	2·5	2·0		2·6
Mean	2·4	2·0	3·0	2·5
Ratio of multinucleate to mononucleate cells	1·0	3·5	8·4	3·1
	0·6	2·0	7·5	9·0
	0·8	3·1		8·4
Mean	0·8	2·9	8·0	6·8

The average number of nuclei per heterokaryon and the ratio of multinucleate to mononucleate cells could be varied by changing the concentration of virus used. Table 1 shows that at lower concentrations of virus the number of nuclei per heterokaryon and the ratio of multinucleate to mononucleate cells fell. Since the proportion of the original cell suspension recovered as multinucleate cells was unaffected by the concentration of virus used (Table 2), the increase in the ratio of multinucleate to mononucleate cells at higher concentrations of virus must have resulted from preferential elimination of mononucleate cells. This was apparently due to the fact that the multinucleate cells were more resistant to the cytotoxic effects of the virus than the mononucleate cells, a conclusion supported by the observation that infective virus eliminated a greater proportion of the mononucleate cells than inactivated virus at the same concentration (Table 1). With high concentrations of virus, however, and especially with infective virus, some of the multinucleate cells showed marked vacuolation. Table 1 also shows that the ratio of HeLa nuclei to Ehrlich ascites nuclei in the heterokaryons was between 2 and 3 irrespective of virus concentration. This preponderance of HeLa nuclei might be due either to the fact that HeLa cells fuse more easily than Ehrlich ascites cells or, more probably, to the fact that cells with

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Table 2. SURVIVAL TIME OF HETEROKARYONS MADE WITH DIFFERENT CONCENTRATIONS OF VIRUS

Days after production of heterokaryons	No. heterokaryons per coverslip			
	Virus inactivated by ultra-violet light			Infective virus
	80,000 HAU	8,000 HAU	800 HAU	8,000 HAU
1	$4\cdot2 \times 10^3$	$2\cdot7 \times 10^3$	$4\cdot9 \times 10^3$	$4\cdot5 \times 10^3$
	$4\cdot3 \times 10^3$	$3\cdot2 \times 10^3$	$7\cdot1 \times 10^3$	$5\cdot3 \times 10^3$
		Medium changed		
2	$3\cdot3 \times 10^3$	$3\cdot3 \times 10^3$	$5\cdot5 \times 10^3$	$4\cdot8 \times 10^3$
	$2\cdot9 \times 10^3$	$3\cdot6 \times 10^3$	$6\cdot2 \times 10^3$	$6\cdot3 \times 10^3$
3	$2\cdot1 \times 10^3$	$3\cdot2 \times 10^3$	$5\cdot0 \times 10^3$	$8\cdot5 \times 10^3$
	$2\cdot0 \times 10^3$	$3\cdot9 \times 10^3$	$4\cdot4 \times 10^3$	$4\cdot3 \times 10^3$
4	$2\cdot2 \times 10^3$	$5\cdot0 \times 10^3$	$5\cdot1 \times 10^3$	$2\cdot3 \times 10^3$
	$1\cdot1 \times 10^3$	$3\cdot7 \times 10^3$	$5\cdot2 \times 10^3$	$3\cdot5 \times 10^3$
		Medium changed		
5	$1\cdot8 \times 10^2$	$2\cdot3 \times 10^3$	$3\cdot2 \times 10^3$	$3\cdot3 \times 10^3$
	$1\cdot2 \times 10^3$	$2\cdot8 \times 10^3$	$3\cdot1 \times 10^3$	$3\cdot9 \times 10^3$
7	—	$1\cdot4 \times 10^3$	$1\cdot9 \times 10^3$	$5\cdot6 \times 10^2$
			$2\cdot2 \times 10^3$	$2\cdot5 \times 10^2$

surfaces containing a large HeLa contribution adhere more readily to glass. Ehrlich ascites cells adhere poorly to glass, so that heterokaryons containing a preponderance of Ehrlich ascites components might also adhere poorly. In this way, adhesion to glass might select heterokaryons with predominantly HeLa surface characteristics.

While they remained multinucleate the heterokaryons did not multiply, but, under suitable conditions, they remained alive on the coverslips for at least five days. After this time some of them began to degenerate and to lose their attachment to the glass, but others survived for as long as 15 days. Time-lapse cinemicrography revealed that some of the multinucleate cells exhibited sluggish locomotion: they moved a distance approximately equal to their own length in 4 days. The survival times of the heterokaryons produced with different concentrations of inactivated virus and with infective virus are shown in Table 2. The essential reason for the failure of the heterokaryons to multiply appeared to be the fact that the cells contained more than one nucleus. Multinucleate cells containing only HeLa nuclei also failed to multiply, thus indicating that it was the multinucleate state rather than heterokaryosis which was responsible for the deficiency. In mixed cultures containing both mononucleate and multinucleate cells, the mononucleate cells did multiply. It was therefore unlikely that failure of multiplication was due to the injury produced by the virus.

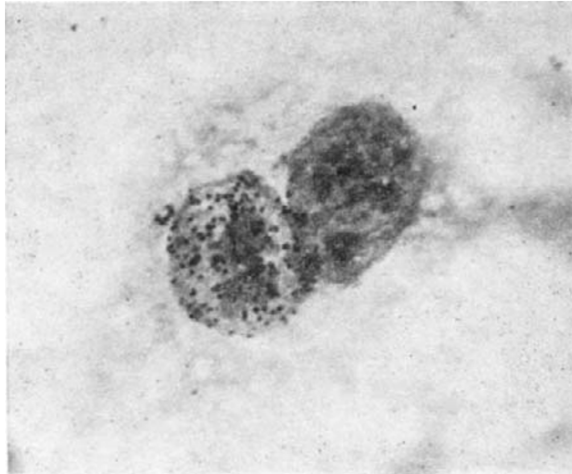

Fig. 3. Autoradiograph of a binucleate cell containing one HeLa nucleus and one Ehrlich ascites nucleus. The HeLa cells had been grown in tritiated thymidine before the heterokaryons were produced. The HeLa nucleus is labelled and the Ehrlich ascites nucleus is not. (× 1,300)

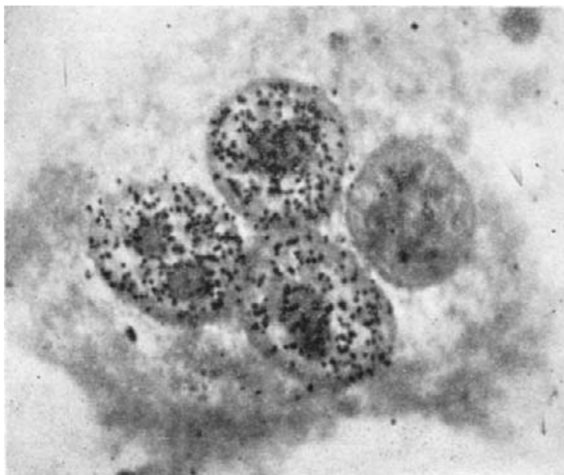

Fig. 4. A tetranucleate cell from the same population as the cell shown in Fig. 3. Three labelled HeLa nuclei and one unlabelled Ehrlich ascites nucleus are shown. (× 1,300)

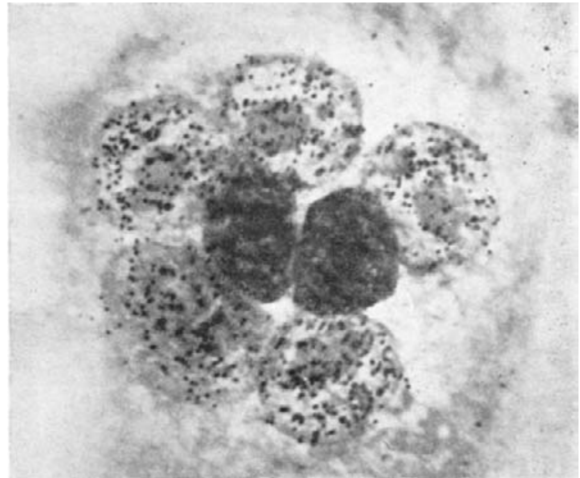

Fig. 5. An octonucleate cell from the same population as the cells shown in Figs. 3 and 4. Six labelled HeLa nuclei and two unlabelled Ehrlich ascites nuclei are shown. (× 1,300)

Since each heterokaryon received a large number of virus particles, the possibility existed that the virus inactivated by ultra-violet light might undergo multiplicity reactivation. In order to examine this possibility the production of infective virus and haemadsorption[6] were investigated. For haemadsorption studies two coverslips were examined each day. They were washed once in Hanks's solution and placed in the cups of a haemagglutination tray: 0·5 ml. of a 3 per cent (v/v) suspension of guinea-pig erythrocytes in Medium 199 was added to each cup and the tray was left at room temperature for 2 h, during which time the erythrocytes settled evenly over the coverslips. These were then removed, washed four times in Hanks's solution, fixed in methanol and stained with May–Grünwald–Giemsa. The percentage of heterokaryons showing adsorption of one or more erythrocytes was then determined. The results are summarized in Fig. 6. This shows that when infective virus was used to make the heterokaryons, all of them exhibited the presence of surface haemagglutinin for at least 5 days. But when the heterokaryons were made with inactivated virus, surface haemagglutinin disappeared from the cells at a rate which depended on the initial virus concentration. Moreover, with infective virus, the haemadsorption was massive and involved the whole of the cell surface; with inactivated virus, haemadsorption, in the decreasing number of cells which showed it, involved a progressively smaller part of the individual cell surface. At the lowest concentration of virus (800 HAU/ml.) less than 0·05 per cent of the heterokaryons exhibited haemadsorption at 24 h. On subsequent days occasional heterokaryons (between 0·5 and 1 per cent) did show haemadsorption, but it is clear that at least 99 per cent of the cells did not produce any viral haemagglutinin during the 5-day period. The

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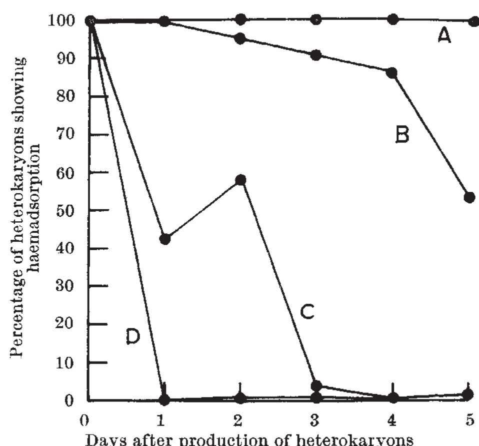

Fig. 6. Rate of loss of surface haemagglutinin in heterokaryons produced by infective virus and virus inactivated by ultra-violet light. *A*, Infective virus at a concentration of 8,000 HAU/ml. *B*, *C*, *D*, Inactivated virus at concentrations of 80,000, 8,000 and 800 HAU/ml., respectively

loss of haemagglutinin from the surface of the heterokaryons produced by inactivated virus no doubt reflects the gradual destruction or elution of the initial inoculum.

In order to examine whether heterokaryons produced by inactivated virus released any infectious virus into the medium, 10 coverslips bearing such heterokaryons were transferred to fresh medium after 24-h cultivation and were then incubated in this medium for a further 48 h. At the end of this period 10-fold dilutions of the medium were made in Hanks's solution and 0·1 ml. of each dilution inoculated into the allantoic cavity of two 10- or 11-day-old fertile hens' eggs. After incubation for 48 h at 37° C the allantoic fluids were collected and their haemagglutinin content titrated. The results of this experiment are set out in Table 3, in which the calculated amount of infectious virus produced per heterokaryon is shown. One unit of infectious virus is defined as the amount which produced 2 HAU/ml. of allantoic fluid in 48 h. It will be seen that, when the heterokaryons were made with inactivated virus at a concentration of 800 or 8,000 HAU/ml., less than one heterokaryon in a thousand produced a single unit of infectious virus. With 80,000 HAU of inactivated virus a yield of 1–10 units of infectious virus per heterokaryon was obtained. This does not, however, necessarily mean that multiplicity reactivation had occurred. Small amounts of virus probably escaped inactivation by ultra-violet light, and these might have been responsible for the yield of infectious virus. When the heterokaryons were made with 8,000 HAU/ml. of virus which had not been inactivated, the yield of infectious virus was also only 1–10 units per heterokaryon. This suggests either that auto-interference had occurred or that the virus, even when it is not inactivated, grows poorly in this system. It is, in any event, clear that with moderate or low doses of inactivated virus at least 99 per cent of the heterokaryons produce neither infectious virus nor viral haemagglutinin.

The usefulness of heterokaryons for the analysis of nucleo-cytoplasmic relationships depends to a large extent on the ability of the two sets of nuclei to synthesize RNA

Table 3. PRODUCTION OF INFECTIOUS VIRUS BY HETEROKARYONS

Concentration of virus used to produce heterokaryons	Haemagglutinin titre (HAU/ml. allantoic fluid) produced in 48 h by 0·1 ml. of culture medium diluted as shown						Units of infectious virus produced per heterokaryon
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
Virus inactivated by ultra-violet light							
800 HAU/ml.	< 2	< 2	—	—	—	—	< 10^{-3}
	< 2	< 2	—	—	—	—	
8,000 HAU/ml.	< 2	< 2	< 2	—	—	—	< 10^{-3}
	< 2	< 2	< 2	—	—	—	
80,000 HAU/ml.	2,048	512	512	< 2	< 2	< 2	1–10
	2,048	—	512	64	< 2	< 2	
Infective virus 8,000 HAU/ml.	512	2,048	< 2	< 2	< 2	< 2	1–10
	512	256	256	256	< 2	< 2	

and on the ability of the hybrid cell to synthesize protein. These two functions were therefore examined by studying the incorporation of tritiated uridine into RNA and tritiated leucine into protein. Heterokaryons, which had been maintained on coverslips for periods up to 5 days, were incubated for 2–6 h with tritiated uridine. The cells were then fixed, digested for 30 min at 37° C with deoxyribonuclease and extracted with 0·3 N trichloroacetic acid at 4° C. Autoradiographs of such preparations revealed that all the nuclei in the heterokaryons were labelled. The cytoplasm became labelled also, but more slowly than the nuclei, as in other nucleated cells. Fig. 7 shows an autoradiograph of a heterokaryon from a 24-h culture exposed for 2 h to tritiated uridine. The cell contains a HeLa nucleus and an Ehrlich ascites nucleus: both are labelled, and there is also slight labelling over the cytoplasm. Fig. 8 shows a similar heterokaryon exposed for 6 h to tritiated uridine: the cytoplasm of the cell is now heavily labelled. There was no marked disparity between the HeLa nuclei and the Ehrlich ascites nuclei in their ability to incorporate tritiated uridine into RNA, or, during the first 5 days, between the amount of nuclear labelling in the heterokaryons and the amount in neighbouring mononucleate cells. It thus appears that synthesis of RNA takes place in the heterokaryons in a manner comparable with that seen in mononucleate cells, and that both sets of nuclei

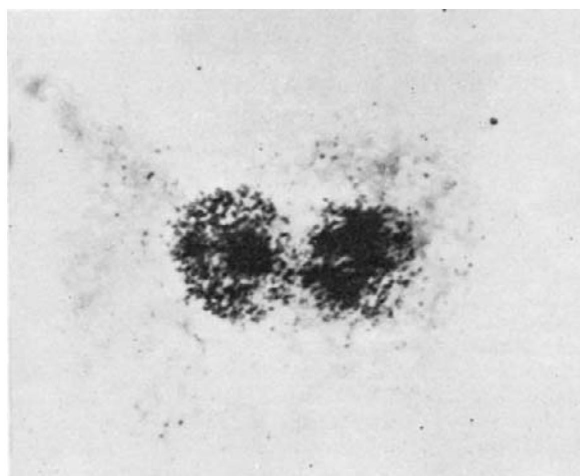

Fig. 7. Autoradiograph of a heterokaryon from a 24-h culture exposed for 2 h to tritiated uridine. The cell contains a HeLa nucleus and an Ehrlich ascites nucleus. Both are labelled, and there is also slight labelling over the cytoplasm. (× 1,100)

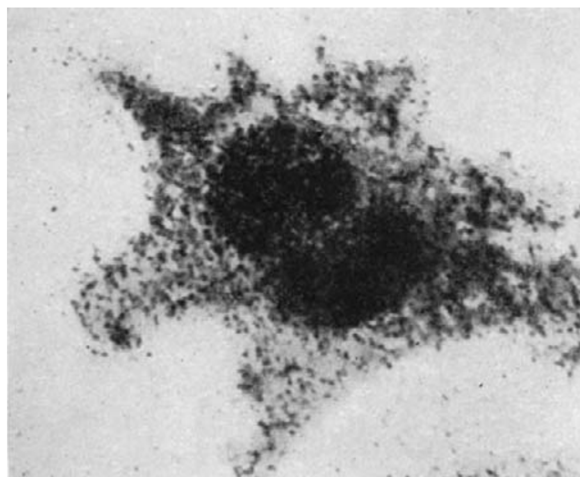

Fig. 8. A similar heterokaryon from the same population as the cell shown in Fig. 7, but exposed for 6 h to tritiated uridine. The cytoplasm of the cell is now heavily labelled. (× 1,100)

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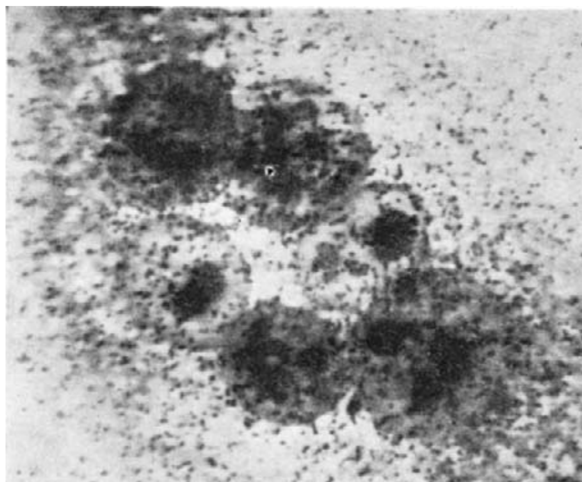

Fig. 9. Autoradiograph of a heterokaryon from a 24-h culture exposed for 6 h to tritiated leucine. There is generalized labelling over nuclei and cytoplasm. (× 1,100)

are involved in this synthesis. The genes of both mouse and man are therefore transcribed.

Autoradiographs of heterokaryons, maintained on coverslips for periods up to 5 days and then exposed for 6 h to tritiated leucine, revealed generalized labelling over nuclei and cytoplasm (Fig. 9). Failure to extract this label with 0·3 N trichloroacetic acid at 90° C confirmed that the tritiated leucine had been incorporated into protein. It is therefore clear that the heterokaryons synthesize protein as well as RNA.

Although heterokaryons in the multinucleate state did not multiply, they did synthesize DNA. When cultures which had been maintained on coverslips for 24 h were exposed for 2 h to tritiated thymidine, autoradiographs showed that about 70 per cent of the heterokaryons contained labelled nuclei. In 5 per cent of these labelled heterokaryons all the nuclei in the cell incorporated the precursor, but in the rest labelled and unlabelled nuclei were present in varying proportions. In any one cell some nuclei of each type might be labelled and others not. Fig. 10 shows a multinucleate cell in which all the nuclei are synthesizing DNA, and Fig. 11 shows a multinucleate cell in which only two of the Ehrlich ascites nuclei are synthesizing DNA. In these labelled heterokaryons about 80 per cent of the Ehrlich ascites nuclei were synthesizing DNA, but only about 30 per cent of the HeLa nuclei. The percentage of heterokaryons showing labelling after 2 h exposure to tritiated thymidine fell from 70 at 24 h to 55 at 48 h, 44 at 4 days and 31 at 7 days. But in those heterokaryons which did show some form of nuclear labelling, the proportion of Ehrlich ascites nuclei and of HeLa nuclei which were synthesizing DNA underwent little change over 7 days. An exponentially growing population of Ehrlich ascites or HeLa cells exposed to tritiated thymidine for 2 h would show labelling in about a third of the nuclei, an expression of the duration of the phase of DNA synthesis relative to the total intermitotic time. The very high proportion of labelled Ehrlich ascites nuclei in the heterokaryons, therefore, means that these nuclei have become partially synchronized in the phase of DNA synthesis. This partial synchronization might have resulted from selection during cell fusion of those Ehrlich ascites cells in which synthesis of DNA was taking place. But the fact that 80 per cent or so of the Ehrlich ascites nuclei in labelled heterokaryons were synthesizing DNA not only at 24 h, when 70 per cent of the heterokaryons were labelled, but also at 48 h, when 55 per cent of the heterokaryons were labelled, suggests another explanation. It seems more probable that many of the Ehrlich ascites nuclei which were not in the phase of DNA synthesis at the time of formation of the heterokaryons passed into this phase during the first 24 h, and that DNA synthesis was not terminated abruptly after a few hours, as occurs in normal cells, but continued for much longer periods. Any alternative explanation would seem to require that at least some of these nuclei underwent two or more normal cycles of DNA synthesis without mitosis. Why the percentage of HeLa nuclei synthesizing DNA at 24 h should be so much lower is obscure, but, since this percentage also shows little change on subsequent days, it is likely that the phase of DNA synthesis was prolonged in at least some of the HeLa nuclei also. These findings, in any event, make one point clear. Whether or not a mammalian cell nucleus synthesizes DNA cannot be solely determined by events in the cytoplasm. Even in a common cytoplasm DNA synthesis may, at any one time, be taking place in some nuclei and not in others; and the

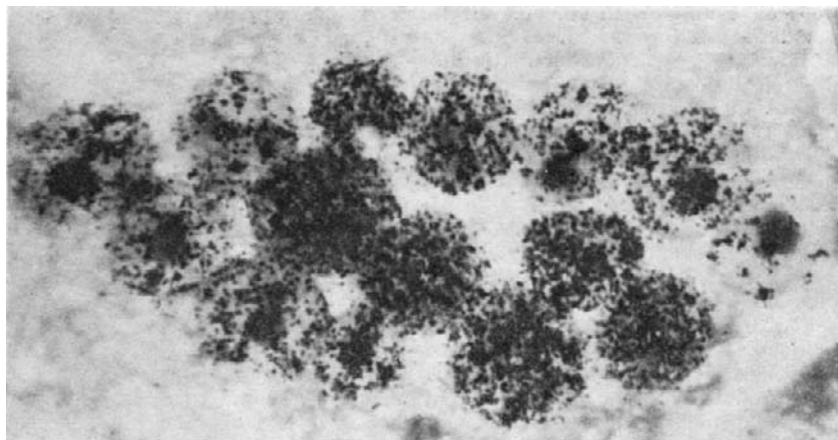

Fig. 10. Autoradiograph of a heterokaryon from a 24-h culture exposed for 2 h to tritiated thymidine. All the nuclei are synthesizing DNA. (× 1,100)

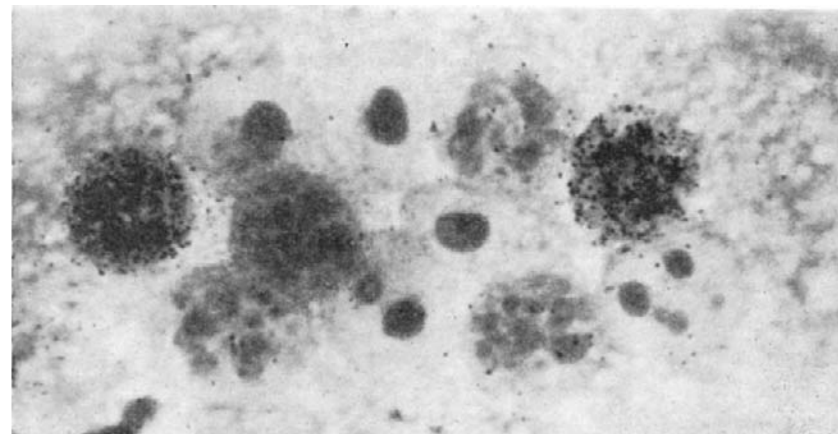

Fig. 11. A heterokaryon from the same population as the cell shown in Fig. 10. Only two of the Ehrlich ascites nuclei are synthesizing DNA. (× 1,300)

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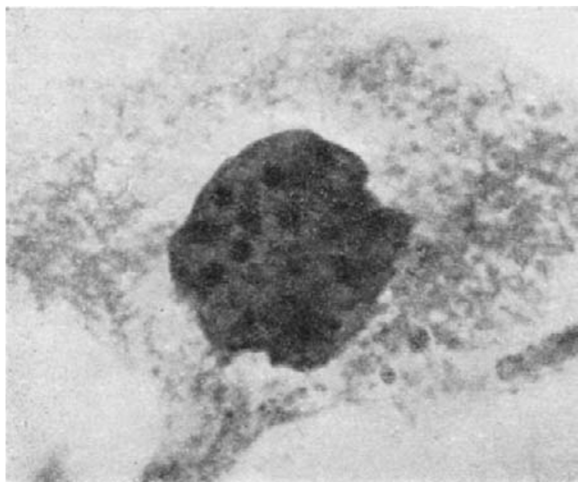

Fig. 12. A cell containing a giant nucleus produced by fusion of a number of discrete nuclei. Several nucleoli or condensations of chromatin are to be seen. (× 1,100)

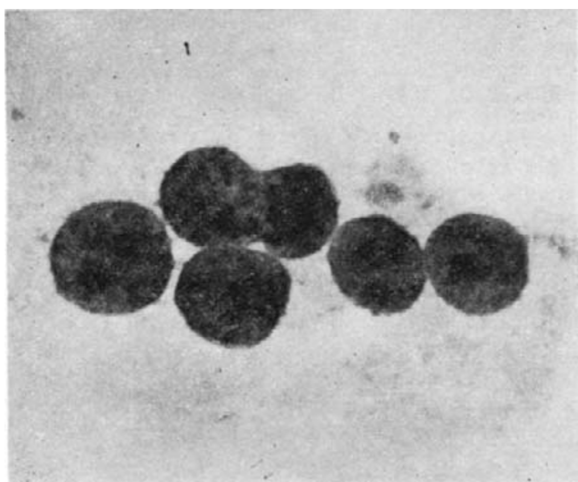

Fig. 13. A heterokaryon in which two of the Ehrlich ascites nuclei are in the process of fusion. (× 1,100)

nuclei from one species may be more active in the heterokaryon than the nuclei from another. The ability of the nucleus to synthesize DNA must therefore depend, at least in part, on factors operating within the nucleus.

Over a 7-day period there was a progressive fall not only in the proportion of heterokaryons showing labelling after exposure to tritiated thymidine, but also in the intensity of the labelling. At 24 h there was no great difference between the intensity of nuclear labelling in the heterokaryons and that found in neighbouring mononucleate cells; but at 7 days the intensity of labelling in the heterokaryons was greatly reduced relative to that found in mononucleate cells. It thus appears that although the phase of DNA synthesis is prolonged in the nuclei of heterokaryons, the rate of synthesis progressively falls, and eventually synthesis stops.

Under certain conditions the nuclei within the heterokaryons underwent fusion. The fused nuclei could usually be recognized by their greater size and by the fact that they contained many large nucleoli or condensations of chromatin. In many of the multinucleate cells only some of the nuclei underwent fusion, but in others all the nuclei in the cell fused together to produce one giant nucleus which sometimes had a bizarre shape (Fig. 12). Fusion took place not only between nuclei of the same kind but also between HeLa nuclei and Ehrlich ascites nuclei. This was demonstrated by fusing HeLa cells which had been grown in tritiated thymidine with unlabelled Ehrlich ascites cells, and vice versa. Autoradiographs of such preparations showed that many of the large nuclei were labelled over only one part of the nucleus. Fig. 13 shows a heterokaryon in which two of the Ehrlich ascites nuclei are in the process of fusion. Fig. 14 shows a giant nucleus in a preparation made by fusing labelled Ehrlich ascites cells with unlabelled HeLa cells: only the right-hand half of the nucleus is labelled.

It might be thought that nuclear fusion represented an extension of the activity of the virus to the nuclear membrane. But experiments on the relationship between the concentration of virus used and the amount and rate of nuclear fusion revealed that this was not the case. Fig. 15 demonstrates that the percentage of cells showing nuclear fusion on successive days is inversely related to the concentration of virus used. With inactivated virus at a concentration of 800 HAU/ml., about 25 per cent of the heterokaryons showed some degree of nuclear fusion at 24 h, and by 5 days nuclear fusion had occurred in almost all the cells. But Fig. 6 demonstrates that at least 99 per cent of the heterokaryons produced by this concentration of inactivated virus had lost all trace of virus haemagglutinin from their surface within 24 h. It therefore appeared unlikely that nuclear fusion, which progressed throughout

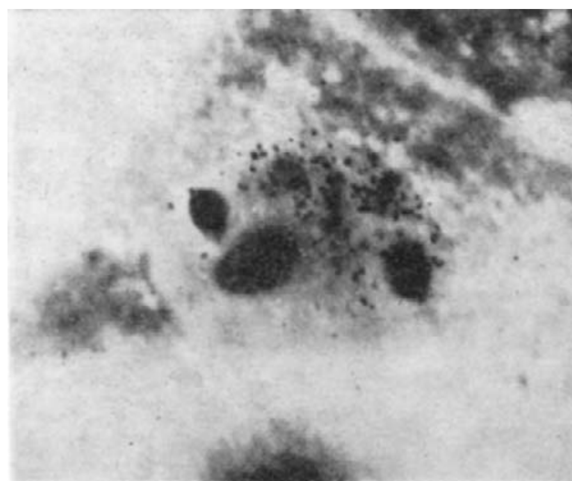

Fig. 14. Autoradiograph of a large nucleus produced by fusion of labelled Ehrlich ascites nuclei with unlabelled HeLa nuclei. The Ehrlich ascites cells were exposed to tritiated thymidine before the heterokaryons were produced. Only the right-hand half of the nucleus is labelled. (× 1,300)

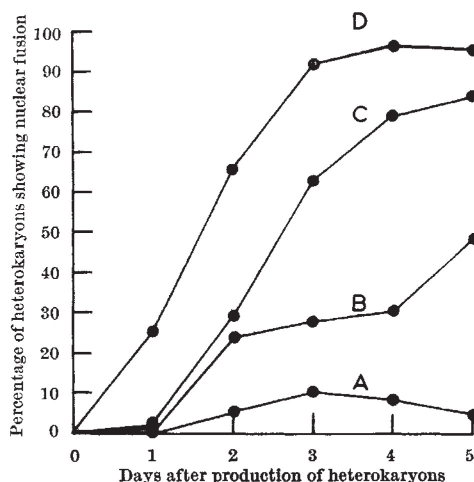

Fig. 15. Relationship between the proportion of heterokaryons showing nuclear fusion and the concentration of virus used to produce the heterokaryons. *A*, Infective virus at a concentration of 8,000 HAU/ml. *B*, *C*, *D*, Inactivated virus at concentrations of 80,000, 8,000 and 800 HAU/ml. respectively

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the 5-day period, was caused by the continued activity of the virus. Indeed, the results indicated that the presence of large amounts of virus inhibited nuclear fusion. With increasing concentrations of virus the proportion of heterokaryons showing nuclear fusion on successive days was reduced; and, at the same dose, infective virus inhibited nuclear fusion more drastically than inactivated virus. It seems as if progressive nuclear fusion is the normal course of events in the heterokaryon, and that the inhibition produced by high doses of virus is a non-specific toxic effect. Preliminary experiments indicate that other toxic substances may also inhibit nuclear fusion. Since the term heterokaryon, as it is generally used, implies a cell with more than one nucleus, multinucleate cells in which the nuclei have fused together to form a single nucleus will be called synkaryons: homosynkaryons where nuclei of the same type have fused together, and heterosynkaryons where nuclei of different types have fused together.

The synkaryons continued to incorporate tritiated uridine into both nuclear and cytoplasmic RNA for at least 5 days at a rate not greatly different from that found in single HeLa cells in the same culture. Some of them also incorporated tritiated thymidine into DNA, although, over a 7-day period, the proportion showing labelling, and the intensity of the labelling relative to single HeLa cells in the same culture, progressively fell. There was, however, reason to believe that some synkaryons, including heterosynkaryons, were able to undergo at least one mitosis. In cultures containing both multinucleate and mononucleate cells it was observed that some of the cells rounded up in mitosis were very much larger than the rest. The cultures were therefore exposed for 18 h to colchicine at a concentration of 0·0025 per cent (w/v), and the karyotypes of the accumulated metaphases examined by the method of Rothfels and Siminovitch[7]. It was found that some of the metaphase figures had up to 300 or more chromosomes, whereas the modal chromosome number for the HeLa and Ehrlich ascites cells was about 80. Moreover, some of these giant metaphases showed large numbers of both metacentric and telocentric chromosomes, including many long telocentrics. HeLa cells normally contain not more than one long telocentric chromosome, and frequently none at all[8]; and Ehrlich ascites cells normally contain very few metacentric chromosomes[9]. The presence of large numbers of both metacentric and long telocentric chromosomes in the one cell therefore suggests that the cell originally contained both HeLa and Ehrlich ascites nuclei. Whether heterosynkaryons can produce clones of cells containing both human and murine chromosomes is the subject of current investigation.

In the experiments which we have described, an inactivated virus has been used to impose a form of artificial sexuality on mammalian tissue cells. Since the resulting hybrid cells synthesize protein, and both sets of nuclei synthesize RNA, these hybrids can be used to investigate problems of nucleo-cytoplasmic relationship which could hitherto be studied only in the heterokaryons of fungi or yeasts. Some features of the mammalian cell system perhaps warrant special comment. The remarkable fact that viable heterokaryons can be made with cells originating from different animal species means that a very wide range of genetic markers can be used to distinguish the two cell types. Indeed, the choice is limited only by the susceptibility of the cells to the virus used to produce fusion. The average number of nuclei per heterokaryon and the proportion of nuclei of each type can, within certain limits, be controlled. Preparations can be made in which 85 per cent or more of the cells are heterokaryons, and the ratio of multinucleate to mononucleate cells can be varied. Unlike fungi or yeasts, mammalian cells use thymidine as a specific precursor of DNA. This property makes mammalian cell heterokaryons eminently suitable for investigations on the regulation of DNA synthesis, as some of the present experiments show. Finally, autoradiographic and chemical techniques for the study of RNA metabolism in mammalian cells have now reached a moderate degree of sophistication: these procedures can be applied without modification to mammalian cell heterokaryons. There is, therefore, reason to hope that these heterokaryons will lend themselves to experiments which have hitherto not been possible in animal cells.

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PROGRESS OF PALAEONTOLOGY IN CHINA

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MODERN scientific investigation of fossils in China dates back to the 1920's. The few palaeontologists made a good start in spite of the difficult working conditions. Many valuable monographic papers were published in *Palaeontologia Sinica*. The memoirs on Fusulinidae (Protozoa), Productidae (Brachiopoda) and others published by the journal are still among the classics of palaeontological literature.

But the investigations were confined to only a few groups of organisms including the fossils of plants, foraminiferas, corals, brachiopods, trilobites and graptolites of the Palaeozoic era, Mesozoic reptiles and Pleistocene mammals. The materials were collected in a few districts with many regions of palaeontological interest unexplored.

Palaeontology has made considerable progress since New China was established in 1949. The number engaged in palaeontological investigations has increased from about 40 in 1949 to the present 400 to meet the growing demand in the fields of geological surveying, exploration, science and education.

In addition to *Palaeontologia Sinica*, two new periodicals, *Acta Palaeontologia Sinica* and *Vertebrate PalAsiatica*, have been published.

At present the Institute of Geology and Palaeontology of the Academia Sinica at Nanking is mainly concerned with the examination of plant and invertebrate fossils and biostratigraphy. The Institute of Vertebrate Palaeontology and Palaeoanthropology of the Academia Sinica

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