The development of feline cell lines for the growth of feline infectious enteritis (panleucopaenia) virus

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The first published account of the successful isolation of feline infectious enteritis (FIE) virus in tissue culture was by Johnson (1964). He used primary kitten kidney tissue cultures and relied upon staining with haematoxylin and eosin (H and E) for the initial recognition of virus isolation and for subsequent studies on intra-nuclear changes in affected cells (Johnson, 1965). Other workers have also used primary kitten kidney tissue culture in studies of FIE virus (King & Croghan, 1965; Gorham *et al.* 1965).

In these laboratories a fairly common finding has been the presence of wild FIE virus in primary kitten kidney tissue cultures, a fact which did not become evident in uninoculated control cultures until the seventh day after seeding. Since cultures are normally inoculated with virus on the second or third day after seeding, when the cell sheet is three-quarters confluent, and harvesting takes place 2 or 3 days later, warning of contamination with wild virus by its effect on control cultures comes too late. Apart from the interruption to research work, the possible presence of wild virus in primary kitten kidney tissue cultures is clearly a serious hazard in any attempt to develop a modified vaccine virus by serial passage in this tissue.

The cost of rearing cats in complete isolation for the provision of a regular supply of susceptible kidney tissue is prohibitive. Accordingly, the development of feline embryonic cell lines for the study of FIE virus was undertaken, and this also provided an opportunity for a detailed investigation of the chromosomes of the domestic cat.

MATERIALS AND METHODS

Cell culture

Feline embryos were removed by Caesarian section from normal healthy pregnant queens and washed in sterile phosphate buffered saline (PBS) containing 200 units of penicillin and 100 μg. of streptomycin per ml. After removal of the embryos from their membranes, various organs were taken and chopped into very small fragments. The tissue was trypsinized as described by Dulbecco & Vogt (1954) and the resulting suspension seeded at the rate of 5×10^6 cells per 4 oz. medical flat containing 10·0 ml. of medium. The medium consisted of 80 % Eagle's Medium Basal modified to have twice the usual amount of amino acids and vitamins, 10 % tryptose phosphate broth, 10 % adult bovine uninactivated serum, 200 units of penicillin and 100 μg. of streptomycin per ml.

After 2–3 days incubation at 37 °C. a confluent monolayer developed which was

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subcultured in the following manner. The medium was removed and the cells were gently washed for 30 sec. in 3·0 ml. of 0·1 % trypsin in 0·05 % versene. This was quickly followed by pouring off the trypsin–versene mixture and the bottles were placed cell-sheet down on the bench. Several minutes later the cells began to detach from the glass and the bottles were again shaken until all the cells were free. The cells were then resuspended in 5·0 ml. of medium and divided equally between two 4 oz. medical flats. Eight ml. of medium were added to each bottle, which was then incubated at 37° C.

Viruses

Although most studies were done with a strain (12 B 1) of FIE virus isolated from the kidneys of an infected cat, other strains recovered from infected spleens and intestines were used. A virus isolated from a leopard (Johnson, 1964) and a strain of mink enteritis virus (Johnson, 1967 a) were also tested.

Virus propagation and assay

For the isolation of virus, infected tissues were ground in a mortar and a 1/10 suspension in PBS prepared. After centrifuging at 800 rev./min. for 5 min., tenfold dilutions of the supernatant were made and 0·2 ml. of each dilution inoculated into test-tubes containing coverslips and $2·0 \times 10^5$ of freshly versenized cells in 2·0 ml. of medium. The tubes were incubated at 37° C. at an angle of about 10°. On the third day after infection the coverslips were fixed in Bouin's solution and stained with H and E. Evidence of viral infection was accepted when the typical intranuclear changes were observed.

Subsequent passaging of virus was done by inoculating 2·0 ml. of infected tissue culture cells and fluid into 4 oz. medical flats containing freshly versenized cells in 10·0 ml. of tissue culture medium. Periodically, coverslip preparations were infected to ensure that the virus was being passaged. Virus-infected cultures were frozen at $-30°$ C. after 3 days incubation.

For virus titrations, 0·2 ml. of twofold dilutions in PBS were inoculated into test-tubes containing coverslips. Initially, coverslips from each dilution were removed daily over a period of 5 days, fixed in Bouin's solution and stained with H and E. Latterly, the coverslips from all dilutions were fixed and stained on the third day only after infection. The stained preparations were examined under a $\times 40$ objective and the degree of infection was estimated by counting the proportion of cells showing intranuclear changes in five arbitrarily chosen microscope fields. On average, about 450 cells were counted on each coverslip. The percentage of infected cells was then calculated.

Chromosome preparations

Metaphase chromosome preparations were obtained using a modification of the Rothfels & Siminovitch (1958) technique. Actively growing cultures (usually on the second day) were incubated with 0·04 mg./ml. final concentration colcemide for 3–6 hr. The cells were detached from the glass using trypsin–versene mixture

and sedimented by centrifuging for 5 min. at 800 rev./min. The supernatant was discarded and the pellet resuspended in 0·5 ml. of calf serum. Two ml. of distilled water were added slowly, and after mixing the suspension was left standing for 10 min. It was then centrifuged for 5 min. at 800 rev./min. and 0·5 ml. of freshly prepared fixative (1 volume reagent grade glacial acetic acid and 3 volumes reagent grade ethyl alcohol) added, care being taken not to break-up the pellet. After 20–30 min. the cells were resuspended with a fine pasteur pipette, centrifuged, washed with two changes of fixative, and a drop placed on a clean slide. The slides were air-dried at 37° C. and stained with either Giemsa or Orcein.

RESULTS

Production of cell lines

Embryos from twenty-two pregnancies were used in fifty attempts to produce diploid cell lines from various tissues. In some cases the whole embryos were used, especially when they were 1·0 cm. or less in length; in other cases, portions of the embryos were used, i.e. heart, kidney, liver, lung, skin, small intestine, spleen, tongue, voluntary muscle or combinations of organs such as lung and heart, liver and small intestine, or eviscerated and decapitated carcases. On two occasions amnion was the source of cells.

Table 1. *Development of feline diploid cell lines*
and susceptibility to FIE virus

Embryonic tissue	Growth vigour	Susceptibility to FIE virus
Lung	+	+
Lung and heart	+	+
Embryo whole carcase	+	+
Voluntary muscle	+	+
Kidney	+	+
Heart	+	+
Skin	+	+
Amnion	+	±
Small intestine	+	±
Small intestine and liver	+	±
Tongue	+	±
Liver	−	NT
Spleen	−	NT

+ = satisfactory,   − = unsatisfactory,   ± = Poor,   NT = not tested.

Tissue cultures prepared from liver and spleen were very poor and never exceeded four passages. Heart cells grew satisfactorily but were less vigorous by comparison with other cells. Lung cells (FEL) and embryo cells (F Emb), whether or not the latter cells originated from whole or eviscerated and decapitated carcases, grew prolifically (Table 1). Accordingly, for reasons of convenience the work was largely devoted to these two types of cell lines. Of the first five cell lines produced, four became contaminated with mycoplasma and the fifth died at the 20th passage. Another nine attempts to propagate diploid cell lines failed because,

in seven cases the cells did not grow, and in the other two there was bacterial contamination. Eleven embryo and seven lung diploid cell lines exceeded more than five serial passages. One lung cell line, despite contamination with mycoplasma, was passaged more than 100 times and a lung and an embryo cell line from another foetus each exceeded 70 passages.

In general, tissues from embryos of 4·5 cm. or less in length were suitable for the production of diploid cell lines and 3·0–4·0 cm. appeared to be optimal for diploid lung cell lines (Table 2).

A number of cell lines were stored frozen in liquid nitrogen at −170° C. and have been successfully revived.

Table 2. *Relationship of feline embryo size and production of diploid cell lines*

Embryo size (cm.)	Proportions of litters producing cell lines exceeding 5 passages	Type of cell lines	
		Embryo	Lung
0·5	3/6	3	ND
1·0	2/4	2	ND
1·5	1/1	1	1
3·0	2/2	ND	2
4·0	5/6	4	3
4·5	1/1	1	1
Full term	0/2	0	0
Totals	14/22	11	7

ND = not done

Cell Morphology

After the first four or five passages all the diploid cell lines were clearly fibroblastic in morphology (Pl. 1, fig. 1.) and remained so for the next thirty passages. At this point it was noted that the time taken for the cultures to become fully confluent lengthened from 2–3 to 5–6 days. By the 45th passage the cells had regained their vigour and needed subculturing every 3 days. At about the same time they began to assume a polygonal shape (Pl. 1, fig. 2), which remained a dominant feature until about the 60th passage when a further change was noted. The cells now showed uneven distribution with foci of relatively few cells surrounded by dense areas of cells stacked in a disorganized arrangement (Pl. 1, fig. 3).

Karyology

The incidence of tetraploidy in cultures before the 35th passage was 106 (3.4 %) in 3150 metaphases examined. Of 364 nuclei counted accurately, 33 had less than 38 chromosomes; thus revealing 9 % subdiploidy (Table 3). Sixteen (4·4 %) gaps or breaks were detected. However, these deviations fall within the accepted limits for cultured human cells as laid down in the Revised Standards for Karyology of Human Diploid Cell Strains (1966).

Chromosomes from fifty metaphases were photographed, measured and two values calculated. The first, the arm-length ratio, is expressed as the length of the

longer arm relative to the shorter one. The second, the percentage mean index length, expresses the average length of a pair of chromosomes as a percentage of the sum of the average lengths of all the pairs of chromosomes. The chromosomes (Table 4) and the karyotype (Pl. 2, figs. 4, 5) are arranged to follow, in general, the recommendations of the Denver Conference on human chromosome karyotype (Book *et al.* 1960). The classification of chromosomes by Cranmore & Alpen (1964)

Table 3. *Chromosome frequency distribution in 364 cells*

Cells ...	1	4	8	20	328	2	2	1
Chromosomes	30	35	36	37	38	39	40	41

Table 4. *Quantitative characteristics of the feline mitotic chromosomes*

Group	Chromosome no.	Arm-length ratio	Percentage mean index length
A	1	1·9	9·7
	2	3·3	8·2
	3	3·0	6·2
	4	3·2	5·8
	5	3·4	5·5
B	6	1·1	8·7
	7	1·4	6·6
	8	1·3	6·0
	9	1·4	5·3
	10	1·4	4·8
	11	1·5	4·2
C (i)	12	1·2	3·8
	13	1·2	3·7
	14	1·0	3·3
C (ii)	15	∞	3·0
	16	∞	2·8
C (iii)	17	1·0	2·7
	18	1·0	2·3
Sex chromosomes	X	1·5	5·6
	Y	1·0	1·9

has been simplified into three main groups. Group A comprises five large sub-telocentric chromosomes having an arm-length ratio greater than 1·9. There are six large metacentric chromosomes in group B, whose arm-length ratios do not exceed 1·6. Group C contains the smaller chromosomes, whose percentage mean index lengths are less than 4·0. This group can be divided into three subgroups. *Chromosomes 12, 13 and 14 are difficult to identify individually, except when the* satellites on chromosome 14 are visible. Chromosomes 15 and 16 are telocentric and 17 and 18 are small median metacentric. The X chromosome is difficult to distinguish from numbers 8, 9 and 10, but the Y chromosome is easier, because it is the smallest and is metacentric.

Between the 40th and 50th cell passages significant deviations in the karyotype were noted. The modal chromosome number of a cell line derived from the whole

embryo dropped from 38 to 37, and by the 55th passage 73 % of the nuclei examined had 37 chromosomes (Pl. 2, fig. 6), while cell lines derived from lung tissue showed more striking deviations from the normal. Among translocations affecting a large number of chromosomes, ring formation of chromosome 6 and translocation of part or all of small chromosomes on to the number 2 chromosome were frequently observed (Pl. 2, fig. 7). In one 48th passage culture only 10 % of the nuclei examined appeared to have a normal chromosome complement.

Fig. 1

Fig. 2
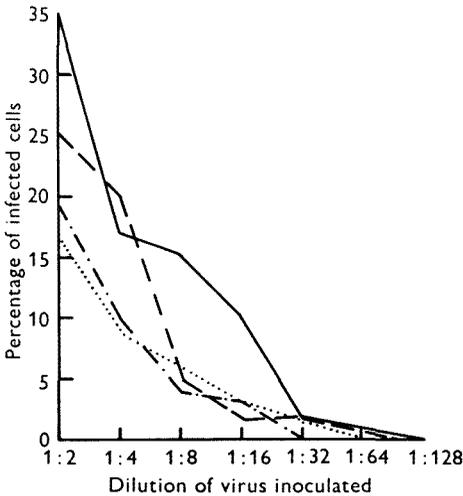
Text-fig. 1. Percentage of cells infected with dilutions of 15th passage of FIE virus 72 hr. after inoculation of three feline embryo diploid cell lines. ———, 4th passage of embryo 17 cell line. — —, 10th passage of embryo 17 cell line. —·—, 8th passage of embryo 20 cell line. .... 3rd passage of embryo 21 cell line.
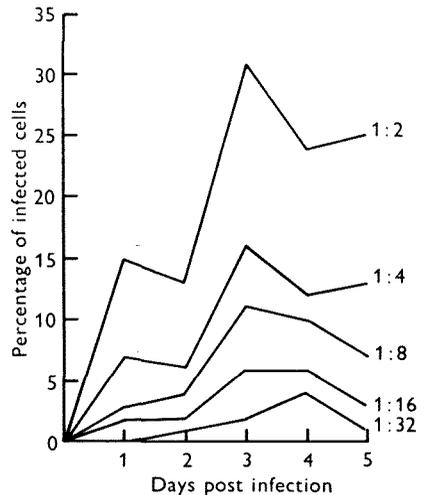
Text-fig. 2. Growth curves of dilutions of FIE virus in 45th passage of feline embryo lung cell line.

Virus susceptibility

All the cell lines were tested for susceptibility to FIE virus. Only one, FEL 1 A, was insusceptible, probably because it was contaminated with mycoplasma. Five different FIE viral isolates as well as the leopard and mink enteritis viruses were grown and passaged and one FIE virus strain (12 BI) was serially passaged more than 25 times. The passage level of the cells tested ranged from the 2nd to the 70th. Some variation in viral susceptibility between cell lines of different tissues and between cell lines of different embryos was observed (Table 1; Text-figs. 1, 2).

The intranuclear changes in these cell lines following virus infection resembled the changes described by Johnson (1965) in primary kitten kidney monolayers. The earliest nuclear changes appeared about 16 hr after infection, with a peak of infection 48 hr. later (Pl. 3, figs. 8, 9). A CPE in unstained preparations was only observed, and then inconsistently, when large inocula of undiluted virus were used (Pl. 4, figs. 10, 11).

Effect of Mycoplasma *on viral susceptibility*

The first cell line (FEL 1A) tested for susceptibility to virus infection became contaminated with *Mycoplasma hominis*. This fact was not evident initially, and numerous unsuccessful attempts were made to passage the virus serially in these cells used between the 21st and 100th passages. The first passage of virus produced cellular infection on 21 of 23 (91 %) occasions. The infection rates at the second and third serial passages of virus were 71 % and 25 % respectively. In no instance was there any evidence of infection at the fourth and fifth passages. Proof that infection of the cells occurred at the first virus passages was obtained by observing the characteristic intranuclear changes in monolayer cultures stained by H and E, the development of a positive Feulgen reaction, and the specific staining of the perinuclear haloes with fluorescent labelled FIE antiserum.

The virus survived five passages when the cells were alternately primary kitten kidney cells and FEL 1A cells. During the next three alternate passages no virus was detected.

In three of the next four diploid cell lines developed, mycoplasma contamination was detected at the 12th passage of two of them and at the eighth passage of the third. These cells in their early passages yielded infective virus, but after a few passages in a laboratory adjacent to a mycoplasma infected area they became incapable of producing fully competent new virus.

Although there are no reliable methods of eliminating *Mycoplasma* from tissue cultures, contaminated virus suspensions were freed of the organism by heating the suspensions at 50° C. for 30 min.

DISCUSSION

Because primary kitten kidney cells may be infected with wild FIE virus it was necessary to search for a virus-free source of tissue culture. In this laboratory it did not prove possible to passage FIE virus serially in the following tissue culture cells: BHK, calf bone marrow, calf buffy layer, calf thyroid, chick embryo, dog kidney, HeLa, patas and pig kidney.

Both the lung and embryo cell lines were susceptible to infection with FIE virus. A comparison of the percentage cellular infection recorded in this paper with those of Johnson (1967b), who used primary kitten kidney monolayers, suggests that the cell lines are possibly more susceptible to infection than primary tissue.

Contamination of the cells with *Mycoplasma* interfered with the production of infective virus. Since no mycoplasmas were recovered from very low passages of any of the cell lines, the inference must be that the contamination was of laboratory origin. Strict application of good tissue culture technique and the exclusion from the laboratory of all other tissue culture systems have prevented a repetition of the earlier experiences. Similar interference with virus replication in tissue cultures contaminated by *Mycoplasma* has been reported to occur with adenovirus type 2, Rous sarcoma virus and Rous associated virus (Rouse, Bonifas & Schlesinger, 1963; Somerson & Cook, 1965). Viral infection in the second and third passages in FEL 1A cells may have been due to residual virus in the inoculum used

at the first passage, since we have found that FIE virus will withstand 70° C. for 30 min.

Recent publications by Nakanishi (1960), Matano (1963) and Cranmore & Alpen (1964) show disagreement in the karyotype of the domestic cat. Identification of individual chromosomes from the karyotypes of these workers was difficult and in no case were detailed measurements given. The simplified karyotype presented here, together with the calculation of arm-length ratio and percentage mean index length, should go some way to improving the position.

The significance of the abnormal karyotypes described is not obvious, but transformation to an established cell line results in several different chromosomal abnormalities. These findings support the theory of Sandberg (1966) that transformation occurs when cells break free from the normal genetic control and that changes in karyotype are a mere epiphenomenon of abnormal growth rather than its cause.

SUMMARY

Primary kitten kidney cultures are frequently contaminated with wild feline infectious enteritis (FIE) virus and this led the authors to develop feline embryo diploid cell lines. Monolayer cultures were prepared from the lungs or from eviscerated and decapitated carcases of embryos obtained by Caesarian section from healthy pregnant queens. At about the 30th passage, these cells lost their fibroblastic morphology to become polygonal. After a further thirty passages the monolayers exhibited foci of low cell density circumscribed by bands of cells stacked in disorganized arrangement. All the developed cell lines were susceptible to infection with FIE virus and produced intranuclear changes resembling those described by Johnson (1965) in primary kitten kidney monolayers.

On four occasions the cell lines became contaminated with *Mycoplasma* and although there was evidence that the virus could infect the cells, there was no production of infective virus.

A simple karyotype was devised in which the 38 chromosomes were arranged in three groups according to the arm-length ratio and the percentage mean index length. After the 50th passage many of the nuclei of lung-derived cultures exhibited abnormal chromosomes resulting from ring formation or translocation, whilst those of embryo culture demonstrated a new modal chromosome number of 37.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. F Emb diploid cell line at 12th passage. The fibroblastic morphology of cells which have been passaged less than 30 times is clearly evident. × 170.

Fig. 2. FEL cell line at 42nd passage. These cells have lost their fibroblastic morphology and have become polygonal. × 170.

Fig. 3. F Emb cell line at 67th passage. After sixty passages these cells with a modal chromosome number of 37 (see also fig. 6) show foci of low cell density circumscribed by bands of cells stacked in a disorganized arrangement. × 170.

PLATE 2

Fig. 4. Normal chromosomes of the domestic cat from 3rd passage F Emb culture. × 1130.

Fig. 5. Karyotype prepared from fig. 4. × 1500.

Fig. 6. Karyotype prepared from 55th passage F Emb culture. × 1000.

Fig. 7. Karyotype prepared from 48th passage FEL culture. This nucleus contained 36 chromosomes. In addition to translocation on to chromosome 2 and ring formation at chromosome 6, a further translocation has resulted in the formation of a marker chromosome. × 1330.

PLATE 3

Fig. 8. FEL diploid cell line at 26th passage 40 hr. after infection with FIE virus. Three stages of intranuclear change are shown: *A*, early; *B*, more advanced and *C*, terminal at which time the cells become detached from the glass. There is also thinning of the cell sheet. × 250.

Fig. 9. F Emb cell line at 67th passage 48 hr. after infection with FIE virus. The three stages of intranuclear destruction described in fig. 8 are shown. With high passage cells, the nuclear changes following infection occur earlier and are more widespread than with low passage cells. × 250.

PLATE 4

Fig. 10. FEL diploid cell line at 25th passage 72 hr. after infection with FIE virus. There is no observable CPE in this Roux bottle which cannot be distinguished from an uninfected control bottle. × 50.

Fig. 11. FEL diploid cell line at 25th passage 72 hr. after infection with FIE virus. This is another Roux bottle inoculated simultaneously with same dose and virus used to infect the bottle in Fig. 10. In this bottle there is thinning of the cell sheet and a tendency for the cells to string out and show discrete clusters of degenerate cells.

Fig. 1   Fig. 2

Fig. 3

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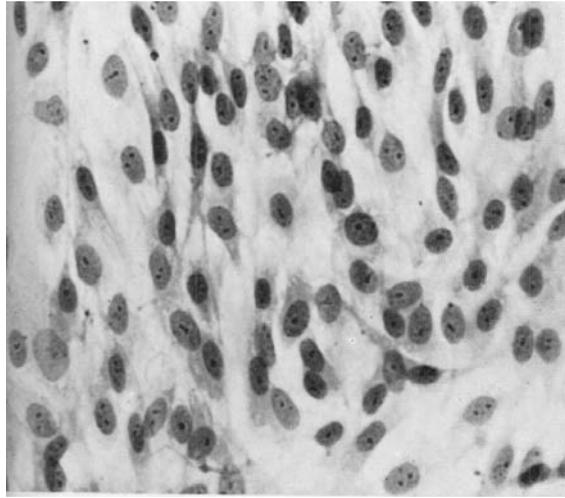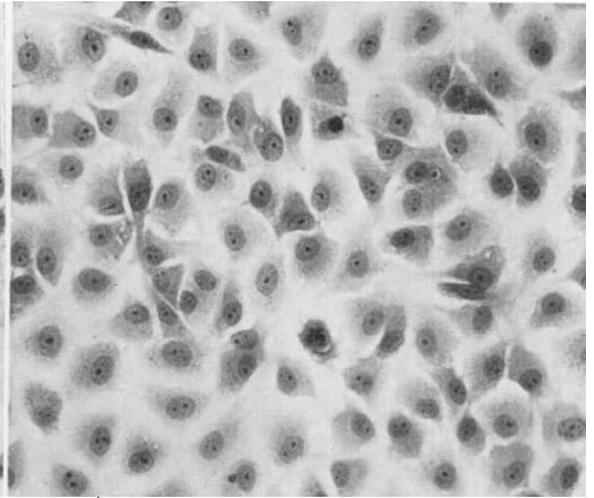
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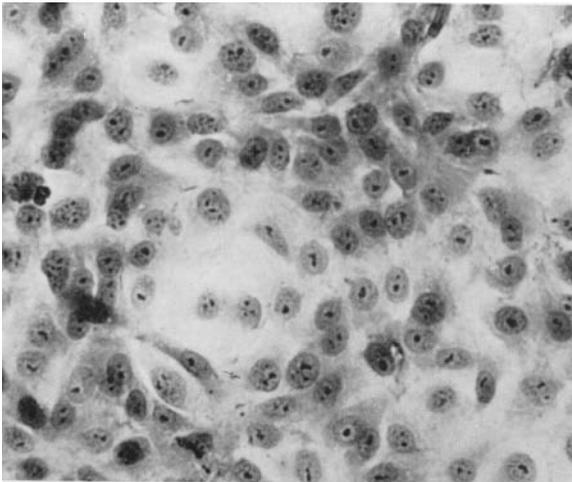
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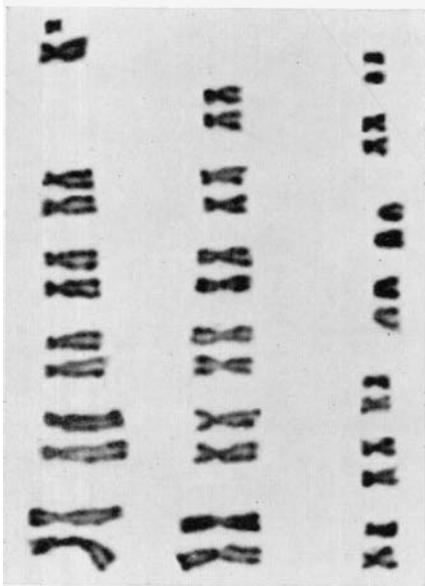
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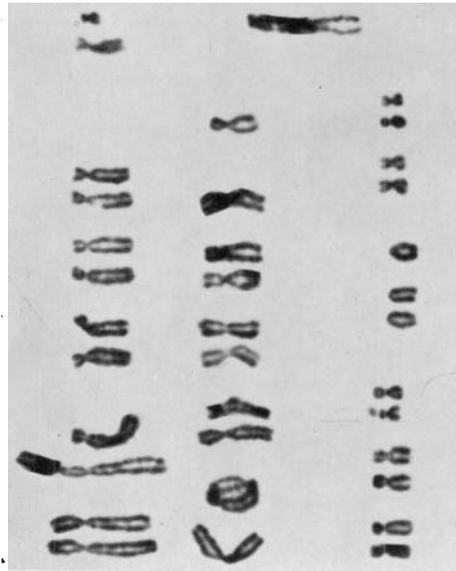
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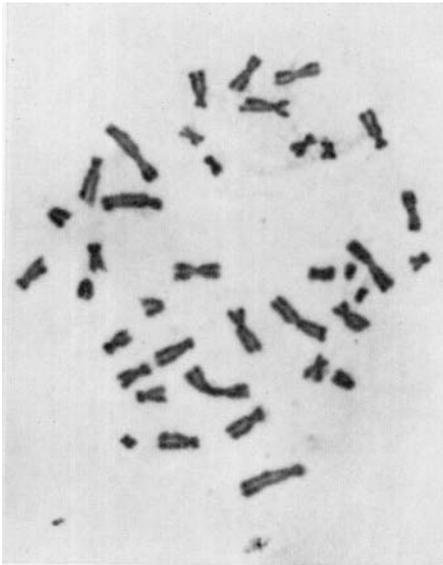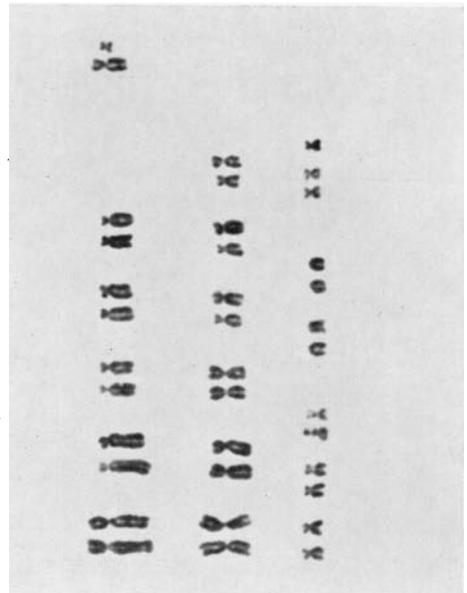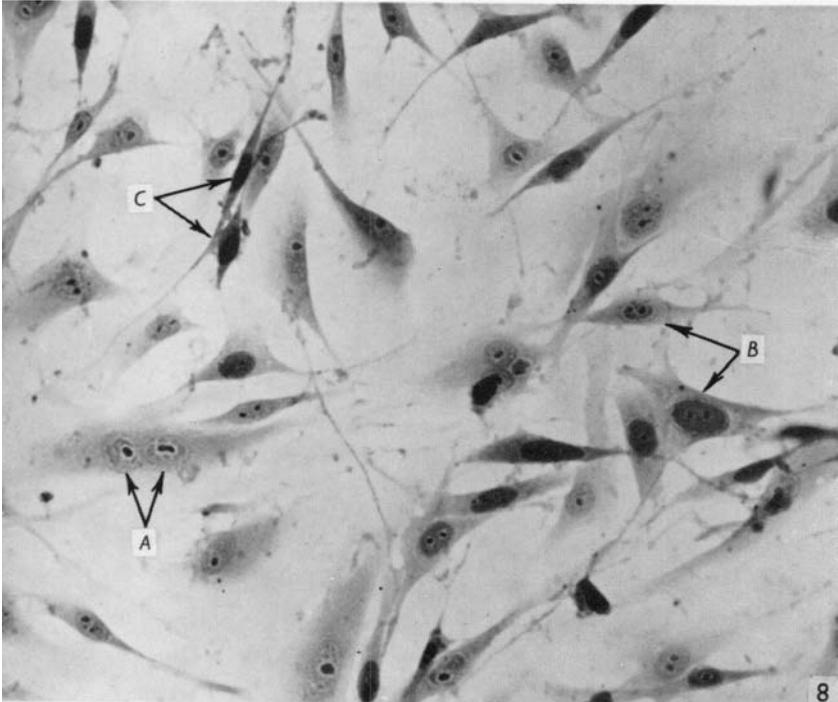
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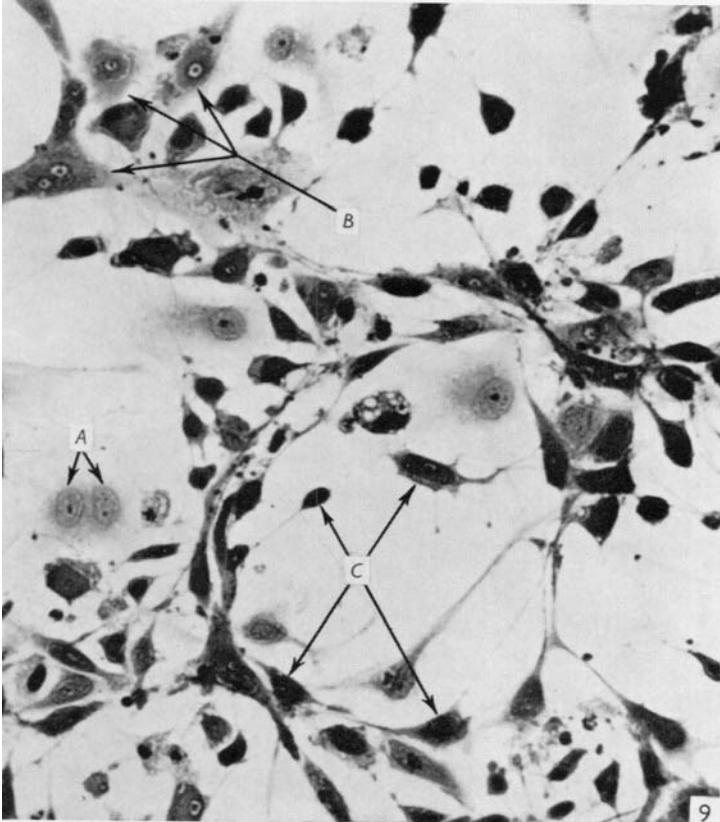
Fig. 9

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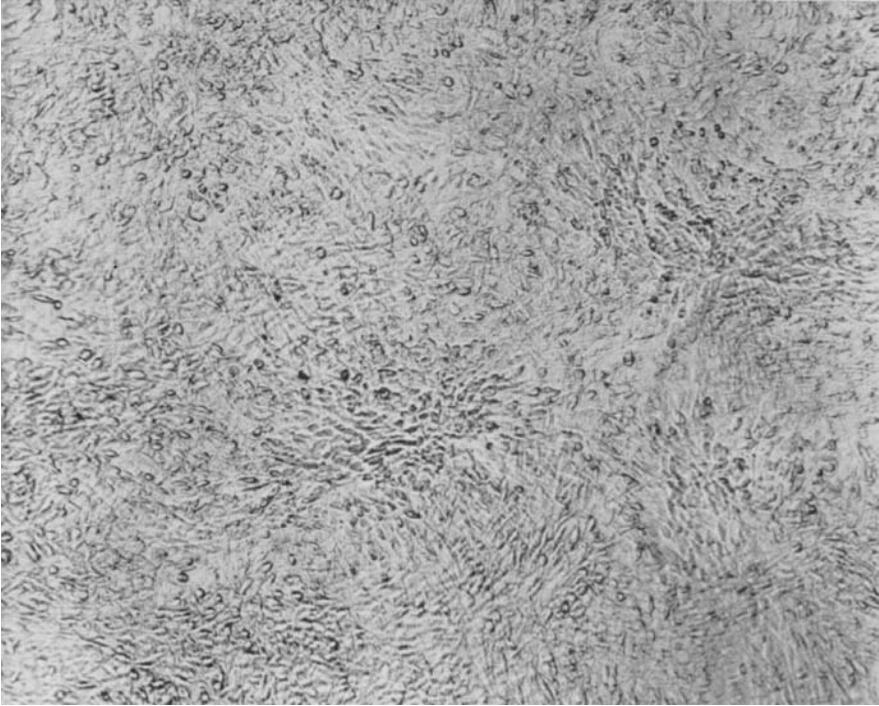
Fig. 10

Fig. 11

K. J. O'REILLY AND A. M. WHITAKER