Table 3. ATTEMPTED REVERSAL OF DEPRESSED PFC RESPONSE IN IRRADIATED MICE BY INJECTION OF NORMAL THYMUS OR BONE YARROW CELLS

Group No.	Donor cell inoculum	No. of mice	PFC/spleen (mean ±s.e.)	Significance levels 1†	2‡
1	10' control spleen cells + SRRC	9	3,187 ± 411	—	P < 0.001
2	10^7 spleen cells from DMBA-treated mice + SRBC	12	681 ± 147	P < 0.001	—
3	10' spleen cells from DMBA-treated mice + 5 x 10' normal thymus cells + SRBC	7	967 ± 334	P < 0.001	N.S.*
4	10^7 spleen cells from DMBA-treated mice + 5 x 10^7 normal bone marrow cells + SRBC	5	3,663 ± 885	N.S.*	P < 0·001
5	As in group 4 but with only 2.5 x 10^7 bone marrow cells	5	3,768 ± 717	N.S.*	P < 0.001
6	5 x 10^7 normal bone marrow cells + SRBC (no spleen cells)	3	119 ± 44	P < 0·001	—

* Not significant: P > 0.05.
† With respect to group 1.
‡ With respect to group 2.

depleting only a rapidly dividing marrow-derived lymphocyte population.

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Characteristics of a Human Diploid Cell Designated MRC-5

THE stability and integrity of the human foetal cell strain WI-38, which has been well demonstrated in the past ten years[1–3], and its susceptibility to viruses infective for man[1,4], explain the value of such material for the isolation of viruses and in the development of vaccines[6–8]. We have developed another strain of cells, also derived from foetal lung tissue, taken from a 14-week male foetus removed for psychiatric reasons from a 27 year old woman with a genetically normal family history and no sign of neoplastic disease both at abortion and for at least three years afterwards. The criteria used for characterizing the cells are those recommended internationally[2,9].

The serial propagation of cells was effected essentially according to the method of Hayflick and Moorhead[1]. When the cells reached about the seventh cell doubling, all except a few cultures were harvested and frozen to form a cell stock. Those not subjected to freezing continued to be passaged serially to determine their in *vitro* life span and other characteristics. Cells used to prepare a stock were harvested and suspended in a preserving medium which comprised seventy-five parts Eagle's basal medium, seventeen parts unheated calf serum and eight parts dimethyl sulphoxide. The concentration of cells in the final suspension was adjusted so that 1 ml. contained about 3 x 10^6 cells before being distributed in volumes of 1 ml. to glass ampoules of 1·2–1·5 ml. capacity. Immediately before the cells were distributed and cooled, and before the addition of dimethyl sulphoxide to the medium, samples were removed which were tested subsequently for the presence of microbial contaminants, including mycoplasma. No viable agents were isolated. For storage the cells were cooled at about 2° C per min to −80° C or lower, when they were immersed in liquid nitrogen. A total of 481 ampoules of cells was prepared and stored in this way. Neither the growth medium nor the preserving medium contained antibiotics.

Cells that were cultivated serially without being subjected to freezing and storage attained 48 cell doublings before showing the customary signs of decline exhibited by cells of normal human foetal origin[1]. Later studies on cells reconstituted from the frozen state have shown that storage of the cells in these conditions for about three years does not alter their in *vitro* life span. Comparative figures for cells propagated serially without being subjected to freezing and cells propagated similarly after restoration from a frozen stock are shown in Table 1.

Table 1. COMPARATIVE NUMBER OF POPULATION DOUBLINGS REACHED BY MRC-5 CELLS BEFORE THE ONSET OF THE STAGE OF DECLINE (CELLS RESTORED FROM STOCK FROZEN AT 'PASSAGE' 7)

Storage time (months)	Number of population doublings
0*	48
1	43
9	42
14	45
18	46
23	44
35	45

* Cultured continuously.

Observations during the concurrent propagation of MRC-5 and WI-38 cells reveal differences in their rates of growth and their sensitivities to minimal environmental (mainly medium) factors. MRC-5 cells replicate more rapidly than WI-38 cells—they attain the same degree of confluency in 3 days as is reached by WI-38 cells in 4 days. Confirmation of this difference in the rate of multiplication has been obtained at comparable time intervals after seeding by doing cell counts. Moreover, MRC-5 cells more readily tolerate environmental changes—in glassware or serum, for example—which can adversely influence the growth of WI-38 cells. For karyological investigation, cells taken from cultures 2 days after subculture, and after 12, 19, 23, 27 and 36 "passages", were arrested in metaphase by adding colchicine and examined for exact chromosome counts and for analysis of karyotype (Table 2).

Table 2. KARYOLOGICAL DATA ON YRC-5 CELLS

Population doubling ("passage")	Frequency of: Structural abnormalities	Breaks or gaps	Aneuploidy	Polyploidy	Hyperdiploidy
12	0/100	5/100 (5%)	5/42 (12%)	1/265 (0·4%)	0/100
19	0/100	5/100 (5%)	5/50 (10%)	4/290 (1·4%)	0/100
23	1/100 (1%) (dicentric)	3/100 (3%)	12/85 (14%)	7/232 (3%)	0/100
27	0/100	5/100 (5%)	8/67 (12%)	6/326 (1·8%)	0/100
36	0/100	1/52 (2%)	4/42 (10%)	8/194 (4·1%)	0/100

Since the completion of these initial studies on the karyology of MRC-5 cells almost 3 years ago, more data have accumulated from the results of analyses on cells reconstituted and propagated after removal from the frozen stock. These long-term results are combined with those obtained earlier (Table 2) in Table 3.

Table 3. CUMULATIVE DATA ON THE KARYOLOGY OF MRC-5 CELLS

Population doubling ("passage")	Frequency of: Structural abnormalities	Breaks or gaps	Aneuploidy	Polyploidy	Hyperdiploidy
12–26	3/2,878 (0·1%)	64/2,878 (2%)	235/1,573 (15%)	283/20,829 (1%)	6/1,573 (0·05%)
27–33	0/400	14/400 (3·5%)	20/186 (11%)	67/2,133 (3%)	0/186
34–40	0/390	8/390 (2%)	27/192 (14%)	105/2,670 (4%)	0/192

These results confirm initial observations and indicate that the karyological properties of MRC-5 cells conform to those required of a diploid cell of human origin to be used for producing viral vaccines intended for human use[9]. The population doublings shown in Table 3 are so arranged because the WI-38 cells at present used for the manufacture of vaccines are infected with virus up to, but not later than, the thirtieth population doubling and the karyology of the control cells is examined between 27 and 33 doublings. Thus the results for this "middle" group are directly related to cells commonly used in the manufacture of vaccines. The results suggest that with increasing age the karyology of the cells varies only in respect of polyploidy, which seems to increase with age.

Fig. 1 illustrates the characteristic fibroblastic morphology of MRC-5 cells and Fig. 2 shows that chromosomes taken from a metaphase plate have normal appearance.

Cells after 15 to 35 population doublings were used to determine susceptibility to viruses, as shown by cytopathic effects. Rubella virus was grown in MRC-5 and WI-38 cells and titrated in RK_{13} cells[10]. For comparison, concurrent titrations were set up using WI-38 cells. All assays were conducted in rolled tube-cultures kept at 37° C, except for the rhinoviruses, when the assays were done at 33° C. Serum-free Eagle's basal medium containing 0·1 per cent $NaHCO_3$ was used, except that for the rhinovirus titrations the initial pH was lowered to 6·8 by reducing the amount of $NaHCO_3$ to 0·02 per cent and adding calf serum (final concentration 2 per cent). Stock suspensions of viruses were used at 0.6 log, steps, with five tubes used at each dilution. Assays were carried out in duplicate using polioviruses, several ECHO viruses, arboviruses, adenoviruses, rhinoviruses and herpes viruses, and those of vaccinia, measles, rubella, and Coxsackie A9. The results show that MRC-5 cells are widely susceptible and that their sensitivity to these viruses is remarkably similar to that of WI-38 cells.

At several stages during serial propagation we tested for the presence of extraneous agents. Cells and supernatant fluids were taken from cultures after 14, 24, 32 and 42 population doublings and were used to attempt the isolation of microbial agents *in vivo* and *in vitro*. For the *in vivo* studies, about 10^7 cells were inoculated intramuscularly into each of the following groups of animals: five rabbits, five guinea-pigs, ten adult mice, one litter of suckling mice, one litter of newborn hamsters, and ten embryonated eggs (11 days old). The animals were observed twice weekly for a period of four weeks and the embryonated eggs were examined after four days. Complementary *in vitro* tests were done to detect the presence of viruses. Cells or spent medium were added to primary cell cultures prepared from patas monkey kidney, human embryo kidney, rabbit kidney, HeLa cells and WI-38 cells. These cultures were incubated at 36°–37° C for 21 days and were examined periodically for any change in morphology. Tests to detect haemadsorbing viruses were done by using red cells from humans, guinea-pigs and chickens. Samples were removed from cultures on days 3–5 and on days 15–21. At the end of the period of incubation, cells were stained and examined for the presence of inclusion bodies. Suitable media were used to test for the presence of bacteria, fungi and mycoplasmas, but no extraneous agents were detected.

To ascertain whether the cells had any neoplastic properties, between 10^6 and 10^7 cells removed from cultures during the log phase of growth were implanted into Syrian hamsters aged six weeks, using both cheek pouches of each animal, and into the lateral thoracic subcutaneous layer. More recently, mice treated with rabbit anti-mouse lymphocyte serum were also inoculated subcutaneously with cells. The cells used were taken from cultures after 16 and 31 population doublings for the test done in hamsters and at the twentieth doubling for those done in mice. All animals were examined at weekly intervals for one year. Animals not surviving the full period were examined post mortem, and observations for neoplastic growth were made on these as well as on those which survived the full term. None of the animals showed evidence of cells which effected the growth of an invasive

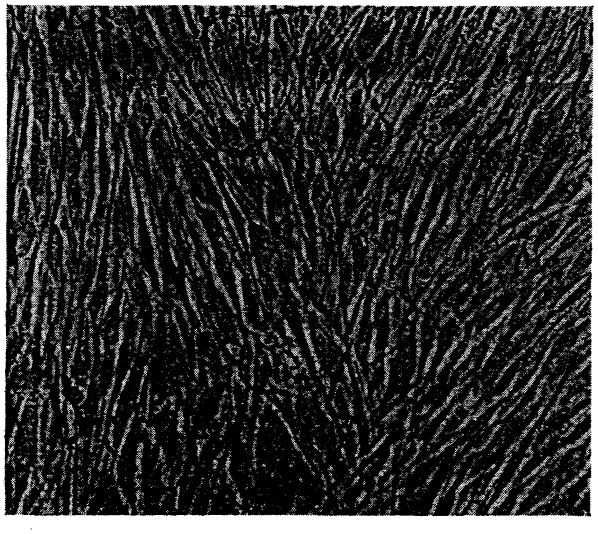

Fig. 1. Normal YRC-5 cells, phase photograph (x 162).

Fig. 2. Chromosomes from an MRC-5 cell showing typical diploid appearance (x 1,296).

nodule, whereas positive control (HeLa) cells did produce such a growth both in cheek pouches and subcutaneously when about 10^5 cells were implanted.

Our studies indicate that by presently accepted criteria, MRC-5 cells—in common with WI-38 celle of similar origin—have normal characteristics and so could be used for the same purposes as WI-38 cells.

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Proteolytic Enzymes Initiating Cell Division and Escape from Contact Inhibition of Growth

BRIEF treatment with very low concentrations of proteolytic enzymes can bring about a change in the cell surface similar to that occurring in the chemical or viral transformation of normal to malignant cells[1]. This suggests to us that proteolytic enzymes may not only convert the surface structure into the type seen in malignant cells but that the whole cell may respond to the proteolytic enzyme with initiation of new rounds of cell division and a concomitant escape from contact inhibition of growth, as is the case for malignant cells in tissue culture[a].

Two observations led to this hypothesis. First, an agglutination site containing N-acetylglucosamine has been found to be exposed in all virally transformed tumour cells tested so far[3,4], but not in the untransformed parent cell. Brief proteolytic treatment can unmask this site in untransformed cells[1]. Many carbohydrate containing sites, as well as other sites, are known to become exposed in the course of transformation[2,5]. Second, a non-malignant (3T3 fibroblast) cell line has been found to proliferate better and lose some of its contact inhibition of growth when grown in mixed culture together with a malignant cell (L1210) (unpublished observations of M. M. B.). Because the leukaemia cell produces proteases, it may be that these enzymes act as mediators for growth stimulation like the growth factor—which also may have proteolytic activity[6]—produced by cells infected with Rous virus. Increases in the levels of peptidases in tumour cells have, moreover, been reported[7]. We wish to report evidence that proteolytic enzymes can initiate cell division and escape from contact inhibition of growth.

By adding small amounts of trypsin to a non-malignant mouse fibroblast culture (3T3) which has reached confluence, we have found that most cells will escape contact inhibition of growth, divide further and eventually reach densities at which, as in the case of malignant or transformed cells, some cells come to lie on top of each other. Such an effect is illustrated in Fig. 1*b* with only 0·007 per cent of crystalline trypsin. In the conditions in which these cells are grown (3 per cent serum), much more trypsin is necessary to bring about microscopically visible effects or release from the substratum. The trypsin effect is based on proteolytic activity and does not simply result from adsorption of the enzyme to the cell surface. Our evidence for this is, first, that diisopropylfluorophosphate substituted trypsin is inactive (Fig. 1); second, that the trypsin inhibitor ovomucoid prevented the growth stimulatory effect while ovomucoid alone in a control experiment had no effect; third, heat-inactivated trypsin was not effective; and fourth, the trypsin effect could be overcome with massive doses of serum from conditioned medium containing trypsin inhibitory activity.

Because the cell surface change described earlier[1] is brought about by any protease, the question arises whether the growth stimulatory effect is also non-specific or can only be induced with trypsin. Pronase (Figs. 1a and 2) and ficin (Fig. 1a) were both found to trigger escape from contact inhibition of growth in a confluent culture. Each of these enzymes acts at ten to fifty-fold lower concentrations as compared with trypsin. For a growth stimulatory effect, one would expect both cell numbers and the mitotic index to increase, reflecting the increased rate of cell division. This is borne out by Fig. 2.

Do cells have to be continually exposed to the proteolytic enzyme or will a brief exposure initiate growth and cell division later on? Exposure for 45 min to a 0.0005 per cent solution of pronase is sufficient to initiate cell divisions occurring a few days later (Fig. 1a). Once the proteolytic enzyme has exerted its effect, the cells go through at least one division. To this extent the effect is irreversible, but it is certainly not an inheritable effect similar to viral or chemical transformation, for the same cells grew to usual saturation density levels after sub-

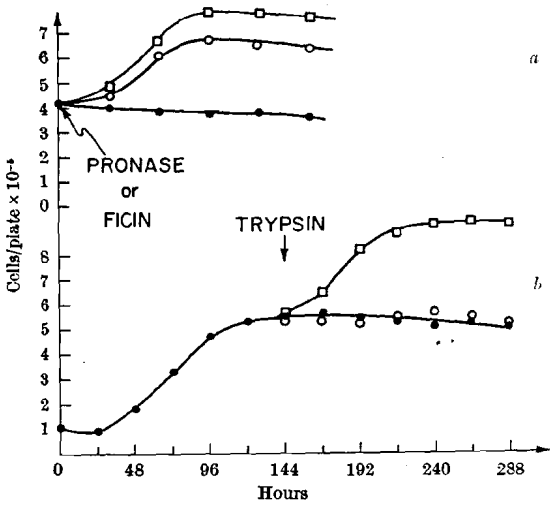

Fig. 1. *a*, 3T3 mouse fibroblasts were grown on 3·5 cm Falcon dishy in Dulbecco–Vogt medium with 3 per cent serum to saturation density; pronase (Calbiochem) was then added to a final concentration of 5 μg/ml. for 45 min. Incubation medium was removed from both experimental and control dishes and the cultures were rinsed three times with 2 ml. of medium drawn off from 2 day old cultures (wndltioned medium) to prevent a stimulatory effect of fresh serum. The same conditioned medium was used for continuous incubation of the treated and the control culture. Dally, thereafter. the cell content per plate was counted after release with EDTA or 0.01 per cent trypsin with a Levy–Hausser chamber. (The Coulter counter gave less reliable results.) One point corresponds to six different experiments. ○, Pronase; ●, control; □, 0·0005 per cent flcin. b, 3T3 cells were grown as in *a* except that the plates were inoculated at day 0 rather than three days previously. At the arrow, a solution of sterile trypsin in saline was added to bring the trypsin concentration to 0·007 per cent and saline was added to the control. In general Calbiochem trypsin (chymotrypsin-free) was used: otherwise Sigma (2× crystallized) or Grand Island Biological preparations. □, Trypsin; ○, control with 0.01 per. cent diisopropylfluorophosphate inactivated trypsin (Worthington), ●, control without protease. For b, 48 per cent chick and 0.2 per cent calf serum were used.