

Viral-induced Fusion of Human Cells

II. HETEROKARYOCYTES BETWEEN HUMAN CELLS FROM DIFFERENT DONORS, AND BETWEEN HUMAN CELLS AND THOSE FROM OTHER SPECIES: FORMATION AND PROPERTIES^{1,2}

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ABSTRACT A simple method is described for effecting the formation of heterokaryocytes between different lines of human diploid fibroblasts, and between human diploid fibroblasts and cultured cells derived from other species. In the case of mixed monolayer cultures of human diploid fibroblasts exposed to UV-inactivated Sendai virus, the proportion of nuclei in heterokaryocytes is between 25 and 35%. The heterokaryocytes engage in *de novo* protein synthesis. No evidence of hybrid enzymes was found in mixed cultures of human and mouse cells which had been exposed to Sendai virus and which therefore presumably contained mouse-human heterokaryocytes. However, with the available data, it is not possible to distinguish between the absence of synthesis of hybrid enzymes and the synthesis of hybrid enzymes in amounts insufficient to permit their detection.

In a previous paper (Velázquez et al., '71) a simple method was described which yields a high frequency of polykaryocytes in cultures of human diploid fibroblasts. This method involves the addition of UV-inactivated Sendai virus to a cell monolayer, without removing the medium, and exposing the cells to the virus for about 12 hours. When this technique is used, the frequency of nuclei which are present in multinucleated cells (the polykaryocytic index) is about 50%. One of the purposes of the present study was to determine the degree of heterokaryocytosis induced by this method, when the virus is added to a mixed culture containing different strains of human diploid fibroblasts, or cultures containing a mixture of human diploid fibroblasts and cultured cells derived from other species. Other reports have described the use of the conventional method (which involves exposing a mixed cell suspension to the virus for a short length of time) for the induction of fusion between different strains of human diploid fibroblasts (Siniscalco et al., '69), or human diploid fibro-

blasts and fowl erythrocytes (Guggenheim et al., '68; Harris and Cook, '69). An additional objective of this study was to inquire whether or not hybrid enzymes could be detected in cultures containing interspecific heterokaryocytes.

MATERIALS AND METHODS

Some of the materials and methods used have been described earlier (Velázquez et al., '71). Only those not previously employed are summarized below.

Composition of culture media

The composition of automedium (Krooth, '64) is similar to nucleomedium (Velázquez et al., '71) with two exceptions: auto-

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medium contains fetal bovine serum which has been dialysed for 21 hours against tap water and for three hours against distilled water. Secondly, it does not contain nucleosides or NCTC 135.

Cell lines

MIS is a human fibroblastic line developed from a skin biopsy on a normal Caucasian adult female. 3T3 was derived from a Swiss mouse embryo (Todaro and Green, '63), and was kindly supplied by Dr. George J. Todaro. CHA₂ was developed from the lung of a male Chinese hamster (V-explant) by Ford and Yerganian ('58). A subline (V79-379A) from the original line was kindly supplied by Dr. Norman Salzman. The R00 line was derived from a male rat-kangaroo (Shaw and Krooth, '64). MIG is a clonal line of human mouse hybrid cells, which was kindly supplied by Dr. Barbara Migeon.

Formation of heterokaryocytes

One of the two cell lines to be fused was labelled with thymidine-methyl-H³ (19.2 c per mM; New England Nuclear Corp., Boston, Mass.) at a final concentration of 0.3 μ C/ml for 72 hours. The cultures were then washed twice with a nucleomedium containing non-radioactive thymidine at a concentration of 52.4 mg per liter. This concentration of thymidine is about 10⁴-fold higher than the concentration of the tritiated compound used to label the cells. This modified nucleomedium containing unlabelled thymidine was used throughout the rest of the experiment. After being washed with medium, the cultures were trypsinized, suspended in medium and mixed in a 1:1 ratio with cells of the unlabelled strain. The total concentration of cells in the suspending medium was 4 \times 10⁵ per milliliter. One milliliter of this suspension was then added to each of six Leighton tubes and cells were allowed to attach to the surface of the tubes. In addition, six pure cultures of each parental line were plated at the same initial cell density. The cells in each culture were then fused by the procedure described previously (Velázquez et al., '71): half of the cultures were exposed to virus (and half to Hanks' balanced salt solution) for 12 hours and were later subcultured at low population density to Petri dishes con-

taining coverslips. After six to eight hours, the coverslips were fixed, washed in 0.3 N trichloroacetic acid at 4°C for one hour, rinsed several times with distilled water, dipped briefly in 70% ethanol and air-dried. Finally, the coverslips were mounted on slides and autoradiographs were made.

Labelling of cultures with tritiated leucine

DL-leucine- 4,5-H³ (5 c per mM; New England Nuclear Corp., Boston, Mass.) was added to the medium to give a final concentration of 4.12 μ C per milliliter. This quantity of radioactivity corresponds to a concentration of 0.108 μ g of leucine per milliliter, which is less than 1/500th the concentration of leucine present in nucleomedium or in complete automedium.

Acrylamide gel electrophoresis

The cells were harvested by trypsinization and were suspended in distilled water and disrupted by sonication (Krooth et al., '62). The cell sonicate was extracted twice with toluene and then centrifuged at 23,000 g for 30 minutes. Sucrose was added and the extracts were dehydrated by vacuum evaporation. The final protein concentration was 10 mg per milliliter and the final sucrose concentration was 20 gm %. The solutions were then frozen and stored at -60°C.

Acrylamide gels were prepared by adding 14 mg of Cyanogum-41, 0.2 ml of TMED (N,N,N',N'-tetramethylethylenediamine) and 200 mg of ammonium persulfate) all from EC-Apparatus Corp., Philadelphia, Pennsylvania) to 200 ml of a buffered solution (*vide infra*). The electrophoretic migration of lactic acid dehydrogenase (LDH) and malic acid dehydrogenase (MDH) in acrylamide gels was measured using a Survey Model Vertical Gel Cell (EC-Apparatus Corp.). The buffered solution used for LDH was tris-Na₂ EDTA-boric acid (0.1 M), pH 9.5 (EC-Apparatus Corp., technical bulletin no. 134). In the case of MDH, the buffer employed was tris-Na₂ EDTA-boric acid, prepared as described by Peacock et al. ('65), except that in the present experiments, the pH was adjusted to 8.0 (from pH 8.4) with additional amounts of boric acid.

The electrophoresis of LDH was performed at 4°C for three hours using a current of 400 v and 180 ma. In the case of MDH, the current was run for two hours 30 minutes using 300 v and 150 ma. The gels were stained for LDH by the method of Brewer ('70) and for MDH by the procedure of Weitkamp et al. ('69).

RESULTS AND DISCUSSION

A. Quantitative studies of heterokaryocyte formation

In order to measure the degree of heterokaryocytosis obtained in a given experiment, a parameter has been chosen which I shall call the *heterokaryocytic index*. It is defined as the proportion of nuclei, among all nuclei observed, which are present in heterokaryocytes. The heterokaryocytic in-

dex is therefore computed by dividing the number of nuclei in heterokaryocytes by the number of nuclei in all cells observed.⁴ If one assumes that, on the average, there is the same amount of cytoplasm associated with those nuclei which participate in cell fusion as with those which do not, then the heterokaryocytic index estimates the proportion of the total cytoplasm in the culture which is present in heterokaryocytes. Figure 1 shows a heterokaryocyte formed by the fusion of labelled CR cells with unlabelled MIS cells, both human diploid fibroblasts.

When mouse or Chinese hamster cells were exposed to tritiated thymidine under the conditions described earlier, 96 and

⁴ Estimation formula for the standard error of the heterokaryocytic index has proven very difficult to derive and is not yet available.



Fig. 1 An example of a heterokaryocyte formed by fusion of two lines of human diploid fibroblasts. Male cells (CR line) were labelled with H^3 -thymidine (labelling efficiency: 78.6%) and were mixed in a 1:1 ratio with unlabelled female cells (MIS line). Afterwards, the procedure described under MATERIALS AND METHODS was followed. The coverslips were then fixed, washed with cold TCA and autoradiographed. The heterokaryocyte shown in this picture contains three labelled and three unlabelled nuclei. Phase contrast microscopy. Magnification is approximately $\times 270$.

100% of their nuclei, respectively, became labelled. On the other hand, only 65 to 90% of the nuclei of human diploid fibroblasts incorporated the radioactive precursor to a measurable extent.⁵ A statistical method for estimating the heterokaryocytic index when the labelling of one of the parental lines with tritiated thymidine is incomplete has been described elsewhere (Velázquez, '70).

Figure 2 summarizes the results of several fusion experiments. When different strains of human diploid fibroblasts were fused with one another, the heterokaryocytic index was 0.238 in the first experiment (a), and 0.361 in the second (b). These figures suggest that roughly between 25 and 35% of the cells in mixed cultures of human diploid fibroblasts are incorporated into heterokaryocytes when exposed to Sendai virus under the present conditions. However, it should be added that this inference can rigorously be drawn only on the following assumptions: (1) no multinucleate cells arise by nuclear division;

(2) no cells or nuclei undergo division after the virus is added; and (3) no nuclear fusion takes place. These assumptions have been discussed in more detail by Velázquez et al. ('71).

While these experiments were in progress, Siniscalco et al. ('69) reported on the formation of hybrid cells between human diploid fibroblasts derived from two different donors. These authors presented evidence for the formation of heterokaryocytes between the two cell lines. However, they did not present data bearing on the degree of heterokaryocytosis obtained.

There is an obvious analogy between mammalian heterokaryocytes and the heterokaryons of higher fungi. The fungal heterokaryons, however, will grow and bear spores, whereas human heterokaryocytes

⁵ As human fibroblastic homonuclear cells age *in vitro* (Hayflick and Moorhead, '61; Hayflick, '65), the proportion of cells which engage in DNA synthesis, during a 24-hour interval, decreases (Macieira-Coelho et al., '66; Macieira-Coelho and Ponten, '69). Merz and Ross ('69) have shown that, over a period of months, as human homonuclear cells grow *in vitro*, the percentage of cells capable of undergoing division decreases exponentially.

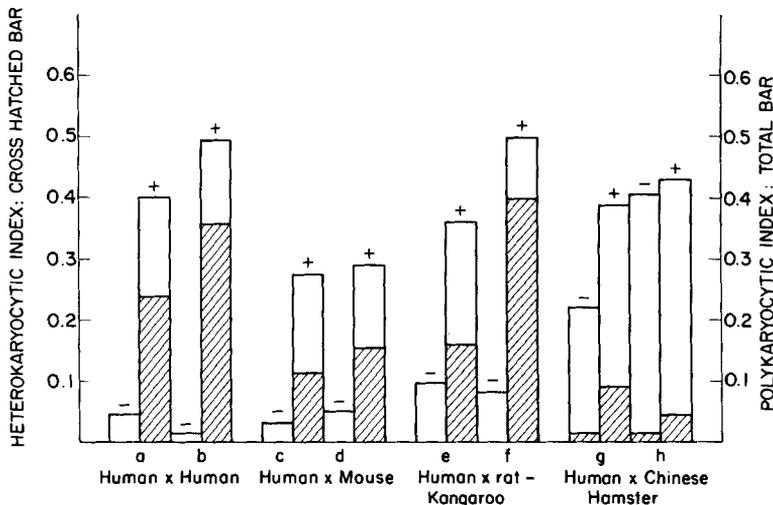


Fig. 2 Summary of the experiments measuring the degree of fusion of human diploid fibroblasts with other human cell strains and with cells from other species. The total area included in each bar represents the polykaryocytic index. The area included in the cross hatched segment of a bar represents the heterokaryocytic index. (—): cultures exposed to BSS; (+): cultures exposed to Sendai virus. (a) Labelled human cells, CR, mixed with unlabelled human cells, MIS. (b) Labelled human cells, JDU, mixed with unlabelled human cells, CR. (c) Labelled human cells, CR, mixed with unlabelled mouse cells, 3T3. (d) Labelled mouse cells, 3T3 mixed with unlabelled human cells, CR. (e,f) Two different experiments, having mixed labelled human cells, CR, with unlabelled rat-kangaroo cells, ROO. (g) Labelled human cells, CR, mixed with unlabelled Chinese hamster cells, CHA₂. (h) Labelled Chinese hamster cells, CHA₂, mixed with unlabelled human cells, CR.

TABLE 1

Frequency of CR multinucleate cells (homokaryocytes) relative to all CR cells observed in pure cultures and in mixed cultures containing CR and CHA₂ cells. No Sendai virus added

	Pure CR cultures	Mixed CR + CHA ₂ cultures
Experiment 1	3.0	5.1
Experiment 2	4.8	13.0

do not grow. Hence, it is important to maximize the frequency with which human heterokaryocytes are formed. They cannot, unfortunately, be selected by devising media which would favor their overgrowth. However, a heterokaryocytic index for human-human mixtures, of 25 to 35%, which was obtained in the present work, is quite appreciable and may enable us to study complementation between different human genes. It is of interest to contrast heterokaryocytosis with somatic cell hybridization. This latter process occurs at frequencies lower than 1%, even after the addition of Sendai virus (Coon and Weiss, '69; Kao et al., '69).

The heterokaryocytic index observed after fusing human and mouse cells varies between 0.10 and 0.15 (fig. 2c,d). The heterokaryocytic index observed when human and rat-kangaroo cells were fused, was very different in the two experiments performed (fig. 2e,f); the index was 0.161 in the first experiment, and 0.398 in the second. The reason for this variation is unknown. In both of the above experiments, incidentally, the human nuclei were the ones labelled with tritiated thymidine. Experiments in which the rat-kangaroo cells were labelled suggest that the cultured cells of this animal are very sensitive to tritiated thymidine; after the cells are labelled, they do not survive throughout the fusion experiment. The Chinese hamster line used in these experiments normally contains 20 to 30% multinucleated cells. This is one of the reasons why the *polykaryocytic* index of the control human-hamster cell cultures is high.

As shown in figure 2, the mere co-cultivation of human and Chinese hamster cells seems to induce the formation of a small proportion of heterokaryocytes, which is further increased, though not to a high level, when Sendai virus is added. Although the polykaryocytic indices of mixed human-Chinese hamster cultures exposed to Sendai virus were similar in both experiments, the heterokaryocytic index varied over a two-fold range in these two experiments. I am presently unable to explain this difference. It should also be pointed out that the frequency of CR *homokaryocytes* seems to increase when cells of this line are co-

cultivated with CHA₂ cells in the absence of virus (table 1).

Perhaps the fusion which takes place between human and hamster cells (and between different human cells co-cultivated with Chinese hamster cells) in the absence of added Sendai virus, might be due to a virus carried by the Chinese hamster cells — a virus whose presence is covert. This idea is supported by electron microscopic observations which show that many lines of hamster cells contain virus-like particles (Bernhard and Tournier, '64; Compans et al., '66). Recently, Jensen et al. ('70) have reported the isolation of a paramyxo-like virus from a line of SV-40 transformed hamster cells. They found that this virus induces the formation of polykaryocytes in cultures of other lines of hamster cells.

B. Studies on the electrophoretic mobility of the lactate and malate dehydrogenase activities recovered from mixed human-mouse cultures following exposure of the cultures to Sendai virus

Two experiments were performed in order to study the electrophoretic mobility of the LDH and MDH activity recovered from mixed human-mouse cultures. Cells were added to flasks at appropriate density to form a confluent monolayer. One set of flasks received a mixture of CR and 3T3 cells in a 1:1 ratio. Two other sets contained pure cultures of CR or 3T3 cells. Each set was subdivided into two groups, one which received the virus, the other receiving BSS instead. In the first experiment, the cells were harvested one day after addition of the virus. In the second experiment, the cells were harvested on the ninth day. The polykaryocytic indices, for these two experiments were 0.311 and 0.296, respectively. It was decided not to label the nuclei of one of the cell lines in

order to estimate the heterokaryocytic index, because H^3 -thymidine may damage or kill cells which incorporate it (Drew and Painter, '59), and thus interfere with studies on the cellular protein synthesis. Furthermore, the electrophoretic experiments required large quantities of cells, and labeling one of the parental lines would be very costly.

In both experiments, the electrophoretic patterns of mixed cultures exposed to Sendai virus were indistinguishable from the control ones (mixed but without virus). The patterns of the virus-exposed cultures were also indistinguishable from those produced by a 1:1 mixture of cell extracts from control cultures of both parental lines. As shown in figures 3 and 4, no additional bands were detected. On the other hand, new LDH bands, not observed in extracts from the parental cultures, were observed in extracts of cells from a human-mouse hybrid clone (MIG). These new bands very likely correspond to hybrid isozymes (fig. 3). No hybrid MDH bands were observed in extracts from MIG cells (fig. 4). This could well be due to the loss of human chromosomes containing the locus for MDH in this clone, since most human chromosomes are eventually lost in human-mouse hybrids (Weiss and Green, '67).

The absence of hybrid enzymes in cultures containing heterokaryocytes was unexpected, since hybrid isozymes of LDH and MDH have been observed in clones of human-mouse hybrid lines (fig. 3; Nabholz et al., '69; Boone and Ruddle, '69). Moreover, there is evidence that, at least for some loci, genes of both nuclei are expressed in mammalian heterokaryocytes. In HeLa-Ehrlich ascites tumor cell heterokaryocytes, antigens of both man and mouse are present on the cell surfaces, and seem to be randomly mixed (Watkins and Grace, '67).

In the present study some of the cultures, at the time of harvest, had been exposed to virus as long as nine days earlier. One would think this interval provided ample time for the cytoplasm of the heterokaryocytes to become thoroughly mixed.

There is the possibility that the heterokaryocytes did not synthesize protein and that the protein present in the parental cells at the time of cell fusion was not

replaced by *de novo* synthesized protein and hence no hybrid enzymes were formed. An experiment was therefore performed in order to determine whether or not the cultures containing human-mouse heterokaryocytes are able to engage in protein synthesis. In cultures containing a 1:1 mixture of human (CR) and mouse (3T3) cells exposed to Sendai virus, the ability of all cells, both mono and polykaryocytes (which presumably include the heterokaryocytes) to incorporate tritiated leucine into their respective acid-insoluble pools was measured. In this experiment, the polykaryocytic index of the cultures exposed to virus was 0.258.

At days 1, 4 and 7 after addition of the virus, the cells were subcultured at low cell population density into dishes containing coverslips and nucleomedium. Fifteen hours later, the cells were washed twice in leucine-free automedium and labelled with tritiated leucine for two hours. Two sets of dishes (each set containing control and virus-treated cultures) were exposed to tritiated leucine in automedium containing no cold leucine, one of these sets also containing cycloheximide (Acti-dione, Nutritional Biochemical Corp., Cleveland, Ohio) at a final concentration of 100 μ g per milliliter. A third set of cultures was exposed to tritiated leucine in complete automedium, which contained about 500 times more cold than radioactive leucine. Afterwards they were washed twice, fed with nucleomedium and incubated at 37°C for 45 minutes. The coverslips were then washed, fixed and submerged in 0.3 N trichloroacetic acid at 4°C for one hour. The coverslips were washed in distilled water and 70% ethanol before being mounted and studied autoradiographically. In all the cultures to which H^3 -leucine was added to leucine-free automedium, all the cells, both mono and polykaryocytes, became labelled. It seems likely that most of the radioactivity was indeed in leucine at the time of being incorporated, because cultures in complete automedium rather than in leucine-less automedium did not become labelled. The incorporation of tritiated leucine to the acid insoluble pool of the cells is almost certainly a consequence of protein synthesis. No cell was labelled when

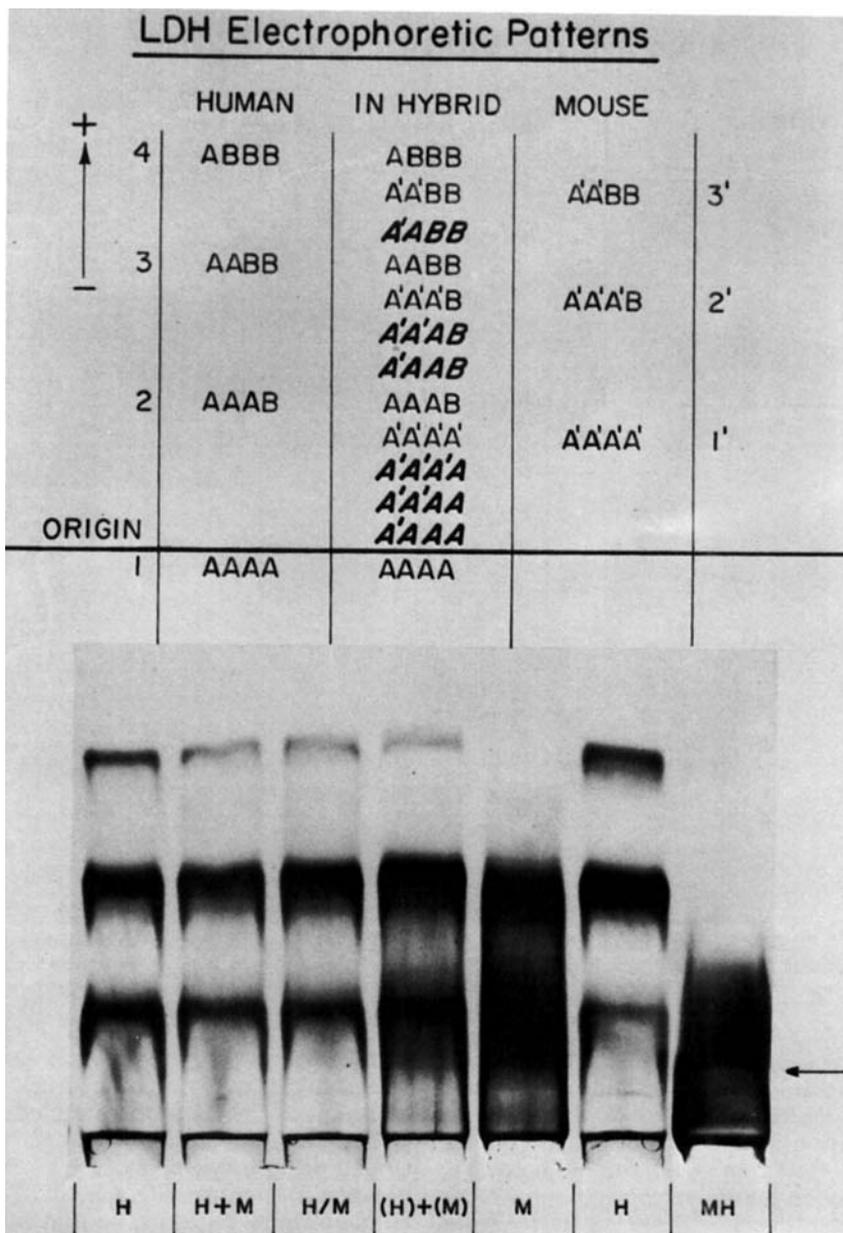


Fig. 3 LDH electrophoretic patterns of extracts from human, mouse and human-mouse cell cultures. Upper half: Diagrammatic representation of the mobility and composition of LDH isozymes from pure and hybrid human-mouse cultures in acrylamide gels (from Nabolz et al., '69; Boone and Ruddle, '69). B human and mouse subunits have the same mobility; A human subunit moves slower than A' mouse subunit. Theoretically distinguishable hybrid isozymes are represented by bold-face letters. Lower half: Observed patterns of extracts prepared as described under MATERIALS AND METHODS, and electrophoresed in acrylamide gel. Cells were harvested after nine days of exposure to BSS or virus. H: extracts from human cells; M: extract from mouse cells; H + M: extract from mixed culture of human and mouse cells exposed to BSS; H/M: extract from mixed human-mouse culture exposed to virus; (H) + (M): 1:1 mixture of human and mouse cell extracts; MH: extract from a clone of human-mouse hybrid cells. Putative hybrid bands are designated by the arrow in the lower part of the figure.

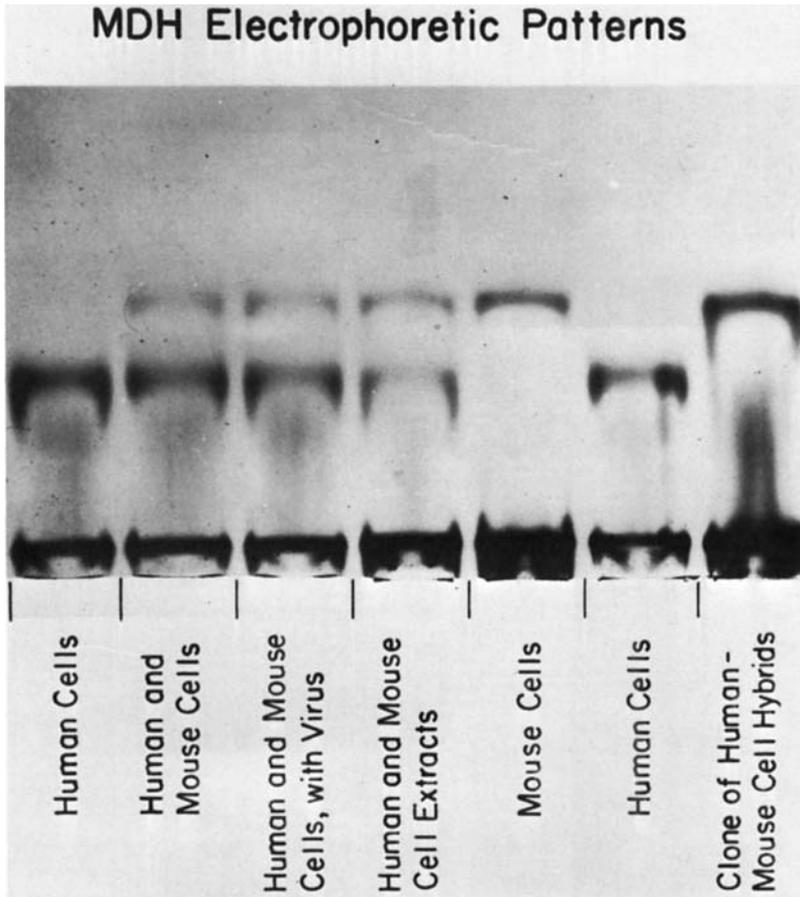


Fig. 4 MDH electrophoretic patterns of extracts from human, mouse and human-mouse cell cultures. Cells were harvested after nine days of exposure to BSS or virus, and extracts were prepared as described under MATERIALS AND METHODS.

the pulse was administered in the presence of cycloheximide, an inhibitor of protein synthesis, which appears to act by blocking the formation of peptide bonds (Wettstein et al., '64; Colombo et al., '65). Therefore, human-mouse heterokaryocytes appear capable of synthesizing protein, at least as far as can be determined from the behavior of the polykaryocytes recovered from mixed cultures. Moreover, this synthesis can be demonstrated for at least seven days after the culture has been exposed to virus. Harris et al. ('66) have also demonstrated that heterokaryocytes formed by human cells and cells derived from other species, including fowl, are capable of incorporating tritiated leucine.

There are two alternative possibilities which might explain the absence of hybrid isozymes in virus-treated mixed cultures, in spite of their apparent capacity for continued protein synthesis. The genes of each nucleus may retain the control of their corresponding cytoplasm, so that polypeptide subunits coded by genes in different nuclei will not form part of the same molecule. Alternatively, hybrid enzymes may indeed be formed, but not in detectable amounts, due to the relatively low frequency of heterokaryocytes. Some support for the first possibility comes from an observation by Siniscalco et al. ('69). These authors found that even 48 hours after virus exposure a mixed culture of normal and deficient cells

for glucose-6-phosphate dehydrogenase contained binucleated cells in which the enzyme could be detected histochemically around only *one* of the two nuclei. Pontecorvo ('52) has suggested that genes combined at different levels of cellular organization may exhibit different phenotypes. For example, in *Didymium iridis* complementation between two non-allelic pigment mutants occurs in the heterozygous state, but not in diploid heterokaryons (Collins, '69).

On the other hand, it is possible that hybrid enzymes are indeed formed in human-mouse heterokaryocytes, but because the heterokaryocytic index of the cultures is not sufficiently high their concentration in the extract is below the limits of detection. A possible means of deciding between these two possibilities might be by fractionating the cell population on the basis of cell size, employing sedimentation velocity techniques (Miller and Philips, '69), and in this way obtaining a fraction rich in heterokaryocytes.

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