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A Series of Hybrid Cells Containing Different Ratios of Parental Chromosomes Formed by Two Steps of Artificial Fusion

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ABSTRACT **Double hybridization of Ehrlich ascites tumor cells (ETC) and L cells was performed. In the first step, a hybrid (LE) of ETC and L_{AG} (a mutant of L cells, resistant to 10 μg/ml of 8-azaguanine) and a hybrid (LL) of L_{AG} and L_{BrdU} (a mutant of L cells, resistant to 100 μg/ml of 5-bromodeoxyuridine) were prepared by the use of artificial fusion by UV-irradiated HVJ (Sendai virus). In the second step the LE hybrid and ETC, or the LL hybrid and ETC, were fused again by UV-HVJ. The hybrids (LEE and LLE) were segregated during culture. Thus, the series of hybrids L, LLE, LE, LEE, and ETC was obtained. The morphological feature and karyological characters of these hybrids and the distribution of antigens corresponding to each parent on their cell surfaces supported the above identification of the series of hybrids obtained by double hybridization.**

All three hybrids acquired new characters, such as the ability to form large colonies in soft agar and large tumors on the chorioallantoic membrane of chicken eggs, unlike either parent. The tumor-forming capacity of the series was ETC > LEE > LE > LLE; L cells formed no tumor in mouse abdomen under the test conditions.

Hybrid cells can be formed artificially (1–4) by fusion of somatic cells by UV-irradiated HVJ (Sendai virus). Thus when treated with HVJ, cells fuse together and form polykaryocytes containing various numbers of nuclei: di-, tri-, tetra-,... karyons. The hybrid cells formed by this technique seem to be derived mainly from the di-heterokaryons and usually contain nearly all the chromosomes of both parents. Yamanaka and Okada (5, 6) reported that among the polykaryons formed by fusion, the dikaryons divided into daughter cells with the highest frequency and the frequency decreased with increase in the number of nuclei in the polykaryocytes. The results obtained by Coon and Weiss (7) on the frequency of hybrid cell formation and fusion efficiency supported this possibility. Murayama and Okada (8) found that among the various heterokaryons, di-heterokaryons formed hybrid daughter cells most rapidly. On the basis of these findings, experiments were made on hybridization of two parent cells and then double hybridization with one of the parents by secondary fusion using HVJ. In this way, we obtained a series of hybrids with different ratios of the two parental chromosomes.

MATERIALS AND METHODS

Ehrlich ascites tumor cells (ETC, hyper-diploid line) and two mutant lines of L cells (9), L_{AG} (resistant to 10 μg/ml of 8-azaguanine) and L_{BrdU} (resistant to 100 μg/ml of 5-bromodeoxyuridine) were used as parents. The HVJ-Z strain of Sendai virus, propagated in embryonated eggs, was dialyzed and exposed to UV-radiation as described previously (5). A standard method for the fusion of cells used in our laboratory (10) was employed. For quantitative determination of parental surface antigens on the hybrids, the following cytotoxicity test was used. Xenogenic rabbit antisera against L cells and ETC were absorbed with ETC and L cells, respectively. The number of cells required for 50% absorption of the antisera was estimated by the cytotoxicity, using L cells or ETC as the targets (8). Syngeneic antigens expressed on the hybrids were determined by an immune adherence test (11).

Abbreviations: TFD_{50}, 50% tumor-forming dose; HAT medium, selective medium containing hypoxanthine, aminopterin, and thymidine.

RESULTS

First, hybrids of L_{AG} and ETC (the LE hybrid) or L_{AG} and L_{BrdU} (the LL hybrid) were prepared from cultures in HAT medium after fusion by UV-irradiated HVJ (9). Next, double hybridization of ETC and L cells was performed. LE and ETC or LL and ETC were chosen as parent combinations. With LE and ETC, we used a selective medium (HAT medium) in which LE cells and the double hybrid cells would grow rapidly while ETC would not grow. A mutant resistant to 45 μg/ml of 8-azaguanine (LE_{AG}) was segregated from the LE hybrid clone prior to fusion of the hybrid cells with ETC. After double hybridization of LL and ETC, the two lines and double hybrid colonies could be identified directly under the microscope during culture of the sample of LL and ETC after fusion by UV-HVJ. The two double hybrid lines were named LEE and LLE, respectively.

The morphological features of the parent and hybrid lines in culture are shown in Fig. 1. L cells, one of the original parents, grew in culture in an extended form attached to the plate, while the other parent, ETC, floated on the medium. The three hybrids showed morphological characters intermediate between those of the two parents: LLE resembled L more than LE, and LEE resembled ETC more than LE.

Karyological characters of the hybrids

In the parents, the chromosomes numbered 57 in L_{AG}, 50 in L_{BrdU} and 45 in ETC. In the hybrids the numbers were 118 in LLE, 88 in LE, and 103 and 123 in LEE (a mixed population). The hybrids contained fewer than the sum of the numbers of chromosomes of the parents, namely 78% of the expected number in LLE, 86% in LE, and 71 and 84% in LEE.

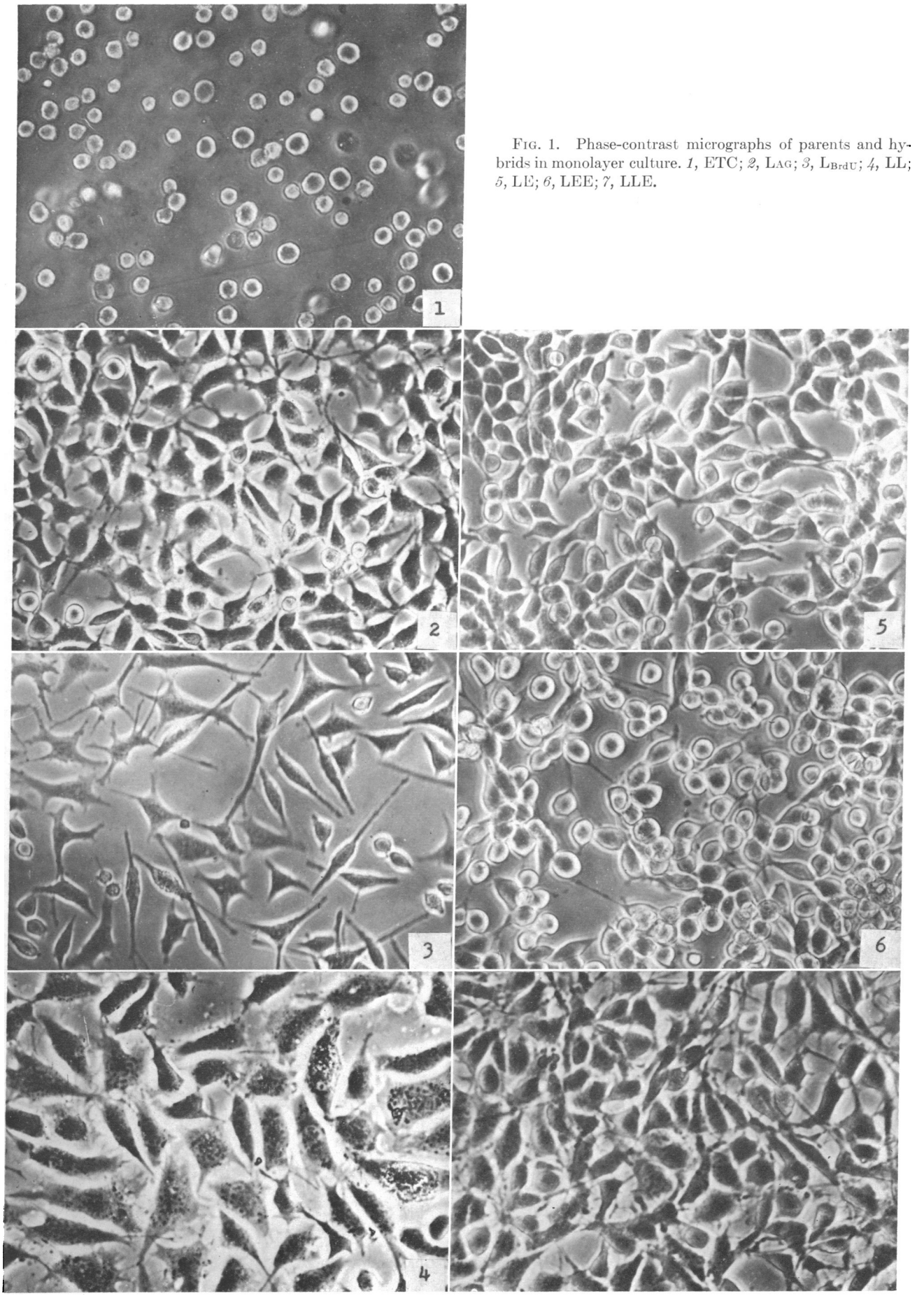

FIG. 1. Phase-contrast micrographs of parents and hybrids in monolayer culture. *1*, ETC; *2*, L_{AG}; *3*, L_{BrdU}; *4*, LL; *5*, LE; *6*, LEE; *7*, LLE.

TABLE 1. *Number of chromosomes of parents and hybrids*

Cells	Chromosome no.	History
L_{AG}	57	A mutant of L cells, resistant to 10 μg/ml of azaguanine
L_{BrdU}	50	A mutant of L cells, resistant to 100 μg/ml of 5-bromodeoxyuridine
ETC	45	Ehrlich ascites tumor cells of hyper-diploid line
LE_o	88	A hybrid clone of L_{AG} and ETC, cultured only *in vitro*
LE_d	83	A progeny of LE_o, passaged 5 times alternately *in vitro* and in dd0 mice
LE_c	81	A progeny of LE, passaged once in a C3H mouse
LE_{AG}	83	A clone of LE_d, resistant to 45 μg/ml of azaguanine
LLE_o	118	A hybrid clone of LL and ETC, cultured only *in vitro*
LLE_d	108	A progeny of LLE_o, passaged once in a dd0 mouse
LLE_c	109	A progeny of LLE_o, passaged once in a C3H mouse
LEE_o	103 and 123	A hybrid line of LE_{AG} and ETC, cultured only *in vitro*
LEE_{d1}	103 and 123	A progeny of LEE_o, passaged once in a dd0 mouse
LEE_{d2}	103 and 123	A progeny of LEE_o, passaged twice in dd0 mice
LEE_c	103 and 115	A progeny of LEE_{d1}, passaged once in a C3H mouse
C	40	Embryo skin fibroblast from a normal C3H mouse, secondary culture
CE_o	81	A hybrid line of C and ETC, cultured only *in vitro*
CE_d	83	A progeny of CE_o, passaged once in a dd0 mouse

However, the pattern of the numbers in the hybrid series indicated that double hybridization was possible, because the chromosome numbers of the hybrids were higher than those of the parents and the numbers of the double hybrid lines were higher than those of the single hybrid line (Table 1).

The karyological characters of the hybrids were compared with those of the parents on the basis of the percentage of long metacentric chromosomes to the total; 14.1 long metacentric chromosomes in L_{AG} (24.8% of the total), 13.4 in L_{BrdU} (26.7%) and 2 in ETC (4.45%). The LL hybrid contained all the chromosomes of the parents, L_{AG} and L_{BrdU}, and the long metacentric chromosomes constituted 25.5% of the total, that is exactly the expected value. Thus, this seemed a suitable method of analysis. The observed values in the hybrids were 20.8% in LLE, 16.6% in LE, and 11.0% in LEE. From these values, it seems that LLE contained the highest proportion of chromosomes from L cells and LEE the lowest. The observed values were compared with values calculated (*A* in Table 2) on the assumption that the hybrids were the expected types LLE, LE, and LEE and contained all the parents' chromosomes without loss or (*B* and *C* in Table 2), on the assumption that hybrids contained the complete set of chromosomes from one parent (those of ETC for column *B* and those of L for column *C*), the additional chromosomes being derived from the other parent, and the ratio of long metacentric chromosomes derived from the latter parent being the same as in the parent. Of these calculated values, the first (*A*) were rather similar to those observed while the other values (*B* and *C*) were very different. This similarity to the observed values suggests that the series of hybrids was formed as expected and that loss of chromosomes from the hybrids occurred without apparently affecting the percentage of long metacentric chromosomes in the total. From this it seems that LLE may contain 152% or more L chromosomes and 76% or fewer ETC chromosomes, LE may contain 81% or more L chromosomes and 81% or fewer ETC chromosomes, and LEE may contain 142% or more ETC chromosomes and 71% or fewer L-chromosomes.

Distribution of surface antigens on the hybrids

Second, surface antigens were used as a marker to see whether hybrid cells were formed as expected. For quantitative analysis of the expression of the antigen complex of L cells and ETC on the hybrids, rabbits were immunized with L or ETC. It was thought that xenogenic antiserum probably reacted with more surface antigens than allogeneic or syngeneic antiserum. Antiserum against L cells was absorbed completely with ETC and *vice versa*. The number of hybrid cells required for 50% absorption of the antiserum was estimated by the cytotoxic test using L or ETC as targets. From the result, the ratio of L-antigen complex to ETC-antigen complex expressed on each type of cells was calculated. The values were 1.0/0.0 on L cells, 0.62/0.38 on LLE cells, 0.56/0.44 on LE cells, 0.22/0.78 on LEE cells, and 0.0/1.0 on ETC. The distribution of both parental antigen complexes on the hybrids is highly consistent with the expected form of them.

Characteristics of the hybrids as tumors

L_{AG} did not form any tumor, whereas ETC grew well as an ascites tumor, when injected into the abdomen of 4- to 5-week old C3H or dd0 mice. All the hybrids showed the character of tumors and formed solid tumors adhering to the peritoneum, not floating in the ascites fluid. The character of the hybrids as tumors may be derived from that of ETC. The change of the tumors from the ascites form to the solid form implies that the fibroblastic character of L is also present in the hybrids.

The hybrids all formed tumors that were lethal in mice, death occurring most rapidly with LEE and slowest with LLE.

TABLE 2. *Karyological character of the hybrids*

Cells	Observed value: No. of chromosomes	Observed value: Long metacentric chromosomes (%)	Calculated number of long metacentric chromosomes (%): *A*	*B*	*C*
L_{AG}	57	24.8			
L_{BrdU}	50	26.7			
ETC	45	4.45			
LL	106	25.8			
LE_o	88	16.6	15.8	14.4	17.7
$LE_{d.AG}$	83	16.4	15.8	13.7	18.5
LEE_o	123	11.0	12.3	8.4	13.8
	103	11.2	12.3	5.1	15.6
LLE_d	108	20.8	19.4	16.9	25.5

* See text for explanation of assumptions leading to values under *A*, *B*, and *C*.

After injection of the hybrids, observation of the mice for 1 month sufficed to determine whether the cells had taken. The tumor-forming capacities of the hybrids were stable and were retained even after several passages *in vivo*, as shown in the preceding paper (8).

To examine the quantitative differences between the tumor-forming capacities, the 50% tumor-forming doses (TFD_{50}) of the hybrids were determined in 4- to 5-week old C3H or dd0 mice. The values were highest for LLE and decreased in the order LE, LEE, and ETC, as shown in Table 3. This result also seems to support the proposed arrangement of the series of hybrids.

Acquisition of new characters by hybridization

L cells grew well in standard medium (minimum Eagle's plus 10% calf serum) but did not grow in soft agar. ETC does not grow either in the standard medium or in soft agar. All the hybrids grew well in the standard medium and acquired the character of forming large colonies in soft agar (Table 3) (8).

All the hybrids formed large tumors on the chorioallantoic membrane of chicken eggs. Monolayers of the hybrids were trypsinized, and a small drop of the packed cells was put on the chorioallantoic membrane of 10-day old embryonated eggs and incubated at 37°C. In every hybrid, a large tumor (about 10 mm in diameter) was observed after 8 days of incubation. Microscopic examination of sections of these tumors showed that the hybrid cells had penetrated into the mesodermal layer of the membrane, where they grew well. L_{AG} cells also penetrated the mesodermal layer, but the growth was slow and the tumor was about 1 mm in diameter 8 days after inoculation. Unexpectedly, ETC formed only a small tumor (about 1 mm in diameter) on the membrane, but the cells in the mesodermal layer showed frequent mitotic figures.

From these findings, it seems (*a*) that the ability of the hybrid cells to survive in soft agar or attach to chorioallantoic membrane and penetrate into the mesodermal layer was derived from the L cells rather than the ETC, (*b*) that the characters of the hybrids to grow well in soft agar or in chorioallantoic membrane may be derived from the ETC.

Expression of H-2 antigen or tumor antigen on hybrid cells

L_{AG} cells originating from a C3H mouse bear the $H\text{-}2^k$ antigen complex. ETC shows a remarkable loss of the H-2 antigen complex, but possesses the surface antigens, cross-reactive with syngeneic mouse (C3H/He) antiserum against mammary tumor surface antigen (MM), prepared by Nishioka *et al.* (13). The expression of the $H\text{-}2^k$ antigen complex on the hybrids was largely suppressed; this is similar to the results of Harris *et al.* (12). The ratio of dose on cells to dose on L_{AG} was 0.14 in LLE, 0.20 in LE, 0.05 in LEE, and 0.03 in ETC, determined by the number of cells required for 50% absorption of the reactivity of a standard A.CA anti A (containing anti $H\text{-}2^k$). The expression dose of MM antigen on each line of cells was determined in the same way, using C3H/HE anti-MM serum. The ratio of dose on cells to dose on ETC was 0.0 in L_{AG}, 0.23 in LLE, 0.63 in LE, and 0.26 in LEE. The decreased expression of both antigens on the hybrids prepared by double hybridization compared with those on LE cells seems compatible with the fact that surface antigen is expressed less strongly on polyploid cells than on diploid cells (16).

TABLE 3. *Biological characters of the hybrids*

Cells	50% tumor-forming dose (TFD_{50})		Colony formation in soft agar	Tumor size on CAM	Generation time (hr)
	In C3H mouse	In dd0 mouse			
L_{AG}	—	—	—	Small	18
LLE	$\geq 6.6 \times 10^7$ cells	$>6.0 \times 10^7$	Large	Large	10
LE	8.5×10^6	1.7×10^6	Large	Large	12
LE_{AG}	NT	Same as LE			
LEE	4.3×10^5	2.9×10^5	Large	Large	16
ETC	5.0×10^2	1.0×10^2	—	Small	11
CE*	1.4×10^4	6.8×10^3			
dE*	5.0×10^5	4.0×10^4			

CAM, chorioallantoic membrane.

* Hybrids of ETC and embryo skin fibroblasts (secondary culture) from a C3H mouse or dd0 mouse.

DISCUSSION

Malignancy of the hybrids

Recently, Harris *et al.* reported (12) that the "malignancy" of ETC (hypo-tetraploid line) was suppressed by hybridization with A9 cells (a mutant from L, segregated by Littlefield), and the suppression was reduced by loss of L chromosomes. The results were discussed by Ephrussi *et al.* (14) and again by Harris and Klein (15). Independently of Harris' group, we (Murayama and Okada) observed (8) that the tumor-forming capacity of ETC (hyper-diploid line) decreased sharply on hybridization with L_{AG}. We analyzed this phenomenon further by double hybridization as described in this report. Our results will be discussed in relation to the above discussion.

All our hybrids showed the characters of forming large colonies in soft agar and large tumors on chorioallantoic membrane, and the three types exhibited these characters to about the same degree. These findings suggest that the hybrids express abnormal characters under these conditions—that is, in the absence of the immune mechanism that would operate *in vivo*. All of them were malignant *in vivo* and even tumors of LLE, which contained only 76% or fewer ETC chromosomes and 152% or more L chromosomes, were lethal. However, their TFD_{50} values differed. The individual values seemed to be determined by the ratio of L components to the total in the hybrids; the effect of L components of the hybrids was quantitative but not qualitative. Thus, the effect of L cells on the tumor-forming capacity of the hybrids in our system cannot be explained simply as "suppression of the malignancy of ETC by "L," but probably involves several other factors *in vivo*. "Malignancy," estimated as tumor formation *in vivo*, must be understood as the sum of several factors, not as a simple character of the cells.

During our experiments we also prepared other hybrids, such as CE and dE, which were the hybrids of ETC with embryo skin fibroblasts (secondary culture) of C3H and dd0 mice, respectively. Their TFD_{50} values were higher than that of ETC, but clearly lower than those of L–E hybrids (Table 3). For example, the TFD_{50} of CE, which containing about 100% of the parents' chromosomes, was clearly lower than that of LEE, which contained 71% or fewer L chromosomes and 142% or more ETC chromosomes. These results, together with those of refs. 14 and 15, suggest that the character which decreases the tumor-forming capacity of ETC is peculiar to

L cells. We consider that this character of L cells may not be due to a repressor capable of suppressing the expression of the oncogene(s) of ETC.

On *in vivo* passage of the hybrids, loss of chromosomes was observed (5/88 in LE, 10/118 in LLE). However, the ratio of long metacentric chromosomes to the total was fairly constant, there was scarcely any variation in the TFD_{50}, and in no case did this variation result in overlap of the TFD_{50} values with those of the other hybrids. LEE formed a population with two peaks in chromosome number, but this population did not change on *in vivo* passage. Thus both types of cells grew well both *in vivo* and *in vitro*.

In the present work, we used C3H and dd0 mice as recipients for determining the TFD_{50}. The dd0 line is an inbred line, commonly used in Japanese laboratories, in which ETC has been passaged serially for over 10 years. The susceptibility of this line to L–E hybrids and CE hybrid was rather higher than that of C3H mice, as in the case of dE hybrid. Lymphocytes from dd0 adult mice reacted strongly with A.CA anti A($H\text{-}2^f$ anti $H\text{-}2^a$), A.CA × C3H anti C57 BL($H\text{-}2^f \times H\text{-}2^k$ anti $H\text{-}2^b$), A.BY × DBA anti C3H($H\text{-}2^b \times H\text{-}2^d$ anti $H\text{-}2^k$) and A × C57 BL anti A.SW($H\text{-}2^a \times H\text{-}2^b$ anti $H\text{-}2^s$) sera (Tachibana, T., and X. Suzuki, unpublished data). Thus dd0 mice are a heterozygous strain with respect to the H-2 histocompatibility antigen. However, the difference in the susceptibility to L-E hybrids or CE hybrid of the two recipients does not seem to be due to the H-2 system.

The double hybridization

By double hybridization, three kinds of hybrid were formed. Their morphological and karyological characters, the distribution of parental antigens on them, and the TFD_{50} values supported this interpretation. Recently, further support has been obtained. The beating rhythm of two muscle cells from mouse embryo heart, attached to a plate at a distance, is synchronized by bridge formation with other kinds of cells, but not with L cells. The capacity for such mediation was zero in LLE, but 17% of LE cells and 47% of LEE cells that formed the bridge between two muscle cells synchronized their beating (17).

The double hybridization technique thus seems to be useful in decreasing chromosomal balance in a hybrid.

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