The Enhanced Transfer of Drug-Resistant Genes in NIH-3T3 Cells Transformed by the EJras Oncogene

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The spontaneous transfer of drug resistance genes has been shown to take place between cultured mammalian NIH-3T3 cells and occurs with a hierarchy of transfer efficiencies, transformed cells being more efficient than non-transformed cells. This experiment was accomplished by co-cultivating two NIH-3T3 sublines, each transfected by standard plasmid methods with a different drug resistance gene, subjecting the mixed population to double selection by adding both drugs to the mixed cell culture, and isolating single cells which were resistant to both drugs. The genes used were the *neo* gene and *gpt* gene which conferred resistance to the drugs G418 and mycophenolic acid, respectively. DNA analysis confirmed the presence of both resistance genes in the cells which were resistant to both drugs. The mechanism of this gene transfer was by cell fusion rather than by chromosomal DNA uptake. The efficiency of gene transfer, as indicated by the number of double-resistant colonies standardized by number of cells cultured, was much higher between two sublines of cells transformed by the EJras oncogene than between one transformed and one non-transformed subline, which in turn was higher than between two non-transformed sublines. The higher efficiency of gene transfer between the transformed cells also occurred when these cells were injected into nude mice, thus demonstrating that the same process occurred *in vivo*. It would appear that drug resistance genes may be transferred spontaneously in cultured mammalian cells by cell fusion, and that transformed cells have a higher efficiency of gene transfer compared to non-transformed cells.

INTRODUCTION

The rapid emergence of multiple drug resistance is often observed clinically during the drug treatment of patients with malignancies. The mechanisms of drug resistance development are poorly understood, though various physiologic, pharmacologic, and cellular factors are presumably quite important [1]. On the cellular level, the emergence of drug resistance may be a function of the mutation rate and genetic instability of tumor cells [2]. We consider that cell fusion and the ensuing gene transfer and subsequent chromosomal segregation may be very important and contribute to the development of genetic instability in tumors. The increasing genetic instability of a malignant tumor has been associated with increasing tumor heterogeneity and metastatic potential [3] and the development of drug resistance [2,4].

The observation that clinical drug resistance in human tumors is common and generally occurs at all tumor sites simultaneously would suggest that either malignant cells have a great tendency to develop resistance or that the genetic alterations responsible for resistance can be transferred among malignant cells. A second hypothesis is that since drug resistance does not routinely appear in normal, non-malignant tissue, for example, bone marrow, then if gene transfer were to occur, it

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TABLE 1
Drug-Resistant NIH-3T3 Cell Lines

Cell Lines	Drug Resistance Gene	Transformation by EJras Oncogene
pEJ103	*gpt* (mycophenolic acid)	Yes
pCV103	*gpt* (mycophenolic acid)	No
pEJ2.9A	*neo* (G418)	Yes
pAVCV007	*neo* (G418)	No

would preferentially take place among malignant cells. It has been shown that genes from pleiotropic drug resistance cells can be placed on the surface of non-resistance cells and confer on these non-drug-resistance cells the pleiotropic drug resistance phenotype, supporting the concept of gene transfer as an important contributing factor of the emergence of clinical drug resistance [5].

To study the spontaneous transfer of drug resistance genes from one mammalian cell to another, two NIH-3T3 cell lines, each transfected with a different drug resistance gene, were co-cultivated, and the cell mixture subjected to double selection with both drugs. Only cells that either contained both drug resistance genes as a result of gene transfer or shared the needed drug resistance enzymes could survive and grow. Southern blot analysis [6] of genomic DNA from double-resistant cell colonies cloned from single cells, which were derived from the original double drug-resistant colonies, was performed to confirm that drug-resistant gene transfer was indeed responsible for the development of double drug resistance. Comparison of the apparent efficiencies of gene transfer between two sublines of drug-resistant cell lines which had been transformed by the insertion of an EJras oncogene was also completed.

METHODS

Cell Culture and Engineering of Cell Lines

Four cell lines (Table 1) were created by introducing the engineered plasmids depicted in Fig. 1 into mouse fibroblast NIH-3T3 cells by using DNA-calcium phosphate transfection [7] followed by colony selection with either G418 [8] or mycophenolic acid [9]. Subclones were passaged weekly without drug, indicating the stability of the integrated resistant genes. All these cell lines were grown in Dulbecco's Modified Eagle's Medium with 10 percent fetal calf serum, sodium bicarbonate buffer, penicillin-streptomycin, in 5 percent CO_2 at 37°C. For the G418 resistant cell lines (pEJ2.9A and pAVCV007), G418 at 200 μg/ml was added to the medium.

Co-Cultivation of Cell Lines and Selection of Double-Resistant Colonies

As single-cell suspensions, each of the two cell lines containing the *gpt* gene was mixed with each of the two cell lines containing the *neo* gene (refer to Table 2) and the cell mixtures seeded to a confluent monolayer density on to 60 mm plates (total of 2 $\times$ 10^6 cells per plate). In some experiments fewer cells were plated, so that drug treatment could be applied before confluence was achieved. After seven days in non-selective medium, the cell mixtures were trypsinized into single-cell suspensions and 5 $\times$ 10^5 cells were seeded on to 100 mm plates (four plates for each cell mixture). At this cell density, there was very little cell-to-cell contact as determined by microscopic inspection. Double selection with G418 and mycophenolic acid was then
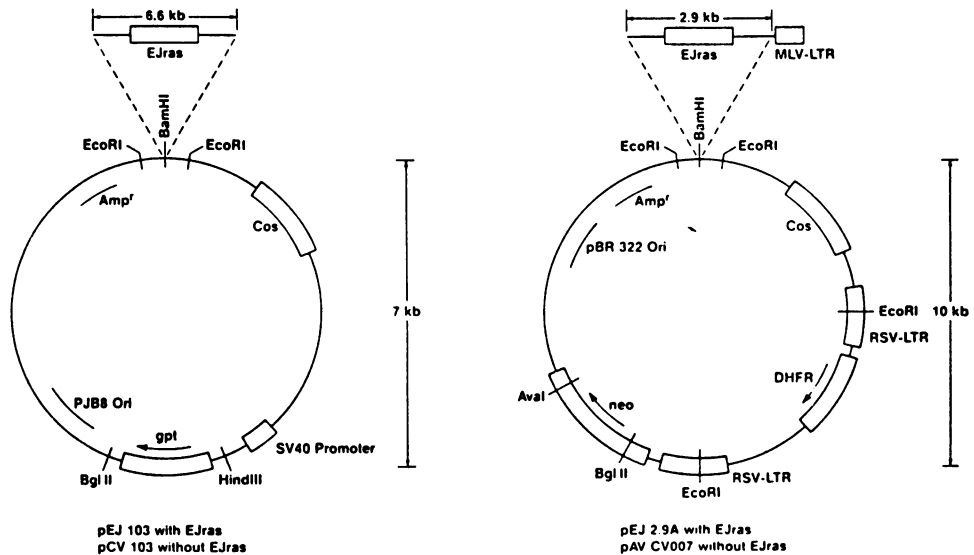
FIG. 1. Structure of plasmids. pAVCV007 is a cosmid vector carrying the *neo* gene driven by a Rous sarcoma virus LTR (kindly provided by K.H. Choo and Y.W. Kan). pEJ2.9A is a derivative plasmid with the 2.9 kb Sac I-Sac I fragment of the EJras oncogene which encompasses the entire coding region inserted into the Bam HI cloning site of pAVCV007 after Bam linkers were attached to the fragment, immediately 3' to the EJras insert in the U3 portion of the murine leukemia virus LTR. We have shown that this plasmid efficiently transforms 3T3 cells upon transfection and expresses the aberrant c-Ha-ras mRNA [Liu E; unpublished observations]. pCV103 is a cosmid vector which harbors the *gpt* gene driven by the SV40 early promoter (a gift of Chris Lau). Its derivative, pEJ103, has the 6.6 kb Bam HI fragment of the EJras gene inserted into the Bam HI cloning site of pCV103. pAVCV007 and pCV103 do not transform 3T3 cells upon transfection, whereas pEJ2.9A and pEJ103 do.

applied to select double-resistant colonies. Care was taken not to dislodge cells during medium change. After three to four weeks, macroscopically evident colonies (0.5–1.0 mm diameter) were counted. A separate set of cell mixtures was subjected to treatment with polyethylene glycol (PEG), an agent known to enhance cell fusion [10]. The day following the plating of the cells, a one-minute exposure to a 50 percent w/w

TABLE 2

Spontaneous Gene Transfer as Shown by the Development of Double-Resistant Cell Colonies

Transformed		Non-Transformed		Double-Resistant Colonies 1×10^6 Cells	
gpt Gene	*neo* Gene	*gpt* Gene	*neo* Gene	Spontaneous	PEG
		pCV103	pAVCV007	0, 1, 0 (0.3 ± 0.6)	23 (±1)
pEJ103			pAVCV007	4, 3, 4 (3.7 ± 0.6)	122 (±4)
	pEJ2.9A	pCV103		3, 4, 5 (4.0 ± 1.0)	117 (±4)
pEJ103	pEJ2.9A			42, 53, 43, 49 (47 ± 5.2)	148 (±7)

Result of three to four experiments, using two different clones of each of the single-resistant parent cell lines for each condition. Parentheses indicate mean ± SD. The efficiency of gene transfer as indicated by the number of double-resistant colonies was much higher with the two transformed lines than with one transformed and one non-transformed line, which in turn was higher than with two non-transformed lines ($p < 0.001$ by student *t*-test). PEG treatment increased efficiency of gene transfer, more so with the non-transformed cells.

TABLE 3
Spontaneous Gene Transfer *In Vitro* and *In Vivo*

Condition	Cell Mixture 1× pEJ103 (Cells Mixed with:)	Double-Resistant Colonies (per 1 × 10^6 pEJ103 Cells)
1. Monolayer Cell Culture	20× pAVCV007	4
	5× PAVCV007	2
	1× pAVCV007	3
	1× pEJ2.9A	42
2. Spheroid Cell Culture	5× pAVCV007	0
	1× pEJ2.9A	38
3. Nude Mice	4× pAVCV007	0
	1× pAVCV007	0
	1× pEJ2.9A	115

pEJ103 cells mixed with pAVCV007 (in various proportions) or pEJ2.9A, with the following experimental conditions: 1, monolayer cell culture; 2, spheroid cell culture; 3, *in vivo* in nude mice

PEG-1000 in calcium-free buffer was used. The subsequent methods of double selection after seven days were the same. Appropriate controls of single drug-resistant cells and non-drug-resistant cells were performed with all experiments.

Spheroid Cell Culture Experiments

In order to test whether enhanced cell-to-cell contact in a three-dimensional mixed-cell spheroid system [11] would increase gene transfer, cell mixtures (Table 3, Condition 2: 5 × 10^6 pEJ103 cells with 2.5 × 10^7 pAVCV007 cells or 5 × 10^6 pEJ2.9A cells) were placed into 200 ml non-selective medium in spinner bottles. After spheroids were seen macroscopically (0.5–1.0 mm diameter) in seven to ten days, they were trypsinized back to single-cell suspensions and 5 × 10^5 cells were seeded on to 100 mm plates (four plates for each cell mixture) and double selection was applied, as above.

Nude Mice Experiments

To show that transfer of drug resistance genes also occurs *in vivo*, a mixture of 1 × 10^6 pEJ103 cells with pAVCV007 or pEJ2.9A cells, as shown in Table 3, Condition 3, were injected subcutaneously into nude mice. After two weeks, the resultant tumors were harvested, trypsinized into single-cell suspensions, plated, and subjected to double selection immediately, as described above. Autopsies on the nude mice revealed no metastasis.

Southern Blot Analysis of Genomic DNA

Genomic DNAs from parent NIH-3T3 cells, pAVCV007 cells, pEJ2.9A cells, pCV103 cells, pEJ103 cells, and seven double-resistant cell lines, as described in the legend of Fig. 2, were digested with EcoR1, electrophoresed on 0.8 percent agarose gels, and blotted onto Gene Screen Plus filters. The 0.5 kb Hind III/EcoR1 fragment from pSV-gpt was nick-translated and used as a probe for the *gpt* gene; the 0.9 kb Pst I fragment from pSV2-neo was nick-translated and used as a probe for the *neo* gene.

Cellular DNA Content Estimate

The relative DNA contents per cell of three double-resistant lines and three single-resistant parent lines (refer to Table 4) were measured by a fluorescence-
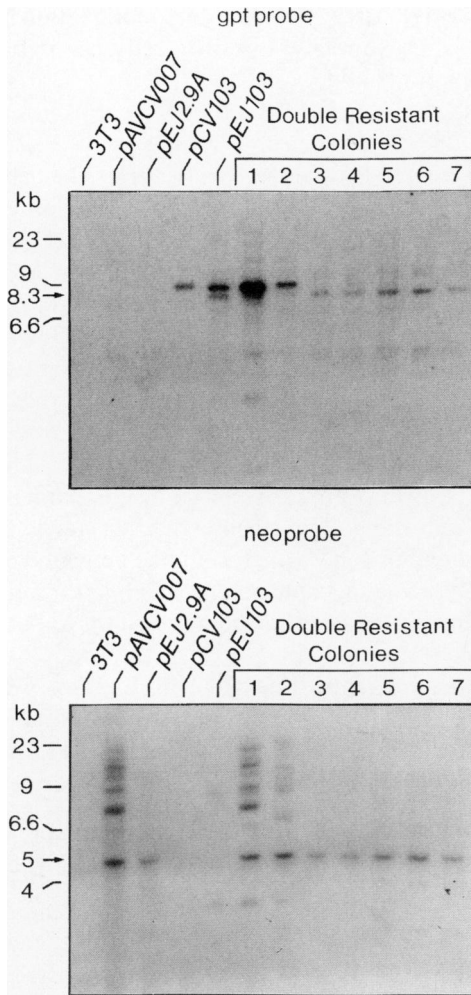
FIG 2. Southern blot analysis of genomic DNA, using the cloned *gpt* (A, *above*) and *neo* (B, *below*) genes as probes.   Genomic DNAs from parent 3T3 cells, pAVCV007 cells, pEJ2.9A cells, pCV103 cells, pEJ103 cells, and seven double-resistant cell lines were analyzed. The double-resistant cell colonies were from: 1, PEG-treated pEJ103 and pAVCV007 cell mixture in monolayer culture; 2, pEJ103 and pAVCV007 cell mixture in monolayer culture (spontaneously developed without PEG treatment); 3, pEJ103 and pEJ2.9A in spheroid culture; 4 and 5, pEJ103 and pEJ2.9A in nude mice; 6 and 7, pEJ103 and pEJ2.9A in monolayer culture (spontaneously developed without PEG treatment).   A: The pCV103, pEJ103, and all the double-resistant cells contained the *gpt* gene, while the 3T3, pAVCV007, and pEJ2.9A cells did not. The pCV103 lane showed one 9 kb band, pEJ103 showed two bands, at 9 kb and 8.3 kb, indicating more than one integration site for the *gpt* gene; double-resistant colony number 1 showed both the 9 kb and the 8.3 kb bands, number 2 the 9 kb band only, numbers 3 through 7 the 8.3 kb band only. The loss of one band in double-resistant colonies numbers 2 through 7, as compared to the parent pEJ103 cells, suggested that cell fusion was followed by chromosome segregation of the fused cell.   B: The pAVCV007, pEJ2.9A, and all the double-resistant cells contained the *neo* gene as shown by the 5 kb band, while the 3T3, pCV103, and pEJ103 cells did not.

TABLE 4
Relative DNA Contents of Single- and Double-Resistant Cell Lines

Cell Lines	Relative DNA Content
Single-Resistant "Parent" Lines	
pEJ103	59
pAVCV007	62
pEJ2.9A	57
	Average, 59
Double-Resistant Lines	
From pEJ103 and pAVCV007 in monolayer culture	91
(cells on lane 2 of Southern blot analysis)	
From pEJ103 and pEJ2.9A in nude mice (lane 4)	103
From pEJ103 and pEJ2.9A in monolayer culture (lane 6)	97
	Average, $97 = 1.65 \times 59$

activated cell sorter (Becton Dickinson FACS II), after 70 percent ethanol fixation of single-cell suspensions of these cell lines (approximately 1×10^6 cells per cubic centimeter) and labeling the DNA with propidium iodide.

RESULTS

Four cell lines, as listed in Table 1, were created by transfection of NIH-3T3 cells with four engineered plasmids (pAVCV007, pEJ2.9A, pCV103, pEJ103) and named according to the plasmid. The structures of the plasmids are depicted in Fig. 1. The plasmids pAVCV007 and pCV103 confer resistance to G418 and mycophenolic acid, respectively, whereas pEJ2.9A and pEJ103 are their counterparts, which also carry the transforming EJras oncogene. All cell lines derived from transfecting NIH-3T3 cells with the above constructs were clonally derived. The pAVCV007 line is resistant to G418 but is not transformed; the pEJ2.9A line is resistant to G418 and is transformed. Similarly, the pCV103 line is resistant to mycophenolic acid but not transformed, while the pEJ103 line is resistant to mycophenolic acid and is transformed. The advantage of using these drug resistance genes which are of bacterial origin, namely, the *neo* gene conferring resistance to G418 and the *gpt* gene conferring resistance to mycophenolic acid, is that there are no mammalian genes which could be activated or mutated to circumvent the toxic actions of G418 and mycophenolic acid. Only through gene transfer between the cell lines could some of these cells become resistant to both drugs and grow under the condition of double selection.

The number of double-resistant colonies from each of the four cell mixtures was determined and is shown in Table 2. There were 42 to 53 double-resistant colonies per 1×10^6 cells of each cell line when the two transformed lines (pEJ103 and pEJ2.9A) were used; only three to five double-resistant colonies resulted from one transformed and one non-transformed line (pCV103 and pEJ2.9A, or pEJ103 and pAVCV007) and zero to one colonies from two non-transformed lines (pCV103 and pAVCV007). These differences are statistically significant ($p < 0.001$ by student t-test).

If one or both drugs (G418, mycophenolic acid) had been applied before the end of the seven-day period of co-cultivation in non-selective medium, the apparent efficiency of gene transfer decreased (data not shown). If the cell mixture was seeded sparsely and cell-to-cell contact was never attained (as determined by microscopic examination) prior to the addition of both drugs (selective media), no double-resistant colonies were obtained. Polyethylene glycol (PEG) also increased the apparent efficiency of gene transfer (Table 2). It is noteworthy that, for the two transformed cell lines, the number of double-resistant colonies following the addition of PEG was only increased threefold, as compared to 30- to 40-fold increase when one transformed and one non-transformed cell line were used.

Although cell-to-cell contact may be enhanced in a three-dimensional mixed-cell spheroid system, the apparent efficiency of gene transfer, represented by the number of double-resistant colonies, was not higher in the mixed-cell spheroid system as compared to monolayer growth, as shown in Table 3.

As negative controls, pEJ2.9A and pAVCV007 cells were treated with mycophenolic acid, and pEJ103 and pCV103 were treated with G418. No cell growth of tumor colonies resulted, although a few scattered pEJ2.9A and pAVCV007 cells remained as isolated single cells after four weeks.

The doubling times as a measure of growth rate of the cells were as follows: Parent NIH-3T3 (in non-selective medium)—24 hours; pCV103 (with mycophenolic acid in

medium)—28 hours; pEJ103 (with mycophenolic acid in medium)—22 hours; pAVCV007 (with G418 in medium)—24 hours; pEJ2.9A (with G418 in medium)—22 hours. The double-resistant cells (in double selection medium with G418 and mycophenolic acid) grow more slowly, with a doubling time between 35 to 44 hours.

In nude mice, 115 double-resistant colonies were obtained from the culture of 2×10^6 tumor cells (i.e., 1×10^6 pEJ2.9A cells) obtained from the tumor which formed at the injection site of the cell mixture of both transformed lines, pEJ103 and pEJ2.9A. No double-resistant colonies occurred when the injected cell mixture contained the transformed pEJ103 and the non-transformed pAVCV007 used instead of the transformed pEJ2.9A. Thus the same process of double drug resistance transfer occurs *in vivo* and *in vitro* with transformed cells showing a higher efficiency compared to non-transformed cells.

Southern blot analysis of genomic DNA isolated from expanded double-resistant cell colonies confirmed that the double-resistant cells contained both the *neo* gene and the *gpt* gene. Thus, gene transfer, rather than an epigenetic mechanism, appeared to be responsible for the double drug resistance (Fig. 2).

The relative DNA content of the double-resistant lines is, on the average, 1.65 times that of the single-resistant parent lines (Table 4) consistent with cell fusion as the mechanism of gene sharing in these experiments.

DISCUSSION

The observation that double-resistant cells might result from some form of cell-to-cell interaction dates back more than 20 years [12]. One could not, however, exclude the possibility that the single-resistant cells might have acquired a second resistance by mutation or gene amplification. Furthermore, an epigenetic phenomenon, rather than gene transfer, could be the basis of double resistance conferring cell survival. In the experiments presented, the possibility of mutation or amplification of an endogenous gene as the basis of double resistance is highly unlikely, since both drug resistance genes are bacterial in origin, and there are no normal mammalian counterparts. Southern blot analysis confirmed that gene transfer, rather than an epigenetic mechanism, was responsible for the double resistance.

Gene transfer between cells may be by cell fusion [13] or uptake of extracellular chromosomal DNA [14]. Conceivably, one cell may release its DNA into the microenvironment during its death, and the DNA be taken up by surrounding cells. Cell fusion was, however, most probably the mechanism of gene transfer in this study. The following points support this contention: *(1)* there was no apparent gene transfer if the cell mixture was seeded sparsely and cell-to-cell contact did not occur; *(2)* when either one of the drugs was applied earlier to the co-cultured cells to induce cell necrosis and DNA release of one cell line, before the second drug was applied, the efficiency of gene transfer decreased rather than increased (data not shown); *(3)* the relative DNA content of the double-resistant lines is 1.65 times that of the single-resistant parent lines. The number of chromosomes of a fused cell is somewhat less than the sum of those in the two parent cells, probably due to chromosome segregation and loss [13,15]. Hybrid cells might be more prone to chromosome rearrangement as compared to their parent cells [16]. This finding is consistent with cell fusion followed by chromosome loss. The efficiency of spontaneous uptake of extracellular chromosomal DNA is quite low [14]. With uptake of extracellular chromosomal DNA, the overall DNA content probably would not have increased appreciably.

Spontaneous cell fusion, *in vitro* as well as *in vivo* between injected tumor cells and host cells, has been well documented [13,15,17]. Cell fusion as part of tumor-tumor cell interaction has, however, not been well studied. Careful quantification of cell fusion efficiency comparing transformed cells and non-transformed cells has not been done.

The apparent efficiency of the transformed cell lines to fuse and form double-resistant cells approached one per 1 to 2×10^4 cells of each cell line. We speculate that this finding may be important clinically. As an example, let us consider the cancer patient being treated with two non-cross-resistant drugs (or two drug combinations). Drug 1 would presumably kill the tumor cells which had not mutated to become resistant to this drug; it would even kill cells that are resistant to drug 2. Similarly, drug 2 would kill cells, including those resistant to drug 1. This reasoning is behind combination chemotherapy and non-cross-resistant alternating drug therapy. In a tumor, however, the cells which are susceptible to both drugs are likely to be killed in greater proportion than the cells resistant to either drug 1 or drug 2 because there would be two cytotoxic agents acting concurrently on these double drug-sensitive cells. The proportion of these "single-resistant" cells to either drug 1 or drug 2 would increase at a time when the entire tumor is shrinking. Eventually a substantial proportion of the remaining tumor would be composed of these single-resistant cells. Since the tumor would be smaller because of the death of the sensitive cells, the single drug-resistant cells would be more likely to come into cell-to-cell contact with each other. If cell fusion were to occur between cells resistant to drug 1 and cells resistant to drug 2, then the newly formed fused cells would now be resistant to both drugs and continue to grow despite treatment with these drugs. This process could be a mechanism of treatment failure in patients who show a good response initially even to the point of a clinical "complete remission."

Furthermore, cell fusion with gene transfer and subsequent chromosomal segregation may contribute to the development of genetic instability in tumors. Goldie and Coldman have postulated that the emergence of drug resistance may be a function of the genetic instability of tumor cells [2]. The rate of apparent cellular mutation is believed to be about one in 10^6 to 10^7 cells. The results of the experiments presented in this study show that new genetic material may be acquired through cell fusion with an apparent efficiency as high as one per 2×10^4 transformed cells. It is conceivable that this gene transfer results in greater genetic instability, thus encouraging the emergence of drug resistance. Certainly these results are not incompatible with the Goldie-Coldman hypothesis and in fact validate its potential importance.

The observation that transformed cells have a much higher fusion efficiency than their non-transformed counterparts may be important clinically. This difference may be due to a difference in the cell membrane properties between malignant and non-malignant cells [18]. Tumor cell membrane properties have been considered to be a critical factor in tumor progression and metastasis and in tumor cell heterogeneity [17,19,20]. Kerbel et al. addressed the fact that there are problems in assessing the importance of spontaneous *in vivo* tumor-host cell fusion to tumor progression, tumor heterogeneity, and metastasis, using lectin-resistant membrane mutants [17,20]. Mutagenesis may induce a variety of genetic changes. In the experiments presented here, the difference between the cell membranes of the transformed and non-transformed lines is probably attributable to the action of the EJras oncogene alone and can thus be more easily studied. Future experiments will attempt to elucidate this difference biochemically and physiologically.

It may be argued that since the transformed cells are less anchoraged-dependent, increase in cell-to-cell contact alone might account for the apparent increase in gene transfer. This possibility is unlikely, however, because: *(1)* in the spheroid system, though cell-to-cell contact was higher, the efficiency of gene transfer was no higher than that of monolayer; *(2)* increase in the proportion of the non-transformed pAVCV007 to the transformed pEJ103 cells, thus increasing the contact of each pEJ103 cell with the surrounding pAVCV007 cells, did not increase the number of double-resistant colonies per 1×10^6 pEJ103 cells (Table 3). These observations imply a qualitative difference between the cell membrane properties of a transformed cell compared to those of a non-transformed cell as a potential cause of the increased fusion potential.

Pleiotropic drug resistance to a wide range of amphiphilic drugs in association with the development of a cell surface glycoprotein has been documented [1,21]. This study described another mechanism that may contribute to drug resistance development, and that is through the spontaneous gene transfer which may enhance genetic instability, thus encouraging the development of drug resistance, as well as perhaps directly affecting the transfer of drug resistance genes among tumor cells. Furthermore, the much greater efficiency of gene transfer between transformed cells as opposed to non-transformed cells may help to explain why multiple drug resistance is clinically observed in tumor cells but not in the normal host cells such as bone marrow cells.

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