TRANSFECTION OF DNA FROM CHORIOCARCINOMA CELL LINES AND SPERM CELLS: DNA METHYLATION PREVENTS THE EXPRESSION OF GENES FOR THE MAJOR HISTOCOMPATIBILITY COMPLEX (HLA) CLASS I AND THE T CELL DIFFERENTIATION ANTIGEN, LEU-2@

SAVERIO ALBERTI* and LEONARD A. HERZENBERG
Department of Genetics, Stanford University, Stanford CA 94305 (U.S.A.)
*On leave from the Istituto Mario Negri, Milano, Italy

ABSTRACT

Trophoblast cells are apparently among the few nucleated cells in the body which do not express class I MHC antigens (1). Since the trophoblast is a layer of the placenta which is fetal-derived and closest to maternal tissue, this lack of expression may be essential for the survival of the fetus, which otherwise might be rejected as a foreign graft. Because even occasional expression of HLA or other (tissue-specific) membrane antigens on trophoblast might cause maternal rejection of the fetus, we suggested that the genes for these antigens in the trophoblast might not simply be unexpressed because of potentially leaky regulatory mechanisms as is the case for protein mediated regulation (2). Rather, they might not be expressed because they are methylated in control regions. Methylation seems to be a method that cells use to "turn off" genes irreversibly (3).

We had earlier shown (4) that some choriocarcinoma cell (CC) lines expressed no HLA Class I, like cytotrophoblast from term placenta, while other CC lines expressed low, intermediate or high (normal) levels of HLA Class I. To test the methylation hypothesis, we investigated the transfectability of HLA Class I and a T cell membrane expressed differentiation antigen, Leu-2, with purified DNA isolated from trophoblast-derived CC lines.

The transfection system we used was a cotransfection with a plasmid containing the thymidine kinase (TK) gene into mouse L cells lacking TK genes (LTK⁻) using the calcium phosphate precipitation method (5). We found, as predicted by our hypothesis, that DNA from CC lines which express HLA transfected for HLA and Leu-2 with an efficiency more or less proportional to HLA expression on these lines. But DNA from JAR, a CC line which expresses no HLA Class I, does not transfect for HLA or Leu-2. DNA from sperm, which also do not express HLA, as well as DNA from leukocytes, which do express HLA,

transfected efficiently for HLA and Leu-2. A trophoblast-|specific antigen, Trop-1, which is expressed on all our CC lines but which is not expressed on sperm or leukocytes is efficiently transfected with DNA from all these cell sources.

These results suggest that the DNA segments containing the genes for HLA or Leu-2 in the JAR cell line are covalently modified so that purified DNA cannot transfect HLA or Leu-2. Methylation is the only covalent modification of which we are aware that can affect gene expression (3). We would suggest from our results that these genes are not methylated in any sites critical for expression in sperm or leukocytes, even though they are not necessarily expressed in these cells. Therefore, DNA from these sources can transfect for these molecules. The Trop-1 results show that the lack of expression of HLA and Leu-2 is not due to general poor expression of transfected genes and are consistent with the observation that DNA from various cell types which do or do not express a particular gene can nevertheless transfect the gene with expression of the gene product (6).

To test the idea that methylation is responsible for the nontransfectability of HLA and Leu-2 with DNA from JAR, we treated JAR cells with azacytidine, an inhibitor of the DNA hemimethylase (3) and then tested transfecting activity of DNA from these cells after culture for various lengths of time. We found that DNA taken from cells cultured for short and intermediate periods transfected for both HLA and Leu-2, whereas cells cultured for 4-6 months reverted to the situation with untreated cells: DNA from them is unable to transfect for HLA and Leu-2. DNA from treated and untreated cells transfected Trop-1 antigen, albeit with somewhat different efficiencies.

MATERIALS AND METHODS

Cell lines: The choriocarcinomas cell lines Jar, Ima, Bewo and Enami (4) were cultured in Waymouth's medium. Thymidine-kinase (LTK⁻) cells (5) were cultured in DMEM. Spermatozoa and peripheral blood leukocytes (PBL) were obtained from the same donor to avoid restriction fragment length polymorphism due to DNA sequence differences.

Cell DNA extraction: High molecular weight DNA was extracted with either conventional methods of sodium dodecyl sulfate/proteinase K (Boehringer) lysis followed by banding on a cesium chloride gradient (5) or by a method developed in our laboratory of cell lysis by guanidinium thiocyanate (Kodak) followed by cesium chloride banding (7). Since we obtained higher transfection efficiencies with L cells using the guanidinium method than with the conventional method (2000-3000 colonies/dish versus an average of 1000

colonies/dish respectively) most transfections were performed using DNA prepared with guanidinium.

DNA transfection: Cotransfection by the calcium phosphate (CaPO) precipitation technique was followed (5). Briefly, the day before a transfection 10^6 LTK cells/dish were seeded. A $CaPO_4$ precipitate of 20 ug of cell DNA per 1 ug of TK plasmid was added to each dish and incubated at 37^0 C for 48 h before HAT selection. HAT resistant colonies were analyzed after two weeks of selection.

Immunofluorescence: Fluorescence analyses and sorts were made on a greatly modified fluorescence-activated cell sorter (FACS II, Becton Dickinson, Sunnyvale, CA) and used essentially as described (8). To improve the detection of transfectants stained with antibodies, a new method of subtraction of cell autofluorescence was used as described (9).

Antibodies: Fluorescein-isothiocyanate conjugated (FITC) anti-Leu-2a (CD8) antibody was provided by Becton Dickinson (BD, Mountain View, CA). The anti Trop-1 (4) and W6/32 (anti HLA, Class I) (6) antibodies were FITC-conjugated using conventional methods. Cell staining was performed as described (8).

DNA blot analysis was performed following the method of Southern (10).

Azacytidine (aza-C) treatment: Jar cells were cultured with 10 uM aza-C in Waymouth's medium for four days, that is two doubling times and then cultured in the same medium without Aza-C. DNA was extracted at various times after the treatment as shown in Table 2 and used in transfection experiments.

RESULTS

Table 1 shows the frequencies of transfection for the surface antigens studied (HLA class I, Leu-2 and Trop-1) with DNA from several choriocarcinoma cell (CC) lines as well as from sperm and leukocytes. As can be seen, all DNA sources transfected Trop-1, although this antigen is expressed only on the CC lines. Similarly, DNA from sperm, which expresses neither HLA nor Leu-2 (data not shown), transfects as efficiently for both these antigens as does leukocyte DNA, although leukocytes express both HLA and Leu-2. DNA from other tissue sources, all of which express HLA, transfect efficiently for these three surface antigens as well as a variety of other membrane differentiation antigens, whether or not they express the latter molecules (6, and data not shown).

TRANSFECTION EFFICIENCY OF VARIOUS SOURCES OF DNA

		Sources of DNA					
		Choriocarcinoma cell lines				Sperm	Leukocytes
		Jar	Bewo	Ima	Enami		
HLA expression		0	low	low	intermediate	0	high
Transfected antigens	HLA	0/16 a	0/14	5/8	3/4	9/12	6/7
	Leu-2	0/16	3/14	0/8	3/6	14/14	15/15
	Trop-1	25/29	8/8	8/8	6/6	7/9	15/15

a: number positive/ number transfected dishes; cumulative results of 9 experiments.

The interesting finding is that DNA from the CC lines transfect according to their expression of HLA. Thus, DNA from JAR, which expresses no HLA, does not transfect for HLA or Leu-2; DNA from BEWO and IMA, which express quite low levels of HLA, transfect inefficiently or not at all for either HLA or Leu-2; and DNA from ENAMI, which has normal levels of HLA expression (data not shown), transfects efficiently for both these antigens.

Southern blots performed after digesting the DNA with various restriction endonucleases reveal that the genes for Leu-2 and HLA are present in all the CC lines (data not shown). However, using the enzymes Msp I and Hpa II, which are isoschizomers that are differentially able to cut dependent upon methylation of their recognition sequence, different patterns of methylation of Leu-2 and HLA were found for each of the sources of DNA (Figure 1). Although we found differences, we could not discern an obvious correlation between gene expression or gene transfection efficiency and particular DNA fragments.

In order to find whether methylation affects the efficiency of transfection, we used azacytidine (Aza-C) treatment of JAR. Culture in Aza-C randomly decreases methylation of DNA (3). The hypomethylated DNA thus produced slowly becomes remethylated in cells growing in the absence of Aza-C and can even become more methylated than the original untreated cells (data not shown).
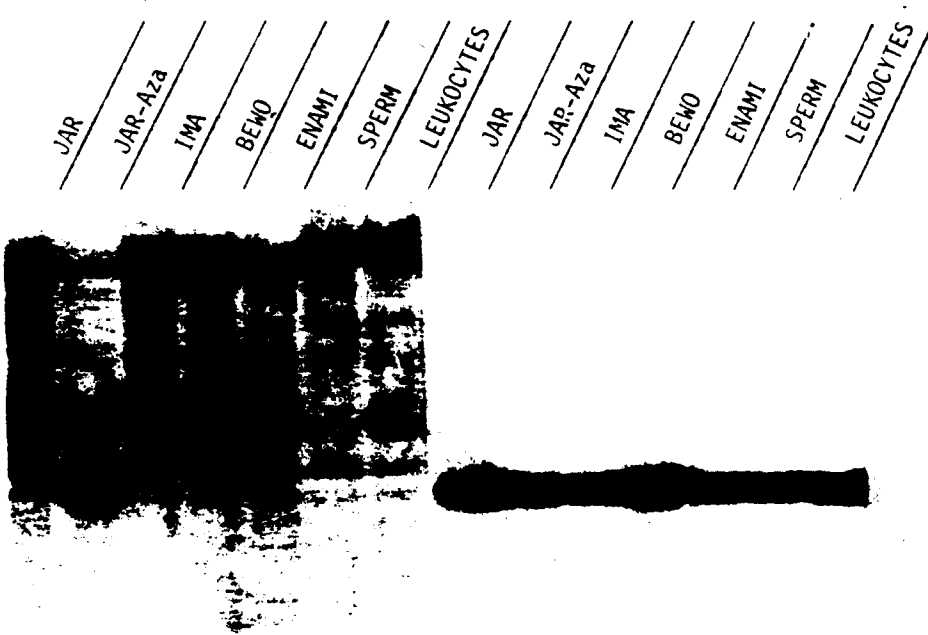
Figure 1: Southern blot of the high molecular weight DNA used for this study. DNA were cut with Hpa II (left seven lanes) or Msp I (right seven lanes). The lane labeled Jar-Aza contains DNA obtained 5 days after Aza-C treatment. The nitrocellulose filter was hybridized with cDNA coding for the Leu-2 gene.

In Table 2 we show that DNA from Aza-C treated JAR becomes able to transfect for HLA and Leu-2. At short and intermediate periods after Aza-C treatment, there is efficient transfection for these antigens, as well as for Trop-1. Eventually (4-6 months), however, DNA from these cells can no longer transfect for HLA or Leu-2 and the transfection efficiency for Trop-1 decreases. These transfections efficiencies for HLA and Leu-2 correlate fairly well with the general degree of methylation (data not shown).

TRANSFECTION EFFICIENCY OF DNA FROM JAR CELLS TREATED WITH AZACYTIDINE

		untreated	5 days[a]	12 days	6-8 weeks	4-6 months
Transfected antigens	HLA	0/16[b]	1/9	4/10	6/13	0/14
	Leu-2	0/16	1/9	4/11	1/11	0/13
	Trop-1	25/29	3/9	10/12	12/13	3/12

a: time after the treatment with azacytidine.

b: number of positive/ number of transfected dishes.

DISCUSSION

These experiments have shown that certain choriocarcinoma cell (CC) lines and, by implication cytotrophoblast from which tissue they are derived, are unable to serve as sources of DNA which will transfect for various polymorphic and T cell differentiation surface molecules. The CC lines which fit in this category are precisely those which most resemble cytotrophoblast, in that they do not express any HLA surface molecules or mRNA for HLA.

We postulated from these results that the genes controlling HLA and T cell differentiation antigens, as well as other membrane differentiation antigens expressed on leukocytes, would be methylated so that they could not accidentally be expressed. Methylation is a covalent, well maintained modification of DNA which appears to be used to permanently shut off genes which are no longer needed by a given cell type (3). Our postulation includes an extension of this idea to genes whose expression would be detrimental to the organism. Clearly, expression of HLA or polymorphic T cell differentiation antigens on trophoblast could readily lead to an immune response by the mother against her own fetus in an outbred species like man.

Methylation of DNA, including of the genes for HLA and Leu-2, a T cell differentiation antigen, was indeed seen in CC lines and the observed pattern was different from that of leucocytes. This is consistent with our postulate, but is by no means proof of it. We sought and found direct evidence in favor of it by using Aza-C to reversibly change methylation patterns in a CC line which did not serve as a source of DNA for transfecting HLA or Leu-2.

After four days of growth in Aza-C and then varying periods of culture in ordinary medium, DNA from the JAR CC line became active in transfecting HLA· and Leu-2. Transfecting ability was absent in untreated JAR cells, became evident immediately after the Aza-C treatment and then peaked between two and eight weeks. Furher culture lead to decreasing ability to transfect the antigens studied. Crude estimation of degree of methylation of these same two genes indicated correlation between degree of demethylation and ability to transfect. We interpret this as support for our hypothesis: The nontransfectability is due to hypermethylation, presumably of certain regulatory DNA sequences, and the occurrence of transfectability followed by nontransfectability as a function of time after azacytidine treatment is due to hypomethylation followed by remethylation.

These findings are consistent with the idea that to prevent rejection of the fetus as an allograft, and thereby allow the perpetuation of mammals, the layer of the placenta closest to the mother and which is fetal-derived utilizes hypermethylation as a means to irreversibly prevent expression of surface antigens which might serve as targets for rejection.

ACKNOWLEDGEMENTS

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