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Genetics of Somatic Mammalian Cells: Lethal Antigens as Genetic Markers for Study of Human Linkage Groups*

(lactic dehydrogenase/auxotrophic mutations/Chinese hamster cells/single cell plating/human cells)

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ABSTRACT **The antigen that causes killing of at least 98% of a human cell population treated with a 1% solution of a specific rabbit antiserum in the presence of complement is a sensitive genetic marker. The rapid loss of human chromosomes in human–Chinese hamster cell hybrids makes possible a convenient test of linkage relationships with this marker. Hybrid clones with and without the lethal antigen were isolated and analyzed. In 76 clones and subclones studied, 41 carried both the lethal antigen and the lactic dehydrogenase-A marker, 35 carried neither, and no clones contained only one of the two markers. In contrast to this clear demonstration of linkage, absence of linkage was found between the lethal antigen and the following markers: Lactic dehydrogenase B, NAD-dependent malic dehydrogenase, NADP-dependent malic dehydrogenase, glucose-6-phosphate dehydrogenase, phosphoglucomutase, glutamate oxaloacetate transaminase, indophenol oxidase, glucose phosphate isomerase, proline, inositol, hypoxanthine B, and glycine A. This lethal antigen appears to be carried on a single human autosome.**

Previous work from this laboratory has shown that hybrids between human and Chinese hamster cells are especially convenient for studying linkage between selected human genes because of the various well-characterized auxotrophic mutations available in the Chinese hamster cell, and because of the rapidity with which these hybrids lose unnecessary human chromosomes. When such hybrids derived from Chinese hamster cells with multiple auxotrophies are grown in media deficient for some but not all of the nutrilites required by the original auxotroph, more human chromosomes become expendable and are lost than when the minimal nutritional medium is used. By selection of clones from such hybrids, which have grown up in partially reinforced media and investigation of their nutritional requirements, it becomes possible to determine whether various markers are lost simultaneously or independently so that the presence of linkage can be determined (1). Similarly, by investigation of whether new genes initially contributed by the human chromosomes are lost simultaneously or independently, linkage among such genes can also be established.

In the present paper, study of antigenic sites capable of killing human cells when treated with an appropriate antiserum in the presence of complement (2) is presented. Other investigators have studied human antigens using techniques of mixed agglutination or measurement of increased cell permeability occasioned by action of antibody plus complement (3, 4). Such studies have revealed that human antigenic genes may be distributed among several different chromosomes. The present study was undertaken to determine whether use of selection techniques afforded by single cell plating in the presence of lethal concentrations of antibodies would permit the identification of specific antigenic markers that are associated with single chromosomes.

METHODS AND MATERIALS

The Chinese hamster cells used were the CHO-K1 parental cell (5) and its auxotrophic mutants whose production, properties, and mode of cultivation *in vitro* have been described (6–8). All these mutants have been shown by complementation analysis to involve single gene changes (7, 9). Single cell platings were performed either in complete F12 medium (10) or in F12 from which specific omissions were made as required by the needs of each experiment. In either case, the medium was supplemented by the macromolecular fraction of fetal calf serum as described (5). The mutants used here are auxotrophic for glycine (gly^-A), hypoxanthine (hyp^-B), inositol (ino^-), and proline (pro^-).

The human cells used for fusion consisted of fibroblasts obtained from skin biopsies as described (11), cells from amniocentesis samples, and lymphocytes (12). UV-irradiated Sendai virus was used for the promotion of hybridization as described (13). Selective media that permit growth of human–Chinese hamster hybrid cells only, but not of either parental cell alone, were formulated as described (1).

Some hybridization experiments were performed in the presence of 1.0 μg/ml of 8-azaguanine and in the absence of proline, glycine, hypoxanthine, and thymidine, or in the presence of 1.0 μg/ml of 5-Fluorouracil. The rationale for this procedure lay in the presumption that increasing environmental stress might increase the number of human chromosomes retained by the hybrid cells. Such an effect was suggested by our earlier demonstration that hyperdiploid Chinese hamster cells survived in concentrations of L-azetidine-2-carboxylic acid that were lethal to cells with lower multiplicities of the proline-synthesizing gene (14).

Abbreviations: AHC, antisera to human cells; A_L, human lethal antigen.

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Specific antisera were obtained by injection of appropriate tissue culture cells into rabbits along with Freund adjuvant as described (2). Antisera prepared against either human fibroblasts or the human S3 HeLa cell behaved in a similar manner. These antisera, designated as antisera to human cells (AHC) or antisera to Chinese hamster cells were collected and stored in the frozen state until used. Clones were tested for their possession of human lethal antigen (A_L) by plating 200 cells in a Petri dish, in standard growth medium containing 1% of standard AHC. This concentration of AHC kills 99.9% or more of human tissue culture cells but has little or no effect on CHO-K1 cells. Clones were scored as A_L^+ if their plating efficiency in the presence of 1% of AHC was 2% or less of that of a control plate without antiserum. In the experiments described here, the antisera were used without heating, so that it was unnecessary to add extraneous complement. Survival curves were expressed in terms relative to simultaneously prepared control platings from which AHC was omitted. The absolute plating efficiency in the absence of antiserum was between 70–80% for the CHO-K1, 30–70% for hybrids, and about 10–15% for the normal human fibroblast.

Tests for particular isozymes were performed by gel electrophoresis by the procedures of Dietz (15) and Davis (16). Readily distinguishable patterns between human and Chinese hamster cell isozymes were found for the following nine enzymic reactions: Lactic dehydrogenase A (LDH-A, EC 1.1.1.27); Lactic dehydrogenase B (LDH-B, EC 1.1.1.27); NAD-dependent malic dehydrogenase (MDH–NAD, EC 1.1.1.37); NADP-dependent malic dehydrogenase (MDH-NADP, EC 1.1.1.40); glucose 6-phosphate dehydrogenase (G6PD, EC 1.1.1.49); phosphoglucomutase (PGM, EC 2.7.5.1); Glutamate oxaloacetate transaminase (GOT, FC 2.6.1.1); Indophenol oxidase (IPO); glucose phosphate isomerase (GPI, EC 5.3.1.9).

EXPERIMENTAL RESULTS

A. Demonstration of the loss of lethal antigen in certain human–Chinese hamster cell hybrids

Oda and Puck (2) demonstrated that the antisera produced in the rabbit by injection of tissue culture cells exhibit a high degree of species specificity when tested by the single-cell plating method. Table 1 illustrates: (*a*) Chinese hamster cells are virtually unaffected by 1% AHC; (*b*) human cells are destroyed by 1% AHC; (*c*) a stable human–Chinese hamster cell hybrid, produced by fusion of an ino$^-$ Chinese hamster cell with a human fibroblast is not appreciably killed by 1% AHC. This particular hybrid appears to have lost all but two of the human chromosomes (1). Since this cell is not killed by the standard AHC, it has presumably lost the human chromosomes that control the development of the particular lethal antigen studied here.

TABLE 1. *Demonstration of the survival of colony formation by various types of cells when treated with 1.0% of a standard, unheated rabbit antiserum prepared against human tissue culture cells as antigen*

	Cell tested	Relative plating efficiency, % (compared to a control with no antiserum).
(*a*)	CHO-K1	98.1
(*b*)	Human fibroblast	0
	Human S3-HeLa	0
(*c*)	A stable human–Chinese hamster hybrid containing two human chromosomes (1)	94.0

TABLE 2. *Demonstration of the loss of A_L marker in clones of hybrid human-Chinese hamster cells, which have lost all but one or two of the human chromosomes*

Hybrid clone number	Auxotrophic requirement of the Chinese hamster parental cell that is relieved in the hybrid	A_L status
83-3c	gly$^-$A	−
83-3g	gly$^-$A	−
56-A5	gly$^-$A	−
7-6	ino$^-$	−
73-4	ino$^-$	−
68-5	hyp$^-$B	−
68-8	hyp$^-$B	−
68-10	pro$^-$	−

Other stable, hybrid clones derived from human and auxotrophic Chinese hamster cell fusion (1) were tested and found also to have lost the A_L marker (Table 2). The simplest interpretation of these data is that the human gene(s) controlling expression of A_L is not linked to the genes for glyA, ino, pro, or hypB.

B. Production of hybrid clones containing the A_L gene

Human cells were hybridized with the doubly auxotrophic CHO-K1, pro$^-$, gly$^-$A, in medium lacking proline, glycine, hypoxanthine, and thymidine, and containing 1.0 μg/ml of azaguanine. The resulting hybrids were cultivated for two weeks in this same medium. Single cells were then plated in standard growth medium and clones were picked for further study. Some properties of these clones are shown in Table 3. The two that have retained the lethal antigen of the human cell have also retained more human chromosomes than the hybrids that had lost A_L gene.

The four primary hybrid clones listed in Table 3 were also tested for the presence of the nine human isozymes used in this study. Data indicative of possible linkage of such isozymes with the A_L gene were obtained only for LDH-A and LDH-B; the other isozymes are apparently unlinked to A_L. Fig. 1 illustrates the different phenotypic responses of the two

TABLE 3. *The chromosome number distribution and presence of A_L characteristic in hybrid clones grown up in the presence of 8-azaguanine as described in the test**

Hybrid clone	Range of chromosome numbers in central 75% of the cell population	A_L
78–1	26–45	+
78–3	18–21	−
78–4	19–20	−
78–5	25–32	+

* The Chinese hamster parental cell has a modal chromosome number of 20, and the human parental cell has 46 chromosomes.

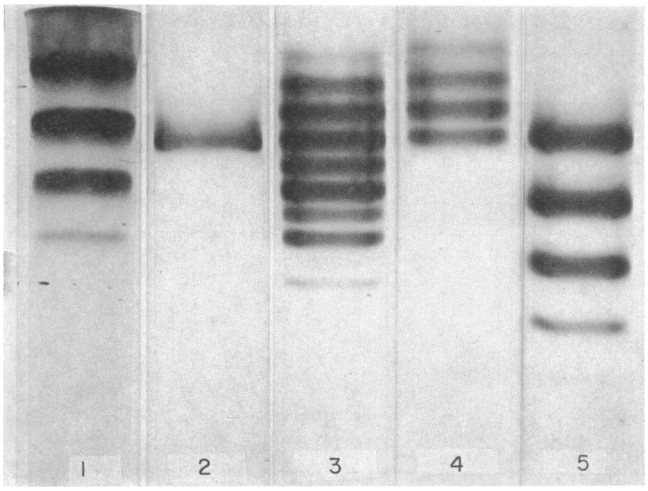

FIG. 1. Typical zymograms for LDH patterns from the clonal cells here studied: (*1*) Normal human fibroblast. Four bands are visualized here, the fifth requiring a more intensive staining, which obscures resolution of the other bands. The pure A tetramer is the topmost band. (*2*) CHO-K1. Only one band, the A tetramer occurs. (*3*) Human–Chinese hamster cell hybrid, which possesses the human A^+B^+ phenotype 10 of the 15 theoretically possible bands are readily visualized, and other nonoverlapping bands can be developed by more intensive staining. (*4*) Human–Chinese hamster cell hybrid, which is A^+B^- for the human components. Four of the theoretical five bands are shown. (*5*) Human–Chinese hamster cell hybrid, which is A^-B^+ for the human components. All five bands can sometimes be visualized on the same gel, although sometimes as here only three or four become prominent with the standard staining procuedure.

parental cells and of hybrids A^+B^+, A^+B^-, and A^-B^+ in the human lactic dehydrogenase genes.

The two primary A_L^+ clones, 78-1 and 78-5, were cultivated in complete medium to permit further chromosome loss. Cultures were also grown up in the presence of 1% AHC for selection for cells which have lost the A_L marker. Colonies were picked from both kinds of plates yielding new clones, some of which had retained and some of which had lost the A_L marker.

C. Tests for linkage

A series of primary and secondary clones was picked, grown up into new stocks, and tested for various genetic markers (Table 4). These clones exhibited variations in chromosomal number, growth rate, and cell morphology, as well as in the specific genetic markers tested. The identification of the human chromosomes retained by these various hybrid clones is the subject of a separate study. Seventeen clones were selected from platings in the presence of 1% AHC of the cells from the A_L^+ clones, 78-1 and 78-5. Eight of these were found to be A_L^-. One clonal isolate from 78-1 that had lost its A_L marker was also picked without antiserum selection. These clones were then tested for presence of the two LDH genes. The data presented in Table 5 reveal definite linkage between A_L and *LDH-A* and no linkage between A_L and *LDH-B*.

The linkage of the human A_L with *LDH-A* implies that A_L is not on the X-chromosome, since *LDH-A* was shown by other investigators to be autosomal (4, 17–19). The data of Table 4 also indicate no linkage between *glyA* and *LDH-A*, but a possible linkage between *glyA* and *LDH-B*, a situation which is receiving further study.

DISCUSSION

The data of this paper indicate that unless complex phenomena are operative, human genes for A_L and LDH-A lie on the same linkage group. Moreover, in contrast to mammalian cell antigens studied by other techniques (3, 4), the human lethal antigen as defined operationally here appears to be limited to a single chromosome. This conclusion follows from the data of Table 5, in which no instance was obtained in which a cell exhibited only one of the two markers, A_L and *LDH-A*.

A further indication of consistency in the simple relationships proposed for interpretation of these data is the fact that no secondary clone with positive A_L, LDH-A, or LDH-B phenotype was ever obtained from a parental clone negative for the particular characteristic.

While Nabholz *et al.* (4) obtained data that were interpreted as consistent with one or more antigenic genes being located on the same chromosome as the LDH genes, their system cannot be related in a simple fashion to ours. Thus, the *LDH-A* and *LDH-B* genes have now been shown not to be on the same chromosome (17–19). Further, certain hybrid clones obtained by Miggiano *et al.* (12), like those with antigen but no LDH activity, cannot be reconciled with the simple picture presented here, and must therefore involve different genetic determinants.

The high resolving power of our experiments appears to derive at least in part from the method adopted for scoring clones as positive or negative for lethal antigen. Apparently, this method can select genetic markers in a single linkage group for consideration. Clones with definite but smaller immunologic responses have also been observed by us. Presumably, these involve other antigenic situations. Study of these may furnish additional single-gene antigenic loci suitable for mapping purposes.

The linkage information about human genes gathered so far by means of the human–Chinese hamster cell hybridization experiments described in this and the previous paper of this series (1) may be summarized as follows: *LDH-A* and A_L are linked. *LDH-B* and *glyA* are possibly linked. *Ino* is unlinked to *pro*, A_L, *LDH-A*, *LDH-B*, *MDH-NAD*, *MDH-NADP*, *G6PD*, *PGM*, *GOT*, *IPO*, and *GPI*. *Pro* is unlinked to *glyA* and A_L. *GlyA*, *hyp B*, *LDH-B*, *MDH-NAD*, *MDH-NADP*, *PGM*, *IPO*, *GPI*, and *G6PD* are all unlinked to A_L.

Identification of linkage groups is the first step in gene mapping, and should permit subsequent positioning of the genes upon a common chromosome by techniques such as somatic crossing-over or use of various agents to yield chromosome breakage. As has been indicated (20), gene mapping provides the information needed for understanding the nature of the metabolic defects underlying the human chromosomal aberrations, and for unravelling the role of geometric factors in the regulatory processes governing activation of different genetic loci.

The data presented here suggest that the rapid and massive loss of human chromosomes that occurs during the first week of hybrid cell growth reflects an initial chromosomal instability which later disappears from these cells. Thus, the A_L^+ clones produced in this study have remained stable throughout many months of propagation in media with no apparent

TABLE 4. *Summary of genetic characteristics tested in hybrid clones of human and Chinese hamster cells**

Primary clones	Secondary clones	Total no. of clones	Human LDH-A	Human LDH-B	A_L	Gly A
551c		1	−	+	−	+
55d		1	−	+	−	+
83-3c		1	−	−	−	
83-3g		1	−	−	−	
7-6		1	−	−	−	
68-5		1	−	−	−	
68-8		1	−	−	−	
56-A5		1	−	−	−	
	56-A5: a,b,c,d,e,f,g,h	8	−	−	−	
73-4		1	−	−	−	
	73-4: a,b,c,d,e	5	−	−	−	
68-10		1	−	−	−	
78-1		1	+	+	+	+
	78-1: a,b,c,d,e,f,g,h,j	9	+	+	+	+
	78-1c: R1	1	−	+	−	+
	78-1d: R2,R3,R4,R5	4	−	+	−	+
	78-1: i	1	−	+	−	+
78-3		1	−	−	−	
	78-3: d	1	−	−	−	−
78-4		1	−	−	−	−
78-5		1	+	+	+	+
	78-5: c,f,g,h,j,k,x,R9,R11	9	+	+	+	+
	78-5: b,i,y,z,R8	5	+	−	+	−
	78-5f: 2,3	2	+	+	+	+
	78-5g: 2	1	+	+	+	+
	78-5g: R12	1	−	−	−	−
	78-5g: R16	1	+	−	+	−
	78-5j: 1,2,4,5	4	+	−	+	−
	78-5j: R18,R19,R20,R21	4	+	−	+	−
	78-5j: 3	1	+	+	+	+
	78-5j: R15	1	−	−	−	−
	78-5x: R13	1	−	+	−	+
	78-5z: 1,2,R22	3	+	−	+	−

* Clones of the R series were isolated from plates treated with AHC.

selective advantage for this characteristic. The present system is also convenient for study of the dynamics of chromosome loss, since loss of the A_L marker can be accurately measured. In preliminary experiments, the rate of such loss was shown to be clone specific, the frequency of loss of A_L^+ was about 50 times greater in hybrid 78-1 than in 78-5. This system also makes possible ready isolation of clones that have lost the LDH-A marker for which selective media have not been available.

The use of this cell system makes possible rapid isolation of hybrid cells containing small numbers of specific human chromosomes. These cells offer possibilities for a variety of different kinds of experiments. For example, a hybrid cell with a hemizygous human chromosome complementing a gene deficiency of the Chinese hamster cell offers the possibility of readily testing mutagenesis of various agents for this particular human gene on a human chromosome. This mutagenic efficiency can be compared with that of the corresponding gene on the Chinese hamster chromosomes. Equivalence in this respect would lend further support to use of the latter cell as a model for mutagenic processes in human cells, as has been proposed (8). Studies of specific gene and chromosomal repair processes in the parental cells and the hybrids might also illuminate aspects of these mechanisms. Mutations from A_L^+ to A_L^-, either spontaneous or mutagen-induced, may also be used as a direct, highly selective method to quantitate forward mutagenesis and to serve as a highly efficient screening test for monitoring environmental agents to which human populations may be exposed.

Study is in progress to determine whether single or multiple genes are involved in the A_L characteristic, where the corresponding antigenic structures are located in the cell,

TABLE 5. *Tests for linkage between A_L and the LDH isozymes, A and B*

	A_L:	+	+	−	−	A_L:	+	+	−	−
Cell phenotype	LDH-A:	+	−	+	−	LDH-B:	+	−	+	−
No. of clones found		41	0	0	35		24	17	9	26

whether these are related to histocompatibility antigens, and what role the antigens concerned play in the metabolic behavior of the cell.

The use of various stresses in addition to auxotrophic nutritional requirements for selective purposes would appear to expand the range of human chromosomes which can be specifically retained in hybrid cells, making possible convenient isolation of large numbers of hybrid cells with particular human chromosomes. These should find application in many kinds of studies including those dealing with differentiation and cancer.

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1. Kao, F.-T., and T. T. Puck, "Genetics of Somatic Mammalian Cells: Linkage Studies with Human-Chinese Hamster Cell Hybrids," *Nature*, **228**, 329 (1970).
2. Oda, M., and T. T. Puck, "The Interaction of Mammalian Cells with Antibodies," *J. Exp. Med.*, **113**, 599 (1961).
3. Weiss, M. C., and H. Green, "Human-Mouse Hybrid Cell Lines Containing Partial Complements of Human Chromosomes and Functioning Human Genes," *Proc. Nat. Acad. Sci. USA*, **58**, 1104 (1967).
4. Nabholz, M., V. Miggiano, and W. Bodmer, "Genetic Analysis with Human-Mouse Somatic Cell Hybrids," *Nature*, **223**, 358 (1969).
5. Kao, F.-T., and T. T. Puck, "Genetics of Somatic Mammalian Cells IV. Properties of Chinese Hamster Cell Mutants with Respect to the Requirement for Proline," *Genetics*, **55**, 513 (1967).
6. Kao, F.-T., and T. T. Puck, "Genetics of Somatic Mammalian Cells VII. Induction and Isolation of Nutritional Mutants in Chinese Hamster Cells," *Proc. Nat. Acad. Sci. USA*, **60**, 1275 (1968).
7. Kao, F.-T., L. Chasin, and T. T. Puck, "Genetics of Somatic Mammalian Cells X. Complementation Analysis of Glycine-requiring Mutants," *Proc. Nat. Acad. Sci. USA*, **64**, 1284 (1969).
8. Kao, F.-T., and T. T. Puck, "Genetics of Somatic Mammalian Cells IX: Quantitation of Mutagenesis by Physical and Chemical Agents," *J. Cell. Physiol.*, **74**, 245 (1969).
9. Kao, F.-T., and T. T. Puck, "Linkage Analysis in Human Somatic Cells by Hybridization with Selected Chinese Hamster Mutants," *Proc. 4th International Congress of Human Genetics*, Paris, France, 1971, in press.
10. Ham, R. G., "Clonal Growth of Mammalian Cells in a Chemically Defined Synthetic Medium," *Proc. Nat. Acad. Sci. USA*, **53**, 288 (1965).
11. Puck, T. T., S. J. Cieciura, and A. Robinson, "Genetics of Somatic Mammalian Cells III. Long-term Cultivation of Euploid Cells from Human and Animal Subjects," *J. Exp. Med.*, **108**, 945 (1958).
12. Miggiano, V., M. Nabholz, and W. Bodmer; in "Hybrids between Human Leukocytes and a Mouse Cell Line: Production and Characterization," *Heterospecific Genome Interaction*, ed. V. Defendi (The Wistar Institute Press, Philadelphia, 1969), pp. 61–76.
13. Kao, F.-T., R. T. Johnson, and T. T. Puck, "Genetics of Somatic Mammalian Cells VIII: Complementation Analysis on Virus-fused Chinese Hamster Cells with Nutritional Markers," *Science*, **164**, 312 (1969).
14. Puck, T. T., and F.-T. Kao, "Genetics of Somatic Mammalian Cells VI. Use of an Antimetabolite in Analysis of Gene Multiplicity," *Proc. Nat. Acad. Sci. USA*, **60**, 561 (1968).
15. Dietz, A. A., and T. Lubrano, "Separation and Quantitation of Lactic Dehydrogenase Isoenzymes by Disc Electrophoresis," *Anal. Biochem.*, **20**, 246 (1967).
16. Davis, B. J., "Disc Electrophoresis II. Method and Application to Human Serum Protein," *Ann. N.Y. Acad. Sci.*, **121**, 404 (1964).
17. Sanlachiara, A. S., M. Nabholz, V. Miggiano, A. J. Darlington, and W. Bodmer, "Genetic Analysis with Man-Mouse Somatic Cell Hybrids: Linkage between Human Lactate Dehydrogenase B and Peptidase B Genes," *Nature*, **227**, 248 (1970).
18. Ruddle, F. H., V. M. Chapman, T. R. Chen, and R. J. Klebe, "Genetic Analysis with Man-Mouse Somatic Cell Hybrids. Linkage between Human Lactate Dehydrogenase A and B and Peptidase B," *Nature*, **227**, 251 (1970).
19. Ruddle, F. H., V. H. Chapman, F. Ricciuti, M. Murnane, R. Klebe, and P. Meera Khan, "Linkage Relationships of Seventeen Human Gene Loci as Determined by Man-Mouse Somatic Cell Hybrids," *Nature New Biol.*, **232**, 69 (1971).
20. Puck, T. T., "Some Considerations on Genetic Analysis at the Single Gene Level in Man," *Ann. N.Y. Acad. Sci.*, **171**, 406–421 (1970).