

SURFACE ANTIGENS OF MAMMALIAN CELLS AS GENETIC MARKERS. II*

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It was previously shown that cultured human and Chinese hamster cells are respectively killed when treated with a 1% solution of the homologous rabbit antiserum in the presence of complement, but not by the heterologous antiserum. Cell killing is accompanied by cytoplasmic leakage from the cell, so presumably involves lysis at antigenic sites on the cell surface (1). Hybrid clones formed from the fusion of auxotrophic Chinese hamster cells and human fibroblasts retain or lose this capacity to be killed, in accordance with a pattern to be expected of a simple genetic characteristic. This lethal human antigenic activity, which was named A_L , was shown to be linked to the lactic dehydrogenase A marker (2).

In the present paper, the distribution of A_L on various human cells has been studied and another lethal antigenic marker, B_L , has been shown to be present on certain human cell surfaces.

Materials and Methods

Tissue Culture Media and Cells.—The CHO-K1 Chinese hamster ovary cell and auxotrophic mutants isolated from it were employed in these experiments. The production, characterization, and mode of cultivation of these mutants have been described (3, 4). These cells and the human-Chinese hamster cell hybrids obtained from them were grown in F12 medium (5) or F12 from which specific omissions were made as required by the needs of each experiment. All growth media were supplemented either with 5–10% of fetal calf serum or with an equivalent amount of its macromolecular components. Hybridization experiments utilized the appropriate, deficient media required to isolate the desired hybrids and were supplemented by the macromolecular fraction of fetal calf serum (6). Most hybrids were propagated in complete media after 1 mo. Some, however, required continuous cultivation in deficient media to maintain chromosomal constancy. Standard F12 (5) supplemented with 10% fetal calf serum (F12FC10) was used for the single-cell platings involved in the antigen assay experiments.

The cultured human cells were the S3 clone of HeLa, normal human skin fibroblasts, and normal fibroblast-like cells derived from amniotic fluid, the latter two cell types behaving similarly in all the experiments reported here. HeLa cells were grown in plastic culture dishes

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or as suspension cultures in F12 supplemented with 10% of fetal calf serum. Human fibroblasts were cultivated in F12 supplemented with 10% fetal calf serum and 5% human cord serum. For the single-cell plating in the antigenic assay experiments the human cord serum was omitted, since it slightly neutralized the effects of antiserum and was found not to be necessary for the growth of colonies of the human fibroblasts in short-term experiments.

Hybrid clones, selected from virus-mediated fusions of Chinese hamster auxotrophic mutants and human cells from a variety of sources, have been described (2, 7). These previously isolated primary clones, plus newly formed similar primary clones, and a variety of subclones selected from both these primary sources were employed in the present study. The term "hybrid clone" in this paper always refers to the result of hybridization of a human cell with an appropriate CHO-K1 auxotroph.

Preparation of Antisera.—Most of the tissue culture cells utilized as antigens were harvested with 0.05% trypsin. Some HeLa cells and Chinese hamster cells were harvested without trypsin from suspension cultures. One rabbit was immunized with human fibroblasts harvested from plastic plates by scraping. No differences attributable to method of harvesting were noted. Red blood cells (RBCs)¹ were purified by careful removal of the buffy coat. In subsequent washings, red cells were taken from the bottom of the tube to minimize leukocyte contamination. White blood cells used for immunization were obtained by removal of the buffy coat from human blood. Residual RBCs were subjected to hypotonic lysis and repeated washing to remove membranous debris. All cells were washed at least three times in physiological saline.

Antigens were prepared by suspending tissue culture cells in a concentration of 5×10^7 cells/ml of saline, or blood cells prepared as described in a concentration of 2×10^8 /ml of saline. The larger number of the latter cells was used to compensate approximately for their smaller surface area. An equal volume of Freund's adjuvant (Difco Laboratories, Inc., Detroit, Mich.) was emulsified with each cell suspension just before injection. Similar antigens were used for both sheep and rabbit immunizations. The rabbits received injections of 1.0 ml of antigen intramuscularly while the sheep received 5.0 ml. A second injection of the same antigen was given to each immunized animal after 2 wk. Serum titers were checked at 4 wk. Those animals with low titers received a third injection of the same antigen.

Horse antiserum to pooled, cultured human cells of "lymphocytic" origin (HAHL) was supplied by Dr. Noboru Kashiwagi (Department of Surgery, Veteran's Administration Hospital, Denver, Colo.). The immunization was effected by one subcutaneous injection of the cell suspension plus adjuvant. Weekly booster shots were given intravenously without additional adjuvant (8).

A standard pooled, rabbit antihuman cell serum (RAHC) with a high titer of A_L antibody was prepared by combining pretested lots of rabbit antisera to cultured A_L⁺ human fibroblasts and HeLa cells.

Adsorption of Specific Cell-Lethal Antibodies from Antisera.—Tissue culture cells were grown in monolayers in F12 medium plus 10% of fetal calf serum. Confluent plates were harvested with trypsin that was inactivated with 5% fetal calf serum after the cells were released from the surface. The cells were then washed three times in saline before adsorption. In one experiment HeLa cells were grown in suspension so that no trypsinization was required.

Human RBCs for adsorption were obtained from heparinized blood. Human lymphocytes from heparinized blood were prepared with modifications of the differential sedimentation method of Thorsby and Bratlie (9). Preliminary RBC removal was aided by the addition of dextran. The leukocyte-rich plasma was centrifuged at 280 g. The pellet containing leuko-

¹ Abbreviations used in this paper: HAHL, horse antihuman lymphocyte serum; RAHC, rabbit antihuman cell serum; RBCs, red blood cells; WBCs, white blood cells.

cytes and some RBCs was suspended in isotonic saline, pH 6.5, and layered over a 1.077 g/ml Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.; Winthrop Laboratories, New York) cushion in the bottom of a centrifuge tube. Centrifugation was carried out for 40 min at 380 *g*. The purified lymphocyte layer was removed from the interface of the saline and the Ficoll-Hypaque high-density cushion. Harvested lymphocytes were then washed three times in saline.

The antisera were heated to 56°C for 30 min before adsorption. Cells and heated antisera were placed together in plastic tubes (Falcon no. 2003; Falcon Plastics, Division of BioQuest, Oxnard, Calif.) 12 mm in diameter to a depth not to exceed 1 cm. Control tubes of the sera were included to which no cells were added. Tubes with adsorbing cells and antisera were incubated in a 37°C water bath shaker for 2 h with vigorous shaking (200 excursions of 1.5 cm amplitude per minute). Cells adsorbing antibodies usually agglutinate while nonagglutinated cells remain in suspension under these conditions. At the end of the incubation an equal volume of saline G (10) was added to each tube to maximize serum collection. All the cells were then sedimented by centrifugation and the diluted serum was removed and sterilized by Millipore filtration (Millipore Corp., Bedford, Mass.). Experiment demonstrated that tissue culture cells in a concentration of 5×10^7 cells per ml removed all detectable killing activity from homologous rabbit antisera under these conditions. This number of cells was used in all the standard adsorption tests, unless specified to the contrary. Other cell numbers were employed in special experiments as indicated.

Complement.—Initial experiments were performed with fresh, unheated rabbit antiserum (2). In the results here reported fresh complement was added to the heated sera. Guinea pig complement as reported by Oda and Puck (1) was satisfactory for HeLa cell assays but was toxic to the CHO-K1 parental cell and most hybrid cells. Normal rabbit serum, though occasionally toxic, was found to be a superior source of complement for these experiments (11).

Normal rabbits were bled by heart puncture and the blood was permitted to clot at room temperature. The serum was collected, filtered for sterility, and frozen for storage in 1- and 2-ml samples at -70°C . Such handling precluded activity loss by thawing and re-freezing. At the time of filtration the serum was tested for complement activity and non-specific cytotoxicity. Lots that showed toxicity or substandard activity were discarded. Frozen complement was stored for a maximum of 4 wk.

Assay of Antigens by Single-Cell Plating.—The basic techniques for screening of clones for the presence of A_L antigen consisted in making the following additions to each of a series of 35-mm plastic culture dishes: 1 ml of F12 medium supplemented with 10% fetal calf serum, 0.05 ml of a 1:10 dilution of heated test serum, 0.02 ml of rabbit complement, and 200 test cells. The test cells were harvested with trypsin from vigorously growing culture plates. The plates were incubated for 7–8 days, fixed, stained, and scored for the number of developed colonies. From these counts the plating efficiencies were determined. Controls were set up in the same manner containing only complement on the one hand, and only heated normal rabbit serum on the other in place of the antiserum. Other controls included plates in which susceptible cells consisting of parental cells or hybrids of known A_L^+ character were tested against a standardized antiserum-like RAHC. All platings were made in duplicate.

Plating the cells first and permitting attachment before the addition of antiserum and complement gave the same results as simultaneous addition of cells and reagents. Some hybrids were somewhat sensitive to 2% normal rabbit serum used as complement. In such cases, 1% complement gave clearer results. An antiserum was designated as killing if it decreased the plating efficiency by $\geq 98\%$ under the standard conditions. When finer distinctions were required, killing antisera were classified as +, ++, or +++ depending on whether the amount of antiserum needed for $\geq 98\%$ killing, respectively, lay in the intervals between 2.0 and 1%; 1 and 0.1%; or $< 0.1\%$. Antisera designated as – yielded no detectable cell killing in the standard test even in concentrations of 2%.

The assays of B_L antigen were made in a similar manner. Rabbit complement was used at a concentration of 2%. Tests were made with horse antiserum to cultured human lymphocytes, which was preadsorbed with an A_L^+ hybrid containing only one or two human chromosomes. This particular antiserum had an extraordinarily high potency, such that a solution containing 0.01% of the antiserum completely destroyed susceptible cells.

The assay plates were routinely incubated for 7 days. Colonies were counted and the relative plating efficiency was expressed as a percent of the colony number on control plates without the test antiserum. The absolute plating efficiency of control plates generally ranged from 60 to 80%.

For construction of survival curves, the cell killing titers of various native or adsorbed antisera were determined for each cell type in the same way. A series of serum concentrations was tested, and the logarithm of the relative plating efficiency was plotted vs. the serum concentration. All single-cell platings were performed at least in duplicate, and each experiment was repeated with similar results.

Mutagenesis of a Hybrid Clone.—Clone 78-5z1 (2), a typical, stable A_L^+ hybrid containing approximately two human chromosomes, was chosen as a standard A_L^+ clone. Cells of this clone were treated with the mutagen, ethylmethanesulfonate, 385 $\mu\text{g}/\text{ml}$ for 24 h (12), and then cultivated for 1 wk in standard growth medium. The resulting culture was then exposed to 1% solution of an antiserum specifically lethal to A_L^+ cells, and new A_L^- subclones that grew out were isolated. These were examined for LDH-A isozyme. One of these clones to be discussed later was found to be LDH-A⁺. Another had the more typical LDH-A⁻ phenotype. This latter clone, 66-G9, had a modal chromosome number one less than its parental cell, 78-5z1. In all other characteristics tested it was similar to the standard A_L^+ clone. Therefore it was chosen as a standard A_L^- clone.

Isozyme Analysis.—The methods previously described (2, 7) were employed with the following additions: Isocitrate dehydrogenase, 6-phosphogluconate dehydrogenase, peptidase A, adenosine deaminase, and nucleoside phosphorylase isozymes were electrophoretically separated by adopting procedures described by Meera Khan (13). Staining methods for the following enzymes were adopted from those described by the investigators cited: isocitrate dehydrogenase and 6-phosphogluconate from Meera Khan (13); peptidases from Lewis and Harris (14); adenosine deaminase from Spencer, Hopkinson, and Harris (15); and nucleoside phosphorylase from Edwards, Hopkinson, and Harris (16).

EXPERIMENTAL RESULTS

Linkage and Segregation between the A_L and LDH-A Genetic Determinants.—In the previous paper the linkage between A_L and LDH-A was inferred from examination of 76 clones of which 14 were primary and the rest secondary (2). Further hybrids have now been prepared so that by this time a total of 161 primary and secondary clones have been examined. The data shown in Table I would appear to establish the linkage between the A_L and LDH-A markers beyond any question.

No spontaneously occurring clone containing only one of the two markers A_L and LDH-A has ever been found. However, a segregation of the two markers was obtained by treatment with the mutagen, ethylmethanesulfonate. As described in the Materials and Methods section, a hybrid A_L^+ clone, 78-5z1, containing both markers was treated with the mutagen by the standard technique, and the surviving cells were plated in medium containing antiserum so as to select only A_L^- colonies. These survivors were then tested for the presence

TABLE I
Evidence for Linkage Obtained from Distribution of A_L^+ and LDH-A Markers Among Primary and Secondary Clones of Human-Chinese Hamster Cell Hybrids

	A_L	+	+	-	-
	LDH-A	+	-	+	-
Primary clones		5	0	0	20
Secondary clones					
From A_L^+ clones		62	0	0	37
From A_L^- clones		0	0	0	37
Total		67	0	0	94

of the human LDH-A marker. One clone was found that is A_L^- but LDH-A⁺. This experiment is of particular interest because it presumably demonstrates how mutagenesis of genes on a human chromosome that is carried in the Chinese hamster hybrid can be carried out.

Demonstration of Differences in Available A_L Antigen on Cells in Different Differentiation States.—

Demonstration of differences in cellular ability to elicit killing antisera: Different kinds of human cells were injected by the standard methods described into experimental animals and the resulting antisera collected. Table II presents a summary of the effectiveness of such antisera as killing agents when tested against different cells. The rabbit demonstrates clear differences in antigenic response to different human cells. Injection of HeLa cells, human cultured fibroblasts, or human RBC results in antisera that are indistinguishable by the procedures used in the experiments of Table II. The human WBC, however, produce an antiserum that can kill HeLa cells, but not cultured human fibroblasts or A_L^+ hybrids of human and Chinese hamster cells. This finding suggests that the WBC possess little or no A_L activity, but that it and the HeLa share one or more other killing antigens not present on the cultured human fibroblast or the A_L^+ hybrid.

Results obtained with sheep antiserum were similar to those obtained from the rabbit except that in the sheep the human RBC appears less potent an antigen for the A_L marker than does the S3-HeLa or the fibroblast. The most potent antiserum of all was obtained in a horse in response to administration of multiplying human cells of lymphocytic origin. Its antigenic behavior, however, resembles that of actively multiplying fibroblasts, rather than the nonreproducing human WBC.

Demonstration of differences in cellular ability to adsorb killing activities from different antisera: Better definition of the presence of available antigen is afforded by experiments in which standard antisera are adsorbed in a uniform fashion by different cell populations. The killing activity against different

TABLE II
*Qualitative Demonstration of the Existence of Differences in Ability of Different Cells to Produce Specific Cell-Lethal Antibodies**

Animal immunized	Cell used as antigen	Killing of test cells treated with each antiserum in presence of standard complement				
		S3-HeLa	Human fibroblasts	CHO-K1	Standard A _L ⁺ hybrid	Standard A _L ⁻ hybrid
Rabbit	S3-HeLa	++	++	-	++	-
	Human cultured fibroblast	++	++	-	++	-
	Human RBC	++	++	-	++	-
	Human WBC	++	-	-	-	-
Sheep	S3-HeLa	++	++	-	++	-
	Human fibroblast	++	++	-	++	-
	Human RBC	+	+	-	+	-
Horse	Long-term cultured cell of human lymphocytic origin	+++	+++	-	+++	-

* In the rabbit experiments two to six animals were injected with each antigen. The antisera, produced from different rabbits similarly tested, behaved similarly. One animal for each antigen was used in the case of the sheep and horse. The table records the killing activity of each antiserum for each cell in the standard plating efficiency experiment. - = no detectable cell killing titer; + = 0.02-0.01 ml antiserum/ml of solution required to kill 98% of the cells; ++ = 0.01 - 0.001 ml antiserum required; +++ = 0.001 ml or less antiserum required.

standard test cells of the adsorbed antisera can then be determined by direct single-cell titration.

Fig. 1 presents a set of survival curves so obtained. These data illustrate that three cell types—HeLa, actively growing human fibroblast, and the A_L⁺ hybrid—are all highly potent in adsorbing killing activity for the standard A_L⁺ hybrid, from the standard, pooled, RAHC antiserum prepared against HeLa and human fibroblast cells. On the other hand, the human nonmultiplying lymphocyte, the human nonmultiplying RBC, the CHO-K1, and the A_L⁻ hybrid are all ineffective in adsorption tests carried out under these specified conditions. These data are consistent with those of Table II except in the case of the RBC, which showed A_L⁺ activity in the qualitative experiments of Table II, but not in the quantitative experiments of Fig. 1.

In order to inquire more deeply into this apparent discrepancy, experiments were carried out in which the number of adsorbing cells was varied, but in which all other conditions of the adsorption test remained unchanged.

In Fig. 2 is shown a typical set of survival curves obtained when A_L⁺ cells are exposed to standard RAHC antiserum adsorbed with varying numbers of HeLa cells. From such curves, one can determine the percentage concentration

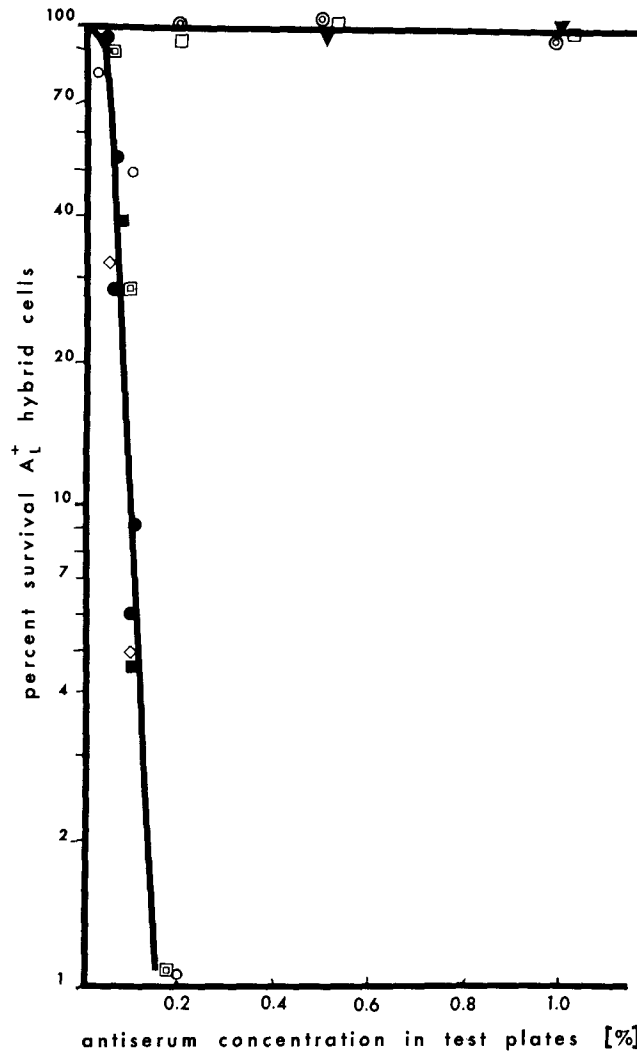


FIG. 1. Demonstration that the standard pooled antiserum against human cells (RAHC) kills the standard A_L^+ hybrid cell in its unadsorbed state (●) and is unchanged after adsorption by the standard technique with 5×10^7 of each of the following cells: human RBCs (○); human lymphocytes (◻); CHO-K1 (■); and the standard A_L^- hybrid cell (◊). Note that adsorption with any of the four cells leaves the serum indistinguishable by this test from its native state. In contrast to this behavior, the standard antiserum loses all of its killing potency for the standard A_L^+ hybrid cell after adsorption with: the standard A_L^+ hybrid itself (▼); the S3-HeLa cell (⊙); and the cultured human fibroblast (□).

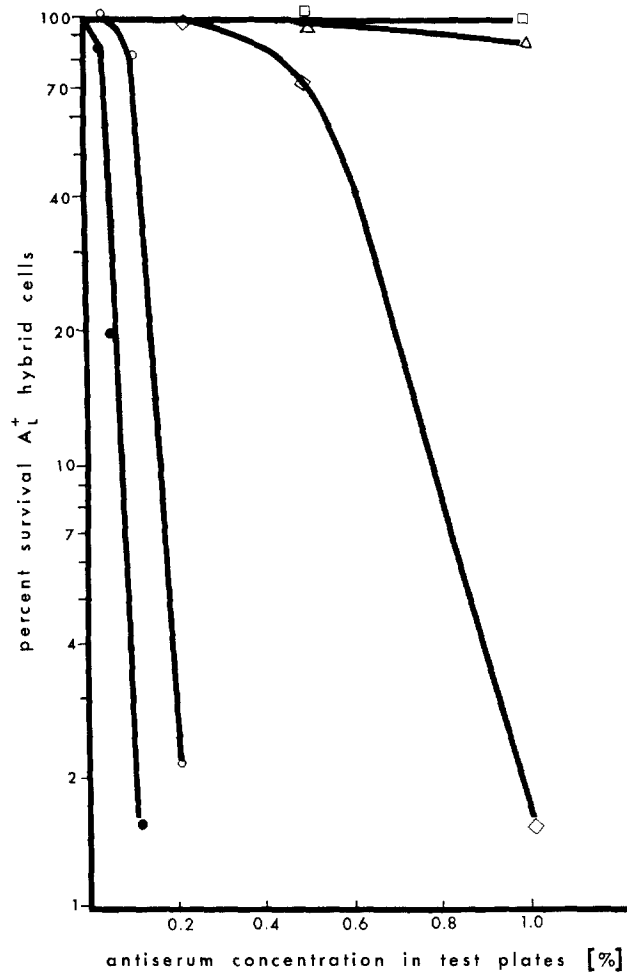


FIG. 2. The effect of changing the concentration of adsorbing cells on the killing titer. HeLa cells were used in the concentrations indicated for adsorption of RAHC antiserum, which was then titrated for its killing action on the standard A_L^+ hybrid: unadsorbed serum (●); 3×10^5 cells/ml (○); 1×10^6 cells/ml (◊); 3×10^6 cells/ml (Δ); 1×10^7 cells/ml (□).

of unadsorbed serum to which a partially adsorbed serum is equivalent in killing potency.

Experiments similar to those in Fig. 2 were carried out, using human RBC and WBC as the adsorbing cells. These experiments revealed that human lymphocytes that display no A_L activity in the procedures of Table II or Fig. 1 can indeed remove anti- A_L activity from the standard antisera if their

concentrations are raised sufficiently.² If one employs 300 times as many human lymphocytes as HeLa cells, the apparent adsorptive capacity for A_L antibody is as great for the former as the latter. This effect cannot be due to the smaller surface of the lymphocyte compared to the HeLa cell since the surface area of the latter is only about six times larger than that of the former. The behavior of the human lymphocyte then is consistent with the hypothesis that it contains about $\frac{1}{300}$ th of the available A_L antigen as the HeLa cell. We therefore designate this cell as $A_L^{(\pm)}$.

The behavior of the human RBC is more complex. The human RBC when injected into a rabbit or sheep produces an antiserum that exhibits some killing activity against A_L^+ cells and none against A_L^- cells (Table II). The former killing activity can be adsorbed out almost completely by 3×10^7 /ml A_L^+ hybrid cells or by 1×10^9 /ml RBC, indicating that the latter cell is only about 3% as effective as the former in this specific adsorption. However, when all other conditions of the adsorption procedure are kept constant and only the cell density is varied, even concentration as high as 3×10^9 /ml of human RBC do not adsorb out the A_L killing activity from antisera produced against HeLa or other A_L^+ cells. This anomalous behavior of the human RBC remains to be explored further. One hypothesis among several is that the RBC contains antigenic sites that are only partially but not sufficiently similar to A_L sites to duplicate all the activity of the latter. We therefore designate the human RBC as $(A_L^+)^+$. A summary of the relative adsorbing potency of the various human cells and cell hybrids is shown in Table III.

Demonstration of B_L Antigenic Sites by Adsorption Tests.—The highly potent horse antiserum against human tissue culture cells of lymphoid origin was selected for these experiments. This serum was adsorbed in separate experiments with various A_L^+ cells until no demonstrable toxicity remained when tested with cells of the same clone as used for the adsorption. Each of the various adsorbed sera was then tested to see if any killing activity remained against cells other than that used in its own adsorption.

In Table IV are demonstrated the results obtained when the standard (HAHL) antiserum was respectively adsorbed by S3-HeLa and by the standard A_L^+ hybrid. The resulting sera were then tested against these same clones and against another A_L^+ cell hybrid. The data indicate that the S3-HeLa cell has removed all killing antibodies capable of affecting any of the three test cells employed. However, whereas the one A_L^+ hybrid, 78-5z1, can remove all of the antibodies capable of killing itself, it leaves behind activity capable of killing the other two test cells. Therefore, S3-HeLa and the 78-1f2 hybrid contain killing antigens different from their common A_L antigen.

² In the case of the human white cells, the total WBC complement was used in preparation of the antigen, but only lymphocytes in the adsorption experiments. Tests demonstrated that all of the killing action of the WBC antiserum for the test cells used here could be removed by adsorption with lymphocytes.

TABLE III
Relative Adsorbing Ability of Various Human Cells and Cell Hybrids

Cell type	No. of cells needed to adsorb 80% of the anti-A _L activity from RAHC
HeLa	1×10^6
Human fibroblast	3×10^6
78-5z1 (A _L ⁺ hybrid)	1×10^7
Human lymphocyte	3×10^8
Human RBC	No adsorption at any cell concentration tested
66-G9 (A _L ⁻ hybrid)	No adsorption at any cell concentration tested

TABLE IV
*Demonstration That Killing Antibodies Affecting Some but Not Other Cells Remain When A_L⁺ Antiserum (HAHL) Is Selectively and Completely Adsorbed with Specific Cells**

Adsorbing A _L ⁺ cells	Killing effectiveness of each specifically adsorbed antiserum on the following test cells		
	S3-HeLa	78-5z1 hybrid (A _L ⁺)	78-1f2 hybrid (A _L ⁺)
S3-HeLa	—	—	—
78-5z1 hybrid	+	—	+

* — means that the adsorbed antiserum in 2% concentration has no effect on the test cell, while + means that it kills at least 98% of the test cells under the standard conditions.

Since adsorption of the standard serum with S3-HeLa also removes all of its killing activity for the 78-1f2 hybrid, these two cells must possess a common killing antigen. We name this antigen B_L. Experiments revealed that the S3-HeLa and the 78-1f2 hybrid A_L⁺ clones behave similarly with respect to B_L activity, in their susceptibility to killing by specific antisera with and without prior adsorption. Therefore these two cells can be designated as A_L⁺B_L⁺, while the 78-5z1 hybrid would be A_L⁺B_L⁻. By standard adsorption tests, the human lymphocyte was also shown to be able to remove antibodies to B_L, as well as to produce these, as indicated in Table II. We designate the lymphocyte as A_L[±]B_L⁺.

The cultured human fibroblast has been demonstrated both by antibody elicitation and by specific adsorption to contain B_L, but preliminary tests indicate it to have less of this activity than the HeLa cell or the human lymphocyte. However, all experiments searching for B_L activity on the human RBC have been negative. Quantitative evaluation of these cell activities is in progress.

It is possible to select B_L⁻ subclones from originally B_L⁺ clones by growing up populations of the latter in killing concentrations of B_L antiserum. Such subclones have been picked and were found to be stable on continued cultivation. Typical plates are shown in Fig. 3. Thus B_L like A_L appears capable of behaving like a useful genetic marker.

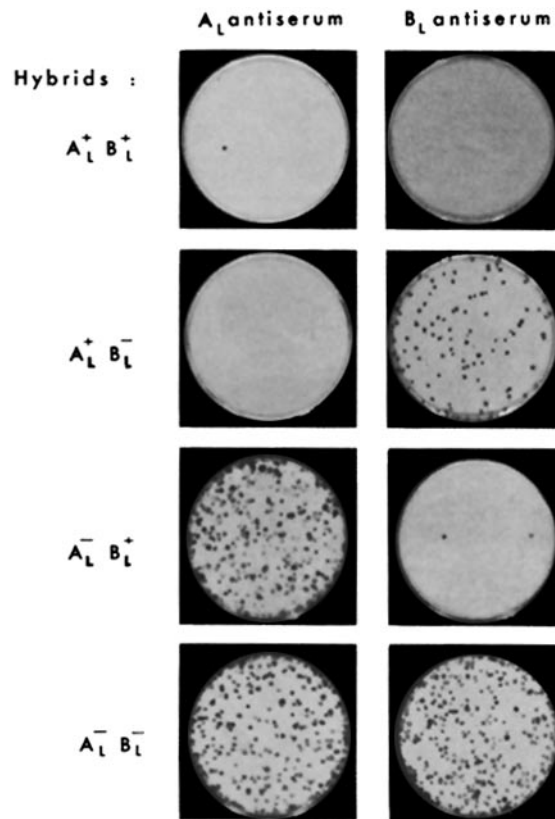


FIG. 3. The specific killing of human-Chinese hamster hybrid cells with A_L or B_L antisera. Control plates with normal serum typically had 100–150 colonies from 200 cells plated. With the appropriate antiserum the killing of (+) hybrids is virtually complete but there is no killing of the (–) hybrids. The $A_L^- B_L^-$ clone was obtained from the $A_L^- B_L^+$ clone by selection of an occasional surviving colony in the presence of B_L antiserum.

Table V illustrates some of the different kinds of hybrid clones that are obtained directly or through selection by the use of specific antisera. All possible combinations of the two antigenic markers have been prepared in different hybrids. It may be noted that whereas A_L^- and B_L^- clones are often obtained by the use of the appropriate selective antiserum from the corresponding + clones, we have never yet obtained either an A_L^+ or a B_L^+ subclone from a clone that was originally lacking the respective antigen, a fact to be expected if simple genetic relationships are obeyed. The data of Table V appear to support the conclusion that A_L and B_L are unlinked since these markers are readily lost independently of each other.

It should be possible to prepare specific antisera against A_L and B_L separately not only by specific adsorption of multivalent antisera with the appro-

TABLE V
*Demonstration of the Killing Antigen Designation of Various Hybrid Clones and Subclones
 Based on Ability of Cells to Be Killed by Specific Antisera**

Cell clones tested		A _L	B _L
Human-Chinese hamster hybrid subclones			
Original clone	Derived by selection with specific antiserum		
78-5z1		+	-
	66-G9	-	-
78-1f2		+	+
	78-1f2-R1	-	+
	78-1f2-R1A	-	-
	78-1f2-R1B	-	-
	78-1f2-R1C	-	+
	78-1f2-R1D	-	+
	78-1f2-R1E	-	-
	78-1f2-R1F	-	+
	78-1f2-R1G	-	-
	78-1d-R4	-	+
	78-1d-R5	-	+
78-1i		-	-

* The data indicates independent segregation of A_L and B_L markers.

priate cells, but also by direct immunization of animals with A_L⁺B_L⁻ and A_L⁻B_L⁺ clones, respectively. Such an immunization has been carried out with an A_L⁺B_L⁻ clone in a rabbit and resulted in an antiserum with a high lethal titer for human A_L⁺ cells. This antiserum is, of course, also lethal for the parental CHO-K1 and for all the hybrids, because the cell used as antigen contained all or most of the CHO-K1 genome. However, by exhaustive adsorption with an A_L⁻B_L⁻ hybrid clone, virtually all of the lethality for CHO-K1 was removed. As was expected, the killing titer of the antiserum for the A_L⁺ S3-HeLa cells was unchanged. It also now kills only A_L⁺ hybrids. The resulting antiserum presumably then contains antibodies only to those substances that are due to genes on the human chromosomal complement of the hybrid. This procedure offers promise of providing reasonably pure antibody reagents to gene products of particular human chromosomes. The preparation of similar specific antisera for the case of the B_L marker is in progress.

Further linkage studies on B_L comparable to those already reported for A_L have been undertaken. B_L is unlinked to glucose 6-phosphate dehydrogenase and therefore is presumably autosomal. B_L also appears to be unlinked to the following: 6-phosphogluconate dehydrogenase, phosphoglucomutase, NAD-dependent malate dehydrogenase, glutamic oxaloacetic transaminase, lactic dehydrogenase A, lactic dehydrogenase B, isocitrate dehydrogenase, NADP-dependent malate dehydrogenase, glucose phosphate isomerase, adenosine deaminase, peptidase A, peptidase D, nucleoside phosphorylase, the glutamic

reductase leading to proline synthesis, and serine hydroxymethylase. Cytogenetic and further isozymic examination of the B_L^+ and B_L^- hybrids is under investigation and will be described in forthcoming reports.

By extension of these experiments it has been shown that sera from which all A_L and B_L antibodies have been removed still possess the ability to kill S3-HeLa cells and cultured human fibroblasts. Therefore these cells appear to possess other lethal antigens in addition to A_L and B_L . Preliminary experiments show that some of these additional antigens are and some are not identical to each other. In Table VI is presented a summary of the lethal antigens so far identified on the various human cells used in this study.

The concentration of complement used in these experiments is critical, since if the amount is altered, the apparent amounts of the antigens carried by

TABLE VI
Current Status of Lethal Antigens on the Human Cells Here Studied

Cell type	A_L	B_L	Evidence available for existence of additional lethal antigens
S3-HeLa	A_L^+	B_L^+	+
Cultured human fibroblasts	A_L^+	B_L^+	+
Human RBC	$(A_L^+)^+$	B_L^-	-
Human lymphocyte	$A_L^{(\pm)}$	B_L^+	+

these cells are altered. Therefore, all experiments must be carried out on a carefully standardized source of complement whose concentration is kept constant throughout the series. The role of complement in altering these responses is under study.

DISCUSSION

Other investigators have demonstrated different categories of specific antigens on cultured cells (17, 18), on various differentiated cells (19-21), and on interspecies hybrid cells (22, 23). Differences have been demonstrated both in production of specific antibodies and their adsorption to cells of different organs in both allo- and heteroimmunologic systems.

The study described in the papers of this series differs from previous studies in that the following combination of methodologies has been used: (a) Hybrids between human and Chinese hamster cells have been employed which permit incorporation of one or a small number of human chromosomes into a stable clone whose genome is otherwise that of the Chinese hamster cell. This effects an enormous simplification in the range of human antigens produced by each clone. The Chinese hamster antigens can be disregarded in these experiments by virtue of the demonstrated lack of cross-reactivity of the antigens from cells of these two species under the carefully controlled conditions here described.

(*b*) Single-cell plating has been used as the technique for demonstrating the presence of lethal antigens. It has provided clear distinction between antibody-sensitive and resistant cell populations and permitted selective isolation of resistant clones from an initially sensitive parental population. (*c*) Genetic tests have been applied with the result that the human antigens studied appear to furnish excellent genetic markers.

Genetic markers like A_L and B_L appear to be particularly useful in these hybrid cells since they can readily be made the basis for selection procedures. These are human genes and should be capable of being studied for mutagenicity in these cells by methods described earlier. By the use of agents like caffeine, which we have shown to introduce chromosome breaks with few or no gene mutations, it might be possible to produce terminal deletions in the human chromosome carrying a given gene so as to be able to map the distance between such a gene and other human genes contained on the same chromosome. The presence or absence of specific lethal antigens on mammalian cells can be shown by the (*a*) ability of specific cells to elicit antibodies capable of killing other cells with known antigenic constitutions; (*b*) ability of the cell to adsorb out specific killing antibodies from particular standardized antisera; and (*c*) ability of a cell to be killed specifically and quantitatively by standardized antisera. The second method is more quantitative than the first and is more widely applicable than the third since it is applicable to nonmultiplying cells like RBC and WBC. The results so far obtained by all three methods are reasonably consistent within the limitations of uncertainty of each procedure.

The experiments reported here indicate that the use of lethal antigens, which behave as good genetic markers, can be extended. Moreover, specific differentiated human cells like the RBC and the lymphocyte have antigenic constitutions different from those on particular human clones grown in tissue culture. These methods may permit studies on cells whose differentiation states involve different surface antigens.

The use of single-cell plating provides a precise and quantitative measurement of the potency of lethal antigens on various cell types. It also makes possible ready isolation of clones resistant to specific antisera so that genetic variants can be readily obtained and studied. By hybridization with a variety of Chinese hamster auxotrophs in appropriate selective media, the human chromosome on which the gene responsible for this antigenic activity should be identifiable, and the linkage between this and other human genes determined. By methods described earlier (24), it may be possible to identify the control genes that affect the operation of specific antigens. This approach might be useful in characterization of specific cell antigens of various normal and malignant differentiation states.

SUMMARY

A second surface antigen, B_L, lethal in the presence of specific antibody and complement has been identified on some human cells and shown to behave as a good genetic marker. It is autosomal, unlinked to the human A_L antigen previously described, and unlinked to 15 other human genes. The A_L antigen, which is linked to the lactic dehydrogenase A gene, is found on the HeLa, the cultured human fibroblast, and in small amounts on the human lymphocyte. B_L occurs on HeLa cells, on cultured human fibroblasts, and on human lymphocytes, but not on human RBCs. Hybrid cells formed by fusion of human and Chinese hamster cells have been prepared containing each of the four possible combinations of these two markers. Highly selective antisera sensitive to each marker separately can be obtained. The use of single-cell plating to demonstrate the presence of the antigens and of hybrid cells containing desired combinations of the markers facilitates study in this system.

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