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Genetics

Genetic analysis of the human cell surface: Antigenic marker for the human X chromosome in human–mouse hybrids

(somatic cell/antibody/indirect immunofluorescence/fluorescence-activated cell sorting)

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ABSTRACT **Somatic cell hybrids of human fibroblasts and mouse A9 cells, carrying only a portion of the human X chromosome in a mouse chromosome background, were injected into C3H mice. The resulting mouse anti-hybrid cell antisera contain antibodies found to be human specific and to react with only those hybrid cells carrying the human X chromosome, as confirmed by essentially perfect concordance between antibody binding assayed by indirect immunofluorescence and presence of the human X-linked enzyme hypoxanthine phosphoribosyltransferase determined by autoradiographic assay of [^{3}H]hypoxanthine utilization. Heterogeneous mixtures of hybrid cells may be analyzed into fluorescent (X plus) and nonfluorescent (X minus) subpopulations and fractionated viably by using a fluorescence-activated cell sorter.**

Numerous properties characteristic of the human cell surface are found to be expressed in a dominant or codominant mode on the surface of interspecific somatic cell hybrids. That is to say, they are expressed if and only if the hybrid cell retains the necessary human genetic information. Consequently, such properties may be viewed as constitutive markers for the presence of human genes, and it has been possible to use somatic cell hybridization methods to assign the chromosomal linkage group controlling their expression. If the assigned markers are analyzed with respect to immunological considerations, three classes may be discerned. First, one finds surface markers whose biological role is clearly a part of or related to functioning of the immune system. Examples are the surface expression of immunoglobulin heavy chain controlled by chromosome 2 (1), the major histocompatibility loci on chromosome 6 (2), and β_2-microglobulin production which occurs in the presence of chromosome 15 (3–5). In a second class are markers that confer upon the cell sensitivity to specific biological agents, due presumably to the presence of cell surface receptors coded for by specific human chromosomes but whose immunogenic potential is generally unknown. Examples include sensitivity to herpes simplex virus (6), diphtheria toxin (7), poliovirus (8, 9) and interferon (10–13) which have been associated with the presence of chromosomes 3, 5, 19, and 21, respectively. For the third class of surface marker, the biological role may be unclear but the antigenic properties, such as sensitivity to cytotoxic selection, have been demonstrated. These include the so-called lethal antigen(s) associated with chromosome 11 (14–16) and others associated with chromosomes X (17–20), 7 (21–23), and 17 (23).

Whatever their intrinsic biological role, the antigenic potential of these human cell surface characters and their presence on interspecific hybrid cells hold considerable significance for somatic cell genetics. Workers in several laboratories have now demonstrated that antibodies against the human cell surface components expressed on human–mouse hybrid cells can be elicited by injecting the hybrids into mice syngeneic with the mouse parent of the hybrid line (15–18, 20, 21, 23). In the syngeneic mouse, hybrid cell surface components derived from the mouse parent should in principle stimulate no immune response. However, cell surface components associated with human genes present in the hybrid may stimulate the mouse immune system. If so, the mouse should produce antibodies directed against the human cell surface components specifically. Such antibodies should be expected to bind any human cell, or any human–mouse hybrid expressing the associated cell surface antigen. They could be used to stain individual hybrid cells via indirect immunofluorescence as a diagnostic for the presence of the human genes governing expression in the hybrid. The immunofluorescence staining reaction would permit determination of the frequency of a particular human linkage group in an inhomogeneous hybrid cell population. And, inasmuch as the immunofluorescence assay can be done on living cells, it should be possible to use a fluorescence-activated cell sorter to fractionate mixed populations into subpopulations homogeneous with respect to a given human chromosome. Homogeneous fractions derived in this way can substantially facilitate genetic analysis.

As a model for this approach, we report here the production, assay, and initial cell sorter utilization of antisera capable of labeling specifically those hybrid cells carrying the human X chromosome. We are happy to acknowledge that the present work was prompted initially by an earlier report of antisera against an X-linked lymphocyte antigen obtained by Buck and Bodmer (18).

MATERIALS AND METHODS

Cell Culture. The human, mouse, and human–mouse hybrid cell lines used (24–37) are listed in Table 1. Conventional techniques were used for maintenance of hybrid cells (38), including selection for cells expressing the human X-linked enzyme hypoxanthine phosphoribosyltransferase (HPRT; IMP: pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) in culture medium supplemented with 13.6 μg of hypoxanthine, 0.19 μg of aminopterin, and 3.9 μg of thymidine per ml (HAT) according to Littlefield (39), and back-selection against cells expressing HPRT in medium containing the purine analog antimetabolites 8-azaguanine and (or) 6-thioguanine (38). Conventional cytological procedures were used for autoradiography (40), chromosome analysis (33, 41–44), and starch gel electrophoretic analysis of human isozymes (45).

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Abbreviations: HPRT, hypoxanthine phosphoribosyltransferase (EC 2.4.2.8); PGK, phosphoglycerate kinase (EC 2.7.2.3); G6PD, glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

Table 1. Cell lines assayed with anti-11d antisera

Cell line	Origin (refs.)	I[a]	II[b]
Human or mouse lines:	(24–32)		
GM 144[c]	Human skin fibroblasts (24)	+	NT[d]
HeLa S-3[e]	Human cervical tumor (27)	+	+
CCL 1 (A9)[f]	C3H mouse L-cell (28–30)	−	−
GM 347 (B82)[c]	C3H mouse L-cell (31)	−	−
Human–mouse hybrids:	(12, 13, 33–36)		
AHA-11a[g]	GM 144–A9	+	+
AHA-11d-75-20-1[h]	GM 144–A9; see text (33)	+	+
AHA-3d[g,h,i]	GM 144–A9	+/−	+/−
AHA-3d-14[h,j]	Subclone of AHA-3d	+/−	+/−
AHA-16d[g,i]	GM 144–A9	+/−	+/−
AHA-16e[g,i]	GM 144–A9	+/−	+/−
WAIa[j]	CCL 75 (WI 38)[f]–A9 (34)	+/−	+/−
WAII (WAIIa)[j]	CCL 75 (WI 38)[f]–A9 (34)	+/−	+/−
41pT-4[k]	GM 126[c]–tsClAGOH[l] (35)	+/−	+/−
157BnpT-1[k]	GM 126[c]–tsClAGOH[l] (35)	+/−	+/−
AHA-11d-75-13-20	AHA-11d subclone; HPRT−	−	−
AHA-11aAGThG	AHA-11a derived; HPRT−	−	−
WAVR4d, -F94a[m]	CCL 75 (WI 38)–A9 (12–13)	−	−
CT11c	HeLa chromosomes–A9 (36)	−	−

[a] Scored for human X chromosome, by karyotype and isozyme analysis; intact X except as noted. Scoring: ~100% positive, +; ~100% negative, −; mixture, +/−.
[b] Scored for immunofluorescence. Scoring: ~100% positive, +; ~100% negative, −; mixture, +/−.
[c] Human Genetic Mutant Cell Repository, Camden, NJ.
[d] Not tested.
[e] From J. R. Allen and J. E. Hearst, University of California, Berkeley.
[f] American Type Culture Collection, Rockville, MD.
[g] Primary hybrid from R. S. Kucherlapati.
[h] Contains presumptive X chromosome fragment; HPRT-positive.
[i] Karyotype data from ref. 37.
[j] Karyotype data from R. S. Kucherlapati.
[k] Karyotype data from ref. 35.
[l] See ref. 32.
[m] Subclones and karyotype data from M. Dennis and J. B. Lawrence.

Immunological Procedures. Antisera were produced in strain C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) by three or four injections, at weekly intervals, of 10^7 intact cells per animal. The initial injection was split between foot pads and the intraperitoneal route; subsequent injections were exclusively intraperitoneal. Indirect immunofluorescence assay of antibody binding was performed by incubating test cells with mouse antisera at dilutions ranging from 1:5 to 1:40 for either 45 min at 37° in the case of attached-cell assays or 15–30 min at 4° for test tube assays. Cells were washed with serum-free medium and incubated with either fluorescein- or rhodamine-conjugated rabbit or goat immunoglobulins against mouse gamma globulin (Cappel Laboratories, Downington, PA), under the same conditions as the first antiserum incubation. Assay cells were washed free of unbound conjugated antiserum and fixed on slides by 30-sec exposure to 95% ethanol at 4°. For some experiments (see Table 2), antisera were pre-absorbed with cells of HPRT-negative hybrid line AHA-11aAGThG (1 μl/10^6 cells) by incubation for 1–2 hr at room temperature or overnight at 4° with agitation.

Cell Sorting. Fluorescence-labeled (stained) and unstained cells were fractionated on a fluorescence-activated cell sorter (FACS II, Becton-Dickinson Electronics Lab, Mountain View, CA). Cell sorter modifications utilized for aseptic handling of somatic cell hybrids will be detailed elsewhere.

Table 2. Anti-11d fluorescence vs. [^{3}H]hypoxanthine labeling

	Single cell scoring (fluorescence/^{3}H)[a]					
Hybrid line	+/+	−/−	−/+	+/−	NS[b]	Total
WAIa	52	54	6	7	NS	119
AHA-16e	532	70	10	1	95	708
WAII	39	465	4	2	12	522
WAII-1636[c]	500	0	NS	NS	NS	500

[a] Anti-11d antiserum was unabsorbed (WAIa) or absorbed on clone AHA-11aAGThG (AHA-16e, WAII, WAII-1636). Positive (+) score, fluorescence ring reaction at cell margin or grain count at least 3 times autoradiographic background (the majority of autoradiographic positives were labeled by 5 to 10 times the grain count of mouse A9 or HPRT− hybrid cell controls); broken or ambiguous cells were not scored; others were scored negative (−).
[b] Not scored.
[c] Fluorescence-positive subpopulation derived from cell sorter experiment; autoradiographic and fluorescence analysis after second passage in culture.

RESULTS

AHA-11d, the immunizing cell line used in these experiments, is a human fibroblast–mouse fibroblast hybrid cell clone which, on the basis of cytological and isozyme analysis, had lost at an early passage all detectable human chromosomes except the X chromosome and the X-linked markers HPRT, phosphoglycerate kinase (PGK; EC 2.7.2.3) and glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49). Assays for one or more isozyme markers each for human chromosomes 1, 2, 4, 5, 6, and 8 through 21 were negative. A subclone derived in hypoxanthine/aminopterin/thymidine medium at passage 13, AHA-11d-75-20-1, is homogeneous for the presence of a human–mouse translocation chromosome (see ref. 33, figure 3); no other human chromosome has been detected. On the basis of its concordant segregation with human isozymes HPRT and PGK (G6PD is not expressed) in back-selection experiments, the translocation presumably involves a fragment of the human X, including the known Xq loci for HPRT and PGK but not G6PD (46, 47). This report will treat the sera raised in C3H mice against subclone AHA-11d-75-20-1, which we designate "anti-11d antisera."

Typical immunofluorescence reactions for cells stained with anti-11d antisera are depicted in Figs. 1 and 2. These antisera stain human cells, do not stain mouse cells, and, when tested on hybrid cells grown on nonselective medium, stain a subset of the population. The frequency of antigen-positive cells is readily scored, and positive cells present at low frequency can be specifically labeled and detected (Fig. 2). When cells of hybrid clones including AHA-11a or AHA-11d-75-20-1 were grown in hypoxanthine/aminopterin/thymidine medium to select for retention of the X chromosome and then stained, essentially every cell in the population exhibited positive fluorescence (data not shown). Analysis of the anti-11d antiserum specificity has been conducted on a series of hybrid clones and parental cell lines. The results for the cell lines tested (Table 1) show an exact correlation between the presence of fluorescence staining and the presence of the human X chromosome or X-linked isozyme markers in the cell population.

Specificity of fluorescent staining with anti-11d antisera can be analyzed not only with respect to the human chromosome composition of cell lines but also at the level of individual hybrid cells. Mouse A9 cells are deficient in HPRT. Consequently, only those human–A9 hybrid cells retaining the human X-linked gene for HPRT will be capable of incorporating exogenous hypoxanthine. HPRT-positive cells can be identified by

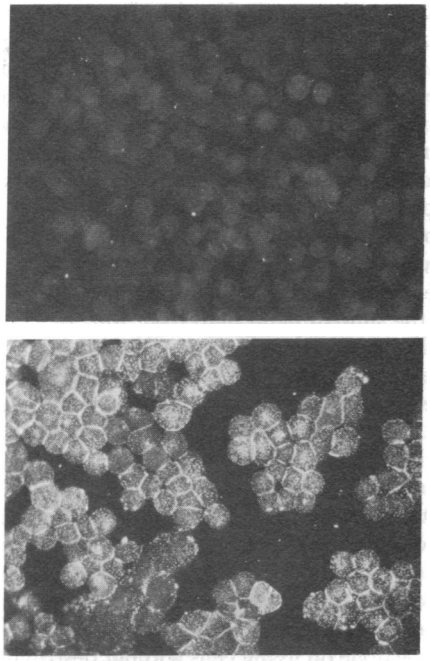

FIG. 1. Mouse A9 cells (*Upper*) and human HeLa S-3 cells (*Lower*) assayed by indirect (fluorescein conjugate) immunofluorescence for reaction with antibodies in unabsorbed anti-11d antisera. A9 and HeLa cells were grown in separate chambers of the same slide and assayed together by incubation with antisera under a single cover slip to ensure uniform reaction conditions. Photographic exposure time (90 sec) and all subsequent processing were identical. Fixation of cells after the second antibody reaction produces the low-level (auto-) fluorescence on the A9 cells, even on control slides not exposed to antisera. Assayed cells are viable prior to fixation. Both panels also show a number of bright spots due to incomplete removal of debris from the fluorescent-conjugated (second) antibody preparation; such debris appears even if the first antibody treatment has been omitted. The fluorescence observed as speckling over the surface of every HeLa cell and, more brightly, at the intercell margins is taken as a positive antibody reaction. Positive reactions have not been observed with control sera (including sera from preimmune C3H mice or C3H mice injected with A9 cells or with fetal bovine serum) produced by the same immunization procedures used to obtain anti-11d antisera.

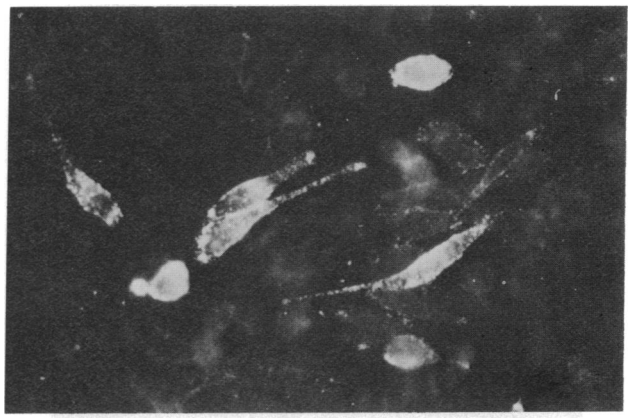

FIG. 2. Human–mouse hybrid cells from line WAII assayed for reaction with unabsorbed anti-11d antisera by indirect (fluorescein conjugate) immunofluorescence. Assay was performed on cells growing attached to sterile chamber slides in nonselective medium. Positive antibody reaction is indicated by fluorescent patches over the cell surface, including the long cellular extensions. In clone WAII, positive reactions are restricted to groups of cells distributed among the predominantly negative hybrid cell population. The positive cells found in a given group tend to possess similar cellular and fluorescence stain morphology, suggesting that they may be clonally related.

growing the hybrids in medium containing [^{3}H]hypoxanthine and then doing autoradiography. In this context, the autoradiographic result constitutes an independent, single-cell assay for the presence of the human X chromosome. After growth in the presence of isotope, but prior to autoradiographic processing, test cell populations can be treated with antisera for immunofluorescence assay. We can subsequently examine, on a single cell basis, the correlation between the presence of radioactive grains on the isotopically labeled HPRT-positive cells and fluorescent staining with anti-11d antisera. For certain of these single-cell correlation experiments, the anti-11d antisera were preabsorbed with cells from line AHA-11aAGThG (derived from line AHA-11a by selection in 6-thioguanine and 8-azaguanine). The results (Table 2 and Fig. 3) indicate that no fewer than 90% (using unabsorbed anti-11d antisera) and as many as 98% (using absorbed anti-11d antisera) of the cells are concordant in that they are either isotopically labeled and fluorescent or unlabeled and nonfluorescent. These results demonstrate that the antibodies in anti-11d antisera are capable of specifically recognizing those hybrid cells carrying the human X chromosome. It may be deduced from these results that the human–mouse hybrid cells tested here carry on their surface one or more components that are antigenic upon presentation to the mouse immune system and whose expression is controlled by a gene (or genes) syntenic with the X-linked HPRT gene. And it may be inferred from the high correlation between the presence of HPRT activity and of fluorescence staining with anti-11d antisera that expression of the X-associated antigenic determinant(s) for these hybrid cells is essentially constitutive. Thus, not only is staining with anti-11d antiserum diagnostic for hybrid cells that have retained the human X chromosome but also, under the assay conditions used here, the absence of staining can be taken as diagnostic for cells that have eliminated the X chromosome.

The preceding results suggest that separation of the fluorescence-positive and fluorescence-negative subclasses from a human–mouse hybrid cell population treated with anti-11d antisera should lead to concomitant assortment of other genetic traits determined respectively by the presence or absence of the human X chromosome. One approach available to test this notion involves use of instruments capable of analyzing and fractionating cells on the basis of the fluorescence elicited by flow through a beam of visible laser light (see ref. 48 for review). We have used a commercially built fluorescence-activated cell sorter of the Herzenberg design (49). In our hands, hybrid cell populations treated by immunofluorescence assay and fractionated on the cell sorter remain highly viable. The sorted hybrid cells may be grown out under standard culture conditions, provided that serum complement has been heat inactivated. Cultured subpopulations may at any time thereafter be analyzed to confirm the degree of population homogeneity achieved by sorting. Resorting may be done if necessary. Sorted subpopulations judged to be sufficiently homogeneous may be analyzed for genotypic or phenotypic traits that have segregated with the chromosomal determinant of the fluorescent antibody reaction. Results are presented for a model experiment of this type, involving line WAII hybrid cells fractionated on the basis of immunofluorescence due to treatment with anti-11d antisera (Table 2). It has been possible to fractionate the X chromosome-containing cells from hybrid cell line WAII without recourse to biochemical selection. Analogous separations involving antisera exhibiting different chromosomal specificity can also be achieved and will be reported elsewhere.

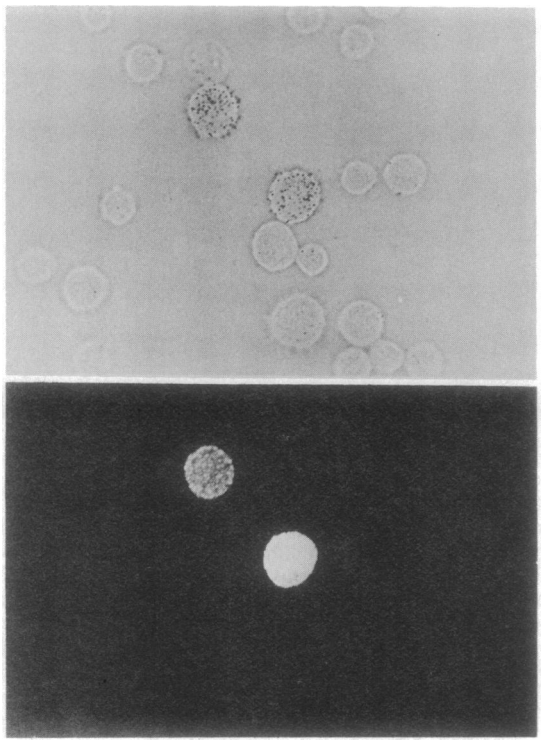

FIG. 3. Comparison of autoradiographic labeling (*Upper*) due to uptake of [^{3}H]hypoxanthine and indirect (rhodamine conjugate) immunofluorescence labeling (*Lower*) due to treatment with absorbed anti-11d antisera, for cells of clone WAII. Cells were grown in the presence of [^{3}H]hypoxanthine (0.2 μCi/ml; 5 Ci/mmol) for 7 days to obtain homogeneous labeling of HPRT-positive cells. Indirect immunofluorescence staining reaction was performed in the test tube prior to spreading of cells on slide for autoradiography. Selected fields were sequentially photographed under bright field (*Upper*) and epi-illumination (*Lower*). In this example, note two ^{3}H-positive cells labeled by about 50 silver grains each; the remaining negative cells carry 5 grains or less. The same two cells, and no others, are labeled by immunofluorescence. The substantial difference in fluorescence intensity of the positive cells presumably reflects variation in antigen titer, the basis for which has not been studied. However, the distinction between fluorescence-positive and -negative hybrid cells in this preparation is unambiguous. The correlation between autoradiographic labeling and fluorescence labeling depicted here is representative of the hybrid cell populations tested, as indicated by data in Table 2.

DISCUSSION

We have found that one or more X chromosome-associated cell surface antigens are constitutively expressed on human fibroblasts and on hybrids between human fibroblasts and mouse A9 cells. We have not succeeded in demonstrating the presence of the antigen(s) by a positive immunofluorescence reaction on human peripheral blood lymphocytes. These results contrast with those of Buck and Bodmer (18) who, using a human lymphocyte–mouse L cell hybrid, have raised antisera capable of reacting specifically with human B lymphocytes but not with T lymphocytes or fibroblasts. Buck and Bodmer suggested that the X chromosome-associated cell surface antigen on human lymphocytes be designated "SA-X". Inasmuch as the lymphocyte and fibroblast antigens may be distinct, we have proposed that the X-associated cell surface antigen(s) present on human fibroblasts and their hybrids be designated "SA-X2". We note that expression of lymphocyte antigen SA-X has been associated with the long arm of the X chromosome at a gene locus between the known loci for HPRT and G6PD (19).

It is noted that no data have been obtained in the present study regarding the physical or biochemical nature of the human X chromosome-associated cell surface antigen(s), and we can draw no conclusions regarding their distribution among animal species. For example, it is not known whether the antigenic human cell surface component on a human–mouse hybrid is unique to human cells in particular or is shared by other primates, or whether absence of the antigen(s) might be a mouse-specific or rodent-specific trait.

Numerous reports concerning human-specific cell surface antigens expressed on interspecific hybrid cells have appeared in the recent past (1–5, 10–16, 18–23). All such antigens contribute to the human gene map (50). But, in the context of somatic cell genetics, the paramount importance of antigenic cell-surface markers resides in their use for scoring the presence (or absence) of an associated human chromosome in *individual, living* cells. In particular, antibodies raised against a cell surface antigen whose presence is constitutively associated with a specific human chromosome offer two crucial advantages. First, fluorescent antibody labeling that can be scored on single cells confers the ability to do rapid, convenient, and statistically meaningful assays for the frequency of antigen-bearing cells and the associated linkage group in a heterogeneous hybrid population. Second, and equally important, the fact that the assay may be done on living cells without destroying viability carries with it the potential for "chromosome-specific" selection in hybrid cell systems. Negative selection is possible in those cases in which the antisera exhibit complement-dependent cytotoxic killing activity (14, 15, 22). Independent of cytotoxic activity, either positive or negative selection is possible via fluorescence-activated cell sorting techniques. Hybrid cell populations obtained from a flow sorter, homogeneous for the presence or absence of a particular human chromosome, are analogous to mass populations derived from biochemical selection systems. It might be noted that sorter experiments may be extended in principle to simultaneous selection of pairwise combinations of cell surface markers (51), including "chromosome-specific" cell surface antigens of the type reported here (52). Such selection potential can contribute significantly to the genetically useful manipulation of somatic cell hybrids.

The antisera utilized in this study were obtained by injecting intact human–mouse hybrid cells into animals syngeneic with the mouse parent. Interspecific hybrid cell immunogens are unusual in two respects. First, they represent a composite of species-homologous and species-heterologous antigenic determinants. Second, the subset of determinants associated with the presence of any heterologous chromosome(s) will be included (or excluded) depending upon which chromosomes have been retained (or eliminated) in the hybrid. It is not particularly surprising that such immunogens would elicit antibodies against heterologous determinants on the hybrid cell surface. But it is fairly remarkable to contemplate that *essentially any* component of the human cell surface might become antigenically active in the hybrid cell context. Upon elimination of antigenic or steric competition along with various subsets of the human genome, significant antigenic potential might devolve onto any of the remaining human-specific architectural proteins or more specialized surface structures. Consequently, hybrid cell immunogens and the antibodies that they stimulate hold tremendous potential for genetic, biochemical, and physiological analysis of the human cell surface.

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