Isoantigenic Variants: Isolation from Human Diploid Cells in Culture

Abstract. *Variant human fibroblast substrains, resistant to a cytotoxic human isoantiserum, were isolated from sensitive strains by repeated exposure to isoantiserum and rabbit complement. The resistant phenotype was stable, apparently occurred at low frequency, and was associated with loss of surface isoantigens.*

The application of cell culture techniques to human genetic analysis depends on the availability of means to isolate variants arising in vitro. There is increasing use of human cell cultures for genetic investigation, and a relatively large number of markers is known for these cells (*1*). However, not all of the markers discovered thus far can be used for selection, and no variants arising in vitro have been isolated from human diploid cultures. Our interest in this area was stimulated by the prospect of detecting somatic recombination and applying it to determine human linkage. We now describe an isoantigenic selective system for human diploid cultures and the isolation of isoantigenic loss variants from these cultures.

To detect isoantigenic loss variation, a cytotoxic antiserum is needed, and the cell strain used must be heterozygous at the selective locus—in a homozygote, antigenic loss resulting from a single genetic event would not lead to loss of sensitivity to antiserum. We obtained a selective antiserum reactive with heterozygous human diploid strains by taking advantage of maternal isoimmunization by fetal cells (*2*). Maternal isoimmunization results from immune stimulation by paternally inherited antigens present on fetal blood cells and not present in the maternal genome (*3*). Thus cells of the offspring which react with maternal antiserum are obligate heterozygotes for the reactive locus. We established fibroblast cultures from newborn infants and tested serum from their mothers for cytotoxicity. Several reactive cell strain–antiserum combinations were obtained.

Reconstruction experiments were performed initially to test the efficiency of selection. Reactive and nonreactive strains were mixed in varying proportions and exposed to antiserum and rabbit complement (C′). These studies indicated that only about 90 percent of an initial sensitive population of 10^6 to 10^7 cells could be killed by a single exposure to antiserum and C′. Because of the inefficiency of selection and the probability that variants arising from genetic events would occur at low frequency, an enrichment method was employed to isolate variants (*4*). Sensitive cell populations were exposed to antiserum and C′, the survivors were regrown, and this process was repeated until variant substrains were obtained.

Isoantigenic selection was carried out with a maternal isoantiserum (AS) and a cell culture derived from the offspring; 5×10^6 cells from a monolayer culture were suspended by washing with $5 \times 10^{-4}M$ ethylenediaminetetraacetic acid and brief incubation with 0.05 percent trypsin. The trypsin was removed by centrifugation, and the cells were incubated in 0.4 ml of undiluted AS at 0°C for 30 minutes with vortex mixing at 5-minute intervals. The AS was removed by centrifugation, and the cells were resuspended in 12 ml of C′ (*5*) diluted to 20 to 25 percent in medium 199 (*6*). The cells were returned to culture and regrown to a monolayer; they were then resuspended and exposed to AS and C′ as before. As a control, cells were treated as above except that the exposure to AS was omitted.

Dose response assays of cell killing by AS, performed following each selective cycle by a modification of the method of Gorer and O'Gorman (*7*), indicated that a progressive and ultimately complete loss of sensitivity to AS occurred. The initially sensitive starting population (Fig. 1, curve A) became considerably less sensitive after five cycles of exposure to AS and C′ (curve B). After six exposures to AS and C′, a substrain completely resistant to the cytotoxic effect of AS was obtained (curve C). For comparison, the control culture exposed only to C′ (curve D) remained fully sensitive. Selection experiments were also performed on two other cell strains, which were sensitive to the AS, obtained from unrelated donors. Variant substrains resistant to AS were isolated from each after six to eight selective cycles.

The basis of the resistant phenotype could be loss of a reactive isoantigen or a nonantigenic change. Absorption experiments were performed to distinguish between these possibilities. In a preliminary experiment, AS was absorbed with varying packed-cell volumes of sensitive cells (measured by capillary hematocrit) to determine the minimum volume of cells that would completely remove cytotoxic antibody from AS. Equal volumes of AS were then absorbed with this minimum packed-cell volume of sensitive cells, with equal packed-cell volumes of resistant variant substrain cells, and with cells from a nonreactive strain from an unrelated individual. The residual AS titer after absorption was determined by assay against a sensitive test strain. Equal packed-cell volumes of absorbing cells, rather than surface areas or cell numbers, were used for absorption because of the following consideration. Although the extent of absorption is a function of the total surface area of absorbing cells, surface area cannot be measured. It can be calculated from packed-cell volume and cell number if cell shape is known. Microscopic examination of suspended cells in these experiments revealed considerable heterogeneity in shape, rendering its assumption uncertain. Of the parameters that could be measured, total cell volume was used as the unit of absorption rather than cell number because cell volume reflects differences in cell surface area, whereas cell number does not.

The resistant variant substrain did not remove antibody from the AS, as evidenced by cytotoxicity remaining in AS after absorption (Fig. 2, curve G). An equal packed volume of the sensitive cell strain absorbed out the antibody completely (curve H). Cells from a nonreactive strain failed to remove antibody from the serum (curve I), indicating that significant nonspecific absorption did not occur. It is concluded from these data that AS resistance is associated with loss of surface antigenic receptors reactive with AS. If we assume that the cell shape was spherical, the ratio of total surface areas calculated from the packed-cell volumes and cell numbers would have been 1.0, 0.78, and 0.88 for sensitive, resistant, and neutral cell strains, respectively. Because of the possible discrepancy (22 percent) in surface areas of sensitive and resistant cells used for absorption, we cannot be certain from the present data whether the loss of the reactive

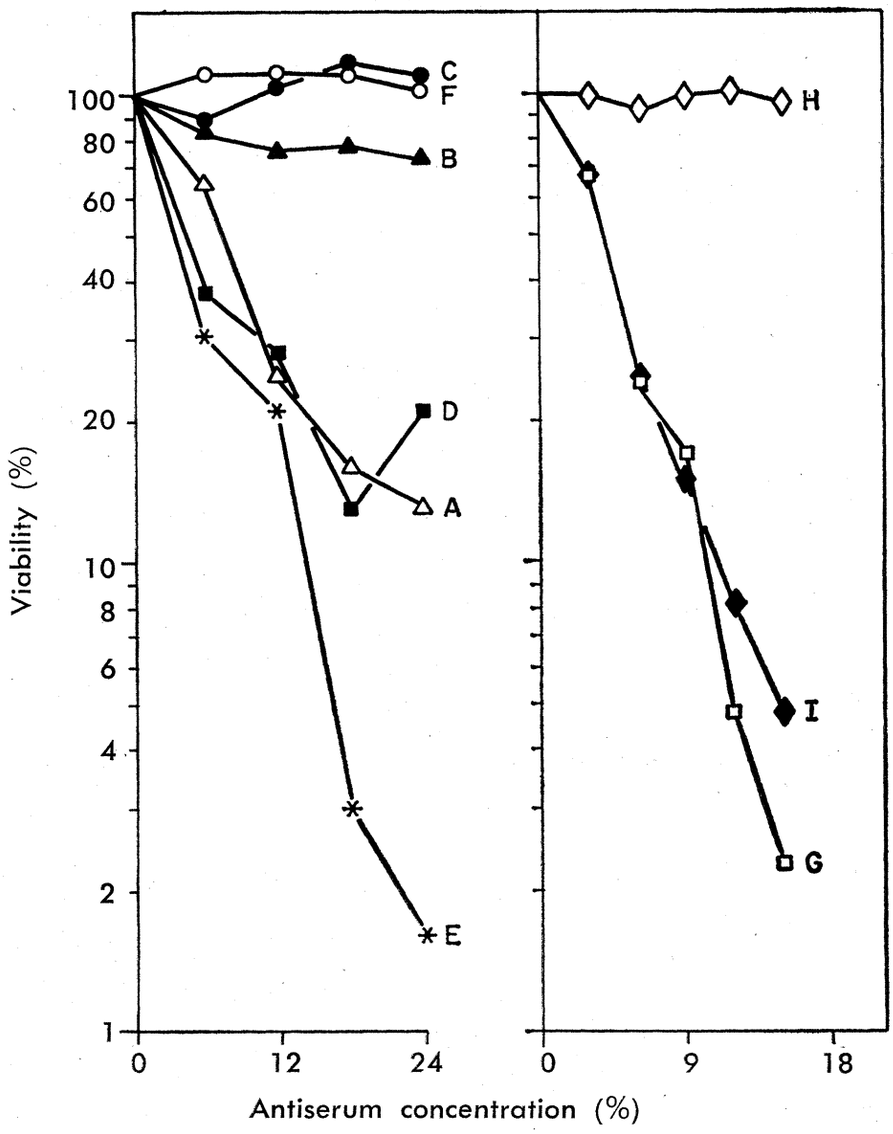

Fig. 1 (left). Dose response of cycled cell strains to AS, which was determined as follows. The indicated concentration of human AS, 10^3 cells, and C′ at a final concentration of 33 percent in a total volume of 30 μl were incubated for 90 minutes at 37°C. Incubations were carried out under an atmosphere of 97 percent air and 3 percent CO_2, with mixing at 20-minute intervals; 10 μ1 of 1 percent trypan blue dissolved in 0.85 percent NaCl was then added and mixed, and the incubation was continued for 30 minutes at 37°C. The tubes were then set in ice and the proportion of unstained (or viable) cells to total cells was determined with a hemacytometer (100 to 200 total cells were counted per point). (Curve A) Cell strain prior to selective cycling; (curve B) cells cycled five times with AS and C′; (curve C) cells cycled six times with AS and C′; (curve D) cells cycled with C′ only; (curve E) cells cycled with C′ only and grown for an additional 20 generations in the absence of C′; and (curve F) cells cycled with AS and C′ and grown for an additional 20 generations in the absence of AS and C′. Fig. 2 (right). Dose response with absorbed AS against a sensitive test strain. Packed cells (0.1 ml) obtained after centrifugation for 10 minutes at 3000*g* were used to absorb the AS. The packed cell volume was determined by centrifugation of the cell suspension in a capillary hematocrit tube. To the cell pellet was added 0.3 ml of 50 percent AS with mixing, initially by pipette, and subsequently at 5-minute intervals with a vortex mixer. The serum was absorbed for 20 minutes at 0°C, the cells removed by centrifugation, and the AS passed through a Millipore filter (pore size, 0.8 μm) to remove unsedimented cells. The absorbed AS was assayed as given for Fig. 1. The AS was absorbed with the parent sensitive cell strain (curve H), the resistant variant substrain isolated from the parent strain (curve G), and a nonreactive cell strain obtained from an unrelated individual (curve I).

isoantigens in the resistant substrain is complete.

In order to be certain that the loss of surface isoantigens found in the resistant substrain did not merely reflect a loss by senescent cultures of the ability to express isoantigens, cells that had been cycled in C′ as controls were grown in nonselective media to near the end of their capacity to grow in culture. These cells retained full sensitivity to AS (Fig. 1, curve E), which indicates that in vitro aging cannot account for the resistance of the selected substrain. It was also of interest to determine whether the resistant phenotype of the variant substrain would be retained in the absence of additional selection after isolation. For this purpose, cells from the resistant variant strain were tested immediately following selection (Fig. 1, curve C) and after 20 additional cell doublings in normal growth medium (curve F). Resistant cells remained resistant in the absence of further selection following isolation.

Assuming that antiserum selects for preexisting variants, an estimate of the resistant fraction present in the original sensitive population can be made, based on the efficiency of selection and the size of the selected population. In the reconstruction experiments, a tenfold enrichment for resistant variant cells occurred with each selective cycle, that is, 90 percent of the sensitive cells were killed each time, while none of the resistant cells were affected. At least six cycles were needed to isolate stable resistant subpopulations of cells. Since there were 10^7 cells in the original population selected against, the apparent frequency of resistant cells in the original sensitive population was of the order 10^{-5} to 10^{-7}.

Several aspects of the observed phenotypic change are consistent with a genetic change. The resistant phenotype apparently occurs with low frequency, and, in addition, it is stable within the limit of growth remaining after selection for these cells. The possibility that antiserum induces an alteration in the expression of the reactive isoantigens is less likely but not excluded by these studies. In the case of modulation of mouse TL antigenic expression by antiserum, the continued presence of antiserum is required to maintain the altered state (*8*); whereas in these experiments, the human isoantigen selected against did not re-

appear after 20 cell doublings in the absence of isoantiserum. Fluctuation analysis, generally used to distinguish between inductive and selective mechanisms of variation (*9*), cannot be applied to these experiments because of the inability of antiserum to eliminate all sensitive cells in a single exposure. An alternative means of making this distinction is to determine the antiserum sensitivity of clones isolated from strains showing partial resistance after several selective cycles (for example, Fig. 1, curve B). If induction is the mechanism, the clones isolated from partially resistant strains should belong to one population with intermediate resistance; whereas if the mechanism is selection for preexisting variants, the clones should form two populations, either fully sensitive or fully resistant. These studies are in progress.

Although the locus specifying the isoantigen reactive with AS in the cell strains used in these experiments is not known, it is of interest that monospecific antiserums of HL-A specificity are cytotoxic for human fibroblasts (*10*). By using these antiserums in an isoantigenic selective system in conjunction with the approximately 30 other markers known to be detectable in human fibroblasts, it may be possible to detect mitotic recombination in these cells and to determine human linkage.

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Acetylcholine Sensitivity of Muscle Fiber Membranes: Mechanism of Regulation by Motoneurons

Abstract. *Inhibitors of RNA and protein synthesis prevent the development of acetylcholine supersensitivity in organ-cultured rat diaphragm muscle but do not affect established acetylcholine sensitivity. The restriction of this sensitivity in innervated muscle apparently involves neuronal restriction of gene activity in muscle fibers.*

The distribution of active acetylcholine (ACh) receptors in skeletal muscle fiber membranes (ACh sensitivity) is controlled by motoneurons (*1*). This neuronal influence is a prime example of "neurotrophic effects" (*2*). Innervated muscle fibers are highly sensitive to ACh only in the area of neuromuscular junction but, following denervation, become quite sensitive to ACh over their entire length. The mechanism by which motoneurons regulate the distribution of ACh sensitivity and the mechanism by which ACh sensitivity is increased in denervated muscle fibers have remained unknown. I now report that the increase in ACh sensitivity in denervated muscle fibers is prevented when actinomycin D or puromycin or cycloheximide is used to inhibit RNA or protein synthesis. I conclude that the neuronal restriction of ACh sensitivity in muscle fibers involves regulation of gene activity in muscle fibers.

The left phrenic nerves of female Sprague Dawley rats weighing 100 to 180 g were cut approximately 5 to 10 mm from the diaphragm surface. At appropriate times after denervation diaphragms were transferred to organ culture (*3*). For culture each hemidiaphragm was cut to form two radial straps 6 to 8 mm wide. Each strap was pinned, slightly stretched, through a single layer of cheesecloth to a stainless steel grid. The ends of the grid were bent so that the diaphragm strap rested upon the surface of the culture medium (7 ml of medium in a 15- by 60-mm disposable plastic culture dish). Trowell T-8 medium (without chloramphenicol) was supplemented with 1 percent rat or horse serum and equilibrated at 37°C with 5 percent CO_2 in oxygen. When needed, actinomycin D

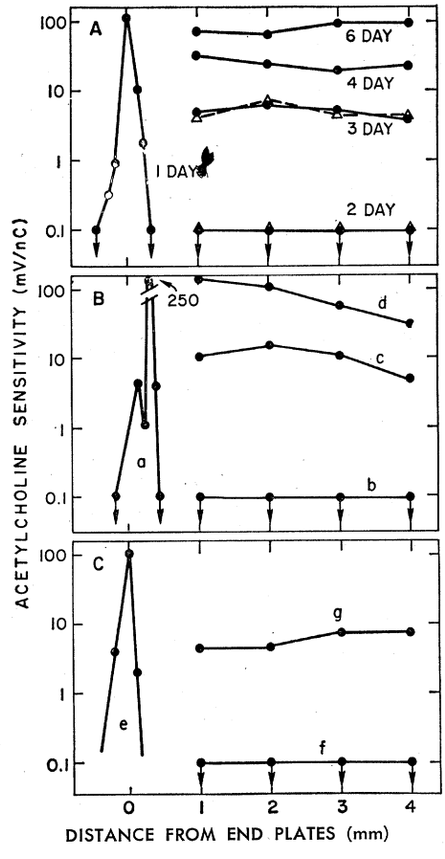

Fig. 1. (A) Acetylcholine sensitivity of rat diaphragm muscle fibers 1, 2, 3, 4, and 6 days after section of the phrenic nerve (•—•) or 2 and 3 days after transfer of normal muscle to organ culture (△—△). The arrows indicate that the sensitivity was less than 0.1 mv/nC and usually below the limit of detection. (B) Acetylcholine sensitivity of muscle fibers: (*a*) single end plate after 3 days' culture in 1 μg of actinomycin D per milliliter (ACh sensitivity was demonstrated in 25 such end plates); (*b*) after 2 days' denervation in vivo and subsequently 1, 2, or 3 days' culture in 1 μg of actinomycin D per milliliter (data from 105 fibers in 12 cultures); (*c*) after 3 days' denervation in vivo and subsequently 2 days' culture in 1 μg of actinomycin D per milliliter; (*d*) after 4 days' denervation in vivo and subsequently 1 day in culture in 1 μg of actinomycin D per milliliter. Data in (*c*) and (*d*) are representative of 152 fibers in 19 control cultures. (C) Acetylcholine sensitivity of muscle fibers: (*e*) single end plate after 3 days' culture in 10 μg of cycloheximide per milliliter (ACh sensitivity was demonstrated in 22 such end plates); (*f*) after 2 days' denervation in vivo and subsequently 1, 2, or 3 days' culture in 10 μg of cycloheximide per milliliter (data from 121 fibers in seven cultures); (*g*) after 3 days' denervation in vivo and subsequently 2 days' culture in 10 μg of cycloheximide per milliliter (data from 27 fibers in two cultures).