IN VITRO STUDIES OF MAMMALIAN SOMATIC CELL VARIATION

II. Isoimmune Cytotoxicity with a Cultured Mouse Lymphoma and Selection of Resistant Variants*, ‡

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Plates 5 and 6

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The search for suitable markers for genetic analysis of variation in cultured mammalian cell populations has led us to investigate the use of mouse isoantigens. Antigens directly reflect gene specificity and are sensitive indicators of change at their respective controlling chromosomal loci. Of the 15 or more histocompatibility (isoantigenic) loci in the mouse, genetic and immunologic information is most extensive for the H-2. This locus, which lies on the ninth chromosome, linked to genes controlling visible tail characteristics (1), specifies a complex of isoantigenic components on the cell membrane, and component composition of an H-2 isoantigen varies with each of 19 alleles presently known (2). The H-2 antigenic complex plays a prominent role in homograft rejection and induces hemagglutinins (3), leukoagglutinins (4), and cytotoxic antibody (5) in mice of a differing H-2 genotype. Although the component isoantigens specified by a single allele generally segregate as a unit, meiotic crossing over has been found, indicating that the locus is a pseudoallelic complex (2, 6). With heterozygous mouse tumor cell populations, Klein *et al.* have demonstrated heritable isoantigenic variation which is consistent with mitotic recombination within the H-2 locus (7, 8).

We have established that the H-2 isoantigenic expression can be detected in cells of long term cultured mouse lines (9), indicating that the phenotype is heritable *in vitro* and thus might be useful as a cell marker. In this paper we report that this antigenic phenotype also provides the basis for an *in vitro* immunoselective procedure—isoimmune cytotoxicity—in which cultured mouse lymphoma cells possessing the isoantigenic marker are killed by isoantibody and complement. The selection of variants resistant to the cytotoxic effects of isoantibody is described.

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Methods and Materials

Cultured Cell Line.—Clone 2B-2 (isolated July, 1960) from ML388 (10), a line of cells growing continuously in culture since its establishment, in 1956, from a methylcholanthrene-induced lymphoma of a DBA/2 mouse (homozygous for the allele H-2^d). Cells are maintained in Eagle's medium (11) supplemented with 5 to 6 per cent calf serum, 10^{-3} M pyruvate, and 10^{-4} M L-serine, under an atmosphere of 5 per cent CO_2 and 95 per cent air at 37°C, and grow as monolayers attached to the flat side of silicone-stoppered prescription bottles. The H-2d phenotype has been detected in this cell line by quantitative absorption studies (9) and by immunofluorescence (12). Karyotypic studies have established a modal number of 55 chromosomes (13).

Growth Measurements.—(*a*) *Population growth.* 4 ml aliquots of cells in growth medium (usually 10,000 cells) were inoculated into replicate 1 ounce prescription bottles and incubated. After an appropriate interval(s) the medium was aspirated, the cells suspended in isotonic saline containing formalin, 1 per cent by volume (formalin-saline), and duplicate cell counts performed using an electronic (Coulter, model B, 100 micron orifice) cell counter. Agreement between duplicate counts was usually within 5 per cent. Agreement among replicate bottles averaged within 15 per cent of the mean.

(*b*) *Viable cell count.* 500 or 1,000 cells in 4 ml growth medium were incubated for 8 days in each of four T-15 culture flasks. Cell colonies adherent to the glass were fixed with acetone and stained with 0.05 per cent crystal violet in isotonic saline. All colonies visible at a magnification of approximately 3 were counted (Fig. 1). In these experiments an average plating efficiency of 27 per cent was found for 2B-2 under normal conditions. Agreement among replicate flasks averaged within 25 per cent of the mean.

Isolation of Clonal Lines.—1 ounce prescription bottles were inoculated with approximately 60 cells per bottle. After 10 to 14 days' growth, about 10 to 20 large separate colonies were visible. With a platinum bacterial loop containing a droplet of medium, cells from a single, well isolated colony were picked up and transferred to a bottle with fresh growth medium to establish a clonal line.

Mouse Strains.—

C57BL/6J	H-2^b	Retired breeder females from incross, Jackson Memorial Laboratories, Bar Harbor, Maine
C57BL/10JHz	H-2^b	Supplied by Jackson Memorial Laboratories in 1961 and bred at Stanford
C57BL/10SnHz	H-2^b	Supplied by Dr. G. D. Snell in 1961 and bred at Stanford
C3H·SW/SnHz	H-2^b	Supplied by Dr. G. D. Snell in 1961 and bred at Stanford
C3H/SnHz	H-2^k	Supplied by Dr. G. D. Snell in 1961 and bred at Stanford
DBA/2J	H-2^d	Retired breeders *via* Simonsen Laboratories, Gilroy, California
B10·D2/SnHz	H-2^d	Supplied by Dr. G. D. Snell in 1961 and bred at Stanford
BALB/CTu	H-2^d	From the colony of the late Dr. F. C. Turner

The H-2 allele carried by any strain mentioned in this paper will be listed, in parenthesis, after the strain designation.

Isoantisera.—Hyperimmune isoantisera were prepared by repeated injections of spleen and thymus as previously described (14). Isoantisera used were

C57BL/6J(H-2^b) anti DBA/2J(H-2^d)

C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d)
C3H·SW/SnHz(H-2^b) anti C3H/SnHz(H-2^k)
B10·D2/SnHz(H-2^d) anti C3H/SnHz(H-2^k)

Unimmunized C57BL10/SnHz(H-2^b) and C57BL/6J(H-2^b) mice which had not been outbred served as sources for normal serum. Sera were not heated at 56° C prior to use in these experiments as cytotoxicity was negligible without added complement.

Complement.—Lyophilized guinea pig serum obtained from Hyland Laboratories, Los Angeles, was reconstituted with sterile distilled water and used as complement. Aliquots were frozen in sterile ampules and stored no longer than 4 weeks at −20°C.

Isoimmune Cytotoxicity.—1 ml aliquots of growth medium containing the desired number of cells were pipetted into sterile, 16 × 150 mm, screw cap, silicone[1]-coated, culture tubes containing a mixture of 5 per cent CO_2 and air. 0.2 ml of isoantiserum or normal serum, diluted appropriately with growth medium, and complement were added. The tubes were tightly capped and placed at 37°C for 30 minutes in an inclined, revolving, test tube rack (30 revolutions per minute). The cell, serum, and complement mixtures were then diluted 1:40 with growth medium and 4 ml aliquots were inoculated into replicate T-15 flasks and/or 1 ounce prescription bottles which were incubated at 37°C for an appropriate period. All growth medium used in these experiments was heated at 56°C for 30 minutes to inactivate any complement present in calf serum. Heat-inactivated medium was not inferior to unheated medium in supporting growth of cells.

Preparation of H-2 Antigen-Rich Cell Membrane Fraction of Mouse Liver.—As described (14) except that the membrane fraction was floated on the KBr solution during a 1 hour centrifugation at 37,000 *g* in the cold, resuspended in KBr, and again centrifuged at 37,000 *g*. After the second centrifugation the flotsam was transferred into phosphate-buffered saline (PBS) containing, per liter, 0.1 gm $CaCl_2$, 0.1 gm $MgCl_2$, 0.06 gm penicillin G U.S.P. and 0.1 gm streptomycin sulfate U.S.P., washed twice, stored overnight in this solution at 6–8°C, and then used for isoantiserum absorption.

Absorption of Cytotoxic Activity from Isoantiserum.—As described (14) except that isoantiserum diluted 1:5 with growth medium was absorbed twice consecutively, each time with that amount of membrane fraction containing 5 mg protein.

Quantitative Absorption of Isoantiserum with Cultured Cells.—As described in reference 9. In a typical quantitative absorption experiment 5×10^7, 2×10^7, 1×10^7, and 5×10^6 2B-2 cells were used and the number of variant cells chosen to correspond to the surface area of the absorbing 2B-2 cells.

Determination of Relative Surface Area of Variant Cells.—Suspensions of washed 2B-2 and variant cells were introduced into matched Wintrobe hematocrit tubes and packed cell volumes were determined by centrifuging in an International clinical centrifuge at 3000 RPM for 15 minutes. These determinations were performed in duplicate on different samples of the same cell suspension. The surface area of variant cells relative to 2B-2 cells was calculated from packed cell volume values.

Hemagglutination.—As described in reference 14.

Protein Determination.—Protein was determined by the method of Lowry *et al.* (15) using crystalline bovine serum albumin (Armour) as a standard.

RESULTS

Isoimmune Cytotoxicity. Isoimmune cytotoxicity is quite pronounced after exposure of 2B-2 cells to anti H-2d isoantibody and complement; 99.3 per cent of cells so treated were unable to form colonies, compared to control cells

[1] Desicote, Beckman Instruments, Inc., Fullerton, California.

exposed to normal serum and complement (Table I). Treatment with only isoantibody or only complement did not kill cells. Fig. 1 shows the cytotoxic effect evident in a typical experiment.

Conditions for Maximal Cytotoxicity.—(a) Concentration of complement. To determine the complement concentration which allows maximal cytotoxic effect of isoantibody while causing minimal cell killing with normal serum, we examined the effect of increasing complement concentrations in the presence of excess antibody (Table II). Marked cell killing resulted with 4 per cent or more

TABLE I

Requirements for Isoimmune Cytotoxicity

1 ml aliquots containing approximately 10,000 2B-2 cells were treated with normal serum (C57BL/6J(H-2^b), 1:12), isoantiserum (C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d), 1:12) and/or complement, 2 per cent. After 30 minutes at 37°C cells were diluted 1:40 and inoculated into prescription bottles (for cell counts) and T flasks (for colony counts), approximately 1,000 cells per inoculum.

Additions to cells	Colonies per flask per 1000 Cells inoculated*	Cells per bottle at 8 days referred to inoculum as 1‡
None	238	250
Normal serum	212	79
Anti H-2d isoantiserum	208	320
Complement	164	150
Normal serum + complement	181	260
Anti H-2d isoantiserum + complement	1	6

* Average of 4 flasks.

‡ Average of 4 bottles.

TABLE II

Optimum Concentration of Complement for Isoimmune Cytotoxicity

1 ml aliquots containing approximately 100,000 2B-2 cells were treated with normal serum (C57BL/10SnHz(H-2^b), 1:12) or isoantiserum (C57BL/10JHz(H-2^b) anti B10·D2/SnHz-(H-2^d), 1:12) and complement, as indicated. After 30 minutes at 37°C cells were diluted 1:40 and inoculated into prescription bottles, approximately 10,000 cells per bottle.

Concentration of complement	Cells per bottle at 6 days referred to inoculum as 1*	
	Isoantiserum	Normal serum
per cent		
0	27	37
4	1.4	55
11	1.2	8.6
25	0.9	1.4

* Average of 4 bottles.

complement, but above 4 per cent the normal serum-treated controls were seriously affected. Thus in all experiments a concentration of 4 per cent complement was not exceeded. In numerous experiments, isoantibody was as effective in killing cells with either 2 or 4 per cent complement. When the concentration was reduced to 1 per cent, cytotoxicity was submaximal.

(*b*) *Concentration of isoantibody.* Exposure to a 1:60 dilution of anti H-2d isoantiserum, in the presence of complement, was sufficient to achieve the maximal cytotoxic effect (Table III). Cell killing was halved when the isoantiserum was diluted to 1:300.

TABLE III

Titration of Cytotoxic Activity in Isoantiserum

1 ml aliquots containing approximately 10,000 2B-2 cells were treated with isoantiserum (C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d)), as indicated, and complement, 2 per cent, and aliquots of 5,000 cells were treated with normal serum (C57BL/6J(H-2^b), 1:12) and complement. After 30 minutes at 37°C cells were diluted 1:40 and 4 ml aliquots were inoculated into prescription bottles (for cell counts) and T flasks (for colony counts).

Serum dilution		Cells per bottle at 8 days referred to inoculum as 1*	Colonies per flask per 1000 cells inoculated‡	Decrease in viable cells relative to control
				per cent
Isoantiserum	1:12	6.2	16	94.0
	1:60	6.4	8	97.0
	1:300	73	134	50.2
	1:1200	180	260	3.3
Normal serum	1:12	220	269	0

* Average of 4 bottles.

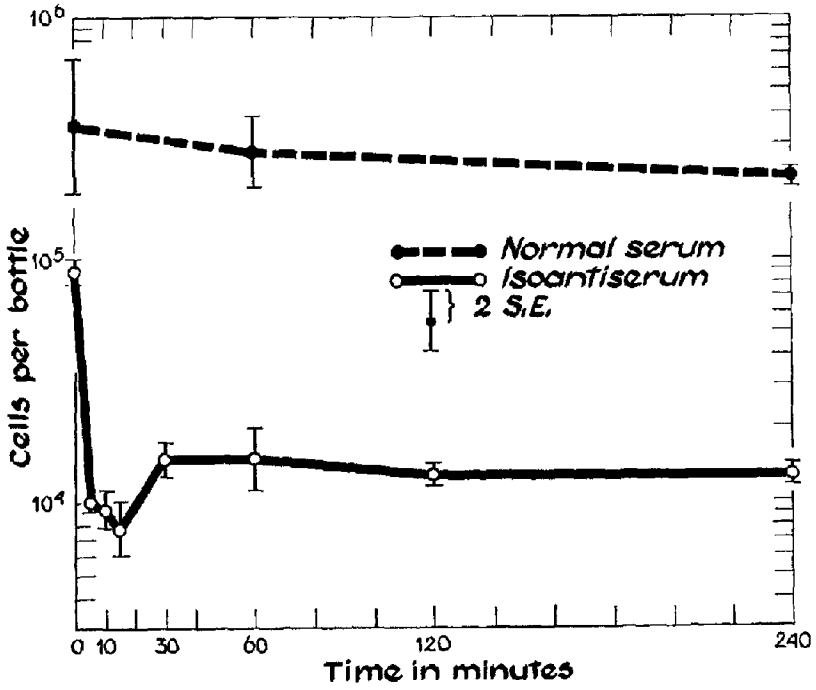
‡ Average of 4 flasks.

(*c*) *Kinetic studies of isoimmune cytotoxicity.* Attempts to maximize cell killing led to experiments in which the time of exposure of cells to isoantiserum and complement was varied prior to dilution and plating (Text-fig. 1). Aliquots were removed from mixtures of cells, complement, and isoantiserum at various intervals after mixing, and then diluted and inoculated into replicate prescription bottles. Maximal cell killing (as reflected by the number of cells in each bottle after incubation for 6 days at 37°C) occurred after exposure to isoantiserum and complement 5 to 15 minutes; longer exposure periods (varying from 30 to 240 minutes) were no more lethal.

Effect of Cell Density on Cytotoxicity.—The proportion of cells killed by isoantibody and complement is independent of the density of the cell population exposed over an 100-fold range (Table IV). There was no difference in the percentage of survivors from treated populations containing 10,000 through 1,000,000 cells per ml. Although it was not possible to count the large number

of colonies arising from the population treated at 1,000,000 cells per ml, we can infer that there was no effect on the proportion of survivors by comparing the 8 day cell counts.

Growth Kinetics after Exposure to Isoantibody.—Following treatment with normal serum and complement, 2B-2 cells grew at an exponential rate over the



TEXT-FIG. 1. Time course of cell killing as measured by population growth. Approximately 100,000 2B-2 cells per ml medium were treated with normal serum (C57BL/6J(H-2^b), 1:12) or isoantiserum (C57BL/6J(H-2^b) anti DBA/2J(H-2^d), 1:12) and complement, 2 per cent. At times indicated 1 ml aliquots were diluted 1:40 and inoculated into prescription bottles, approximately 10,000 cells per bottle. Each point is the average of four bottles 6 days after inoculation.

next 6 days after an initial slight drop in cell number (Text-fig. 2). Dilute (1:300) antiH-2d isoantiserum and complement caused a marked drop in cell number within 24 hours of exposure after which a recovery phase was noted. Cells which were not killed began to multiply and by the 5th and 6th days the population was growing at a rate similar to that of control cells. The population treated with high concentrations of isoantibody (1:30 isoantiserum) did not recover during the 6 day observational period after the initial drop in cell count.

Association of Cytotoxic Activity with Anti H-2 Isoantibody.—To date the

most effective cytotoxic isoantisera have contained anti H-2d antibody. Although a preliminary experiment with an anti "non-H-2" isoantiserum (B10·D2/SnHz(H-2^d) anti DBA/2 lymphoma L-1210 (H-2^d)) showed some cell killing (less than 20 per cent of 2B-2 cells killed), under the same conditions an antiserum containing only anti H-2d isoantibody (C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d)) killed 99 per cent of the population.

The predominant role of anti H-2 isoantibody in cytotoxicity is further demonstrated by absorption of cytotoxic activity from an isoantiserum potentially containing both H-2 and non-H-2 antibodies with an antigen-rich preparation which would be expected to absorb only the anti H-2 antibody. In

TABLE IV

The Proportion of Cells Killed by Isoantiserum and Complement is Independent of the Number of Cells Treated

1 ml aliquots containing the indicated number of 2B-2 cells were treated with isoantiserum (C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d), 1:12) or normal serum (C57BL/10SnHz(H-2^b), 1:12) and complement, 4 per cent. After 30 minutes at 37°C, cells were diluted 1:40 and 4 ml aliquots were inoculated into prescription bottles (for cell counts) and T flasks (for colony counts).

No. of cells treated	Serum	Cells per bottle at 8 days referred to inoculum as 1*	Colonies per flask per 1000 cells inoculated‡	Decrease in viable cells relative to control
				per cent
5,000	Normal serum	300	312	0
10,000	Isoantiserum	5.4	6	98.1
100,000	"	1.2	2	99.4
1,000,000	"	1.2	§	—

* Average of 4 bottles.

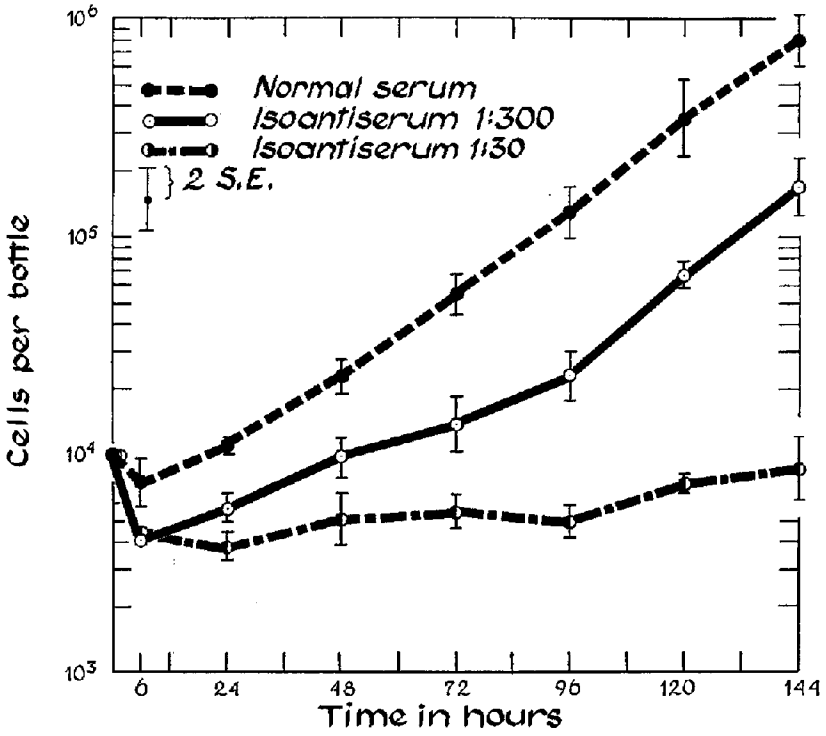
‡ Average of 4 flasks.

§ Too many colonies to count.

this experiment a C57BL/6J(H-2^b) anti DBA/2J(H-2^d) isoantiserum was absorbed with cell membrane fractions of livers (14) from C57BL/6J(H-2^b) or B10·D2/SnHz(H-2^d) mice. The fraction prepared from C57BL/6J(H-2^b) liver removed no cytotoxic activity, no difference noted between the proportion of cells killed after exposure to H-2b absorbed or unabsorbed isoantiserum diluted 1:60 (Table V). However, the antigen preparation from B10·D2/SnHz, a strain genetically similar to C57BL/6J except that it carries the H-2^d allele, removed all cytotoxic activity. The anti H-2d hemagglutinin titer of this isoantiserum (1:2560) dropped markedly after absorption with the H-2d fraction (1:20) but was unaffected by absorption with the H-2b preparation.

Isoimmune Cytotoxicity with Bicomponent Isoantiserum.—Even an isoantiserum containing antibody directed against only two of the component antigens of the H-2d isoantigenic complex was found to be cytotoxic. Approximately 82

per cent of 2B-2 cells were killed, under standard conditions, by an isoantiserum which would react with components C and H (Table VI). This isoantiserum is C3H·SW/SnHz(H-2^b) anti C3H/SnHz(H-2^k) made with a coisogenic mouse



TEXT-FIG. 2. Growth kinetics of lymphoma cells after treatment with isoantiserum and complement. Approximately 100,000 2B-2 cells per ml medium were treated with normal serum (C57BL/10SnHz(H-2^b), 1:30 or isoantiserum (C57BL/10JHz(H-2^b) anti B10·D2/SnHz (H-2^d) as indicated and complement, 2 per cent. After 30 minutes at 37°C, cells were diluted 1:40 and inoculated into prescription bottles, approximately 10,000 cells per bottle. Each point is the average of three or four bottles at the indicated time after inoculation.

pair (16), therefore containing only anti H-2k antibody. As the H-2d isoantigenic complex is known to share two components, C and H, with the H-2k complex, this isoantiserum is bicomponent with respect to H-2d isoantigens.

In Vitro Selection of Variant Cells Resistant to Cytotoxic Effects of Anti H-2d Isoantiserum.—Early in the course of study of isoimmune cytotoxicity, 100,000 2B-2 cells were exposed to C57BL/6J(H-2^b) anti DBA/2J(H-2^d) isoantiserum and complement, and surviving cells allowed to multiply. The resulting cell population was treated with isoantibody and complement and, again, survivors were propagated. This cycle was repeated 12 additional times. During initial

cycles, only a few cells survived but by the fourteenth selective procedure more cells (approximately 50 per cent) survived treatment with isoantiserum and

TABLE V

Association of Cytotoxic Activity with Anti H-2d Isoantibody

1 ml aliquots containing approximately 5 or 10 thousand 2B-2 cells were treated with normal serum (C57BL/10SnHz(H-2^b)), isoantiserum (C57BL/6J(H-2^b) anti DBA/2J(H-2^d)) or isoantiserum absorbed as indicated. Complement, 4 per cent, was added to each cell serum mixture. After 30 minutes at 37°C cells were diluted 1:40 and 4 ml aliquots were inoculated into T flasks.

Serum	Serum dilution	Absorbed with	Colonies per flask per 1000 cells inoculated*
Normal serum	1:30	—	270
Isoantiserum	1:30	—	65
"	1:60	—	95
"	1:30	C57BL/6J(H-2^b)‡	91
"	1:30	B10·D2/SnHz(H-2^d)‡	269

* Average of 4 flasks.

‡ H-2 antigen-rich cell membrane (14) fraction containing 5 mg protein each absorption.

TABLE VI

Isoimmune Cytoxicity with Bicomponent Isoantiserum

1 ml aliquots containing the indicated number of 2B-2 cells were treated with anti H-2d (C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d), 1:12), anti C,H(C3H·SW/SnHz(H-2^b) anti C3H/SnHz(H-2^k), 1:12) or normal serum (C57BL/6J(H-2^b), 1:12) and complement, 4 per cent. After 30 minutes at 37°C, cells were diluted 1:40 and 4 ml aliquots were inoculated into prescription bottles (for cell counts) and T flasks (for colony counts).

No. of cells treated	Serum	Cells per bottle at 8 days referred to inoculum as 1*	Colonies per flask per 1000 cells inoculated‡	Decrease in viable cells relative to control
				per cent
5,000	Normal	58	263	0
10,000	Anti H-2d	7.3	9	96.6
10,000	Anti C,H	20	48	81.7
100,000	"	13	§	—

* Average of 4 bottles.

‡ Average of 4 flasks.

§ Too many colonies to count.

complement. From this partially resistant population eight cell colonies were chosen at random, and clonal lines were established from them in the absence of isoantibody and complement.

Exposure, 2 months later, of these eight clonal lines to anti H-2d isoantibody

and complement revealed varying degrees of resistance to cytotoxicity (Table VII). Cells from five lines grew as well or almost as well following treatment with isoantibody as after exposure to normal serum. Descendents from some of these colonies were again tested 3 and 5 months after isolation and found to possess partial to almost complete resistance.

TABLE VII

Resistance of Variant Lines to Isoimmune Cytotoxicity

1 ml aliquots containing approximately 100,000 cells were treated with serum and complement, 2 per cent. After 30 minutes at 37°C, cells were diluted 1:40 and inoculated into prescription bottles, approximately 10,000 cells per bottle.

Variant line	Cells per bottle at 6 days referred to inoculum as 1*					
	2 months after isolation		3 months after isolation		5 months after isolation	
	Normal serum‡	Isoantiserum§	Normal serum‡	Isoantiserum§	Normal serum‖	Isoantiserum¶
1	10	12				
2	11	8.8	18	15	52	28
3	26	23	31	13	21	13
4	17	7.8				
5	9.3	12				
6	13	7.4				
7	19	14	16	20	32	28
8	14	7.2				
2B-2 cell line			30**	0.9**	57‡‡	1.6‡‡

* Average of 4 bottles.

‡ C57BL/6J(H-2^b), 1:12 normal serum.

§ C57BL/6J(H-2^b) anti DBA/2J(H-2^d), 1:12 isoantiserum.

‖ C57BL/10SnHz(H-2^b), 1:12 normal serum.

¶ C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d), 1:12 isoantiserum.

** Average of 12 bottles in three separate experiments testing variant lines 3 months after isolation.

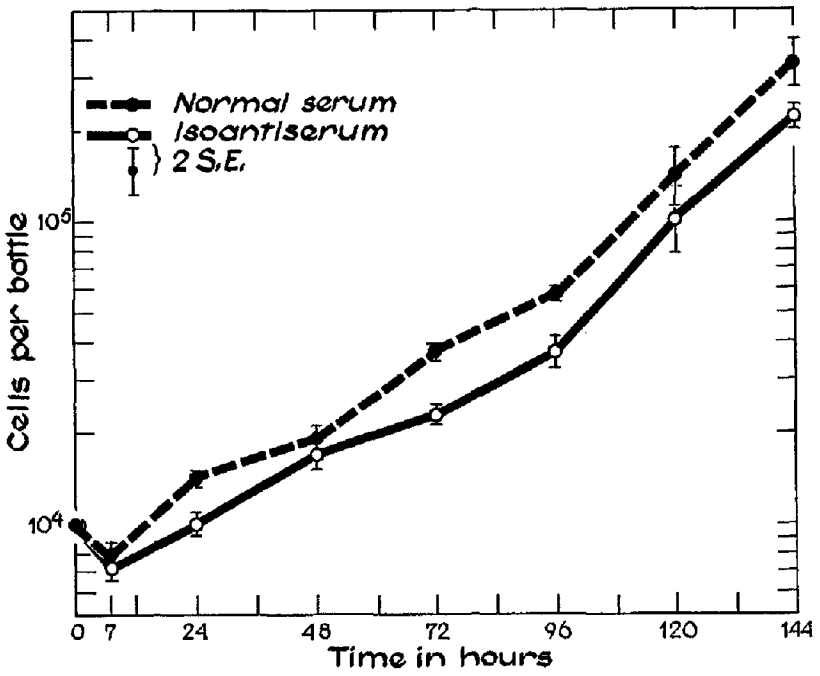
‡‡ Average of 12 bottles in three separate experiments testing variant lines 5 months after isolation.

A more detailed analysis of the growth of one clonal line after treatment with isoantibody, under standard conditions, is presented in Text-fig. 3. Only a slight depression in growth rate is noted.

Characteristics of Variant Lines.—In mass culture the growth rate of untreated variants is 25 to 30 per cent slower than that of untreated cells of the 2B-2 line.

Certain gross morphologic characteristics distinguish 2B-2 cells from the isolated variant lines. When attached to glass, cells of the 2B-2 line are round and grow in tightly packed colonies (Fig. 2 A). Fusiform cells comprise less than

1 per cent of a 2B-2 monolayer at any cell density. When visible in the gross, cell colonies are round, dense, and of smooth contour. Variant cell populations contain a high proportion of fusiform cells (Fig. 2 B), this proportion varying with the variant line and the density of the monolayer. In comparison to 2B-2, visible variant cell colonies are less dense and possess a rough contour.



TEXT-FIG. 3. Growth kinetics of variant cells after treatment with isoantiserum and complement. Approximately 100,000 variant cells (variant line 7) per ml medium were treated with normal serum (C57BL/10SnHz(H-2^b), 1:12) or isoantiserum (C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d), 1:12) and complement, 2 per cent. After 30 minutes at 37°C, cells were diluted 1:40 and inoculated into prescription bottles, approximately 10,000 cells per bottle. Each point is the average of three or four bottles at the indicated time after inoculation.

The variant cells appear larger than 2B-2 cells under phase microscopy (×400). For two variant lines determination of packed cell volume showed that variant cells occupy a larger volume than do equal numbers of 2B-2 cells (Table VIII). Further, there is approximately 1.3 times more protein per variant cell, so that the protein concentration per unit cell volume is approximately 75 per cent of the value for 2B-2 cells.

Quantitative Absorption Studies.—To investigate the possibility that isoantibody resistance of the variants resulted from complete loss of the H-2d isoantigenic complex, we tested two lines for their ability to absorb anti H-2d

antibody, as compared with the parent 2B-2 line. Table IX summarizes the results of such a quantitative absorption experiment. Both variant lines tested possess the H-2d antigenic complex. Absorption with each lowered the hemagglutinin titer of a C57BL/6J(H-2^b) anti DBA/2J(H-2^d) isoantiserum. However

TABLE VIII

Comparison of 2B-2 Cell Line with Two Variant Lines Resistant to Cytotoxic Isoantibody for Various Parameters Studied

	2B-2 line	Variant line 3C*	Variant line 7
Degree of resistance‡	2.8§	89§	88§
Packed cell volume relative to 2B-2 line‖	1	1.8	1.7
Cell surface area relative to 2B-2 line	1	1.5	1.4
Cell protein relative to 2B-2 line¶	1	1.4	1.3
Cell morphology	Predominantly round cells	Predominantly fusiform cells	Predominantly fusiform cells

* This line was isolated from cells of variant line 3 which had survived two further exposures of anti H-2d isoantiserum and complement.

‡ $\frac{\text{Cells per bottle 6 days after isoantiserum exposure}}{\text{Cells per bottle 6 days after normal serum exposure}} \times 100.$

§ These data are derived from tests of variant lines for resistance to anti H-2d isoantibody, performed 5 months after isolation.

‖ Average of two determinations with different samples of each cell suspension.

¶ Average of two determinations with different samples of each cell suspension.

TABLE IX

Quantitative Absorptions with 2B-2 Line and Variant Lines Resistant to Anti H-2d Isoantibody

Absorbing cell population	Serum absorbed	Erythrocytes	Reciprocal hemagglutinin titer of serum after absorption with		
*Surface area units**			2B-2	3C	7
2	C57BL/6J(H-2^b) Anti DBA/2J(H-2^d)	H-2^d‡	<20	40	80
1	" "	"	20	80	160
0.5	" "	"	40	1280	320
0	" "	"	2560	2560	2560
2	B10·D2/SnHz(H-2^d) Anti C3H/SnHz(H-2^k)	H-2^k§	2560		
0	" "	"	5120		

* One unit is the surface area of 1×10^7 2B-2 cells, equivalent to 6.7×10^6 cells from variant line 3C or 7.1×10^6 cells from variant line 7.

‡ B10·D2/SnHz(H-2^d) erythrocytes.

§ C3H/SnHz(H-2^k) erythrocytes.

there appears to be less isoantigen per cell in the variant lines than in the 2B-2 line. Variant cells absorbed less hemagglutinating activity than did populations of 2B-2 cells equivalent in surface area to that of absorbing variant populations.

Absorption of B10·D2/SnHz(H-2^d) anti C3H/SnHz(H-2^k) isoantiserum with 30 to 50 million cells from the variant lines tested did not affect the hemagglutinin titer. Thus the absorption by these cells of anti H-2d hemagglutinins is immunologically specific.

DISCUSSION

The studies presented here represent the first in which *in vitro* growth has been used to assess the cell killing effect of *isoantibody*. Previously cytotoxic activity has been demonstrated by supravital staining of normal or tumor mouse cell suspensions to which isoantiserum and complement had been added (5, 17). Amos and Wakefield (18) measured cytotoxicity *in vivo* by counting, at appropriate intervals, the number of mouse lymphoma cells growing in diffusion chambers implanted in the peritoneal cavities of mice injected with anti H-2 isoantibody. Using more potent *heterologous antibody*, Oda and Puck (19) have measured colony formation from single HeLa cells plated in medium containing antibody, and Roizman and Roane have developed an assay for cytotoxicity in culture based on failure of killed cells to support virus multiplication (20). The *in vitro* cytotoxic action of antibody has been shown by various workers to require complement (5, 17, 19–21), and quantitative studies presented here have again established this requirement.

We have applied the cytotoxic properties of isoantibody to cell culture to develop an immunoselective tool for the study of somatic cell variation. The marked sensitivity of lymphoid cells to cytotoxic isoantibody has persisted in the cultured cell line used for these studies. The line, established from a mouse lymphoma, possesses transplantation and morphological characteristics similar to the original neoplasm and is quite sensitive to the complement-dependent, cytotoxic action of anti H-2 isoantibody. Slightly more than 99 per cent of these cells are killed under optimal conditions. If the frequency of isoantigenic variation is governed by rates of mutation and/or genetic recombination of the magnitude found in populations of microorganisms or by rates of variation recently reported for some markers in mammalian cell culture (22, 23), the selection of isoantigenic variants will have to be achieved by repeated passages of populations and survivor populations through isoantibody and complement.

For analysis of isoantigenic variation, the importance of studying the phenotypic expression of a single strong histocompatibility locus, *e.g.* the H-2 locus, has been stressed (24). The major role of the H-2 locus in the potential *in vitro* immunoselective method presented here has been suggested by past studies of cytotoxic isoantibody (5, 17) and in the present study by (*a*) loss of cytotoxic activity from an isoantiserum containing anti H-2 and anti non-H-2 antibodies

by absorption with an H-2 antigen-rich fraction and (*b*) achievement of maximal cell killing with isoantiserum containing only anti H-2 antibody. Cytotoxicity with even anti H-2 bicomponent isoantiserum further suggests the possibility of selecting for variation of single or few isoantigenic components offering hope for *in vitro* exploration of the fine structure of a mammalian chromosomal locus.

The advantages attending the use of the H-2 antigenic phenotype as a marker for analysis of somatic cell variation have been discussed by Klein (24). Extensive *in vivo* genetic studies of this locus serve as references for interpretation of *in vitro* findings and for correlative studies of *in vivo* and *in vitro* phenomena. Detection, by hemagglutinin absorption, of the H-2d isoantigenic complex on 2B-2 cells growing in long term culture indicates the *in vivo* phenotype is stable and heritable under the stringent conditions imposed by the *in vitro* environment (9). As shown here the H-2 phenotype provides the basis for a potential immunoselective technique, similar to the homograft reaction in that cells possessing an incompatible antigen are killed, the selective advantage existing for alteration of the antigen.

Employing the homograft reaction to study variation at the H-2 locus, Klein and coworkers have selected from tumor cell populations, heterozygous at the H-2 locus, variants which have lost the antigen(s) specified by one or the other H-2 allele (7, 8, 24). The basis for this loss is not known, but a genetic phenomenon is suggested by the stable and heritable nature of the change. As in Klein's experience, selection of isoantigenic loss variants should be most frequent from cell lines heterozygous at the H-2 locus. The susceptibility of such cells to isoantibody can be altered by mutation or loss of a single H-2 allele or by change in its phenotypic expression. If alteration in the cell genetic material affecting the relevant allele is compatible with viability, variants will survive exposure(s) to isoantiserum and complement and, upon plating and incubation, will grow into a population. With cultured 2B-2 cells, presumably homozygous for the $H\text{-}2^d$ allele, alteration in both alleles is required to overcome the selective pressure imposed by isoantibody. Thus, establishment of heterozygous cell lines in long term culture—from lymphomas, other tumors, and from normal tissue—is being pursued in this laboratory.

Should established lines of heterozygous cultured cells be sensitive to cytotoxic isoantibody, the problem of detecting and selecting for alteration at a single H-2 locus in an aneuploid cell may be encountered.

Selection of variants from the homozygous 2B-2 cultured cell line is reminiscent of the isolation of non-specific variant tumors from near diploid tumor cell populations homozygous at the H-2 locus (25). These variant tumors grew in hosts of several H-2 genotypes, possessed serologically detectable but reduced isoantigen and were karyotypically near tetraploid (25, 26). Our cultured variant cell lines have retained at least partial resistance to isoantibody after 5 months (subcultured every 7 to 10 days) in the absence of isoantibody and

complement, suggesting that the change, whatever its basic nature, resulting in resistance is heritable. The H-2 isoantigenic complex is detectable serologically but in reduced quantity in the cultured cell variants. Recently Roizman and Roane selected human cultured cell variants resistant to the action of heterologous antibody and complement. These investigators were unable to explain the basis for the altered reactivity of variant cells (20).

The consistent finding, by quantitative absorption, of less H-2d isoantigen on cells of two resistant lines than on cells of the 2B-2 parent cell line could be a consequence of increased variant size. When correcting for differences in absorbing surface area, populations of larger cells with the same amount of antigen per cell as on the smaller 2B-2 cells will possess less total absorbing antigen. The slight increase in cell size might even account for resistance to cytotoxic antibody by altering the spatial relationships for optimum binding of antibody and complement. Should the concentration of isoantigenic sites on the surface of variant cells be reduced below that of 2B-2 cells, resistance might be overcome by exposing variants to complement and mixtures of isoantisera containing antibody to all components of the H-2d antigenic complex, if the concentration of reacting antigenic sites is critical for the cytotoxic phenomenon (27).

It is unlikely that resistance to isoimmune cytotoxicity represents contamination with another cell type. During the period of selection a number of cultured mouse cell lines was being carried in the laboratory. Cells from three of these lines, established from animals which did not possess the H-2^d or H-2^a allele, would not be expected to absorb anti H-2d hemagglutinins as did the variants. Three cell lines were established from heterozygous mice, H-2^d/H-2^b, and should absorb both anti H-2b and anti H-2d isohemagglutinins; variants absorbed only anti H-2d antibody. One cell line, derived from an animal homozygous for the H-2^d allele, can easily be distinguished morphologically from variant lines. Cells of this line are fibroblastic, are larger than variant cells and grow slowly, as a syncytium.

SUMMARY

When long term cultures of mouse lymphoma cells, known to possess the isoantigenic phenotype determined by the H-2^d allele, are incubated with anti H-2d isoantibody and guinea pig complement, slightly more than 99 per cent of cells are killed under optimal conditions. Growth in mass culture and colony formation by single cells after incubation with isoantibody and complement are employed to assess the cytotoxic effect. The cytotoxic action of isoantibody is complement-dependent, for viability of cells exposed to antibody alone is unaltered. When excess isoantibody and optimum concentrations of complement are used, killing begins as soon as these reagents are mixed with the cells, and no further killing occurs after 5 to 15 minutes at 37°C. About 80 per cent

of cells are killed with an isoantiserum containing antibody to two isoantigenic components of the H-2d complex. That the cytotoxic action is mediated through the H-2 isoantigen is shown by (*a*) isoantiserum containing only anti H-2d antibody produces maximal cell killing, and (*b*) isoantiserum from which anti H-2d antibody has been removed by absorption loses all cytotoxic activity.

Variant cells resistant to the cytotoxic action of anti H-2d isoantibody were isolated from lymphoma cell populations surviving multiple exposures to isoantibody and complement. These variants can be distinguished morphologically from the isoantibody-sensitive parent cell line. Although variants are resistant to anti H-2d isoantibody, these cells possess H-2d isoantigen but in a lower concentration than found in cells of the parent line. The basis for resistance to cytotoxic isoantibody is discussed.

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EXPLANATION OF PLATES

PLATE 5

FIG. 1. Appearance of cell colonies in T flasks 8 days after exposure to isoantiserum or normal serum and complement.

Left. The T flask was inoculated with approximately 1000 2B-2 cells from a population exposed to C57BL/10JHz(H-2^b) anti B10·D2/SnHz(H-2^d) isoantiserum, 1:12, and complement, 2 per cent. × 3.

Right. The T flask was inoculated with approximately 500 2B-2 cells from a population exposed to C57BL/10SnHz(H-2^b) normal serum, 1:12, and complement, 2 per cent. × 3.

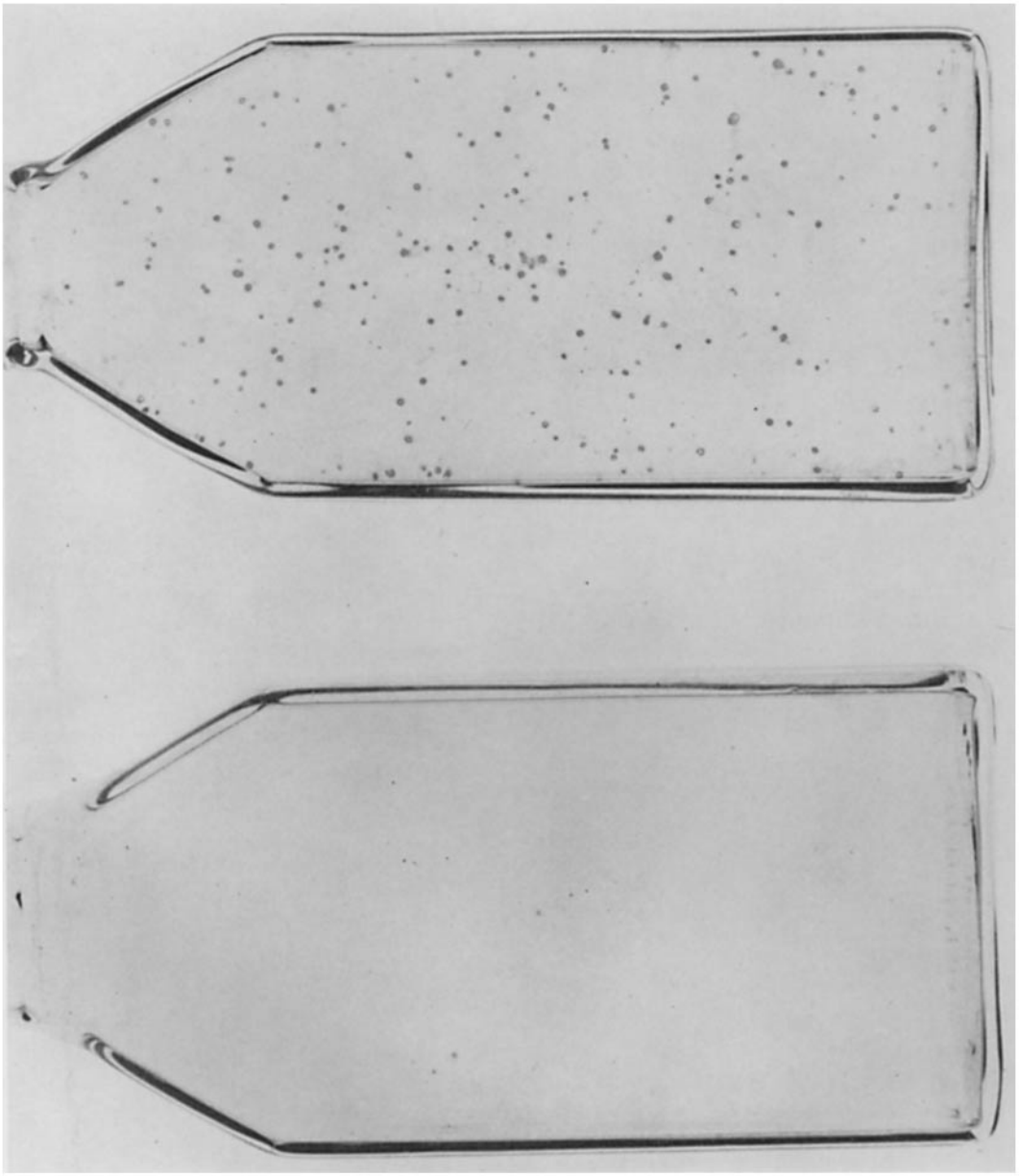


FIG. 1

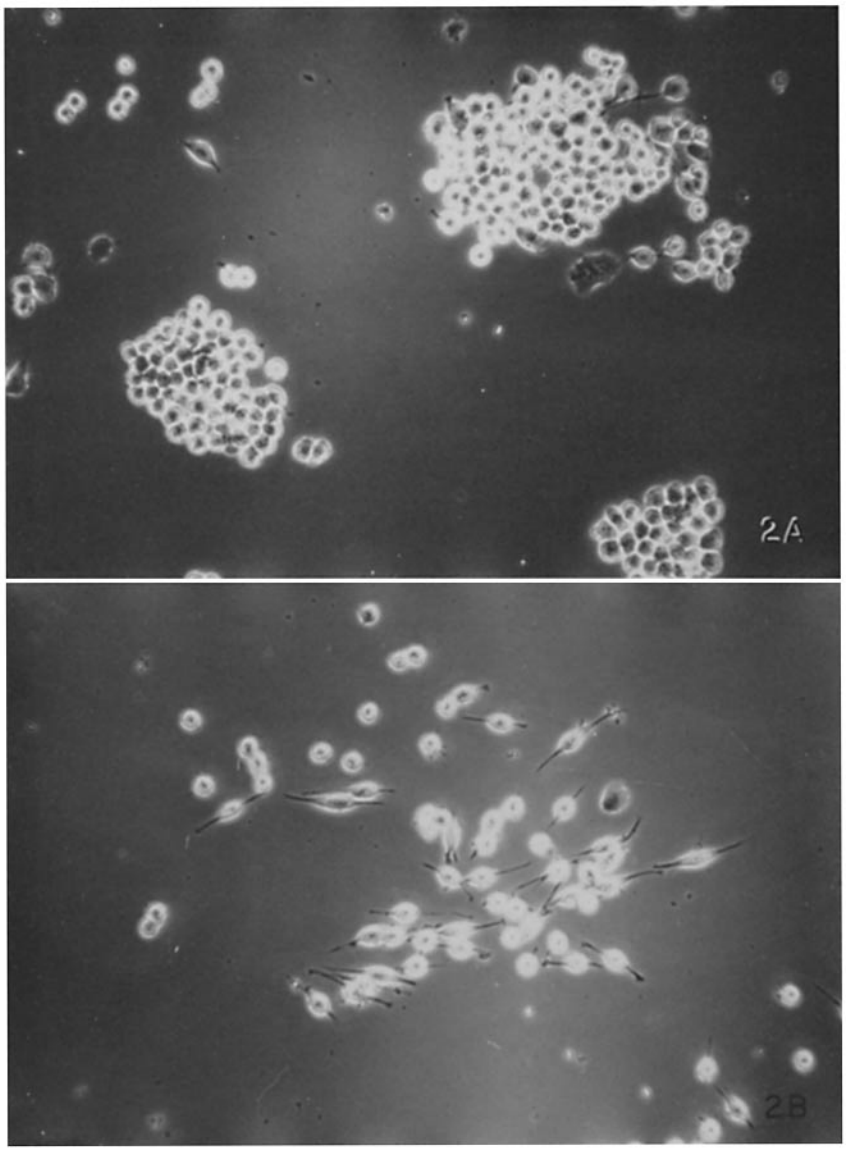
(Cann and Herzenberg: Mammalian somatic cell variation. II)

PLATE 6

Phase contrast photomicrographs of 2B-2 cells (parent line) and variant cells growing in culture.

FIG. 2 A. The predominantly round cell morphology of the 2B-2 line is evident. Cells are growing in a tightly packed colony. × 80.

FIG. 2 B. Variant cells from clonal line 3. Note that these cells are fusiform, long, and stretched. Cells are growing in a loosely packed colony. × 80.



(Cann and Herzenberg: Mammalian somatic cell variation. II)