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Isolation and Characterization of an Isoantigenic Variant from a Heterozygous Mouse Lymphoma in Culture [1]

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ABSTRACT A cell line from a mouse lymphoma heterozygous at the chromosome region for the H-2^d and H-2^k alleles was originally obtained from a transplantable lymphoma in the (C3H × DBA/2)F_1 hybrid (H-2^d/H-2^k) and cultured *in vitro*. The original cultured line, termed parent line, was susceptible to the cytotoxic action of antibodies directed against antigenic components of both the d and k alleles. The parent line also absorbed hemagglutinins from both anti-d anti-k antisera. A resistant, variant subline was selected from the original population by immunoselection *in vitro* with anti-H-2^d antibody and complement in a cytotoxic system. After one year in continuous culture in the absence of selecting antisera, the variant subline was still resistant to the cytotoxic action of anti-H-2^d antibody. Serologic analysis of the variant indicated that it had lost the D antigenic component of the d allele, had a reduced amount of the H component, controlled by both the d and k alleles, and had retained the K component of the k allele. Possible genetic mechanisms that might account for the emergence of the variant line are discussed. While the results do not necessarily support an analysis based on mitotic recombination, ascribing other mechanisms is also difficult because of aneuploidy in the cell line. Finally, the experiments point out the advantages of using *in vitro* immunoselective methods in the genetics of mammalian somatic cells.

Genetic analysis of mammalian somatic cell variation *in vitro* is dependent upon the availability of appropriate markers (Herzenberg, '62; Spencer et al., '64). For this purpose the mouse H-2 histocompatibility antigens are particularly suitable since they are located on the cell surface of red blood cells and other tissues (Herzenberg and Herzenberg, '61; reviewed in Amos, '64). There is also an extensive background of information on genetic variation at the H-2 locus in mice (Snell, '63; Stimpfling and Snell, '62) which is in linkage group IX (Allen, '55), and is the strongest of the loci affecting tissue transplantation immunity. The H-2 antigens induce a vigorous homograft reaction and concomitant hemagglutinin and ctyotoxic antibody formation in mice of a differing genotype (Amos, '64).

In a previous communication Cann and Herzenberg ('63a) reported that H-2 antigens could be detected on mouse cell lines maintained *in vitro* for long periods. This report established that the *in vivo* phenotype was stable and heritable under *in vitro* culture conditions. In additional studies, using a cytotoxic system as an immunoselective method, variant sublines resistant to the action of anti-H-2^d antibody and complement were isolated from the parent line-lymphoma ML-388, homozygous at the H-2 locus (Cann and Herzenberg, '63b). Analysis of these variants indicated they had a detectable decrease but not a complete loss of H-2 antigens. Thus the resistant variants isolated from this cell line could not be considered qualitative isoantigenic variants.

The H-2 antigens specified by an allele at the H-2 locus can be divided further into a set of individually detectable components which generally segregate as a unit (Stimpfling and Snell, '62). However, a number of crossovers have been reported, suggesting that the locus is complex and best described as the H-2 region (Stimpfling and Snell, '62; Amos, '64). Selection of isoantigenic variants should be most

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readily accomplished with cell populations heterozygous at the H-2 region (Lederberg, '56), and this concept has been tested by a number of laboratories using *in vivo* techniques. Since the antigens of the H-2 region are inherited as co-dominants, the homograft reaction in the incompatible parent line has been used as a selection system for tumor cells originating in an F_1 hybrid (Mitchison, '56; Klein and Klein, '56; E. Klein, '61; Hellström, '61; Klein and Klein, '64; Dhaliwal, '64; Ozer et al., '66). Variant lines were selected *in vivo* from mouse tumor cell populations heterozygous at the H-2 region, by growth in the incompatible, homozygous, parent-line host. The Kleins' studies have demonstrated that heritable isoantigenic variants could be isolated from the parent population and that these variants had lost some of the antigenic components specified by one of the alleles. The apparent polar loss of antigenic components has been cited as evidence that the variants have arisen by mitotic recombination within the H-2 region (E. Klein, '61; Hellström, '61; Klein and Klein, '64; Ozer et al., '66).

Selection of isoantigenic variants *in vitro* should be more readily accomplished with cell lines heterozygous at the H-2 region as in the Kleins' studies using *in vivo* methods. With the cell line studied previously by Cann and Herzenberg, which was originally homozygous at the H-2 region ($H\text{-}2^d/H\text{-}2^d$), alterations in both alleles would be required to give rise to an antigenic loss variant. Susceptibility of heterozygous cells to isoantibody and complement can be altered by mutation, mitotic recombination, or loss of a chromosome or chromosomal portion in the H-2 region. Viable cells arising from alterations affecting the relevant allele would be expected to survive exposure to an isoantiserum directed against antigens controlled by that allele and give rise to a variant population. In the present study we have derived a cultured cell line from a lymphoma heterozygous at the H-2 region for the $H\text{-}2^d$ and $H\text{-}2^k$ alleles. The parent cell line was obtained by culturing a lymphoma which was a transplantable tumor in the F_1 hybrid between the C3H and DBA/2 strains. A variant line was selected from the original population by immunoselection *in vitro*, and was found to be resistant to the action of antibody directed against the "D" antigenic component of the $H\text{-}2^d$ allele in a cytotoxic test system. Serologic analysis indicated that it lost the "D" antigenic component of the H-2 allele.

MATERIALS AND METHODS

Isolation and propagation of the lymphoma DH_1 in culture. Lymphoma DH_1 was obtained from Dr. K. E. Hellström, of the Karolinska Institute, Stockholm, Sweden. At the time of receipt in May 1962, the tumor grew as a solid subcutaneous lymphoma and was readily transplantable in compatible mice. It was induced by methyl-cholanthrene (Hellström, personal communication) in the $(C3H \times DBA/2)F_1$ ($H\text{-}2^d/H\text{-}2^k$) hybrid and was maintained by serial transplants in the hybrid in our laboratory. The tumor was excised and put into Eagle's MEM medium (Microbiological Associates) supplemented with 6% calf serum, and made into a cell suspension by passage through a syringe fitted with a stainless steel screen. The resulting suspension was placed into a culture in a modified Eagle's medium (Medium 11) containing added pyruvate (10^{-3} M) L-serine (10^{-4} M) (Herzenberg and Roosa, '60) and 10% calf serum under an atmosphere of 5% CO_2 and 95% air at 37°C. Initial inocula consisted of approximately 10^6 cells/ml, and the lymphoma cells grew as a suspension in the medium above a fibroblast monolayer. Repeated subculturing of heavy suspensions (approximately 10^6 cells/ml) of the free-growing lymphoma cells resulted in the propagation of a line of cells which would grow in the medium described above (Medium 11) supplemented with a 6% calf serum, and as a lymphoma when transplanted back into $(C3H \times DBA/2)F_1$. Lymphoma cells harvested from mice inoculated with the *in vitro* propagated cells grew easily when put back into culture. The behavior of this cell line *in vitro* is described below.

Measurement of cell growth in vitro. Cell growth was measured by counting the number of cells per milliliter in a Coulter Model B electronic cell counter. The 100-μ aperture tube was used, and optimal counts for this cell line were obtained at an amplification setting of eight and aperture

current of one-fourth. Replicate growth bottles (1 oz, 6 oz, or 8 oz prescription bottles) were inoculated with 10,000 cells/ml, and growth was followed by counting aliquots of the cell suspension diluted appropriately into a counting solution containing 0.15 M NaCl and 1% Formalin.

Cell size distributions were obtained by the threshold difference method as described by Mattern et al. ('57). Mean counts and standard deviations were obtained by the use of the LINC computer, and a program for small population samples prepared by Lee Hundley and Tim Coburn, of the Instrumentation Research Laboratory, Department of Genetics, Stanford University, was developed from a formula for standard deviation in small samples:

$$\sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}{n-1}}$$

where X = cells/ml and n = sample size.

The generation time was calculated from the logarithmic portion of the growth curve.

Mouse strains and antiserum. Hyperimmune antisera were prepared by spleen cell injections as described previously (Herzenberg and Herzenberg, '61; Cann and Herzenberg, '63b). The inbred mouse strains were obtained either from the Jackson Memorial Laboratories, Bar Harbor, Maine, or from stocks maintained by brother × sister matings in this laboratory from breeders supplied by Dr. George D. Snell, of the Jackson Memorial Laboratories. Antisera specific for H-2 antigens were made by immunization of congenic strains (reviewed in Snell, '63) which differ only by the H-2 alleles they carry.

Isoantisera monospecific for the H-2 components, E and D, were prepared by Dr. Harvey Ozer, of this laboratory, and monospecific sera against components, C, D, H, and K, were obtained from Dr. Jack H. Stimpfling of the Jackson Memorial Laboratories. Antiserum against the antigenic specificity Z, which is linked to, but not a part of the H-2 region, was obtained from Dr. G. Hoecker, of the University of Chile, Santiago, Chile.

Complement. Guinea pigs were used as a source of fresh complement. The blood was collected by heart puncture into glass tubes and allowed to clot. The clot was separated from the glass and allowed to contract in the cold for 24 hours. Following centrifugation the separate serums were pooled and stored in 2 ml aliquots at − 70°C in a frozen CO_2 chest.

Isoimmune cytotoxity. Cytotoxic tests were performed by a modification of the technique described by Cann and Herzenberg ('63b). Cells were harvested in the log phase of growth, counted and suspended in a pH 7.4 buffered diluent containing NaCl, Mg^{++} and Ca^{++}, as described by Mayer (Kabat and Mayer, '61). Cells were then added to duplicate reaction tubes containing buffer, 10% guinea pig serum as a source of complement, and antiserum diluted in calf serum or normal (non-immune) mouse serum. The usual incubation suspension contained 3×10^5 cells. The reaction mixture was incubated for 30 minutes at 37°C in a rotor, and the tubes were then chilled on ice to stop the reaction. The cells in the cooled reaction tubes were diluted one-thirtieth into growth bottles (three replicates for each reaction tube), so that the initial cell count in each growth bottle would be 10,000/ml. Growth was then measured by counting cells per milliliter in growth bottles with the Coulter counter at specified intervals after inoculation. Cytotoxicity was also determined by the trypan blue test as a measure of cell viability. Following incubation, cells were centrifuged at 1,000 rpm in a clinical, tabletop centrifuge, resuspended in 0.25% trypan blue dissolved in Eisen's balanced salt solution (Eisen et al., '59), and counted in a hemocytometer for the number of stained and unstained cells. As in other studies (Hellström, '61) uptake of dye correlated well with cell lysis and dye exclusion indicated viability.

Hemagglutination and quantitative absorption of isoantiserum with cultured cells. Hemagglutination was performed by a modification of a polyvinyl pyrrolidone method (Stimpfling, '61). Tubes were read after incubation for 90 minutes at room temperature and gentle shaking against a diffuse, fluorescent light background; titers were expressed as the last tube showing 1 + agglutination on a scale from 0 to 4 +.

Quantitative hemagglutinin absorption was performed as described previously (Herzenberg and Herzenberg, '61) except that the cells and antiserum were allowed to remain at room temperature throughout the absorption and centrifugation.

RESULTS

Lymphoma DH_1 was propagated serially in Medium 11 by inocula of 10–20 thousand cells per milliliter of fresh medium, as described under materials and methods. Six months after establishment of the lymphoma as a primary culture, the cells grew as round cells in suspension without attaching firmly to glass, and they have retained this characteristic for nearly two years in continuous culture. The growth kinetics and generation time of 30 hours during the logarithmic phase of growth have remained constant throughout the course of these studies.

Cells obtained from the logarithmic phase and inoculated at concentrations of 10^4 cells/ml, or above, into fresh medium would continue to grow at the same rate. Below 10^4 cells/ml the lag was in excess of seven days. By the time the cell line had been in culture for two years (May, '64) occasional inocula of 100 cells or less would grow into a population and a larger proportion appeared to stick to glass, although loosely. However, attempts to clone cells on glass in 6–20% calf serum and Medium 11 or Fischer's Medium (Fischer and Sartorelli, '64), with or without conditioning or by immobilizing them in a soft agar overlay of 0.4% Ionagar containing Medium 11 supplemented with 10% beef embryo ultra filtrate or chick embryo extract, were unsuccessful.

Cytotoxicity studies. Preliminary studies were performed to assess the effect of anti-H-2^d and anti-H-2^k antisera on the parent line. Tests using trypan blue dye uptake indicated that effective cytotoxicity could be achieved in the presence of complement with various antisera directed against the antigens of the H-2^d allele (table 1). Neither normal mouse serum alone or in combination with complement, nor complement alone, was cytotoxic to any significant degree with this cell line (tables 1 and 2).

The cytotoxic effect of certain isoantisera directed against antigens of the H-2^d allele was observed in dilutions of 1/400 (table 2). Two antisera directed against the antigens of the H-2^k allele were not so effective as the antisera against H-2^d antigens (table 2).

Cytotoxicity could also be demonstrated with an antiserum directed against non-H-2 antigens. A C3H-SW (H-2^b) anti-

TABLE 1

Cytotoxic effect of isoantisera on cultured DH_1 lymphoma cells as measured by trypan blue uptake [1]

Reaction mixture	No. cells counted	Per cent dead cells
Buffer control	27/378 [2]	7.1
Complement only	27/231	11.6
DBA/2 normal serum + complement	31/252	12.3
C3H·SW anti-DBA/2 serum (H-2^b anti-H-2^d) + complement	302/315	95.8
C3H·SW anti-DBA/2 serum + complement inactivated at 56°C for 30 minutes	17/133	12.7
C3H anti-DBA/2 serum (H-2^k anti H-2^d) + complement	148/150	98.6
C3H·SW anti-C3H serum (H-2^b anti-H-2^k) + complement	143/198	72.2
C3H·SW anti-C57Bl/10 (H-2^b anti-H-2^b — non-H-2 antiserum) + complement	34/107	31.7

[1] Reaction tubes contained suspended cells in barbital buffer pH 7.4, antiserum or normal serum 25%, and complement 25%. Following incubation, cells were centrifuged at 1000 rpm in a clinical, table-top centrifuge, resuspended in trypan blue solution at one-half volume, and counted in a hemocytometer.

[2] Fraction refers to number of stained cells/unstained cells.

TABLE 2

Titration of cytotoxic activity of different isoantisera [1]

Reaction mixture consisted of buffer, 10% guinea pig serum as a source of complement, and antiserum diluted in calf serum or control normal, non-immune mouse serum. Control tubes contained calf serum or normal serum only. Duplicate reaction tubes contained 3×10^5 cells suspended in the reaction mixture and were incubated for 30 minutes at 37°C in a rotor, chilled on ice, and then diluted into replicate growth bottles.

Reaction mixture	Cell growth at 7 days after inoculation (cells/ml × 10^4)				
	k anti-*d* [2]	*b* anti-*d* [3]	*b* anti-*d* [4] r (congenic)	*d* anti-*k* [5]	*b* anti-*k* [6] r (congenic)
Non-incubated; cells diluted in barbital buffer only	6.41(±1.95) [7]	23.2(±1.1)	15.6(±3.40)	8.05(±4.30)	— [8]
Calf serum, 10%	8.58(±2.37)	53.7(±6.4)	20.1(±2.00)	5.58(±1.54)	—
(C3H × DBA/2)F_1 normal serum, 10%	4.69(±0.34)	18.6(±1.6)	10.8(±2.10)	9.30(±4.20)	—
Antiserum and dilution					
1/10				1.08(±0.46)	
1/20			0.9(±0.50)		
1/30					1.02(±0.23)
1/100	0.87(±0.21)	2.6(±0.9)	1.3(±0.40)		
1/200	0.70(±0.12)	2.2(±0.6)	18.0(±0.55)		
1/400	0.69(±0.17)	3.2(±0.7)			
1/800	1.21(±0.32)	10.2(±3.0)			
1/1600	1.87(±0.69)	17.7(±1.6)			
1/3200	4.59(±2.18)	12.8(±1.6)			

[1] *Antisera*:
[2] C3H anti-DBA/2.
[3] C3H·SW anti-DBA/2.
[4] C57Bl/10 anti-BIO·D2 (specific anti-H-2^d from immunization in a congenic strain combination; see materials and methods.)
[5] DBA/2 anti-C3H.
[6] C3H·SW anti-C3H (specific anti-H-2^k from a congenic combination, as in (4)).
[7] The figures in parentheses refer to the standard deviation for the mean cell count indicated in each row.
[8] The solid lines refer to control values which were part of the same experiment as the d anti-k antiserum (5).

C57B1/10 (H-2^b) antiserum showed moderate cytotoxic activity in the trypan blue exclusion test (table 1).

Selection of an isoantigenic, resistant, variant subline. Selection experiments were begun, using a specific anti-H-2^d antiserum prepared in C57B1/10 mice against its congenic partner, the BIO·D2 strain. The reaction mixtures and experimental conditions were similar to those in table 2 with the following exceptions: after 90 minutes incubation at 37°C the cells were diluted 1:100 and further incubated at 37°C in 3 oz prescription bottles. By 12 days incubation, populations of cells had grown up from the tubes containing normal serum or buffer only. However, growth bottles planted from the tubes containing antiserum and complement showed only slight growth by 18 days. There was much cell debris in these cultures, and it was removed by passing the cells through sterile glass wool filters and planting the cells into new media. From 10 to 100 cells were picked up in Pasteur pipettes and subcultured into T-15 flasks. Ten days later the populations were ready for further subculture. Following two further passages in Medium 11, the two sublines derived respectively from antiserum and normal serum exposure were again subjected to cytotoxic testing. Both sublines at this time

TABLE 3

Growth of anti-d resistant variant in anti-k sera

Reaction mixture	Cell growth at 7 days after inoculation
	cells/ml × 10^4
Non-incubated; cells diluted in buffer only	45.93(±0.52)
Calf serum diluent	14.93(±0.10)
d anti-*k* (1/10) (DBA/2 anti-C3H)	3.57(±0.12)
b anti-*k* (1/10) (C3H·SW anti-C3H)	4.99(±0.09)

[1] Reaction mixture and conditions are the same as in table 2.

appeared to be more resistant than the parent line to cytotoxic lysis by specific antiserum. However, further testing of the normal serum-treated sublines indicated that their resistance to further cytotoxic action of antiserum was *not heritable*. The various sublines were repeatedly subcultured at weekly intervals in Medium 11 over a period of eight months. Growth studies made at this time indicated that

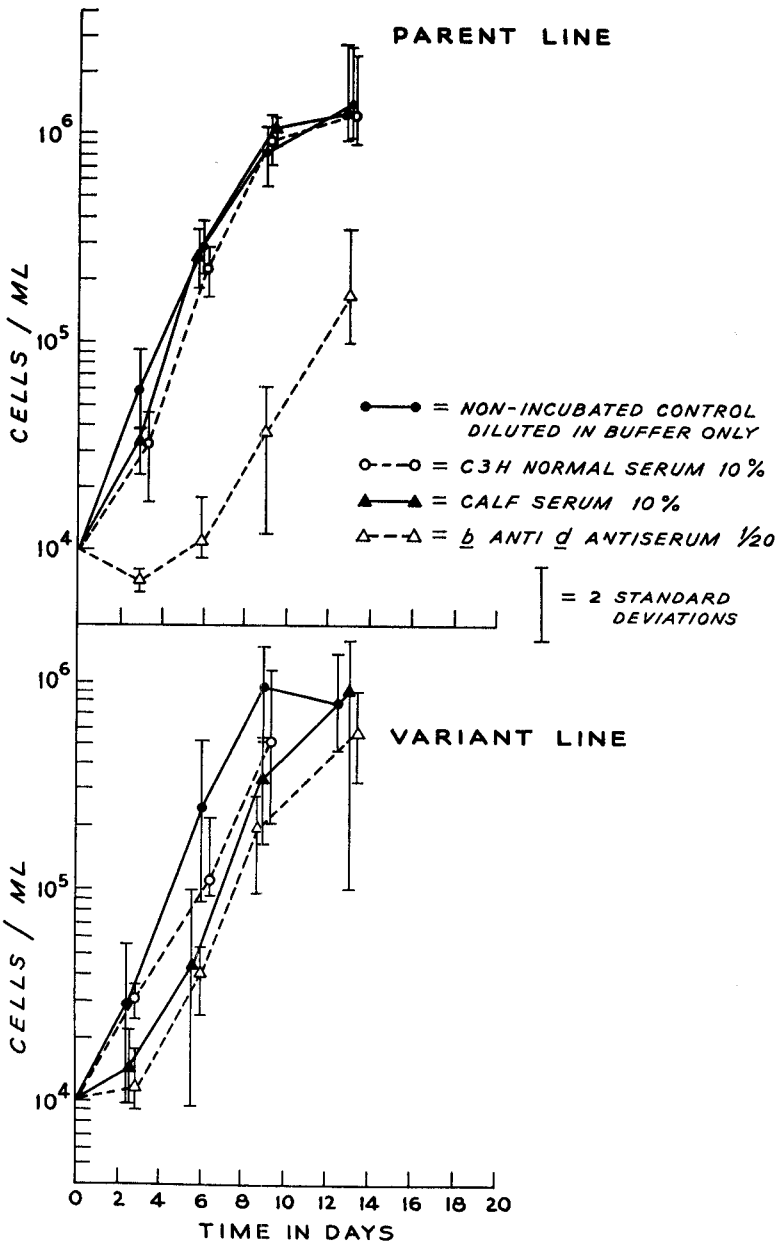


Fig. 1 Growth of parent line cells (top) and resistant variant line cells (bottom) after treatment with C57Bl anti-BIO·D2 (H-2^b anti-H-2^d) antiserum diluted 1/20 as compared with controls.

only the antiserum-treated sublines had remained heretably resistant to isoimmune cytotoxicity. This subline was termed the *variant*. Final tests performed on the variant indicated a resistance to the action of specific anti-*d* antiserum one year after its original isolation. In figure 1 the growth of the parent line of cells is compared with the variant subline after treatment with anti-*d* antiserum and complement.

The variant is not resistant to the cytotoxic action of anti-*k* antiserum (table 3). Microscopic comparison of the variant with parental cells did not reveal obvious morphologic or size differences. Figure 2 illustrates that the cell size distribution plots obtained in the Coulter counter for the parent cell line and the variant cell line do not differ. The growth rates of the two also do not differ significantly (fig. 3).

Attempts were made to isolate antigenic resistant variants after treatment with anti-*k* antisera. In these experiments a *d* anti-*k* (DBA/2 anti-C3H) serum and a *k* specific anti-H-2^k serum made in the C3H·SW strain (H-2^b) against C3H (H-2^k) spleen cells were used. Populations were recovered in some cases after treatment with anti-*k* antisera, but resistance was not maintained after repeated subculture in antiserum free medium. The resistance observed was both to anti-*k* and *d* cytotoxicity. Thus these sublines were comparable to the temporarily resistant lines isolated after treatment with normal serum and complement in the previous experiment. Several other attempts to isolate permanently resistant sublines following treatment with anti-*d* antisera were also unsuccessful.

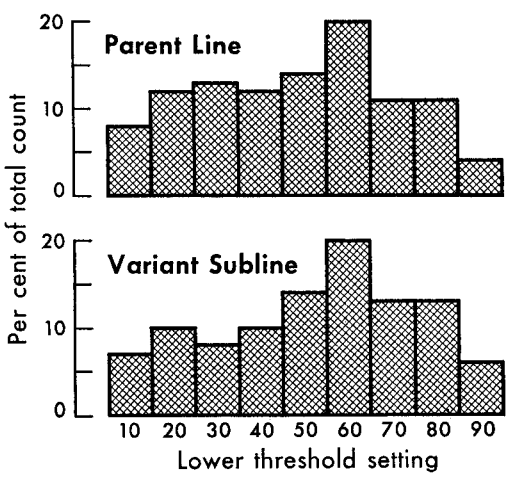


Fig. 2 Cell size distribution plots of the parent and variant sublines. The two curves were obtained by difference counts at successively higher threshold settings in the Coulter Model B electronic cell counter. The distributions reflect the broad range of cell sizes in both the parent and variant lines.

Although a heritable variant which was resistant to the action of anti-*d* antibody and complement was isolated, and could be maintained for at least a year in serial culture in standard medium, the variable findings in cytotoxic studies with this cell line deserve further comment. During the course of these studies spontaneous resistance to isoimmune cytotoxicity developed in parental line cultures on two occasions approximately six months apart. These cultures were fully susceptible to the cytotoxic action of a rabbit anti-B10·D2 mouse spleen cell antiserum, using the same source of complement as in the mouse studies. Newly thawed cultures that had been stored frozen in solid CO_2 or liquid nitrogen were always susceptible to isoimmune cytotoxicity. The growth of the cells following incubation with antiserum was always quite variable (tables 2 and 3). Therefore in each experiment the following controls were always included: C3H × DBA/2 normal and/or calf serum in the barbital buffer diluent, and barbital buffer diluent alone. Cells in the control reaction mixture containing calf serum or normal mouse serum were incubated in the rotor at 37°C, just as in the antisera-treated group, and were then diluted into growth bottles. Growth was measured as in the antiserum-treated groups. The variability in total growth and growth rates of the control groups incubated in normal serum only or buffer only is indicated in tables 2 and 3 and figure 1. Poor or erratic growth of cells from the control reaction tubes was a continuing problem necessitating many repeats of each experiment.

Hemagglutinin absorption studies of the parent line and variant subline. By the use of appropriate antiserum and red cell combinations and quantitative hemagglutinin absorption tests the H-2 antigenic components of the parental and variant

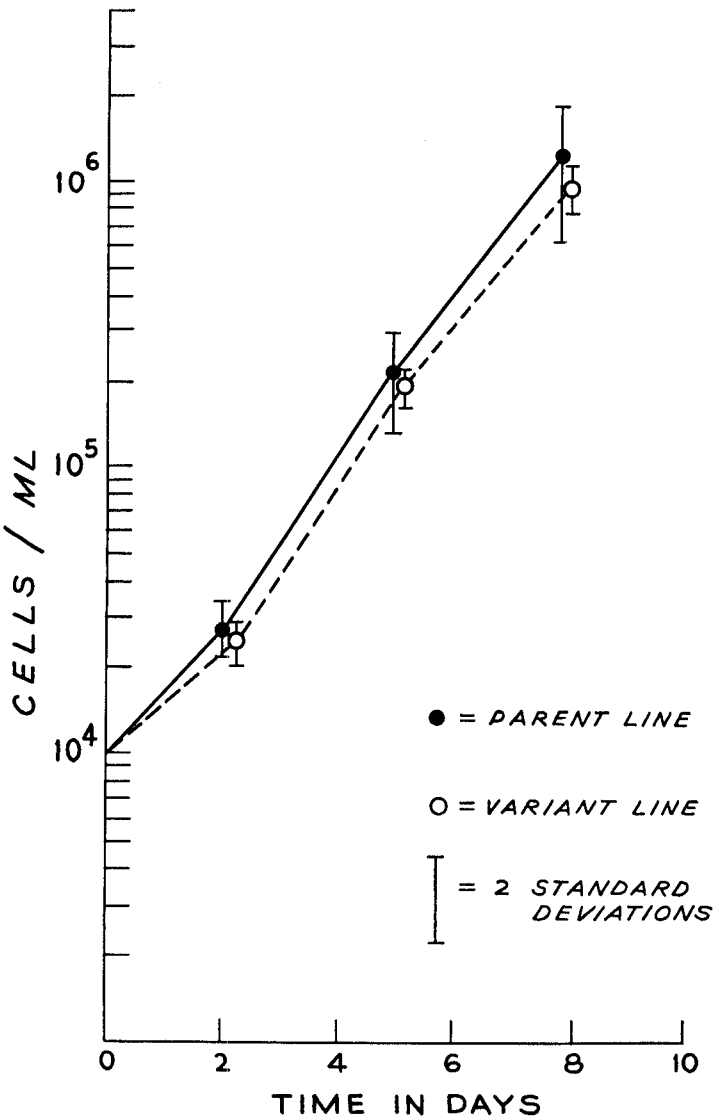


Fig. 3 Growth of parent and variant lines in the absence of antiserum. The generation time of the two does not differ significantly in Medium 11.

lines were studied. A *phenotypic* map indicating the H-2 antigenic components which have been described for the *d* and *k* alleles is illustrated below. Table 4 lists the antiserum and red cell combinations used in this study, along with appropriate combinations of antisera and red cells for detecting antigenic components of the *d* and *k* alleles. From the residual titers it can be seen that

H-2^d	CDE^{d}F	H	J	MN	A′B′C′
H-2^k	ACE	H	K	Y	

(After Stimpfling and Snell, '62)

antibodies directed against both the *d* and *k* antigenic specificities are absorbed by the

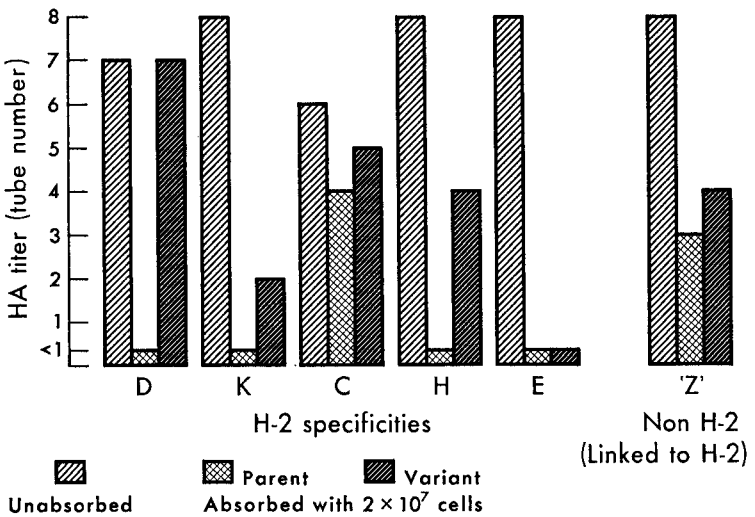


Fig. 4a Absorption of hemagglutinins from unicomponent sera by the parent line and variant subline. The vertical bars indicate residual titers after absorption with 2×10^7 cells of the parent or variant lines compared to the unabsorbed control.

parent line cells. Multicomponent and unicomponent antisera were used to detect the antigenic components of the *d* and *k* alleles. A further indication of the specificity of absorption was obtained when the parent line cells failed to absorb an A anti-A·SW serum ($H\text{-}2^a$ anti-$H\text{-}2^s$).

In contrast to the parent line, the variant subline failed significantly to absorb antibodies to the *d* specificities in these antisera. However, nearly comparable absorption of anti-*k* activity was obtained when the variant subline was compared to the parent line except with a B10·D2 anti-C3H serum. Specificity of absorption was also indicated by the variant subline when the absorbing capacity of the variant subline cells was tested with the anti-A·SW sera.

A much more reliable and complete characterization can be obtained when unicomponent sera made by specific absorption (Stimpfling and Snell, '62) or appropriate combinations of mouse strains for immunization (Stimpfling and Snell, '62) are used for these studies, as in the work of Stimpfling and of previous work from this laboratory (Cann and Herzenberg, '63b). Two unicomponent sera made by Dr. Harvey Ozer in this laboratory were used — one with specific activity against the component D, another with specific activity against the component E when used with C3H red cells. In addition unicomponent antisera against components C, D, H, and K were obtained from Dr. Jack Stimpfling and used in these studies. As with the multicomponent sera, the variant subline failed to absorb anti-D activity but it did absorb anti-K antibodies. Comparable amounts of hemagglutinin activity from the antiserums directed against the C component and the E component were absorbed by both the parent and variant lines. However a significant difference was noted in the absorbing capacity between the parent and variant sublines in absorbing anti-H activity, as can be seen in table

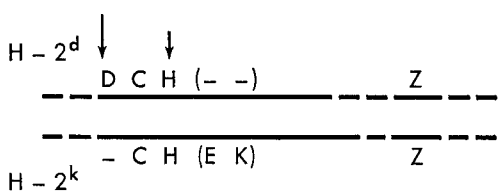
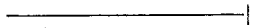
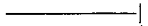
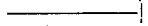
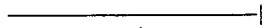
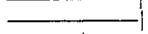


Fig. 4b Genotypic map of the antigenic components arranged in the linear order proposed by Stimpfling and Snell ('62). The components indicated are those detected on the parent and variant sublines as in figure 4a. The arrows point to the components absent or reduced in the variant.

TABLE 4

Hemagglutination absorption of isoantisera by parent and variant cell lines

Antiserum and dilution	Red cell panel [1]	Components [2]	H-2 allele [3]	Cell concentration for absorption [4]	Relative titer (log_2 units) [5]	
					Parent line	Variant subline
					0 1 2 3 4 5 6 7 8	0 1 2 3 4 5 6 7 8
b anti-d (1/100) C3H·SW anti-DBA/2	B10·D_2	CDHJME^d	d, k	—	——————————|	——————————|
				10^7	——|	——————————|
				2×10^7	—|	——————————|
				4×10^7	–|	————————|
				8×10^7	NT [6]	——————|
b anti-d (1/100) C3H·SW anti-DBA/2	C3H	CH	d, k	—	——————|	————————|
				10^7	–|	——————|
				2×10^7	–|	——————|
d anti-k (1/20) DBA/2 anti-C3H	C3H	AEKYD^k	k	—	——————————|	——————————|
				10^7	——|	————————|
				2×10^7	—|	——————|
				4×10^7	–|	————|
				8×10^7	NT	—|
d anti-k (1/20) B10·D_2 anti-C3H	C3H	AEKYD^k	k	—	——————————|	——————————|
				10^7	–|	——————————|
				2×10^7	–|	——————————|
k anti-b (1/100) C3H anti-C3H·SW	B10·D_2	FNA′B′C′	d	—	——————————|	——————————|
				10^7	——————|	——————————|
				2×10^7	——————|	————————|
k anti-d (1/100) C3H anti-DBA/2	B10·D_2	DFJMNA′B′C′E^d	d	—	——————————|	——————————|
				10^7	——|	NT
				2×10^7	—|	——————————|
				4×10^7	NT	——————————|
				8×10^7	NT	————————|
d anti-b [7] (1/10) B10·D_2 anti-C3H·SW	C3H	E	k	—	——————————|	——————————|
				5×10^6	——|	——————|
				10^7	——|	——————|
				2×10^7	–|	–|

k anti-d [7] (1/20) C3H anti-DBA/2 absorbed with AKR	D1·C	D	d	—	
				10^7	
				2×10^7	
				4×10^7	
				8×10^7	
anti-"D" [8] (1/50)	B10·D_2	D	d	—	
				10^7	
				2×10^7	
				4×10^7	
				8×10^7	
anti-"K" [8] (1/20)	C3H	K	k	—	
				10^7	
				2×10^7	
				4×10^7	
				8×10^7	
anti-"C" [8] (1/50)	C3H	C	d, k	—	
				10^7	
				2×10^7	
				4×10^7	
anti-"H" [8] (1/50)	C3H	H	d, k	—	
				10^7	
				2×10^7	
				4×10^7	
anti-"ZW" [9] (1/50)	B10·D_2	Z	non-H-2	—	
				5×10^6	
				10^7	
				2×10^7	
				4×10^7	

[1] The red cells used for testing were obtained from the mouse strains listed in this column. Hemagglutination was performed by the PVP technique described in the materials and methods section.

[2] The capital letters refer to the maximal number of antigenic components which could react with the particular antiserum-red cell combination used, according to Stimpfling and Snell ('62).

[3] The H-2 allele is listed which specifies each set of phenotypic components in the second column of the table. Two of the antigenic components, C and H, are shared by the d and k alleles.

[4] This refers to the number of DH_1 lymphoma cells from the parent and variant lines used to absorb the antiserum. Unabsorbed controls are indicated by —.

[5] The results of the hemagglutination tests are expressed as relative titers in $\log_2$ units. The beginning dilution is indicated at zero and the final titer at the end of the bar.

[6] NT absorptions were not tested when the next lowest cell concentration absorbed hemagglutinins completely, or failed to absorb at all.

[7] Sera prepared by Dr. Harvey Ozer, Department of Genetics, Stanford University.

[8] Sera obtained from Dr. Jack Stimpfling, of the Jackson Laboratories, Bar Harbor, Maine.

[9] Serum obtained from Dr. G. Hoecker, University of Santiago, Chile.

4. A further study was carried out to determine the absorbing capacity of the parent and variant sublines against the anti-Z antiserum obtained from Dr. G. Hoecker. The Z antigen is linked to, but not a part of the H-2 locus. Both the parent and variant sublines absorbed equal amounts of anti-Z antibody.

The important differences in the H-2 antigenic components of the parent and variant lines are summarized in figure 4a, where the hemagglutinin reduction of the unicomponent sera are compared at a concentration of 2×10^7 cells for absorption. In figure 4b these antigenic components detected on the parent line of cells are arranged in a *genotypic* map in the linear order proposed by Stimpfling and Snell ('62) on the basis of recombination analysis obtained from appropriate backcrosses in mice.

These results are entirely consistent with a decrease in the D antigenic component below detectable levels of hemagglutinin-absorbing activity by the variant subline in our experiments. The discrepancy in the absorbing power of the variant line with two antisera directed against antigens of the H-2^k allele, DBA/2 anti-C3H and B10·D2 anti-C3H (table 4) cannot be explained without unicomponent sera directed specifically against the antigens A, Y, and D^k. There is no significant difference between the parent and variant lines in absorbing anti-E antibodies and an insignificant difference in absorption of anti-K (table 4 and fig. 4a). Thus the apparent difference may be related to differences in the amount of the antigens A, Y, or D^k controlled by the H-2^k allele in the variant subline. Nevertheless we have placed greatest reliability on the results of absorption of unicomponent sera, and these would indicate that the variant has lost the D antigenic component and has a reduced amount of H. As is illustrated in figure 4b, the D antigenic component is a product of the H-2^d allele only, while H is controlled by both *d* and *k* alleles.

DISCUSSION

This study demonstrates that a heritable isoantigenic variant subline was isolated *in vitro* by immunoselection from a parent population of lymphoma cells heterozygous at the H-2 region. The variant maintained stable resistance to anti-*d* cytotoxicity in a test system containing isoantiserum against antigenic components of the H-2^d allele, and lacked anti-"D" absorbing power in hemagglutinin absorption tests. There was no possibility that the variant could be a contaminating cell line. The only other H-2^d containing cell line in culture in the laboratory was the homozygous (H-2^d/H-2^d) lymphoma ML-388 (clone 2B2) which differed from the variant described here in growth rate, morphology, and the fact that it could be cloned.

Variant isolation *in vitro* has certain advantages over *in vivo* techniques. Specific isoantisera can be used to select variant sublines from parent populations. These antisera can be made to react to a narrow range of antigenic components or even a single component. Thus selection can be directed more specifically to certain antigens than is possible *in vivo* using the homograft reaction as a selective agent. The rate and amount of population growth, as well as maintenance of uniform conditions for growth, and measurement of the degree of effectiveness of the cytotoxic antiserum are obviously more easily controlled *in vitro*. Disadvantages with *in vitro* methods and approaches at present include: the relatively few cultured mouse cell lines that are heterozygous for different alleles at the H-2 region, and the observation that very few cell lines in culture are susceptible to isoimmune cytotoxicity.

The antiserum used for selecting the variant, C57Bl/10 anti-B10·D2 (H-2^b anti-H-2^d), was made in a congenic strain combination (Stimpfling and Snell, '62) and had very little reactivity to the components C and H controlled by both the *d* and *k* alleles at the dilutions tested. Therefore this selective antiserum was essentially a unicomponent anti-"D" antiserum. The variant failed to absorb anti-"D" antibodies in hemagglutinin tests and also had reduced amounts of "H"-absorbing power. Further study of the effects of immunoselection *in vitro* with unicomponent sera will provide much more reliable data on antigenic loss variants than *in vivo* methods.

Previous studies from the Kleins laboratories (Eva Klein, '61; Hellström, '61; Klein and Klein, '64) have suggested that populations of tumor cells, heterozygous for the H-2 antigenic components D and K in *cis* and *trans*, can give rise to antigenic loss variants which can be isolated in parent line, homozygous mice. With D and K in *cis*, as in H-2^a, D-K- and D+K− variants were readily obtained, while D−K+ variants were never found. When D and K were in *trans*, as in H-2^d/H-2^k, the previously absent D−K+ variants were readily obtained (Klein and Klein, '64). They have postulated that the variant cell population arose by mitotic recombination within the H-2 region. In a recent study by Ozer et al. ('66) with (A × A·SW)F_1 lymphoma (H-2^a/H-2^s), a variant was isolated *in vivo* which was D+H−K−. They interpreted this result as demonstrating that H lies between D and K.

The cell line used in our study was obtained from K. E. Hellström and was heterozygous for D and K in *trans* (H-2^d/H-2^k). The variant isolated by *in vitro* selection with anti-D antiserum was also D−K+. Loss of the D component without loss of other components would be consistent with mitotic crossing over in the region to the left of D on the map, as indicated in figure 4b. However a decrease in the H component was also observed; since H is present on both chromosomes, this variant could also have arisen by a deletion in the chromosome carrying the *d* allele, or even total loss leading to monsomy of the IXth chromosome.

Although a genetic change is a likely explanation for the emergence of a D−K+ variant in our studies, the aneuploid chromosomal number of the cells prevented us from distinguishing between alternative genetic mechanisms. Other factors influencing interpretation included failure of the cells to stick to glass surfaces and failure to clone in the time period studied. Despite limitations encountered with this cell line, prospects for further studies of somatic cell genetics *in vitro* with immunoselective methods seem promising. Increasing evidence for the possibilities of mitotic crossing over in mammalian cells is accumulating (German, '64). Recombinants arising by cell fusion which contain the H-2 antigens of the parents have been analyzed (Spencer et al., '65). Induction of mitotic recombination in the fungal species *Ustilago* and *Saccharomyces* has recently been reported (Holliday, '64) and is being studied as a means of inducing mitotic crossing over in mammalian cells (Shaw and Cohen, '65).

Definitive mapping of the H-2 region by means of *in vitro* methods would, therefore, require cell lines which can be propagated in culture, and cloned while maintaining a diploid chromosome number. The paucity of cell lines which are susceptible to the cytotoxic action of isoantiserum and complement and which have an appropriate genotype for study would seem to be only a temporary problem. Linkage of the antigen Z with the H-2 region allows detection of two serologically distinct phenotypic marker groups on cell surfaces controlled by separate loci. Proof of mitotic crossing over by means of genetic markers could most readily be obtained with cells doubly heterozygous for the H-2 and Z loci, thereby facilitating fine structure exploration of the H-2 region.

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Note added in proof: After this paper was submitted for publication, a paper by Stimpfling and Richardson was published analyzing recombination within the H-2 locus. Their study presents additional data based on large numbers of backcross animals for three distinct regions of H-2; D, C, and K. (Stimpfling, J. H., and A. Richardson, '65. Recombination within the histocompatibility-2 locus in the mouse. Genetics, *51:* 831–846.)