IN VITRO STUDIES OF MAMMALIAN SOMATIC CELL VARIATION

I. Detection of H-2 Phenotype in Cultured Mouse Cell Lines*, ‡

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The phenotype specified by the histocompatibility-2 (H-2) locus in mice is an isoantigenic complex found in normal and tumor tissue and on red blood cells. Genetic analysis of the H-2 locus indicates at least 19 alleles among inbred mouse strains, each allele specifying a characteristic combination of antigenic components (1). The H-2 locus might serve as a marker for genetic analysis of variation in cultured mammalian cells if its phenotype can be readily detected at the cellular level and if this expression can identify a rare isoantigenic variant in a large cell population. Since H-2 antigens stimulate isohemagglutinin production (2), phenotypic expression should be detectable by hemagglutinin absorption, the specific removal of anti H-2 isohemagglutinins from an antiserum by cells bearing the antigen. This serologic method has been employed to detect H-2 isoantigen (3) and to confirm isoantigenic variation (4) in transplantable tumor cell populations. The initial phase of an inquiry into somatic cell variation, *in vitro*, has been concerned with detection of the H-2 phenotype in cultured cell lines. The results presented here indicate that the phenotype can be readily detected in these lines by hemagglutinin absorption.

Materials and Methods

Cultured Cell Lines.—Clone 2B-2 (isolated July, 1960) from ML388, 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) (5). These cells grow as a monolayer attached to the flat side of silicone-stoppered prescription or culture bottles. On microscopic examination attached cells are seen to be round. Cell suspensions are prepared by scraping the cells from the glass.

B-th, a line of cells growing continuously in culture since its establishment, in 1961, from the thymus of a C57BL/Ka leukemic mouse (homozygous for the allele H-2^b). These cells

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attach firmly to glass and can be released with trypsin. On microscopic examination attached cells are fibrobastic in appearance and grow as a syncytium.

C-th, a line of cells growing continuously in culture since its establishment, in 1961, from the thymus of a B10·D2/SnHz × C57BL/10JHz F_1 embryo (heterozygous at the H-2 locus, H-2^d/H-2^b). These cells also attach firmly to glass and can be released by trypsin or by vigorous scraping. A microscopic picture similar to cells of line B-th is shown by these cells.

All cell lines were maintained in Eagle's medium (6) supplemented with 5 to 6 per cent calf serum, 10^{-3} M pyruvate, and 10^{-4} M L-serine (5) under an atmosphere of 5 per cent CO_2 and 95 per cent air at 37°C.

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
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
C3H/Tu	H-2^k	From the colony of the late Dr. F. C. Turner
C3H/SnHz	H-2^k	Supplied by Dr. G. D. Snell in 1961 and bred at Stanford

Isoantisera.—Hyperimmune isoantisera were prepared by repeated injections of spleen and thymus as previously described (7). Isoantisera used were

C57BL/6J(H-2^b) anti DBA/2J(H-2^d)
BALB/CTu(H-2^d) anti C3H/Tu(H-2^k)
C3H/SnHz(H-2^k) anti C3H·SW/SnHz(H-2^b)
C3H·SW/SnHz(H-2^b) anti C3H/SnHz(H-2^k).

Antisera to individual H-2 components were a gift from Dr. Jack Stimpfling, Jackson Memorial Laboratories, Bar Harbor, Maine.

Unimmunized C57BL/6J(H-2^b), C57BL/10SnHz(H-2^b), and C3H/Tu(H-2^k) animals which had not been outbred served as sources for normal serum.

Solutions.—Phosphate-buffered saline (PBS),[1] polyvinylpyrrolidone in PBS (PVP),[2] citrate saline, and citrate-phosphate dextrose (CPD)[3] as described (7, 8).

Hemagglutination.—As described (7) except that in recent experiments erythrocytes were collected in CPD containing merthiolate 1/10,000, and 1.35 per cent PVP replaced 1.5 per cent PVP as serum diluent.

Hemagglutination Absorption and Elution.—Cells were grown in 1 liter Blake culture bottles, each containing 50 ml of medium, scraped from the glass surface, and suspended in, washed three times with, and resuspended in cold PBS. Cell numbers were determined with an electronic cell counter (Coulter Electronics, Chicago, model B, 100 micron orifice). 0.25 ml aliquots of serum diluted 1/20 with PVP were absorbed with cells for 30 minutes at 6–8°C. Serum was then separated by centrifugation in the cold.

Elution was carried out after washing the absorbing cells three times with cold PBS. The cells were then suspended in 0.25 ml normal serum, diluted 1/20 in PVP, and incubated at 56°C for 30 minutes. The serum was recovered by centrifugation at room temperature.

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[1] PBS, phosphate-buffered saline.

[2] PVP, polyvinylpyrrolidone in PBS.

[3] CPD, citrate-phosphate dextrose.

RESULTS AND DISCUSSION

We have detected the isoantigenic phenotype of the H-2 locus in three mouse cell lines growing continuously in culture. One cell line, 2B-2, derived from a lymphoma, has retained morphological and transplantation characteristics similar to the original neoplasm after 6 years of *in vitro* propagation, cells assuming a round shape when attached to glass, growing in tightly packed colonies, and forming tumors upon injection into histocompatible mice. The other two cell lines show a fibroblastic morphology and grow as a syncytial monolayer approximately 1 year after being established in culture. Lines 2B-2 and B-th each were established from animals homozygous at the H-2 locus, while C-th originated from a mouse heterozygous at this locus, H-2^b/H-2^d.

TABLE I

Detection by Quantitative Absorption of H-2d Isoantigen in 2B-2 Line of Cultured Mouse Lymphoma Cells

No. of absorbing cells in millions	Serum	Erythrocytes	Reciprocal hemagglutinin titer
0	H-2^b anti H-2^d*	H-2^d‡	5120
4	H-2^b anti H-2^d	H-2^d	5120
10	H-2^b anti H-2^d	H-2^d	640
40	H-2^b anti H-2^d	H-2^d	80
0	Normal serum	H-2^d	<20
0	H-2^d anti H-2^k§	H-2^k‖	5120
40	H-2^d anti H-2^k	H-2^k	5120

* C57BL/6J(H-2^b) anti DBA/2J(H-2^d) isoantiserum.
‡ DBA/2J(H-2^d) erythrocytes.
§ BALB/CTu(H-2^d) anti C3H/Tu(H-2^k) isoantiserum.
‖ C3H/SnHz(H-2^k) erythrocytes.

As seen in Table I, 10 million or more 2B-2 cells absorbed hemagglutinating activity from anti H-2d isoantiserum, reflected in a decreased hemagglutinin titer of the absorbed serum. To control for a possible non-specific effect of the cultured cells on the hemagglutinin titer of mouse isoantisera, a non–cross-reacting anti H-2 antiserum was always absorbed in parallel. As shown in Table I, 40 million 2B-2 cells did not affect the titer of such a serum. Thus the removal of activity is due to a specific absorption by the H-2 isoantigen on the cells.

A more sensitive demonstration of absorption of agglutinins is by the elution of absorbed antibodies. In this procedure, although a titer decrease in the absorbed serum cannot be detected, a readily detectable titer may be present in the eluate. Four million 2B-2 cells did not change the hemagglutinin titer of the anti H-2d isoantiserum (Table I), yet on elution a titer of 1/640 was found.

Each H-2 allele specifies a characteristic isoantigen composed of a particular

combination of antigenic components. As the various isoantigenic complexes share one or more components it is possible, by using mice carrying appropriate H-2 alleles, to prepare antisera directed at single or a few components. Thus we

TABLE II

Detection of H-2 Isoantigenic Components C and D in 2B-2 Line of Cultured Mouse Lymphoma Cells

Serum	No. of absorbing cells in millions	Reciprocal hemagglutinin titer*
H-2^b anti H-2^d‡	0	5120
H-2^b anti H-2^d	19	80
Anti C, S§	0	160
Anti C, S	10	<20
Anti D‖	0	320
Anti D	10	<20

* DBA/2J(H-2^d) erythrocytes.
‡ C57BL/6J(H-2^b) anti DBA/2J(H-2^d) isoantiserum.
§ C57BL/10J(H-2^b) × A·CA(H-2^f) F_1 anti A·SW(H-2^a) isoantiserum.
‖ DBA/1J(H-2^q) anti DBA/1·C(H-2^e) isoantiserum.

TABLE III

Detection of H-2b Isoantigen in B-1k Line of Cultured Mouse Cells

No. of absorbing cells in millions	Serum	Erythrocytes	Reciprocal hemagglutinin titer	
			Absorbed isoantiserum	Serum* eluate
0	H-2^k anti H-2^b‡	H-2^b§	1280	<2
4	H-2^k anti H-2^b	H-2^b	1280	80
10	H-2^k anti H-2^b	H-2^b	1280	160
20	H-2^k anti H-2^b	H-2^b	5120	320
40	H-2^k anti H-2^b	H-2^b	5120	320
0	H-2^b anti H-2^k‖	H-2^k¶	5120	<2
40	H-2^b anti H-2^k	H-2^k	1280	20

* C3H/Tu(H-2^k) normal serum.
‡ C3H/SnHz(H-2^k) anti C3H·SW/SnHz(H-2^b) isoantiserum.
§ C3H·SW/SnHz(H-2^b) erythrocytes.
‖ C3H·SW/SnHz(H-2^b) anti C3H/SnHz(H-2^k) isoantiserum.
¶ C3H/SnHz(H-2^k) erythrocytes.

detected not only the expression of the H-2d isoantigenic complex in 2B-2 cells by absorption, but also some individual antigenic components (C and D) of the complex (Table II). These cells removed agglutinating activity for H-2^d red blood cells from an isoantiserum containing antibody to only H-2 component, D, and from an isoantiserum with antibody to components C and S. (S is a component not present in the H-2^d complex).

In contrast to the 2B-2 cell line, cells of the fibroblastic lines B-th and C-th possess relatively poor capacities to absorb hemagglutinins from appropriate isoantisera (Tables III and IV). However, the H-2b phenotype of both lines and the H-2d phenotype of the C-th line were detected by elution. A roughly quantitative relationship of absorbing cell number and elution of hemagglutinins was demonstrated for B-th. Absorption of anti H-2b isoantiserum with 40-million cells from either line and of anti H-2d isoantiserum with 40 million C-th cells failed to lower the hemagglutinin titer for appropriate erythrocytes but the elution data indicate that B-th and C-th cells absorbed roughly equivalent amounts of anti H-2b isoantibody and C-th absorbed some anti H-2d

TABLE IV

Detection of H-2b and H-2d Isoantigens in C-th Line of Cultured Mouse Cells

No. of absorbing cells in millions	Serum	Erythrocytes	Reciprocal hemagglutinin titer			
			Isoantiserum absorbed with		Serum* eluate from	
			C-th	2B-2	C-th	2B-2
0	H-2^b anti H-2^d‡	H-2^d§	>10,240	>10,240	<2	<2
10	H-2^b anti H-2^d	H-2^d	2,560	80		
40	H-2^b anti H-2^d	H-2^d	5,120	<20	640	
0	H-2^k anti H-2^b‖	H-2^b¶	5,120	5,120	<2	<2
10	H-2^k anti H-2^b	H-2^b	2,560			
40	H-2^k anti H-2^b	H-2^b	5,120	5,120	320	40

* C57BL/10SnHz(H-2^b) normal serum.

‡ C57BL/6J(H-2^b) anti DBA/2J(H-2^d).

§ B10·D2/SnHz(H-2^d) erythrocytes.

‖ C3H/SnHz(H-2^k) anti C3H·SW/SnHz(H-2^b).

¶ C3H·SW/SnHz(H-2^b) erythrocytes.

isoantibody. The marked contrast between the quantity of anti H-2d hemagglutinins absorbed by 2B-2 cells and that absorbed by C-th cells, or, although different isoantisera were used, the anti H-2b hemagglutinins absorbed by either fibroblastic cell line suggests that 2B-2 possesses a higher concentration (or surface availability) of H-2 isoantigenic sites than do cells of the other two lines. The absorbing capacities cannot be attributed to differences in absorbing surface areas (9) since microscopic examination of cell suspensions revealed C-th and B-th cells to be larger than 2B-2 cells. Recently Möller and Möller have reported differences in absorbing capacity for various normal and tumor mouse cells, concluding that the concentration of isoantigenic sites varied in different cell types tested (9).

To eliminate the possibility that the H-2^b erythrocyte hemagglutinins removed from H-2^k anti H-2^b isoantiserum by C-th were absorbed by H-2d anti-

genic components F and N (found also in the H-2b complex), an aliquot of this antiserum was also absorbed with 2B-2 cells. Less antibody (3 tubes dilutions less) which agglutinated H-2^b erythrocytes was separated by elution from 2B-2 cells than from C-th cells, suggesting that the latter possess H-2b components other than F and N. Elution of hemagglutinins from 2B-2 cells resulted in 2 tube dilutions of weak hemagglutinating activity for H-2^b erythrocytes which, on the basis of many experiments with various isoantisera, is not considered a significant elution titer.

Prior to these studies human blood group isoantigens were demonstrated on HeLa cells cultured 8 years *in vitro* (10). Heterologous antibody or plant agglutinins were required for detection of H, M, and N antigens by mixed agglutination; only Tj^a was detected by isoantibody. Isoantigens possessed by human leukocytes have also been detected on HeLa cells with human isoantibody (11).

The presence of H-2 antigen on long term cultured mouse cells is being confirmed in this laboratory with a fluorescent antibody labeling method (12). Thus the H-2 isoantigens are heritable and stable marker phenotypes which might be employed for *in vitro* studies of somatic cell variation. We have recently found that the isoantigenic expression of the H-2 locus also serves as the basis for a potential immunoselective procedure in which cultured lymphoma cells are killed by isoantibody and complement. Complete details are presented in the following paper (13).

SUMMARY

The isoantigenic phenotype of the H-2 locus has been detected by isohemagglutinin absorption in a line of mouse lymphoma cells growing continuously in culture for 6 years and in two established lines of fibroblastic mouse cells growing continuously in culture for 1 year. Quantitative absorption studies suggest that the concentration of H-2 isoantigens is higher in the cultured lymphoma cells than in the other two fibroblastic cell lines.

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