I. Steps Toward a Genetics of Somatic Cells in Culture[1]

II. Maternal Isoimmunization as a Result of Breeding in the Mouse

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We are continually confronted with instances of somatic cell variation (e.g., cell changes during differentiation, induction of neoplasms, the immune response, immune tolerance), but as yet have achieved little insight into the mechanisms behind this variation. The hope that genetic analysis of isolated somatic cell populations in culture can yield information pertinent to these questions has been one of the main stimuli to the development of methods for the study of genetics of somatic cells.

With the patterns already established in microbial genetics, the early work in somatic cell-culture genetics has been largely technical centering mostly around the establishment of nutritional requirements, culture conditions, techniques (Marcus, Cieciura and Puck, '56; Lockart and Eagle, '59), etc. Owing to this pioneering work, most cell lines now can be cultured in semi-defined growth media in which dialyzed serum proteins are added to an otherwise synthetic mixture of amino acids, vitamins, salts and glucose. A few can be grown in completely synthetic media. Individual cells can be plated on surfaces, usually glass, where they will form clones. These clones can be counted, separated, and then grown to large populations, each originating from a single cell.

At present, with progress of the search for cellular genetic markers, i.e., stable, heritable characters which differentiate one cell line from a sister line, the analogies with the microbial methodology are becoming more strained. Whereas the same general classes of markers have been established with cell culture lines, (resistance to antimetabolites, gain or loss of nutritional requirements, antigenic variation, etc.), it is more difficult to demonstrate in the animal cell that these differences are due to *genetic* diversity based on structural modification in genetic determinants (DNA) rather than to epigenetic processes controlling the functioning or expression of the genetic determinants. Without being able to transfer genetic material and do recombination studies, the animal cell geneticist can reason to genetic dissimilarity between phenotypic variants only through complicated, often technically difficult, analyses of phenotypical variation. Nevertheless, a wide variety of approaches to the problem are at present under investigation in a number of laboratories. I shall not attempt to cover them all here (see Klein, in press), but I shall instead limit myself to the description of two types of variation under investigation in our laboratory: drug resistance and antigenic markers.

Drug resistance

Our experience with drug resistance is similar to that reported from other laboratories (Fisher, '59; Szybalski and Smith, '59; Harris and Ruddle, '60). We find with a folic acid analogue that we can select, over a year's time, a series of variants showing small stepwise increases in resistance over a 1000-fold range in drug concentration. Based on the number of clones formed as a measure of cell viability, the data in table 1 show that there is a vary narrow concentration range from no inhibition to complete inhibition of growth by the drug. The inhibitory level with amethopterin is independent of the number of cells per unit volume of medium.

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TABLE 1
Multiple stepwise development of resistance to amethopterin

AMT	Parental sens	Resistant steps: Variants r-1	r-2	r-3	r-4	r-5
1×10^{-9}						
2						
5	100[1]					
1×10^{-8}	1					
2	0	100				
5		40				
1×10^{-7}		0	100			
2			41	100		
5			0	30		
1×10^{-6}				0	100	
2					44	
5					0	100
1×10^{-5}						39
2						0

[1] The numbers in the table are percentage of the maximum number of colonies formed at subtoxic concentrations of AMT.

Dr. Robert Roosa and I began this work at the National Institutes of Health. It has been continued by Dr. Roosa at the Wistar Institute and by us at Stanford. The work was done with a mouse lymphocytic neoplasm, P-388, which was initiated as a continuous cell culture line by Dawe and Potter ('57). Three years later its nutritional needs were determined both for growth as a mass population and as single cells (Herzenberg and Roosa, '60). At this point it was designated ML 388, and has subsequently been maintained on Eagle's minimal essential medium supplemented with 5% calf serum (dialyzed for nutritional experiments), 10^{-3} *M* pyruvate and 10^{-4} *M* L-serine.

Development of resistance to the amethopterin deserves some comment. When a minimally toxic level of amethopterin is introduced into a culture of sensitive cells, the entire culture is apparently dead within a few days. Even with careful microscopic examination a week after drug addition, no healthy cells can be seen. Three or more weeks later, small patches of healthy cells become apparent. These increase in size, and if one is picked up with a bacterial loop and transferred to a fresh bottle of drug-free medium a culture can be established which is resistant to the drug level used for selection. This culture remains resistant even after maintenance for more than a year in the absence of AMT (fig. 1).[2]

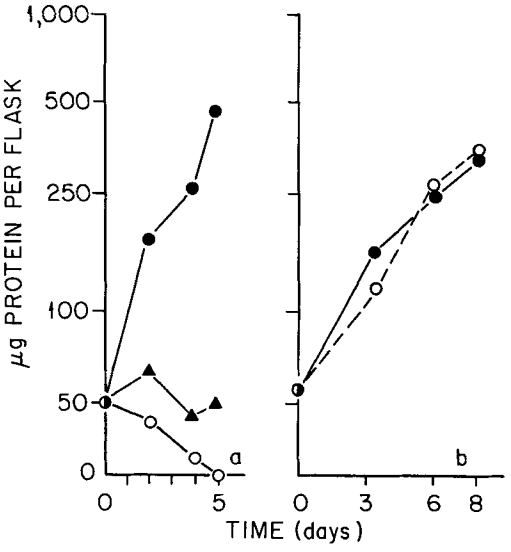

Fig. 1 Growth of parental (sensitive) cells (a) and of resistant (AMT^{r4-6}) cells maintained for 6 months on inhibitor-free medium (b). Eagle's basal media + 5% calf serum + 0.2 m*M* pyruvate was used. ● No inhibitor; ▲ 4×10^{-8} *M* Amethopterin; ○ 1×10^{-7} *M* Amethopterin.

It is difficult to account for the long lag before the resistant cells appear. Although the resistant line finally selected grows somewhat more slowly than the parent population, it gives visible clones within

[2] Abbreviations: AMT, amethopterin; sens, sensitive; FUR, 5-fluorouridine; AzG, 8-azaguanine; ID50, 50% inhibitory dose; AMT^{r-1}, AMT^{r-2}, first step, second step resistant strains, etc.; IR, isogenic resistant.

10 days, even when it is intentionally placed in the presence of large numbers of dead and dying sensitive cells (table 2). The significant point is that it takes a long time to select out by this method a new step of resistance.

TABLE 2

Reconstruction experiment

Additions to medium	Type and no. of cells	No. colonies after 10 days incubation
None	500 AMT^{r-2}	123
10^{-7} *M* AMT	500 AMT^{r-2}	117
10^{-7} *M* AMT	500 AMT^{r-2} + 10^5 AMT^{sens}	129

Aside from the inconvenience this is unsatisfactory for the study of mutation, as it may indicate that many intermediate mutations are necessary before the arbitrarily chosen level of resistance is attained. Although it is certainly not proved that this is the case, it is suggestive that no matter how small the increase in drug concentration, the selected resistant variants are always resistant to that precise concentration and no higher.

This is quite different from the pattern of the development of resistance to the purine analogue, 8-azaguanine. Here, over the entire solubility range of AzG, only three steps of resistance can be demonstrated (see table 3). The clear definition of resistant steps would make these lines useful for genetic transfer studies, except for the fact that the spontaneous mutant frequency is often as high as one per 1000. Unfortunately, this high background makes it difficult to detect events occurring at lesser frequencies. Experience in microbial genetics leads us to expect transfer frequencies in the range of one per million or less.

TABLE 3

Development of AzG resistance

Strain	ID 50 (*M*)
Sens	5×10^{-7}
AzG^{r-1}	5×10^{-6}
AzG^{r-2}	2×10^{-5}
AzG^{r-3}	$>1\times10^{-3}$

We also studied resistance to fluorinated pyrimidines and encountered still another kind of problem. In this case the selection of resistant lines appears to proceed more on a population basis. Distinct patches are not found; instead, after a lag the whole population appears to start to grow. The explanation for this peculiar behavior became apparent when we found that the inhibitory drug level depends to a great extent on the number of cells in the initial inoculum. In other words, we were probably dealing with cells excreting an "antagonist of the antagonist" into the medium and thus feeding other cells (see table 4), which makes selection at the cell level difficult.

TABLE 4

Dependence on inoculum size of FUR inhibition

Cell type	ID 50 FUR (*M*)	
	200 Cells/ml	40,000 Cells/ml
Sens	4×10^{-9}	2×10^{-8}
FUR^{r-1}	2×10^{-8}	2×10^{-7}
FUR^{r-2}	1×10^{-7}	—

Antigenic variation

Dissatisfaction with the inherent limitations of the drug-resistance system led us, eventually, to the decision that a better set of markers needed to be developed to study the genetics of somatic cells in culture. The Kleins had already begun to use the isoantigens of mouse tumors to follow somatic cell variation of tumor cell populations *in vivo*, and found the isoantigens to be under stable hereditary control (Klein and Klein, '56). Because these antigens (Gorer, '37; Snell *et al.*, '53) are responsible for interstrain rejection of tissues, the fact that ML 388 could grow progressively only in the mouse strain in which it arose, DBA/2, made it reasonable to suppose that at least some of the DBA/2 histocompatibility antigens were still present on the cultured cells.

We were fortunate to be able to show, rather quickly, that ML 388 cells are killed by exposure to isoantiserum prepared against DBA/2 tissues. As shown in table 5, both the isoantiserum and complement (guinea pig serum), are essential for cytotoxicity.

TABLE 5

Requirements for isoimmune cytotoxicity

Additions to cells	Colonies per flask (per 1000 cells inoculated)	Cell per bottle at 8 days (referred to inoculum as 1)
None	238 ± 24	250 ± 52
Normal serum	212 ± 12	79 ± 23[1]
Anti *H-2*^{d} isoantiserum	208 ± 19	320 ± 23
Complement (guinea pig serum to 5%)	164 ± 37	150 ± 30[1]
Complement + normal serum	181 ± 21	260 ± 25
Complement + anti *H-2*^{d} isoantiserum	1 ± 0.5	5.8 ± 0.5

[1] ± Numbers indicate one standard error.

Cells, antiserum and complement are incubated in standard medium at 37°C. for 30 minutes. The mixture is then diluted 1:40 with medium and aliquots are planted in 1-ounce prescription bottles and incubated at 37°: (1) to determine the number of cells capable of giving rise to visible colonies after 8 days and (2) to measure the growth rate of the population. (Growth is estimated by counting the number of cells attached to the glass in replicate flasks on successive days, with the aid of a Coulter electronic cell counter.) By these measurements, we find up to 99% of cells killed under optimal conditions with potent antisera (table 6 and figs. 1, 2).

As our degree of sophistication increased, we tried to identify the particular antigen(s) responsible for the sensitivity of the cells to the antiserum. Since we already knew that our cytotoxic sera contained a high titer of anti-*H-2d* hemagglutinins, it was not very difficult to make use of the available genetic and immunologic methods to establish the presence of *H-2d* antigens on the cells (Hoecker, '56; Gorer, '61; Herzenberg and Herzenberg, '61).

It is well known that, of the 15 or more histocompatibility loci of the mouse, the *H-2* plays a predominant role in serology and graft rejection (Barnes and Krohn, '59). It is the only known mouse histocompatibility antigen detectable by hemagglutination (Gorer and Mikulska, '61; Stimpfling, '61) and leukoagglutination (Mishell and Herzenberg, in preparation) although other additional isoantigens can now be detected by these techniques and much work has been done on the elucidation of the cross-reactions of the antigens determined by the various *H-2* alleles (Gorer, '59; Hoecker, Pizarro and Ramos, '61; Stimpfling and Pizarro, '61). Snell has developed a number of isogenic resistant (IR) strains, pairs of which differ from each other only at the *H-2* locus (Snell, '58), which permit sera to be prepared containing only antibodies specific for the *H-2* type of the tissue donor.

The evidence for the presence of *H-2d* antigens has been obtained in our laboratory by Dr. Howard Cann, who also worked out the details of the cytotoxicity methods just described. He showed that not only are anti-*H-2d* hemagglutinins in a hyperimmune serum completely removed by

TABLE 6

Titration of cytotoxic activity in anti-H-2^d *isoantiserum*

Dilution of isoantiserum	Cells per bottle at 8 days (Referred to inoculum as 1)	Colonies per flask (Per 1000 cells inoculated)	% Decrease in colony-farming cells relative to control
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

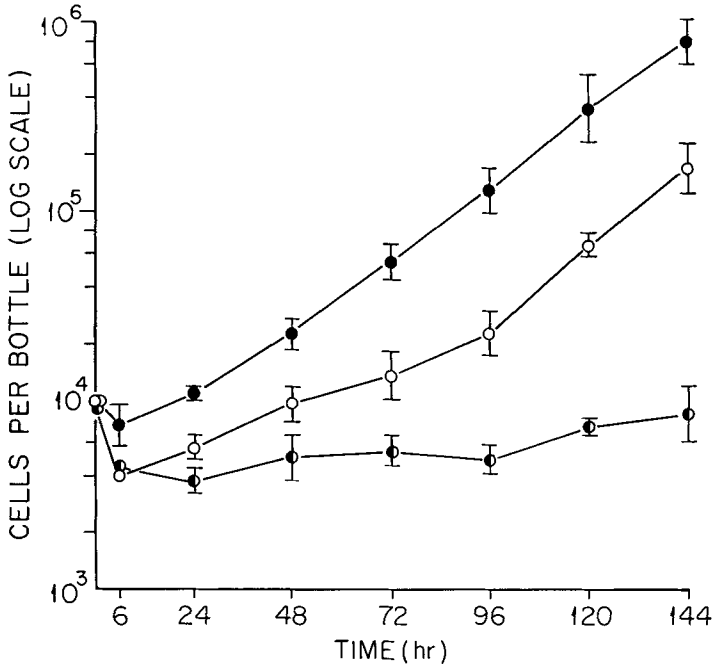

Fig. 2 Growth of ML 388 after exposure to C57BL/6J anti-DBA/2J. Conditions described in text. ● Normal serum; ○ isoantiserum, 1:300; ◐ isoantiserum, 1:30. The vertical lines indicate two standard errors on each side of the point estimate.

absorption with ML 388 (Cann and Herzenberg, '61) (table 7, first column), but that the cells are killed by a serum containing only anti-*H-2d* antibodies (table 8, bottom row, last column).

TABLE 7

Removal of anti-H-2^d antibodies by ML-388 and an isoantiserum cytotoxicity-resistant variant

Number of absorbing cells	Reciprocal hemagglutinin titer after absorption[1]	
	Parent cell line	Clone 7
5×10^7	<20	<20
2×10^7	<20	20
1×10^7	80	640
5×10^6	640	1280
0	2560	2560

[1] Serum: C57BL/6J anti DBA/2J. Erythrocytes: B10.D2.

Since about 99% of cells are killed by isoantisera under appropriate conditions, Dr. Cann began cycling a population through cytotoxic exposure and regrowth of survivors with the hope of deriving a population resistant to the antiserum. Initially, he was unsuccessful, but finally, after 14 cycles, he found his population had become partially resistant to the antiserum level which kills 99% of the parent population (Cann and Herzenberg, in preparation). He chose 8 clones at random from the resistant population, and found 5 of these have partial to complete resistance to cytotoxicity.

When tested by quantitative absorption of hemagglutinins, cells from a resistant variant appear to contain, with reference to the serum used, all the *H-2d* antigenic components represented in the parent population. The efficiency of absorption, relative to cell number, is slightly less (approximately one-half) with the variant population. However, as the variant tested has approximately twice the cell surface area, the efficiency of absorption calculated per unit cell surface is closer to one-quarter that of the parent (table 7).

The variants are morphologically distinguishable from the parent cell type. As shown in figure 3a, b, they form looser clones that are more spread out. The in-

TABLE 8

Stable heritable resistance to isoserum cytotoxicity of selected variant clones

Clone no.	Cells per bottle at 6 days (referred to inoculum as 1)					
	(December 1961)		(January 1962)		(March 1962)	
	Normal serum	C57BL/6J (*H*-2^b) anti DBA/2J (*H*-2^d)	Normal serum	C57BL/6J (*H*-2^b) anti DBA/2J (*H*-2^d)	Normal serum	C57BL/10 Sn (*H*-2^b) anti B10·D2 (*H*-2^d)
2	11 ± 1	8.8 ± 1.8[1]	18 ± 3	15 ± 2	52 ± 1	28 ± 2
7	19 ± 0.3	14 ± 0.3	16 ± 1	20 ± 2	32 ± 1	28 ± 1
Parent cell line			30 ± 2	0.9 ± 0.3	63 ± 6	1.1 ± 0.4

[1] ± Numbers indicate one standard error.

dividual cells are somewhat larger, tend to be more fusiform, and grow slightly more slowly than the parent strain (table 8).

Klein and Klein ('58) have shown that their tumor cell variants are of two types. One type, quite analogous to many long-transplanted tumors, is able to grow progressively in any mouse strain but, nevertheless, like Ehrlich ascites tumors can be shown still to be capable of eliciting an isoimmune response. This result indicates the continued, though masked, presence of histocompatibility antigens. It is probable that Dr. Cann's variants can be grouped with this class. The second type are still specific tumors which, starting heterozygous at the *H*-2 locus have lost the antigen(s) determined by one or the other of the *H*-2 alleles. We are at present putting into culture a number of heterozygous tumors, hoping that selection procedures will yield variants which have lost one of the antigens. The second type of stable variant is far more interesting from the standpoint of the genetic analysis of somatic cells. These variants are selected from tumors which are heterozygous at the *H*-2 locus (that is, arising in F_1 hybrids of a pair of IR lines carrying different *H*-2 alleles) by growth in mice which are homozygous for one or the other of the alleles in the heterozygote. Upon testing, these variants are found to be still quite specific in that they will grow progressively only in the selecting strain or their strain of origin. The Kleins' interpretation, based on these tumor growth characteristics, that the variants have lost antigens determined by the *H*-2 allele of the other parental strain has been well substantiated by direct serological testing (Hellstrom, '61). Thus, they have shown that the heritable stable loss of the products of a specific region of the mouse chromosomes can occur in somatic cells.

Most recently, the Kleins and their coworkers have obtained variants which have lost only some of the antigenic components specified by the *H*-2 locus (Klein, '61). They started with a hybrid tumor which carried the *H*-2^s allele from one parent and the *H*-2^a allele from the other. As grafting and serological work has shown that *H*-2^d plus *H*-2^k specify all of the antigenic components specified by *H*-2^a, the antigenic phenotype of the tumor can be considered "S/DK."

Using the homograft reaction, the Klein group selected variants of the tumor which were phenotypically S/D-S/− −. Significantly, there were no S/-K. This led them to postulate that the antigen(s) loss is due to somatic crossing-over, where K is distal to D relative to the centromere.

If somatic crossing over is frequent enough in mammalian cells, it may be possible to carry out detailed genetic analysis in the absence of cell-to-cell transfer of genetic material (Pontecorvo, '58). Mapping of the *H*-2 region by a combination of the techniques of *in vivo* selection, using the homograft reaction, and *in vitro* selection, using isoantiserum reagents, then seems a feasible problem for the near future.

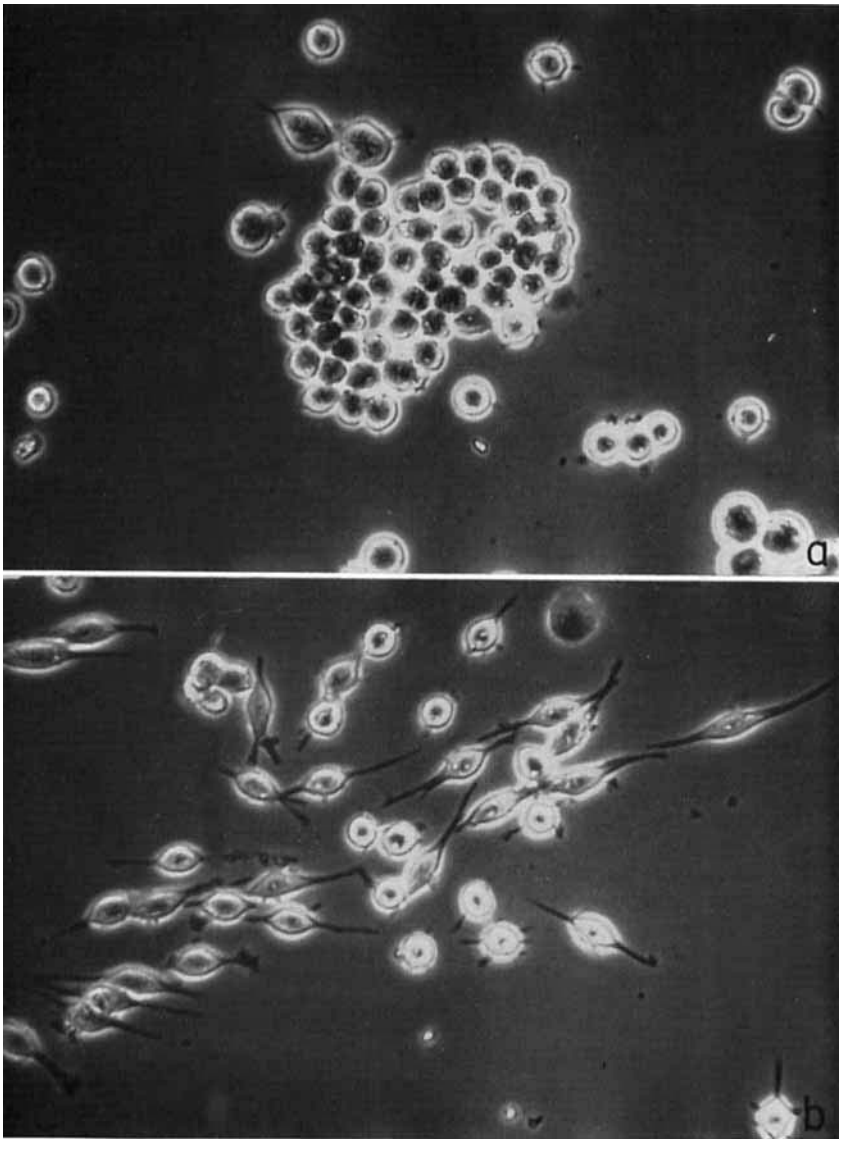

Fig. 3 Cell clones. (a) Parental, (b) Variant.

II. Maternal Isoimmunization as a Result of Breeding in the Mouse

It has been pointed out that although a fetus carries many potential immunogens, maternal immune response to fetal antigens has thus far been observed only in a few antigen systems. In man, the most common maternal response is in the Rh system, where Rh incompatible pregnancies result not only in immunization of the mother, but often in pathological consequences for the developing fetus as well (Diamond and Allen, '57). Other maternal immune responses in man have been demonstrated (Zuelzer and Cohen, '57; Payne, '62), and immunologic disease is also known in certain domestic species (Dujarric de la Riviere and Eyquem, '53). It may be that on closer examination, many more examples of maternal immunization to fetal antigens, with or without accompanying pathology in the offspring, will be found.

Recently, we described maternal immunization in female mice producing an F_1 hybrid (Herzenberg and Gonzales, '62). This was demonstrated by the presence in C57BL/6 females of circulating agglutinins for paternal, DBA/2 erythrocytes. The sera from about half the females retired from this cross contain hemagglutinins (table 9). The fraction of immune females (as shown in fig. 4) rises sharply at 4 months of breeding (6 months of age) and then remains at about 50% throughout the breeding life of the animals.

In general, despite the strong maternal immune response here shown, there is an apparent lack of pathologic effect on the mouse fetus. Production of the F_1 is a routine procedure in many mouse-breeding facilities, and it would seem that if there was a gross effect of maternal immunization on the survival of the hybrid fetus it would have been noted. It may, however, be possible to reveal some strain combinations in which an adverse effect on the fetus can be demonstrated by careful comparison of the fecundity of the incrossed and outcrossed females of the same inbred strain.

There is some reason for believing that fetal hematologic disease may not be a consequence of maternal immunization in the mouse. Adult erythrocytes persist for long periods, *in vivo*, in the presence of isoantibody directed against them, and it is likely that fetal erythrocytes are at least as resistant to immune attack (Erickson, Goor and Herzenberg, unpublished data; Goodman, personal communication; Mitchison, personal communication). Nonetheless, other forms of immunologic disease of the fetus may result from its development in the presence of maternal antibody, and should be looked for in such situations.

The use of hemagglutinin production as an index of immunization in the mouse limits the investigator to study of the response to the *H*-2 antigen alone. Isoim-

TABLE 9

Immune response of outcrossed female breeders[1]

Strain	Cross	Age	Hemagglutinin positive
		months	
C57BL/6J ♀ ♀	Outcrossed to DBA/2J	~ 12	27/50
DBA/2J ♂ ♂	Outcrossed to C57BL/6J	~ 13	0/25
C57BL/6J ♀ ♀	Incrossed	~ 13	never[2]

[1] C57BL/10-*H*-2^d erythrocytes were used to test C57BL/6 sera; C3H-*H*-2^b erythrocytes were used to test DBA/2 sera.

[2] Incrossed breeder sera are routinely tested in this laboratory for use as normal serum controls in hemagglutination assays.

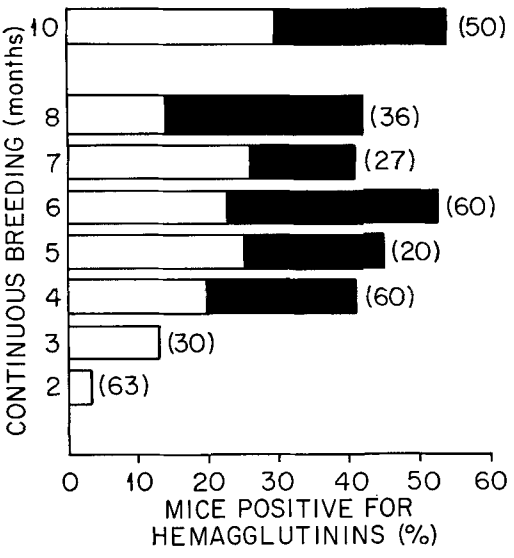

Fig. 4 Fraction of C57BL/6J ♀♀ breeding with DBA/2J ♂♂ having anti-*H*-2^d antibodies. Strong ■ and week □ are arbitrary classifications based on strength of agglutination and titer. Number of animals tested in parentheses.

munization in the mouse could, however, elicit antibodies to other antigens not demonstrable by hemagglutination. The demonstration of isoantibodies directed against non-*H*-2 antigens has recently resulted from the development, by Dr. Robert Mishell, of a method for immune agglutination of leukocytes.

Induction of leukoagglutinins by pregnancy in the human has been demonstrated by Dr. Rose Payne. Mishell has found non-*H*-2 leukoagglutinins in the sera of pregnancy immunized mice. He showed that pregnancy-induced C57BL/6 anti-DBA/2 serum agglutinates both DBA/2 (*H*-2^d) and C57BL/10-*H*-2^d (B10·D2) leukocytes. When this serum is absorbed *in vivo* in DBA/2, all leukoagglutinins are removed, but when it is absorbed *in vivo* in C57BL/10-*H*-2^d, which probably removed only the anti-*H*-2^d antibodies, agglutinins for the DBA/2 leukocytes remain, thus indicating clearly the presence of a non-*H*-2 response (see table 10).

The availability of methods for demonstration of maternal immunization against at least two fetal antigenic systems in the mouse provides a useful experimental model for studying pregnancy-induced immunization. It is possible to systematically investigate such problems as the route(s) of maternal exposure to fetal antigen, pathological effects on the fetus other than hematological disease, and possible therapeutic regimens for averting maternal immunization and/or fetal disease using this model.

The relevance of these findings to the reports of Barrett and Breyere (in press) on the induction of graft tolerance in parous mice should be mentioned. They found that females which have borne two or more litters accept tumor and skin grafts from the paternal strain for periods beyond the normal rejection time. Our finding of both *H*-2 and non-*H*-2 antibodies for paternal antigens circulating in such females raises more sharply the possibility that this "tolerance" is actually immunological enhancement (Kaliss, '57; Snell *et al.*, '60).

TABLE 10

Presence of H-2^d and at least one non-H-2 leukoagglutinin in pregnancy induced antiserum

Serum	Reciprocal of agglutination titer		
	Erythrocytes	Leukocytes	
	B10·D2	DBA/2	B10·D2
Normal C57BL/10	neg[1]	neg	neg
Pool A (C57BL/6 ♀♀ outcrossed to DBA/2 ♂♂)	160	> 128	> 128
Pool A absorbed with DBA/2[2]	neg	neg	neg
Pool A absorbed with B10·D2	neg	> 128	neg

[1] < 5.
[2] Absorptions *in vivo*.

OPEN DISCUSSION

MÖLLER[1] : Did you treat your cultures with antiserum during each of the 14 passages?

HERZENBERG: Yes.

MÖLLER: The change in cytotoxic sensitivity might therefore have occurred in the absence of the selective agent. Did you have any genetical markers on the original cell population, such as drug resistance or chromosomal characteristics, so as to be able to exclude critically the possibility of contamination?

HERZENBERG: I do not think we have contamination. These cells have the *H-2*^{d} antigens on them, as the original parental line did, and we had no other cultures in the laboratory which have only this *H-2* antigen on them. These cells do not have non-*H-2*^{d} components of *H-2*^{b} or *H-2*^{k}. In other words, they are immunologically marked and have proved to be identical to the original cells in that fashion.

HOECKER[2] : Do they take?

HERZENBERG: They have not been tested for ability to form tumors.

HARRIS[3] : We have developed a number of clonal populations of pig kidney cells with characteristic chromosomal patterns, by means of X-radiation. Heritable changes in morphology have clearly taken place in some cases within these clones, with the possibility of contamination excluded by the presence of chromosomal markers.

YERGANIAN[4] : Although Herzenberg discussed somatic cell genetics, we must place it in the category of "aneuploid somatic cell genetics," as apart from that associated with cells maintained in the primary or long-term cultures that retain the diploid chromosome complement. Primary or recently established classic diploid "fibroblast-like" cell cultures are virtually impossible to clone at relatively high frequencies and still retain the diploid chromosome number. Similarly, cells having the classic tetraploid number of chromosomes fail to establish colonies at desired cell dilutions. Cell shape is an excellent indicator of karyological disturbances, as pointed out by Harris.

When employing the term "somatic crossing-over" as a plausible mechanism or explanation for the loss or gain of antigens, as described by Klein, I feel that cytological evidence must be provided to strengthen such a choice, especially when there are a number of anomalies already associated with each tumor, such as "marker" or rearranged chromosomes, aneuploidy, and increased incidence of nondisjunction, that may reflect antigenically. In Klein's experiments, I believe the loss of one of the parent antigens was consistent. That is to say, the F_1-derived tumor loses one of the parental antigens. However, there is cytological evidence to indicate that these two loci or some other (yet to be described) histocompatibility factors may reside on the X chromosome.

It is now well demonstrated that the two X's in somatic cells of the female are morphologically (and genetically) different. Mutations in each of the members may be expressed independently within the limits of its clonal boundary, one member of the X pair differentiates or becomes heterochromatic (in a random fashion during early embryogenesis), thereby permitting the alternate or euchromatic form to function genetically.

The possibility for the existence of X-linked histocompatibility factors may be more readily demonstrated and useful in attempting to clarify antigenic features of malignancies having varied patterns of karyotypes than to simply regard somatic crossing-over as the plausible mechanism. Recently, Bailey (Genetics Society of America, '62) reported the presence of histocompatibility factors on the X chromosome.

HERZENBERG: I don't quite understand your placing the *H-2* locus on the X chromosome, because then it ought to show sex-linked inheritance, which it does not. The genetic evidence places the *H-2* on an autosome. It has been assigned to linkage group IX.

I might also say that none of Klein's evidence before the separation of D and K was relevant to the question of whether or not somatic crossing-over was involved in the loss of these antigens. His demonstration of the loss of only part of the antigenic complex and with a definite po-

[1] Göran Möller, Karolinska Institutet, Stockholm, Sweden.
[2] Gustavo Hoecker, University of Chile, Santiago, Chile.
[3] Morgan Harris, University of California.
[4] George Yerganian, Harvard Medical School, Children's Cancer Research Foundation.

larity to the loss was a strong reason for suggesting that somatic crossing-over was concerned.

MARIN[5] : I believe you said that the spontaneous frequency of drug resistance in the cell strains is around 10^{-3}? How do you estimate this frequency?

I have another question, about the fluorouridine resistance. Can you show that the dependence of this character on cell-concentration is not due to a "concentration effect," rather than to an interaction between cells? I mean a saturation effect. When you increase the number of cells, you may get to the point where the cells saturate all the drug that is available.

HERZENBERG: The frequency is the number of cells forming colonies in the medium containing a drug divided by the number of colonies formers in growth medium alone.

On the second point, I have no information.

RUSSELL[6] : Just a small contribution to the question of somatic crossing-over in the mouse, to show that this idea may not be too far-fetched. A few animals have been observed whose phenotype and breeding could be explained by somatic crossing-over. Thus, Carter (J. Genetics *51:* 1–6, '52) reported a $W^v/+$ mouse which had patches of wild-type fur, but which transmitted an excess of W^v. He suggested as a probable explanation somatic crossing-over with the formation of "twin" spots: $+/+$ in the fur and W^v/W^v in the gonad. In our own laboratory we have had two, or possibly more, cases of c^{ch}/c animals (light gray) that have had areas of white (= c/c?) as well as areas of dark gray (typical c^{ch}/c^{ch} color). Although other explanations are possible for all of these (e.g., somatic nondisjunction, somatic reduction) and although somatic pairing has not yet been cytologically demonstrated in mammals, somatic crossing-over seems the easiest explanation at this time.

ATWOOD[7] : Another word about the somatic crossing-over. We would think that D and K are extremely close together, but we don't know how far they are from the centromere. If they are an average distance from the centromere, then you would expect the double loss of D and K very much more frequently than the single loss of the distal marker. The only way to have somatic crossing-over is that they lie right on the centromere, as well as on each other.

HERZENBERG: I completely agree. We just don't know where the centromere is. We also do not know the relation between meiotic and mitotic map length in the mouse.

GELFANT[8] : I would like to question a basic premise underlying this discussion; that is, the procedure that you use to determine whether you are dealing with a drug-resistant strain which you have isolated, or an immunological strain. You indicated that you were looking for a marker, and that in the first experiment you used amethopterin; if you got something resistant to that, you assumed you had isolated a variant.

Now, in that experiment alone, it seems to me, you are dealing with a genotoxic effect that has nothing to do with the significant drug, amethopterin, or any of the other drugs you used. If you demonstrate a reversible situation — that is, if you used amethopterin, and then folinic acid, and showed a reversible effect — then you could conclude that you are dealing, specifically, with an amethopterin-resistant strain.

When you study some immunologically different strains, I wonder if here, too, you are not dealing with the antiserum as a genotoxic agent.

HERZENBERG: The appropriate control to show that you have not simply selected a cell which is more resistant to all or several toxic effects is simply to show that your resistance is specific. We have done that in each case. For example, when we select an amethopterin-resistant mutant, it is resistant to other antifolic acid antagonists, but is not resistant to 6-mercaptopurine or to the antiserum cytotoxicity.

In addition, we have been able to select double resistant mutants — mutants resistant to both amethopterin and 6-mercaptopurine at the same time.

JACOBSON[9] : I might offer a seldom-appreciated fact to help explain the dif-

[5] Guglielmo Marin, Oak Ridge National Laboratory.
[6] Liane B. Russell, Oak Ridge National Laboratory.
[7] K. C. Atwood, University of Illinois.
[8] Seymour Gelfant, Syracuse University.
[9] K. B. Jacobson, Oak Ridge National Laboratory.

ficulty in obtaining cell lines that are resistant to amethopterin. This inhibitor is usually thought to be an inhibitor of one-carbon transfer by a folic acid system, but Johnson *et al.* (Proc. Soc. Exptl. Biol. Med., *99:* 677, '58) first showed that it is an inhibitor of the acetylation of sulfanilamide by acetyl-CoA. I have confirmed this and amplified it to the point of showing that amethopterin is an inhibitor of the acetyl transfer reaction, competitive with the acetyl donor and noncompetitive with the acetyl acceptor (Jacobson, J. Biol. Chem., *235:* 2713, '60). If amethopterin is indeed inhibiting acetylation reactions as well as folic acid systems, then the likelihood of developing cell lines resistant to this inhibitor is much poorer than if the inhibitor was specific to one class of reactions.

HERZENBERG: To assess this problem one might include folic acid in the medium to prevent the inhibition of folic acid systems and see if the inhibition at 10^{-5} *M* amethopterin is accompanied by aberrations in the acetyl-CoA system.

MÖLLER: The *H*-2^d allele appeared to be remarkably stable in your tissue culture cells. Did you test the possibility that the cells had lost individual isoantigens determined by the *H*-2^d allele by immunizing with the tissue culture cells and testing the serum against these cells after absorption with normal tissue of the *H*-2^d genotype?

HERZENBERG: No. We have prepared serum against the parental cells in culture, and these sera agglutinate *H*-2^d erythrocytes. We have not done absorptions with tissues from an *H*-2^d mouse to see if specificities remain in these sera against other *H*-2 antigens. This should be done.

BILLINGHAM[10] : I should like to comment on evoking transplantation immunity in females, either by sensitization *before* or *during* pregnancy, that would lead to immunological damage or destruction of their fetuses *qua* homografts. The design of the experiments was such that the fetuses confronted their mothers with foreign histoincompatibility factors. No convincing successes have been reported.

In considering the possible routes of isoimmunization in mice it is worth recalling that fetal red cells have repeatedly been demonstrated in the blood of pregnant women, and the presence of tiny fragments of trophoblast cells in pregnant women's lungs is familiar to pathologists.

CEPPELLINI[11] : We are trying to do these experiments with human material. De Carli of my laboratory has obtained a line from thyroid tissue of a group A individual. This line has a high cloning efficiency, and it is possible by using mixed agglutination to see directly that the colonies derived from a single cell have maintained the normal amount of A antigen. The line was maintained *in vitro* for a number of months. Suddenly a loss of the A antigen takes place. At the same time, the chromosome number changes from euploidy to hyperploidy. There is no "bottle infection." The correlation between loss of antigenic specificity and increased chromosome number has been already well-established for mouse tumors (see Hauschka, J. Cellular Comp. Physiol., Suppl. 1, *52:* 197–233, '58).

SALK[12] : I can't make any genetic contribution, but we have used ethylmethane sulfonate (EMS) in cultures of continuously propagating cells originally derived from a tumor from an A/Jax mouse. We could return the cell to the mouse and produce tumors and eventually death.

When EMS in different concentrations was put into the culture medium, striking morphological changes occurred and persisted. We were able to do quantitative studies to show the growth rate in tissue culture of the original and the treated cells. Putting a calculated number of cells into the mouse subcutaneously and then weighing the cell mass at different intervals of time, the same rate of increase in cell mass occurred with the untreated cultures.

After one particular concentration of the EMS, the growth rate in tissue culture remained unchanged but no growth occurred in the mouse, suggesting an antigenic change.

In another concentration of the drug, a different morphological change occurred, but not the change suggestive of an antigenic difference. If this parallels your experience, then, the answer to Hoecker's

[10] R. E. Billingham, The Wistar Institute of Anatomy and Biology.
[11] Ruggero Ceppellini, Università di Torino, Italy.
[12] Jonas Salk, The University of Pittsburgh.

question as to whether or not the change is accompanied by non-take would be in the affirmative as suggested by this experiment. We used the EMS at Benzer's suggestion because it is a strong mutagen.

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