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and it is likely that fetal erythrocytes are at least as resistant to immune attack (Erickson, Goor and Herzenberg, unpublished data; Goodman, personal communication; Mitchison, personal communication).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.

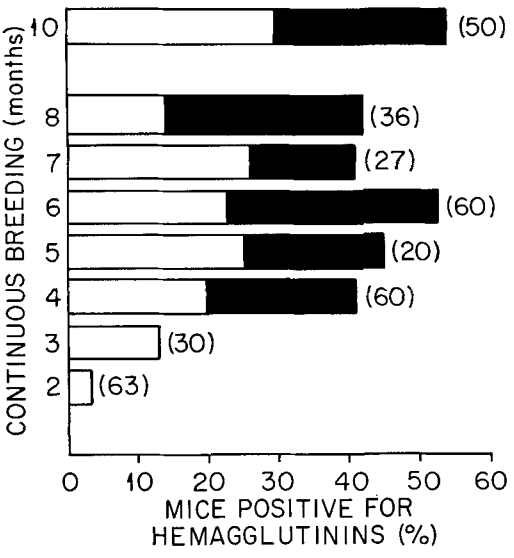

Fig. 4 Fraction of C57BL/6J ♀♀ breeding with DBA/2J ♂♂ having anti-*H*-2^d antibodies. Strong ■ and week □ are arbitrary classifications based on strength of agglutination and titer. Number of animals tested in parentheses.

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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