DIFFERENTIAL IMMUNOFLUORESCENCE OF FERTILIZED MOUSE EGGS WITH H-2 AND NON-H-2 ANTIBODY

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Until recently, interest in the embryonic expression of transplantation antigens has focused primarily upon the question of why pregnancies within genetically heterogeneous mammalian populations are predominantly successful, despite the frequent dissimilarity between mother and fetus for genes determining important histocompatibility antigens (1, 2).

A new impetus for studying H-antigen ontogeny stems from the evidence that these cell substances may play an important role in the processes of cellular differentiation, particularly those events which determine immunological competence or potentiate carcinogenesis. The association of *histocompatibility-2* (*H-2*)[1] antigen phenotype in mice with the capacity to resist oncogenic viruses (3, 4) or to respond to antigen stimulation (5) indicates a biological significance other than that which relates to transplantation of tissues.

Early suggestions that histocompatibility antigens were not expressed in embryos or that they occurred in a nonimmunogenic form have been convincingly refuted by a number of investigations. These studies have shown that in mice, tubal eggs and blastocysts can be prevented from developing when transferred to the kidney capsules of specifically sensitized recipients (6, 7), that 9-day old embryonic tissues are rejected when grafted to preimmunized host (8), and that pregnancy can, in fact, result in maternal sensitization to histocompatibility antigens (9).

Most of the experiments cited were performed before the general availability of congenic resistant (CR) strains of mice (10) which make it possible to study single specific histocompatibility antigen systems. Therefore, conclusions could not be made as to which of the many mouse histocompatibility systems were operative, or whether antigens of the highly immunogenic *H-2* system were present.

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[1] *Abbreviations used in this paper:* $BMOC_2$, Brinster's medium for ovum culture; CR, congenic resistant; *H-2*, *histocompatibility-2*; PBS, phosphate-buffered saline.

Schlesinger (11) reported absorptions of *H-2* agglutinins by tissues from 10.5-day old fetuses, the youngest studied. Attempts to detect *H-2* antigens at earlier embryonic stages have had variable results which may be due, in some instances, to complications of non–*H-2* incompatibilities existing between the strains used for grafting or for production of antibody (12–17).

Heyner et al., taking advantage of antiserum produced by immunizations between CR strains, studied the effects of *H-2* allo-antibody on the development of tubal mouse eggs to blastocysts in vitro (18). Although some antisera known to contain both *H-2* and non–*H-2* antibody destroyed the eggs, those containing *H-2* antibody alone failed to impede blastocyst formation, even in the presence of guinea pig serum as a source of complement. The results suggested a deficiency or absence of *H-2* sites at these early stages.

To test this proposal more directly, immunofluorescence has been used to look for the presence of *H-2* and non–*H-2* specificities on fertilized mouse eggs at the two-cell stage. The results of the study have been presented in preliminary form (19) and are reported in detail here.

Materials and Methods

Mice.—Lymphocytes or eggs from mice of the following inbred strains were used in the immunofluorescence tests: B10.D2/new Sn (B10.D2), A/J, C3H/He, C57BL/10Sn (B10), and DBA/2J. All mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. They were maintained after arrival for periods of 1–3 wk before use.

Target Cells.—Fertilized eggs at the two-cell stage were collected from superovulated mice 8–10 wk old. Female mice received 10 IU of pregnant mare serum (Gestyl, Organon, Inc., West Orange, N.J.) followed 48 hr later by 10 IU of human chorionic gonadotropin (Pregnyl, Organon, Inc.). Females were placed with males immediately after the second injection and were checked for the presence of vaginal copulation plugs next morning. Approximately 24 hr later the oviducts were removed and the two-cell embryos flushed from the tubes, according to the method of Brinster (20). The eggs were washed with Brinster's medium for ovum culture ($BMOC_2$) and the zona pellucida, which may act as a protective barrier around the egg, was removed by gentle digestion with 0.25% pronase. Removal of the zonae was monitored continuously to assure minimum exposure of the blastomeres to the enzyme. When the zonae were so thin as to be only just discernible, the eggs were transferred to culture medium, and this trauma caused the final loss of the zona. Blastocysts were obtained by flushing the oviducts and uterus 3½ days after mating and removing the zona pellucida as described above, or alternatively, four-cell eggs were cultured in vitro to the blastocyst stage, and those blastocysts which were normal in appearance were utilized. Some of these had hatched from the zona and so did not require the pronase treatment; these embryos acted as a control for the reactivity of blastocysts whose zonae were removed enzymatically.

Unfertilized eggs were obtained by flushing the oviduct the morning after the administration of Pregnyl. Cumulus cells were removed by a brief exposure to hyaluronidase, after which the zona was removed as described above.

Lymphocytes were obtained by teasing the axilliary, brachial, and mesenteric lymph nodes. After washing twice in phosphate-buffered saline (PBS) supplemented with 5% normal mouse serum, the cells were suspended at concentrations of 10^6 cells/ml for the immunofluorescence tests.

Serological Methods.—The *H-2*–containing antisera used were obtained after multiple injections of cells from lymphoid tissue or cultured cell lines as summarized in Table I. Ag-

glutinin titers were determined by the human serum-dextran test (21), while cytotoxic activity was determined by a conventional dye-exclusion test, by ^{51}Cr-release (22), or both. To increase the probability of detecting low concentrations of surface *H-2* antigen, antisera obtained from several combinations of donor–recipient immunizations were pooled to broaden the range of cross-reactive antibodies.

Two antisera known to contain only non-*H-2* antibody were available. One of these, M-218, was prepared by immunizations of the B10.LP strain with tissues of B10 mice. These CR strains differ at the *H-3* locus (10) and the resulting antibody is presumably directed toward *H-3* antigens of B10. The second serum, M-220, was obtained by injecting tissues and tissue extracts from the *H-2*-compatible B10.D2 strain into DBA/2 mice. M-220 had a hemagglutinin titer of 1/640, but lacked cytolytic activity for lymphocytes. Tests on segregating populations indicated that antibody was directed to antigens of a single locus. The antibody in tests on 15 inbred strains, reacted with or was absorbed by only those strains possessing the *H-6.A* antigen described by Amos et al. (23). The *H-6* and *H-3* antigens are determined by genes in the same linkage group (24).

Serum absorptions were performed by mixing well-washed lymphoid cells (spleen, lymph node, thymus) with the appropriately diluted antibody and incubating the mixture for 30 min at 37°C before recovering the absorbed serum by centrifugation.

Immunofluorescence Technique.—The procedure used was similar to that originally described by Möller for living cell suspensions (25). Two-cell embryos or cultured blastocysts were incubated with 0.03 ml of undiluted allo-antisera or control (nonimmune or syngeneic immune) sera for 25 min at 37°C in an atmosphere of 5% CO_2 in air. The number of two-cell embryos in each sample varied between 20 and 80, while there were usually under 10 blastocysts/test. After incubation the cells were washed three times with $BMOC_2$ in order to remove unbound serum. The cells were then incubated with 0.05 ml of a suitably diluted fluorescein-conjugated anti-mouse globulin (Microbiological Associates, Bethesda, Md.), after which a minimum of three washings with medium was used to remove excess conjugate. A few blastomeres became separated during the washing and recentrifuging process, but the majority remained intact. For microscopic observation, a drop of 50% medium in glycerol was added to the sediment, and a drop of the suspended cells was placed on a slide under a cover slip. To avoid distortion of the blastomeres, small slivers of glass were used to support the weight of the cover slips. Because the number of mouse embryos surviving the test procedure was limited, all visible embryos were counted on every slide. All cells showing any bright green membrane fluorescence were counted, and the distribution over the surface of the cell was noted. One unavoidable difficulty encountered in the study was the propensity for early embryonic cells to incorporate macromolecules, as has been described by Glass (26). This resulted in a faint, nonspecific internal staining of all specimens. However, no difficulty was encountered in distinguishing serologically specific membrane fluorescence from this incorporation or from the diffuse, intense fluorescence indicative of cell death.

RESULTS

Immunofluorescence of Two-Cell Eggs.—The tests gave three kinds of results: no visible surface membrane fluorescence, staining which appeared as intense fluorescent patches evenly distributed over the surface of the blastomeres (Fig. 1), or infrequently, staining which occurred as a few sparsely distributed small

FIG. 1. Two-cell mouse embryos exhibiting specific immunofluorescence after treatment with allo-antisera. Supported cover slips permit maintenance of spherical shape of large cells and by focusing one can see typical ring type of staining (*a*) or patchy stain upon surface membrane (*b, c*). Disintegrating polar body visible in (*b*). Figures are of B10.D2 cells from three different tests after treatment with *H-6* antiserum, M-220. × 400.

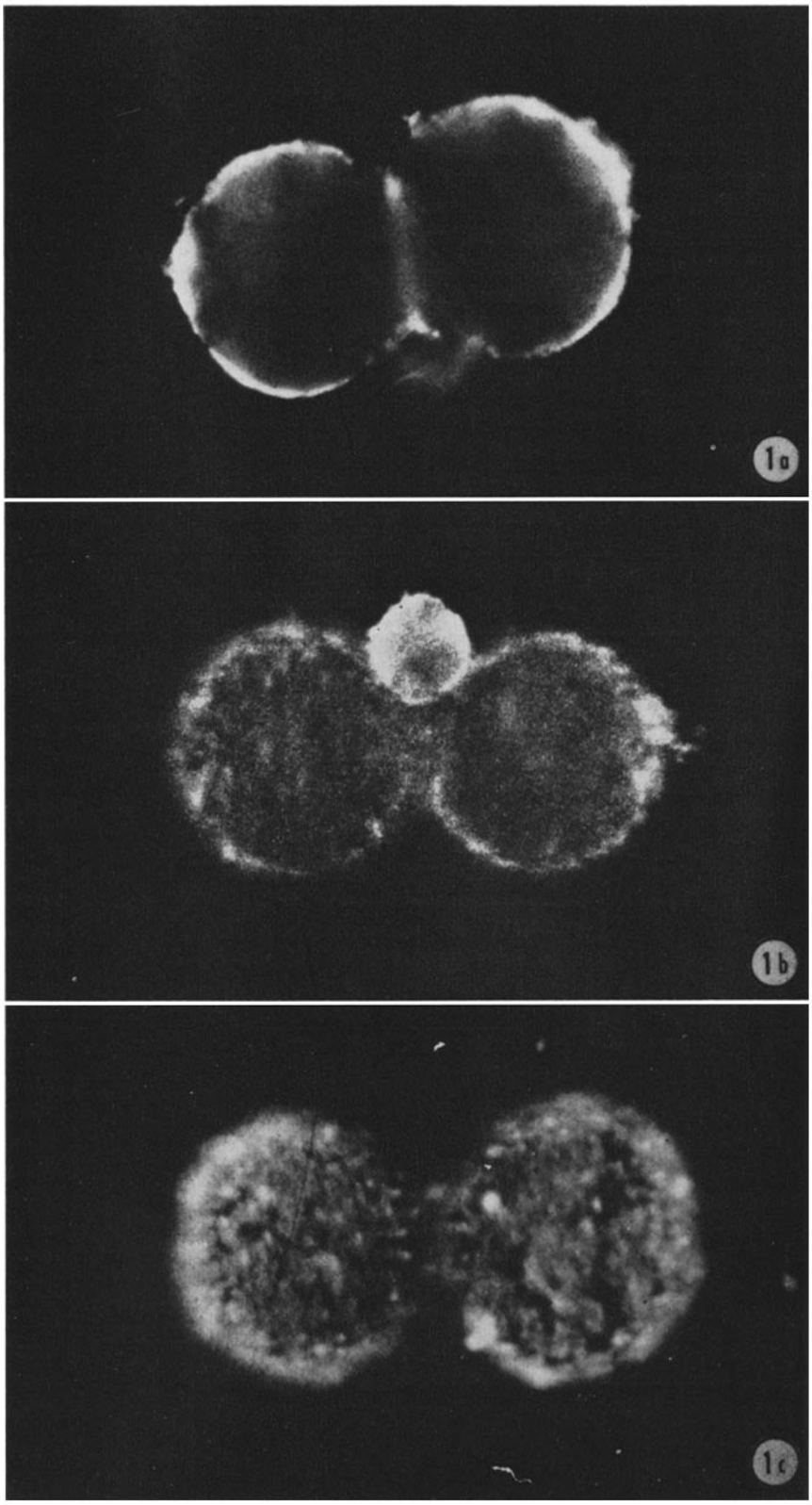

Figure 1.

spots. A nonspecific internal fluorescence, resulting from the tendency of mouse eggs to incorporate macromolecules, occurred in all samples regardless of treatment but there was no difficulty distinguishing this from positive membrane fluorescence. The use of fluorescein-conjugated anti-mouse globulin alone did not result in surface membrane staining of the eggs, nor did treatment with normal mouse serum before exposure to the conjugated fluorescein.

Tests for H-2 Specificities.—Table I indicates the results of tests with sera known to contain *H-2* antibody. The *H-2* cytolytic titers of all antisera were reasonably high and lymphocytes treated with the sera exhibited considerable staining. The antisera were of two types: those (rows 1, 2) which should contain

TABLE I

H-2 Antibody Tests

Antibody	Immunization	Cytolytic titer	2-cell embryos from strain				
			A ($H\text{-}2^a$)	C3H ($H\text{-}2^k$)	B10 ($H\text{-}2^b$)	B10.D2 ($H\text{-}2^d$)	DBA/2 ($H\text{-}2^d$)
H-2	A.SW anti-A	320	0(2)*	0(1)			
	B10 anti-B10.D2	640				0‡(1)	0‡(1)
H-2 plus non-*H-2*	C3H anti-B10	320			+(2)		
	C57BL/6 anti-L5178Y	2560				0(2)	+(2)
	Pool 1 (anti-$H\text{-}2^d$+)§	2560				+(2)	
	Pool 2 (anti-$H\text{-}2^k$+)§	640		+(2)			

* Numbers in parenthesis indicate test replicates. 0 = No reactivity, + = Reactivity.

‡ Occasional embryo with minimal stain.

§ Mixtures of equal volumes of antisera containing *H-2* cytotoxic antibody from following immunizations: *Pool 1*: C3H anti-DBA/2; C57BL anti-DBA/2; C3H anti-A; C57BL anti-A; *Pool 2*: BS/VS anti-C3H; DBA/2 anti-C3H; C57BL/6 anti-C3H; A anti-C3H.

H-2 antibody only, because they resulted from immunizations in which the donor and recipient were *H-2* CR strains (10), and those (rows 3–6), which could, and in some instances were known to, contain various non–*H-2* antibodies in addition to *H-2*.

Five tests (10–25 specimens/test) were performed with the two CR antisera, using embryos from four inbred strains. The tests were classified as negative because the majority of embryos exhibited no staining. However, in each test with the CR serum a few specimens were observed exhibiting small, isolated stained areas. This kind of reactivity was not observed in control specimens and, therefore, may be of significance.

In contrast, all those antisera coming from immunizations in which non–*H-2* antibody might develop in addition to *H-2* specificities gave evidence of reactivity, including two with *H-2* hemagglutination titers comparable to the two

CR *H-2* antisera that had failed to react. This observation, plus the failure of anti-L-5178Y sera to react with eggs of the B10.D2 strain which has the same *H-2*^{d} antigen as DBA/2, suggested that the reactions observed were non-*H-2*.

Support for this possibility came from absorption analysis of the high-titered anti-*H-2*^{d} serum, pool No. 1, as shown in Table II. For the absorptions, tissues of the CR lines B10 (*H-2*^{b}) and B10.D2 (*H-2*^{d}) were used. The strains differ for the *H-2* chromosome region only; consequently, absorption with B10 tissues should remove all antibodies cross-reactive with B10.D2 tissues except those detecting the *H-2*^{d} antigen of this strain. The B10 tissues removed reactivity for B10.D2 eggs, despite the remaining presence of *H-2*^{d} antibody, as indicated by the reactivity of B10.D2 lymphocytes. Absorptions with B10.D2 lymphoid tissue removed all activity for both eggs and lymphocytes.

Tests for Individual Non-H-2 Specificities.—Embryos from three of the four

TABLE II

Tests for Specificity of Reactivity with Antiserum (Pool 1) Containing H-2 and Non-H-2 Antibody*

Absorption with lymphoid cells of	Cells tested		
	2-cell embryo B10.D2	Lymphocytes	
		C57BL/10	B10.D2
—	+(2)‡	+	+
C57BL/10	0(2)	0	+
B10.D2	0(1)	0	0

* Mixture of antisera produced in five different strains (see Materials and Methods). See footnote Table I.

‡ Number of tests.

strains tested reacted with the putative *H-6* antibody, whereas eggs from the serum donor strain, DBA/2, were consistently negative in two tests (Table III). The serological specificity of the reactions was confirmed by absorption analyses (Table IV).

M-218, the antiserum produced by immunization of CR lines differing for *H-3* antigens, had a low agglutinating titer and no obvious cytolytic antibody, but it produced marked positive reactions with B10 and B10.D2 eggs (Table III).

Unfertilized Eggs.—Three tests were done on unfertilized eggs of the B10.D2 strain, using CR *H-2* antisera elicited in B10 donors. A total of 30 eggs was examined, all of which were completely negative.

Immunofluorescence of Blastocysts.—The failure to obtain *H-2* reactivity at the two-cell stage prompted a study of more mature embryos. The blastocyst, a multicellular form occurring in vivo at about 72 hr after fertilization, may also be obtained by culturing early embryos in vitro. From 4 to 10 blastocysts

in each of eight experiments were tested for the presence of *H-2* antigens. Interpretation of the results was hampered by nonspecific fluorescent staining, resulting both from uptake of fluorescein and from what appeared to be entrapment within the blastocoele cavity. Despite this, it was clear that in normal specimens immunofluorescence of most cellular surface membrane was absent and that the majority of outer exposed cells were not obviously reactive with *H-2* antibody (Fig. 2).

An interesting feature of the tests was the occurrence in all antibody-treated

TABLE III

Non–H-2 Antibody Tests

Antiserum	Antibody	Non-*H-2* agglutinin titer	2-cell embryos from		
			B10 ($H\text{-}2^b$)	B10.D2 ($H\text{-}2^d$)	DBA/2 ($H\text{-}2^d$)
DBA/2 anti-B10.D2	*H-6**	640	+	+	0
B10.LP anti-C57BL/10	*H-3**	40	+	+	0
C57BL/6 anti-L-5178Y	*H-2* + unknown non-*H-2*	None detected‡	0	0	+

* See text for immunization procedure used.

‡ Tests on DBA/2 RBC after absorption with B10.D2 tissues.

TABLE IV

Serological Specificity of H-6 Antibody Tests (Antiserum: DBA/2 ($H\text{-}2^d$) anti-B10.D2 [$H\text{-}2^d$])

Absorption with lymphocytes of	2-cell embryos from			
	A	B10	B10.D2	DBA/2
—	+(1)*	+(2)	+(4)	0(2)
DBA/2		+(1)	+(2)	
B10.D2			0 (1)	
B10		0 (1)	0 (1)	

* Number of tests.

specimens of a few localized areas of intense staining (Fig. 2). This was found after treatment with both the pooled anti-$H\text{-}2^d$ and CR *H-2* antiserum, for a total of eight apparently normal blastocysts. It was not observed in tests of three blastocysts treated with B10 normal serum before exposure to the fluorescein conjugate.

DISCUSSION

More than 40 tests with fertilized mouse embryos of the two-cell stage have shown (*a*) that serologically-specific tests for histocompatibility allo-antigen on the surface of mammalian two-cell embryos are feasible, (*b*) that *H-2* anti-

gens, if they are expressed at this stage, are beyond detection with the test system as employed, and (*c*) that the immunofluorescent reactivity of eggs to specific allo-antiserum seems not to be correlated with antibody activity as detected by other serological tests. Sera with weak agglutinating titers and

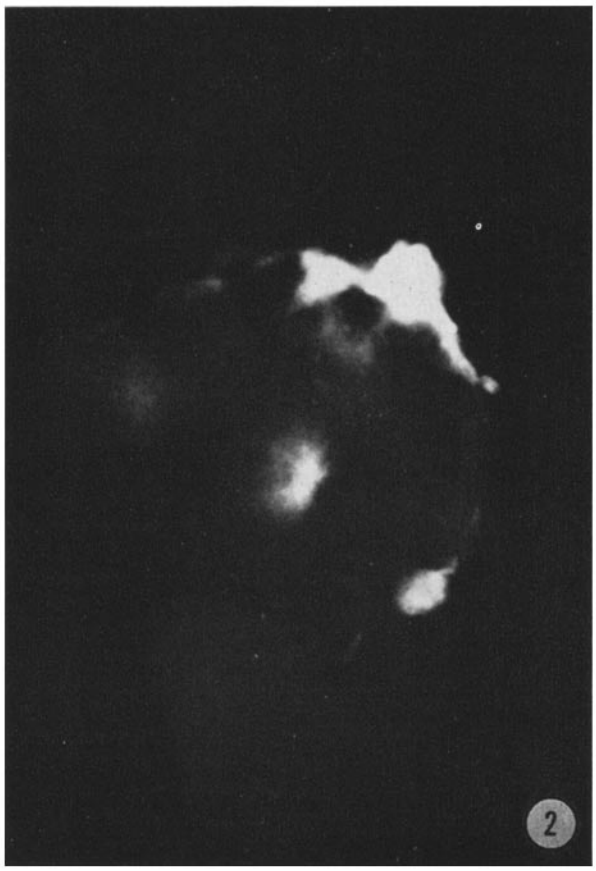

FIG. 2. Cultured B10.D2 blastocyst exhibiting localized, intensely stained area after immunofluorescent treatment. Most surface areas unstained. × 400.

with no cytolytic activity for lymphocytes exhibited marked reactivity with eggs, while potent *H-2* antibody failed to react.

The ease with which evidence was obtained of non–*H-2* expression at this early stage was surprising. Reactivity to both *H-3* and *H-6* antisera appeared as patchy staining over the entire surface of the cells. The *H-3* antigens are considered to be in the category of weak transplantation antigens in adults (10) and as such may have fewer sites exposed on the surface of cells. *H-6*, a blood

group antigen, is not known to be involved in transplantation reactions but does occur in variable amounts on some adult tissues (23). The apparent abundance of reactive sites on the blastomeres is therefore of interest.

The two non–*H-2* antigens detected in these studies are determined by genes located in close proximity within the fifth chromosome linkage group (10, 24). It may be relevant that this region has been implicated in two kinds of antigen-associated phenomena of probable embryonic initiation: a maternal–fetal interaction that causes a prenatal selective disadvantage to *H-3* homozygous individuals (27) and the development of immunological competence to respond, by producing antibody, to a genetically-defined red cell antigen of mice (28).

All evidence available indicates that, when present on cells, *H-2* antigens are more easily detected serologically than are the various non–*H-2* specificities. Moreover, the antisera detecting the latter in these studies exhibited considerably less antibody by several conventional serological tests than did the *H-2*–containing antisera. In light of these facts, the failure to detect *H-2* sites seems unlikely to be due to insensitivity of the test system.

The possibility that the pronase used to disrupt the zona pellucida caused enzyme degradation of *H-2* sites is more difficult to exclude as an explanation for the results. Nevertheless, several lines of evidence do argue against this. These include the very limited exposure of the blastomeres during zona disruption, the cleanly negative surfaces of most cells treated with *H-2* antibody, and the observation that similar concentrations of enzyme were ineffective in diminishing the reactivity of lymphocytes to *H-2* antibody. Furthermore, in our earlier studies (19), culturing of eight-cell embryos with *H-2* antibody plus complement for 2 days did not impede development to blastocysts. Even if pronase treatment in these experiments had been sufficiently damaging to have cleaved *H-2* sites, as has been reported to occur after papain treatment of some tumor cells (29), the subsequent regeneration of these sites, by analogy, should have occurred sufficiently early for the antibody to have an effect.

Perhaps the most compelling argument against pronase digestion as a reason for the absence of reactivity to *H-2* antibody was the failure to detect surface *H-2* on cells of the hatched blastocysts; these had not been subjected to pronase treatment. The results support the conclusions from the earlier studies (19) that expression of *H-2* antigens is either nonexistent or very deficient on the surface of the developing zygote during early stages of embryogenesis. Similarly, unfertilized eggs also appear to be lacking in *H-2* sites that are detectable either by the immunofluorescence tests described here or in the cytotoxic tests reported by Baranska et al. (30).

The results are in contrast to reports that *H-2* determinants are sufficiently concentrated on sperm to allow detection with cytotoxic antibody (31–33). Speculation concerning the meaning of this apparent difference in *H-2* expression on surface membranes of the male and female gametes is hampered by the

absence of unequivocal evidence of the haploid or diploid nature of the *H-2* phenotype of sperm. It is conceivable that the difference may reflect dissimilar opportunities for adsorption of *H-2* substances from body fluids during maturation (33).

Whatever its physiological basis, the lack of readily detectable *H-2* sites on the early zygote is convenient. In the absence of *H-2* complications, for example, hyperimmune anti-tumor sera may be readily analyzed for antibodies detecting other cell surface antigens such as those that may be common to embryonic and malignant cells (30, 34–36), or those representing defined non–*H-2* systems whose role in differentiation may be of greater biological significance than is their effectiveness in invoking transplantation immunity.

SUMMARY

Mouse embryos at the two-cell and blastocyst stages, as well as unfertilized eggs, have been studied by indirect immunofluorescence for the expression of *H-2* and non–*H-2* histocompatibility antigens on surface membranes. Serologically-specific reactivity to non–*H-2* antibody (*H-3* and *H-6*) was observed as diffuse, patchy staining over the entire surface of the blastomeres at the two-cell stage. In contrast, no reactivity of two-cell or unfertilized egg embryos of four inbred strains was observed when antisera containing only multispecific *H-2* cytolytic antibody were used. Antisera containing *H-2* along with non–*H-2* antibody of unknown specificity showed varying degrees of reactivity, which could be shown by absorption studies to be due to the non–*H-2* content of the serum. The results suggest that the initial expression of histocompatibility genes varies and support the hypothesis that the appearance of these cell components may relate to specific stages of differentiation.

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