Embryonic differentiation and organogenesis may depend on the expression of particular surface macromolecules by cells at different stages of development. Cell surface antigens specific for early mouse embryos have been identified, including antigens present on teratomas and antigens regulated by the t complex. While the expression of these and other alloantigens varies during development, the function of these molecules remains to be defined (1-6). The expression of histocompatibility antigens by embryos is of particular interest because of the possible involvement of these antigens in rejection of the fetus as an allograft and because of the possible function of these antigens in mediating cell-cell interactions in adult tissues (7, 8).

It is generally agreed that products of the major histocompatibility complex (H-2) are expressed by 7-1/2 day and older embryos (1, 2, 4) on tissues derived from the inner cell mass of the blastocyst. Also, in blastocyst outgrowths in culture, presumed cells of the inner cell mass are positive for H-2, while the trophoblastic giant cells are negative (9, 10).

Expression of histocompatibility antigens by preimplantation mouse embryos has been examined by several approaches, including immunofluorescence (5, 11), mixed agglutination (12), complement-mediated cytotoxicity (13), cell-mediated cytotoxicity (14), and ectopic transplantation (15). The majority of these tests failed to detect H-2 antigens, although products of the minor histocompatibility loci (non-H-2) have been detected on all cleavage-stage embryos and blastocysts (5, 10-15). Recently, experiments using immunoperoxidase labeling and electron microscopy indicated that small amounts of H-2 antigens are present on the surface of blastocysts, and that these antigens are lost at the time of implantation (10). The precise time of appearance has not yet been established.

We have investigated the expression of H-2 antigens by preimplantation mouse embryos using sensitive immune precipitation reactions between specific H-2 alloantisera and extracts prepared from embryos grown completely in vitro and radioactively labeled by both external- and internal-labeling procedures. This allowed analysis of the kinetics and locale of expression of these antigens in the absence of contamination by maternal tissues. Our results indicate that H-2 antigens are first expressed at the late blastocyst stage by the cells of the inner cell mass.

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Materials and Methods

Mice. (C57BL/10J x SJL/J)F1 mice bred in our colony and NCS random bred mice obtained from The Rockefeller University colony were used. The F1 hybrids are H-2b/H-2k heterozygotes. The H-2 type of NCS mice is unknown; adult spleen cells from this strain do not react with standard anti-H-2b, H-2s, or H-2k alloantisera.

Antiserum. Antiserum against H-2.33 (H-2Kb) was made by injecting spleen and thymus cells from female B10.A(5R) into female (B10.D2 x A)Fj recipients (16). Antiserum D-19 against H-2Ks was obtained from the Transplantation Immunology Branch of The National Institute of Allergy and Infectious Diseases, Bethesda, Md. Antiserum against H-2.11 (H-2Kk) prepared by injection of cells from B10.RIII mice into (B10.D2 x DBA/1)F1 mice (16) was used as a control for nonspecific precipitation.

In Vitro Fertilization and Culture. The embryos used throughout this study were obtained by fertilization in vitro of ova from superovulated females. Females received 5 IU of pregnant mare serum followed 48 hr later by 5 IU of human chorionic gonadotropin. The conditions for fertilization and the culture medium were according to Hoppe and Pitts (17), except for the concentrations of sodium pyruvate (0.5 mM) and bovine serum albumin (4 mg/ml).

Most of our experiments were carried out using ova from (C57BL/10J x SJL/J)F1 females, fertilized in vitro with epididymal sperm from (C57BL/10J x SJL/J)F1 males. In contrast to experiments using sperm and eggs from inbred strains, this hybrid combination routinely yielded 90% fertilization and greater than 75% of the fertilized eggs grew in culture to the blastocyst stage. The fertilized ova, identified by the presence of both male and female pronuclei at the end of the initial incubation period, were washed in medium and batches of 50 were cultured further in tubes containing 1 ml of culture medium. The day of fertilization is considered to be day 0.

Lactoperoxidase Iodination of Labeled Embryos. Embryos at the appropriate stage of development were collected from culture tubes and washed three to four times by micropipetting under a dissecting microscope with phosphate-buffered saline containing 1 mg/ml polyvinylpyrrolidone. The embryos were then placed in a drop (approximately 50 µl) of this buffer under paraffin oil and 250 µl of a freshly prepared reaction mixture containing 0.1 mg lactoperoxidase (18), 50 µl of 0.0003% hydrogen peroxide, and 0.25 mCi of [125I]NaI (New England Nuclear, Boston, Mass.) in 0.02 M phosphate buffer, pH 7.4, was added. After incubation in this mixture for 10–15 min at room temperature, the embryos were washed by sequential transfer to drops of 10 mM KI in phosphate-buffered saline under oil. They were then transferred in a small droplet to a plastic microfuge tube and extracted with a solution of 0.1% sodium dodecyl sulfate (SDS) in 0.15 M NaCl, 10 mM Tris, pH 7.4, containing 50 U/ml trasyloil to inhibit proteolysis; this concentration of SDS efficiently extracted intact embryos but did not inhibit immune precipitation reactions. The extract was mixed briefly on a Vortex mixer (Scientific Industries, Inc., Bohemia, N. Y.), left on ice for 30 min, and centrifuged for 90 s in a Beckman microfuge (Beckman Instruments, Inc., Fullerton, Calif.). Each group of 100 embryos was extracted in 100 µl.

Incorporation of Amino Acids into Embryos. Radioactive culture medium was prepared by adding 10 µCi/ml of [3H]-leucine or [3H]-amino acid mixture (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) to the embryo culture medium. Embryos were cultured in this medium for 6–12 h, collected, and washed three to four times in phosphate-buffered saline containing 1 mg/ml polyvinylpyrrolidone, and finally transferred to a microfuge tube in a droplet of this buffer. The extraction procedure was the same as for externally labeled embryos. Addition of tritiated amino acids to the medium did not significantly alter the growth rate or development of these embryos from the two-cell stage through the blastocyst at levels of 5–10 µCi/ml. The first cleavage, however, was severely inhibited in the presence of tritiated amino acids at these levels.

Incorporation of Amino Acids into Trophoblast Vesicles. Trophoblast vesicles were produced according to the procedure of Snow (19). Embryos were cultured from the two- to four-cell stage in the presence of 0.35 µCi of methyl-[3H]-thymidine (New England Nuclear), and after blastocysts had formed, 5 µCi of [3H]-leucine were added to the culture medium. The trophoblast vesicles were collected and treated as were the internally labeled embryos.

Labeling of Inner Cell Masses. Inner cell masses were prepared by immunosurgery of blastocysts (20) internally labeled with [3H]-amino acids by treatment with rabbit antiserum against

\^Abbreviations used in this paper: Con A, concanavalin A; SDS, sodium dodecyl sulfate.
OTT6050 embryoid bodies (21) or with rabbit antiserum against (C57BL/10J × SJL/J)F₁ spleen cells. After washing, fresh guinea pig complement was added to lyse the outer trophoblast layer. Inner cell masses were collected, washed, and extracted as for internally labeled embryos.

**Immune Precipitation.** For each assay, 50 μl of extract was mixed with 10 μl of antiserum or normal serum control and left at room temperature for 30 min. The immune complexes were precipitated by addition of 50 μl of a 10% suspension of *Staphylococcus aureus* in saline containing 10 mM Tris, pH 7.4 (22). The precipitates were disrupted by boiling for 1 min in 2% SDS containing 5% (vol/vol) β-mercaptoethanol after which the *S. aureus* was removed by centrifugation (4 min in the microfuge). In some experiments, the immune complexes were precipitated using either goat or rabbit anti-mouse immunoglobulin (Miles Laboratories, Inc., Miles Research Products, Elkhart, Ind.). We selected batches of goat or rabbit antiserum that gave negligible precipitation of components in the 45,000 mol wt range judged by control experiments with normal mouse serum. The products of the immune precipitation reaction were analyzed by electrophoresis on SDS-polyacrylamide gels (23). The gels were then sliced and counted for radioactivity. MOPC 21 γ-chain, mol wt 50,000 (24) and MOPC 21 κ-chain, mol wt 23,000 (25) both labeled with 125I using chloramine-T (26) or with 35S by incorporation of 35S-methionine into P3 (MOPC 21) cells were used as internal standards to permit comparison of gels from different experiments. Purified proteins were obtained from Litton Bionetics, Kensington, Md.; P3 (MOPC 21) cells were obtained from The Salk Institute, San Diego, Calif.

**Results**

Eggs and embryos from the two-cell stage through the late blastocyst stage (4-1/2 days) were labeled with iodine by the lactoperoxidase method which has been extensively used to label selectively surface molecules of other cell types. Detergent-solubilized extracts from the labeled embryos were tested for H-2 antigens by immune precipitation with specific alloantisera. H-2 antigens were not detected in the extracts of iodinated embryos prepared throughout the culture period (Fig. 1). To demonstrate that the extracts contained iodinated cellular components, the extracts were tested for concanavalin A (Con A) receptors using immune precipitation with Con A and rabbit antiserum against Con A. The gel profiles obtained (Fig. 1) indicated that some molecules were in fact labeled by this procedure. Furthermore, the patterns differed with the stage of embryonic development. Because H-2 antigens are easily labeled by lactoperoxidase iodination of adult cells, the lack of detectable H-2 in the immune precipitates suggested that these antigens were lacking from the surface of early embryos.

In an alternative approach, we analyzed extracts of embryos internally labeled by culture in medium containing radioactive amino acids. These experiments demonstrated the synthesis of H-2 antigens by cells in the late blastocyst stage. The results of immune precipitation reactions on extracts of embryos grown for 12 h in the presence of 3H-leucine during the period of blastocyst expansion and hatching, i.e., from 4 to 4½ days postfertilization, are shown in Fig. 2. Immune precipitates from extracts of F₂ embryos yielded peaks of apparent mol wt of 45,000 and 12,000, as expected for H-2 heavy and light chains (27). These peaks were detectable with antisera against the parental H-2 types, namely H-2K⁺ and H-2K⁻ (Fig. 2a and b), but not with antiserum against an irrelevant H-2 type (H-2K⁺, Fig. 2c) nor with normal mouse serum (Fig. 2d). As a further control, parallel experiments with extracts of NCS embryos, which do not have a known H-2 type, also yielded negative results (Fig. 2e). These results
H-2 ANTIGENS OF PREIMPLANTATION EMBRYOS

FIG. 1. Analysis of extracts of cleavage-stage embryos iodinated with lactoperoxidase. Immune precipitates using anti-H-2K^b (solid line) or 10 μg of Con A followed by 100 μg of rabbit anti-Con A immunoglobulin (dashed line) were analyzed by electrophoresis on 10% polyacrylamide gels in SDS. (a) unfertilized eggs; (b) four-cell embryos; (c) morulae; (d) late blastocysts. γ indicates the position of the MOPC 21 γ-chain, mol wt 50,000; κ indicates the position of the MOPC 21 κ-chain, mol wt 23,000.

...indicate that material specifically reacting with appropriate antisera against H-2 was synthesized by the blastocyst.

Similar experiments using extracts from embryos labeled from 3½ to 4 days postfertilization (during formation of the blastocoel) yielded negative results (Fig. 2d). Extracts from earlier cleavage-stage embryos were also negative. The data indicate that synthesis of H-2 antigens began at about the time of blastocyst expansion.

At the stage of blastocyst formation, the trophectoderm and the inner cell mass become morphologically distinguishable. To determine whether H-2 antigens are synthesized by both cell types or by only one, we examined each of the cell types independently. Embryos were cultured from the two- or four-cell stage in the presence of 0.05 μCi/ml methyl-3H-thymidine, giving rise to trophoblast vesicles, which were then cultured for 12 h in the presence of 3H-leucine. Inner cell masses were obtained by immunosurgery from 3H-leucine-labeled 4½-day embryos. Extracts of the trophoblast vesicles and the inner cell masses were assayed as above for H-2 antigens (Fig. 3). Extracts of trophoblast vesicles lacked any detectable products of the H-2 locus, while the inner cell masses from embryos not treated with 3H-thymidine were able to synthesize these products (Fig. 3a and b). Control precipitations of identical extracts with the irrelevant
anti-H-2K* serum were negative (Fig. 3c). Electrophoresis of the whole extracts before precipitation (Fig. 3d) indicated that both the inner cell mass and the trophectoderm vesicles were able to incorporate $^3$H-leucine into protein. On the basis of these experiments, we conclude that it is the cells of the inner cell mass, rather than the trophoblast, that synthesize H-2 antigens during this period.
**Fig. 3.** Localization of H-2 antigen-producing cells in internally labeled blastocysts. Electrophoresis of immune precipitates of extracts of inner cell masses (solid line) and trophoblast vesicles (dashed line) with: (a) anti-H-2Kb; (b) anti-H-2Ks; and (c) anti-H-2Kk. (d) Electrophoresis of whole extracts of equal numbers of inner cell masses (solid line) and trophoblast vesicles (dashed line). Molecular weight standards as described in Fig. 1.

**Discussion**

The experiments presented above indicate that the synthesis of H-2 antigens by preimplantation embryos is first detectable using immune precipitation assays at the late blastocyst stage, approximately 96 h after fertilization. Furthermore, based on experiments with isolated inner cell masses and trophoblast vesicles, it is the cells of the inner cell mass, rather than the trophectoderm, that synthesize these antigens. Further experiments using electron microscopic labeling techniques and surface iodination of inner cell masses prepared by microdissection will be required to determine whether there is any further restriction in the synthesis of these antigens by individual cells of the inner cell mass.

Conflicting information about the presence of histocompatibility antigens on preimplantation embryos has been obtained using biological assays and immunofluorescence techniques. For this reason we have used a sensitive immune precipitation assay to examine this question. In this assay, identification of H-2 antigens relies on the specificity of the antisera used and on analysis of the products of the serological reaction. We have used highly specific sera prepared in congenic mice and have taken particular care to control for nonspecific
precipitation of cellular components by selecting batches of antisera. In our positive experiments, material with mol wt of 45,000 and 12,000, as expected for \( H-2 \) heavy and light chains (27), was precipitated only with antisera of the appropriate specificity, although the relative magnitudes of the peaks varied. Inasmuch as both the anti-\( H-2K^n \) and the anti-\( H-2K^k \) sera are known to contain activity against certain murine leukemia virus antigens (16), it is unlikely that the material precipitated with anti-\( H-2K^n \) serum is viral in origin. These data, together with the negative results using nonimmune serum and embryos of a different \( H-2 \) type (Fig. 2), indicate that \( H-2 \) antigens are present in the blastocyst extracts. In addition, growth of the embryos in vitro in chemically defined medium eliminated the possibility of contamination by \( H-2 \) antigens from maternal tissues or fluids.

From the strain combinations used to produce the \( H-2 \) antisera, it is possible that these sera may contain activity against products of the \( I \) region. However, the antisera against \( H-2K^k \) did not precipitate products with the molecular weights characteristic of \( Ia \) antigens in previous experiments with adult spleen cells (27), nor did we observe such components in the present experiments. Because the alloantisera were prepared using adult tissues, it is unlikely that these sera would detect products of any genes closely linked to the \( H-2K \) locus that are expressed only during embryonic development. Even though these antisera detect private specificities, we cannot absolutely exclude the possibility that any such embryonic antigens would cross-react with their adult counterparts.

\( H-2 \) antigens were not found in extracts of blastocysts labeled by the lactoperoxidase surface iodination method. This result is consistent with the conclusion that it is the cells of the inner cell mass, rather than the trophoblast, that synthesize these antigens. In intact blastocysts, the inner cell mass is surrounded by the cells of the trophoblast and therefore is inaccessible to lactoperoxidase. While the surface trophoblast cells were iodinated (Fig. 1d), they lacked detectable \( H-2 \) in our assay. Trophoblast vesicles internally labeled with \( ^3\text{H-leucine} \) also failed to yield detectable \( H-2 \) antigens (Fig. 3). While trophoblast vesicles may differ from normal trophoblast because of damage by the \( ^3\text{H-thymidine} \) or, more importantly, because the normal development of the trophoderm may depend on the presence of a viable inner cell mass, the results are consistent with those obtained by lactoperoxidase iodination of normal embryos.

Recent electron microscopic studies using immunoperoxidase labeling have indicated the transient appearance of \( H-2 \) antigens on the surface of blastocysts (10). The relatively low labeling levels obtained suggest that the amount of \( H-2 \) on the surface was possibly lower than could be detected by our techniques. Cells of the inner cell mass were not tested for \( H-2 \) at this stage, although inner cell mass outgrowths in vitro at later stages were heavily labeled.

The initial synthesis of \( H-2 \) antigens correlates with the expansion of the blastocyst and the morphological differentiation of the trophoblast into giant cells. While the exact determination of the onset of \( H-2 \) synthesis requires more detailed experiments, our results suggest that synthesis of the major histocompatibility antigens in embryos is delayed just until the cells synthesizing these
antigens can be protected from possible interactions with the maternal immune system. In the late morula and early blastocyst stages, intercellular junctions develop in the outer cells that form the trophoblast (28), which becomes impermeable to molecules the size of antibodies (20). In addition, the cells of the trophoblast do not serve as targets for cell-mediated lysis (14, 29), which apparently depends on expression of H-2 antigens by the target cell (7, 30). Therefore, the impermeability of the trophoblast layer together with its lack of H-2 antigens may allow both implantation of the embryo and expression of H-2 antigens by the inner cell mass without risk of exposure to immunological rejection. Whether H-2 antigens have any role in the cell-cell interactions required for further differentiation of the inner cell mass remains to be determined.

The techniques we have used to demonstrate the presence of H-2 antigens in embryos may prove valuable for further analysis of early development. While lactoperoxidase iodination, internal labeling, and immune precipitation have been used in other cell systems where greater numbers of cells are available, our results demonstrate that it is possible to use these techniques with the relatively small numbers of embryos that can be obtained. Together with other specific antisera and more sophisticated two-dimensional electrophoresis techniques, it may be possible to define more precisely, at the level of chemical structure, the changes in expression of macromolecules that correlate with cellular differentiation in early embryos.

Summary

Synthesis of H-2 antigens by preimplantation mouse embryos is first detectable at the late blastocyst stage. These antigens were detected using immune precipitation assays of extracts of embryos labeled by incorporation of radioactive amino acids but not by surface iodination. Experiments using isolated inner cell masses and trophoblast vesicles indicate that it is the cells of the inner cell mass that synthesize these antigens. H-2 antigens were not detected in either early blastocysts or at earlier cleavage stages.

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