LOCALIZATION OF *H-2* HISTOCOMPATIBILITY ALLOANTIGENS ON MOUSE EMBRYONIC TOOTH EPITHELIAL AND MESENCHYMAL CELL SURFACES

HAROLD C. SLAVKIN, GARY N. TRUMP, VIRGINIA MANSOUR, PAMELA MATOSIAN, and WILLIAM G. MINO. From the Department of Biochemistry and the Department of Microbiology and Immunology, School of Dentistry, University of Southern California, Los Angeles, California 90007

INTRODUCTION

Recent studies employing a method of grafting mouse embryos to pre-sensitized recipients report that *H-2* first appears on embryonic cell membranes during the 7th day of gestation (1). Serological tests indicated the appearance of *H-2* on various embryonic tissues and organs starting on the 8th day of gestation (2, 3, 4). Immunofluorescent studies of histocompatibility antigens in normal adult mouse tissues suggest their confinement on the cell periphery of spleen, liver, pancreas, cerebral cortex, cerebellum, lymph nodes, thymus, submaxillary gland, and kidney (5–7).

Such observations suggested a potentially useful approach for studying epithelial-mesenchymal interactions (8). Preliminary experiments were designed to search for *H-2* alloantigens on the cell surfaces of both epithelia and adjacent mesenchyme of embryonic tooth rudiments from congenic strains of mice. An unequivocal demonstration of the presence of *H-2* antigens on embryonic epithelia and mesenchyme would not only be of interest for experimental studies on heterotypic cell and tissue interactions (e.g., transfilter embryonic induction studies using intrastrain chimeras), but also could provide an important cell surface marker in membrane isolation procedures. Additionally, *H-2* antigens (cell surface glycoproteins) could provide useful surface markers with which to evaluate enzymatic dissociation of cells and the recovery or resynthesis of cell surface molecules.

In this note we demonstrate the employment of the indirect ferritin-antibody labeling method to detect *H-2* histocompatibility alloantigens on the outer cell surfaces of odontogenic epithelia and mesenchyme isolated from congenic strains of embryonic mice. Ultrastructural demonstration of *H-2* histocompatibility alloantigens on embryonic mouse tissues has not yet been reported.

MATERIALS AND METHODS

Production and Titration of Antisera

Congenic strains, B10.D2/ScSn ($H\text{-}2^d$) and C57BL/10ScSn ($H\text{-}2^b$), differing only at the *H-2* locus, were obtained from the Jackson Laboratory, Bar Harbor, Maine. These congenic strains of mice will hereafter be referred to as B10.D2 and C57BL/10. The mouse anti-$H\text{-}2^d$ antiserum was prepared by injecting C57BL/10 mice (of $H\text{-}2^b$ specificity) with lymphoid spleen preparations from spleens of B10.D2 adults. Mice were bled after six intraperitoneal injections administered every 8 days. Immunoglobulins were isolated from the mouse antisera by precipitation with ammonium sulfate, dialyzed against H_2O and 0.01 M phosphate buffer, pH 7.5, to remove $(NH_4)_2SO_4$, and finally against 0.05 M phosphate buffer, pH 7.5, and stored at 4°C. The hemaggluti-

nation technique of Stimpfling was used to titrate the antisera (9) with minor modifications (M. Takasugi, personal communication). Before use, the serum was adsorbed six times against 5% packed volumes of syngeneic mouse lymphoid cells for 30 min at 4°C in Hanks' basic buffered solution (HBBS) plus 0.5% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.).

Preparation of Ferritin-Conjugated Antibodies

Horse spleen ferritin, recrystallized six times (Miles Laboratories, Inc., Kankakee, Ill.), was further purified by cadmium sulfate crystallization, ammonium sulfate precipitation, and ultracentrifugation after the methods of Nicolson and his colleagues (10). Goat anti-mouse 7S-γ-globulin serum was commercially obtained (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.), and the immunoglobulins were isolated by several precipitations with equal volumes of saturated ammonium sulfate and extensively dialyzed. The goat immunoglobulins and antibodies were subsequently conjugated to ferritin for use as the indirect label. Ferritin conjugates of goat anti-mouse γ-globulin were prepared using toluene-2,4-diisocyanate coupling methods previously published (11). The ferritin conjugates were separated from unconjugated proteins and apoferritin by dialysis and centrifugation, and then stored in sterile vials.

Isolation of Embryonic Tissues

C57BL/10 and B10.D2 embryos during the 18th day of gestation were dissected free of decidua and membranes under low magnification. Days of gestation were assessed after development of the maternal vaginal plug (day 0). They were more exactly staged following the descriptions given by Snell and Stephens (12), Rugh (13), and the in vivo and in vitro morphological descriptions of mouse molar development (14). Mandibular first molar tooth rudiments were carefully dissected from the embryos using microdissection and placed in HBBS, pH 7.5 at 4°C. The isolated molar rudiments were then enzymatically dissociated (1% trypsin [Difco Laboratories, Detroit, Mich.] 1:250 in HBBS) for 10 min, and the enamel organ epithelium was mechanically teased from the adjacent dental papilla mesenchyme (15). Isolated epithelia and adjacent mesenchyme were then thoroughly washed in HBBS and incubated overnight at 37°C in 10% fetal calf serum and Eagle's media without glutamine. The following day (~15 h) the isolated epithelia and mesenchyme from both strains of mice were used for the indirect ferritin labeling experiments. In addition, white blood cell preparations were made from blood samples taken from C57BL/10 and B10.D2 donors. Lymphoid cells were harvested from maternal spleens by techniques previously published (16).

Ferritin-Antibody Labeling

All handling, labeling, and washing of tissues was done in the presence of 0.5% BSA at 4°C (G. Nicolson, personal communication). Embryonic tissues were washed twice with HBBS, pH 7.5 + 0.5% BSA, centrifuged, and pelleted a second time in conical BEEM capsules (130-SPC, Ted Pella Co., Tustin, Calif.). Representative samples of dissociated 18-day embryonic molar tooth tissues, as well as adult spleen cells, red blood cells, and white blood cells were placed in BEEM capsules. Embryonic tissues and cells from both strains of mice were incubated in 200 μl of fractioned, adsorbed C57BL/10 anti-B10.D2 serum for 5-, 10-, 15, or 30-min time periods. Tissues were then washed three times in HBBS containing 0.5% BSA. Each sample was incubated in 200 μl of ferritin-conjugated goat anti-mouse sera for identical times corresponding to the previous incubation. Tissues were then repeatedly washed in HBBS plus BSA to remove excess ferritin label. C57BL/10 embryonic tooth tissues and maternal cell preparations were used as controls in these experiments.

Electron Microscopy

Ferritin-antibody-labeled B10.D2 embryonic tooth epithelia and mesenchyme, adult maternal spleen lymphoid cells, red and peripheral white blood cells, and control C57BL/10 embryonic tissues and maternal cells were fixed in purified glutaraldehyde buffered with 0.2 M cacodylate at pH 7.2 for 20 min, extensively washed and postfixed in 2% osmium tetroxide, dehydrated through a graded alcohol series, and then embedded in Epon 812 (17). Thick sections (0.5 μm) of isolated tissues and cells were stained with 1% toluidine blue for light microscopy. Thin sections with silver to gray interference colors were cut with a diamond knife on a Sorvall MT-2 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.). Some were stained with lead citrate. Stained and unstained sections were examined and photographed in a Zeiss EM-92 electron microscope.

OBSERVATIONS AND DISCUSSION

Ultrastructural observation of labeled antibodies and ligands has been employed to map antigens and receptors on numerous cell surfaces from adult animals and has contributed appreciably to understanding of cell surface molecular topography (18–20). The cell surface serves a major function in regulation of embryonic cell and tissue interactions during morphogenesis, however,

the exact mechanisms of cell recognition and numerous (developmental) intercellular interactions remain unknown.

Our experiments demonstrate that the ferritin-labeled antibody technique can be used to locate *H-2* alloantigens on the outer cell surfaces of embryonic odontogenic epithelial and mesenchymal tissues. A highly specific anti-*H-2* antiserum was prepared by employing congenic strains of mice. The specific staining observed correlated well with results obtained by other investigators (16, 18, 21). The *H-2* alloantigen was distributed diffusely and randomly over the cell surfaces of viable B10.D2 embryonic tooth epithelial and mesenchymal cells (Figs. 1–3), and the observed antigenic sites on outer cell surfaces varied amongst each tissue type (Figs. 2 and 3). Maternal spleen lymphoid cells showed a greater number of *H-2* alloantigens (Fig. 3 *e*) than the embryonic tissue surfaces (Figs. 2 and 3 *a–c*) in accord with our previous findings obtained by the fluoresceine-labeled antibody and immunoferritin cyto-

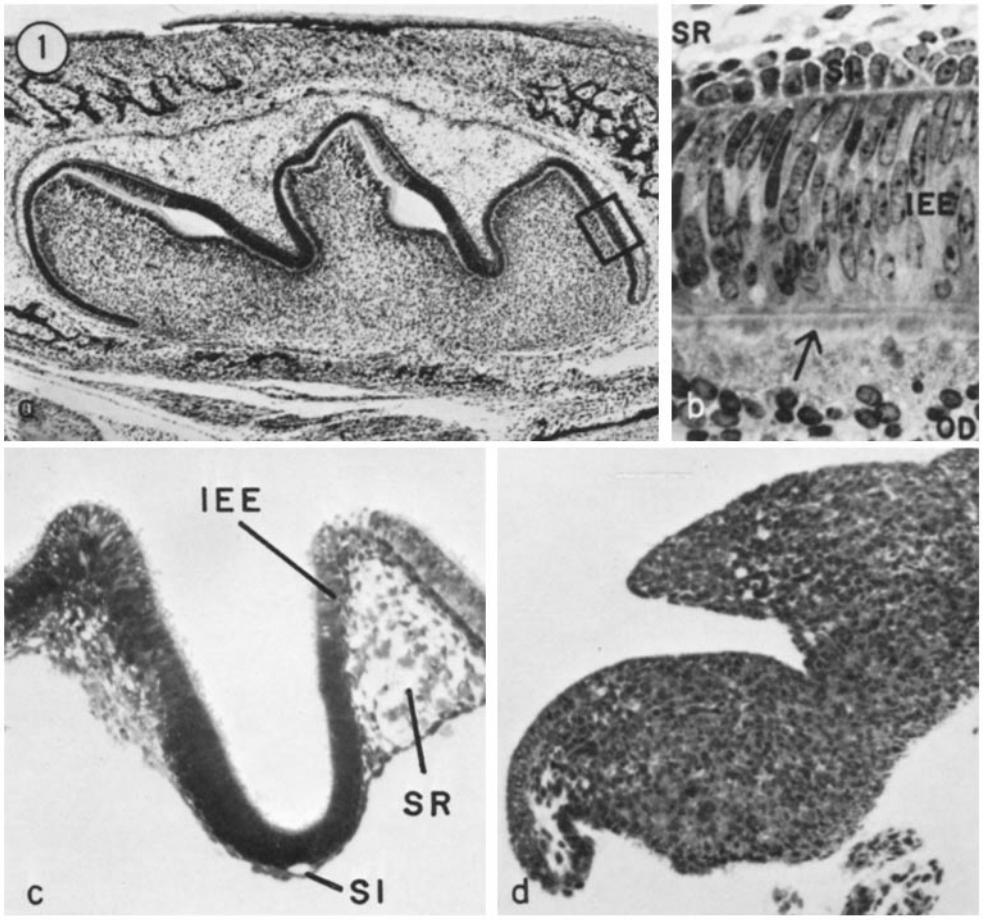

FIGURE 1 Embryonic mouse molar. (*a*) Paraffin-embedded specimen of undecalcified 18-day embryonic mouse mandibular first molar tooth rudiment *in situ*. This light photomicrograph of a 5-μm section, stained with periodic acid-Schiff's reagent, indicates the morphological relationship amongst the odontogenic epithelia and adjacent mesenchyme. × 75. (*b*) Higher magnification of the region within the rectangle shown in Fig. 1 *a*. Note the stratum intermedium (*SI*), and inner enamel epithelium (*IEE*) in juxtaposition to the developing extracellular organic matrix (arrow) and the dental papilla mesenchyme. *Od*, odontoblasts. × 700. (*c*) Trypsin dissociation of embryonic epithelial tissue components. Light photomicrograph of the epithelial tissue containing inner enamel epithelia (*IEE*), stratum intermedium (*SI*) and the stratum reticulum (*SR*). × 125. (*d*) Light photomicrograph of the trypsin-dissociated dental papilla mesenchyme tissue. × 125.

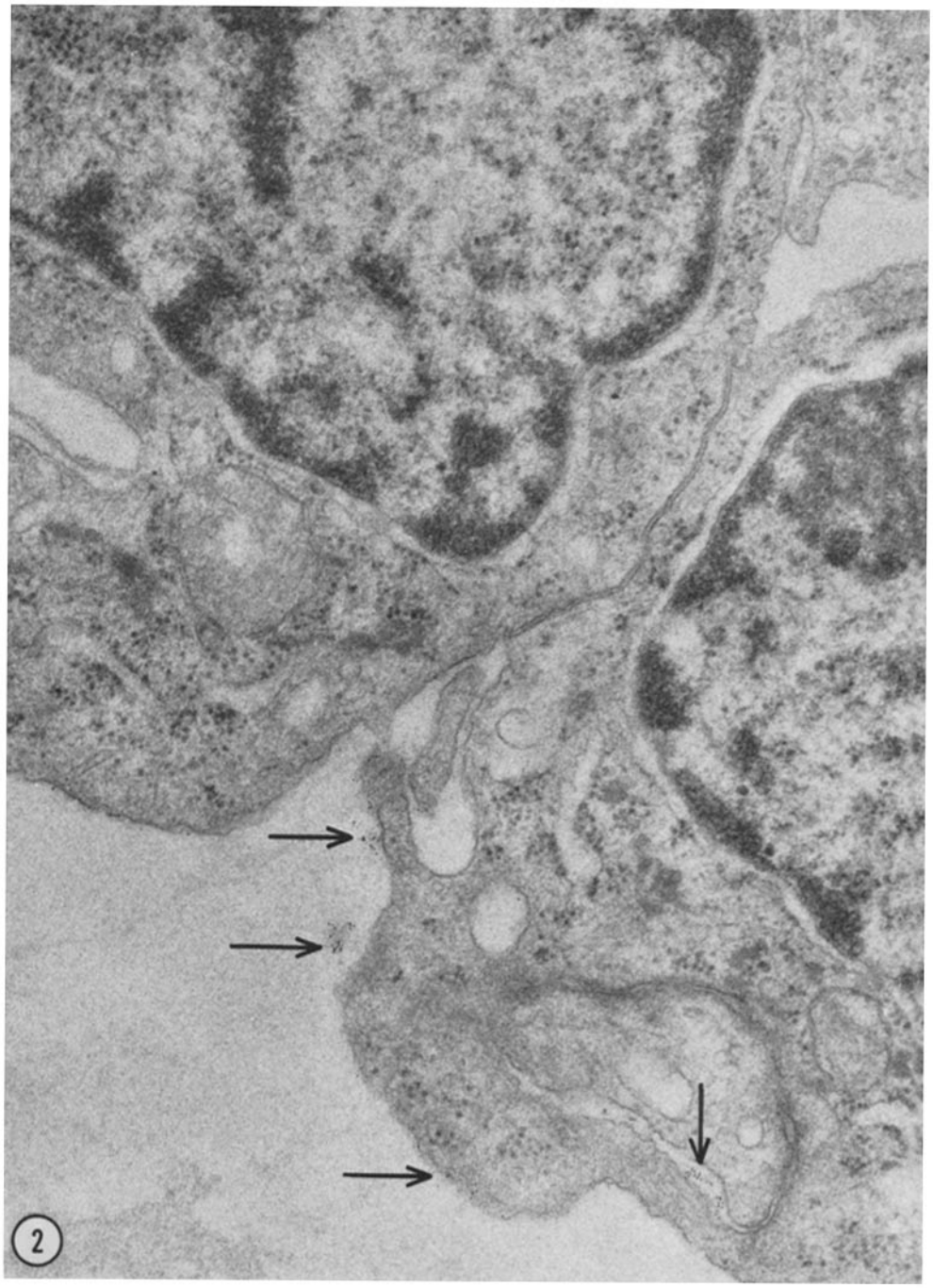

FIGURE 2 The employment of the indirect immunoferritin method demonstrated histocompatibility alloantigens on embryonic outer cell surfaces. This survey electron photomicrograph demonstrates a typical pattern of *H-2*^{d} antigen on the outer cell surfaces of embryonic B10.D2 inner enamel epithelia. Note that the labeled antibodies did not penetrate into the tissues. Only the cell surfaces of the tissue masses were labeled with ferritin (arrows). × 30,000.

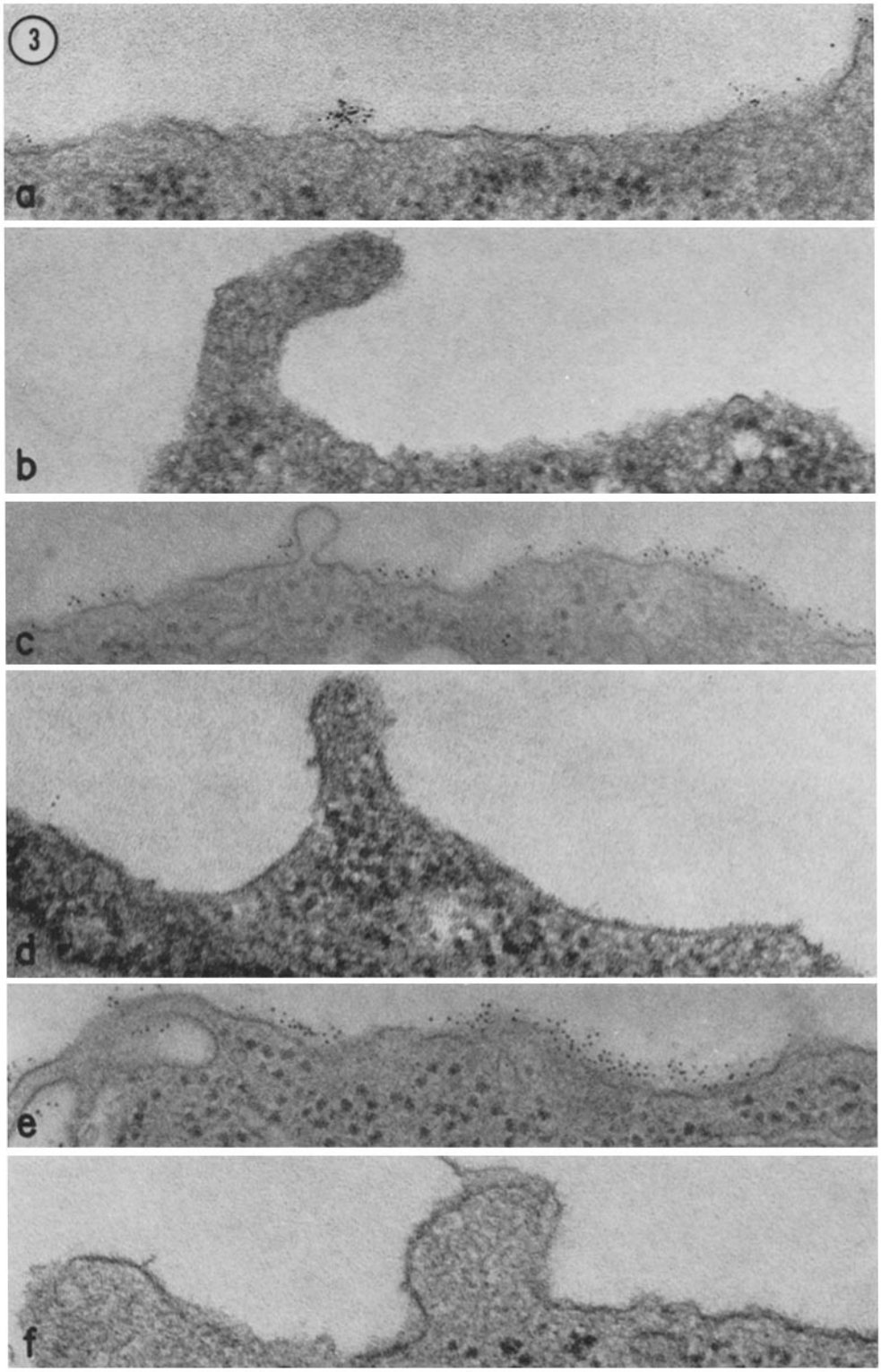

FIGURE 3 Specificity of cell surface labeling on embryonic epithelial and mesenchymal tissues, and on adult spleen lymphoid cell surfaces. (*a*) *H-2*^{d} antigen on embryonic B10.D2 inner enamel epithelia. × 92,400. (*b*) Control using embryonic C57BL/10 epithelia incubated with anti-*H-2* antiserum with *H-2*^{d} specificity. × 92,400. (*c*) *H-2*^{d} antigen on embryonic B10.D2 dental papilla mesenchyme cell surfaces. × 92,400. (*d*) Control using embryonic C57BL/10 mesenchyme incubated with anti-*H-2* antiserum with *H-2*^{d} specificity. × 92,400. (*e*) *H-2*^{d} antigen on adult (maternal) B10.D2 spleen lymphoid cell surfaces. × 92,400. (*f*) Control using adult (maternal) C57BL/10 spleen lymphoid cells incubated with anti-*H-2* antiserum with *H-2*^{d} specificity. × 92,400.

chemistry. The embryonic epithelial cell surfaces showed an appreciably greater number of specifically bound ferritin granules than did the mesenchymal cell surfaces treated under comparable conditions (Fig. 3 *a–d*). Specificity controls using both maternal and embryonic C57BL/10 cells and tissues were employed to assess nonimmunological interaction of ferritin-labeled antibody with the cells.

Application of ferritin-labeled antibodies for study of embryonic cells was effective only with viable cells which had recovered from the trypsinization and dissociation procedures. Dead or injured cells took up ferritin-labeled antibody to such an extent that specificity was masked. Significant nonspecific phagocytosis of label occurred when viable cells were incubated at 37°C (even after only 5 min). However, at temperature employed for this study (4°C), nonspecific phagocytosis of the label was greatly reduced. Moreover, endocytosed ferritin was readily distinguished from specific labeling of outer cell surfaces.

The presence of *H-2* alloantigens on embryonic tooth epithelium and mesenchyme cells obtained from B10.D2 (of $H\text{-}2^d$ specificity) mouse embryos has been demonstrated with immunocytochemistry in this study. Genetic, biochemical, and further developmental functions of the *H-2* histocompatibility alloantigens during embryonic organ formation remains to be elucidated. Moreover, the present results strongly suggest that problems of heterotypic tissue interactions (such as epithelial-mesenchymal interactions during tooth formation) can now be investigated with congenic strains of mice through the development of antisera with exquisite specificity. The use of such antisera in conjunction with studies of odontogenic epithelial-mesenchymal interactions employing interstrain chimeras are now in progress.

The authors wish to especially acknowledge the many suggestions of Dr. Garth L. Nicolson during these studies.

This work is a contribution from the Laboratory of Developmental Biology, Ethel Percy Andrus Gerontology Center. It was supported by grants from the National Institutes of Health, training grant DE-00094, and research grants DE-03569 and DE-02848. H. C. Slavkin was a National Institutes of Health Research Career Development Award Fellow (5-KO4-DE-41739).

Received for publication 21 August 1973, and in revised form 13 November 1973.

REFERENCES

1. PATTHEY, H., and M. EDIDIN. 1973. Evidence for the time of appearance of H-2 antigens in mouse development. *Transplantation.* **15**:211.
2. EDIDIN, N. 1964. Transplantation antigen levels in the early mouse embryo. *Transplantation.* **2**:627.
3. KLEIN, J. 1965. *In* Blood Groups of Animals. J. Matousek, editor. Uitgeverek Dr. W. Junk, The Hague. Netherlands. 405.
4. GRAZIANO, K. D., and M. EDIDIN. 1971. *In* Immunogenetics of the H-2 System. A. Lengerova, and M. Vojtiskova, editors. Karger AG., Basel, Switzerland. 251.
5. GERVAIS, A. G. 1970. Transplantation antigens in the central nervous system. *Nature (Lond.).* **225**:647.
6. GERVAIS, A. G. 1968. Detection of mouse histocompatability antigens by immunofluorescence. *Transplantation.* **6**:261.
7. GERVAIS, A. G. 1972. Further studies on the histological distribution of the H-2 antigens. *Tissue Antigens.* **2**:227.
8. WHITE, E., and G. N. TRUMP. 1972. Immunological determinants in development. *In* Developmental Aspects of Oral Biology. H. C. Slavkin, and L. A. Bavetta, editors. Academic Press, Inc., New York. 35.
9. STIMPFLING, J. H. 1961. The use of PVP as a developing agent in mouse hemagglutination tests. *Transplantation.* **27**:108.
10. NICOLSON, G. L., R. HYMAN, and S. J. SINGER. 1971. The localization of spectrun on the inner surface of human red blood cells by ferritin-conguated antibodies. *J. Cell Biol.* **51**:265.
11. SINGER, S. J., and A. F. SCHICK. 1961. The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin. *J. Biophys. Biochem.* Cytol. **9**:519.
12. SNELL, G. D., and L. C. STEVENS. 1966. *In* The Biology of the Laboratory Mouse. E. L. Green, editor. McGraw-Hill, New York. 205.
13. RUGH, R. 1968. The Mouse, its Reproduction and Development. Burgess Publishing Co., Minneapolis.
14. HAY, M. 1961. The development *in vivo* and *in vitro* of the lower incisor and molars of the mouse. *Arch. Oral Biol.* **3**:86.
15. SLAVKIN, H. C., J. BEIERLE, and L. A. BAVETTA. 1968. Odontogenesis: Cell-cell interactions *in vitro*. *Nature (Lond.).* **217**:269.

16. DAVIS, W. C., and L. SILVERMAN. 1968. Localization of mouse H-2 histocompatability antigen with ferritin-labeled antibody. *Transplantation.* **6**:535.

17. SLAVKIN, H. C., R. CROISSANT, and P. BRINGAS, JR. 1972. Epithelial-mesenchymal interactions during odontogenesis. III. A simple method for the isolation of matrix vesicles. *J. Cell Biol.* **53**:841.

18. NICOLSON, G. L., V. T. MARCHESI, and S. J. SINGER. 1971. The two-dimensional topographic distribution of H-2 histocompatability alloantigens on mouse red blood cell membranes. *J. Cell Biol.* **50**:905.

19. DE PETRIS, S., and M. C. RAFF. 1973. Normal distribution, patching, and capping of lymphocyte surface immunoglobulin studies by electron microscopy. *Nat. New Biol.* **241**:257.

20. SINGER, S. J., and G. L. NICOLSON. 1972. The fluid mosaic model of the structure of cell membranes. *Science (Wash. D. C.).* **175**:720.

21. AOKI, T., U. HAMMERLING, E. DE HARVEN, E. A. BOYSE, and L. J. OLD. 1969. Antigenic structure of cell surfaces. An immunoferritin study of the occurrence and topography of H-2 and TL alloantigens on mouse cells. *J. Exp. Med.* **130**:979.