Localization of H–2K\textsuperscript{k} in developing mouse palates using monoclonal antibody

By MICHAEL MELNICK, TINA JASKOLL\textsuperscript{1} AND MARY MARAZITA\textsuperscript{1}

From the Laboratory for Developmental Biology, University of Southern California

SUMMARY

Using monoclonal antibodies to H-2K\textsuperscript{k} antigen, we sought to develop a reproduceable method of in situ localization in embryonic tissue and to determine whether there are specific patterns of H-2 localization in time and space in the developing palatal tissues of B10.A(H-2\textsuperscript{a}) embryonic mice, with and without corticosteroid pretreatment at 12 days gestation. Our procedure employs ethanol-glacial acetic acid fixation, paraplast embedding, and enzymatic predigestion with purified hyaluronidase and neuraminidase. H-2 antigens were detected in palatal mesenchyme as well as basement membranes but not in oral or nasal epithelium. The pattern of distribution in mesenchyme of untreated embryos changed with progressive shelf development: vertical → horizontal → epithelial fusion → epithelial seam degeneration → mesenchymal confluence. Although the palatal shelves of treated embryos remained vertical, corticosteroid treatment does not appear to alter the detectable spatiotemporal distribution of H-2 antigens in developing palates of embryonic B10.A mice.

INTRODUCTION

There is an explicit and implicit theoretical expectation among many developmental biologists that differentiating cells of vertebrate embryos will be found to have specific cell surface antigens at different stages of development and that cataloguing the localization and potential functions of these antigens will provide a more clear understanding of morphogenesis (Hay, 1980). Major histocompatibility complex (MHC) cell surface antigens of the mouse (H-2) are integral plasma membrane constituents which are associated with allograft rejection and immune response (Klein, 1979). Over the past decade a number of workers have speculated that the MHC codes for cell surface antigens that control critical cell–cell interactions during embryonic development (Bennett, Boyse & Old, 1971; Ohno, 1977; Bonner, 1979). However, prior to any investigation of the function of H-2 antigens in progressive embryonic development one must initially determine (1) when H-2 antigens are first expressed.

\textsuperscript{1} Author's address: Laboratory for Developmental Biology, University of Southern California, Andrus Gerontology Center, P.O. Box 77912, Los Angeles, California 90007, U.S.A.
in developing embryonic tissues, (2) whether the expression of H-2 antigens is restricted to certain cell types, and (3) whether there are specific patterns of H-2 localization in time and space that can be elucidated in situ in embryonic tissues.

Virtually all of the work reported has been confined to addressing the first two questions. The onset of H-2 antigen expression appears to occur in cleavage to blastocyst embryos (see reviews by Ostrand-Rosenberg, 1980; and Johnson & Calarco, 1980). In older embryos H-2 antigens have been detected on 15-day embryonic liver cells in suspension (McClay & Gooding, 1978), on cultured 14-day and 15-day embryonic cells derived from thymus, salivary gland, head and neck skin, thyroid, and lung rudiments (Jenkinson, Owen & Aspinall, 1980), on cultured 18- to 20-day foetal mouse skin cells (Worst & Fusenig, 1973), and on cultured cells of mid-gestation mouse embryonic skin, gut, lung, limb bud and heart (Kirkwood & Billington, 1981). Investigators have failed to detect H-2 antigens on cultured mid-gestation embryonic cells from gonads and kidneys (Kirkwood & Billington, 1981), as well as on suspensions of 15-day embryonic brain cells (McClay & Gooding, 1978). In fact, H-2 in brain has not been detected until 8 days postnatal (Schachner & Sidman, 1973). Even though one interesting study of 12-day otocysts has successfully used immunoperoxidase techniques and Normaski optics (Prystowsky, Khan & Marovitz, 1978), to date, the in situ localization of H-2 antigens in embryonic tissues has been difficult for a variety of reasons, mostly technical (see Discussion below).

Our objectives in this set of experiments were twofold. First, we sought to develop a practical and reproducible method for the in situ localization of H-2 antigens in embryonic tissues; second, using monoclonal antibodies to H-2K\textsuperscript{k} antigen, we sought to determine whether there are specific in situ patterns of H-2 localization in time and space in the developing palatal tissues of B10.A(H-2K\textsuperscript{k}) embryonic mice. Our procedure employs ethanol-glacial acetic acid fixation, paraplast embedding, and enzymatic predigestion with purified hyaluronidase and neuraminidase. H-2 antigens were detected in palatal mesenchyme as well as basement membranes but not in oral or nasal epithelium. The pattern of indirect immunofluorescent staining for H-2K\textsuperscript{k} antigens in mesenchyme changed with progressive palatal shelf development: vertical shelf position \rightarrow horizontal shelf position \rightarrow epithelial fusion \rightarrow epithelial seam degeneration \rightarrow mesenchymal confluence. Since H-2K\textsuperscript{k} is significantly associated with embryonic susceptibility to corticosteroid-induced cleft palate (Melnick, Jaskoll & Slavkin, 1981), we repeated the study in mice treated at 12 days gestation with triamcinolone hexaconetide, a synthetic analogue of cortisol. Although the palatal shelves of treated embryos remained vertical, the patterns of distribution changed with time in a manner similar to untreated embryos. We believe this is the first time specific in situ patterns of H-2 antigen localization have been demonstrated in the progressive differentiation of embryonic tissues.
Fig. 1. Early stage of palate development in which both shelves are vertical: (A) view of the palate as seen with removal of lower jaw, line is the plane of section for B; (B) H & E stained section through the embryonic mouse head showing the relationship between the vertical shelves (v) and the tongue (t) (x 103); (C) immunofluorescent localization of H-2K\(^k\) in one of the vertical shelves, see text for details (x 200).
Fig. 2. Stage of palate development in which one shelf is horizontal and one shelf is vertical: (A) View of the palate as seen with removal of lower jaw, line is the plane of section for B; B. H & E stained section through embryonic mouse head showing relationship of horizontal (h) and vertical (v) shelves to the tongue (t) (x 104); (C) Immunofluorescent localization of H-2K* in the horizontal shelf, see text for details (x 200).
Fig. 3. Stage of palate development in which both shelves are horizontal: (A) View of the palate as seen with removal of lower jaw, line is the plane of section for B; (B) H & E stained section through embryonic mouse head showing the relationship between the horizontal shelves (h) and the tongue (t) (x 95); (C) Immunofluorescent localization of H-2K^k in one of the horizontal shelves, see text for details (x 200).
Fig. 4. Stage of palate development in which both horizontal shelves contact one another: (A) View of palate as seen with removal of the tongue (×100); (C) Immunofluorescent localization of H-2K<sup>K</sup> in the fusing palatal shelves, see text for details of lower jaw, line is the plane of section for B; (B) H & E stained section through embryonic mouse head showing the formation of an epithelial seam (arrow) between the contacting horizontal shelves (×210).
MATERIALS AND METHODS

**Mice**

Virgin female B10.A/SnSg(H-2K^k^) mice, obtained from Jackson Laboratory (Bar Harbor, Maine), were housed in groups of five and were acclimatized to the vivarium environment in our institution during a quarantine period of 2 weeks. When the animals were 12–13 weeks old, groups of three females were placed in a cage with a male overnight. Females were examined daily in the morning for the presence of vaginal plugs. The date of plug detection was designated as day 0 of gestation. Pregnant dams were housed individually in solid-bottom, plastic cages. The vivarium facility is temperature controlled with an average daily temperature of 24 °C. Alternate 12 h periods of light and darkness were maintained daily. All animals were maintained on Wayne's Mouse Breeder Blox containing 20% crude fat and a maximum of 2% crude fibre, and were supplied with drinking water *ad libitum*.

One group of experimental animals received a single intramuscular injection (in the proximal portion of the lower limb) of 2 mg/kg body weight triamcinolone hexacetonide, a long-acting, synthetic analogue of cortisol, at 9.00 a.m. on day 12 of gestation. The remaining pregnant animals were untreated.

**Tissue preparation**

On the morning of days 14–16 gestation, dams were killed by cervical dislocation. By laparotomy, the uterus and all its contents were exposed. Each live embryo was dissected out of its extraembryonic membranes, placed in phosphate-buffered saline (PBS), and staged according to the external morphologic developmental criteria established by Theiler (1972). After staging, embryonic heads were removed with a scalpel and forceps and fixed overnight at 4 °C in absolute ethanol-glacial acetic acid (99:1). The heads were then dehydrated for 1 h in absolute ethanol (4 °C), cleared in xylene for 1 h (4 °C), and embedded in paraplast (56–59 °C). Specimens were serially sectioned at 5 μm in a plane perpendicular to the anteroposterior axis. Sections were placed on clean glass slides and stored at −20 °C for short periods until stained.

**Anti-H-2 McAb**

Monoclonal anti-mouse H-2K^k^ was purchased from Becton Dickenson (Sunnyvale, CA). Their clone 11–4-1 was derived from hybridization of mouse NS-1 myeloma cells with spleen cells from BALB/C(H-2^d^) mice immunized with CKB(H-2K^k^) spleen cells. The monoclonal antibody (McAb) was obtained from serum and/or ascites fluid of tumour-bearing mice and purified by protein A affinity column chromatography. The specificity and purity of each lot of McAb is tested at Becton Dickenson by two-dimensional gel electrophoresis, cytotoxicity, and indirect immunofluorescence staining of spleen cells followed by fluorescence-activated cell-sorter analysis. The McAb was supplied as 0.5 mg
Fig. 5. Stage of palate development in which the horizontal palatal shelves have almost completely fused: (A) View of palate as seen with removal of lower jaw, line is the plane of section for B; (B) H & E stained section through embryonic mouse head showing the degeneration of the epithelial seam (arrow) and the initiation of mesenchymal confluence (×100); (C) Immunofluorescent localization of H-2K\(^{a}\) in the later stages of palatal fusion, see text for details (×220).
purified immunoglobulin in 0.5 ml of PBS containing 0.1% sodium azide and was stored at 2–4 °C. McAb was additionally checked in our laboratory for specificity and radioactivity by indirect immunofluorescent labelling of B10.A(H-2K\textsuperscript{k}) and B10.D2(H-2K\textsuperscript{d}) adult spleen cell suspensions using the methods previously described by Eskinazi \textit{et al.} (1979). B10.D2(H-2K\textsuperscript{d}) cells were routinely negative for fluorescence.

\textbf{Section staining}

Deparaffinized sections were incubated with a 1:5 dilution of rabbit anti-mouse IgG [heavy and light chains, F’ (ab) fragment] (Cappel Laboratories, Cochranville, PA) at room temperature for 30 min and washed 3 times (10 min each) in PBS (pH 7.1) containing 0.025% Triton \times 100 (PBS/TRX). Following this, the sections were incubated with 20 µl/section of essentially protease-free streptomyces hyaluronidase (100 TRU/ml) (Calbiochem, San Diego, CA) in 0.2 M sodium acetate buffer (pH 4.8), washed twice with PBS/TRX (pH 7.1) for 10 min each, and incubated with 20 µl/section of \textit{vibrio cholerae} neuraminidase (1 IU/ml) (Calbiochem, San Diego, CA) in 0.05 M sodium acetate buffer (pH 5.5). Each enzymatic digestion was performed for 2 h at room temperature. Following enzymatic treatment, the sections were washed 6 times (10 min each) with PBS/TRX (pH 7.1) and then incubated at 4 °C with monoclonal anti-mouse H-2K\textsuperscript{k} antibody in PBS (pH 7.1) containing 0.4% Triton \times 100 (10 µg protein/20 µl/section) for 22–24 h. The sections were then washed for
3 h (9 changes) at room temperature in PBS/TRX (pH 7.1) and incubated with 1:40 dilution of FITC-labelled rabbit anti-mouse IgG [heavy and light chains, F' (ab) fragment] (Cappel Laboratories, Cochranville, PA) for 30 min at room temperature. Finally the slides were washed 5 times (10 min each) in PBS/TRX (pH 7.1), washed once for 10 min in PBS (pH 7.1), air dried, and mounted with a coverslip in aquamount. The slides were examined with a Zeiss Epifluorescence Photomicroscope equipped with a high-pressure mercury burner (HBO-50) and tungsten lamp (12 V, 60 W). Photomicrographs were recorded using Kodak Ektachrome 400 film at constant exposure.

In addition to the positive and negative controls described above the adult spleen cell suspensions, a number of other controls were undertaken: (1) unlabelled rabbit anti-mouse IgG alone; (2) unlabelled rabbit anti-mouse IgG followed by FITC-labelled rabbit anti-mouse IgG; (3) monoclonal H-2K\k anti-body alone; (4) monoclonal H-2K\k antibody followed by unlabelled rabbit anti-mouse IgG (1:40 dilution); (5) FITC-labelled rabbit anti-mouse IgG alone; (6) hyaluronidase and/or neuraminidase alone; (7) rabbit anti-mouse unlabelled IgG followed by hyaluronidase and neuraminidase followed by FITC-labelled rabbit anti-mouse IgG. Controls were routinely negative for fluorescence.

RESULTS

Progressive development of the normal secondary palate of the mouse is pictured in the drawings and histologic sections of Figs. 1-5. A number of excellent reviews of normal secondary palate development are extant (e.g. Pourtois, 1972; Trasler & Fraser, 1977). In short, the palatal shelves are initially in a vertical position on either side of the tongue and subsequently reorient themselves to a horizontal position above the dorsum of the tongue. The principal forces causing the 'horizontalization' of the shelves are thought to be hydration of glycosaminoglycans in the mesenchymal extracellular matrix and perhaps contractile elements in the mesenchymal cells themselves. Upon becoming horizontal the shelves contact and strongly adhere to one another. The apposed epithelia fuse to form an epithelial seam which soon breaks down allowing complete fusion and mesenchymal confluence within the palate. The palatal mesenchyme then differentiates into osseous, muscular, and fibrous elements; the palatal epithelium differentiates into pseudostratified ciliated columnar on the nasal surface and keratinized and stratified squamous on the oral surface (Tyler & Koch, 1975).

H-2 and normal palate development

Using monoclonal antibodies and indirect immunofluorescence, H-2K\k antigen was detected in palatal mesenchyme as well as basement membrane regions, but not in oral or nasal epithelium (Figs. 1–6). The fluorescent-staining pattern in the mesenchyme changed with progressive palatal shelf development. Shelves
**H-2K<sup>k</sup> and palate development**

in the vertical position generally showed a diffuse mesenchymal staining (Fig. 1); as the shelves elevated to a horizontal position this staining became more intense but remained somewhat uniform throughout the mesenchyme (Figs. 2, 3). Upon fusion of the apposed epithelia the fluorescent staining began to localize in the direction of the nasal cavity (Fig. 4). As the epithelial seam began to break down the localization beneath the nasal epithelium became more prominent and the staining appeared to intensify (Fig. 5). This staining pattern persisted through the stage of complete mesenchymal confluence.

**H-2 abnormal palate development**

Although the palatal shelves of embryos exposed previously to triamcinolone hexacetonide remained vertical, the patterns of fluorescent staining in the mesenchyme changed with time in a manner similar to untreated embryos. Specifically, mesenchymal staining in early day-16 embryos was found to be more localized in an area beneath what would normally become nasal epithelium (Fig. 6).

**DISCUSSION**

**H-2 antigens and palatogenesis**

Using monoclonal antibodies to H-2K<sup>k</sup> antigen, we were able to demonstrate specific in situ patterns of H-2 localization in time and space in the developing palatal tissues of B10.A(H-2K<sup>k</sup>) embryonic mice. In addition the fluorescent staining appeared to be cell specific, being associated with mesenchymal but not with epithelial cells. It remains obscure as to why the H-2 staining finally localized in the mesenchyme immediately subjacent to the nasal epithelium and not the oral epithelium. Perhaps it is related to the distinctly separate fates of the two epithelia during subsequent differentiation.

Of course, demonstrating changing patterns of specific antigens in embryonic tissues does not necessarily reveal the potential or actual functions of these antigens. In the case of H-2 antigens the function remains an enigma, even though a number of hypothetical models have been proposed which view H-2 antigens as playing a role in cell–cell recognition (Edelman, 1976; Bonner, 1979). There is mounting evidence that embryonic pattern formation depends on strictly local cell–cell interactions (Bryant, French & Bryant, 1981). Although our experiments do not in any way allow us to conclude that this is the function of H-2 antigens during development, it is not unreasonable to think that the changing patterns of distribution of this immunoglobulin-like molecule is more than a fortuitous occurrence in a dynamic mesenchyme.

Although palatal shelves of corticosteroid-treated embryos remained vertical through the beginning of gestation day-16, the patterns of detectable H-2K<sup>k</sup> distribution changed with time in a manner similar to untreated embryos. This is not altogether surprising, since corticosteroid-induced cleft palate in mice is thought primarily to be due to a reduction in shelf force and a resulting delay
in shelf movement both in relation to chronological and developmental age (Walker & Fraser, 1957). Relatively normal elevation and fusion can be achieved in treated foetuses by excising the tongue (Walker & Quarles, 1976). In any event, although there is known to be a strong embryonic effect vis-à-vis the relationship of H-2Kk (or closely linked genes) and high susceptibility to corticosteroid-induced cleft palate (Melnick et al. 1981a), corticosteroid treatment does not appear to alter the detectable spatiotemporal distribution of H-2 antigens in developing palates of embryonic B10.A mice. These data suggest that we should look elsewhere for the mechanism of steroid-induced clefting, perhaps in the transmembrane relationship between H-2 antigen and the microfilament protein actin (Kock & Smith, 1978). Even though it is admittedly dangerous to argue from single instances of association, the investigation of effects on transmembrane relationships may be of particular importance if the effect of the drug is to act on some process subsequent to H-2 expression.

**Methodological considerations**

Previous investigators have reported the indirect immunofluorescent localization of H-2 in tissue sections from placenta (Voisin & Chaouat, 1974) and various adult organs including liver, spleen, pancreas, kidney, and brain (Gervais, 1968; Gervais, 1970; Saunders, Beals & Schultz, 1979). It has been noted by others that these studies are not, however, completely satisfactory for several reasons: (1) there is considerable tissue distortion in the unfixed cryostat sections (Gervais, 1972a; Parr, 1979); (2) there is often high nonspecific background staining (Gervais, 1972b; Saunders et al. 1979); (3) it is almost impossible to make alloantisera specific for individual antigenic components in sufficient quantity with great reproducibility (Swaab, Pool & Van Leeuwen, 1977; Milstein & Lennox, 1980). We believe our protocol has allowed in situ detection of H-2 antigens in embryonic tissue while avoiding the difficulties just enumerated.

We are aware of the extensive study by Gervais (1972a) on the effects of various fixatives and the use of tissue preparations other than frozen sections. It is difficult to compare our results in embryonic tissues with those of Gervais (1972a) in adult tissue. It should be noted, though, that paraffin embedding has proved successful in the immunofluorescent localization of other antigens in embryos, e.g. fibronectin and collagen (Melnick et al. 1981b; Silver, Foidart & Pratt, 1981). Further, although Gervais (1972a) concluded that H-2 antigens are destroyed with various fixatives, Parr & Oei (1973a, b) published results suggesting that paraformaldehyde fixation immobilizes mouse H-2 antigens on cell surface membranes, and thus enhances detection. In addition, Voisin & Chaouat (1974) have also successfully utilized ethanol fixation for indirect immunofluorescent studies of H-2 antigens in tissues.

The primary questions raised in studies of this sort revolve around the specificity of the antigen–antibody reaction. Swaab et al. (1977) have concluded
that if antisera are tested properly, few, if any, of them will be found to be monospecific. Akeson (1979) has noted that although it is generally inferred that the observed pattern of cell and tissue reactivity is due to the presence of a unique macromolecule(s) this is not necessarily the case, particularly when values for positive cells are not greatly above control values (e.g. Saunders et al. 1979). To be sure, there is no substitute for analyzing the suspected or putative macromolecule by biochemical means. Nevertheless, the use of monoclonal antibodies obviously eliminates the problem of multiple antigenic recognition by a mixed antibody population, even though it does not completely eliminate the possibility of cross reactions resulting from antigenic similarities (Milstein & Lennox, 1980). In the view of Milstein & Lennox (1980), this latter possibility, if it occurs at all, is not common enough to blur the recognition of individual ‘differentiation’ antigens.

The interpretation of results obtained with monoclonal antibodies must be made with caution. For example, the fine recognition of McAb may introduce a serious bias when a given antigenic determinant is exposed in one environment but hidden in others (Milstein & Lennox, 1980). This problem may be particularly acute in embryonic tissues which contain large amounts of extracellular matrix macromolecules such as glycosaminoglycans and collagens. These macromolecules are closely associated with cell surfaces and may serve to mask them. It thus seems both reasonable and prudent to ‘unmask’ craniofacial tissues such as palatal mesenchyme, which are known to have large extracellular quantities of hyaluronate associated with their cell surfaces (Toole, 1976). In our laboratory hyaluronidase pretreatment is found to enhance the fluorescent labelling. Similarly, we have pretreated with collagenases but this seems to have no effect. In another context, it is worth noting that Saunders et al. (1979) suggested that the high background staining they observed might actually be due to nonspecific accumulation of FITC-labelled secondary antibody in extracellular matrix. Thus enzymatic pretreatment may serve a dual function.

It has been shown that when over 95% of the sialic acid contained in H-2 glycoprotein is removed the sialic acid-free H-2 siloantigens still retain their activity for the tested specificities (Shimada & Nathenson, 1971). It has also been shown that removal of the sialic acid moiety by *Vibrio cholerae* neuraminidase enhances the immunogenicity of the cells so treated (Currie et al. 1968; Simmons et al. 1971). It has been postulated that perhaps the sialic acid moiety acts by steric hindrance to prevent access to underlying antigenic determinants (Currie, van Doornineck & Bagsharve, 1968; Simmons, Rios & Ray, 1971). This would be particularly so if the antigenic determinants are closely spaced on the surface of the cells (Kent, Ryan & Siegel, 1978). We found that pretreatment of sections with *Vibrio cholerae* neuraminidase also enhanced fluorescent labelling of B10.A embryonic tissue. This result was not altogether unexpected since neuraminidase treatment was necessary to demon-
strate H-2 associated variations in adhesion rates when cells with a B10 genetic background were used, but not with A or C3H genetic backgrounds (Bartlett & Edidin, 1978). In addition it has been reported that B10 erythrocytes contain more sialic acid per cell than do erythrocytes of other strains, and that this interferes with assays of haemaglutinating anti-H-2 antibodies (Rubenstein, Liu, Streun & Decary, 1974).

In conclusion, then, the results of our experiments provide a manageable and a reproducible method for the immunofluorescent labelling of H-2 antigens in embryonic tissue. Initial results with this method seem promising and appear to have the potential for opening up new avenues of investigation relative to specific spatiotemporal patterns of H-2 localization during progressive embryonic morphogenesis.

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REFERENCES


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