ZOOLOGICAL SCIENCE 8: 713–719 (1991)

Effects of Neonatal Diethylstilbestrol Exposure on the Growth of Mouse Vaginal Epithelial Cells in Serum-Free Collagen Gel Culture

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ABSTRACT—Vaginal epithelial cells isolated from ovariectomized *ca.* 40-day-old BALB/cCrgl mice treated with 1 μg diethylstilbestrol (DES) or with oil vehicle alone for the first 5 days after birth were purified by Percoll density gradient centrifugation and grown as primary cultures in collagen gel matrix (CGM) with serum-free medium. Three dimensional colonies of both control and DES-exposed vaginal epithelial cells developed in the CGM. The initial proliferation rate of DES-exposed vaginal epithelial cells was significantly lower than that of the controls. However, after 10 days in culture, approximately 6.3-fold and 7.6-fold increases in cell number occurred in control and DES-exposed vaginal epithelial cells, respectively, a difference that is not significant. Deletion of bovine serum albumin, bovine insulin or mouse epidermal growth factor (EGF) resulted in decreased growth of both control and DES-exposed vaginal epithelial cells. Estradiol-17β (180 fM to 18 nM) did not stimulate growth of control or DES-exposed vaginal epithelial cells. When concentrations of EGF and insulin are increased in the serum-free medium, a dose-related increase in growth was observed in vaginal epithelial cells from both control and DES-exposed mice. DES-exposed vaginal epithelial cells were significantly less sensitive to insulin at two concentrations and to EGF at one concentration than control cells. Thus, neonatal DES exposure results in alterations in vaginal epithelial cells as indicated by decreased initial rate of proliferation and by altered sensitivity to EGF and insulin *in vitro*.

INTRODUCTION

The abnormal development of the genital tract induced by human intrauterine exposure to diethylstilbestrol (DES), a synthetic estrogen, has been studied extensively in animal model systems in attempts to uncover the mechanism by which such exposure results in permanent structural changes [1–3]. Estrogens including DES induce hyperplastic lesions in vagina, uterus and oviduct, cervicovaginal cancer and presistent vaginal cornification in mice when administered perinatally [4–8]. The perinatal period is a time when the development of the mouse reproductive tract occurs [2] and corresponds to the end of the first trimester of human fetal development [9]. DES exposure during this critical period results in the later appearance of a low incidence of cervicovaginal clear cell adenocarcinoma in humans [7, 10] and in abnormal differentiation of the rodent genital tract [9].

Correlation of the onset of structural alterations in the reproductive tract with puberty suggested the direct or indirect involvement of endogenous hormones; as a result, investigators interested in understanding the mechanism by which DES exerted its effects have focused on the ovarian steroids and their receptor systems. The numbers of cytosolic estrogen and progestin receptors in neonatally estrogen-treated mice are altered in the female tract [11–13] as well as in the separated tissue compartments of the vagina [14]. Changes

Accepted February 19, 1991
Received December 11, 1990
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in the synthesis of major cellular proteins occur in the uterus of transplacentally DES-exposed mice [15] and are localized to specific tissue compartments of the neonatally DES-exposed mouse vagina [16]. The present study examines differences in the proliferation and sensitivity of vaginal epithelial cells from normal and neonatally DES-exposed mice when grown in serum-free collagen gel culture.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's and Ham's F-12 (D : H, 1 vol/1 vol, basal), 199, and Waymouth's (10X) media were purchased from Grand Island Biological (Grand Island, NY, U.S.A.); Percoll was from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). Bovine insulin (I), 17β-estradiol (E_2), DES, bovine serum albumin fraction V (BSA), human transferrin (Tr), cholera toxin (CT), deoxyribonuclease I, HEPES buffer, penicillin and streptomycin were from Sigma Chemical (St. Louis, MO, U.S.A.); mouse epidermal growth factor (EGF) was from Collaborative Research (Waltham, MA, U.S.A.).

Animals

Newborn female BALB/cCrgl mice were obtained from the barrier breeding colony at the University of California at Berkeley. Newborn female mice received 5 daily subcutaneous injections of 1 μg DES dissolved in 0.02 ml sesame oil starting within 18 hr after birth. Control animals were injected daily with 0.02 ml sesame oil. The mice were weaned at 3 weeks of age and housed 5 or 6 per cage in a temperature-controlled room (22°C) with 12-hr light and 12-hr dark periods. Animals were provided with pine shavings for bedding, fresh water and Wayne sterilizable rodent block diet *ad libitum*. All animals were ovariectomized at 30 days of age.

Cell dissociation and culture procedure

Vaginae were dissected from *ca.* 40-day-old ovariectomized mice, and separated into tissue compartments by the procedure already described [17] with the following modifications: DES-exposed vaginae were incubated with collagenase (0.12%) for 1.5 hr, whereas the control vaginae were incubated with collagenase (0.12%) for 2 hr as described previously [16]. The keratin layers of the DES-exposed vaginal epithelium were removed with jeweler's forceps under a dissecting microscope. The epithelial sheets separated with forceps were minced with a razor blade on a Teflon block and centrifuged on preformed Percoll density gradients in order to remove fibromuscular elements, cellular debris and proteolytic enzymes and to avoid possible contamination with nonepithelial cells [18]. Cells were rinsed twice with medium 199, embedded in iso-osmotic, neutralized collagen gel matrix (CGM) in 24-well, tissue culture-treated plates (Falcon, Becton-Dickinson, Cockeysville, MD, U.S.A.) and cultured in basal medium (D : H medium buffered at pH 7.2 with 20 mM HEPES containing $NaHCO_3$ (1.2 g/l) with penicillin (50 U/ml) and streptomycin (50 μg/ml), to which I (10 μg/ml), Tr (10 μg/ml), CT (10 ng/ml), BSA (5 mg/ml) and EGF (10 ng/ml) were added.

Media for the deletion experiments were prepared by adding various combinations of 4 of the 5 supplements together to the basal medium. A stock solution of 17β-estradiol (E_2, 500 μg/ml) dissolved in absolute ethanol was prepared, and various concentrations of E_2 were obtained by serial dilution with basal medium. Aliquots of these dilutions were added to the culture medium to attain final concentrations of 180 fM-18 nM. The final concentration of ethanol was less than 0.1% and did not influence cell growth. The serum-free complete (SFc) medium consisted of basal medium supplemented with all five factors [17, 19]. Cultures were incubated at 37°C in a 95% air/5% CO_2 humidified atmosphere, and the medium was changed every 2 days. Each experiment was repeated at least three times, with triplicate wells in a single culture plate used for each point.

Cell number and DNA estimates

Both control and DES-exposed vaginal epithelial cells were plated as $1.5–2.2 \times 10^5$ cells per well. At the end of the experiments, cells were reco-

vered from gels by acetic acid dissolution and stored for later fluorometric determinations of DNA [20, 21]. In order to normalize the differences in the initial seeding density, data were expressed as increases over the initial cell number. The data were analyzed by Student's t-test.

RESULTS

Effects of serum-free media on DES-exposed vaginal epithelial cell growth

The combined mitogenic potencies of the serum-free components of SFc medium were ascertained. Three-dimensional growth of both control and DES-exposed vaginal epithelial cells occurred in serum-free CGM culture, and the respective colonies were of mixed morphology with duct-like, sheet-like, or spherical organization. Histological sections of growing colonies of vaginal epithelial cells from control and DES-exposed mice demonstrated an lumen surrounded by epithelial cells in various stages of differentiation sloughing into the lumen (Fig. 1).

After a 2-day lag period, due to reorganization of the epithelial cell clumps in the CGM, control vaginal epithelial cells increased to 6.3-times the initial seeding density during the 10 days of culture with SFc medium. DES-exposed vaginal epithelial cells showed a 4-day lag period before entering logarithmic phase of growth, but a 7.6-fold increase in cell number was obtained after 10 days of culture (Fig. 2). The initial proliferation rate of DES-exposed cells was significantly lower than

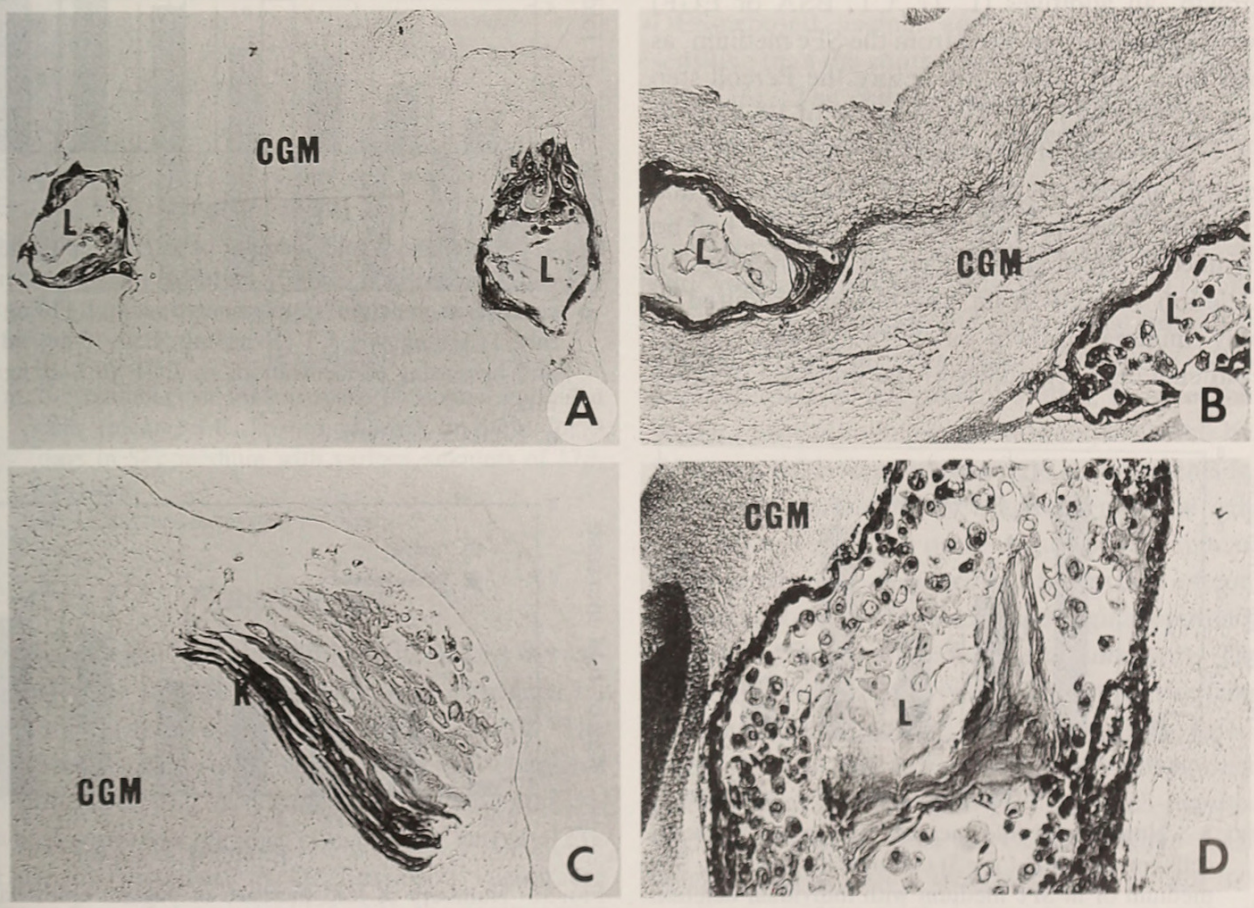

FIG. 1. Histology of control (A, B) and DES-exposed vaginal epithelial cell clumps (C, D) cultured with unsupplemented (D : H) (A, C) and serum-free complete (SFc) media (B, D) for 10 days in neutralized collagen gel matrix (CGM). H & E. ×95. Note that growth in SFc medium and three-dimensional reorganization of the epithelial colony have occurred as indicated by the increased colony size and the formation of a lumen (L) (A, B) and presence of keratin (K) over the dispersed cells grown in D : H medium, whereas colony organization, cell soluthing and growth with lumen (L) formation have occurred in SFc medium (C, D).

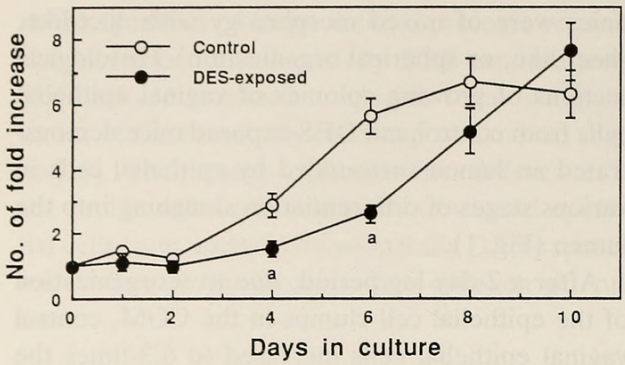

FIG. 2. Numbers of fold increase over the initial cell number of vaginal epithelial cells (mean ± S.E., n = 3) cultured for 10 days in CGM with SFc medium.
a, $P < 0.05$ vs Control.

that of controls, but the final increases were not significantly different.

In order to ascertain the requirement for each component of the SFc medium for optimum growth, all additives (I, Tr, CT, BSA or EGF) were individually deleted from the SFc medium, as had been done previously before the Percoll step was introduced [22]. The deletion of BSA, I, or EGF from the SFc resulted in significantly less growth of both control and DES-exposed vaginal epithelial cells, but deletion of Tr and CT had no significant effect after 9 days of culture (Fig. 3). Deletion of EGF from SFc medium resulted in only minimal growth of DES-exposed vaginal epithelial cells regardless of the presence of other

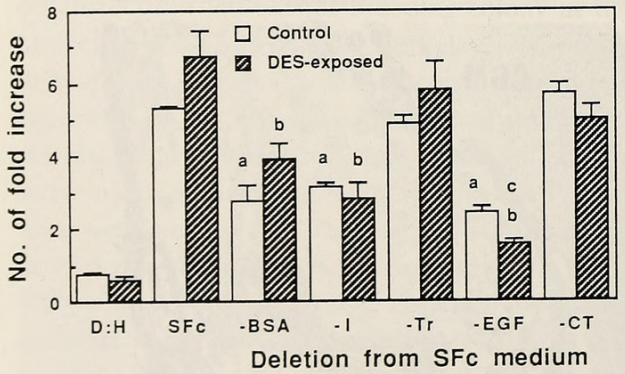

FIG. 3. Numbers of fold increase of vaginal epithelial cells (mean ± S.E., n = 3) cultured for 9 days in SFc medium or in SFc medium with individual deletion of bovine serum albumin (BSA), insulin (I), transferrin (Tr), epidermal growth factor (EGF) or cholera toxin (CT). D:H, unsupplemented medium.
a, $P < 0.05$ vs Control SFc; b, $P < 0.05$ vs DES-exposed SFc; c, $P < 0.05$ vs Control without EGF.

factors. EGF dose-response studies performed in the presence of the other 4 factors showed that concentrations of EGF greater than 0.1 ng/ml resulted in a dose-related proliferation of both control ($Y = 2.17 \log_{10}X + 4.32$, $r = 0.97$, $P < 0.001$, regression analysis) and DES-exposed ($Y = 2.40 \log_{10}X + 3.73$, $r = 0.93$, $P < 0.01$) vaginal epithelial cells. At 1 ng/ml EGF, the increase in DES-exposed vaginal epithelial cells was significantly less than in the controls (Fig. 4). No significant proliferation of either kind of vaginal epithelial cell colonies occurred in the absence of EGF. Insulin dose-response studies showed that there is a dose-

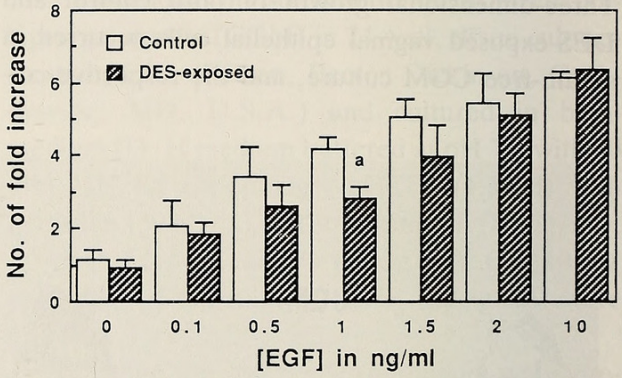

FIG. 4. Numbers of fold increase of vaginal epithelial cells (mean ± S.E., n = 3) cultured for 9 days in serum-free medium supplemented with I (10 μg/ml), Tr (10 μg/ml), CT (10 ng/ml), BSA (5 mg/ml) and increasing concentrations of EGF (0.1–10 ng/ml).
a, $P < 0.05$ vs Control.

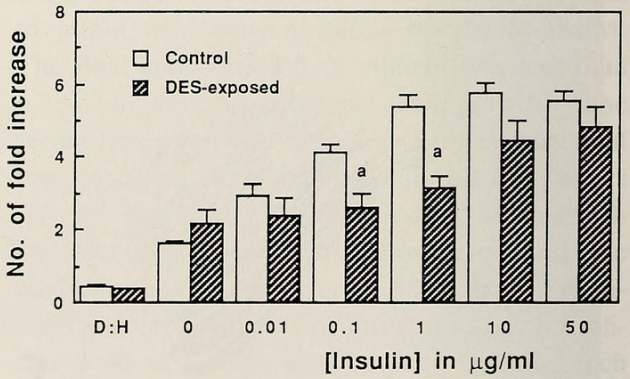

FIG. 5. Numbers of fold increase of vaginal epithelial cells (mean ± S.E., n = 4) cultured for 9 days in serum-free medium supplemented with Tr (10 μg/ml), CT (10 ng/ml), BSA (5 mg/ml), EGF (10 ng/ml) and increasing concentrations of I (0.01–10 μg/ml).
a, $P < 0.05$ vs Control.

related proliferation of both control ($Y=0.74 \log_{10}X+4.74$, $r=0.93$, $P<0.05$) and DES-exposed ($Y=0.71 \log_{10}X+3.55$, $r=0.96$, $P<0.01$) vaginal epithelial cells. Ten μg/ml and 50 μg/ml seem most favorable for growth during 9 days of culture for control and DES-exposed vaginal epithelial cells, respectively. At 0.1–1 μg/ml I, DES-exposed vaginal epithelial cells showed significantly less growth than the controls (Fig. 5). In the complete absence of I, the serum-free medium supported minimal growth of both populations of epithelial cells. E_2 (180 fM-18 nM) in the optimal SFc medium did not stimulate proliferation of either control or DES-exposed vaginal epithelial cells at any concentration tested. At higher concentrations, E_2 tended to inhibit growth only in the control cells (Fig. 6).

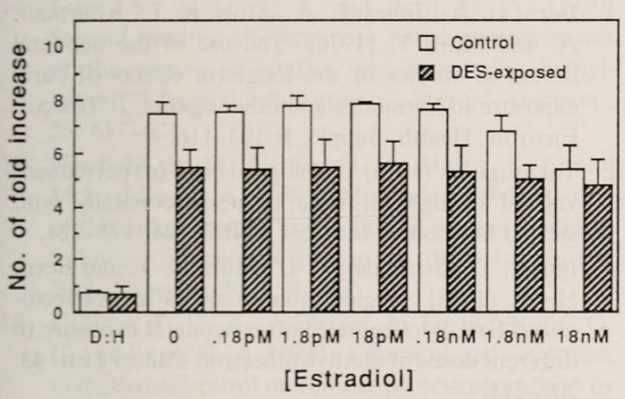

FIG. 6. Numbers of fold increase of vaginal epithelial cells (mean±S.E., n=4) cultured in CGM for 9 days in SFc medium with various amounts of 17β-estradiol.
D:H, unsupplemented medium.

DISCUSSION

Histological and biochemical alterations in vagina of mice treated perinatally with estrogens including DES have been reported [2, 13, 16]. The DES-exposed vagina shows ovary-independent proliferation and keratinization of epithelium [3], which expresses alkaline phosphatase activity [23]. Estrogen receptors in DES-exposed vagina are lower than in the control [11, 12]; the same is true in separated DES-exposed vaginal epithelium [14]. Furthermore, EGF receptors in the DES-exposed vagina are lower than in the control [T. Iguchi, M. Edery, P.-S. Tsai, S. Ozawa and H. A. Bern, submitted]. Responsiveness of neonatally E_2-exposed vagina to exogenously administered E_2 is lower than in the control [2]. Occurrence of major cellular proteins in the epithelial and fibromuscular compartments of the DES-exposed vagina differs from that in the controls [16]. However, differences in the growth of control and DES-exposed vaginal epithelial cells *in vitro* have not been previously reported.

The lag period in growth of control vaginal epithelial cells is 2 days; in DES-exposed cells, it is 4 days. In earlier studies, cell number decreased between 1 and 4 days in culture [17, 22]; the fluorometric DNA assay may not include DNA of dead cells. The occurrence of the longer lag period in DES-exposed vaginal epithelial cells, therefore, may be the result of the non-proliferative nature of the majority of cells, as only basal cells divide in stratified vaginal epithelium [24]. In addition, DES-exposed vaginal epithelial cells are more sensitive than the control epithelial cells to collagenase [16]. The cells eventually enter logarithmic growth indicating that the lag period is reversible *in vitro*.

SFc medium has been shown to support effectively the growth of normal mouse uterine [19, 26] and vaginal epithelial cells [17, 22, 26]. This medium also supports the logarithmic growth of vaginal epithelial cells from neonatally DES-exposed mice in the present study. In an earlier study, I proved to be an absolute requirement, but BSA and EGF were not absolute requirements for growth of normal mouse vaginal epithelial cells [22]. However, in the present study, EGF appears to be an absolute requirement for growth of control and neonatally DES-exposed vaginal epithelial cells, and I was not an absolute requirement. The difference between the two studies may arise from the introduction of the Precoll density gradient step. Tr and CT are not required for optimal growth of either control [22] or DES-exposed vaginal epithelial cells in CGM. When EGF concentrations are increased in serum-free medium, a dose-related increase in growth of vaginal epithelial cells from both control and DES-exposed mice was observed. DES-exposed vaginal epithelial cells were less sensitive to 1 ng/ml of EGF and 0.1–1 μg/ml of I than control vaginal

epithelial cells. Tomooka *et al.* [27] reported that mammary epithelial cells from mice treated neonatally with DES showed a lower growth response to I and EGF than the controls. EGF receptors are lower in DES-exposed vagina than in the controls [Iguchi *et al.*, submitted]; I receptors have not been measured.

Previous studies on growth regulation of normal mouse vaginal epithelial cells using SFc medium demonstrated that estrogenic hormones failed to stimulate enhanced growth of these cells in collagen gel under serum-free conditions [17, 22, 26]. The possible reasons for this have been addressed previously [17, 26], and lack of estrogen stimulation has also been noted in with primary serum-free Matrigel and collagen gel cultures of normal mouse endometrial luminal epithelial cells [25, 28] as well as with immature vaginal epithelial cells [29]. In the present study, E_2 also failed to stimulate growth of DES-exposed vaginal epithelial cells in CGM with SFc medium. However, when a heterotypic recombination of cultured normal vaginal epithelial cells [30, 31] or cultured DES-exposed vaginal epithelial cells [F.-D. A. Uchima, P. Cooke, G. Cunha and H. A. Bern, unpublished data] and cultured normal vaginal stroma were transplanted under the kidney capsule of syngeneic intact female host for 28 days, both normal and DES-exposed vaginal epithelial cells behaved normally by cycling in response to host ovarian hormones. Histologically, alternating layers of keratin and sloughed off cells were observed, indicating a degree of reversibility in the nature of the permanently proliferated DES-exposed vaginal epithelial cells when combined with normal stroma. The histology of colonies of control and DES-exposed vaginal epithelial cells grown in CGM with SFc medium reveals keratin layers in the lumen, indicating spontaneous terminal differentiation without estrogen. These results suggest that vaginal stroma has an important role in induction of ovary-independent proliferation and keratinization of vaginal epithelial cells of mice exposed neonatally to DES [30].

In conclusion, neonatally DES-exposed vaginal epithelial cells can be grown in DCM with SFc medium; the cells show a longer time lag before entering logarithmic growth phase and are less sensitive to I at two concentrations and EGF at one concentration than normal vaginal epithelial cells in this culture system. EGF and I in SFc medium enhance the growth of both control and DES-exposed vaginal epithelial cells, and EGF is essential for growth of both control and DES-exposed cells.

ACKNOWLEDGMENTS

This investigation was supported by Grants CA05388 and CA09041, awarded by the National Institute of Health, DHHS. We thank Dana Wong, Brian Williams, Lisa I. Sewell, Robert Nguyen and Andrew Wong for technical assitance in this study and John Underhill for the photography.

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