Epithelium-specific Response of Cultured Keratinocytes to Infection with Adenovirus Type 2

Brian J. Aneskievich, Ph.D., and Lorne B. Taichman, M.D., M.Sc., Ph.D.

Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, New York, U.S.A.

Adenoviruses are pathogenic for certain stratified squamous epithelia. The sites most frequently involved are the upper respiratory tract and oropharynx. Adenovirus infections of the epidermis are quite rare. We examined the virus-cell interactions of adenovirus type 2 (Ad2) and cultured human keratinocytes grown from a variety of body sites. Our intent was to explore the nature of the apparent epithelium-specific susceptibility to Ad2. In brief, we found that in vitro viral susceptibility of the keratinocytes could be reliably predicted based on whether the cells originated from an epidermal or oropharyngeal surface. Ad2 proceeded through a complete vegetative cycle when used to infect cultured keratinocytes from oropharyngeal sites (e.g., gingiva and soft palate). In contrast, Ad2 infection was severely restricted in keratinocytes from epidermal sites (e.g., foreskin, abdomen, and buttock). These results demonstrate that the in vitro response to infection with Ad2 reflects in vivo tissue-specific susceptibility. In vivo, cervical epithelium is rarely infected with Ad2 and yet in culture, cervical keratinocytes were fully permissive for Ad2 replication. We propose that the permissive or nonpermissive response to Ad2 may be regulated by a particular aspect of cell phenotype. Because the permissive responses seen in this study were all generated in keratinocytes from mucosal sites, it is possible the in vitro response to Ad2 reflects inherent differences between mucosal and epidermal keratinocytes. *J Invest Dermatol 91:309–314, 1988*

Human adenovirus type 2 (Ad2) was first isolated by Rowe and colleagues in 1953 [1] as a serially transmissible agent responsible for the degeneration of cultured epithelial cells [2]. This virus commonly infects the stratified squamous epithelia of the upper respiratory tract and oropharynx [3,4]. However, Ad2 is rarely cited as a pathogen of the epidermis [4]. Two basic mechanisms may underly this epithelium-specific susceptibility to Ad2: 1) Epithelial cells of the oropharynx, but not the epidermis, may possess the surface receptors needed for virus attachment and internalization. 2) Keratinocytes of the oropharynx, but not the epidermis, may provide an internal milieu that allows complete Ad2 expression and thus productive viral replication. Several years ago we observed that Ad2 could infect epidermal keratinocytes cultured from newborn foreskin resulting in the development of cytopathic effects [5]. This result indicated that epidermal keratinocytes a) do present surface receptors capable of binding and internalizing Ad2 and b) do permit some degree of viral expression, but not necessarily vegetative replication.

In this study we further explored the epithelium specific susceptibility to Ad2. We examined the interaction of Ad2 with keratinocytes cultured from different sites in the oropharynx and the epidermis and found a clear difference in the response. Keratinocytes cultured from the oropharynx support high levels of vegetative Ad2 replication; keratinocytes cultured from the epidermis markedly restrict viral expression and thus allow only low levels of vegetative Ad2 replication. In addition, keratinocytes from the uterine cervix provide a fully permissive host for Ad2, suggesting that susceptibility to Ad2 in culture may be a characteristic of mucosal keratinocytes. Because Ad2 is dependent on cellular machinery for its expression [6], it is possible the response to Ad2 in culture is indicative of an inherent difference between mucosal and epidermal keratinocytes.

MATERIALS AND METHODS

Cells Primary cultures of human epidermal and mucosal keratinocytes were grown as described earlier [7] with some modifications. Cultures were initiated by dissecting away the connective tissue and cutting the epithelium into 1-mm^2 fragments. Fragments were arranged in a culture dish, allowed to attach by partial drying, and then maintained with a mitotically inactivated mouse 3T3 fibroblast feeder layer [8,9]. Tissues were from the University Hospital and/or Dental Clinic at the State University of New York at Stony Brook. An explant culture of soft palate epithelium embedded in a collagen gel was kindly provided by J. Brandsma of Long Island Jewish Hospital. Cultures were then derived from keratinocyte outgrowths from explanted tissue. One day before infection, the growth medium [10] was removed and cultures were treated with 0.02% EDTA in PBS to remove feeder layer cells and any human fibroblasts. Tissue sources (Table I) were as follows: cervix, 45-year-old hysterectomy patient; gingiva, 70-year-old male; soft palate, 65-year-old female; abdomen, 19-year-old male; buttock, 40-

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Reprint requests to: Dr. Lorne B. Taichman, Department of Oral Biology and Pathology, School of Dental Medicine, State University of New York at Stony Brook, Stony Brook, New York 11794-8702

Abbreviations:
Ad2: adenovirus type 2
CPE: cytopathic effect
dpi: days post-infection
EDTA: ethylenediaminetetraacetic acid
HEK: human embryonic kidney tubule cells
MOI: multiplicity of infection
PBS: calcium- and magnesium-free phosphate buffered saline
PFU: plaque-forming unit
TCA: trichloroacetic acid

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Table I. Summary of Ad2 infection characteristics in cultures of epidermal and mucosal keratinocytes.

Tissue[a] source	CPE[b]	Cell protein[c] shutoff	Ad2 capsid[d] synthesis	PFU/cell[e] yielded
cx	+/+	++	+++	675
gg	+/+	+	+++	514
sp	+/+	++	++++	549
ab	+/−	−	−	4
bt	+/−	−	+	27
nbfs	+/−	−	+	10
aifs	+/−	−	−	25
aofs	+/−	−	+	42
HeLa	+	(++)	(++++)	531

[a] cx: cervix; gg: gingiva; sp: soft palate; ab: abdomen; bt: buttock; nbfs: new born foreskin; aifs: adult foreskin, inner surface; aofs: adult foreskin, outer surface. See "Materials and Methods" for details of tissues.

[b] Human normal keratinocytes stratify to form basal and suprabasal layers. CPE is therefore reported as occurring for suprabasal/basal layers, respectively. CPE was gauged as +/+ if present in both suprabasal and basal layers or +/− if the suprabasal layer exhibited CPE and the basal layer was CPE-free.

[c] Keratinocyte protein shutoff. Efficiency of virus-induced host protein shutoff is reported as the most severe (++), where synthesis of many cell proteins is inhibited, to the least effect (−), where little or no change was detected.

[d] Synthesis of Ad2 proteins. Synthesis is reported as ranging from the most productive (++++) to where little (+) or no (−) Ad2 capsid protein was detected.

[e] Cultures were harvested at 3 dpi from an input MOI of 40 PFU per cell. Lysates were titered on HeLa cells as described in "Materials and Methods." Results in brackets indicated data not presented in this study.

year-old female; new born foreskin obtained at time of neonate circumcision; adult foreskin, 24-year-old male. When placed into explant culture, the adult foreskin was separated into epidermis from the inner or outer surfaces. Virus expression assays were then done on adult inner surface foreskin (aifs) versus adult outer surface foreskin (aofs). All tissues were clinically normal and had no gross pathology.

Virus An initial stock of wild type Ad2 was provided by B. Stillman, Cold Spring Harbor. Virus stocks were grown in HeLa cells and purified on cesium chloride gradients [11]. Stocks were stored at −70°C in TD (Trizma base 25 mM, NaCl 140 mM, KCl 5 mM, and Na_2PO_4 0.1 mM) pH 7.4, with 10% glycerol and 10 mM $MgCl_2$. Virus titrations of stocks and experimentally infected cells were done via plaque assays on HeLa cells [12]. Cultures of infected cells were lysed by three cycles of freeze-thawing. Cell debris was cleared from the lysate by centrifugation. All infections were done when cultures were approximately 75% confluent with 40 plaque forming units (PFU) per cell. Total PFU needed per dish was calculated based on cell counts done with a Coulter Counter.

Analysis of Protein Synthesis At 3 d post-infection (dpi) mock- and Ad2-infected cultures in 35 mm dishes were labeled with 150 uCi of [^{35}S]methionine for 4 h in 2 ml of methionine-free Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and 0.4 ug/ml hydrocortisone (Sigma). Cells were harvested by scraping, washed with PBS containing 300 ug/ml phenylmethylsulfonyl fluoride (Sigma) [13], and pelleted. Whole cell lysates were prepared by suspension of the pellet in SDS gel sample buffer (Tris-HCl 50 mM, pH 6.8, SDS 3.0%, 2-mercaptoethanol 5.0%, glycerol 10%, and bromophenol blue 0.1%). Equal amounts of TCA-precipitable counts were heated at 100°C for 5 min and then electrophoresed through SDS-polyacrylamide gels [14] as modified for adenovirus protein analysis [15]. The gels were processed for fluorography [16] and exposed to Kodak X-OMAT AR film.

RESULTS

Expression Characteristics of Ad2 in Stratified Squamous Keratinocyte Cultures. Cytopathic Effect Ad2 is recognized as a pathogen of the stratified squamous epithelium found in the upper respiratory tract but not the epidermis. Whether this difference stems from characteristics inherent in the keratinocytes or their

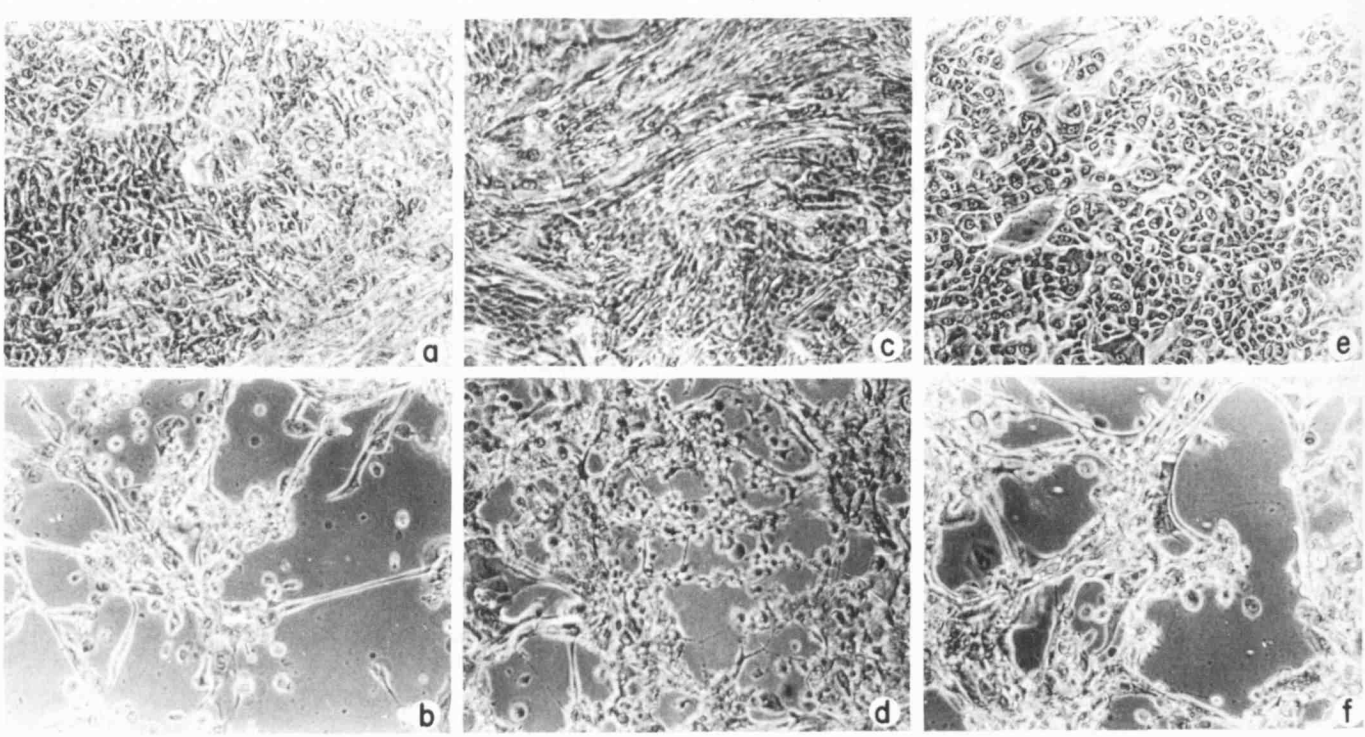

Figure 1. Cytopathic effects of Ad2 in mucosal keratinocytes. While still subconfluent, stratified cultures of soft palate (*a* and *b*), gingiva (*c* and *d*), and cervix (*e* and *f*) keratinocytes were either mock-infected (*a*, *c*, and *e*) or infected with Ad2 at 40 PFU per cell (*b*, *d*, and *f*). Cultures were examined at 3 dpi. Ad2-infected cultures (*b*, *d*, and *f*) exhibit a cytopathic effect in all cell layers. Cells are refractile, rounded, and have retracted, exposing the surfaces of the culture dish. Mock-infected cultures (*a*, *c*, and *e*) show no changes.

tissue environment was examined by keratinocytes cultured from stratified epithelia of various body sites. The epithelium types were broadly referred to as mucosal (gingiva, soft palate, and cervix) or epidermal (abdomen, buttock, and foreskin). Cultures were examined for several signs of cell-virus interactions including induction of a cytopathic effect (CPE), synthesis of viral proteins, inhibition of cellular protein synthesis, and production of progeny virions as determined by quantification of plaque-forming units (PFU).

Subconfluent stratified cultures of mucosal and epidermal keratinocytes were infected at 40PFU per cell and examined for the appearance of virally induced CPE. After infection with Ad2, mucosal keratinocytes retracted from each other and the surface of the plate with many cells becoming highly refractile. By 3 dpi (Fig 1) Ad2-infected cultures from each source of mucosal epithelium exhibited CPE involving all layers of the culture.

Keratinocyte cultures from epidermal sources, in contrast to mucosal keratinocytes, exhibited a limited CPE by 3 dpi with Ad2 (Fig 2). In these cultures only the suprabasal cells developed a CPE; basal cells, though infected [5], remained morphologically unchanged. This is in agreement with a previous report [5] and suggests that the cell-virus interaction between Ad2 and normal epidermal keratinocytes is dependent on the cell's basal or suprabasal nature.

Protein Synthesis Vegetative infection by adenovirus involves production of a number of viral proteins and suppression of host protein synthesis [3]. To assay for these events, cultures of keratinocytes were fed with ^{35}S-methionine and whole cell extracts were examined by gel electrophoresis (Figs 3 and 4). Because hexon, penton, and hexon-associated protein constitute the majority of the virion capsid they serve as easily recognizable markers of adenovirus expression. They are referred to as proteins II (MW 125,000), III (MW 85,000), and III_a (MW 66,000), respectively [3]. An additional non-structural late protein, 100 kd, is present in detectable amounts. Changes in host protein synthesis were gauged by comparing the differences in background labeling of mock- and Ad2-infected cells. Keratinocytes from mucosal sites produced significant quantities of viral specific proteins and experienced a marked shutoff of host cell protein synthesis (Fig 3). Among the mucosal keratinocytes, soft palate cells produced the most viral capsid proteins. These cells also had the greatest default in host protein synthesis (Fig 3 and Table I). Ad2 infection of abdomen keratinocytes had the least effect on host protein synthesis and little capsid protein was produced (Fig 4). In general, no epidermal keratinocytes produced amounts of Ad2 protein or suffered as great a shutoff of cell protein synthesis as seen in mucosal keratinocytes.

Production of Infectious Virus We assayed for the production of infectious virus as a biologic measure of the permissivity of the mucosal and epidermal keratinocytes for Ad2 replication. Mock- and Ad2-infected cultures were collected 3 dpi, lysed, and titered on HeLa monolayers (Table I). Keratinocytes from mucosal sites produced 514-675 PFU per cell. This compared favorably with cells routinely used for permissive infections, e.g., HeLa, where a value of 531 PFU per cell was obtained. Epidermal keratinocytes from various sites produced 4 to 42 PFU per cell. These values were 20- to 100-fold lower than mucosal keratinocytes or HeLa cells. PFU per epidermal keratinocyte production did not increase significantly at later times post-infection (see below).

Restricted Growth of Ad2 in Epidermal Keratinocytes The results presented above demonstrated a clear difference in Ad2 expression in keratinocytes cultured from mucosal epithelia and epidermis. Cells from epidermal sites were obviously less permissive than cells from mucosal sites. Restricted viral expression may often

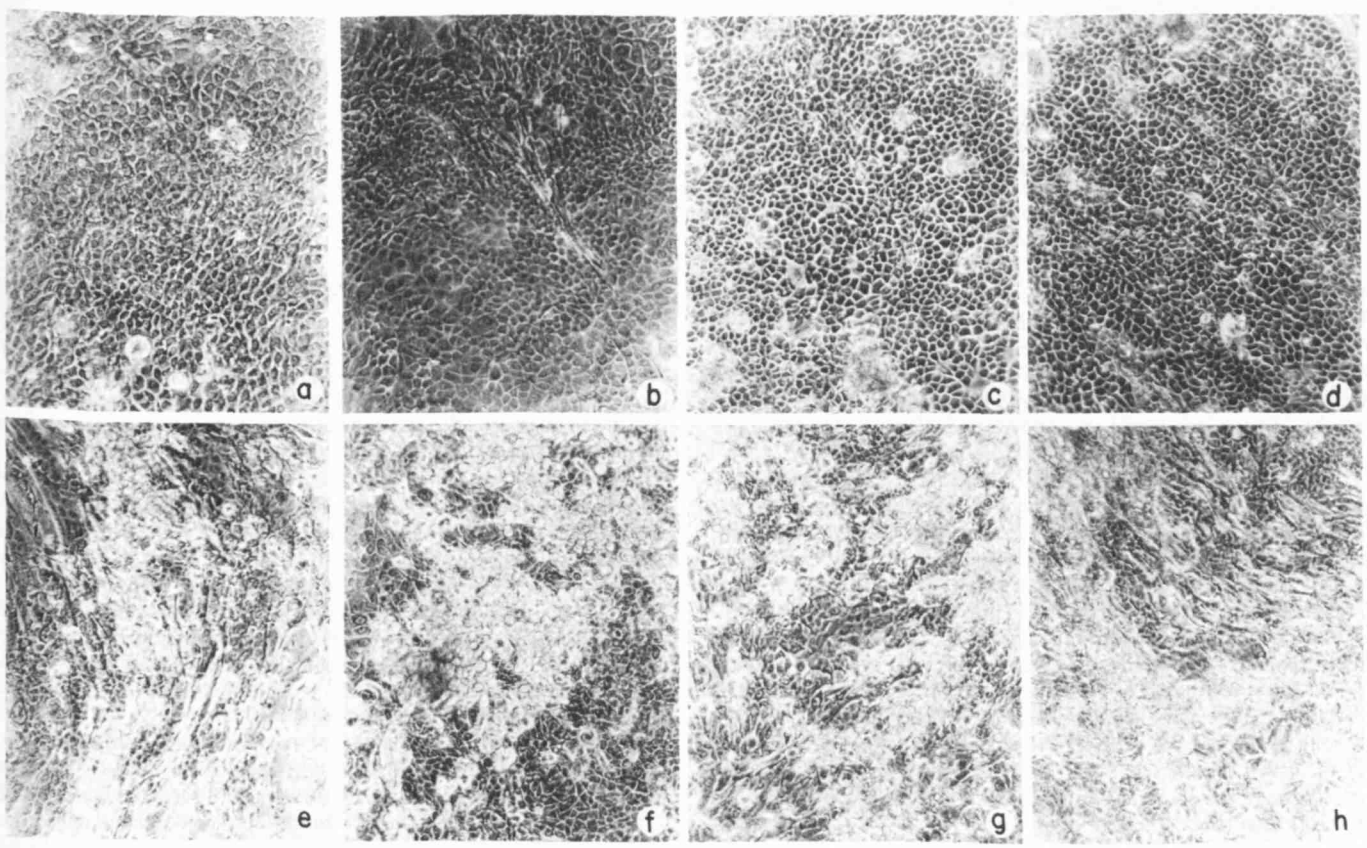

Figure 2. Cytopathic effects of Ad2 in epidermal keratinocytes. Subconfluent, stratified cultures of abdomen (*a* and *e*), buttock (*b* and *f*), adult foreskin inner surface (*c* and *g*), and adult foreskin outer surface (*d* and *h*) were mock-infected (*a, b, c,* and *d*) or Ad2-infected (*e, f, g,* and *h*) at 40 PFU per cell. Cultures were examined at 3 dpi. Cells exhibiting a cytopathic effect are limited to the suprabasal layers. Ad2 infection has not caused a morphologic change in basal cells.

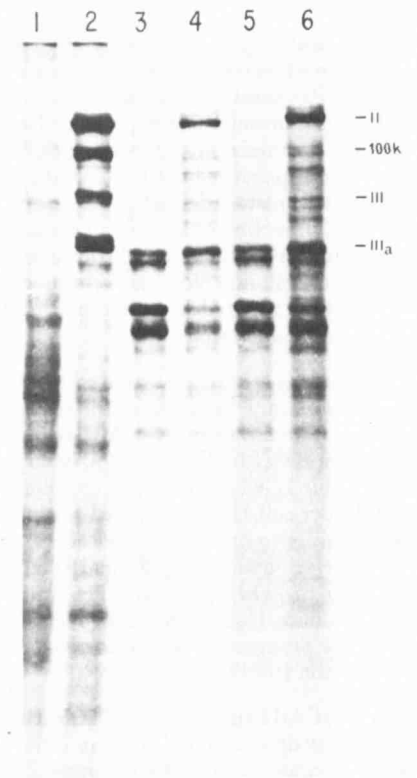

Figure 3. Protein synthesis in mock- and Ad2-infected mucosal keratinocytes. Cultures of cells were labeled with [^{35}S]methionine at 2 dpi. Whole cell lysates were prepared and analyzed by polyacrylamide gel electrophoresis. Production of significant amounts of Ad2 proteins was noted concurrent with a decrease in host cell protein synthesis. The major bands in uninfected cultures are keratin proteins and are not specifically labeled. The most prominent bands in Ad2-infected cultures are viral proteins II, III, IIIa, and 100 kd. These have been labeled. Viral protein IIIa migrates slightly behind the largest keratin protein and these two bands are not readily distinguishable. *Lanes 1* and *2*: soft palate; *lanes 3* and *4*: gingiva; *lanes 5* and *6*: cervix. Lanes *1, 3,* and *5* are from mock-infected cultures. Lanes *2, 4,* and *6* are from Ad2-infected cultures.

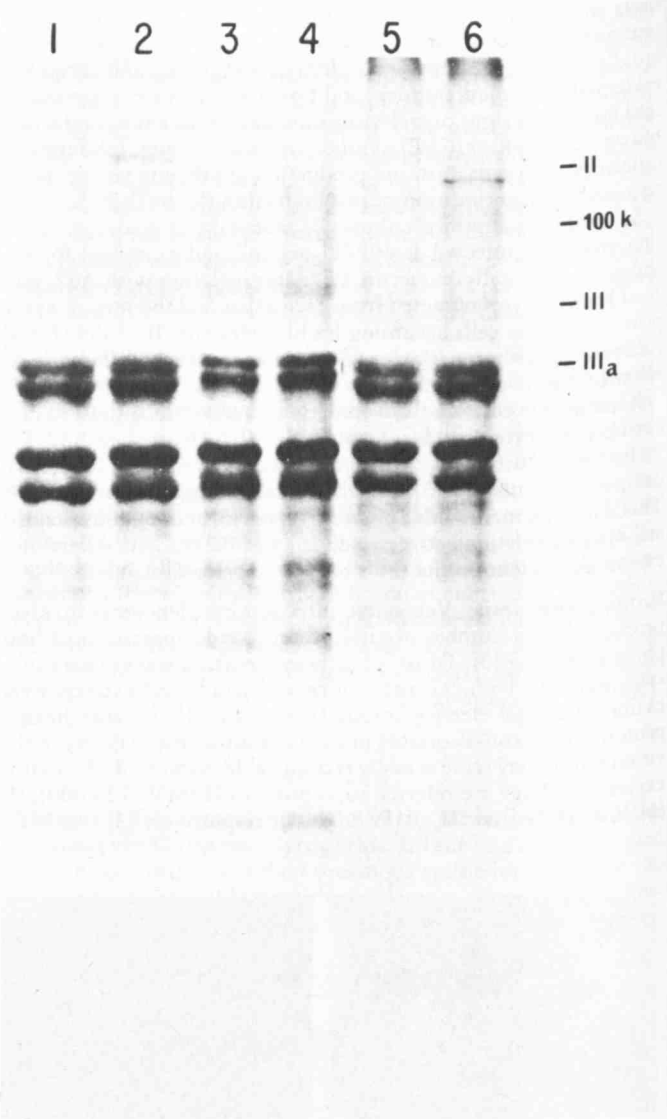

Figure 4. Protein synthesis in mock- and Ad2-infected epidermal keratinocytes. Cultures of cells were labeled with [^{35}S]methionine at 2 dpi. Whole cell lysates were prepared and analyzed by polyacrylamide gel electrophoresis. Ad2-infected cells had little change regarding cellular protein synthesis. Very low levels of Ad2 specific proteins were produced. The locations of some viral late proteins are indicated. *Lanes 1* and *2*: newborn foreskin; *lanes 3* and *4*: abdomen; *lanes 5* and *6*: buttock. Lanes *1, 3,* and *5* are from mock-infected cultures. Lanes *2, 4,* and *6* are from Ad2-infected cultures.

be overcome by prolonged culture periods or high titers of input virus [3]. Sister dishes of subconfluent keratinocytes were infected with increasing amounts of input virus and then maintained for 7 d to determine if the restrictive influence of the epidermal keratinocyte phenotype on Ad2 expression could be overcome. Cultures were examined for CPE induction and for production of infectious virus. Cultures of mucosal keratinocytes were included to provide a profile of virus production in susceptible cells.

Development of CPE in mucosal keratinocytes did not change (results not shown). More interestingly, for epidermal keratinocytes, a 100-fold increase in multiplicity of infection (MOI) did not alter the limited CPE response and low viral output seen at 3 dpi with the routine MOI of 40 PFU per cell (data not shown). A twofold increase in time post infection did not alter the response (Fig 5). It is interesting to note that the shape of the virus growth curve was similar in epidermal and mucosal keratinocyte cultures (Fig 5). This suggests that the kinetics of viral growth in these different keratinocyte types are the same even though the overall output is greatly different. The effect of other tissue characteristics on Ad2 expression were also considered. Newborn and adult (24 years old) foreskin expressed the same infection characteristics (data not shown). Therefore, age is not likely to be an important factor in the limited cell-virus interaction of epidermal keratinocytes and Ad2. In addition, infection of epidermal keratinocytes from the inner and outer aspects of foreskin were similarly restrictive in their expression of Ad2 (Table I). These two sites differ by the presence of a cornified layer in the outer surface [17]. The similar Ad2 expression in keratinocytes from these two sites suggests that epidermal cells need not come from a cornified site to be restrictive for Ad2. Thus, regional variation of keratinization seems to have little effect on Ad2 susceptibility. Donor sex also seemed unimportant as soft palate from a 65-year-old female and gingiva from a 70-year-old male were equally permissive for Ad2 growth. Results independent of parameters such as keratinization pattern and donor age or sex, suggest that the Ad2 expression characteristics were generalized phenomena for mucosal or epidermal keratinocytes.

Tissue from the oropharyngeal cavity can harbor subclinical infections of adenovirus [18]. The presence of the virus is revealed when cells from these sites are maintained and passaged in culture. It is therefore possible that the differences between oral and epidermal cells described in this study were the result of endogenous virus establishing a superinfected state with the experimentally added virus. However, serial passage of mock-infected keratinocytes from each mucosal site did not result in spontaneous development of CPE. Lysates of mock-infected cells from first passage and serially

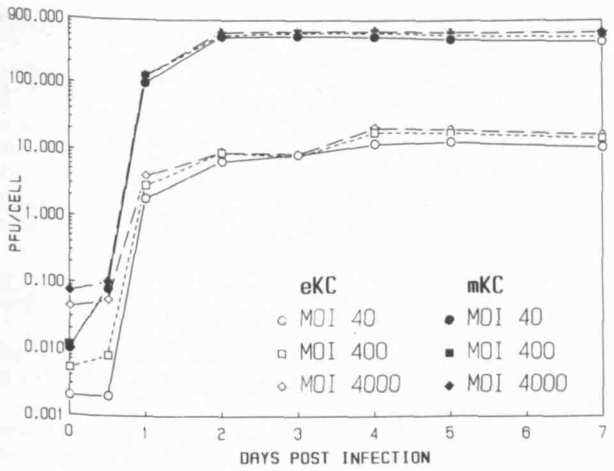

Figure 5. Time course of Ad2 production in epidermal (eKC) and mucosal (mKC) keratinocytes at increasing MOI. Stratified subconfluent cultures of keratinocytes were infected in duplicate at 40, 400, and 4000 PFU per cell. Samples were collected at the times indicated and freeze/thawed three times to release virus. Lysates were titered in duplicate on HeLa cell monolayers. The zero day time point was harvested after the 90-min infection incubation period, during which the virus is internalized and inactivated. Both mucosal and epidermal keratinocytes displayed similar inactivation of input virus followed by logarithmic growth of infectious virus. Near or maximum production in each cell type was achieved at 2 dpi. Epidermal keratinocytes, however, yielded about a 25-fold lower amount of virus at maximum production than mucosal keratinocytes.

passaged cultures did not yield PFUs when titered on HeLa cells (data not shown). This suggests that mucosal keratinocytes cells were initially free from Ad2 and that changes in these cells were due to experimentally added virus.

DISCUSSION

Adenoviruses infect the epithelium of the upper respiratory tract and oropharynx but not the epidermis [3,4]. Using keratinocytes cultured from various body sites, we have explored the apparent epithelium-specific susceptibility to Ad2 infection. Four parameters of cell-virus interactions were measured in cultures 3 d following infection with 40 PFU/cell [19,20]: 1) development of CPE, 2) synthesis of viral proteins, 3) inhibition of host protein production, and 4) yield of infectious progeny virus (Table I). In cultures of keratinocytes from mucosal surfaces (soft palate, gingiva, and cervix), all cells exhibited a CPE, synthesized abundant amounts of viral capsid proteins, exhibited marked suppression of host protein synthesis, and produced 514 to 675 PFU per cell. By these measures, mucosally derived keratinocytes were fully permissive for Ad2. Keratinocytes from the three epidermal sites (abdomen, buttock, and foreskin) exhibited a very different response: CPE was evident only in suprabasal cells, little viral capsid protein was synthesized, no detectable shut-off of host protein synthesis occurred, and only 4 to 42 PFU per cell were produced. By these measures, Ad2 expression in the epidermal keratinocytes was restrictive. Tissue characteristics such as degree of keratinization and donor age or sex had no effect on whether a permissive or restrictive infection was established. Additionally, increases in viral input and duration of infection were not able to overcome the restricted infection in epidermal keratinocytes. These results indicate a clear difference in the response to Ad2 exhibited by epidermal keratinocytes and keratinocytes derived from the oropharynx.

In vivo, the cervix is rarely infected by Ad2 [4]. However, the permissive nature of cultured cervical keratinocytes to Ad2 indicates that keratinocytes other than those derived from the oropharynx may be susceptible in vitro. The basis of this permissivity may stem from the mucosal nature of the cervical epithelium. It is interesting to note that conjunctiva and corneal epithelia, both mucosal surfaces, are also susceptible to Ad2 infection in vivo [3]. Although we have not sampled numerous sites, we feel the data warrant the tentative conclusion that epidermal and mucosal keratinocytes differ in their ability to support Ad2 expression in vitro.

Adenoviruses were originally isolated from throat washings and fragments of tonsilar and adenoid tissue [1]. Because lymphocytes are present in these sources, it is possible that they are also sites for adenovirus replication. In vivo, it seems a latent infection is most often established [18]. In vitro, latent infections may also be established, although lectin-stimulated primary cells [21] or continuous B- and T-cell lines can yield productive infections [22]. While adenovirus can remain latent in lymphoid cells of the oropharynx, it is likely that keratinocytes are the source of large scale production of progeny virus in acute infections.

Although a natural pathogen of oropharynx and upper respiratory tract epithelium, the most exhaustive studies of adenovirus expression have been done in cells from tissues other than sites of natural infection [3]. Investigations involving permissive infections are mostly done with human embryonic kidney tubule epithelium (HEK cells) or continuous cell lines, such as HeLa, derived from carcinomas [3]. Cultures of mucosal keratinocytes would seem to be the most appropriate in vitro model for examining certain aspects of adenovirus biology such as antiviral therapy.

Ad2 does not commonly infect the epidermis [4], and yet in culture epidermal keratinocytes support limited Ad2 expression to a point where CPE is evident in suprabasal cells and low amounts of progeny virus are produced. It is likely that the culture situation [23] itself has altered epidermal keratinocytes in such a way as to permit this limited expression. Whatever alterations take place, they are not sufficient to overrule the restrictive nature of the epidermal keratinocyte and thereby bring about a phenotype fully permissive for Ad2. The failure of basal cells in epidermally derived cultures to exhibit CPE is not the result of a lack of infection of these cells. Previous studies [5] have shown that when suspensions of keratinocytes are infected, the cells that attach and initiate culture growth show no CPE but do harbor the virus. When suprabasal cells arise, CPE appears in these cells. The development of CPE in suprabasal but not basal cells may indicate a reduction in restriction in the more differentiated population of cells. In fact, Ad2 late gene expression has been shown to increase in the larger more differentiated cells of the culture (unpublished observation).

The susceptibility of an epithelial site to infection by a virus may be viewed within the larger context of tissue specificity. How is one epithelial surface distinct from another? Studies of various markers of keratinocyte differentiation have demonstrated that the presence or absence of certain markers as well as the level of their expression is closely associated with tissue specificity. Keratin is one example of these markers. The particular combinations of keratin proteins produced fall into three categories, depending on whether the epithelium is simple, stratified squamous, or stratified squamous keratinizing [24]. Filaggrin is another marker of keratinocyte differentiation. In oral epithelium, the level of filaggrin production correlates with the degree of keratinization found in palate, gingiva, and buccal mucosa [25]. It is thus clear that products normally synthesized by keratinocytes provide an indication of tissue specific differences. We suggest that the response of epithelial cells to infection with certain viral agents may be considered as another indicator of tissue specificity. Because viruses utilize host cell metabolic machinery [6], differences in the response to the same viral agent signal an inherent difference in the makeup of a cell. In this study we have observed differences in the interaction between various keratinocytes and Ad2; these differences correlate with the tissue of origin and suggest a fundamental distinction between mucosally and epidermally derived keratinocytes.

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