

Antigen presentation in the murine oral epithelium

K. ERIKSSON,* E. AHLFORS,* A. GEORGE-CHANDY,* D. KAISERLIAN† & C. CZERKINSKY‡

*Department of Medical Microbiology & Immunology, University of Göteborg, Göteborg, Sweden, †INSERM U404, Institut Pasteur de Lyon and ‡INSERM U80, Hôpital Herriot, Lyon, France

SUMMARY

We have previously reported that the buccal mucosa can support delayed type hypersensitivity (DTH) reactions to contact sensitizers. In the present study, we show that cells isolated from the buccal epithelium are able to present soluble exogenous antigens to specific T cells. Single cell suspensions obtained by enzymatic dispersion of buccal epithelial sheets could present the native protein antigen hen-egg lysozyme (HEL) to the I-A^k-restricted CD4⁺ T-cell hybridoma specific for a.a 46–61 on HEL. T-cell activation resulted in interleukin-2 (IL-2) production which could be inhibited by anti-major histocompatibility complex (MHC) class-II antibodies of pertinent specificity. Immunohistochemical staining of whole buccal epithelial sheets revealed that all MHC II positive cells had a dendritic morphology and expressed ATPase activity, indicating that these cells represent a major antigen-presenting cell (APC) population in this tissue. Furthermore, single cell suspensions isolated from buccal epithelium (BEC) after local *in vivo* administration of either a native soluble protein, a synthetic dodecapeptide, or a contact sensitizer were able to activate antigen-specific T cells *ex vivo*. Kinetic analyses indicated that maximal APC activity in the oral epithelium occurred within 1 hr after local antigen administration, and had essentially vanished after 24 hr. Conversely, APC activity was undetectable in draining cervico-mandibular lymph node cell suspensions recovered 1 hr after local antigen injection but became manifest after 3–24 hr. These observations suggest that dendritic cells can acquire antigens in the buccal epithelium and migrate to draining lymph nodes where they present processed antigen to MHC class II-restricted T cells. This APC population may thus be a critical element in the initiation of Th1-driven DTH responses in the oral mucosa.

INTRODUCTION

The buccal cavity is the most important natural body orifice in contact with ingested and inhaled antigens that gain entry to the aerodigestive tract. Hence, the epithelium lining the buccal mucosa must provide an important barrier function to prevent absorption of undegraded antigens and subsequent development of potentially harmful immune responses. To the latter belong oral lesions of chronic inflammatory disorders, such as lichen planus, gingivitis, periodontal disease, where the major inflammatory cell population infiltrating the buccal epithelium consists of T cells and macrophages.¹

We have shown that the murine buccal mucosa can serve both as an inductive site for local and remote T cell-mediated delayed-type contact hypersensitivity (DTH) reactions.² Such inflammatory reactions involve T cells that produce a Th1 cytokine profile consisting of interleukin-2 (IL-2), interferon- γ (IFN- γ) and tumour necrosis factor (TNF).³ These observations suggested that the buccal mucosa may comprise all necessary elements required for induction of a Th1 type hypersensitivity response. The fact that

epithelial cells from the lower aerodigestive tract can function as authentic, although non-conventional antigen-presenting cells (APC)^{4,5} prompted us to examine the capacity of the buccal epithelium to present antigens both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Animals

Femal CBA/J mice (I-A^k) and BALB/c mice (I-A^d) were obtained from B & K Universal, Sollentuna, Sweden. BALB/c mice expressing a transgenic T-cell receptor (TCR) α/β specific for peptide 111–119 from influenza virus haemagglutinin (HA) were a kind gift from Dr H. von Bohmer, Basel, Switzerland.⁶ All mice were used at 7–10 weeks of age. Animals were housed at the Animal Care Facility of the Department of Medical Microbiology & Immunology, University of Göteborg.

Antigens

Hen-egg white lysozyme (HEL) and oxazolone (4-ethoxymethylene-2-phenyloxazol-5-one) (OXA) were obtained from Sigma Chemical Company (St Louis, MO). A synthetic peptide spanning a.a. 108–119 of influenza virus HA (a.a. SVSSFERFEIFPK) was purchased from Neosystem (Strasbourg, France).

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Correspondence: Dr C. Czerkinsky, Department of Medical Microbiology & Immunology, University of Göteborg, Guldhedsgatan 10A, S-413 46 Göteborg, Sweden.

Antibodies

The following monoclonal antibodies were used in this study: mouse immunoglobulin G (IgG)2b anti-I-A^b monoclonal antibody (mAb) (clone 10.2.16),⁷ a gift from Dr M. Pierres, CIML, Marseille, France; and rat IgG anti-I-E^k mAb, CD311,⁵ a gift from A. Glasebrook, E. Lilly, Indianapolis, IN.

T cells

The CD4⁺ mouse T-cell hybridoma, 3A9, specific for peptide 46-61 of HEL in the context of I-A^k,⁸ was kindly provided by Dr P. Allen (Washington University School of Medicine, St Louis, MO). OXA-specific lymph node cells were obtained from pooled inguinal and axillary lymph nodes of mice previously sensitized on the shaved abdominal skin and on the feet with 100 µl 3% OXA in acetone: ethanol (1:4, v/v), 3 and 4 days prior to sacrifice. Highly purified (>99%) T cells were obtained from spleen and lymph nodes of influenza virus HA peptide-specific TCR transgenic mice by plastic adherence and carbonyl iron (Sigma) treatment, followed by removal of B cells with anti-mouse immunoglobulin-coated beads (Dynal, Norway).

Buccal sensitization

CBA mice were exposed to a total of 20 µl OXA diluted to 1% (w/vol) in acetone: olive oil (1:20, v/v), applied onto the inner surface of both cheeks.² Separate groups of BALB/c mice received 50 µg of HEL or HA peptide in phosphate buffered saline (PBS) and injected into the buccal mucosa (25 µl per cheek).

Preparation of single cell suspensions from mouse buccal epithelium

The inner part of the cheeks was excised and remaining muscle fragments and outer skin were carefully dissected out under a stereomicroscope. The cheeks were then incubated in 20 mM ethylene diamine tetra acetic acid (EDTA)-PBS pH 7.2 for 2-3 hr at 37°, and epithelial sheets were peeled off from the connective tissue.⁹ From these sheets, single cell suspensions were obtained by enzyme dispersion. In the case of animals sensitized with oxazolone or HEL, a 20 min incubation with trypsin (1 mg/ml in PBS-EDTA) at 37° under constant stirring followed by a 20 min incubation with 2 mg/ml DNase (Sigma) in Iscove's medium (Gibco, Paisley, UK) containing 10% fetal calf serum (FCS). Trypsin treatment of adherent lymph node cells previously exposed to HA peptide *in vitro* was found to abrogate antigen presentation to specific T cells. Thus, in the case of experiments involving animals injected with HA peptide, buccal epithelium cells (BEC) were obtained by two consecutive 20 min incubations of buccal epithelial sheets with 1 mg/ml collagenase/dispase (Boehringer Mannheim, Germany) in Hank's balanced salt solution (HBSS) containing 0.5 mg/ml DNase. The above procedures yielded 1-5 × 10⁵ cells nucleated BEC per animal, of which at least 75% were viable.

Preparation of lymph node adherent cells

Single cell suspensions were prepared by mechanical teasing of pooled cervical mandibular and auricular lymph nodes, and were incubated for 90 min in flat-bottomed 96-well tissue-culture plates (Nunc, Roskilde, Denmark, 2 × 10⁶ cells per well). Non-adherent cells were removed by repeated washing with PBS.

Staining of whole epithelial sheets for ATPase and major histocompatibility complex class II-positive cells

Epithelial sheets were fixed in cacodylate-buffered formaldehyde and stained for adenosine triphosphatase as described.¹⁰ After staining, specimens were rinsed and dehydrated in ethanol, followed by xylene, and mounted in EukittTM (O Kindler GmbH & Co, Germany) as described.¹¹ For class II staining, acetone-fixed whole epithelial sheets were incubated with the rat IgG anti-I-E^k mAb, CD311. Specific binding was revealed using biotinylated anti-rat IgG antibodies, avidin/biotinylated horseradish peroxidase complex (ABC, Dakopatt, Denmark), and chromogene substrate (3,3'-diaminobenzidine and H₂O₂). Cells were counterstained with Mayer's haematoxylin and mounted in glycerol-gelatin.

Antigen presentation assays

Freshly isolated cells from buccal epithelial sheets were tested for their antigen presentation function, after *in vitro* or *in vivo* antigen exposure. In the first experimental system (antigen added *in vitro*), various numbers of BEC were cultured with 10⁵ CD4⁺ mouse T-cell hybridoma 3A9 cells in the presence or absence of HEL (300 µg/ml) in a total volume of 200 µl of medium, in flat bottomed microculture wells (Nunc) as previously described.⁵ The medium consisted of Iscove's medium supplemented with 10% FCS, 3 µg/ml L-glutamine (Gibco), antibiotics and 5 × 10⁻⁵ M mercaptoethanol. Supernatants were harvested at 24 and 48 hr of culture and tested for IL-2 production using the IL-2-dependent CTLL2 cell line as described.⁵ In some experiments, anti-Ia (10.2.16 or CD311) mAbs were added at the initiation of the cultures.

In the second system (*in vivo* pulse), BEC from mice painted 45 min earlier with OXA (see above), or from control non hapten-exposed mice, were incubated for 3 days at 37° with lymph node cells from mice sensitized with OXA or from naive control mice. BEC and adherent lymph node cells from mice buccally injected with HEL were cultured with 3A9 cells as described above. Supernatants were collected 24 and 48 hr later and assayed for IL-2 activity.

BEC (10⁵ cells per well) and adherent lymph node cells from mice buccally injected with HA peptide were cultured for 72 hr with purified T cells from TCR transgenic mice (8 × 10⁴ cells per well). Six hours prior to the completion of the culture period, 20 µl of culture medium containing 1 µCi of [6-³H]thymidine (Amersham, UK) was added to each well. The harvesting and subsequent measurement of incorporated radioactivity were performed on an automated filter cell harvester and an argon activated β-scintillation counter (Inotech, Basel, Switzerland). Data are expressed as mean c.p.m. of duplicate or triplicate cultures.

RESULTS

Hapten-derivatized BEC induce IL-2 production by specific T cells

We investigated the ability of single cell suspensions of buccal epithelium, obtained from mice buccally sensitized with the hapten OXA, to induce activation of OXA-specific peripheral lymph node T cells from epicutaneously sensitized mice. BEC obtained 45 min after mucosa painting were able to induce significant IL-2 production when co-cultured with the lymph node T cells from OXA-primed mice (Table 1). This finding indicated that functional APCs were present in the buccal epithelium.

Table 1. Hapten presentation by *in vivo* sensitized BEC IL-2 production* (c.p.m. $\pm$ SD)

T-cells	Cells from buccal epithelial sheets		
	OXA-primed	Naive	None
OXA-primed	3009 $\pm$ 463	1357 $\pm$ 386	573 $\pm$ 19
None	153 $\pm$ 49	113 $\pm$ 27	N.D. [†]

* IL-2 production by lymph node T cells from OXA-primed mice co-cultivated with *in vivo* sensitized BEC was measured in 48 hr culture supernatants using the CTLL-2 assay. The results are expressed as mean $\pm$ SD proliferation of CTLL-2 cells.

[†] N.D. = not done.

Phenotypic characterization of buccal intra-epithelial dendritic cells (DC)

Indirect immunoperoxidase staining of buccal epithelial sheets using the I-E^k-specific mAb CD311 showed staining of cells with a dendritic morphology scattered throughout the epithelium whereas no MHC class-II expression was detected on buccal keratinocytes (Fig. 1a). Likewise, staining of buccal epithelial sheets for ATPase activity revealed the presence of cells with a similar dendritic morphology (Fig. 1b). These data indicate that dendritic-like cells represent the major MHC class-II expressing cell type in the normal murine buccal epithelium.

BEC present HEL to class-II-restricted CD4⁺ T cells

We examined the ability of cell suspensions obtained by enzymatic dispersion epithelial sheets to present the soluble protein antigen HEL to MHC class II-restricted CD4⁺ T-cells *in vitro*. As shown in Fig. 2, single cell suspensions from buccal epithelial sheets exposed *in vitro* to HEL, induced activation of the HEL-specific I-A^k-restricted CD4⁺ T-cell hybridoma 3A9, as monitored by IL-2 production. Omission of either HEL, BEC or 3A9 cells in the culture system resulted in lack of IL-2 production (Fig. 2). Addition of anti-I-A^k (mAb 10.2.16) to cultures of BEC and 3A9 cells strongly inhibited HEL-induced IL-2 production, whereas no inhibition was observed in parallel cultures treated with an irrelevant anti-MHC class II mAb (anti-I-E^k mAb CD311) (Fig. 2).

BEC could also present *in vivo* administered antigen to MHC class II-restricted CD4⁺ T cells. As shown in Fig. 3, single cell

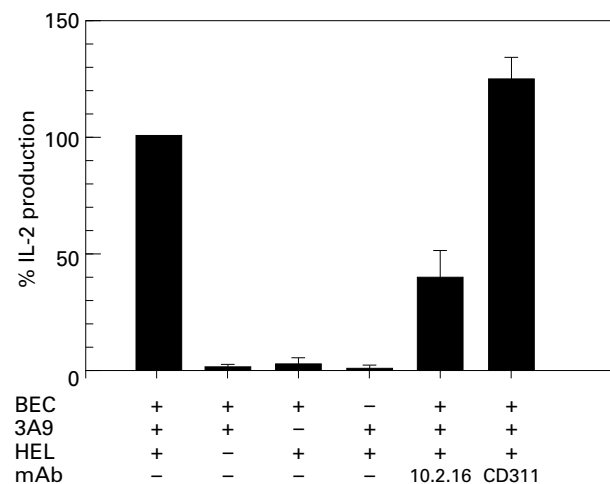


Figure 2. BEC can present HEL *in vitro* to MHC class II-restricted CD4⁺ T cells. Single cell suspensions from buccal epithelial sheets exposed to HEL induced activation of the HEL-specific I-A^k-restricted CD4⁺ T-cell hybridoma (clone 3A9) as monitored by IL-2 production. Data are expressed as mean percentage maximal IL-2 production $\pm$ SD determined by comparison with 3A9 cells cultured with BEC and HEL.

suspensions of BEC obtained 1 hr after intrabuccal HEL injection could activate the 3A9 hybridoma in a dose-dependent and antigen-specific fashion. No activation of 3A9 was induced by BEC from mice injected with bovine serum albumin (BSA).

Kinetics of APC activity in the buccal epithelium and draining lymph nodes after buccal injection of antigen

We compared the kinetics of induction of APC capacity in the buccal epithelium and its draining lymph nodes following injection of antigen into the buccal mucosa. Two experimental systems were used for this purpose. In the first one, HEL was injected into the cheek of CBA mice, and the buccal epithelium as well as draining cervico-mandibular lymph nodes were collected at various times thereafter. BEC suspensions and adherent cells from the lymph nodes were assayed for their ability to activate class II-restricted 3A9 T cells, which have no requirement for accessory signals. A sharp increase in APC activity was observed in the BEC already 1 hr after antigen exposure, rapidly decreased after 3 hr, and had essentially vanished by 24 hr (Fig. 4). In the lymph nodes, maximal APC activity was observed after 3 hr, being still manifest

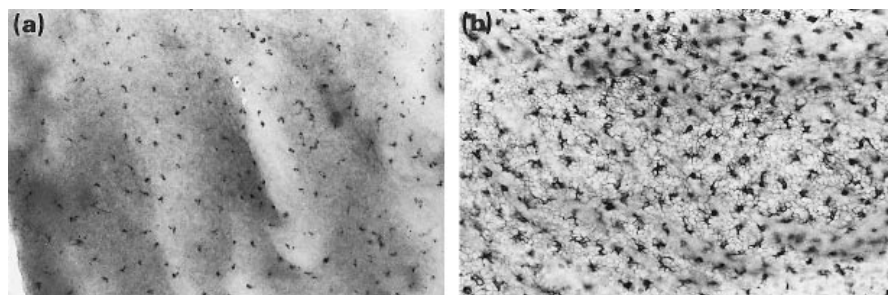


Figure 1. MHC class II (CD311) (a) and ATPase (b) staining of murine buccal epithelial sheets. Staining is exclusively observed on cells with dendritic morphology. Original magnification $\times$ 200.

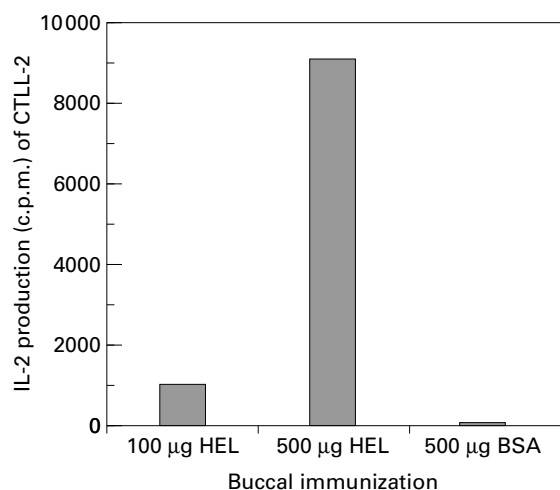


Figure 3. BEC can present HEL *in vivo*. BEC cell suspensions obtained 1 hr after buccal injection of HEL induced activation of the HEL-specific I-A^k-restricted CD4⁺ T-cell hybridoma (clone 3A9). Data are expressed as mean IL-2 production (c.p.m.) of CTLL-2 cells grown with 50% supernatant from co-cultures.

24 hr after HEL injection, and then slowly decreased to become undetectable by 72 hr (Fig. 4).

In the second experimental system, the ability of BEC and adherent lymph node cells to present *in vivo* administered antigen to non-transformed T cells, i.e. T cells that require accessory signals, was investigated. A synthetic peptide mapping a T cell immunodominant epitope on influenza virus HA was injected into the buccal epithelium of BALB/c mice. BEC and adherent cervico-mandibular lymph node cells were assayed for their ability to induce activation of T cells expressing a transgenic TCR specific for the peptide, as measured by the proliferative response of the TCR transgenic T cells. BEC obtained by collagenase dispersion of buccal epithelial sheets recovered as early as 1 hr after peptide

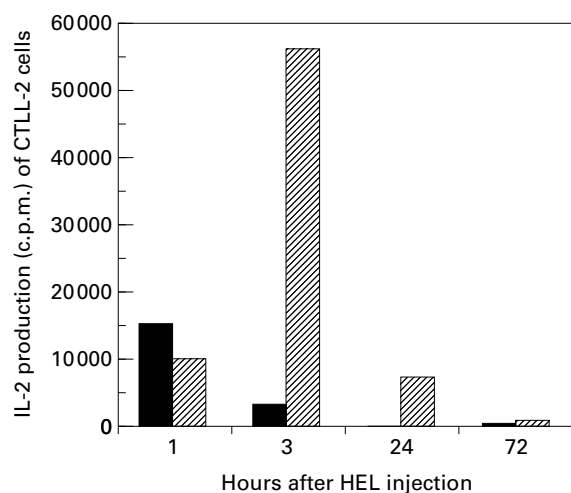


Figure 4. Kinetics of APC activity in the oral epithelium and draining lymph nodes after *in vivo* administration of HEL. Maximal activation of 3A9 T cells by BEC cell suspensions (filled bars) was seen 1 hr after buccal HEL injection. Maximal activation of 3A9 T cells by adherent cervico-mandibular lymph node cells (hatched bars) was observed 3 hr after buccal HEL injection. Data are expressed as per Fig. 3.

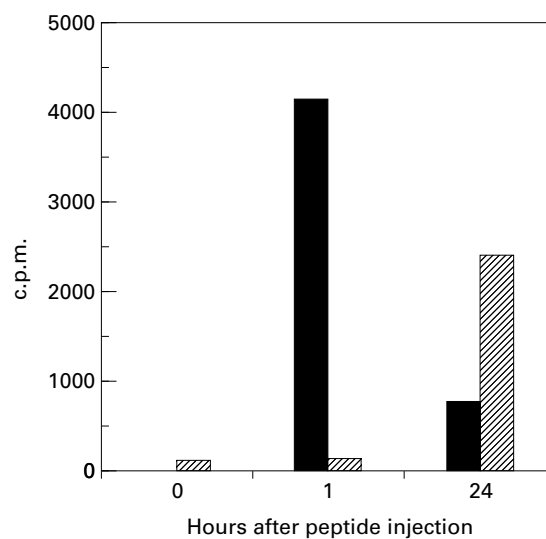


Figure 5. APC activity in the oral epithelium and draining lymph nodes after buccal injection of influenza virus HA peptide. BEC suspensions (filled bars) and adherent cervico-mandibular lymph node cells (hatched bars) were obtained after buccal injection with HA peptide and used as sources of APC for peptide-specific TCR-transgenic T-cells. T cell activation was monitored by ³H-thymidine incorporation. Data are expressed as mean ³H-thymidine incorporation (c.p.m.) of triplicate cultures.

injection, displayed maximal APC activity. In contrast, no APC activity could be detected among adherent cervico-mandibular lymph node cells recovered from the same animals 1 hr after peptide injection, but was manifest by 24 hr, i.e. at a time when APC activity was no longer detectable among BEC. A representative experiment is shown in Fig. 5.

DISCUSSION

In a previous study, the capacity of the murine buccal mucosa to support T-cell mediated inflammatory responses has been documented.² The latter responses are typified by the classical delayed-type contact hypersensitivity reaction and are known to be mediated by Th1 cells.³ This suggested that either (i) the buccal mucosa allowed entry of contact sensitizers into the submucosa and/or its draining lymph nodes, (ii) that the buccal epithelium and/or draining lymphoid tissues were endowed with an antigen-presenting capacity, or (iii) that both properties could account for local and systemic DTH responses seen after buccal sensitization.

Our first aim was to investigate whether the buccal epithelium contains APC. We investigated the ability of single cell suspensions of buccal epithelium, obtained from mice buccally sensitized with OXA, to induce activation of hapten-specific peripheral lymph node T cells. BEC obtained 45 min after oral sensitization were able to induce significant IL-2 production by lymph node T-cells from OXA-primed mice. This finding suggested that cells with antigen-presenting function are present or can be rapidly induced within the buccal epithelium following hapten painting.

The capacity of BEC to present a soluble protein antigen *in vitro* to T cells was also demonstrated. BEC co-cultured with the CD4⁺ T-cell hybridoma 3A9, specific for HEL, were able to induce IL-2 production by 3A9 cells in the presence of antigen. In addition, presentation of HEL by BEC cells to 3A9 T cells was

abrogated by treatment with anti-major histocompatibility complex (MHC) class-II mAb. Since recognition of HEL by this specific T cell hybridoma requires specific processing of HEL and association of processed peptides with the pertinent MHC groove,⁸ this finding indicated that the buccal epithelium comprises a population of MHC class II-restricting APC. The MHC class-II positive dendritic-like cells identified by ATPase and MHC class-II staining of buccal epithelial sheets would appear the most likely candidate APC type in this tissue. This interpretation is consistent with recent reports indicating that oral mucosal Langerhans' cells can present antigen to specific T cells *in vitro*.^{12–14} The finding that BEC from animals injected intrabuccally could present antigen *ex vivo* to a specific T cell supports the notion that the oral epithelium comprises a population of authentic APC. This is at variance with freshly isolated Langerhans' cells from the epidermis, which are considered functionally immature APC.^{15–17}

The finding that *in vivo* OXA-derivatized BEC could activate hapten-specific lymph node T cells may be explained by the fact that contact sensitizing haptens, such as OXA, have been reported to induce the rapid release of pro-inflammatory cytokines from resident keratinocytes. These cytokines are known to be co-stimulatory for T cells (IL-1 and tumour necrosis factor (TNF)- α)¹⁸ or to be involved in the maturation of skin Langerhans' cells (granulocyte-macrophage-colony stimulating factor; GM-CSF)^{19,20} and in their migration from the epidermis to the lymph.^{17,20} In this respect, adherent cells, and thus presumably DC, from lymph nodes draining the oral mucosa were able to present *in vivo* acquired antigen to transformed as well as non-transformed T cells. In the latter situation, APC activity was detected 3 hr after local antigen injection and increased thereafter. This finding is in keeping with a recent report indicating that DC enriched from oral mucosa-draining lymph nodes of hapten painted animals can transfer DTH reactivity.¹⁴ We suggest that the APC activity associated with cervico-mandibular adherent lymph node cells is mainly contributed by dendritic cells having acquired antigen in the oral epithelium and having migrated via the afferent lymph, a process which appears to take approximately 24 hr.^{21,22} In further support to this scenario is the observation that MHC class II- and ATPase-positive DC in the buccal epithelium are increased in numbers 1 hr after HEL injection and have decreased by almost 50% by 24 hr (not shown), i.e. at a time when APC activity is present in the draining lymph nodes but is no longer detected in the oral epithelium.

In conclusion, this study demonstrates that the murine buccal epithelium is endowed with the capacity to process and/or present antigens to specific T cells. This property appears to be mainly contributed by a discrete population of MHC class II-expressing dendritic cells which can process and present antigen on their surface. Upon migration into the draining lymph nodes, these cells can recruit and activate MHC class II-restricted T cells, including DTH T cells. This scenario is compatible with the view that DTH reactivity in the buccal mucosa is initiated mainly by cognate interactions between Th1 cells and dendritic cells originating from the buccal epithelium. We cannot rule out the possibility that other cell types such as keratinocytes which may be induced to express MHC class II molecules at later stages of a DTH reaction²³ can present antigen to T cells, the outcome of which may lead to production of cytokines other than IL-2. This possibility deserves being addressed.

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