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Purified human oral mucosal Langerhans cells function as accessory cells *in vitro*

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SUMMARY

Oral mucosal Langerhans cells (OMLC) may have an important role in the induction of immune responses to oral pathogens. In this study, anti-HLA-DR antibody-coated immunomagnetic beads were used to purify OMLC from suspensions of normal human buccal epithelium and the capacity of the purified cells to function as accessory cells (AC) was investigated. Electron microscopy was used to show that the purified cells possessed all recognized ultrastructural features previously described in epidermal Langerhans cells. Using T lymphocyte proliferation assays in hanging drop microcultures, it was found that purified OMLC could function as AC for responses to concanavalin A by autologous T cells. Purification of OMLC from small biopsies of oral mucosa has enabled us to show that OMLC, like epidermal Langerhans cells, can function as AC *in vitro*.

Keywords Langerhans cells oral mucosa

INTRODUCTION

Langerhans cells (LC) are a population of bone-marrow derived cells with dendritic morphology found in the stratified squamous epithelium of skin and mucous membranes. LC express CD1 molecules [1,2], CR3 (CD11b, CD18) and CD32 (FcRII) [3–5] and the class II MHC-encoded molecules HLA-DR [6,7], DP and DQ [8]. None of these markers is LC-specific, and ultrastructural features, particularly the presence of characteristic Birbeck granules, are necessary for categoric LC identification [9]. Analysis of human epidermis by flow cytometry suggested that LC constituted 2·5% of the cell total [10]. Human epidermal LC can present both exogenous antigens and alloantigens [11], and thus have an important role in the induction of T cell immunity. LC have also been demonstrated in oral mucosa, where they may be important in the induction of immunity to oral pathogens. However, studies of the antigen-presenting capacity of oral mucosal LC (OMLC) have not been possible because of a lack of a method for purification and functional assay. In previous studies, cell suspensions of unfractionated human gingival epithelium from inflamed sites augmented mitogen-driven [12] and alloreactive T cell proliferation [13], but this activity could not be attributed definitely to OMLC; for example, gingival keratinocytes at sites of inflammation also express MHC class II molecules [14], and therefore may possess antigen-presenting capacity *in vitro*, as has been shown for other non-myeloid, MHC class II-positive cells. The aim of this study was to purify human OMLC from biopsies of buccal epithelium and to determine their capacity to function as accessory cells (AC) for autologous T cell responses to concanavalin A (Con A). For T cell proliferation assays, 20 μl hanging drop microcultures were used because of the advantage of this system when only low numbers of cells can be obtained.

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SUBJECTS AND METHODS

Purification of oral mucosal Langerhans cells

Ethical approval was obtained. Biopsies of clinically normal buccal mucosa of approximately 5 × 5 mm were taken from 12 healthy volunteers (four females, eight males, age range 19–34 years) under local anaesthesia (Xylocaine, Astra) and washed in PBS containing penicillin (100 U/ml) and streptomycin (100 mg/ml) (GIBCO) for 1 h. Subjects rinsed with 2% chlorhexidine gluconate immediately before biopsy. Tissue was then immersed in dispase II (Boehringer Mannheim), an enzyme which cleaves epithelium for underlying connective tissue by lysing hemidesmosomes [15]. Each biopsy was immersed in 2 ml of dispase of concentration 2 mg/ml in PBS. Specimens were incubated in the enzyme at 4°C overnight [16]. The epithelial sheet was dissected off the underlying lamina propria and washed in PBS. Cryostat sections of epithelial sheets from two individuals were prepared and stained with an anti-CD1a (NA1/34, Serotec, 1:500 dilution) or DR MoAb (L243, Becton Dickinson, 1:200) and an avidin-biotinylated immunoperoxidase method as previously described [17]. Ten epithelial sheets were immersed in 0·05% trypsin/EDTA (GIBCO) for 15 min at 37°C and then agitated vigorously in a syringe. The released cells were placed in cold RPMI 1640 medium (Northumbria Biologicals) containing

10% heat-inactivated fetal bovine serum (FBS) (Sigma) to inhibit further trypsin activity. Dispersed oral epithelial cells were washed three times, counted on a haemocytometer and viability assessed by trypan blue exclusion. The epithelial cells were then resuspended in 500 μl RPMI/FBS and incubated in Eppendorf tubes with magnetic beads 4·5 μm in diameter coated with anti-DR IgM (Dynal UK Ltd.) for 60 min at 4°C with continuous inversion of the tube. Based on pilot experiments with peripheral blood leucocytes (PBL), a bead : target cell ratio of 10 : 1 was used, and the numbers of beads calculated on the assumption that OMLC constituted 2% of the total epithelial cells. Cells were transferred to a 10-ml tube and placed in a magnet (Dynal). The bead-rosetted cells were washed three times in RPMI/FBS, and the number of cells binding one or more beads was counted. Purified cells were irradiated with 25 Gy before use in T cell proliferation assays.

Phenotypic and ultrastructural analysis of purified cells

Purified oral epithelial cells were fixed in glutaraldehyde and processed for transmission electron microscopy. Alternatively, bead-rosetted cells were added to 100 μl RPMI/FBS containing 10 μl (1 unit) of Detachabeads (Dynal) to separate the cells from the anti-DR coated beads. The mixture was rotated for 60 min at room temperature, after which the suspension was transferred to a 10-ml tube, magnetized and washed as described above. Some cells were pelleted and processed for transmission electron microscopy, others were stained either with anti-CD1a (1 : 10) or anti-DR (1 : 20) MoAbs conjugated with FITC (OKT6, Ortho) and PE (L243, Becton Dickinson) respectively, or with control IgG1/FITC and IgG2a/PE antibodies. Cells were analysed by flow cytometry and fluorescence microscopy.

Preparation of peripheral blood T lymphocytes and accessory cells

Venous blood (20 ml) was obtained from each of eight donors of buccal mucosa. Heparinized blood was layered onto Lymphoprep (Nycomed) and centrifuged at 400 ***g*** for 20 min at room temperature. PBL at the interface were pipetted off and washed three times. T lymphocytes were separated by rosetting with neuraminidase-treated sheep erythrocytes (Sigma) and centrifuging over Lymphoprep. Non-rosetted cells were washed, irradiated with 25 Gy and used as control AC. Sheep erythrocyte-rosetted cells were resuspended in lysing buffer containing 0·17 M Tris HCl (pH 7·6) and 0·83% NH_4Cl, washed and counted. This population contained 93% $CD3^+$ and less than 1% $CD14^+$ cells as determined by flow cytometry. T cell-depleted control AC contained 2% $CD3^+$ and 36% $CD14^+$ cells.

T lymphocyte proliferation assays

T cell responses to Con A were assayed using 20-μl hanging drop microcultures in 60-well Terasaki plates [18], using culture medium containing RPMI 1640, Dutch modification (Flow Laboratories), 10% FBS (Sera-Lab Ltd.), penicillin 100 U/ml, streptomycin 100 mg/ml and L-glutamine 3 mM (GIBCO). This culture system has proved to be valuable for measuring T cell responses using low numbers of cells. Four-day cultures were set up in triplicate, using a range of cell numbers and Con A concentrations, pilot experiments with PBL having shown this to be the optimal culture period. Cultures were incubated in humidified sandwich boxes in a CO_2 incubator, then pulsed with tritiated thymidine (Amersham, specific activity 2 Ci/mM, 1 μl added to each microwell giving a final concentration of 1 μg thymidine/ml) for the final 4 h. Cultures were harvested by blotting onto filter discs using a Flow microharvester. The discs were dried and β radiation measured by liquid scintillation spectroscopy.

RESULTS

Immunoperoxidase staining of oral epithelial sheets removed by dispase digestion demonstrated that the sheet retained rete pegs and that suprabasal $CD1a^+$ and DR^+ cells of dendritic morphology were present (Fig. 1). No other cell type stained with either antibody.

Cell yield of disaggregated biopsy tissue

Ten biopsies of buccal mucosa yielded a mean of $3{\cdot}4 \times 10^6$ total epithelial cells (range $1{\cdot}4$–$8{\cdot}6 \times 10^6$), with a mean viability of 82% (range 73–95%). Most dead cells were large keratinocytes from the higher epithelial strata, and smaller cells were generally of higher viability. The mean number of purified DR^+ cells was $3{\cdot}0 \times 10^4$ (range $0{\cdot}3$–$17{\cdot}0 \times 10^4$) constituting a mean of 0·8% (range 0·1–2·2%) of the total epithelial cell yield (Table 1). In an experiment to test the effect of using Detachabeads, $6{\cdot}6 \times 10^4$ DR^+ cells were obtained from $8{\cdot}4 \times 10^6$ epithelial cells, but only $3{\cdot}3 \times 10^4$ remained after treatment with Detachabeads.

Phenotypic analysis of cells purified with magnetic beads

In two experiments, flow cytometry was used to enumerate DR^+ and $CD1a^+$ cells in disaggregated epithelial suspensions, in magnetic bead-purified populations and in the cells which remained in epithelial suspensions following depletion with magnetic beads. Very few (< 500) bead-purified cells remained at the end of the staining procedure. Although the magnetic bead-purified cells were enriched both for DR^+ and $CD1a^+$ cells, and the depleted epithelial cells contained less than 1% DR^+ cells, flow cytometry suggested significant levels of non-specific staining with the control IgG1/FITC and IgG2a/PE antibodies among purified cells. Direct visualization with fluorescence microscopy was more helpful; 10^3 cells were analysed, except in the case of bead-purified cells when all remaining cells were observed. Four per cent of cells in the unseparated epithelial suspension were $DR/CD1a^+$. Of the purified cells, 88% were $DR/CD1a^+$, compared with 5% which bound stain non-specifically. In the depleted suspension, binding was less than 1% with test and control antibodies. However, to confirm that the bead-purified population consisted primarily of OMLC, transmission electron microscopy was performed to look for cells with ultrastructural features of LC.

Electron microscopy of purified DR^+ cells

Oral epithelial cells rosetted with anti-DR-coated magnetic beads were dendritic in morphology with irregular or lobulated nuclei, and contained mitochondria and endoplasmic reticulum. Many also contained lysosomes. The ultrastructure of a minimum of 50 cells from each sample was analysed, and a mean of 83% contained Birbeck granules (Fig. 2). Desmosomes, tonofilaments, melanosomes and phagosomes were absent. These ultrastructural features are characteristic of LC [9].

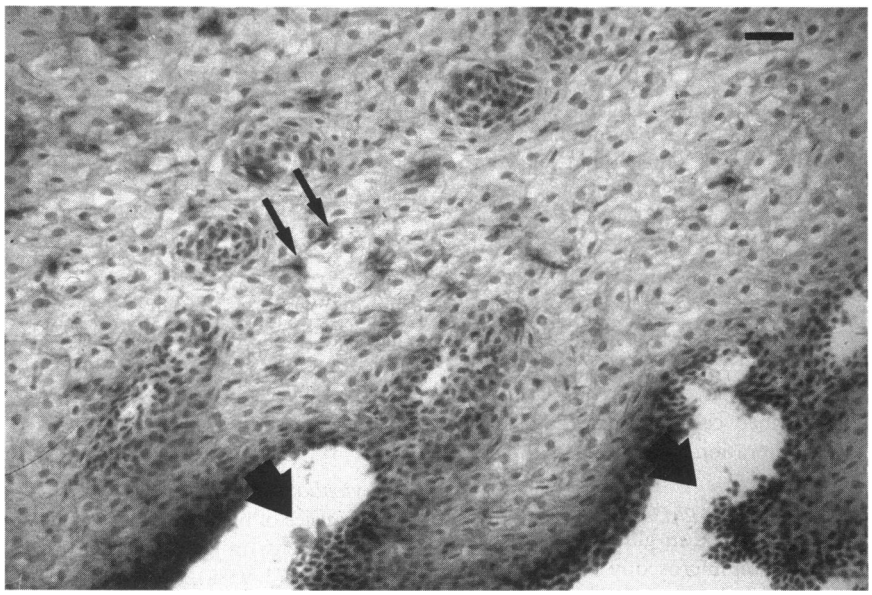

Fig. 1. Sheet of buccal epithelium detached from underlying connective tissue after incubation of the biopsy overnight in dispase. Section reacted with anti-CD1a MoAb. CD1a$^+$ dendritic cells are still present within the epithelium (thin arrows). There are empty spaces occupied by the dermal papillae before digestion (thick arrows). Bar = 100 μm. Avidin-biotinylated immunoperoxidase, $\times$64.

Table 1. Augmentation of T cell responses by purified oral mucosal Langerhans cells (OMLC). Wells contained 20×10^3 T cells and 5 μg/ml Con A

Experiment	Total OMLC purified from biopsy	No. OMLC per well	T cell responses to Con A (mean ct/min)	
			−OMLC	+OMLC
1	6000	60	97	1558
2	2500	25	98	126
3	17500	175	117	4876
4	15000	100	48	449
5	15000	100	34	4062
6	2500	25	86	113
7	30000	50	98	5975
8	12500	100	56	3290

Accessory cell function of purified DR$^+$ cells

Using T lymphocytes at 20×10^3–$2{\cdot}5 \times 10^3$/well, the ability of purified, irradiated epithelial DR$^+$ cells to augment responses to Con A (0·2–5·0 μg/ml) was tested. The number of DR$^+$ cells added to each well varied slightly between experiments, depending on the number obtained from each biopsy (Table 1). In five out of eight individuals, T cell responses to Con A were significantly enhanced in the presence of low numbers of purified DR$^+$ cells (Table 1). Results from representative experiments are shown in Figs 3–5. T cell responses to Con A in the absence of added DR$^+$ cells were minimal and there was no significant T cell response to DR$^+$ cells alone. The presence of as few as 50 DR$^+$ cells significantly enhanced responses to Con A (Fig. 3). In each of the five subjects, purified DR$^+$ cells functioned as AC in these assays, although the T cell number and Con A doses at which this effect was maximal varied between subjects. There was no effect of magnetic beads alone, confirming that DR$^+$ cells were necessary for this activity.

The use of T cell-depleted PBL as controls allowed a comparison of the efficacy of PBL AC with the DR$^+$ cells purified from epithelium. One hundred purified DR$^+$ cells were approximately equivalent to 5×10^3 T cell-depleted PBL (Fig. 4), suggesting that OMLC have similar potency to low density dendritic cells in peripheral blood [19]. Also, epithelial cells depleted of OMLC showed minimal or no AC function (Fig. 5), confirming that the activity of purified DR$^+$ cells in these assays was not due to a minor component of epithelial cells possibly co-purified with the OMLC. These results demonstrate that purified OMLC function as AC *in vitro*.

In three subjects, purified DR$^+$ cells demonstrated no significant AC activity. For two of these, the number of purified DR$^+$ cells was particularly low and a maximum of only 25 OMLC per well was used. It is possible that T cell proliferation had not yet peaked, and that a longer period of culture was necessary. In the third subject, no effect was seen even though 100 OMLC per well were used. There was no apparent technical reason for this—the viability of the epithelial suspension was high (83%) and T cell-depleted PBL demonstrated comparable AC activity to other control AC.

DISCUSSION

OMLC may have an important role in the induction of T cell immunity to oral pathogens. In order to investigate this, we have developed a method for purifying human OMLC to study their function *in vitro*. This method enabled cells with morphological features of OMLC to be purified from small biopsies. Since OMLC comprise a minority of the total intraepithelial cell population, purification methods must be highly efficient in order to produce enough cells for functional studies. For purifying skin LC, sufficient tissue can usually be obtained to render the purification method less critical. For OMLC, relatively little tissue is available and optimal purification procedures are essential. After comparing several approaches,

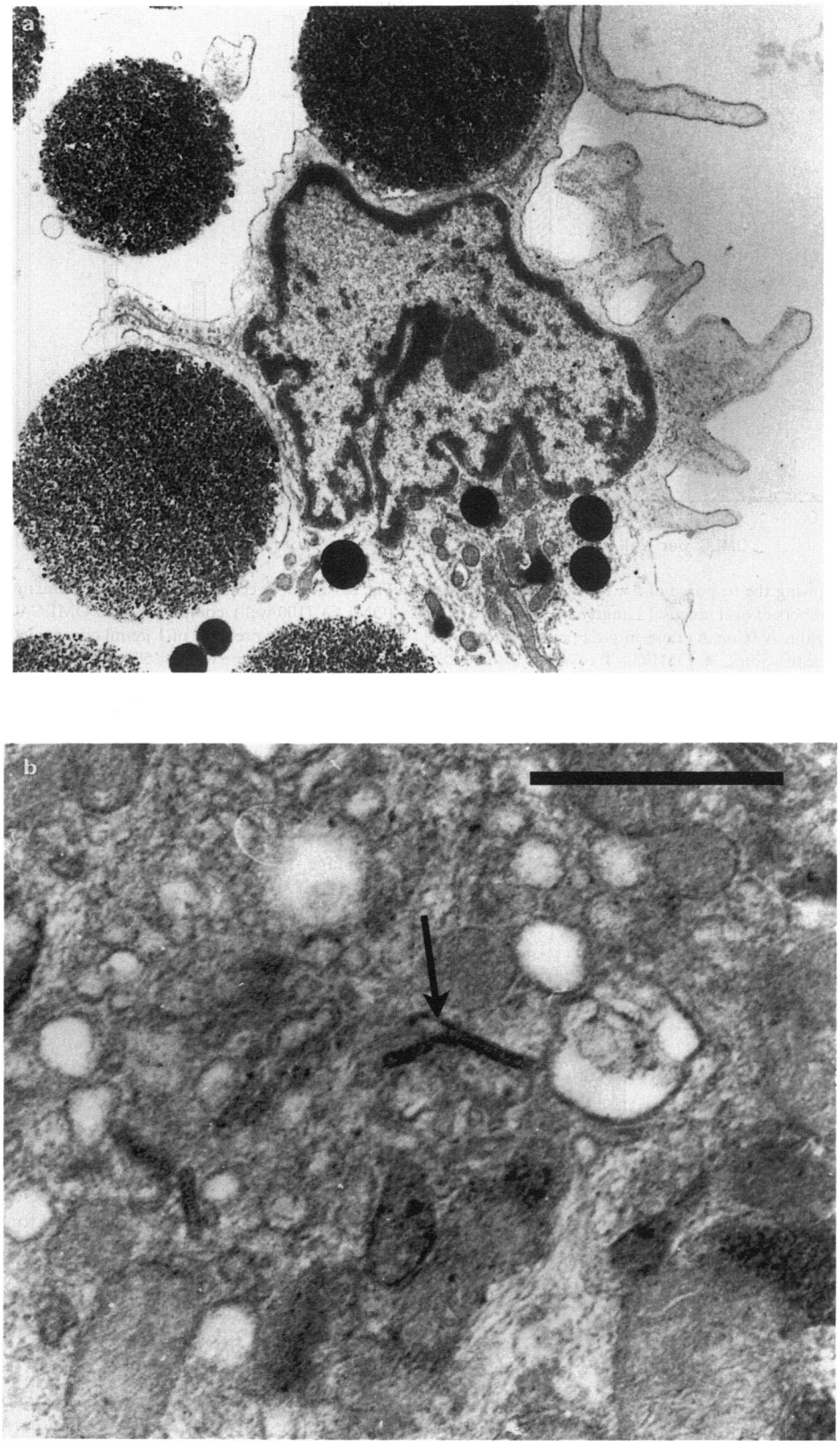

Fig. 2. (a) Dendritic cell isolated from oral epithelium bound by anti-DR-coated immunomagnetic beads. Electron dense lysosomes are present within the cytoplasm. Diameter of beads = 4·5 μm. Transmission electron micrograph, × 8500. (b) Birbeck granule (arrow) present within cytoplasm of isolated DR^+ oral mucosal Langerhans cell (OMLC). Bar = 1 μm. Transmission electron micrograph, × 38 000.

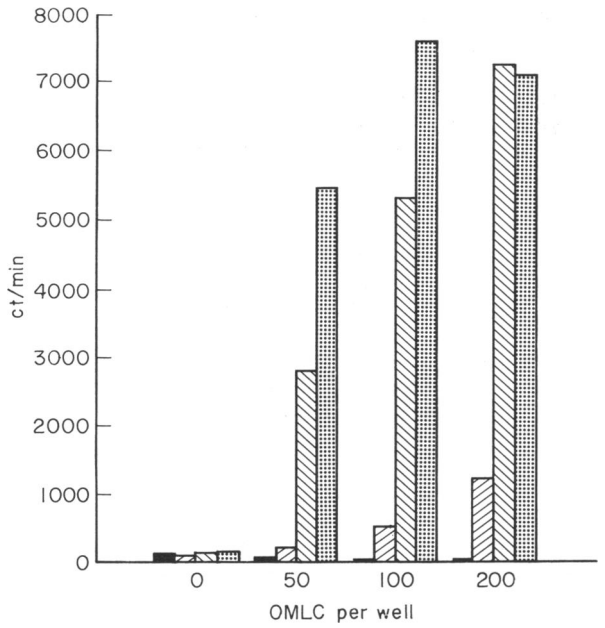

Fig. 3. Experiment showing the response of 5×10^3 autologous T cells per well to varying numbers of oral mucosal Langerhans cells (OMLC) per well and concanavalin A (Con A) dose in 4-day cultures. S.E.M. were within 15% of mean values. ■, OMLC+T cells, no Con A; ■, OMLC+T cells+0·2 μg/ml Con A; ▧, OMLC+T cells+1·0 μg/ml Con A; ▨, OMLC+T cells+5·0 μg/ml Con A.

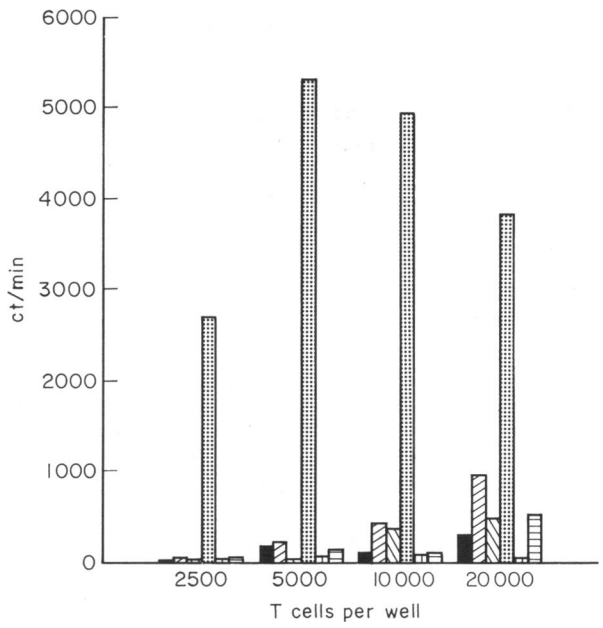

Fig. 5. Accessory cell function of purified oral mucosal Langerhans cells (OMLC) (100/well) compared with OMLC-depleted epithelial cells (10^3/well) in the presence of 1 μg/ml concanavalin A (Con A) in 4-day cultures. S.E.M. were within 15% of mean values. ■, T cells only; ▨, T cells+1 μg/ml Con A; ▧, OMLC+T cells; ▨, OMLC+T cells+1 μg/ml Con A; ▥, OMLC-depleted epithelial cells+T cells; ▤, OMLC-depleted epithelial cells+T cells+1 μg/ml Con A.

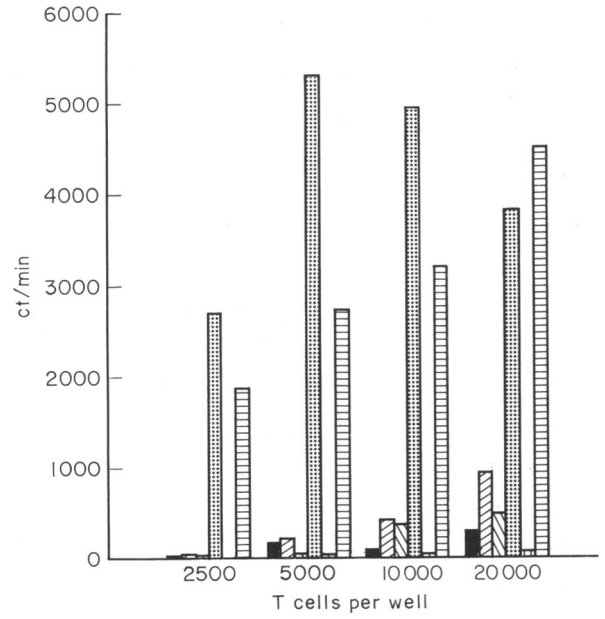

Fig. 4. Accessory cell function of oral mucosal Langerhans cells (OMLC) (100/well) compared with T cell-depleted peripheral blood leucocytes (PBL) (5×10^3/well) in the presence of 1 μg/ml concanavalin A (Con A) in 4-day cultures. S.E.M. were within 15% of mean values. ■, T cells only; ▨, T cells+1 μg/ml Con A; ▧, OMLC+T cells; ▨, OMLC+T cells+1 μg/ml Con A; ▥, T cell-depleted PBL+T cells; ▤, T cell-depleted PBL+T cells+1 μg/ml Con A.

we used magnetic beads coated with anti-DR MoAb to positively select DR^+ cells from enzyme disaggregated oral mucosal biopsy tissue. Use of anti-DR-coated beads permitted a one-stage purification, a considerable advantage since the number of required washing stages, and hence the number of cells lost, was reduced. Cells which rosetted with the anti-DR-coated magnetic beads constituted a mean of 0·8% of the total epithelial cells released following trypsinization. If LC constitute 2·5% of intraepithelial cells in oral mucosa, as they do in skin [10], then this method extracts about one third of the OMLC available. Based on the results of electron microscopy, this method produces a highly purified OMLC population. All purified cells were dendritic, 83% contained Birbeck granules and none resembled keratinocytes. The lack of Birbeck granules in some cells may simply have been the result of the plane of the section taken [20]. Alternatively, some dendritic cells may be 'indeterminate' cells, which have been described in skin [21] and are identical to LC in all respects, except for their lack of Birbeck granules. In skin, the ratio of LC to indeterminate cells has been reported as 4/1 [22], which is similar to that found here.

This method is unlikely to co-purify oral epithelial keratinocytes, since these cells do not express MHC class II molecules in healthy buccal mucosa [14,23]. The immunohistochemical analysis of the detached oral epithelial sheet showed a well defined separation, suggesting that the theoretical possibility of macrophages contaminating the cultures was remote. Previous work has shown that B lymphocytes are absent from oral epithelium or lamina propria [24]. The ultrastructural features of the purified DR^+ cells (including absence of phagosomes), and the need for as few as 50 cells per well confirm that no putative contaminating AC was responsible for T cell prolifera-

tion. Furthermore, epithelial cells depleted of OMLC demonstrated little or no AC capacity. This is the first conclusive demonstration that OMLC function as AC *in vitro*; previous studies have used unfractionated cell suspensions containing other intraepithelial cell populations.

Methods of purifying LC from skin have been reviewed by Schmitt *et al.* [25]. Flow cytometry has been used successfully for both phenotypic analysis of and LC purification from human skin [10], and it is unclear why it was unhelpful in this study. Even with the bead-purified cells, non-specific fluorescence was a persistent feature. Evidence that a high percentage of bead-purified cells were DR/CD1a$^+$ was provided by fluorescence microscopy. In human skin, antibody-coated magnetic beads have been used in two-stage procedures, using anti-CD1a MoAb followed by reaction with magnetic beads. This produced high purity when used for positive selection [26] or efficient removal of LC if used for depletion [27]. The advantage of a one-stage positive selection for OMLC has been alluded to above. Use of Detachabeads to detach the magnetic beads from cell surfaces resulted in a loss of 50% of the OMLC yield. Therefore, DR$^+$ cells were added to culture with beads still attached. Despite concern that the presence of membrane-bound beads may compromise the function of OMLC *in vitro*, we found no evidence to suggest this and our data support those of Hanau *et al.* [26] using human epidermal LC, which retained alloactivating capacity whilst still bound to magnetic beads. Furthermore, the presence of beads added in solution to cultures had no demonstrable non-specific effects on the magnitude of T cell responses to Con A.

Further work is needed to establish whether OMLC also possess allo- and exogenous antigen-presenting capacity, as has been shown with epidermal LC [11]. The method we have used here to demonstrate the capacity of OMLC to function as AC can be applied to these issues, offering the opportunity to study the functional role of OMLC in the protective immunity of the oral cavity, and in inflammatory and neoplastic oral diseases possibly mediated by OMLC.

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