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Dendritic cells from human tissues express receptors for the immunoregulatory vitamin D_3 metabolite, dihydroxycholecalciferol

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SUMMARY

Dendritic cells have been isolated from human tonsillar tissue and shown to act as accessory cells in a mitogenic response. The dendritic cells will induce receptors for the active metabolite of vitamin D_3, $1,25(OH)_2D_3$, in the responder E^+ T cells. The dendritic cells themselves constitutively express receptors for the metabolite, and this distinguishes them from other non-T cells in lymphomedullary tissue. Expression of the $1,25(OH)_2D_3$ receptor may be a dendritic cell property that facilitates their accessory cell role within the tissue microenvironment.

INTRODUCTION

The isolation of dendritic cells from murine tissues (Steinman, Adams & Cohn, 1973) has led to several studies that emphasize the significance of these cells in the induction phase of the immune response. For example, they are recognized as a site of genetic restriction (Sunshine, Katz & Feldmann, 1980); they are stimulators of both proliferative and cytotoxic responses (Czitrom, Katz, & Sunshine, 1982); T-cell hybridomas (Sunshine *et al.*, 1983) and antigen-specific cell lines and clones (Katz *et al.*, 1986) will proliferate when these cells are used as inducer accessory cells; and their effect is demonstrable in the primary initiation of T-cell help (Inaba *et al.*, 1983), possibly in association with T-cell clustering (Inaba, Witmer & Steinman, 1984).

With regard to human studies, a similar cell has been isolated from the peripheral blood (Van Voorhis *et al.*, 1982) and from synovial fluid (Tyndall *et al.*, 1983), but dendritic cells from solid tissues have not been investigated in an analagous fashion. However, the proposed role of the dendritic cell in disease states (Zvaifler *et al.*, 1985) has emphasized the potential significance of these cells in the local microenvironments in which immune responses take place.

In this study we report recent experiments in our laboratory in which we have used a cell separation protocol for human tonsil, which is modified from the protocol used previously, to isolate the murine dendritic cell. This method has allowed us to identify a tonsillar cell population that has *in vitro* properties resembling those associated with the murine dendritic cell, and to examine the interaction of these cells with autologous T cells so as to confirm that they will act as stimulators of accessory cell-dependent mitogen responses. Furthermore, in view of recent findings in several laboratories (Bhalla *et al.*, 1984; Nunn *et al.*, 1986) that the active metabolite of vitamin D_3, 1,25 $(OH)_2D_3$, may play a role in lymphomedullary homeostasis, we have also investigated the dendritic cell–T cell interaction to see whether the metabolite is implicated in this phase of immune response induction.

MATERIALS AND METHODS

Materials

The 5-[^{125}I]-iodo-2-deoxyuridine (^{125}IUdR) and 1,25 [26,27-^{3}H]-$(OH)_2D_3$ were obtained from Amersham International, Amersham, Bucks; ethanol, sucrose, Triton and 2-mercaptoethanol were from BDH, Poole, Dorset; IgG rabbit anti-sheep red cell antibody was from Cappell Labs, Cochranville, PA; rabbit anti-mouse immunoglobulin antibody pre-adsorbed against human cells from Dako Ltd, Stockholm, Sweden; amphotericin, calcium and magnesium free-Hanks' solution, fetal calf serum, L-glutamine, HEPES, penicillin, RPMI, streptomycin were from Gibco Ltd, Paisley, Renfrewshire; concanavalin A (Con A) and Path-O-Cyte 4 were from Miles Labs, Slough, Berks; Tan 199 was from Packard, Lombard, IL; collagenase, (type II), and phytohaemagglutinin (PHA) were from Sigma, Slough, Berks; and sheep red cells were from Tissue Culture Services. Bovine

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Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; Con A, concanavalin A; HD, high density cells from albumin gradient; ID, intermediate density cells from albumin gradient; ^{125}IUdR, 5-[^{125}I]-iodo-2-deoxyuridine; LD, low density cells from albumin gradient; PHA, phytohaemagglutinin.

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red blood cells were supplied by Dr J. Herbert, AFRC Institute of Animal Physiology, Babraham, Cambridge. Monoclonal antibodies used were 3Ac5 and 2H7 (anti-pan-B cell, gift of Dr J. Ledbetter, Genetic Systems Corp., Seattle, WA); Hig78, DA6.231 and DA6.164 (anti-HLA-D region, gift of Drs Guy and Van Huyningen, MRC Clinical and Population Cytogenetics Unit, Edinburgh); and UCHT1 (anti-CD3, gift of Dr P. Beverley, ICRF Human Tumour Immunology Unit, University College, London).

Cell separation

The starting population for the isolation of human tissue cell populations consisted of a digest from human tonsil. Approximately 100 operative specimens were used in the different stages of these experiments. The tissue digest was prepared by incubating the tissue fragments in Collagenase II at a final concentration of 1 mg/ml in calcium- and magnesium-free Hanks' solution for 1 hr at 37°. This was followed by centrifugation on a discontinuous bovine serum albumin gradient (8000 *g*, 0°, 30 min). This procedure resulted in three different cell populations separable by density: low (separating at less than 20% albumin density: LD), intermediate (separating between 23% and 26% albumin density: ID), and high density (separating at greater than 26% albumin density: HD).

Dendritic cell isolation

The LD fraction was incubated overnight at 37° in RPMI, supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 IU), streptomycin (100 mg/ml), amphotericin (2·5 mg/ml), 10 mM HEPES, 0·05 mM 2-mercaptoethanol and 2 mM L-glutamine.

The LD non-adherent cell population was used for further purification of the dendritic cells. The first step was to deplete the population of T cells by rosetting with sheep red cells (E). This resulted in separation into E^+ T cell and E^- non-T cell non-adherent LD populations. In order to purify the dendritic cell population further, the whole E^- cells were preincubated with the monoclonal antibody, 3AC5, directed against a determinant on human B cells (Ledbetter, Martin & Clark, 1985). After incubating for 1 hr in the presence of the antibody, the cells were rosetted with ox red cells that had been coupled to a rabbit anti-mouse immunoglobulin antibody pre-adsorbed against human cells and separated on a Ficoll–Hypaque gradient. As a control for the Ficoll–Hypaque separation, cells were also examined directly in a haemocytometer using methyl violet staining.

The non-T non-B cell population from the interface of the rosetting was incubated at 37° with immune complexes (sheep red cells coated with a rabbit anti-sheep red cell IgG antibody and re-rosetted. This procedure led to the isolation of cells that were non-T, non-B, non-adherent and that either expressed or did not express Fc receptors. The Fc receptor-bearing cells constitute a macrophage-like population but not adherent. The Fc^- cells represent a relatively small fraction (less than 5%) of the total LD cells. Their isolation characteristics suggest that they are homologous with the dendritic cells as previously identified in murine systems (Sunshine *et al.*, 1980). Re-incubation of the LD, non-adherent, non-T, non-B, Fc^- dendritic cell for 1 hr with an anti-HLA-D region, an anti-CD3 antibody, or an anti-B cell antibody at 37° and re-rosetting with the chromic chloride anti-mouse immunoglobulin/red cell complex confirmed their non-T, non-B cell nature and showed that 80% of the cells express HLA-D region antigens on their surface.

T cells

From the three density-defined non-adherent subpopulations (cultured in the same medium as above), the first step in DC purification included an E-rosetting procedure. In order to confirm the T-cell nature of these E^+ cells, they were examined by the chromic chloride coupled ox red cell rosetting method as outlined above, using the CD3 anti-T cell monoclonal antibody. Seventy to eighty per cent of the cells expressed this marker.

Cell proliferation

Cell proliferation assays were performed in RPMI supplemented as outlined above. All cultures were performed in triplicate in 0·2 ml volume in a humidified 5% CO_2 atmosphere at 37°. As a standard positive control an optimum concentration of PHA (0·001 mg/ml) was used. Six hours before harvesting, the cells were pulsed with ^{125}IUdR (5 Ci/mg) and the subsequent incorporation of the radiolabel into macromolecular DNA measured. Results were calculated as the mean c.p.m. $\times 10^{-3}$ of each triplicate plus standard deviation.

In order to evaluate accessory cell function in the induction of T-cell proliferation, the dendritic cells isolated as outlined above were pulsed with an optimum concentration of Con A (0·0225 mg/ml) for 30 min at 37°. The cells were washed three times and resuspended at 10^6 cell per ml. These stimulator cells were irradiated (1200 rads using a calibrated 250 kV Maximar General Electric source). Controls included dendritic cells pulsed with Con A in the presence of alpha-methyl mannoside (0·2 mg/ml) and responder E^+ T cells pulsed in the same fashion. Different concentrations of pulsed stimulator cells were incubated with 10^5 T cells for 96 hr. Proliferative responses were measured (as for the PHA response) by ^{125}IUdR incorporation during the last 6 hr of culture.

Vitamin D_3 metabolites

The active metabolite of vitamin D_3, $1,25(OH)_2D_3$, was used at 10^{-7} M concentration. Previous studies have shown that this concentration inhibits T-cell proliferation, but other D_3 metabolites not hydroxylated in the carbon 1 position (e.g. 25-OH D_3; $24,25(OH)_2D_3$) have no detectable effect on T-cell proliferation at concentrations less than 10^{-6} M and produce a 70% fall in cell viability if used at a concentration of 10^{-5} M. The $1,25(OH)_2D_3$ was dissolved in a final concentration of 0·01% ethanol, and at this concentration ethanol had no effect on either cell viability or proliferation. The concentration of active metabolite present in the serum-supplemented medium was less than 10^{-12} M (Nunn *et al.*, 1986).

$1,25(OH)_2D_3$ receptor

The $1,25(OH)_2D_3$ receptor was measured by a whole cell nuclear association assay. Cell suspensions were incubated for 60 min at 37° with increasing amounts (0·125–2·5 nM) of $1,25[26,27\text{-}^3H]\text{-}(OH)_2D_3$ (specific radioactivity 180 Ci/mol) in serum-free medium and in the presence or absence of a 300-fold excess of cold $1,25(OH)_2D_3$. The cells were then harvested by centrifugation (500 *g* for 2 min) and washed three times in phosphate-buffered saline (PBS). The nuclei were isolated by incubation of the cells for 5 min in a lysis buffer containing 0·25 M sucrose and 1% Triton X-100, followed by centrifugation at 1000 *g* for 5

min. Nuclear pellets were resuspended in 0·2 ml of PBS and 1·0 ml of 95% ethanol, transferred to a scintillation vial, and 8 ml of solubilizing fluor added. The radioactivity was measured by counting for 5 min each in a liquid scintillation spectrophotometer. The affinity (Kd) and number of the receptors were calculated by Scatchard analysis of the specific binding of the tritiated hormone.

RESULTS

The results of a representative experiment using a pulsed dendritic cell population as stimulator for 72-hr ID E^+ T-cell proliferation are shown in Fig. 1. Dendritic cells are effective as

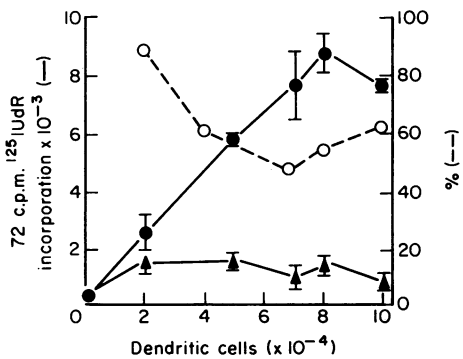

Figure 1. Dendritic cells were isolated from human tonsillar tissues, as outlined in the text. Likewise autologous E^+ T cells were obtained from the ID layer of the same gradient. The dendritic cells were pulsed *in vitro* with Con A (30 min, 0·0225 mg/ml, 37°) and different cell concentrations were used as stimulators for 10^5 T cells in a 72-hr proliferation assay (●—●). Unpulsed dendritic cells were used as a control in this experiment (▲—▲). Six hours before harvesting, 5 Ci/ml ^{125}IUdR were added to the cultures. The results are expressed as incorporation of radiolabel + SD. In parallel the mitogen-pulsed dendritic cells/ID E^+ cells were cultured in the presence of supplementary $1,25(OH)_2D_3$. The 72-hr proliferative response at each stimulator cell concentration without $1,25(OH)_2D_3$ was taken as 100% and the results are expressed as the percentage of the 72-hr proliferation that occurs when the metabolite is included in the culture (o- -o).

mitogen inducers, and this effect is dependent on the number of dendritic cells present in the culture. Similar results have been obtained using HD E^+ cells as responders, but LD E^+ responder cells give a high autologous proliferative reaction (Zvaifler *et al.*, 1985) in the absence of added stimulator cells and a high autologous reaction, even without added mitogen, so these T cells were excluded from the subsequent studies.

In view of previous studies of T-cell mitogenesis, which have shown that activated T cells are susceptible to the inhibitory effect of $1,25(OH)_2D_3$, we used dendritic cells as stimulators to see if a similar effect would be seen in an accessory cell dependent response. Figure 1 shows further that in the same experiment 10^{-7} M $1,25(OH)_2D_3$ inhibits proliferation of T cells when the dendritic cell is used as an inducer cell. This inhibition was $1,25(OH)_2D_3$ dose-dependent in similar fashion to other $1,25(OH)_2D_3$ inhibition systems (data not shown), the degree of inhibition was significant at each concentration.

Figure 2 shows another representative experiment illustrating the use of dendritic cells as inducer cells, but examines the T-cell nuclear receptor expression for $1,25(OH)_2D_3$ rather than

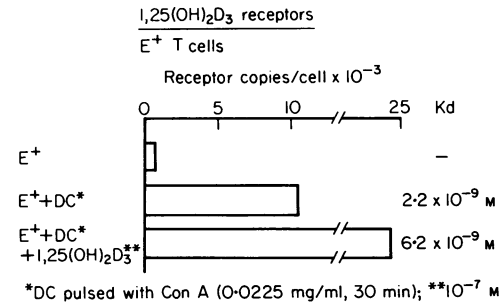

Figure 2. E^+ T cells, T cells plus Con A-pulsed dendritic cells (prepared as outlined in the text), and T cells plus Con A-pulsed dendritic cells plus $1,25(OH)_2D_3$ were incubated in parallel for 72 hr. The cells were harvested, and 5×10^6 cells from each population were examined for receptor expression by a whole cell nuclear association assay as outlined in the text. Results are expressed as the number of receptor copies per cell, and by relative affinity.

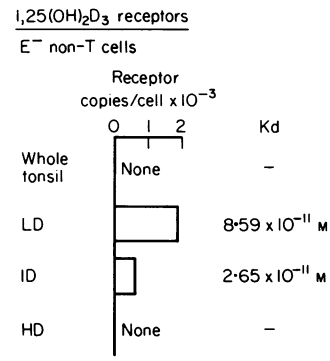

Figure 3. E^- non-T cells from whole tonsil and from all three density layers were isolated as outlined in the text. Cells were harvested and 5×10^6 cells from each population were examined for $1,25(OH)_2D_3$ receptor by a whole cell nuclear association assay as outlined in the text. Results are expressed as number of receptor copies per cell and by relative affinity.

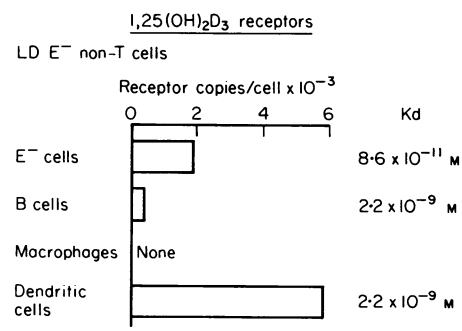

Figure 4. Dendritic cells, B cells and macrophages from the LD E^- non-T population were isolated as outlined in the text. Cells were harvested and 5×10^6 cells from each population were examined for $1,25(OH)_2D_3$ receptor expression by a whole cell nuclear association assay as outlined in the text. Results are expressed as the number of receptor copies per cell and by relative affinity.

proliferation. The dendritic cell/mitogen complex acts as an effective stimulus for a significant induction of nuclear receptors for $1,25(OH)_2D_3$ in the T-cell population. Figure 2 shows further that if the proliferative signal is given in the presence of the metabolite itself, then there is a further significant increase in the number of receptor copies per cell compared to when the mitogenic signal is given alone. The receptor affinity remains unchanged.

As controls for investigation of this receptor status in E^+ T cells, we used the E^- from a whole tonsil cell suspension and from all three density layers. The results of a representative experiment (Fig. 3) show that in freshly isolated tonsillar cells the LD E^- expresses significantly more receptor copies per cell for $1,25(OH)_2D_3$ than the other fractions. Thus, endogenous $1,25(OH)_2D_3$ receptor expression in the human lymphomedullary tissues is a property of a non-T cell population distinguishable from other non-T cell populations by density.

In order to clarify the phenotype of the receptor-bearing cell further, we separated the low density E^- non-T cells into subpopulations of B cells, macrophages, and dendritic cells as outlined above. The results of receptor analysis for these LD E^- subpopulations are shown in Fig. 4. There are significantly more receptor copies per cell on the dendritic cells compared to either the macrophage or the B cell.

DISCUSSION

The isolation of a non-T, non-B, and non-phagocytic subpopulation of cells from within solid murine lymphomedullary tissues has been followed by several studies that evaluate the immunoregulatory role of these cells. In human studies similar dendritic cells have been characterized previously from peripheral blood samples and synovial fluid, rather than from organs such as spleen, lymph node and tonsil. The experiments reported here confirm the hypothesis that there are cells with the properties of dendritic cells present in the human tissue samples and that these cells play a functional role as inducer cells in the immune response, both in terms of T-cell proliferation and in induction of a nuclear receptor known to be associated with T-cell activation.

However, the previous observations concerning the dendritic cell and its accessory cell role do not clarify the mechanism involved in the activity of these cells. The only positive, universally accepted, correlate of dendritic cell function is the presence of abundant class II major histocompatibility complex antigens on their surface. Some recent studies give evidence for a non-lysosomal pathway to operate in the dendritic cell and suggest that this may operate either via an interiorization, or via a membrane enzyme intermediate step (Chain, Kaye & Feldmann, 1986), but no other positive associations have been reported.

In this study we have identified receptors for $1,25(OH)_2D_3$ in the dendritic cell. Since this metabolite is known to be an inhibitor of the T-cell proliferative response, the identification of the receptor on the dendritic cells not only provides a contrast with other potential accessory cells from the low density fraction of human tissues such as B cells and macrophages, but also raises an additional mechanism that might be implicated in dendritic cell function.

One aspect of this is that the dendritic cells that express receptors for $1,25(OH)_2D_3$ are in turn particularly potent inducers of $1,25(OH)_2D_3$-receptor expression on the responder T cells. Although it is impossible to exclude a contribution by the dendritic cells to the very high binding seen in Fig. 4, as reflected in the number of receptor copies per cell, the increase is so striking as to make this alternative inherently implausible. Unfortunately, the perfect control for this type of experiment—i.e. maintenance of the dendritic cells alone for 4 days, and maintenance of the dendritic cells alone but with $1,25(OH)_2D_3$ for 4 days—is technically not possible in our system. To do this successfully would require at least 10^7 viable dendritic cells after 4 days in culture for these control parts of the receptor assays; thus far we have never succeeded in isolating more than 2×10^7 total dendritic cells from an individual tonsil. We are currently investigating the development of a single cell receptor assay, which should permit us to answer this question definitively in the future.

In lymphomedullary tissues the normal site of $1,25(OH)_2D_3$ synthesis is the monocyte/macrophage. These cells have been implicated in inhibitory aspects of immunity previously mediated either via prostaglandin synthesis (Goodwin & Webb, 1980) or via reactive oxygen intermediates (Johnstone *et al.*, 1983). The $1,25(OH)_2D_3$ pathway represents another way for these cells to act as inhibitors, in this instance of activated T cells rather than of the activation itself.

If the immune response was confined to an interaction between a cell that synthesizes an inhibitory metabolite and one that expresses a receptor for, and is inhibited by, the same metabolite, then this would impose marked limitations on the local expansion of the T-cell pool. We suggest that it is in this local microenvironmental context that the dendritic cell receptor for the metabolite may play a role. Thus, during the induction phase of the immune responses in the tissues, if there is a cell type present that not only expresses HLA-D region surface markers and binds antigen but also has nuclear receptors for a macrophage-derived inhibitor, then that cell is likely to be a potent inducer. Once induction has occurred, then the T cells will express autocrine inhibitory receptors, which limit further T-cell expansion.

An example of a potential microenvironment in which dendritic cells might act in this fashion is in the pathogenesis of granulomatous diseases, where secretory macrophages have been shown to be active in producing the inhibitory metabolite (Adams & Gacad, 1985). The presence of a dendritic cell in the T-cell infiltrate surrounding an epithelioid cluster might act as an alternative binding site for the inhibitor and thus serve act to maintain a T-cell response as a reciprocal to the self-inhibitory pathway in the T cells themselves.

There are two further aspects of dendritic cell biology that these observations highlight. Firstly, this proposed mechanism of dendritic cell function implies that dendritic cells will respond to extrinsic signals and that they are able to interiorize these signals. This observation highlights the fact that analysis of the cellular induction mechanisms in immunity should include consideration of intracellular pathways that abrogate negative signals, as well as well-established positive mechanisms such as surface class II major histocompatibility complex expression and macrophage lysosomal enzyme degradation of antigen.

Secondly, it is interesting that $1,25(OH)_2D_3$ receptor expression has a negative association with lymphocytic proliferative responses. The association between receptor expression and

sensitivity to $1,25(OH)_2D_3$ in the myelomonocytic system has been examined previously only in tumour cell lines (Reitsma *et al.*, 1983). In the normal mononuclear phagocyte system the relationship between the dendritic cell's apparent constitutive receptor expression and the observation that these cells do not proliferate may be significant. As for the T cells, so for the normal mononuclear phagocyte system the presence of $1,25(OH)_2D_3$ receptor may be associated with a differentiation (or activation) phase in which decreased proliferative capacity is a feature.

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