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Differentiation of Human Monocytes in Vitro Following Exposure to Canova in the Absence of Cytokines

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ABSTRACT Canova is an immunomodulatory, homeopathic preparation that has been shown to activate macrophages in vitro and in vivo, with resultant enhanced spreading of the cells and formation of microvillus extensions from the cell body. Since monocytes are the precursor cells of macrophages and dendritic cells, the objective of the current study was to investigate the effects of Canova on the differentiation of human blood monocytes in vitro. Monocytes were isolated, grown in culture, and exposed to 10 and 20% Canova without the addition of cytokines. After 48 h, monocytes were prepared for analysis by scanning electron microscopy, while cells kept in culture for 7 days and exposed to Canova on days 1, 3, and 4 were analyzed by flow cytometry for alterations in the levels of expression of CD1a, CD11c, CD14, CD80, CD83, CD86, and HLA-DR. SEM revealed that monocytes exposed to 10% Canova had a morphological appearance similar to that of macrophages. Various cytoplasmic projections were observed with pseudopodia formation. Flow cytometric analysis after exposure of monocytes to 10 and 20% Canova indicated high cell viability and upregulation of CD80, compatible with differentiation into either macrophages or dendritic cells. Exposure to Canova per se causes activation of monocytes with resultant differentiation into large macrophage-like cells of indeterminate phenotype that have increased expression of CD80. Like cytokines, Canova induces differentiation of monocytes, an activity that may underpin the immunomodulatory activity of this product.

KEYWORDS human monocytes, macrophages, dendritic cells, Canova, cytokines

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Immunomodulators are defined as agents that enhance the immunity of an individual to favor a particular immunological response [1]. Recently, several studies have reported that products of natural origin are effective in curing diseases and maintaining the resistance to infections of organisms [2]. Canova is such an immunomodulator of natural origin that stimulates host

defenses against several pathological states [3]. Canova was developed in the Canova laboratory in Argentina and today it is produced as a homeopathic medicine in Brazil [4], according to the Hanemannian homeopathic method. The final product is an aqueous, odorless, and colorless solution that contains *Thuya occidentalis* (Cupressaceae), *Bryonia alba* (Curcubitaceae), *Aconitum napellus* (Ranunculaceae), *Arsenicum* album (arsenic trioxide), and *Lachesis muta* (Veripidae) [5]. It is neither toxic nor mutagenic, is available as drops, and is used by researchers in a wide concentration range from 4 to 50%.

Researchers have shown that Canova affects macrophages in vitro and in vivo [3]. During a typical immune response, macrophages act as coordinators, by presenting antigens and secreting cytokines [6]. Macrophage activation involves an increase in the metabolic state, mobility, and phagocytic activity of the cell [1]. Typical morphology of activated stage macrophages, such as enhanced spreading and numerous cellular projections, was observed in isolated macrophages treated with Canova [5], while administration of Canova was associated with 86% of macrophage activation in comparison with 15% in the control group [1]. A large nucleus with spreading and many microvilli or projections was regarded as morphologically characteristic of activated macrophages when evaluated by SEM and confocal microscopy [1].

Monocytes represent a large population of circulating precursor cells that can differentiate into macrophages or dendritic cells. Typically, dendritic cell morphology is very similar to that of macrophages. These cells appear large with an irregular shape, and cytoplasmic (dendritic) processes can be seen extending from the cell body [7]. As cells mature, e.g., when macrophages and dendritic cells are formed from monocytes, these cells will increase their cell surface expression of adhesion and costimulatory molecules, such as CD80 and CD86 [8].

Although various researchers have shown that macrophages are activated by Canova [1, 5], the effects of this agent on the differentiation of monocytes into antigen-presenting cells or phagocytic cells have not been addressed in previous studies. The aim of this study was therefore to determine whether the immunomodulator Canova causes monocyte differentiation in vitro in the absence of cytokines. SEM techniques were used to investigate the morphological changes that occur in human monocytes after exposure to Canova in vitro, while flow cytometric analysis of monocyte, macrophage, and dendritic cell differentiation markers was used to determine the effects of Canova on monocyte maturation into macrophages or dendritic cells.

MATERIALS AND METHODS

Isolation, Culturing and Exposure of Monocytes to Canova

Peripheral blood was obtained with the informed consent of healthy, adult human volunteers. Peripheral blood mononuclear cells (PBMC) were separated by standard Ficoll-Histopaque (1077, Sigma Diagnostics) density gradient centrifugation as described previously by [9], with some modifications. Briefly, the PBMC were washed in 0.15 M phosphate-buffered saline (PBS, pH 7.4) with 100 μM ethylene glycol tetra-acetic acid (EGTA, pH 7.4) by centrifugation. The supernatant fluid was removed and ice-cold 0.83% ammonium chloride added to the cell pellet to lyse the remaining red blood cells. Following centrifugation, the PBMC were washed again in PBS and the pellet was resuspended in AIM V medium with L-glutamine, 50 μg/mL streptomycin sulfate, 10 μg/mL gentamicin sulfate, and 0.25% human serum albumin (Gibco, The Scientific Group, South Africa).

Monocytes were enumerated using an EPICS XL-MCL Coulter flow cytometer, and plated out in a 6-well plate (CELLSTAR, Greiner bio-one, Lasec, South Africa) at 2×10^6 per 3 mL AIM V medium. Cells were incubated in culture plates for 2 h at 37°C with 5% CO_2, after which nonadherent cells were decanted as described by Romani et al. [10]. Control cells received medium only, while the treatment groups were exposed to concentrations of either 10 or 20% Canova (in the absence of added cytokines) and incubated for 2–7 days as described below.

Scanning Electron Microscopy (SEM)

For SEM studies, the monocytes were plated on plastic stubs in the 6-well plate. Only control groups and groups exposed to 10% Canova were evaluated.

The monocytes were exposed to 10% Canova after the 2-h period of adherence and again after 24 h. The control group received AIM V medium only as treatment. The cells were incubated for another 24 h, after which they were fixed with 2.5% glutaraldehyde in Dulbecco's phosphate-buffered saline (DPBS) buffer with a pH of 7.4 for 1 h and prepared for SEM. The stubs with cells were washed in DPBS and postfixed in osmium tetraoxide (OsO_4) for 20 min. The stubs were washed again with DPBS, serially dehydrated and placed in a critical point dryer. The stubs with monocytes were coated with ruthenium, viewed, and photographed with a JOEL 6000F FEGSEM field emission scanning electron microscope (Tokyo, Japan). This experiment was repeated 3 times.

Flow Cytometry

All flow cytometric analyses were performed on an EPICS XL-MCL flow cytometer (Coulter Immunotech, Beckman Coulter). The instrument was set up to measure forward scatter (FS) and sideways (SS) light scatter, which are parameters of size and granularity of particles, respectively. An unstained sample was used to adjust the forward scatter amplification during analysis. As macrophages/dendritic cells were expected to display a high forward scatter, the amplification used normally for detection of lymphocytes had to be reduced to allow all particles with high forward scatter properties to be distributed in the upper half of the FS vs. SS diagram (Figure 1). Cells were stained using a direct method. A volume of 100 μL of cells (2×10^3 cells/mL) was incubated for 10–15 min at room temperature with 10 μL of each monoclonal antibody (mAb). The following phycoerythrin (PE)-conjugated mouse IgG1 MAbs were used: anti-CD11c (Immunotech, Beckman Coulter, France), -CD80, -CD83, and -CD86 (all from BD Pharmingen, BD Biosciences, France), while IgG2a and IgG1 PE-cyanin 5-conjugated mAbs were used to detect CD1a and CD14, respectively (Immunotech), and an IgG2a-FITC conjugate was used to detect HLA-DR (BD Biosciences). Appropriate isotypic controls were used at the same protein concentration as the test antibody to define the cutoff, which had to be less than 1%. After incubation, the cells were suspended in 0.5 mL DPBS and were ready for analysis. These experiments were performed using cells from 6 different donors.

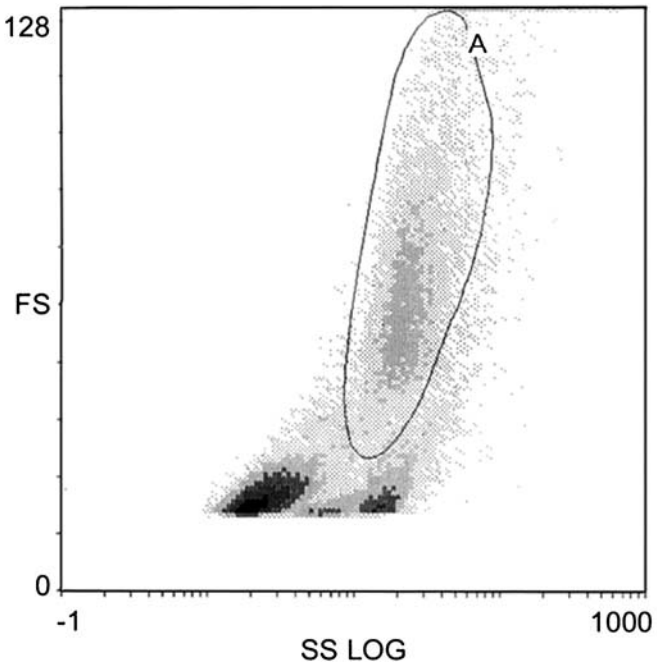

FIGURE 1 **Scattergram illustrating the high forward scatter properties of the monocytes cultured for 7 days in the upper half of the forward scatter versus side scatter axes.**

CD11c (CR4, p150,95) is a β_2-integrin expressed mainly on monocytes, macrophages, and natural killer cells, and to a lesser extent by granulocytes, dendritic cells, and some subsets of T- and B cells [11]. CD14 is expressed predominantly on macrophages, and CD1a on dendritic cells, while HLA-DR is expressed by antigen-presenting cells. CD83 is predominantly expressed by dendritic lineage cells, including Langerhans cells, skin, and peripheral blood dendritic cells [12]. CD83 is also expressed by dendritic cells generated in vitro, but is not expressed by resting peripheral blood leukocytes at detectable levels [13], while CD80 and CD86 are costimulatory molecules expressed on antigen-presenting cells.

Flow cytometric measurement of uptake of propidium iodide was used to determine the viability of control and Canova-exposed monocytes.

Presentation and Analysis of Results

The results of the SEM experiments are presented as representative electron micrographs, while data derived from flow cytometric experiments are presented either in histogram format, or as the mean values ± standard deviation (SD). Statistical analyses were performed using Microsoft Excel–Analysis

ToolPak. All results were analyzed by VBA functions for Analysis ToolPak (Microsoft Office Excel 2007).

RESULTS AND DISCUSSION

Following differentiation, monocytes either develop into macrophages or become dendritic cells [14]. Usually, however, various cytokines are needed before such a transformation can take place [15, 16]. In this study, monocytes received only Canova as a potential activator, which was added to the culture medium; no cytokines were added to induce differentiation.

SEM

Figure 2A indicates a typical monocyte isolated from peripheral blood. Although monocytes were allowed to grow in culture for 48 h, this control monocyte still has a typical monocyte appearance, showing a rounded cell with ruffled membranes and a cytoplasmic veil. Other typical features after 2–3 days growth in culture include some microvillus projections (white arrow) with ruffles and blebs of cell membrane close to the cell body (black arrow) [17, 18].

However, after exposure to 10% Canova for 48 h, the cells had the same appearance as long-term cultured monocytes that differentiated into macrophages [18]. Figure 2B and C are micrographs of monocytes that had been exposed to 10% Canova for 48 h. Flattened cells with micro-extensions (small white arrows) and polarization of the cytoplasm were observed (black arrow), while long cytoplasmic processes extending from one pole of the cell were evident (large white arrow). This is yet another common feature of long-term macrophage-derived monocytes. These projections from the cell body are compatible with the formation of pseudopodia, which assist in the antigen-presenting characteristics of the differentiated dendritic cells/macrophages. A cytoplasmic veil similar to that seen in control cells was also observed (black square). All of these observations indicate that exposure of human monocytes in culture to 10% Canova for 48 h induces the same morphology as that of monocytes that develop into macrophages following prolonged culture in vitro. Monocytes usually develop this type of dendritic/macrophage morphology only in the presence of cytokines such as GM-CSF, IL-4, IL-1β, and TNF-α, or, alternatively, prostaglandin E_2, after

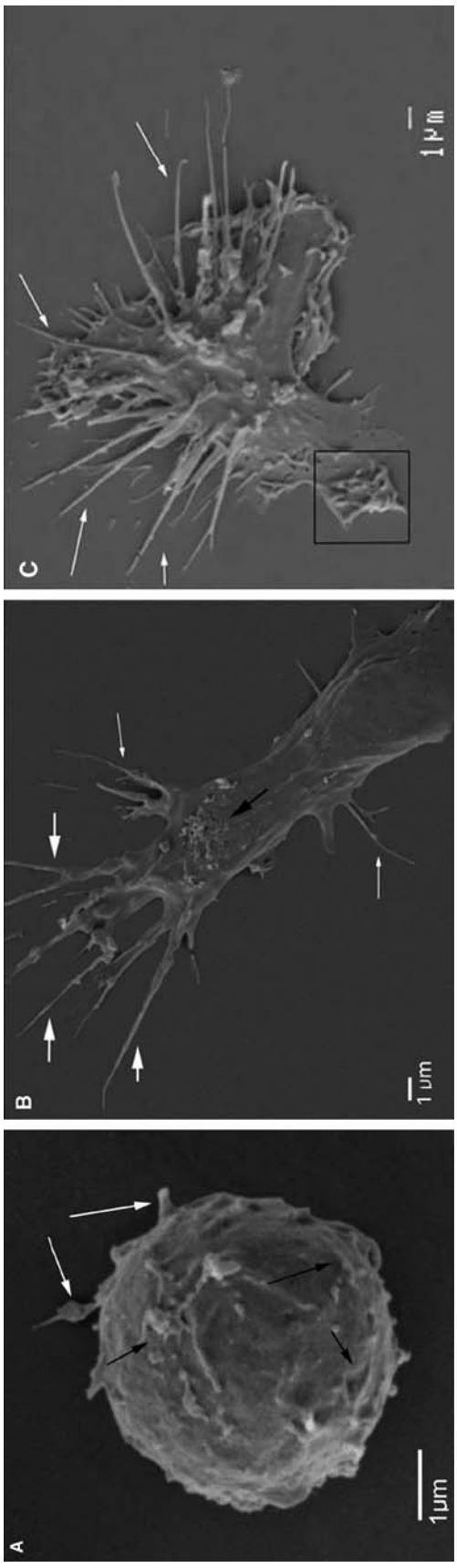

FIGURE 2 (A) SEM micrograph of a control monocyte cultured for 48 h without any cytokines. Some typical monocyte features can be seen, namely: a few microvillous projections (white arrow) with ruffles and blebs of the cell membrane close to the cell body (black arrows). In general, the cell maintained the shape and characteristics found in monocytes cultured for 1–3 days. (B, C) Micrographs of monocytes grown in culture for 48 h and exposed to 10% Canova without cytokines. Numerous microextensions (small white arrows) could be seen with polarization of the cytoplasm (black arrow). Long cytoplasmic processes extending from one pole of the cell were evident (large white arrow). A cytoplasmic veil similar to that seen in the control could also be observed (black square). All these features indicate differentiation of monocytes.

TABLE 1 **The Expression Levels of CD1a, CD11c, CD14, CD80, CD83, CD86, and HLA-DR on Control and Canova-treated Monocytes**

	Percent antigen expressed		
	Control	10% Canova	20% Canova
CD11c	96.33	84.47	81.6
CD83	9.07	9.16	12.08
CD80[a]	13.62	30.51	50.76
CD86	64.64	60.6	68.04
HLA DR	97.5	97.44	95.37
CD14	86.76	75.19	77.07
CD1a	62.76	55.05	43.6

[a]The only cell surface marker that indicated significantly increased levels of expression.

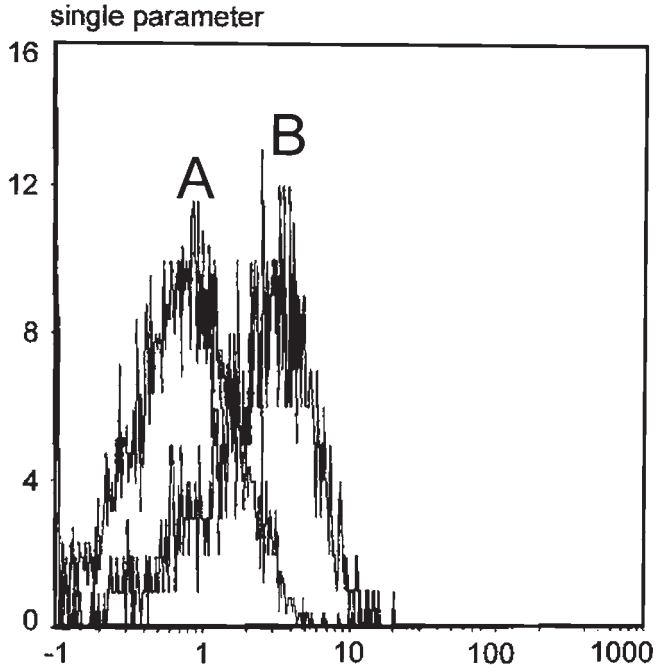

FIGURE 3 **A representative histogram showing the levels of expression of CD80 on (A) control and (B) Canova-treated cells (%).**

5 days of culture [19, 20]. Canova, therefore, has a similar effect to that of cytokines, inducing monocyte differentiation.

Flow Cytometry

Flow cytometric analysis of surface markers of cellular differentiation was used to establish the identity (macrophage/dendritic cells) of the Canova-transformed monocytes. The expression levels of CD1a, CD11c, CD14, CD80, CD83, CD86, and HLA-DR on control and Canova-treated monocytes are shown in Table 1, while one representative histogram depicting expression of CD80 following the 7-day incubation period is shown in Figure 3. The only surface marker that was altered by exposure to Canova was the costimulatory molecule CD80, the expression of which was significantly ($p < .05$) increased. The levels of expression of CD11c and CD14 decreased slightly following exposure of monocytes to Canova, while expression of CD83 was slightly increased. Although compatible with transformation to a dendritic cell phenotype, these differences did not achieve statistical significance. Importantly, exposure of monocytes to Canova for up to 7 days was not associated with alterations in cell viability. To determine cell viability propidium iodide (PI) was used. PI diffuses across plasma membranes that have lost their integrity, enters the cell, and binds DNA. It is therefore an indicator of nonviable cells, when it exhibits fluorescence [21], which was measured by flow cytometry. All the cells were always at a viability of 75% or above.

As observed in the current study, unstimulated monocytes have been reported to exhibit relatively low and high levels of expression of CD80 and CD86, respectively [8]. However, because CD80 is expressed on both dendritic cells and macrophages, the observed upregulation of expression of this costimulatory molecule on Canova-activated monocytes, although compatible with cellular activation and differentiation, does not, in isolation, enable definitive identification of the differentiated monocyte, i.e., macrophage or dendritic cell. In the case of macrophages, as may also be the case with dendritic cells, a spectrum of differentiation exists with the M1 and M2 phenotypes at either extreme [22], corresponding to their TH1 and TH2 counterparts. The Canova-activated, monocyte-derived cell may therefore represent an incompletely differentiated macrophage/dendritic cell, the identity of which will require gene expression profiling [22].

CONCLUSION

Exposure of monocytes to Canova results in cellular activation/differentiation with resultant generation of a cell type that is morphologically similar to macrophages with upregulated expression of CD80, but of indeterminate phenotype. Although these effects of Canova on monocyte activation/differentiation are novel and may underpin the

immunomodulatory properties of this agent, the identity of the Canova-activated monocyte and its functions remain to be established.

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