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Calcitonin Gene-Related Peptide Decreases Expression of HLA-DR and CD86 by Human Dendritic Cells and Dampens Dendritic Cell-Driven T Cell-Proliferative Responses Via the Type I Calcitonin Gene-Related Peptide Receptor

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These studies were performed to establish whether functional receptors for calcitonin gene-related peptide (CGRP) are present on human dendritic cells (DCs) and to investigate potential immunomodulatory effects of CGRP on DCs other than Langerhans cells.

Neuropeptides, including calcitonin gene-related peptide (CGRP), can regulate immunity (1–3). CGRP in addition dilates cerebral, corneal, and peripheral vessels; regulates glucose metabolism; exhibits potent cardiotoxic effects; and may also play a role in migraine-associated neurogenic inflammation due to its localization in the trigeminal ganglia (4). CGRP interacts with specific G-protein-coupled receptors and is synthesized and released by capsaicin-sensitive sensory nerves (4). Langerhans cells (LCs) are bone marrow-derived epidermal cells that are committed to mature into dendritic cells (DCs) (5). Hosoi et al. (6, 16) demonstrated modulation of epidermal LCs by nerves that secrete CGRP. Torii et al. (7) described CGRP-mediated augmentation of LPS-induced IL-10 mRNA and suppression of LPS and GM-CSF-induced IL-12 mRNA in macrophages and the epidermal LC-like cell line XS52.

Although neuropeptides can influence many cell types in the immune system including LCs, their effects on human blood-derived DCs have not been extensively characterized. These professional APCs express the two signals (8) required by T cells: one signal consisting of processed Ag presented on MHC class I or II; and second signals for T cell binding and activation via such molecules as ICAM-1 (CD54), LFAs (CD2, CD11a, CD58), and B7s (CD80, CD86). DCs derived from monocytes exposed to IL-4 and GM-CSF express MHC class II and CD86 but revert to a macrophage phenotype if GM-CSF or IL-4 is withdrawn (9). Further culture with monocyte conditioned medium (MCM) yields fully mature DCs. We show that immature and mature DCs possess type 1 CGRP receptors and that treatment with CGRP during the maturation phase can dampen DC stimulation of T cells.

Materials and Methods

Culture medium

We used RPMI 1640 (Cellgro, Fisher Scientific, Springfield, NJ), supplemented with 2 mM L-glutamine (Life Technologies, Grand Island, NY), 50 μM 2-ME (Sigma, St. Louis, MO), 10 mM HEPES (Life Technologies), penicillin (100 U/ml)-streptomycin (100 μg/ml) (Gemini Bio Products, Calabasas, CA), and 1% human plasma (heparinized).

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Cytokines

Recombinant human GM-CSF was purchased from Immunex (Seattle, WA), and recombinant human IL-4 was purchased from R&D (Minneapolis, MN).

Monocyte-conditioned medium (MCM)

IgG-coated bacteriologic plates (100 mm, Falcon, Lincoln Park, NY) were prepared immediately before use by addition of 4 ml human γ-globulin (10 mg/ml; Cappel Laboratories, Organon Teknika, Westchester, PA) for 3 min. Plates were washed three times with PBS before use. Peripheral blood was obtained from buffy coats purchased from the New York Blood Center (New York, NY). PBMCs were isolated by sedimentation in Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden). T cell-depleted (erythrocyte rosette (ER)–) fractions were prepared by rosetting with neuraminidase-treated sheep RBC as described previously (10). T cell-depleted (ER–) cells (5 × 10⁶) or 9 × 10⁶ PBMCs were cultured on IgG-coated plates for 1 h at 37°C. Nonadherent cells were washed off with gentle aspiration. The IgG-adherent cells were cultured with 10 ml fresh medium at 37°C for 24 h. The medium was collected and frozen at −20°C before use.

Monoclonal Abs

mAbs to the following Ags were used: HLA-DR; CD3; CD14; CD20; CD25 (PE conjugate, Becton Dickinson, Mountain View, CA); CD83 (IgG1, unlabeled or PE conjugate, Coulter, Miami, FL); and CD86 (PE conjugate, PharMingen, San Diego, CA). Cell populations were phenotyped with these mAbs using a FACScan (Becton Dickinson Immunochemistry Systems, San Jose, CA).

Generation of DCs from human blood

Peripheral blood was obtained from normal human donors in heparinized syringes or from buffy coats purchased from the New York Blood Center. T cell-enriched (ER+) or T cell-depleted (ER–) fractions from PBMCs were prepared by rosetting with neuraminidase-treated sheep RBC as described (10). ER– cells (2.5 × 10⁷/well) were plated in each well of a six-well plate (Falcon) or adherent monocytes on day 0 of culture and cultured for 8 days with MCM (100 ng/ml). Alternatively, adherent monocytes were derived by plating 8 × 10⁶ PBMCs/well in 3 ml culture medium for 1 h at 37°C. Nonadherent cells were removed, and adherent cells washed with warm RPMI. GM-CSF (1000 U/ml) and recombinant human IL-4 (100 U/ml) were added to the ER– or adherent monocytes on day 0 of culture and replenished on days 2, 4, and 6 by removal of 0.3 ml medium followed by addition of 0.5 ml medium with cytokines. To generate mature DCs, cultures were supplemented with cytokines on day 8 but were not supplemented with MCM.

Purification of DCs

In all experiments, CD14-negative, immature, and mature DCs were purified by negative selection by incubation with anti-CD2 and anti-CD19 Dynabeads (Dynal, Oslo, Norway) per manufacturer’s instructions. After two cycles of separation as described (11), purity was >99% and was confirmed by FACS analysis.

Generation of T cells

PBMCs from heparinized blood from normal human donors or from buffy coats were isolated by sedimentation in Ficoll-Hypaque as described for generation of DCs. T cell-enriched (ER+) fractions were prepared by rosetting with neuraminidase-treated sheep RBC (10). Alternatively, T cells were purified from nonadherent fractions by negative selection by incubation with Dynabeads coated with anti-HLA-DR according to the manufacturer’s instructions. Purity was confirmed by FACS analysis.

Neuropeptides and neuropeptide treatment

CGRP and CGRP type 1 receptor antagonist, CGRP-R1, were purchased from Phoenix Pharmaceuticals (Belmont, CA), and CGRP type 2 receptor agonist, diacetaxymethylcysteine CGRP (CYS (ACM 2, 7) CGRP) was purchased from Sigma. The neuropeptide treatment protocol was a modification of the one described by Fox et al. (12). Immature DCs, after 7 days of culture with IL-4 (100 U/ml) and GM-CSF (1000 U/ml), were cultured with CGRP (10⁻⁷ M) for 2 h at 37°C. Then cells were cultured for 2 additional days in medium supplemented with MCM and CGRP. As mentioned earlier, addition of MCM to the culture media for 2 days promotes DC maturation. We refer to this cell population as CGRP-treated, mature DCs. In parallel plates, DCs were cultured for additional 2 days with CGRP, but without addition of MCM in the culture medium. Under these conditions, DCs do not mature and remain as immature DCs. We refer to this cell population as CGRP-treated, immature DCs. At day 9, CGRP-treated, mature DCs and CGRP-treated, immature DCs were cultured again with CGRP (10⁻⁷ M) for 2 h at 37°C and washed twice before phenotypic analysis and functional assays.

Allogeneic mixed leukocyte reactions

To test for allogeneic T cell-stimulatory function, 50–5000 purified CGRP-treated or untreated, mature or immature, purified DCs were added as stimulators for 2 × 10⁶ allogeneic T cells in 96-well flat-bottom plates (Costar, Cambridge, MA) for 5 days at 37°C. Each combination was set up in triplicate. Proliferation was determined by day 5 by measuring the uptake of [³H]TdR (4 μCi/ml) during the final 8 h of coculture. Results are expressed as the mean cpm incorporated from triplicate wells (mean cpm). In some experiments, CGRP (10⁻⁷ M in each well) was added to cocultures on days 0, 2, and 4.

Staphylococcal enterotoxin B (SEB) assay

As another assay for DC presenting function, 300–10,000 CGRP-treated or untreated, mature or immature, purified DCs were added to 1 × 10⁷ T cells with 5 ng/ml SEB (Toxin Technology, Sarasota, FL) in 96-well flat-bottom plates and cultured for 3 days at 37°C. Each combination was set up in triplicate, and proliferation was determined on day 3 using [³H]TdR (4 μCi/ml) uptake during the final 8 h (mean cpm). In some experiments, CGRP (10⁻⁷ M in each well) was added to cocultures on days 0 and 2.

Assay for intracellular calcium

Mature or immature, purified DCs (2–8 × 10⁶) were collected in 17–20-mm conical tubes (Falcon), pelleted at 2000 rpm for 2 min, washed twice with EBSS-H (125 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM NaH₂PO₄, 26 mM HEPES, 5.6 mM glucose, 0.1% w/v BSA, 2 mM CaCl₂), and resuspended in 1 ml EBSS-H at 1–4 × 10⁶ cells/ml. The fluorophore fluo-3-acetoxymethyl ester (fluoro-3-AM) (Molecular Probes, Eugene, OR) was added to 2 μM, and the DCs were incubated for 60–75 min at room temperature on a nutator. The fluo-3-AM-loaded DCs were pelleted and resuspended in EBSS-H, washed again, and incubated in EBSS-H for 30–45 min. The DCs were pelleted and transferred to cuvettes at 2–4 × 10⁶ cells/ml, and the fluorescence due to intracellular calcium was measured at room temperature in a Hitachi F-2000 fluorescence spectrophotometer using excitation and emission wavelengths of 505 and 525 nm, respectively.

RT-PCR for detection of CGRP-R1 transcripts

Total RNA from 1–2 × 10⁶ purified mature or immature DCs was extracted in 2 ml RNAzol (Tel-Test, Friendswood, TX) mixed with 0.2 ml chloroform and centrifuged at 12,000 × g for 15 min. The aqueous phase was transferred to a fresh tube, isopropanol was added to precipitate the RNA, and the pellet was dissolved in 20 μl double-distilled H₂O and stored at −20°C. First-strand cDNA was reverse transcribed from 5 μg total RNA in a 20-μl reaction volume in 20 mM Tris-HCl, 50 mM KCl, 2.5 mM MgCl₂, 10 mM DTT; 500 nM oligo(dT); 500 μM each dATP, dCTP, dGTP, dTTP; and 200 U Super Script II RT (Life Technologies, Gaithersburg, MD) for 50 min at 42°C. Two μl of the 20 μl of RT reaction mixture were PCR amplified. Primers for human CGRP-R1 were synthesized based on a published sequence (13). The sense primer 5’-TCT GGT TCT TTT GCC TTT TTT TAT G-3’ corresponded to nucleotides 581–605; and the antisense primer 5’-GTC CAT GTT CTG TTG CCT GCT G-3’ was complementary to nucleotides 898–919. Thirty cycles of PCR were run at 94°C for 1 min, 56°C for 1 min, and 72°C for 1 min with 2.5 U Taq polymerase (Perkin-Elmer, Norwalk, CT).

Automated DNA sequencing

This was done by Taq FS dye terminator cycle sequencing using a Model 377 stretch sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA) (14).

Results

Immature and mature DCs express CGRP-R1 mRNA.

Because immunomodulatory effects of CGRP on LCs had been described, we set out to identify CGRP receptors on DCs derived from human PBMCs and then study their immunoregulatory potential. Total RNA was isolated from purified blood-derived, mature, and immature DCs. Results of RT-PCR experiments indicated that mRNA specific for CGRP type 1 receptor (CGRP-R1)
(705 bp) was expressed in mature and immature DCs (Fig. 1). Sequence analysis confirmed 98 and 99% identity with the human CGRP-R1, as recorded in GenBank, for mature and immature DCs, respectively.

**CGRP triggers calcium flux in immature and mature DCs**

To determine the functional potential of CGRP-R1 on DCs, we measured the ability of CGRP to trigger calcium fluxes in mature and immature cells. The DCs were loaded with fluo-3 AM, a dye that exhibits increased fluorescence in the presence of calcium. When either mature or immature, fluo-3-AM-loaded DCs were treated with CGRP ($10^{-7}$ M), intracellular calcium mobilization was noted as determined by fluorometry (Fig. 2). Mobilization of intracellular calcium was abrogated by the presence of equimolar concentrations of CGRP$_{8-37}$, a CGRP-R1 antagonist, whereas calcium flux was not triggered by diacetoamidomethylcysteine CGRP (CYS (ACM 2, 7) CGRP), an agonist for the type 2 CGRP receptor (CGRP-R2) (data not shown). Mobilization of intracellular calcium by CGRP confirmed the functionality of the CGRP-R1 receptor on both mature and immature DCs.

**Decreased surface expression of CD86 and HLA-DR on CGRP-treated, mature DCs**

As shown previously, CD25 and CD83 are expressed on mature DCs, whereas the levels of the MHC class II molecule, HLA-DR, and the costimulatory molecule, CD86, are increased (9). Previous studies indicated that CGRP suppressed CD86 in the murine, LC-like, XSS52 cells (6), so we conducted similar studies on human DCs, as a model for the in vivo situation in which DCs are exposed to neurogenic stimuli before or concomitant with the exposure to Ag. Treatment of purified DCs with CGRP ($10^{-7}$ M) for 2 h before and during culture with MCM, and for an additional 2 h after the MCM-induced maturation phase, yielded CGRP-treated, mature DCs and resulted in decreased expression of CD86 and HLA-DR (Fig. 3). Expression of CD25, the α subunit of the IL-2 receptor,
and CD83, a maturation marker, were unchanged by CGRP treatment. Treatment of immature DCs with CGRP (10^{-7} M) subsequently cultured with GM-CSF and IL-4 but without MCM, yielded CGRP-treated, immature DCs with decreased expression of CD86 but unchanged expression of HLA-DR (not shown). CD25 and CD83 expression on immature DCs remained negative after CGRP treatment. Decreased expression of CD86 and HLA-DR was observed with DCs from 60% of the buffy coats and blood donors we studied. In all cases in which regulation was observed, the changes in expression of HLA-DR and CD86 occurred conjointly. In the case of CGRP-treated, mature DCs, these phenotypic changes correlated with diminished induction of allogeneic T cell proliferation.

Decreased allogeneic T cell stimulation by CGRP-treated, mature DCs

The down-regulation of CD86 on CGRP-treated, mature, and immature DCs suggested that CGRP treatment might modulate their Ag-presenting capability. This was investigated using the allogeneic MLR assay. Mature DCs vigorously stimulate allogeneic T cells and at low DC:T cell ratios (9), whereas previous studies revealed a decrease in allogeneic T cell stimulation by CGRP-treated LCs (6). Therefore, purified, CGRP-treated or untreated, mature or immature DCs were used to stimulate allogeneic T cells in the MLR assay. The proliferative responses were lower than those induced by untreated, mature DCs (25–50%), especially at low DC:T cell ratios (1:360 in Fig. 4). Addition of CGRP (10^{-7} M) to DC:T cell cocultures on days 0, 2, and 4 of coculture had no additional effect on T cell-proliferative responses. In contrast, allogeneic T cell-proliferative responses driven by CGRP-treated, immature DCs were not different from those driven by untreated, immature DCs.

CGRP treatment of DCs does not affect T lymphocyte responses to SEB

Finally, we examined presentation in a superantigen (SEB) system that is less sensitive to subtle changes in surface expression of HLA-DR. Varying numbers of purified, CGRP-treated or untreated, mature or immature DCs were cocultured with syngeneic T cells and SEB for 3 days, and proliferation was determined by tritiated thymidine uptake. Data are from one of three experiments with similar results and are expressed as mean [^3]H]Tdr cpm \times 10^{-3} \pm SEM. ■ Control DCs; □ CGRP-treated DCs; ○, control DCs with CGRP added during coculture; ◀, CGRP-treated DCs with CGRP added during coculture.

**FIGURE 4.** CGRP treatment of DCs decreases their allogeneic stimulatory capacity. A, Mature DCs. B, Immature DCs. DCs were generated as described in Materials and Methods. Varying numbers of CGRP-treated or untreated, pure mature or immature DCs were cocultured with allogeneic T cells for 5 days, and proliferation was determined by tritiated thymidine uptake. In some experiments, additional CGRP (10^{-7} M in each well) was added at days 0, 2, and 4 of coculture. Data are from one of three experiments with similar results and are mean [^3]H]Tdr cpm \times 10^{-3} \pm SEM. ■, Control DCs; □, CGRP-treated DCs; ○, control DCs with CGRP added during coculture; ◀, CGRP-treated DCs with CGRP added during coculture.

**FIGURE 5.** CGRP treatment of DCs does not affect their ability to stimulate T lymphocyte responses to SEB. A, Mature DCs. B, Immature DCs. Varying numbers of CGRP-treated or untreated, pure mature or immature DCs were cocultured with syngeneic T cells and SEB for 3 days, and proliferation was determined by tritiated thymidine uptake. Data are from one of three experiments with similar results and are expressed as mean [^3]H]Tdr cpm \times 10^{-3} \pm SEM. ■, Control DCs; □, CGRP-treated DCs.
CGRP (10^{-7} M) to the cocultures on days 0 and 2 did not affect the response to SEB (data not shown).

Discussion

Several points have become apparent in these studies: 1) mature and immature DCs express mRNA specific for CGRP-R1; 2) treatment of mature and immature DCs with CGRP leads to the mobilization of intracellular calcium; 3) cell surface expression of CD86 and HLA-DR are decreased on “CGRP-treated, mature DCs”; 4) expression of CD86, but not HLA-DR, is decreased on CGRP-treated, immature DCs; and 5) CGRP-treated, mature DCs are less effective at driving allogeneic T cell proliferation than untreated DCs but are unaffected in stimulating syngeneic T cell responses to SEB.

Our results extend the findings that some neuropeptides may regulate immune responses (15). Neuropeptides, including CGRP, pituitary adenylate cyclase-activating peptide, vasoactive intestinal peptide, and substance P, can regulate the function of B cells, T cells, and macrophages (1). The interaction of LCs and neuropeptides, in particular CGRP, has been described (3). Hosoi et al. (16) demonstrated that LCs often lie in apposition with epidermal nerves containing CGRP. Also CGRP is required for UVB-mediated suppression of contact hypersensitivity (CH) (17, 18). Other studies support the potential suppression of delayed-type hypersensitivity as well as CH by CGRP (19). Finally, Fox et al. (12) reported that treatment of human monocytes with CGRP for 2 h decreased their presentation of tetanus toxoid.

The expression of CGRP-R1-specific mRNA (Fig. 1) by DCs alludes to the potential for functional regulation of DCs by neuropeptides. At least two subtypes of CGRP receptors have been identified, termed CGRP-R1 and CGRP-R2 (20). Sequence identity with human CGRP-R1 supports the presence of the type 1 receptor on human blood-derived DCs, consistent with other data of CGRP-R1 on Lc (21).

CGRP-R1 mobilizes intracellular calcium in human DCs (Fig. 2). The CGRP receptor is a member of a superfamily of transmembrane receptor proteins. On ligand binding, the receptor couples to a membrane-associated heterotrimeric G protein that initiates signaling pathways, ultimately leading to increased intracellular calcium (22). The inhibition of calcium fluxes by the CGRP-R1 antagonist and the lack of a calcium flux to CY (ACM 2, 7) CGRP, a CGRP R-2 agonist, further supports the presence of a functional CGRP R1 on DCs. The identity of specific G protein subunits involved with CGRP-mediated signaling in DCs remains to be determined.

Dampening of DC-driven immune responses by CGRP may involve regulation of costimulatory molecules, alterations in cytokine induction, or direct effects on Ag uptake. We observed a decreased expression of CD86 and HLA-DR by CGRP-treated, mature DCs (Fig. 3) but only a decrease in CD86 in CGRP-treated, immature DCs. Because the stimulatory activity for allogeneic T cells was decreased only for mature DCs (Fig. 4), the down-regulation of HLA-DR may be more significant. CGRP also did not reduce DC-driven T cell proliferation to SEB, a phenomenon that is less susceptible to subtle down-regulation of HLA-DR (23), supporting a role for HLA-DR down-regulation in the dampening of allogeneic T cell proliferation to CGRP-treated, mature DCs.

As stated earlier, the phenomenon of phenotypic regulation was observed in only 60% of blood donors. The reasons for this are not clear and may involve differences in the level of expression of CGRP-R1 on DCs or the presence of defective receptors in some individuals. However, in all cases in which regulation was observed, the changes in expression of HLA-DR and CD86 occurred in concert, and, in CGRP-treated, mature DCs, phenotypic changes correlated with reduced allogeneic T cell stimulation.

Suppression of CD86 on human monocytes by CGRP has been described and has been reported to involve IL-10 (12). Other studies have demonstrated CGRP-mediated inhibition of IL-10 and suppression of IL-12 and CD86 in the epidermal LC-like cell line XSS52 (7). The dampened DC function that we observed still needs to be addressed at the levels of these cytokines and Ag uptake. Dampened immune responses in another system has been suggested by Khachatryan et al., who showed that CGRP expression in pancreatic β cells suppresses development of diabetes in nonobese diabetic mice (24).

We considered the idea that CGRP could have a direct effect on T cells. CGRP potentiates T cell proliferation in the presence of IFN-γ-treated, class II-bearing, intestinal smooth muscle cells (25) but suppresses mitogen-stimulated murine T cells (26). In our studies, addition of CGRP (10^{-7} M) to DC:T cell cocultures had no effect on DC-driven allogeneic T cell proliferation or responses to SEB (Figs. 4 and 5). The expression of CGRP-R1 mRNA by immature and mature DCs, coupled with effects of CGRP on intracellular calcium, HLA-DR, and CD86, suggests that CGRP acts on DCs directly.

On the basis of previous and current findings, we speculate that the release of neuropeptides in the face of inflammatory stimuli, including certain inflammatory skin disorders, can suppress APCs including DCs. The cellular mechanisms involved in regulation of CH induction by UV radiation, especially the role of LCs, have been studied (17–19, 27). The potential therapeutic effects of UV radiation in inflammatory disorders, including atopic dermatitis and psoriasis, are also described (28). Because CGRP is required for the suppression of CH by UVB (18, 19), CGRP may influence responses to phototherapy. Accordingly, selective activation of CGRP-R1 on DCs might prove useful in the treatment of inflammatory skin disorders.

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References


