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Localization and Expression of CCR3 and CCR5 by Interleukin-1β in the RIN-5AH Insulin-Producing Model System: A Protective Mechanism involving Down-Regulation of Chemokine Receptors

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ABSTRACT

Context and objective The inflammatory cytokine interleukin-1β has been considered to be an immune effector molecule in insulin dependent diabetes mellitus. As such, we examined its role on chemokine receptors which, when expressed in the pancreas, have also been associated with the development of type I autoimmune diabetes.

Design and main outcome measures The presence of membrane and cytoplasmic levels of CCR3 and CCR5 expression is assessed by immunofluorescence in control and interleukin-1β-treated RIN-5AH cells. The cytoplasmic expression is also shown by confocal microscopy as assessed by the brightness of the cells whereas enzyme-linked immunosorbent assay detects secreted CCR3 and CCR5 molecules by comparing optical density values as these derive from the control and the treated cells. Cell-fractionation experiments show the exact location of the intracellular pools of the chemokine receptors by using the rab7 monoclonal antibody as a guiding molecule.

Results Interleukin-1β down-regulates constitutively expressed surface CCR3 and CCR5 levels implying receptor internalization for re-utilization or destruction, secretion or both. Cytoplasmic immunofluorescence and confocal microscopy demonstrate cellular retention of chemokine receptors by interleukin-1β which may be released in the absence of interleukin-1β as assessed by enzyme-linked immunosorbent assay. Finally, cell-fractionation shows the presence of both receptors in endosomes exhibiting an increasing density after interleukin-1β treatment.

Conclusions Given the association of chemokine receptors with progression to diabetes, it appears that interleukin-1β-induced down-regulation of CCR3 and CCR5 promotes a protective mechanism against cellular destruction. The major role of interleukin-1β is to maintain these molecules within the endosomes. Thus, interleukin-1β modulates the movement and the expression of constitutively expressed chemokine receptors and does not accentuate the total destructive effect suffered by the cells.

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is a chronic autoimmune disease characterized by the specific destruction of the insulin-secreting β cells of the pancreas [1, 2]. Pancreatic Langerhans islets are composed of

a heterogeneous population of secretory cells (α, β, δ and PP cells) as well as non-endocrine cells (macrophages, endothelial cells, dendritic cells and fibroblasts) [3]. The presence of such a variety of cellular components renders the investigation of causes leading to β cell destruction extremely difficult and, although studies on IDDM started decades ago [4], the exact pathway(s) from insulitis to the end-stage disease still remains unknown [5, 6, 7].

Early experiments dating back to 1985 demonstrated that cytokines released from peripheral blood mononuclear cells were able to damage isolated islets of Langerhans [8]. Since then, many studies have shown how IDDM is advancing and how different pathways of regulation may interfere with this autoimmune disease in a positive (corrective) or negative (destructive) manner. The inflammatory spectrum of interleukin-1 (IL-1) activities has led many laboratories to focus their attention on its role and particularly on the destruction of β cells [9]. Even though IL-1 modulates insulin release and (pro)insulin biosynthesis probably leading to β-cell death, its inhibitory and toxic effects are at least in part due to early apoptotic signals and the induction of nitric oxide radicals all mediated by specific receptor binding [7, 8, 9, 10]. Although IL-1α and IL-1β bind to different receptors [10, 11, 12, 13], it has been demonstrated that IL-1β is capable of transducing different signals whereas IL-1α appears to be more important in the initiation of the destructive events on β cells [14]. Therefore, IL-1β, although not cytotoxic to the RIN-5AH population in the logarithmic growth phase even at very high concentrations [15], induces class II antigen expression, promotes proliferation and causes production of NO which may be an important co-factor in IL-1β-mediated β cell damage [14]. In addition, IL-1β generates early apoptotic signals, and induces Fas expression and DNA damage as is manifested by chromatin condensation [16, 17, 18].

Recently, laboratories have undertaken research regarding chemokines (CC) as these molecules mediate innate and adaptive immune responses by recruiting and activating T lymphocytes and monocytes/macrophages [19, 20]. Certain members of the CC family of chemokines, such as macrophage inflammatory protein-1α (MIP-1α), have been implicated in experimental autoimmune diseases [21]. Such regulatory scenarios have been shown to be modulated by Th1- and Th2-type cytokines [22, 23]. Moreover, a differential expression of CC chemokines and the CCR5 receptor in the pancreas has also been reported in the sense that such an outcome is associated with progression to type I diabetes [24]. In particular, the non-obese diabetic mice which spontaneously develop a form of IDDM [25] express MIP-1α and its receptor, CCR5 implying that CC chemokines as well as their receptors constitute novel intermediates in the regulation of diabetes.

Given thus the association of chemokine receptors (CCRs) with type I diabetes and taking into account the role of IL-1β in the pathogenesis of IDDM, we investigated whether IL-1β's action is mediating or is mediated upon these molecules. Since the expression of CCRs depends on the state of activation/differentiation of T and other immune cells, we chose two representative members of this family, CCR3 and CCR5. CCR3 expression is regulated by Th2-type cytokines and CCR5 by Th1-type cytokines [26, 27]. Thus, the expression of CCR5 [24], and possibly CCR3, on insulin-producing cells could attract T cells as well as other inflammatory cytokine-secreting cells to the tissue and initiate the local destruction events. It is also possible that IL-1β is able to affect CCR3 and CCR5 whose presence would most likely suggest a complex system of CC-CCR interactions facilitating, hindering or even promoting the action of IL-1β as this has been described to date. By employing simple experimental procedures such as immunofluorescence and ELISA as well as more complex approaches such as sub-cellular fractionations and confocal microscopy, the possible reciprocal relationship of IL-1β and CCR3/CCR5 was studied using the former as an inducing agent

and the latter as a read-out parameter leading to the delineation of this question which will contribute to a better understanding of the regulation of diabetes.

MATERIALS AND METHODS

Cells and Culture

Dr. Herbert Oie provided the β cell-like line RIN-5AH used in this study [28]. Cells were grown in plastic culture flasks (Sarstedt, Numbrecht, Germany) in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and maintained at 37 ^oC in a 5% CO_2 atmosphere.

Experimental Design

Based on the hypothesis that expression of chemokine receptors on β-pancreatic cells could initiate the process of autoimmune diabetes by attracting immune cells including T lymphocytes and macrophages to this site, we studied the expression of CCR3 and CCR5 on the experimental model of RIN-5AH cells with or without the presence of IL-1β. This approach is supported by the fact that T cells and macrophages, in addition to the genetic predisposition of the individual, facilitate auto-antigen recognition resulting in β-cell destruction. Moreover, IL-1β is known to be involved in the pathway of IDDM pathogenesis [7] and is, therefore, a major candidate molecule for such study. Since chemokine receptors demonstrate a rapid turn-over regarding their appearance on the membrane, internalization to the cell and, in some cases, extra-cellular release [29], we determined the presence of CCR3 and CCR5 on the cell membrane, intracellular compartments and medium after 2, 4, 8 and 24 hours of culture with or without the presence of IL-1β. Thus, the experiments of immunofluorescence, ELISA and confocal microscopy were performed with cells of the same seeding concentration (see below), induction and passage of culture. For the sub-cellular fractionation approach, only the cell numbers were modified, as this procedure requires a high number of cells. All experiments were performed in the presence of 10% FBS since serum-free conditions do not allow or largely delay the formation of the confluent layer needed for further induction and experimentation.

Reagents and Induction Protocol

The human recombinant IL-1β was a generous gift from Dr. Steven Gillis (Immunex Corp., Seattle, WA, USA) and was used at a concentration of 25 ng/mL [14, 18]. An IL-1β preparation from the National Cancer Institute (Mc Kesson BioServises, Rockville, MD, USA) was also used at the same protein concentration and yielded identical results. Monoclonal antibodies to human but also rat-reactive CCR3 (LS63 7B11) and CCR5 (LS100 2D7) were delivered by the AIDS Project (UNAIDS Repository Programme EVA Centralised Facility, National Institute for Biological Standards and Controls, Hertfordshire, UK) and used at a dilution of 1/100 for the immunofluorescence experiments and at a dilution of 1/1,000 for the ELISA. Mouse (also rat-reactive) anti-rab7 monoclonal antibody (Santa Cruz, CA, USA. 200 µg/mL) was used in ELISA experiments at a dilution of 1/1,000. Goat anti-mouse IgG coupled with horseradish peroxidase (Sigma, St. Louis, MO, USA) was used at a dilution of 1/1,000. Goat anti-mouse IgG antibodies coupled with fluorescein isothiocyanate (FITC) or rhodamine B isothiocyanate (RITC) (Boehringer Mannheim GmbH, Mannheim, Germany) were used in immunofluorescence experiments at a dilution of 1/250.

As previously shown [18], IL-1β was added to the cell cultures at time zero and measurement of CCR3 and CCR5, for the majority of the experiments described, took place at 2, 4, 8 and 24 hours. In short, for the immunofluorescence, ELISA and confocal microscopy experiments, RIN-5AH cells were seeded at a concentration of 10^5 cell/mL and were allowed to form a confluent layer for 2 days before the addition of IL-1β (considered

as time zero). Cells or supernatants were accordingly employed for each type of experiment.

Indirect Immunofluorescence and ELISA Experiments

RIN-5AH cells were subjected to immunofluorescence experiments as previously described [30, 31]. For the detection of surface and intracellular CCR3 and CCR5 molecules, double immunofluorescence experiments were performed. Thus, for surface staining, the cells were first reacted with anti-CCR3 and anti-CCR5 at 4 ^{o}C (1:100) and they were revealed with an anti-mouse IgG-FITC antibody. For intracellular detection, the cells were fixed with ice-cold paraformaldehyde (PFA, 4%) for 5 minutes and incubated with the same primary antibodies diluted in HBSS-saponin solution (HBSS: 0.01 M; HEPES: 1% saponin) since saponin was found to have a milder effect than the 20% ice-cold methanol usually employed on the cell membranes. After washing in PBS-Saponin, the anti-mouse IgG-FITC antibody was added to the cells. Finally, the samples were washed, fixed in 25% glycerol, mounted on slides and examined for surface or intracellular staining. Bright to very bright cells were counted as positive using a microscope (Olympus, Hamburg, Germany). All experiments were repeated five times.

Indirect ELISA was performed as previously described [30, 31]. In short, samples were coated in 96-well flat bottom plates (Sarstedt, Numbrecht, Germany), incubated overnight at 4° C and washed four times in 5% Tween-20. The remaining protein free sites in the plate were blocked by 2% PBS-BSA solution after an incubation of 2 hours at room temperature. After washing four times, 100 μL of test antibodies diluted in 0.1% PBS-BSA were added and incubated for 1 hour at room temperature. Extensive washing of the plate was followed by the addition of 100 μL of goat anti-mouse IgG coupled with horseradish peroxidase (Sigma, St. Louis, MO, USA. 1/1,000 dilution) and incubation for 1 hour at room temperature, in the dark. Finally, the reaction was developed by adding 100 μL/well of tetramethyl benzidine-H_2O_2 (Sigma, St. Louis, MO, USA) for 20 minutes. The enzymatic reaction was stopped with 50 μL H_2SO_4 (4N). Optical density (OD) was measured at 450 nm using a Titertec ELISA photometer (Digiscan, ASYS Hitech GmbH; Engendorf, Austria). Each experiment was repeated at least four times. The results are expressed as the percentage of OD increase over background levels (±SE, calculated from four or more experiments).

Sub-Cellular Fractionation

For the experiments of sub-cellular fractionation, RIN-5AH cell cultures were carried out in 75 mm^2 flasks (Corning/Costar Co, Cambridge, MA, USA). After an appropriate incubation of 4 hours in 50 mL of culture medium with or without 25 ng/mL of IL-1β, the cells were scraped off the flasks and submitted to sub-cellular fractionation experiments following the technique described by Qiu *et al.* [32]. In brief, 70x10^6 cells treated as described above were collected, re-suspended in 2 mL of homogenization buffer (HB; 10 mM Tris, 1mM EDTA, 0.25 M sucrose, pH 7.4) and gently treated in a Dounce Tissue Grinder (Wheaton 357542; Wheaton Industries, Millville, NJ, USA). The homogenate was centrifuged at 900 *g* and, after collecting the supernatant in a clean tube, the pellet was re-suspended in 1 mL HB and the 900 *g* spin was repeated. The resulting supernatant was combined with the first one and all were centrifuged at 10,000 *g* to remove the mitochondria. Two mL of the resulting supernatant were loaded on a 9-mL Percoll gradient (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA. 1.05 g/mL) at 34.809 g_{max}. After collecting 0.5-mL samples from bottom to top, the resulting 18-20 fractions were tested by ELISA for detection of CCR3 and CCR5 activity and rab7 using an anti-rat secondary antibody coupled with horseradish peroxidase. The results are expressed as the percentage of OD increase over background values.

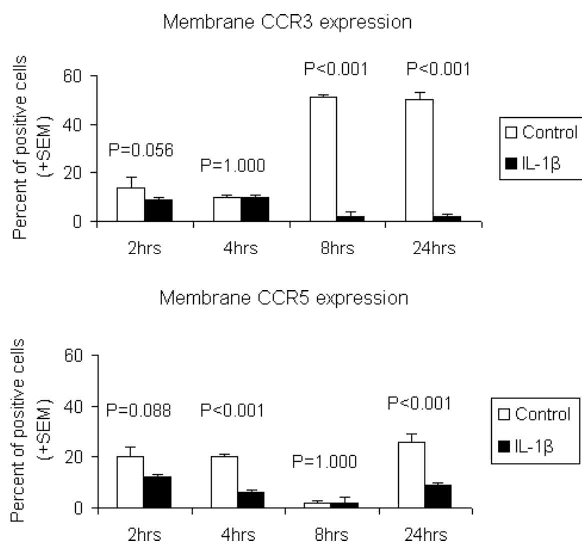

Figure 1. Surface CCR3 and CCR5 expression as assessed by immunofluorescence in control and IL-1β-treated RIN-5AH β-like cells. IL-1β significantly down-regulated CCR3 membrane expression at 8 and 24 hours. Statistical significance was obtained for CCR5 at 4 and 24 hours.

Confocal Microscopy

Confocal microscopy was employed in order to detect the expression of CCR3 and CCR5 in the cytoplasmic compartments of the RIN-5AH population. The experimental procedure was identical with the one described above for internal immunofluorescence staining. The only difference in the method was the final dilution of the cell pellets in 25 μL/mL Mowiol (Sigma, St. Louis, MO, USA) before observation. The induction of cells with IL-1β lasted 4 hours.

STATISTICS

The Student's *t* test was employed in order to evaluate the significance level of the comparison between the control and the test values. Two-tailed P values less than 0.05 were considered statistically significant.

RESULTS

The expression of surface CCR3 at 2 and 4 hours of culture is apparent only in 14% and 10% of the cells respectively, whereas, after 8 and 24 hours of culture, 51% and 50% of the cells express the receptor, respectively (Figure 1). CCR5 follows a different pattern of expression, since it is present in 20% of the cells after 2 and 4 hours of culture, but is hardly detected on the cell surface after 8 hours (2%) and then reappears in 26% of the cells after 24 hours of culture. The presence of IL-1β significantly down-regulates surface expression of both CCR3 and CCR5, where CCR3 expression is reduced by 96% ($P<0.001$) after 8 and 24 hours of culture and CCR5 by 70% and 65% after 4 and 24 hours of culture, respectively ($P<0.001$; Figure 1).

The differential expression of CCR3 and CCR5 on the cell membrane and their down-regulation by IL-1β can be explained either by internalization to the intracellular compartments for re-utilization or destruction, or release to the extra-cellular matrix or both. Therefore, as an initial attempt to localize the CCR3 and CCR5 molecules, we performed cytoplasmic immunofluorescence experiments under the same experimental conditions. The results show a complementary as compared to a surface pattern of expression. Thus, although low expression of CCR3 is obtained at 2 hours of

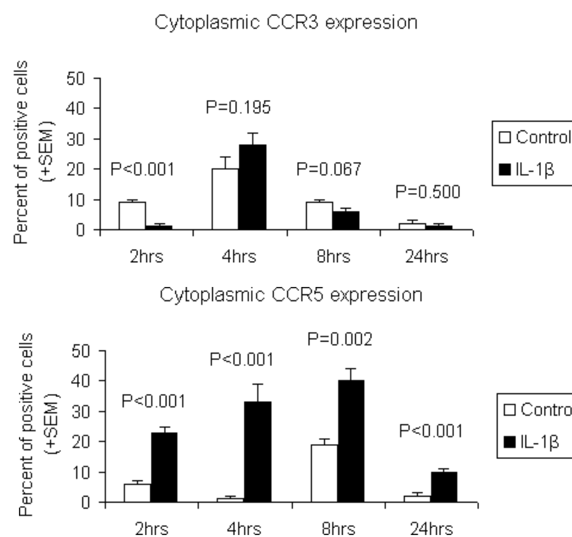

Figure 2. Cytoplasmic CCR3 and CCR5 expression as assessed by immunofluorescence in control and IL-1β-treated RIN-5AH β-like cells. IL-1β significantly modified the cytoplasmic pools of CCR3 at 2 hours only, whereas significant modifications were obtained at 2, 4, 8 and 24 hours when cytoplasmic CCR5 expression was evaluated after IL-1β addition.

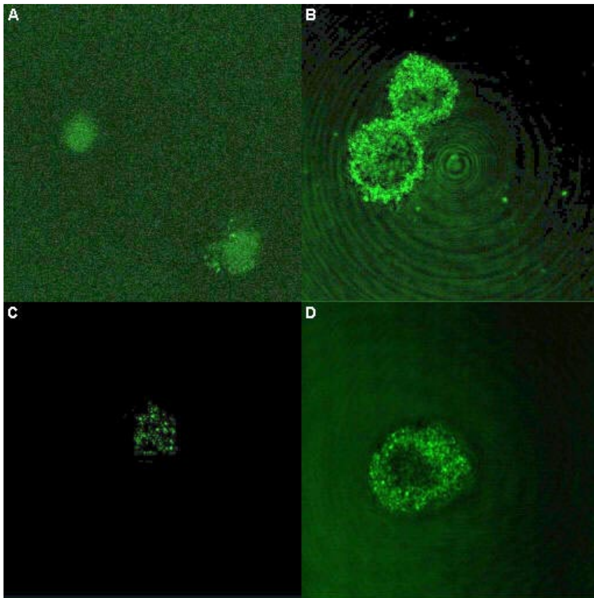

Figure 3. Cytoplasmic CCR3 and CCR5 staining as assessed by confocal microscopy in control and IL-1β-treated RIN-5AH cells: A) cytoplasmic CCR3 without IL-1β; B) cytoplasmic CCR3 with IL-1β; C) cytoplasmic CCR5 without IL-1β; D) cytoplasmic CCR5 with IL-1β.

culture, 20% of the cells contain cytoplasmic pools of this receptor at 4 hours, which drops to 9% and 2% at 8 and 24 hours of culture, possibly because these molecules migrate to the membrane (Figure 2). Only a small percentage of cells contain cytoplasmic CCR5. The highest percentage is obtained after 8 hours of culture, which corresponds to the lowest percentage of surface CCR5 expression (Figure 2). The presence of IL-1β does not seem to significantly modify the cytoplasmic pools of CCR3 at 4, 8 and 24 hours, while a significant decrease of cytoplasmic CCR3 was observed at 2 hours ($P<0.001$). However, IL-1β seems to block the migration of CCR5 to the membrane, since a significant number of cells express cytoplasmic CCR5 ($P<0.001$ at 2, 4 and 24 hours; $P=0.002$ at 8 hours), which is almost undetectable on the cell membrane under the same experimental conditions.

These results were confirmed by confocal microscopy experiments. As shown in Figure 3, IL-1β causes CCR3 and CCR5 to stay in the cytoplasm whereas, in the absence of the cytokine, intracellular CCR5 is almost undetectable after 4 hours of culture while CCR3 shows only a faint staining.

ELISA experiments were performed, in order to evaluate whether some portion of CCRs is released to the culture medium,. Detectable amounts of both CCR3 and CCR5 were obtained after 8 hours of culture (Figure 4). Although CCR3 is only found in small quantities in the culture supernatants, corresponding to 23% of OD increase over background, CCR5 is found in quantities corresponding to 81% OD increase over background. In the presence of IL-1β, only CCR5 is decreased by 55% ($P<0.001$), whereas the amount of CCR3 increases in the culture supernatants ($P=0.038$), indicating that IL-1β does not promote secretion of CCR3.

Cell fractionation experiments were performed in order to evaluate whether the intracellular pools of CCR3 and CCR5 are localized in the endosomal compartments for re-usage/secretion or the lysosomal compartments for degradation (Figure 5). Thus, control or IL-1β treated cells were cultured for 4 hours (the time that gave the best results in the intracellular staining experiments) and fractionated into sequential portions ranging from lysosomal to variably sized endosomes. Using rab7 as an indicative marker for lysosomal and endosomal compartments [31], we were able to detect a

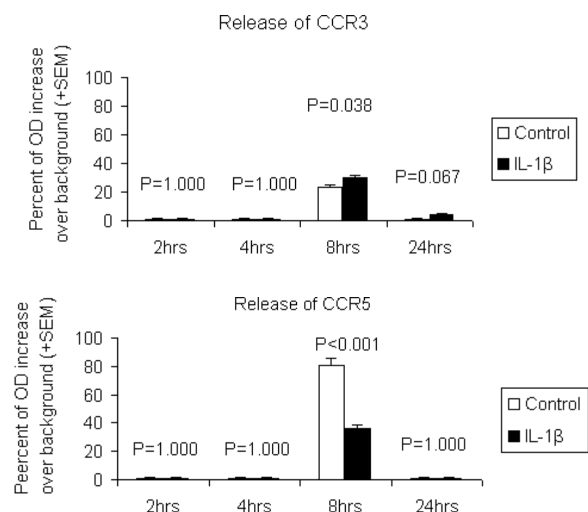

Figure 4. Soluble CCR3 and CCR5 activity in the supernatants of control and IL-1β-treated RIN-5AH cells as assessed by ELISA. At 8 hours, a significant inhibition of CCR5 release was obtained after IL-1β addition, whereas no inhibition of CCR3 release was observed.

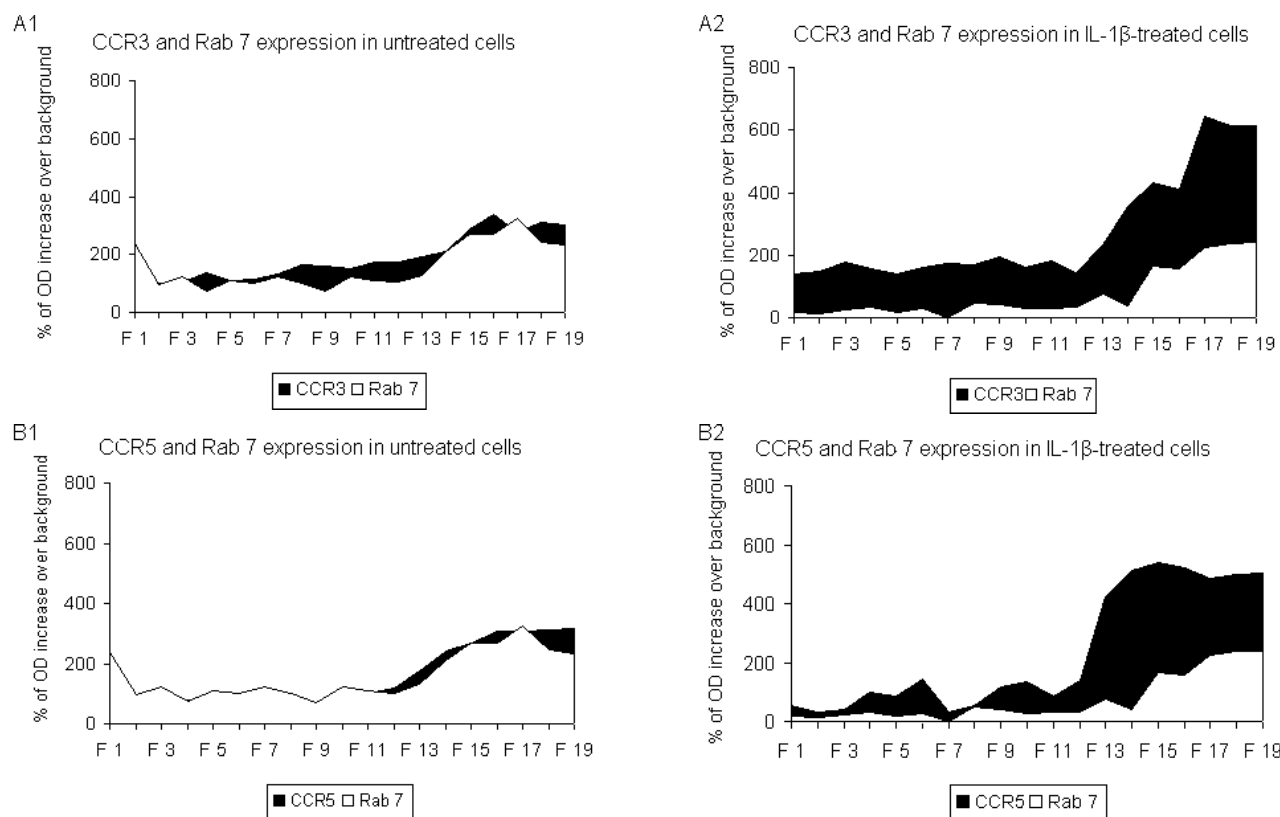

Figure 5. Localization of CCR3 (panels A1 and A2) and CCR5 (panels B1 and B2) intracellular pools in control RIN-5AH cells (panels A1 and B1) and their re-distribution after treatment with IL-1β (panels A2 and B2). The lysosomal and endosomal cytoplasmic compartments are detected with the rab7 monoclonal antibody, employed as a guiding marker. Fractions 1 to 3 show lysosomal vesicles whereas fractions 14 to 19 show the endosomal compartments.

significant amount of CCR3 in the endosomal compartments (fractions 14-19) but not the lysosomal compartments (fractions 1-3). In the presence of IL-1β, the distribution of lysosomal/endosomal compartments was changed (absence of rab7 in fractions 1-3 and decreased reaction in fractions 14-19). However, a higher accumulation of CCR3 was detected in the endosomal fractions (Figure 5). A similar pattern of distribution was seen in CCR5 where, in control cells, CCR5 was accumulated in the endosomal fractions and considerably increased after IL-1β treatment of the cells (Figure 5).

DISCUSSION

Although IL-1β has been shown to exert diverse actions on β-cells in conjunction with type I autoimmune diabetes [7, 15], only a few reports exist about the role and/or regulation of another parameter, namely the chemokine receptors, also involved in the development and progression of the disease [24]. Since both IL-1β and CCR5 have been reported to be involved in the pathogenesis of IDDM, this study was undertaken in order to examine the relationship, if any, between an inflammatory cytokine and CCR3 as well as with CCR5.

Our results show that CCR3 and CCR5 are constitutively expressed on the cell surface of the RIN-5AH insulin-producing, pancreatic β-cell model-system. This membrane expression is down-regulated by 25 ng/mL of IL-1β, a concentration sufficient to initiate early destructive events on the cell population used [18]. The decrease of the CCRs, however, is subject to regulation since recent publications report down-regulation of CCR3 by IL-3 [33] and CCR5 by RANTES [34] and IL-10 [35] in various cellular systems. Therefore, and in view of the initiation of diabetes by CCR5 [24], any decline of the CCRs would imply a protective mechanism against IDDM. Zimmermann *et al.* [36] and Elsner *et al.* [37]

report prolonged CCR3 internalization in human eosinophils by aminooxypentane-RANTES, an event similar to what IL-1β might have caused in the RIN-5AH population. It is known that an internalized receptor will either be destroyed or re-expressed/secreted depending on the intracellular compartment in which it ends up, i.e. lysosomal or endosomal, respectively [31, 38]. For this reason, we examined cytoplasmic CCR3 and CCR5 expression with and without IL-1β. It was shown that at 2, 4, 8 and 24 hours of culture, the cytoplasmic expression of both CCRs was variable and complementary to the surface levels supporting the "rapid turn-over" hypothesis which demonstrated very early chemokine receptor recycling according to the stimulus provided [29]. The most important observation, however, was the ability of IL-1β to predominantly retain CCR5, and to a lesser degree CCR3, within the cytoplasmic compartment of the cells, a finding also confirmed by confocal microscopy.

Since surface and cytoplasmic CCR3 and CCR5 expression whether under the influence of IL-1β or not is shown to be a time-dependent event, we inquired whether the CCRs retained by IL-1β are secreted or destroyed. To answer the above question, we measured soluble CCR3 and CCR5 levels by ELISA and showed that both a small portion of CCR3 and a significant amount of CCR5 were released in the extracellular matrix in the absence of IL-1β. When IL-1β is present, the secretion of CCR5 is hindered whereas soluble CCR3 is not affected. We performed cell-fractionation experiments in order to localize and further analyze whether a portion of the intracellular pools of the two CCRs under study end-up in the lysosomal vesicles or endosomal compartments to be destroyed or re-utilized respectively. It is shown that neither CCR3 nor CCR5 are wasted by the IL-1β treatment. The highest concentrations are detected in the endosomal compartments and are apparently ready to be re-utilized. This latter finding explains and confirms the cytoplasmic fluorescence, confocal microscopy and ELISA for the secretion experiments presented in this work.

Thus, an inhibitory effect of IL-1β on surface CCR3 and CCR5 expression using the RIN-5AH β-like cell model is shown for the first time. To date, surface and mRNA CCR3 down-regulation has been demonstrated in human eosinophils by IL-3 and RANTES but not by IL-5, IL-1β, IL-4, IL-10, IL-13, interferon-γ, TNF-α or the granulocyte-macrophage colony-stimulating factor whereas CCR5 may be decreased by IL-10 and RANTES [33, 34, 35, 36]. Most importantly, the retention of both CCR3 and CCR5 in the endosomes of the cell and their release are novel findings ascribing a role in IDDM to these two chemokine receptors.

Since CCR5 has been associated with the progression to type I diabetes, the unexpected action of IL-1β on CCRs, contrary to its known destructive effects on β-cells [15, 18], exhibits a protective potential never described or considered before. It is our estimate that the key element playing a pivotal role in this kind of regulation is the extremely fast action of this interleukin. This leads us to the hypothesis that the existence of sub-optimal or even optimal IL-1β concentrations *in vivo* is apparently protective in inhibiting CCR expression and thus the influx of inflammatory cells. Such action, however, can be easily overcome when the infiltrating cells dramatically increase in numbers and their products (including IL-1β) modify the local conditions thus rendering this factor toxic.

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Key words Cytoplasmic Structures; Diabetes Mellitus, Insulin-Dependent; Disease; Endosomes; Interleukins; Lysosomes; Receptors, Chemokine; Subcellular Fractions

Abbreviations CCRs: chemokine receptors; FITC: fluorescein isothiocyanate; HB: homogenization buffer; IDDM: insulin dependent diabetes mellitus; IL: interleukin;

MIP: macrophage inflammatory protein; RITC: rhodamine B isothiocyanate

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