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Downregulation of Beta Adrenergic Receptor Expression on B Cells by Activation of Early Signals in Alloantigen-Induced Immune Response

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Key Words

β-Adrenoceptor · Intracellular signaling · Allostimulation · B cell activation · T cell-soluble factors

Abstract

Previously we described a decrease in β-adrenergic receptor expression in B lymphocytes as a consequence of in vivo alloimmunization. This decrease correlates with the highest response of alloantibody production by B cells. In the present report we examined the participation of intracellular signals elicited after alloimmune stimulation. We showed that in vitro stimulation of B cells with mitomycin C-treated allogenic cells induced a reduction in the number of β-adrenoceptors. This downregulation correlated to changes in basal and in isoproterenol-stimulated intracellular cAMP levels. We found that calcium mobilization and protein kinase C activation triggered after direct allogenic stimulation and/or by the action of T cell-soluble factors induced the reduction in β-adrenoceptor sites. These findings could be of interest to understand the neuroendocrine mechanisms involved in the regulation of B cell activation.

Introduction

Over the past several years strong evidence has been accumulated indicating the participation of the autonomic sympathetic system in the modulation of lymphocyte activity. The immunological effects of neurotransmitters are mediated via specific receptors localized in lymphoid organs [1, 2] or in circulating lymphoid cells [3, 4]. Several studies have identified adrenergic receptors that are functionally coupled to a cAMP-adenylyl cyclase signalling pathway [3, 5]. Moreover, it has been suggested that cellular activity could be regulated by the adrenergic expression in the cell, exerting a negative neuroimmune control of cellular response.

Various earlier studies have supported the notion that the immune system is constitutively suppressed by the sympathetic nervous system [6, 7]. In this context, Fuchs et al. [8] demonstrated that lymphocytes from norepinephrine-depleted animals have an overexpression of surface β-adrenergic receptors and showed a reduced ability to respond to sheep red blood cells in vitro. Accordingly, we have previously reported that β-adrenergic sites are diminished in activated B lymphocytes purified from animals subjected to one or two immunological challenges with alloimmune cells [9]. This decrease correlates to low intracellular cAMP levels and with a higher response of

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alloantibody production [9]. A strong interrelationship between lymphocyte adrenoceptors, noradrenaline content of the spleen, and antibody response has been revealed in different conditions [7, 10]. Furthermore, it was described that antigenic challenge induces an increase on sympathetic activity in the spleen which would account for the downregulation of β-adrenergic receptors on lymphocytes during the immune response [8, 11]. Another possibility is that the alteration of receptor density on antigen-driven lymphoid cells does not respond to splenic catecholamine levels; this effect might be a direct consequence of the activation of immune cells by antigen stimulation. In fact, Cazaux et al. [12] demonstrated that β-adrenergic receptor expression and function were diminished in in vitro concanavalin-A-stimulated T lymphocytes.

The present study was undertaken to analyze whether downregulation of β-adrenergic receptors induced by an in vivo allogenic stimulus on B cells could be a direct consequence of B cell activation via antigen stimulation and/or a secondary effect mediated through soluble factors produced by antigen-activated cells. By performing in vitro studies, here we show that both events modulate the β-adrenoceptor expression. We also describe the intracellular pathways triggered during lymphocyte activation that are involved in this phenomenon.

Materials and Methods

Mice

Inbred female BALB/c and C3H mice were purchased from the Instituto Dr. A.H. Roffo. All animals were between 60 and 100 days of age. Mice were housed in standard conditions of light (on from 7.00 a.m. to 7.00 p.m.) and temperature (22 ± 2 °C). Food and water were provided ad libitum. The animals were cared for in accordance with the principles and guidelines of the Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996.

Cell Suspensions

Mice were killed by decapitation and spleens were removed and disrupted through a 1-mm metal mesh, and the cell suspension was filtered through a 10-μm nylon mesh. Non-lymphoid cells were removed by centrifugation over Ficoll-Hypaque. After three washes in RPMI-1640, the cells were resuspended in RPMI-1640 supplemented with 10% of batch-tested nonstimulatory fetal calf serum, 2 m*M* glutamine, 100 U/ml of penicillin and 100 μg/ml of streptomycin (supplemented medium; Gibco Co). Cell viability was estimated according to the trypan blue exclusion criteria [13] and was higher than 90%.

B Cell Purification

Spleen cell suspension in a concentration of 10^7 cells/ml was treated with a monoclonal anti-Thy 1.2 (Sigma) plus pig complement (Gibco) at 37 °C for 1 h in order to produce lysis of T cells. After washing, cells were incubated twice in supplemented medium at 37 °C in 5% CO_2/air for 45 min in 60-mm plastic tissue culture dishes to remove adherent cells. The B cell population purity was assessed by direct immunofluorescence using a monoclonal antibody to mouse κ and λ chains (Sigma) and resulted in the expression of more than 95% Ig-bearing cells.

T Cell Purification

T cell-enriched populations were obtained by passage of the cell suspensions through a nylon wool column following the method of Julius et al. [14]. Briefly, 5×10^7 cells in 1 ml of phosphate-buffered solution (PBS) with 5% of inactivated fetal calf serum were placed in a 6-ml nylon-wool column. After 1 h of incubation at 37 °C, cells were eluted by washing the column twice with 10 ml of supplemented PBS. A purification of more than 97% was obtained as checked by direct immunofluorescence using a monoclonal antibody to mouse Thy 1.2 (Sigma).

Culture Conditions

2×10^6 BALB/c cells/ml were cultured in RPMI-supplemented medium in T-25 culture flask (Corning, N.Y.) at 37 °C with 5% CO_2 at 100% humidity for different times. Spleen cells were allostimulated with 4×10^5 C3H cells/ml. In order to obtain one-way stimulation, allogenic cells were treated with 25 μg of mitomycin C at 37 °C for 30 min in the absence of serum followed by three washes in a 20-fold excess of supplemented medium, according to the method of Bach and Voynow [15]. Control stimulation cultures were performed using mitomycin C-treated BALB/c cells. Purified B cells were incubated alone or in the presence of lipopolysaccharide (LPS; 20 μg/ml), phorbol myristate acetate (PMA; 10^{-9} *M*), or ionophore A23187 (2×10^{-6} *M*), or stimulated with C3H cells pretreated with mitomycin C (2×10^{-5} *M*) as indicated before. Where indicated, more than one stimulator were used or different enzymatic pathway blockers involved in cellular activation were added, at the beginning of cultures, at the concentrations indicated in the Results section.

Preparations of T-Cell Supernatants

Purified T cells (2×10^6 cells/ml) were incubated with C3H (allogenic cells) or BALB/c cells (singenic cells) treated with mitomycin C as indicated above. After incubation at 37 °C in humidified air with 5% CO_2 for 2 days, cell suspensions were centrifuged twice at 500 *g* for 15 min. Fresh supernatants were used to stimulate BALB/c cells.

Proliferation Assays

Proliferative assays were performed in 0.2-ml aliquots of macrocultures separated at the indicated times and pulsed for the last 18-hour period of culture with 1 μCi of ^{3}H-thymidine (Amersham, 15 Ci/mmol). The thymidine incorporation was measured by scintillation counting after retention over GF/C glass fiber filters of the acid-insoluble macromolecular fraction. The maximal proliferative response for each condition was determined by testing the thymidine incorporation on a daily basis. For cells stimulated with LPS, the maximal proliferation was observed after 72 h of culture, whereas the peak of response to allogenic stimulus was observed after 5–6 days of culture. Results are expressed as stimulation index (SI) calculated as the rate between dpm values in experimental cultures and dpm from control values obtained with unstimulated cells.

^{125}I-Cyanopindolol Binding to Intact Cells

Cells (4×10^6 cells/tube) were added to ^{125}I-cyanopindolol (Amersham, 2,000 Ci/mmol) solutions containing the ligand at concentrations from 1 to 300 p*M* in a final volume of 200 µl of 20 m*M* HEPES buffer pH 7.4 containing 12 m*M* $MgCl_2$ and made isotonic with NaCl. After 30 min incubation at 30°C, the reaction was stopped by the addition of 12 ml of ice-cold PBS pH 7.4 and rapid vacuum filtration on Whatman GF/C filters. The filters were washed 3 times with 4 ml of buffer and counted in a Packard gamma counter. Total binding curves from the performed experiments were analyzed using the computer program LIGAND which fits parameters for B_{max}, K_d and nonspecific binding [16]. The nonspecific binding parameters fitted by LIGAND from the total binding curve did not differ from those determined using 1 µ*M* l-propranolol.

c-AMP Production in Intact Cells

10^7 cells were incubated in 1 ml RPMI-1640 with 3-isobutyl-1-methyl xanthine (1 m*M*) at 37°C for 20 min and were incubated alone (basal values) or in the presence of the β-adrenoceptor agonist isoproterenol (10^{-7} *M*) for 10 more minutes. At the end of the incubation time, 2 ml of chilled ethanol were added. The cells were then homogenized and centrifuged at 3,500 *g* at 4°C for 15 min. The supernatants were collected and pellets were rehomogenized in 1 ml of ethanol:water (2:1) and centrifuged. Supernatants were joined and evaporated at 55°C under N_2 stream. The cAMP contained in the residue was dissolved in 0.3 ml of assay buffer (sodium acetate Tris-HCl 50 m*M*, pH 7.4, theophylline 8 m*M*, 2-mercaptoethanol 6 m*M*, EDTA 1 m*M*) and stored at −20°C until the assay was carried out. Aliquots of 100 µl were taken for nucleotide determination using the procedure described by Brown et al. [17].

Determination of Intracellular Ca^{2+} Concentration

Calcium concentration was measured by flow cytometry in B cells loaded with indo-1/AM in a FACStar Plus flow cytometer (Becton Dickinson) equipped with a 5-watt argon laser. Free indo-1 was measured by BP 488/10, and indo-1/Ca^{2+} fluorescence was recorded by BP 395/10, and a DM 425 was used to separate both fluorescences. Autofluorescence was measured by BP 530/30green, 5×10^3 cells per sample were obtained and analyzed in a C-30 Hewlett-Packard computer.

Determination of Protein Kinase C Activity

10^7 B cells were incubated for different times alone or in the presence of 2×10^6 mitomycin C-treated cells in a final volume of 1 ml of supplemented medium or in the presence of 1,000 µl of T cell supernatants. After incubation cells were immediately centrifuged (5,000 *g* for 30 s) and the cell pellets chilled on liquid N_2. The homogenization was performed in 2 ml of an ice-cold medium containing 300 m*M* sucrose, 10 m*M* β-mercaptoethanol, 2 m*M* EGTA, 2 m*M* EDTA, 1 m*M* PMSF, 10 µg/ml leupeptin and 20 m*M* HEPES, pH 7.4 (buffer A). After centrifugation at 100,000 *g* for 30 min, the supernatant (cytosolic fraction) was removed and the pellet (membrane fraction) carefully rinsed with 0.5 ml of buffer. To solubilize the particulate enzyme, the pellet was resuspended in 2 ml of buffer A containing 0.1% Nonidet P-40 and 2 m*M* EGTA and maintained for 30 min in an ice-cold bath. Under these conditions, maximal recovery of membrane-bound enzyme was obtained. Thereafter, the homogenate was centrifuged and the supernatant collected. The supernatants were filtered through a DE52 column (3.5 × 0.5 cm) equilibrated with buffer A. After washing with buffer A, the enzyme was eluted in a buffer containing 120 m*M* NaCl, 10 m*M* β-mercaptoethanol, 0.5 m*M* EGTA and 10 m*M* HEPES, pH 7.4 (buffer B).

Protein kinase C (PKC) activity was assayed by measuring the incorporation of ^{32}P from [γ-^{32}P]-ATP into the selective PKC substrate peptide, MBP (4–14) (Gibco), according to the method of Yasuda et al. [18]. Incubations were conducted for 10 min at 30°C in a final volume of 50 µl. In a final concentration, the assay mixture contained 20 µ*M* ATP, 50 µ*M* Ac-MBP (4–14), 1 m*M* $CaCl_2$, 20 m*M* $MgCl_2$, 20 m*M* Tris pH 7.5 and 1 µCi [γ-^{32}P]-ATP. After incubation, 25 µl of each tube were spotted onto phosphocellulose discs. Discs were washed twice in 500 ml of water with 1% (v/v) phosphoric acid and the radioactivity retained was determined by counting the disc in 2 ml of scintillation fluid. PKC activity was determined after substracting the ^{32}P incorporation in the presence of the PKC pseudosubstrate inhibitor peptide PKC (19–36) (Gibco). Data were expressed as picomoles of phosphate incorporated into the substrate per minute and per 10^7 cells.

Drugs

The following drugs were used in culture at the final concentrations indicated in the Results section. The protein kinase inhibitors 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) and *N*-(2-guanidinoethyl)-5-isoquinoline sulfonamide hydrochloride (HA 1004) were obtained from Seikagaku Kogyo (Tokyo, Japan). The protein kinase inhibitor H-7, ionophore A23187, calcium blocker verapamil, LPS and PMA were purchased from Sigma.

Statistical Analysis

After analysis of variance the Student-Newman-Keuls test was applied to determine the level of significance.

Results

Effect of in vitro Alloantigen Stimulation on B Cell β-Adrenoceptor Expression

In order to determine if the downregulation of β-adrenoceptors observed on B cells consecutively of in vivo alloimmunization could be obtained by in vitro allostimulation of B cells, we prepared macrocultures of spleen cells from BALB/c mice stimulated with mitomycin C-treated allogenic cells from C3H mice. Then, saturation assays on B cells purified from macroculture were performed. Figure 1 shows a representative binding study.

As it can be seen, the maximal binding capacity (B_{max}) decreased in allostimulated B cells. This phenomenon was not due to culture conditions, as B cells cultures with mitomycin C-treated BALB/c lymphocytes did not show a reduction in the β-adrenergic receptor number (fig 1). It is worth noting that modifications in the B_{max} values were observed on the 3rd day of culture, prior to the peak of proliferation (day 5), and remained between these levels at least until day 7. Furthermore, we found a significantly lower response to isoproterenol-stimulated cAMP pro-

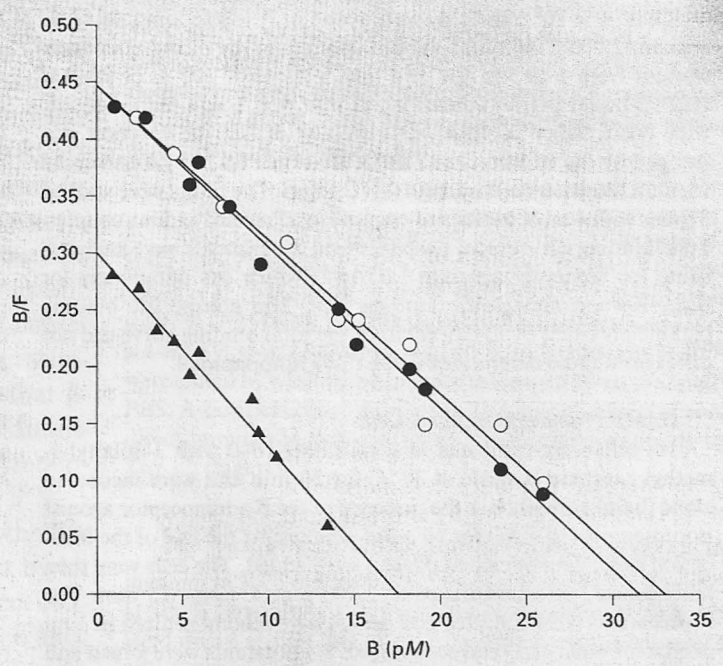

Fig. 1. ^{125}I-cyanopindolol (^{125}I-CYP) binding to B cell-enriched populations. Stimulated or unstimulated spleen cells from BALB/c mice were kept in culture for 3 days. ^{125}I-CYP binding was performed on intact purified B cells obtained from cultures as described in the Materials and Methods section. Scatchard plots are representative of 4 experiments performed in duplicate. Mean values of K_d and B_{max} ± SD are shown in the table below the figure together with cAMP levels obtained from 10^7 cells incubated alone or with isoproterenol 10^{-7} *M* for 10 min (n = 3 independent experiments performed in triplicate). * $p < 0.01$ vs. control values (unstimulated B cells).

Cell stimulant	Binding of ^{125}I-CYP		AMP levels, pmol/mg protein	
	K_d ± SE p*M*	B_{max} ± SE sites/cell	basal	stimulated
None (●)	68 ± 7	949 ± 58	15 ± 3	148 ± 15
Syngenic cells (○)	75 ± 9	994 ± 63	16 ± 2	154 ± 16
Allogenic cells (▲)	58 ± 6	536 ± 48*	11 ± 2	82 ± 9*

duction in in vitro allostimulated B cells than on control or on syngenic stimulated cells (fig. 1).

Participation of Soluble Factors in the Down Regulation of β-Adrenoceptors

We evaluated whether the direct activation of B cells by allogenic lymphocytes or the indirect action of soluble factors derived from alloreactive T cells induced changes in the sympathetic activity of B cells. When purified B cells were cultured with mitomycin C-treated allogenic cells a reduction in the number of β-adrenergic receptors was also obtained on day 3 of culture (table 1). In order to study the effect of T-cell-soluble factors, purified B cells were resuspended in supernatants from macrocultures of allostimulated T cells. In this experimental conditions we observed that T cell supernatants are capable of inducing the downregulation of β-adrenoceptor expression on purified B cells on days 2–3 of culture (table 1). However, in both situations, the observed reduction in B_{max} values was lower than that obtained on B cells purified from spleen mixed lymphocyte cultures. Given these facts, we performed cultures of purified B cells in the presence of allogenic cells and supernatants from allostimulated T cells. Under these experimental conditions the B_{max} values were similar to those obtained on B cells purified from spleen mixed lymphocyte cultures (table 1). Moreover, cAMP levels correlated to the changes observed in β-adrenoceptor expression (table 1). It is worth noting that we did not observe a proliferation of purified B cells in any case (data not shown).

PKC and Calcium Participation in the Downregulation of β-Receptors

We determined the involvement of early signals triggered after B cell activation, i.e. calcium mobilization and PKC activation in this phenomenon. As shown in fig-

Table 1. β-Adrenoceptor expression on B cells: effect of supernatants from allostimulated cultures

Cell stimulant	Binding of ^{125}I-CYP		cAMP levels, pmol/mg protein	
	K_d p*M*	B_{max} sites/cell	basal	stimulated
None	62±5	938±57	14±4	153±13
SS	59±4	965±75	16±3	143±11
AS	64±7	724±46*	12±4	109±11*
C3H cells	58±4	663±64*	11±4	99±11*
BALB/c cells	68±7	950±71	16±4	157±17
C3H cells plus SS	53±6	693±45*	12±3	102±10*
C3H cells plus AS	61±5	493±55**	9±3	77±8**

B cell-enriched populations purified from BALB/c mice were stimulated with T cell supernatants and/or with cells obtained as indicated in the Materials and Methods section and kept in culture for 3 days. ^{125}I-cyanopindolol (^{125}I-CYP) binding studies and cAMP level determination were performed on cells as indicated in figure 1. Data represent the mean value ± SD of three experiments performed in duplicate.

* $p < 0.05$; ** $p < 0.01$ vs. basal values. SS = Syngenic supernatants; AS = allogenic supernatants.

Table 2. Participation of PKC and calcium mobilization in β-adrenoceptor expression

Treatment[a]	^{125}I-CYP binding[b]		SI[c]
	K_d p*M*	B_{max} sites/cell	
None	56±6	915±48	–
Allostimulated	53±5	499±39*	1.1±0.3
Allostimulated + H-7 (5 μ*M*)	60±9	914±51	1.2±0.2
Allostimulated + staurosporine (1 n*M*)	59±7	968±58	1.1±0.1
Allostimulated + HA-1004 (5 μ*M*)	62±6	548±49*	1.4±0.2
PMA (2 n*M*)	62±5	1,052±73	1.3±0.2
A23187 (2 μ*M*)	65±7	900±63	1.1±0.3
Allostimulated + verapamil (10^{-5} *M*)	68±5	905±51	1.5±0.3
A23187 (2 μ*M*) + PMA (2 n*M*)	59±4	516±56*	3.3±0.5
LPS (20 μg/ml)	65±7	826±69	66±8*

Values represent mean ± SD. * $p < 0.01$ vs. basal values. ^{125}I-CYP = ^{125}I-cyanopindolol. SI = stimulation index.

[a] B cell-enriched populations were cultured for 3 days according to the indicated treatment. Allostimulated refers to cells stimulated with mitomycin C-treated allogenic cells plus supernatants from allostimulated T cells. Similar effects were observed when B cells were stimulated with allogenic cells or with the supernatants alone.

[b] B_{max} and K_d values were calculated as indicated in figure 1 and are from three independent experiments performed in duplicate.

[c] SI was calculated from the peak of proliferation.

ure 2a when fura 2-loaded BALB/c B cells were incubated with mitomycin C-treated C3H lymphocytes a change in the cytosolic Ca^{2+} was observed. A similar response in calcium mobilization was also obtained after incubating the cells with supernatants from allostimulated T cells. Concerning PKC involvement, it was observed that stimulation of BALB/c B cells with allogenic cells induced a rapid and transient PKC translocation from cytosolic to membrane fraction (fig. 2b). Similar results were obtained after treatment of B cells with supernatants from allostimulated T cells (fig. 2b). Control experiments were performed with mitomycin C-treated BALB/c cells and supernatants from syngenic culture observing no effect in both calcium mobilization or PKC activation (fig. 2a, b).

In order to evaluate the participation of calcium mobilization and PKC activation in the downregulation of β-receptors inhibitors and activators of both pathways were used. We found that H-7 (PK inhibitor) and staurosporine 10^{-9} *M* (PKC inhibitor) [19] impaired the decrease of β-adrenoceptor sites obtained on purified B cells (table 2). In contrast, HA-1004, in a concentration that does not affect PKC activity (less than 20 μ*M*) but inhibits cyclic nucleotide PK [20], did not show any effect (table 2). It is worth noting that inhibitor treatment alone in the absence of allostimulation did not modify the number of β-receptors (data not shown). On the other hand, treatment of B cells with the PKC activator, PMA, did not induce a reduction on β-adrenergic receptor expression, and even a sligth increase in these receptor sites was found on the 3rd day of culture (table 2). In order to study calcium participation B cells were treated with the calcium ionophore A23187. As shown in table 2, on day 3 we did not find a reduction in β-adrenergic sites. However, treatment of B cells with the calcium blocker verapamil abrogated the effect on the β-adrenoceptor number. It is important to note that the lack of effect of PMA or calcium ionophore alone was not due to a kinetic difference in the induction of receptor downregulation, as similar results were obtained on cells cultured at different times (data not shown). Finally, when B cells were treated with both calcium ionophore and PMA, a significant reduction of the β-adrenoceptor number was observed (table 2). Moreover, stimulation of B cells with LPS, mitogen that directly activate PKC without inducing calcium mobilization

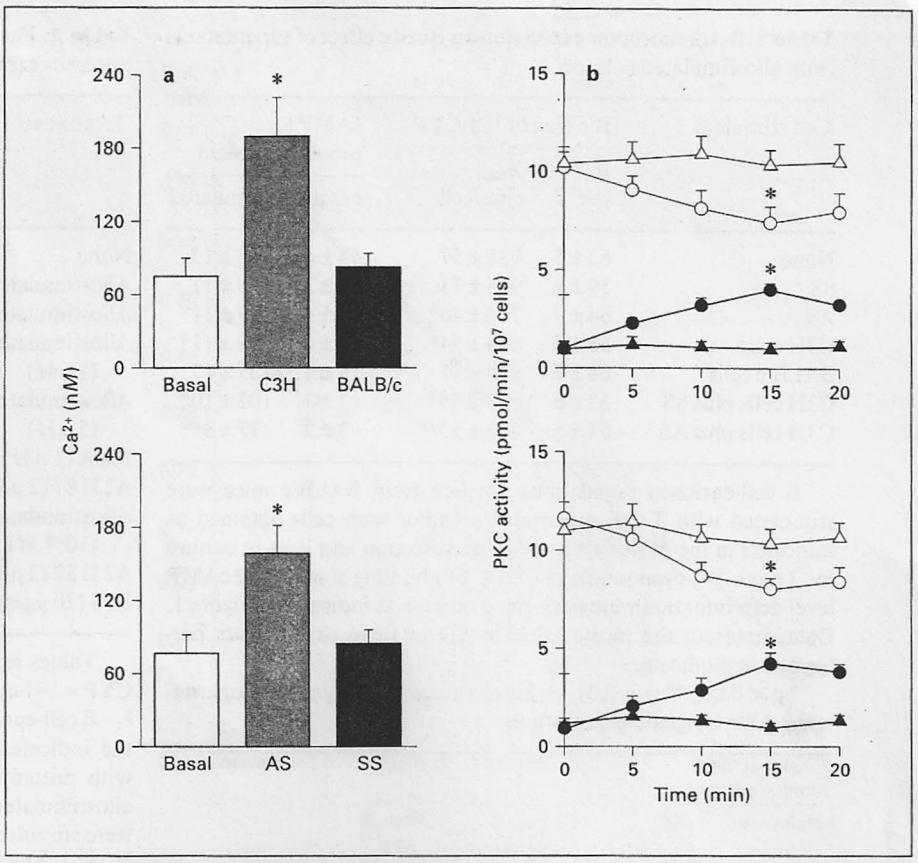

Fig. 2. Calcium mobilization and PKC activation in allostimulated B cells. **a** 10^6 B cells were loaded with indo-1/AM and analyzed by flow cytometry after stimulation with 2×10^5 C3H cells or BALB/c cells (upper panel) or with supernatants from allogenic (AS) or syngenic (SS) T cell culture (lower panel). The maximal calcium increase and the percentage of responding cells (6–8%) were quantified by this technique. Results are the mean ± SD of three independent preparations. **b** PKC was purified from subcellular cytosol (△, ○) and membrane (▲, ●) fraction from 10^7 B cells after allogenic (○, ●) or syngenic (△, ▲) stimulation with cells (upper panel) or supernatants (lower panel). PKC activity was measured using MBP [4–14] as substrate as indicated in the Materials and Methods section. Results are the mean ± SD of three experiments performed in duplicate. * $p < 0.05$ vs. basal values.

[21, 22], did not induce β-adrenoceptor downregulation on the 3rd day of culture (table 2) or at different tested culture times (data not shown). It is important to note that we did not observe any correlation between cell proliferation (see SI) and B_{max} values (table 2).

Discussion

We have previously shown that in vivo alloimmunization induced changes in β-adrenoceptor expression and cAMP levels on B lymphocytes. In addition, the antibody synthesis induced by allogenic stimulus was inversely proportional to the number of β-adrenoceptor sites on B cells [9]. Accordingly, Fuchs et al. [11] reported a downregulation of β-receptors after a challenge with sheep red blood cells and they suggested that this effect is secondary to an increased sympathetic outflow that occurs during the immune response [8].

The aim of the present work was to investigate whether this phenomenon is necessary under the control of the symphathetic system depending on the catecholamine levels or whether it could also be a direct consequence of the immune cell activation by antigen stimulation.

According to our results, the downregulation of β-adrenoceptor related to the lower cAMP levels occurs as a direct consequence of antigen-responding cell activation. A significant decrease in β-receptor sites in B cells, purified from in vitro allostimulated spleen cells, was observed. Changes in the expression of various pharmacological receptors, including β-receptors, induced by mitogen and antigenic stimulation in vitro have been reported [12, 23–25]. It has been proposed that hormone and neurotransmitter receptor variation could be a regulatory mechanism for cellular activity [8, 9]. Thus, it was demonstrated that antibody formation from stimulated B cells is inversely related to β-adrenoceptor expression [9]. It is reasonable to think that antigen recognition by immune cells would be a more specific mechanism involved in β-adrenergic site downregulation than the release of catecholamines. These effects could be a consequence of the direct activation of B cells by antigen recognition and/or secondary to the action of lymphokines released by other activated cells. As alloreactive T cells play a key role in the

alloimmune response [26], we analyzed the participation of soluble factors derived from allostimulated T cells on B cell downregulation of β-adrenoceptors. According to our results a decrease in β-adrenoceptor expression was obtained on purified naive B cells directly stimulated with alloantigen or exposed to the action of T cell-derived soluble factors, althought both effects were quantitatively lower than the downregulation observed on B cell purified from mixed splenocyte cultures. However, when purified B cells were allostimulated in the presence of T cell soluble factors, a downregulation was observed, resembling the one obtained by performing mixed lymphocyte cultures.

In previous reports we demonstrated that the stimulation of naive B cells from BALB/c mice with allogenic lymphocytes from C57BL/6 mice induces the activation of the phosphatidylinositol-specific phospholipase C that in turn promotes an increase in calcium mobilization and a rapid and transient activation of PKC, but fails to induce proliferation [27]. In the present study, we confirm that B cells from BALB/c activated with allogenic cells from C3H mice exhibited an early Ca^{2+} increase as well as translocation of PKC. According to this observation, supernatants from allostimulated T cells were able to induce calcium mobilization and PKC activation. Some T cell-derived soluble factors such as interleukins IL-2, IL-4, IL-5 and IL-6 have a number of biological effects on B cell lineage and the existence of an intricate interplay of agonistic and antagonistic cytokine effects that modulate B cell responses was demonstrated [28]. Besides, both PKC activation and calcium mobilization were reported to be involved in the signal transduction mechanisms triggered by some interleukins [28–30]. Given these findings, we studied whether these intracellular events were involved in the downregulation of β-adrenoceptors. The results obtained in the presence of PK inhibitors indicate that PKC, but not protein kinase A, participate in this phenomenon. However, treatment of B cells with the PKC activator PMA had no effect in the expression of β-adrenergic sites. In order to study the participation of calcium mobilization, B cells were incubated with the calcium ionophore A23187 but it did not have any effect. However, the calcium blocker verapamil abrogated the effects induced by allogenic cells. Moreover, when B cells were incubated with both A23187 and PMA, a significant reduction in β-adrenoceptor sites was achieved. Furthermore, the incubation of B cells with the mitogen LPS, which induces PKC translocation but not calcium mobilization, did not induce downregulation of β-receptors. Considering our results, it is reasonable to conclude that both early signals, calcium mobilization and PKC activation, elicited in B cells after direct allogenic stimulation and/or by the action of soluble factors derived from T alloreactive cells, are necessary in order to induce a reduction in β-adrenoceptor sites. It is probable that a specific PKC calcium-dependent subtype may be involved in the downregulation. It has been demonstrated that activated mouse lymphocytes can synthesize catecholamines with a capacity for regulating lymphocyte function [31]. This system may be activated in allocultures and accounts for the downregulation of β-receptors. However, according to the results obtained using protein kinase activity inhibitors, this possibility would not be involved in the phenomenon. Thus, inhibition of PKA did not modify the alloantigen-induced downregulation and the inhibition of PKC activation impaired this effect.

The downregulation observed after allostimulation was not correlated to proliferation of B cells, at least for naive B cells. It is possible that these findings represent a regulatory mechanism that is necessary to elicit an efficient secondary response. In fact, it was demonstrated that allostimulation promotes changes on B cells leading to cell proliferation [27]. Moreover, it was reported that downregulation of β-receptors in B cells is associated with a significant increase in alloantibody formation [9]. It is possible to postulate that the genetic signals induced by the biochemical cascade of intracellular messengers triggered after alloantigen recognition by B cells would be involved in downregulation of β-adrenoceptors as a mechanism to prepare the cell for an optimal B cell activity. In fact evidence of β-adrenoceptor downregulation as a change in the synthesis of receptors at the gene level was demonstrated in thymocytes after an immunogenic challenge [32]. Furthermore, the marked decrease in the β-adrenoceptor number and their mRNA levels found early after immunization were related to the regulation of immune reactivity in response to an antigenic signal [32]. Finally, the present report could be of interest to understand the neuroendocrine mechanisms involved in the regulation of B cell activation.

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