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Differential regulation of mouse B-cell activation by β-adrenoceptor stimulation depending on type of mitogens

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SUMMARY

We investigated the effects of the β-adrenoceptor agonist isoproterenol (ISO) and the α- and β-adrenoceptor agonist norepinehrine (NE) on murine B-cell activation. Cells were stimulated either by anti-mouse μ-chain antibodies (anti-μ), or by lipopolysaccharide (LPS), or a membrane proteoglycan of *Klebsiella pneumoniae* (Kp MPG), a T-independent polyclonal activator distinct from LPS, which induces B-cell proliferation and Ig synthesis. ISO and NE enhanced LPS- and Kp MPG-induced B-cell proliferation and maturation into IgM-, IgG- and IgA-secreting cells. The enhancement was prevented by prior addition of the β-adrenoceptor antagonist propranolol but not by the α-adrenoceptor antagonist phentolamine. Earlier events in the LPS- and Kp MPG-stimulated B-cell activation, such as increases in Ia antigen expression and RNA synthesis, were not modified by the catecholamines. Unlike ISO and NE, the membrane-permeant cyclic adenosine 3′,5′-monophosphate (cAMP) analogue dibutyryl cAMP (dbcAMP), and the potent adenylate cyclase activator forskolin did not enhance but even inhibited DNA synthesis and Ig secretion stimulated by LPS and Kp MPG. In addition, ISO and NE did not enhance but strongly inhibited anti-μ-induced B-cell proliferation, and these effects were mimicked by dbcAMP and forskolin. Collectively, the data demonstrate that β-agonists differently modulate B-cell activation depending upon the polyclonal activator, and provide additional evidence for distinct biochemical mechanisms of B-cell activation by anti-μ and LPS. Moreover, our results indicate that β-adrenergic stimulation up-regulates B-cell responses to LPS and Kp MPG by a novel and cAMP-independent pathway.

INTRODUCTION

Immune responses may be regulated by catecholamines released by autonomic nerve fibers in both primary and secondary lymphoid organs (Felten *et al.*, 1985). Inhibitory effects of the catecholamines have been generally observed on lymphocyte and accessory cell functions in human as well as in animal models, as a result of negative signals delivered via interaction with β-adrenoceptors. The β-adrenoceptor-mediated immunosuppressive effects include inhibition of mitogen-induced T-cell proliferation (Hadden, Hadden & Middleton, 1970), interleukin-2 (IL-2) synthesis and IL-2 receptor expression (Feldman, Hunninghake & McArdle, 1987), suppression of interleukin-1 (IL-1) production by activated macrophages (Koff *et al.*, 1986) and inhibition of Ia antigen expression on interferon-gamma (IFN-γ)-stimulated astrocytes (Frohman *et al.*, 1988). Some positive effects of β-agonists have also been reported, including potentiation of the cytotoxic T-lymphocyte response (Felten *et al.*, 1987), and enhancement of IgM anti-sheep erythrocyte plaque-forming cell response *in vitro* (Sanders & Munson, 1984). Virtually all these stimulatory and inhibitory effects of the β-agonists are classically related to activation of the adenylate cyclase complex, which leads to rapid and transient accumulation of intracellular cyclic adenosine 3′,5′-monophosphate (cAMP) levels (Coffey & Hadden, 1985).

Recently, we have shown that the α- and β-adrenoceptor agonist norepinephrine (NE) enhanced lipopolysaccharide (LPS)-induced mouse B lymphocyte proliferation and Ig secretion through stimulation of β-adrenoceptors, with concomitant increase in intracellular cAMP levels in the B cells (Kouassi *et al.*, 1988). Since B cells can be activated by different mitogens via distinct biochemical pathways, it was of interest to determine whether the up-regulation effects of β-adrenoceptor stimulation were selective to the LPS model. This study was designed to investigate the effects of NE and of the β-adrenoceptor agonist isoporoterenol (ISO) on mouse B cells activated by anti-μ

Abbreviations: Anti-μ, goat anti-mouse μ-chain antibodies; cAMP, cyclic adenosine 3′,5′-monophosphate; dbcAMP, dibutyryl cAMP; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; IL-1, interleukin-1; IL-2, interleukin-2; ISO, isoproterenol; Kp MPG, membrane proteoglycan of *Klebsiella pneumoniae*; LPS, lipopolysaccharide; NE, norepinephrine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C.

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antibodies, or by LPS or a membrane proteoglycan of *Klebsiella pneumoniae* (Kp MPG), which induces B-cell proliferation and Ig production without T-cell help (Millet *et al.*, 1987), and to assess the role of cAMP as second messenger in these systems. The results demonstrate that stimulation of β-adrenoceptors down-regulates anti-μ-induced B-cell proliferation via elevated intracellular levels of cAMP, and up-regulates LPS- and Kp MPG-induced B-cell proliferation and Ig production by a cAMP-independent mechanism.

MATERIALS AND METHODS

Mice

Male BALB/c mice, 2–3 months old, were bred in our laboratory.

Reagents

Isoproterenol hydrochloride, norepinephrine bitartrate (Winthrop, Clichy) and phentolamine methanesulphonate (Ciba-Geigy, Basle, Switzerland) were obtained in commercially available ampules. Propranolol hydrochloride, pargyline hydrochloride, dbcAMP and forskolin were purchased from Sigma Chemical Co. (St Louis, MO). LPS from *Escherichia coli* 0111:B4 was obtained from Difco laboratories (Detroit, MI). The membrane proteoglycan from a mutant non-encapsulated strain of *Klebsiella pneumoniae* (Kp MPG, batch GN 415) was kindly provided by Pierre Fabre SA (Castres). Affinity-purified F $(ab')_2$ goat anti-mouse IgM (anti-μ) antibodies were purchased from Cappel (West Chester, PA), concanavalin A (Con A) from IBF (Villeneuve la Garenne), and pokeweed mitrogen (PWM) from Gibco (Grand Island, NY).

B-cell preparation and culture

Mice were killed by cervical dislocation and spleens were aseptically removed and placed into cold Hanks' balanced salt solution (HBSS). Cell suspensions were obtained by gentle teasing and filtered through nylon-wool to eliminate aggregates. Erythrocytes were lysed by treatment with Gey's solution. T lymphocytes were depleted by treating the splenocytes with a cocktail of rat mAb, consisting of the anti-Thy-1.2 mAb HO.13.4 (Marshak-Rothstein *et al.*, 1979), the anti-L3T4 mAb GK1.5 (Dialynas *et al.*, 1983) and the anti-Lyt-2 mAb AD4.15 (Raulet, Gottlieb & Bevan, 1980), plus 1:5 Low tox M rabbit complement (Cedarlane Laboratories, Hornby, Ontario, Canada). Dead cells were eliminated by centrifugation over Lympholyte M cell separation medium (Cedarlane Laboratories) followed by washing in HBSS. The resultant cell suspension, henceforth referred to as B cell, contained less than 3% of Thy-1.2-positive cells determined by immunofluorescence staining with a fluorescein isothiocyanate (FITC)-conjugated anti-Thy-1.2 antibody, and there was no proliferation after Con A and phytohaemagglutinin stimulation.

B cells were cultured at 10^6/ml in RPMI-1640 medium (bioMérieux, Charbonnières-les-Bains, France), supplemented with L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (50 μg/ml) and 10% of FCS (Boehringer, Mannheim, FRG), in 96-well flat-bottomed plastic culture plates (Costar, Cambridge, MA). Cells were incubated for different times at 37° in an humidified air containing 7% CO_2, with or without various mitogens (LPS or Kp MPG 50 μg/ml of each, or anti-μ 40 μg/ml) and with different concentrations of drugs.

Assays for B-cell activation

Expression of MHC class II (Ia) antigen at 24 hr after stimulation with various mitogens was determined by indirect staining with a monoclonal anti-mouse I-A^d antibody of IgG isotype (Becton-Dickinson, Mountain View, CA) followed by FITC-conjugated goat anti-mouse IgG antibody (Southern Biotechnology, Birmingham, AL).Relative fluorescence was analysed with a Cytofluorograf 50H (Ortho Instruments, Westwood, MA). Cells incubated with the FITC-conjugated goat anti-mouse IgG second antibody alone served as controls.

RNA and DNA syntheses were measured by quantifying levels of [^{3}H]uridine ([^{3}H]UdR) and [^{3}H]thymidine ([^{3}H]TdR) incorporation, respectively. [^{3}H]UdR (1 μCi) was added per 0·2 ml of culture for the last 6 hr of a 24-hr incubation period, and [^{3}H]TdR (1 μCi) for the last 24 hr of a 3-day culture period, and incorporation into the nuclei was measured by liquid scintillation with a Packard scintillation counter.

The amount of Ig in 6-day culture supernatants was determined by a sandwich-type enzyme-linked immunoadsorbent assay. Briefly, microtitre plates (Nunc, Copenhagen, Denmark) were coated with goat anti-mouse IgM, IgG or IgA (Zymed, San Francisco) in veronal-buffered saline (VBS), pH 8·6. After three washings in VBS containing 0·1% BSA, 100 μl of serial dilutions of culture supernatants were added to duplicate wells. After incubation for 1 hr at 37° and three washings, 100 μl of alkaline phosphatase-conjugated isotype-specific goat anti-mouse IgM, IgG and IgA (Zymed) were added to appropriate wells. After incubation and washing, substrate, *p*-nitrophenyl phosphate (Sigma) was added to each well. Microplates were incubated 1 hr at 37° and optical densities were measured at 405 nm on a Kontron SLT 210 reader. Calibration curves were simultaneously prepared in each experiment by using normal mouse serum containing 0·504 mg/ml IgM, 7·04 mg/ml IgG and 3·41 mg/ml IgA, and Ig isotype concentrations were calculated by interpolation.

RESULTS

ISO and NE enhance DNA synthesis and Ig secretion by LPS- and Kp MPG-stimulated B cells

As shown in Fig. 1, ISO and NE enhanced DNA synthesis by mouse B cells stimulated by LPS (50 μg/ml) or Kp MPG (50 μg/ml) in a dose-dependent fashion. ISO was more potent than NE. The enhancing effects of ISO on Kp MPG-induced B-cell proliferation could be blocked by the β-adrenoceptor antagonist propranolol (Fig. 2). NE effect was also blocked by prior addition of propranolol, but not by the α-adrenoceptor antagonist phentolamine (Fig. 2) showing the β-adrenoceptor specificity of the phenomenon. ISO (10^{-5}M) and NE (5×10^{-5}M) also enhanced LPS and Kp MPG-induced IgM, IgG and IgA secretion (Fig. 3). The catecholamines had no effect on the background [^{3}H]TdR incorporation and Ig secretion by B cells in the absence of mitogenic stimulus (not shown).

Effects of ISO and NE on B-cell response to other mitogens

Because anti-Ig can activate B lymphocytes to proliferate by mechanisms distinct from that of LPS (Bijsterbosch *et al.*, 1985), it was of interest to assess the effects of ISO and NE on anti-μ-induced B-cell proliferation. As shown in Fig. 4, ISO and NE did

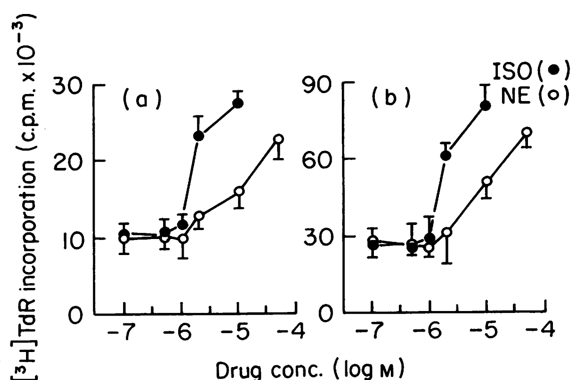

Figure 1. Enhancement of LPS- (a) and Kp MPG (b)-induced proliferative response by ISO and NE. Spleen B cells were cultured at 10^6/ml with LPS or Kp MPG in the presence or absence of ISO or NE at the indicated concentrations. Uptake of [^{3}H]TdR was measured on Day 3 of culture. Values are means ± SD of triplicate cultures.

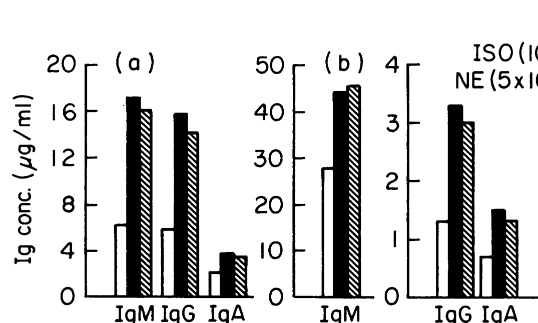

Figure 3. Enhancement of LPS (a) or Kp MPG (b) induced polyclonal Ig secretion by ISO and NE. B cells were cultured in the same conditions as indicated under Fig. 1, and Ig secretions (μg/ml) were measured by isotype-specific ELISA in the supernatants of 6-day culture.

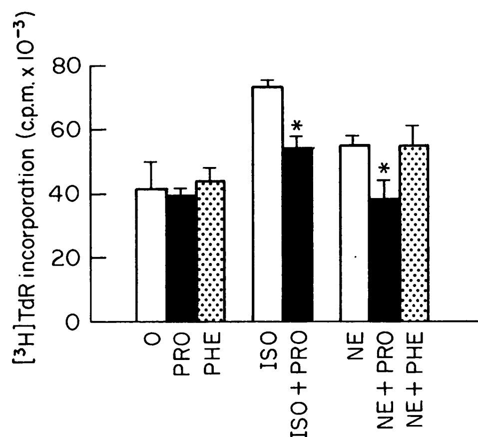

Figure 2. β-adrenoceptor specificity of the enhancing effects. Spleen B cells (10^6/ml) were cultured with Kp MPG in the presence or absence of ISO (10^{-5}M) or NE (10^{-5}M) with or without propranolol (PRO, 10^{-5}M) or phentolamine (PHE, 10^{-5}M) and [^{3}H]TdR incorporation was measured at 72 hr of culture. Values represent means ± SD of triplicate cultures. Controls with PRO (10^{-5}M) or PHE (10^{-5}M) alone are also shown. * $P < 0{\cdot}01$ compared with cultures without PRO, Mann–Witney *U*-test.

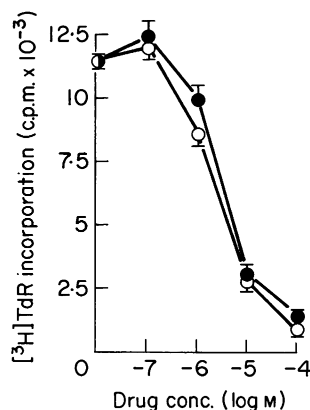

Figure 4. Inhibitory effects of ISO and NE on anti-μ-induced B-cell proliferation. Spleen B cells (10^6/ml) were cultured with anti-μ (40 μg/ml) in the presence or absence of ISO (○) or NE (●) at the indicated concentrations. Incorporation of [^{3}H]TdR was measured at Day 3. Mean values ± SD of triplicate cultures are shown. Background incorporation was 302 ± 26 c.p.m.

not enhance but dose-dependently and quite completely abolished [^{3}H]TdR incorporation into B lymphocytes stimulated by anti-μ. ISO and NE also dose-dependently inhibited DNA synthesis and IgM and IgG secretion by total spleen cell stimulated by Con A and PWM (data not shown).

Effects of ISO and NE on early stages of B-cell activation

Proliferation and maturation are late events in B-cell activation. To determine whether β-agonists influence earlier activation events, we measured their effects on increases in Ia antigen expression which accompanies the transition from G0 to G1 in the cell cycle (Mond *et al.*, 1981), and on RNA synthesis, which also occurs during G1 phase (Darzynkiewicz *et al.*, 1980). Concentrations of ISO (10^{-5}M) and NE (5×10^{-5}M), which were optimal to enhance B-cell proliferation and Ig production, had no effect on increases in Ia antigen expression and RNA synthesis induced by LPS and Kp MPG (Table 1).

The enhancement of B-cell activation by β-adrenoceptor agonists is not related to elevation of cAMP level

In view of the possibility that the enhancement of LPS- and Kp MPG-induced B-cell activation by β-adrenergic stimulation may be mediated by cAMP, the effects of two other cAMP-elevating agents, dbcAMP and forskolin, were assessed in this functional model. As shown in Fig. 5, dbcAMP and forskolin did not enhance but actually and dose-dependently inhibited [^{3}H]TdR incorporation stimulated by LPS and Kp MPG, though seemingly less efficiently than that stimulated by anti-μ. In addition, secretion of the three major Ig classes stimulated by LPS and Kp MPG was inhibited by dbcAMP and forskolin (Table 2).

Table 1. Lack of effect of ISO and NE on mitogen-induced increases in Ia Ag expression and [^{3}H]UdR incorporation*

Mitogens	Drugs	Mean fluorescence intensity† 12 hr	24 hr	[^{3}H]UdR‡ (c.p.m. × 10^{-3})
0	0	7·7	8·1	9·1 ± 1·6
LPS	0	9·1	9·6	31·0 ± 1·6
	ISO	9·7	9·3	32·1 ± 1·8
	NE	9·3	9·5	30·7 ± 1·1
Kp MPG	0	9·4	9·6	38·7 ± 2·5
	ISO	9·1	10·7	46·1 ± 2·7
	NE	9·6	10·4	45·1 ± 2·6

*Spleen B cells were cultured with 50 μg/ml LPS or Kp MPG in the presence or absence of ISO (10^{-5}M) or NE (5 × 10^{-5}M) for the periods indicated.

†Ia antigen expression was detected by indirect immunofluorescence with a mouse anti-I-A^d mAb (IgG) and FITC-conjugated goat anti-mouse IgG. Fluorescence intensity (log scale) was analysed by cytofluorometry.

‡RNA synthesis was measured as [^{3}H]UdR incorporation during the last 6 hr of a 24-hr incubation period. Mean ± SD values of triplicate cultures are shown.

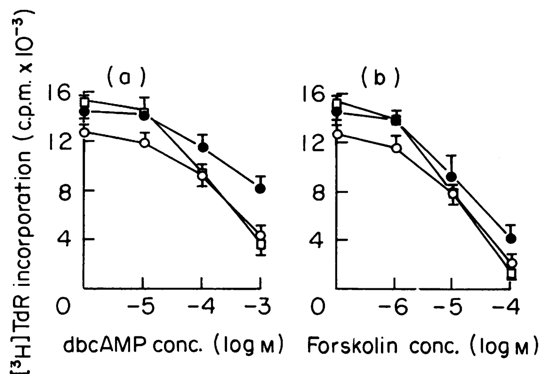

Figure 5. Dose-dependent inhibition of mitogen-induced B-cell proliferation by dbcAMP and forskolin. Spleen B cells were cultured with or without LPS (○) (50 μg/ml), Kp MPG (●) (50 μg/ml), or anti-μ (□) (40 μg/ml) in the presence or absence of increasing concentrations of dbcAMP (a) and forskolin (b). Incorporation of [^{3}H]TdR was measured at Day 3. Values are means ± SD of triplicate cultures,and background incorporation averaged 540 ± 48 c.p.m.

DISCUSSION

We have previously shown that the physiological catecholamine NE increases intracellular cAMP levels in mouse B lymphocytes and amplifies their proliferation and maturation into IgM-, IgG- and IgA-secreting cells induced by LPS (Kouassi *et al.*, 1988). These effects are specifically mediated by β-adrenoceptors. In the present study, we undertook further analysis of the effects of β-adrenoceptor stimulation and of cAMP as second messenger on mouse B-cell activation by different polyclonal activators. We found that ISO and NE enhance B-cell growth and differentiation induced not only by LPS but also by Kp MPG, another T-independent polyclonal B-cell activator, and IL-1 inducer distinct from LPS (Millet *et al.*, 1987). The β-adrenoceptor antagonist propranolol but not the α-adrenoceptor antagonist phentolamine prevents the enhancement.These effects concern the S/G2 phase in the B-cell cycle, but not the transition from G0 to G1 because activation events characteristic of the G1 phase such as increases in Ia Ag expression and RNA synthesis are not modified by the β-agonists. In contrast to their enhancing effects on LPS and Kp MPG responses, β-agonists suppress anti-μ-induced B-cell proliferation. These results indicate that β-adrenergic modulation of B-cell activation may be cell cycle-specific and dependent upon the type of polyclonal activator. They also suggest that LPS and Kp MPG may use similar biochemical signalling pathways but distinct from that of anti-μ to induce B-cell activation.

There is considerable evidence that cross-linking of surface Ig by anti-Ig antibodies mimics antigen binding and triggers, in the initial stages of B-cell activation process, the phospholipase C-catalysed phosphatidylinositol 4, 5-bisphosphate (PIP_2) hydrolysis, with formation of two second messengers, diacylglycerol which activates protein kinase *C* (PKC) and inositol trisphosphate which mobilizes intracellular calcium (Coggeshall & Cambier, 1984; Bijsterbosch *et al.*, 1985). In contrast, LPS does not induce PIP_2 hydrolysis or calcium mobilization in resting B cells (Bijsterbosch *et al.*, 1985; Grupp & Harmony, 1985), but it may directly activate PKC (Wightman & Raetz, 1984; Bosca & Diaz-Guerra, 1988). However, other mechanisms might be involved in B-cell stimulation by LPS because of the following evidence: (i) PKC activation alone by phorbol esters is not sufficient to drive resting B cells into the cell cycle (Klaus *et al.*,1986); and (ii) LPS still induces DNA synthesis by B cells in which PKC has been desensitized (Mond *et al.*, 1987). LPS signalling may also involve the activation of an inhibitory guanine nucleotide-binding (G_i)-like protein (Defranco, Gold & Jakway, 1987). The concordance of the inhibitory effects of β-agonists on the anti-μ response with that of other substances

Table 2. Inhibition of Ig synthesis by dbcAMP and forskolin

Mitogen	Drug	Ig (μg/ml)* IgM	IgG	IgA
0	0	0·3	0·19	0·15
LPS	0	29·4	3·55	2·08
	dbcAMP (5 × 10^{-4}M)	11·5	1·88	0·98
	Forskolin (10^{-5}M)	11·4	0·99	0·83
Kp MPG	0	33·5	2·54	2·00
	dbcAMP (5 × 10^{-4}M)	6·6	0·53	0·87
	Forskolin (10^{-5}M)	18·8	1·81	1·58

*Spleen B cells were cultured with or without LPS (50 μg/ml) or Kp MPG (50 μg/ml) in the presence or absence of dbcAMP and forskolin at the indicated concentrations, and Ig concentrations in 6-day culture supernatants were measured by ELISA. Values are means of triplicate cultures.

which raise cAMP implies a role for cAMP. These data correlate those of a previous study (Klaus, Vondy & Holman, 1987) showing a high susceptibility of anti-Ig-stimulated B-cell proliferation to inhibition by various cAMP-elevating agents. It has been shown that elevated intracellular cAMP levels inhibit PIP_2 turnover in anti-Ig-stimulated B cells (Coggeshall & Cambier, 1984), in activated T cells (Lerner, Jacobson & Miller, 1988), and in a variety of other cells, thus precluding full cellular activation and entry into the cell cycle.

Elevated intracellular levels of cAMP may also act at different levels to inhibit B-cell responses to LPS and Kp MPG. However, the finding that β-agonists up-regulate LPS- and Kp MPG-induced B-cell growth and maturation, while dbcAMP and forskolin are inhibitory in the same conditions, is striking. In addition, the potentiating effects of the β-agonists occurs despite elevated cAMP levels induced in the B cells (Kouassi *et al.*, 1988). Altogether the data suggest that, in the LPS and Kp MPG model, β-agonists are not acting through the classical adenylate cyclase pathway. Further studies are needed to elucidate the mechanisms involved.

The enhancing effect of β-agonists on LPS- and Kp MPG-induced B-cell activation may be exerted either directly on the B cells, or indirectly on regulatory cells via their secretory products. In this context, any role of T cells can be ruled out because of extensive depletion of Thy-1.2^{+}, CD4^{+} and CD8^{+} cells in the preparation of our B cells. Furthermore, the enhancing effects of β-agonists on LPS-stimulated B-cell activation are similar in athymic nude spleen cells and in the presence of T lymphocytes in unseparated BALB/c spleen, lymph node and Peyer's patch cells (Kouassi *et al.*, 1988). The contribution of adherent cells is also unlikely because their depletion does not affect the enhancing effect of NE on the LPS response (Kouassi *et al.*, 1988). Furthermore, β-agonists reportedly inhibit IL-1 production by mouse peritoneal macrophages activated by LPS plus IFN-γ (Koff *et al.*,1986). This effect, if present, would result in inhibition of LPS- and Kp MPG-induced B-cell activation, as opposed to the observed data.

Many of the immune regulatory effects of β-agonists are in keeping with those of glucocorticoids which also suppress T lymphocyte and accessory cell functions, and enhance B-cell responses (Akahoshi, Oppenheim & Matsushima, 1988). Glucocorticoids can up-regulate β-adrenoceptor number and responsiveness on mammalian lymphocytes (Cotecchia & De Blasi, 1984). In conclusion, our results support the concept that glucocorticoids and catecholamines induced by various stress-stimuli may act in conjunction to down-regulate T-cell functions, T-dependent and antigen-specific B-cell responses, and to up-regulate Ig production.

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