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A role for histamine in cytokine modulation by the adenosine A_3 receptor agonist, 2-Cl-IB-MECA

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Abstract

The effects of adenosine receptor agonists on cytokine production in vivo were investigated in mouse models of endotoxemia. Selective adenosine A_3 (2-chloro-N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide) (2-Cl-IB-MECA) and A_{2A} (2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamido adenosine hydrochloride) (CGS 21860) receptor agonists were found to modulate endotoxin-induced cytokine responses in mice sensitized to D-galactosamine or primed with *Corynebacterium parvum*. The adenosine receptor agonists had similar effects in these models of endotoxemia, suppressing the production of tumor necrosis factor α (TNF-α) and interleukin-12 while enhancing that of interleukin-10. However, 2-Cl-IB-MECA also caused a dramatic increase in circulating histamine levels shortly after its injection into mice. The cytokine modulatory activities of 2-Cl-IB-MECA were mimicked by the mast cell depleting compound 48/80 and both drugs only produced such effects at doses that caused an elevation in circulating histamine levels. Furthermore, the capacity of 2-Cl-IB-MECA to modulate cytokine responses was greatly diminished when the drug was administered to mast cell deficient (WBB6F-W/W^V) mice. Together, these results strongly suggest a role for histamine in cytokine modulation by 2-Cl-IB-MECA. Cimetidine, a histamine H_2 receptor antagonist, did not reverse cytokine modulation by 2-Cl-IB-MECA and pyrilamine, a histamine H_1 receptor antagonist, prevented the increase in serum histamine that was induced by 2-Cl-IB-MECA. This effect of pyrilamine and other histamine H_1 receptor antagonists confounded attempts to determine a role for the histamine H_1 receptor in cytokine modulation by 2-Cl-IB-MECA. However, under some experimental conditions, pyrilamine appeared to antagonize the modulatory effects of the adenosine A_3 receptor agonist on cytokine responses. The apparent antagonism of pyrilamine was unrelated to its suppressive effects on histamine release and appeared to reflect activity at the level of the histamine H_1 receptor.

Keywords: 2-Cl-IB-MECA; Histamine; Pyrilamine; Adenosine receptor; Cytokine modulation

1. Introduction

The ability of adenosine and adenosine analogs to alter inflammatory responses and various immune functions has stimulated interest in developing therapeutic modalities that target specific adenosine receptors. The anti-inflammatory activities of adenosine compounds have been observed in multiple experimental models. In a mouse model in which air pouches were formed and injected with carrageenan, leukocyte accumulation in the pouches was inhibited by {4-amino-1-(5-amino-5-deoxy-1-β-D-ribofuranosyl)-3-bromo-pyrazol[3,4-d] pyrimidine}, GP515 an adenosine kinase inhibitor. Exudates harvested from the pouches of inhibitor-treated mice contained elevated levels of adenosine and leukocyte accumulation was not inhibited, when adenosine levels were reduced by injecting adenosine deaminase into the pouch with carrageenan (Cronstein et al., 1995). These observations suggested that the anti-inflammatory effects of GP515 were caused by adenosine, which appeared to block leukocyte accumulation through activation of adenosine A_2 receptors, since the effect was reversed by treatment with a specific adenosine A_2 receptor antagonist. In a subsequent study, aspirin and sodium salicylate were also found to block leukocyte accumulation in a carrageenan injected mouse air pouch. The decrease in leukocyte accumulation did not result from the inhibitory effects of the drug on prostaglandin synthesis, was largely mediated by adenosine and involved the adenosine A_2 receptor (Cronstein et al., 1999). In an in vitro study, adenosine receptor agonists were shown to

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inhibit degranulation and superoxide anion (O_2^-) generation by human neutrophils stimulated with the chemotactic peptide *f*-methionyl-leucyl-phenyalanine (FMLP) (Schrier et al., 1990). Other findings suggest that adenosine analogs act via adenosine A_2 and A_3 receptors to inhibit the release of granule proteins from activated human neutrophils (Bouma et al., 1997).

Adenosine receptor agonists can have pro-inflammatory effects as evidenced by the ability of selective adenosine A_1, A_2 and A_3 receptor agonists to induce an edematous response when injected into the hindpaw of rats (Sawynok et al., 1999). The edema caused by adenosine A_1 (N^6-cyclopentyladenosine) and $A_{2B/3}$ (N^6-benzyl-5′-*N*-ethylcarboxamidoadenosine: N^6-B-NECA) receptor agonists was blocked by mepyramine and ketaserin, histamine H_1 and 5-HT_2 receptor antagonists, respectively. These findings implicated mast cell derived mediators in the pro-inflammatory effects of the agonists which is consistent with the reported expression of adenosine A_{2B} and A_3 receptors by rat mast cells (Ramkumar et al., 1993; Linden, 1994; Reeves et al., 1997).

The regulatory effects of adenosine and adenosine agonists on cytokine responses have been widely investigated. In general, adenosine receptor agonists were found to down-regulate the production of inflammatory cytokines (tumor necrosis factor α and interleukin-12) and to up-regulate the production of interleukin-10. The exact adenosine receptor(s) involved were not identified in some of the early studies with monocytes/macrophages and monocytic cell lines (Le Vraux et al., 1993; Le Moine et al., 1996). However, results obtained in studies with more selective adenosine analogs indicate that the activation of adenosine A_1, A_2 or A_3 receptors can lead to modulated cytokine responses by monocyte/macrophage cells (Hasko et al., 1996, 2000; Link et al., 2000; Khoa et al., 2001). Similar effects have been observed in vivo and selective adenosine A_{2A} and A_3 receptor agonists have both been shown to inhibit the production of tumor necrosis factor α and to augment the production of interleukin-10 in endotoxemic mice (Hasko et al., 1996, 1998; Bowlin et al., 1997). Because of questions surrounding the selectivity of some of the agents that were used as adenosine A_3 receptor agonists, it was not clear until recently that cytokine responses could indeed be modulated through that receptor. A highly selective adenosine A_3 receptor agonist (2-chloro-N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide: 2-Cl-IB-MECA) was shown to suppress an endotoxin-induced tumor necrosis factor α response in D-galactosamine sensitized wild-type ($A_3R^{+/+}$) mice, but not in mice with a targeted disruption of the adenosine A_3 receptor gene ($A_3R^{-/-}$ mice). By contrast, a selective adenosine A_{2A} receptor agonist, 2-*p*-(2-carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamido adenosine hydrochloride: CGS 21680) suppressed the response in both $A_3R^{+/+}$ and $A_3R^{-/-}$ mice (Salvatore et al., 2000). These experiments confirmed the selectivity of 2-Cl-IB-MECA for the adenosine A_3 receptor and the ability to achieve cytokine modulation through activation of that receptor.

2-Cl-IB-MECA has been shown to cause an elevation in the levels of plasma histamine when injected into rats (Van Schaick et al., 1996). Since histamine has also been found to alter cytokine responses in human whole blood and monocyte cultures (Elenkov et al., 1998; Kohka et al., 2000), the present studies were undertaken to examine the potential role of histamine in cytokine modulation by 2-Cl-IB-MECA. We monitored the effects of 2-Cl-IB-MECA on serum histamine levels and cytokine responses in two well-characterized mouse models of endotoxemia. We also present evidence suggesting that while 2-Cl-IB-MECA and the selective adenosine A_{2A} receptor agonist, CGS21680 have similar modulatory effects on cytokine responses, they differ in their mode of action.

2. Materials and methods

2.1. Mice

Specific pathogen-free BDF1 male mice, genetically mast cell deficient $WWB6F_1$-W/W^V and wild-type $WBB6F1^{+/+}$ control mice, 8–12 weeks of age (Jackson Laboratories, Bar Harbor, ME) were used in this study.

2.2. Cytokine production in experimental models of endotoxemia

Mice were made hypersensitive to endotoxin by treatment with D-galactosamine (Galanos et al., 1979) or *Corynebacterium parvum* (Smith et al., 1994). When D-galactosamine was used as the sensitizing agent, the drug (800 mg/kg) was injected by the intraperitoneal (i.p.) route together with endotoxin (10 μg, lipopolysaccharide, *Escherichia coli* serotype 055:B5). For sensitization to *C. parvum*, heat-killed cells (0.5 mg) of ATCC strain 11827 (American Type Culture Collection , Rockville, MD) were injected into mice by the intravenous (i.v.) route. The mice were challenged 1 week later by the i.p. route with 20 μg endotoxin. With both models of endotoxemia, sera were obtained 1.5 h after the endotoxin challenge to measure circulating levels of tumor necrosis factor α and 3 h after the challenge to measure circulating levels of interleukin-12 and interleukin-10. Serum histamine levels were also examined at these time-points (see below).

2.3. Determination of serum cytokine and histamine levels

Histamine and interleukin-10 levels in sera were measured with enzyme-linked immunosorbent assay (ELISA) kits purchased from Immunotech (Marseille, France) and R&D Systems (Minneapolis, MN), respectively. TNF-α and interleukin-12 in sera were quantified by enzyme-linked immunosorbent assays (ELISAs) in which monoclonal anti-TNF-α (hamster clone TN3-19.12) and anti-interleukin-12 (rat clone c15.6) antibodies from Pharmingen (San Diego, CA)

were paired with biotinylated goat polyclonal immunoglogulin G (IgG) antibodies. The goat polyclonal antibodies were purchased from R&D Systems and included anti-mouse TNF-α, (cat #BAF410) and anti-mouse interleukin-12 (cat #BAF419). Standard curves were developed with recombinant mouse cytokines from Pharmingen; TNF-α (cat #19321T) and interleukin-12 (cat #19401w).

2.4. Drugs

2-Chloro-N^6-(3-iodobenzyl) adenosine-5′-*N*-methyluronamide (2-Cl-IB-MECA, adenosine A_3 receptor agonist), 4-(2-[7-amino-2-(2-furyl)[1,2,4] triazolo[2,3a][1,3,5] triazin-5-ylamino] ethyl) phenol (ZM241385, adenosine A_{2A} receptor antagonist) and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, adenosine A_1 receptor antagonist) were purchased from Tocris (Ballwin, MO). 2-*p*-(2-Carboxyethyl) phenethylamino-5′-*N*-ethylcarboxamido adenosine hydrochloride, (CGS 21680, adenosine A_{2A} receptor agonist); 1-cyano-2-methyl-3-(2-(((5-methyl-4-imidazolyl)methyl)thio)ethyl) guanidine (cimetidine, histamine H_2 receptor antagonist); *N*-[(4-methoxyphenyl)methyl]-*N*′, *N*′-dimethyl-*N*-2-pyridinyl-1,2-ethanediamine (pyrilamine, histamine H_1 receptor antagonist) and 2-diphenylmethoxy, *N*,*N*-dimethylthaneamine (diphenhydramine, histamine H_1 receptor antagonist) were purchased from Sigma (St. Louis, MO). 2-Cl-IB-MECA and CGS 21680 were prepared as stock solutions in dimethyl sulfoxide (DMSO) and further diluted in saline for i.v. injections. The final concentration of DMSO never exceeded 0.2% and was usually $<0.1\%$. DPCX and ZM241385 were suspended in 1:1:18 emulphor/ethanol/saline and were administered to mice by the i.p. route.

2.5. Data analysis

Data were analyzed by one-way analysis of variance (ANOVA) and the unpaired *t*-test using StatView for Windows version 5.0.1. *P* values <0.05 were considered as statistically significant.

3. Results

3.1. Effects of selective adenosine A_{2A} and A_3 receptor agonists on cytokine responses in D-galactosamine and C. parvum sensitized mice

CGS-21680 (adenosine A_{2A} receptor agonist) and 2-Cl-IB-MECA (adenosine A_3 receptor agonist) were administered intravenously to mice at the time of sensitization with D-galactosamine and challenge with LPS. As shown in Fig. 1, the production of TNF-α (Fig. 1B) and interleukin-12 (Fig. 1C) was decreased while that of interleukin-10 (Fig. 1D) was increased when the responses of CGS21680-treated mice (200 and 400 μg/kg, i.v.) were compared to those of vehicle-treated controls. There were no significant changes

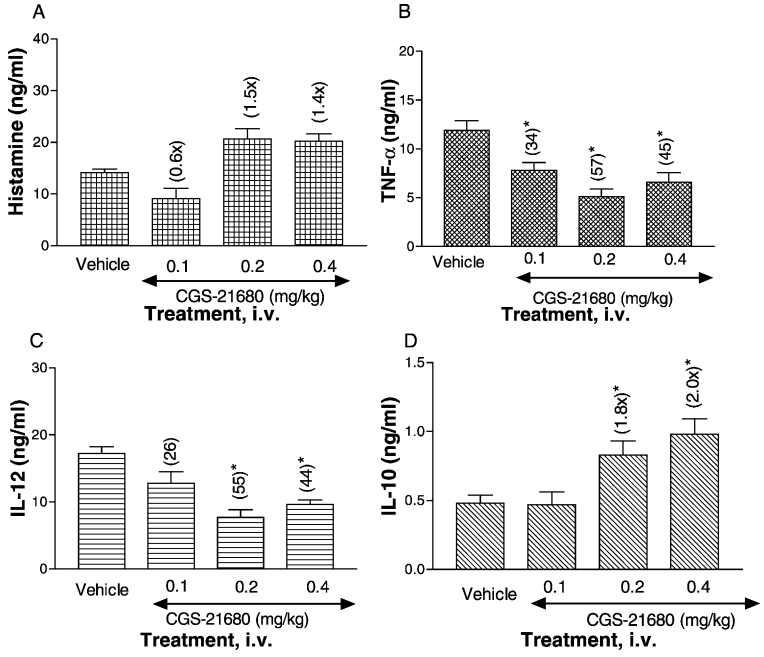

Fig. 1. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in BDF1 mice treated with CGS-21680 by the i.v. route. The adenosine A_{2A} receptor agonist was administered to mice at the time of sensitization with D-galactosamine (800 mg/kg, i.p.) and challenge with endotoxin (10 μg, i.p.). Serum histamine and tumor necrosis factor α levels were measured 1.5 h after the challenge and the interleukin-12 and interleukin-10 responses 3 h after the challenge. The values shown represent the mean ± S.E.M. of six mice per treatment group and the results presented are representative of those obtained in three independent experiments. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $*P<0.01$.

in circulating histamine levels in mice treated with the drug (Fig. 1A). The adenosine A_3 receptor agonist, 2-Cl-IB-MECA (50–200 μg/kg) produced similar changes in the cytokine responses (Fig. 2). However, the changes in TNF-α (Fig. 2B), interleukin-12 (Fig. 2C) and interleukin-10 (Fig. 2D) caused by 2-Cl-IB-MECA were more striking and dose-dependent than those produced by CGS 21680. In addition, mice treated with 2-Cl-IB-MECA exhibited a marked and dose-related elevation in serum histamine levels (Fig. 2A) which did not occur when mice were given the adenosine A_{2A} receptor agonist (Fig. 1A). The histamine levels shown were obtained 1.5 h after simultaneous treatment with the drug and challenge with endotoxin, which corresponded to the time of the peak production of TNF-α. Serum histamine levels were also measured 3 h after the challenge at the time of peak production of interleukin-10 and interleukin-12. The circulating levels of histamine actually reached a maximum approximately 30 min after treatment with 2-Cl-IB-MECA, declining slowly thereafter. These findings were similar to those obtained in an earlier study where the highest levels of plasma histamine were detected 30 min after treatment with 2-Cl-IB-MECA (Van Schaick et al., 1996). However, when compared to vehicle-treated control mice, there were always substantially higher levels of histamine in sera from 2-Cl-IB-MECA-treated mice, when measured 0.5–5.5 h after the treatment. It was determined in preliminary experiments that endotoxin itself had no effect on circulating histamine levels.

In comparison to D-galactosamine sensitized mice, mice sensitized to *C. parvum* exhibit a stronger and more diverse systemic inflammatory cytokine response (TNF-α, interleukin-1, interleukin-6, interleukin-12, interleukin-18 and interferon-γ) when challenged with endotoxin. The selective adenosine receptor agonists had similar effects on cytokine responses in *C. parvum* mice as in D-galactosamine sensitized mice. Thus, in *C. parvum* mice, GCGS 21680 had no effect on serum histamine levels (Fig. 3A), while causing a reduction in TNF-α (Fig. 3B) and interleukin-12 (Fig. 3C) and an increase in interleukin-10 (Fig. 3D) subsequent to the endotoxin challenge. The selective adenosine A_3 receptor agonist, 2-Cl-IB-MECA, modulated cytokine responses in a similar manner (Fig. 4B–D) and also caused a dramatic elevation in circulating histamine levels as observed in D-galactosamine sensitized mice. In addition to the failure of CGS 21680 to trigger a histamine response, both selective adenosine A_1 (DPCPX) and A_{2A} (ZM241385) receptor antagonists were found to have no effect on the increase in serum histamine or cytokine modulation caused by 2-Cl-IB-MECA (data not shown). Therefore, 2-Cl-IB-MECA did not appear to interact with other adenosine receptors in producing its effects. Interleukin-10 is a widely recognized inhibitor of proinflammatory cytokine production. However, in both models of endotoxemia, an anti-interleukin-10 monoclonal antibody was found to have no effect on the reductions in TNF-α and interleukin-12 that were caused by 2-Cl-IB-MECA and CGS 21680. Thus, the elevated levels of interleukin-10 appeared to have no role in the suppressive effects of the adenosine receptor agonists on pro-inflammatory cytokine production. Subsequent experiments were performed in *C. parvum* mice because of their stronger systemic inflammatory cytokine response.

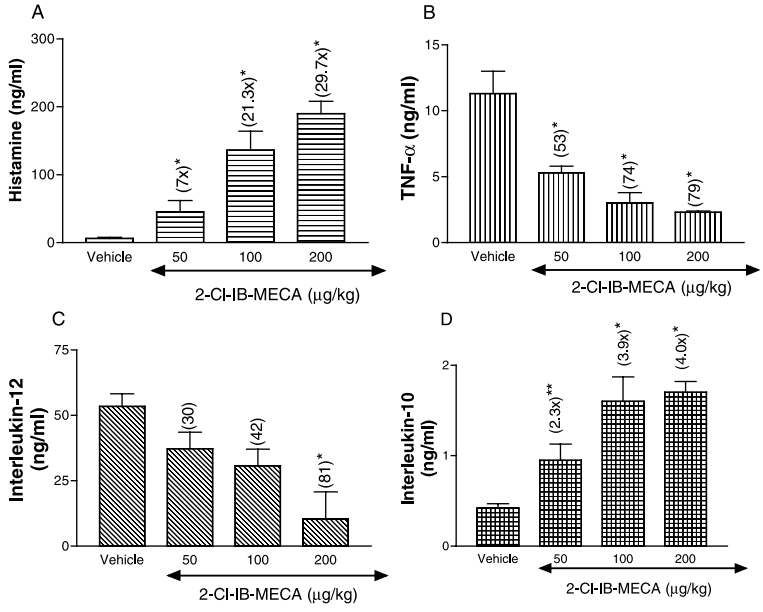

Fig. 2. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in BDF1 mice treated with 2-Cl-IB-MECA by the i.v. route. The adenosine A_3 receptor agonist was administered to mice at the time of sensitization with D-galactosamine (800 mg/kg, i.p.) and challenge with endotoxin (10 μg, i.p.). The values shown represent the mean ± S.E.M. of six mice per treatment group and the results presented are representative of those obtained in three independent experiments. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $*P<0.01$.

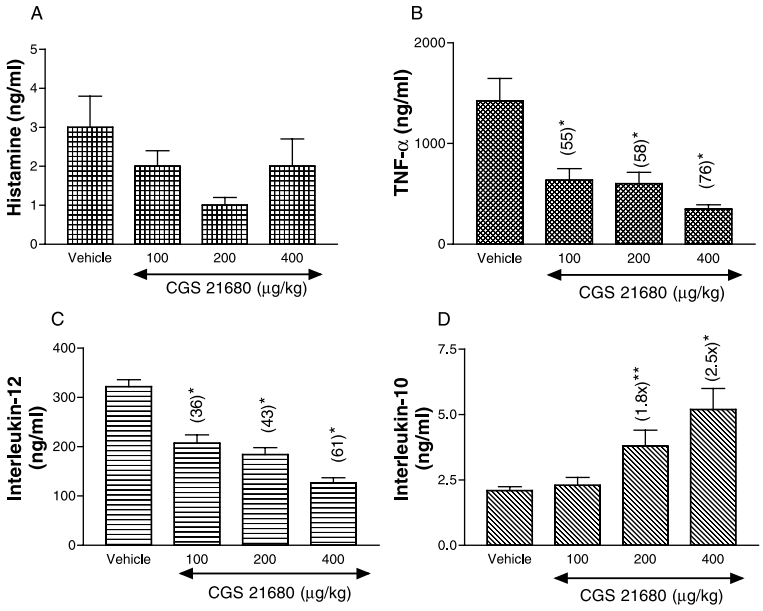

Fig. 3. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in *C. parvum* primed BDF1 mice treated with CGS-21680. Mice were challenged with endotoxin (20 μg, i.p.) 1 week after priming and the adenosine A_{2A} receptor agonist was administered to mice by the i.v. route at the time of challenge. The values shown represent the mean ± S.E.M. of six mice per treatment group and the results presented are representative of those obtained in two independent experiments. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $*P<0.01$ and $**P<0.05$.

3.2. Cytokine modulation by the mast cell depleting agent compound 48/80

Histamine has been reported to inhibit interleukin-12 and increase interleukin-10 production in endotoxin-stimulated human whole blood cultures (Elenkov et al., 1998). As shown above, interleukin-12 and interleukin-10 were similarly modulated in mice that had high circulating levels of histamine following treatment with 2-Cl-IB-MECA. Therefore, it was of interest to determine whether histamine had a role in the cytokine modulatory activities of the adenosine A_3 receptor agonist. If so, the results would suggest

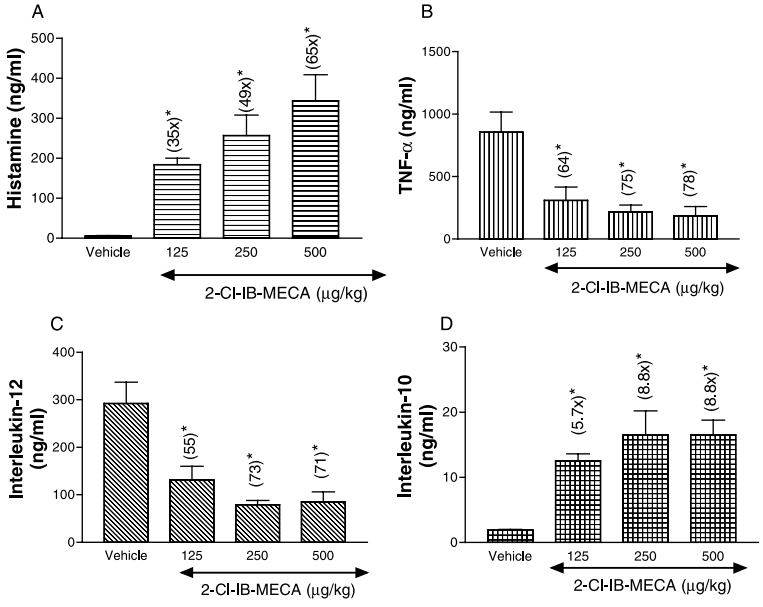

Fig. 4. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in *C. parvum* primed BDF1 mice treated with 2-Cl-IB-MECA. Mice were challenged with endotoxin (20 μg, i.p.) 1 week after priming and the adenosine A_3 receptor agonist was administered to mice by the i.v. route at the time of challenge. The values shown represent the mean ± S.E.M. of six mice per treatment group and the results presented are representative of those obtained in two independent experiments. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $*P<0.01$.

differences between 2-Cl-IB-MECA and CGS 21680 in their mode of action on cytokines, since the latter agent did not trigger a histamine response. If other mast cell depleting agents were found to produce similar effects as 2-Cl-IB-MECA, such evidence would strongly support a role for histamine as the mediator of cytokine modulation by the adenosine A_3 receptor agonist.

An experiment in which the mast cell depleting agent, compound 48/80 was administered to *C. parvum* mice is shown in Fig. 5. When injected into mice by the i.v. route, at a dose of 0.9 mg/kg, compound 48/80 did not increase serum histamine levels or modulate endotoxin-induced cytokine responses. However, at a higher dose (2.7 mg/kg), the drug caused a substantial increase in serum histamine (Fig. 5A), inhibited the production of TNF-α (Fig. 5B) and interleukin-12 (Fig. 5C) and increased that of interleukin-10 (Fig. 5D). The main objective of this experiment was to administer a dose of compound 48/80 that would yield serum levels of histamine that were comparable to those elicited with 2-Cl-IB-MECA (200 μg/kg). The serum histamine levels in mice given 2.7 mg/kg compound 48/80 were comparable (1.6-fold higher) to those of mice that had received a 200 μg/kg dose of 2-Cl-IB-MECA. Cytokine responses were also modulated to a similar extent by the two compounds at their respective doses. This ability of compound 48/80 to mimic the effects of 2-Cl-IB-MECA only when administered to mice at doses that caused an increase in serum histamine supported the hypothesis that histamine was the mediator of cytokine modulation by 2-Cl-IB-MECA.

3.3. A second dose of 2-Cl-IB-MECA, given shortly after the first, has no effect on histamine levels or cytokine responses

In the experiment presented in Fig. 6, groups of *C. parvum* mice were treated with 2-Cl-IB-MECA (200 μg/kg), 2.5 h before endotoxin, at the time of endotoxin or at both time-points. All groups were bled for histamine levels 1.5 h after the endotoxin challenge. Thus, the total time of exposure to drug was 4 h for mice treated with 2-Cl-IB-MECA, 2.5 h before challenge with endotoxin and 1.5 h when it was administered to the animals at the time of challenge. The mean circulating levels of histamine were 262 ± 46 ng/ml in mice given a single dose of 2-Cl-IB-MECA simultaneously with endotoxin (Fig. 6A). The mean histamine levels were 95 ± 11 ng/ml in mice that received a single dose of 2-Cl-IB-MECA 2.5 h before endotoxin and 85 ± 12 ng/ml in mice that were treated with the drug both 2.5 h before and simultaneously with endotoxin (Fig. 6A). Thus, the circulating histamine levels in mice that received two doses of 2-Cl-IB-MECA, 2.5 h apart were almost identical to those of mice that had been given a single dose of drug, 2.5 h before endotoxin (Fig. 6A). The TNF-α (Fig. 6B), interleukin-12

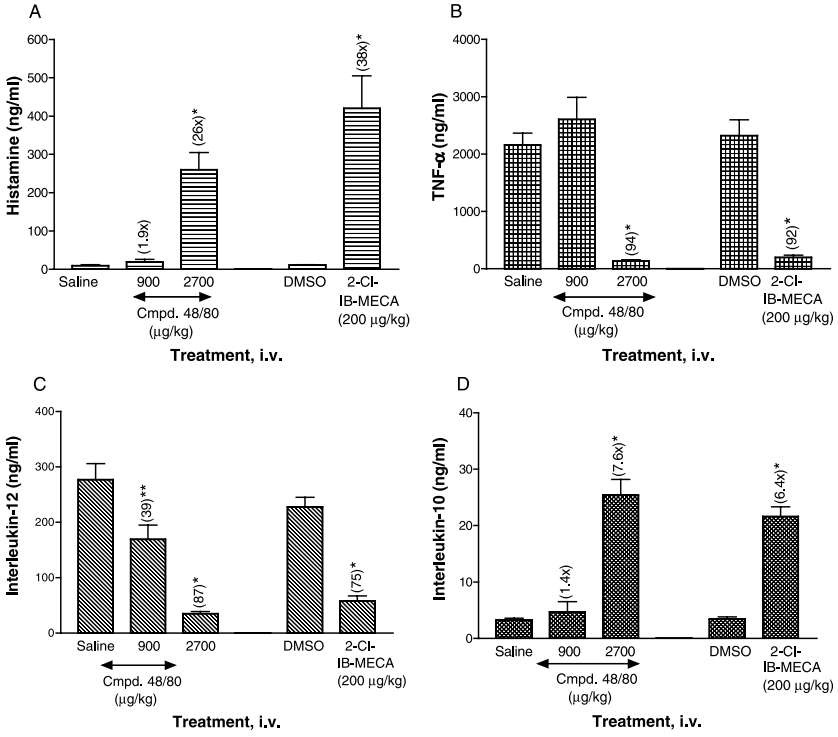

Fig. 5. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in *C. parvum* primed BDF1 mice treated with 2-Cl-IB-MECA or compound 48/80. The drugs were administered to the mice by the i.v. route at the time of the endotoxin challenge. The values shown represent the mean ± S.E.M. of six mice per treatment group and the results presented are representative of those obtained in two independent experiments. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $*P<0.01$ and $**P<0.05$.

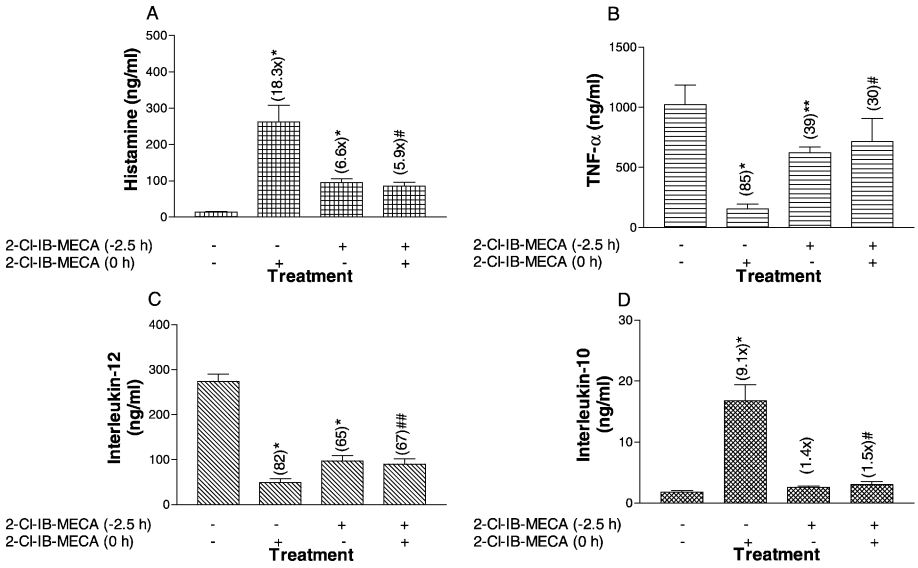

Fig. 6. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in *C. parvum* primed BDF1 mice that were treated with 2-Cl-IB-MECA (200 μg/kg, i.v.), 2.5 h before endotoxin (− 2.5 h), simultaneously with endotoxin (0 h) or at both timepoints. The values shown represent the mean ± S.E.M. of six mice per treatment group and the results presented are representative of those obtained in two independent experiments. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $^{*}P<0.01$ and $^{**}P<0.05$. Significantly different from the levels in mice that received a single dose of 2-Cl-IB-MECA simultaneously with endotoxin, $^{\#}P<0.01$ and $^{\#\#}P<0.05$.

(Fig. 6C) and interleukin-10 (Fig. 6D) responses were also modulated to a similar extent in the two groups of mice indicating that as with histamine, the second dose of 2-Cl-IB-MECA had no additional effect on the cytokine responses. Thus, as previously shown for 2-Cl-IB-MECA-induced histamine release and associated hemodynamic effects in rats (Van Schaick et al., 1996), endotoxemic mice developed a rapid tachyphylaxis to the drug as evidenced by the failure of a second dose to cause additional changes in serum histamine levels or cytokine responses. It is also important to note that cytokine responses were most effectively modulated when 2-Cl-IB-MECA was given as a single dose simultaneously with endotoxin. As indicated above, this latter group of mice exhibited the highest circulating levels of histamine. Taken together, the results presented in Fig. 6 strongly support the hypothesis that histamine is a direct mediator of cytokine modulation by 2-Cl-IB-MECA.

3.4. Effects of histamine H_1 and H_2 receptor antagonists on cytokine modulation by 2-Cl-IB-MECA

To further demonstrate a role for histamine in cytokine modulation by 2-Cl-IB-MECA, attempts were made to block the effects of the adenosine A_3 receptor agonist using histamine H_1 and H_2 receptor antagonists. Results typical of those obtained in several experiments in which pyrilamine and cimetidine were used as histamine H_1 and H_2 receptor antagonists, respectively, are shown in Fig. 7. In this experiment, mice that had been treated simultaneously with 2-Cl-IB-MECA and endotoxin exhibited serum histamine levels of 395 ± 42 ng/ml when bled 1.5 later (Fig. 7A). The levels were 9 ± 0.9 ng/ml in mice similarly treated with vehicle, indicating that the drug caused a 44-fold increase in the response. The mean serum histamine concentration was only 41 ± 2 ng/ml in the group of mice that received pyrilamine (20 mg/kg, p.o.) prior to treatment with 2-Cl-IB-MECA (Fig. 7A). This was an indication of the ability of the histamine H_1 receptor antagonist to block the release of histamine from mast cells that had been exposed to the adenosine A_3 receptor agonist. Despite its capacity to markedly inhibit the release of histamine into the circulation, pyrilamine was only able to reverse the 2-Cl-IB-MECA-mediated changes to TNF-α (Fig. 7B) and interleukin-10 (Fig. 1D) by about 50% and the changes to interleukin-12 by 20% (Fig. 7C).

Previous reports have suggested that the cytokine modulatory activities of histamine can be blocked by histamine H_2 receptor antagonists (Elenkov et al., 1998; Kohka et al., 2000). However, in endotoxemic mice, cimetidine did not antagonize the changes in cytokine responses that were caused by 2-Cl-IB-MECA (Fig. 7). This was the case even when the drug was administered to mice at doses up to 150 mg/kg (data not shown). In contrast to the effects of pyrilamine, the high circulating levels of histamine caused by 2-Cl-IB-MECA remained elevated in the presence of cimetidine.

Despite the complicating factor of mediator release inhibition, histamine H_1 receptor antagonists were considered to be pivotal reagents in trying to discern the receptor subtype through which histamine evoked its cytokine modulatory activity. Monocytes/macrophages are the major cellular sources of TNF-α, interleukin-10 and interleukin-12 in endotoxemic mice. While these cells express histamine H_2

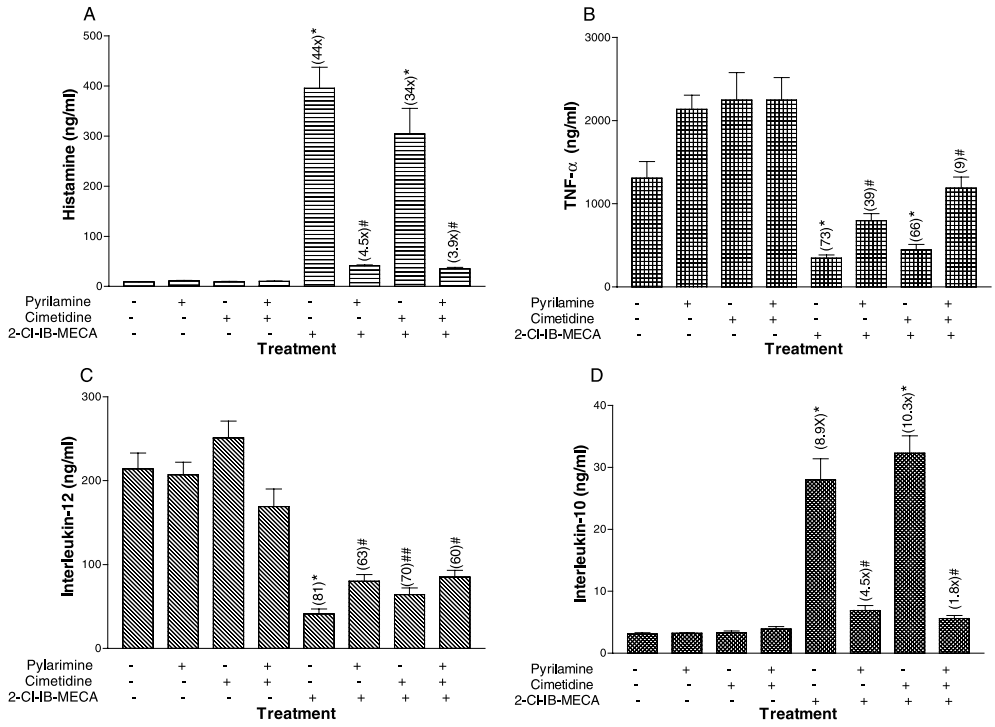

Fig. 7. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in *C. parvum* primed BDF1 mice that were given cimetidine (50 mg/kg, i.p.) or pyrilamine (20 mg/kg, i.p.) 1 h before treatment with 2-Cl-IB-MECA (200 μg/kg, i.v.). Data were pooled from two independent experiments and the values shown represent the mean ± S.E.M. of 12 mice per treatment group. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $*P < 0.01$.

receptors (Elenkov et al., 1998; Azuma et al., 2001), such receptors appeared to have no role in 2-Cl-IB-MECA-mediated cytokine modulation because the effect could not be blocked by cimetidine, a highly selective histamine H_2 receptor antagonist. Monocytes/macrophages have also been reported to express histamine H_1 receptors (Pyneart et al., 1999), but histamine H_3 receptors appear to be absent or present at very low levels (Elenkov et al., 1998). Therefore, in postulating a role for histamine in cytokine modulation by 2-Cl-IB-MECA, it became critically important to determine whether its activity was mediated through the histamine H_1 receptor.

We then attempted to identify a histamine H_1 receptor antagonist that did not block the histamine response to 2-Cl-IB-MECA-treated mice. The antihistamine diphenhydramine (5–20 mg/kg), not only prevented the release of histamine in response to 2-Cl-IB-MECA, but also exhibited an ability to modulate cytokine responses itself. All of the other histamine H_1 receptor antagonists evaluated (terfenidine, ceterizine, chlorpheniramine and nor-astemizole) exhibited an ability to inhibit 2-Cl-IB-MECA-induced histamine release at doses that had been used successfully to block the receptor in other experimental systems (data not shown). This phenomenon was previously shown to be an inherent property of some agents that act as antagonists at the histamine H_1 receptor (Sekardi and Freidberg, 1989).

A major consideration in trying to demonstrate antagonism of cytokine modulation by a histamine H_1 receptor antagonist was the observation that mice were being exposed to vastly different levels of histamine in the presence and absence of an antihistamine like pyrilamine that exhibited mediator release inhibitory properties. In pyrilamine-treated mice, the histamine levels caused by 2-Cl-IB-MECA never approached those that could be obtained in the absence of the antihistamine. The peak histamine response occurred 30 min after treatment with 2-Cl-IB-MECA, whether administered to mice in the presence or absence of the antihistamine. However, even at the peak of the response, the histamine levels induced by 2-Cl-IB-MECA in pyrilamine-treated mice were only two- to threefold higher than the background levels that were detected in normal control mice (data not shown). This raised an issue of whether mice given a similar dose of 2-Cl-IB-MECA in the absence of the antihistamine, in which markedly higher circulating levels of histamine were induced, represented appropriate controls for detecting antagonism at the histamine H_1 receptor. Theoretically at least, it should be easier to observe antagonism when the levels of agonist are similar in the presence and absence of the antagonist.

If the cytokine modulatory effects of histamine were being mediated through the histamine H_1 receptor, it was considered likely that the cytokine responses obtained with 2-Cl-IB-MECA in the presence of pyrilamine actually reflected some degree of antagonism. That was the case, but that there was a need to minimize the differences in the circulating histamine levels of untreated and pyrilamine-treated mice in order to

detect the antagonism of 2-Cl-IB-MECA-mediated cytokine modulation by the antihistamine is shown in Fig. 8. In this experiment, 2-Cl-IB-MECA was injected into mice 1 h after pyrilamine, but 2.5 h before the endotoxin challenge. When bled 1.5 h after endotoxin, the histamine levels induced by 2-Cl-IB-MECA in untreated and pyrilamine-treated mice were 98 ± 9 and 21 ± 3 ng/ml, respectively (Fig. 8A). This was in contrast to the much larger difference that had been observed in the serum histamine levels of these groups (395 ± 42 and 42 ± 2 ng/ml, respectively), when 2-Cl-IB-MECA was given simultaneously with endotoxin and bled 1.5 h later (Fig. 7A). Thus, when 2-Cl-IB-MECA was given to mice 2.5 h before endotoxin, the circulating histamine levels in mice that had not been treated with pyrilamine, more closely approximated those in mice that had also received the antihistamine. It was under these latter experimental conditions that the modulatory effects of 2-Cl-IB-MECA on TNF-α (Fig. 8B), interleukin-12 (Fig. 8C) and interleukin-10 (Fig. 8D) production appeared to be less pronounced in the presence of pyrilamine, reflecting the antagonistic activity of the antihistamine. It should be emphasized here that pyrilamine-treated mice exhibited similar levels of circulating histamine when treated with 2-Cl-IB-MECA whether or not there was evidence of antagonism of cytokine modulation by the antihistamine (compare Figs. 7 and 8). From these observations, it became evident that pyrilamine did not antagonize cytokine modulation by blocking the 2-Cl-IB-MECA-mediated release of histamine into the circulation.

The data presented in Fig. 9 further demonstrate the capacity to observe a reversal of 2-Cl-IB-MECA-mediated cytokine modulation by antagonists at the histamine H_1 receptor, under conditions where the differences in circulating histamine levels in the absence and presence of the antagonist are less pronounced. In the experiment shown, 2-Cl-IB-MECA was administered to mice simultaneously with endotoxin at a dose that was two- to fourfold lower than the 200 μg/kg dose used in previous experiments. Mice given the 50 μg/kg dose of 2-Cl-IB-MECA at the time of challenge had circulating histamine levels of 37 ± 5 ng/ml when measured 1.5 h after endotoxin (Fig. 9A). This amount of circulating histamine had no effect on the production of TNF-α (Fig. 9B), but caused modest changes in interleukin-12 (30% decrease, Fig. 9C) and interleukin-10 (twofold increase, Fig. 9D). Pyrilamine-treated mice exhibited circulating histamine levels of 26 ± 2 ng/ml when administered this dose of 2-Cl-IB-MECA (Fig. 9A) and there was no evidence of the changes to interleukin-12 (Fig. 9C) and interleukin-10 (Fig. 9D) that had occurred in the absence of the antihistamine. The serum histamine was 102 ± 32 ng/ml in mice that received a 100 μg/kg dose of 2-Cl-IB-MECA in the absence of pyrilamine. This increase in circulating histamine was accompanied by a 58% decrease in TNF-α (Fig. 9B), a 62% decrease in interleukin-12 (Fig. 9C) and a fivefold increase in interleukin-10 (Fig. 9D). The mean serum histamine concentration was 33 ± 3 ng/ml in pyrilamine-treated mice that were given the same dose of 2-Cl-IB-MECA (Fig. 9A). These circulating histamine levels did not differ from those obtained when a 200 μg/kg dose of the adenosine A_3 agonist was administered to pyrilamine-treated mice (Fig. 7A). Yet, in contrast to the 200 μg/kg dose of 2-Cl-IB-MECA, where there was no evidence of an antagonism of cytokine modulation by pyrilamine, the antihistamine

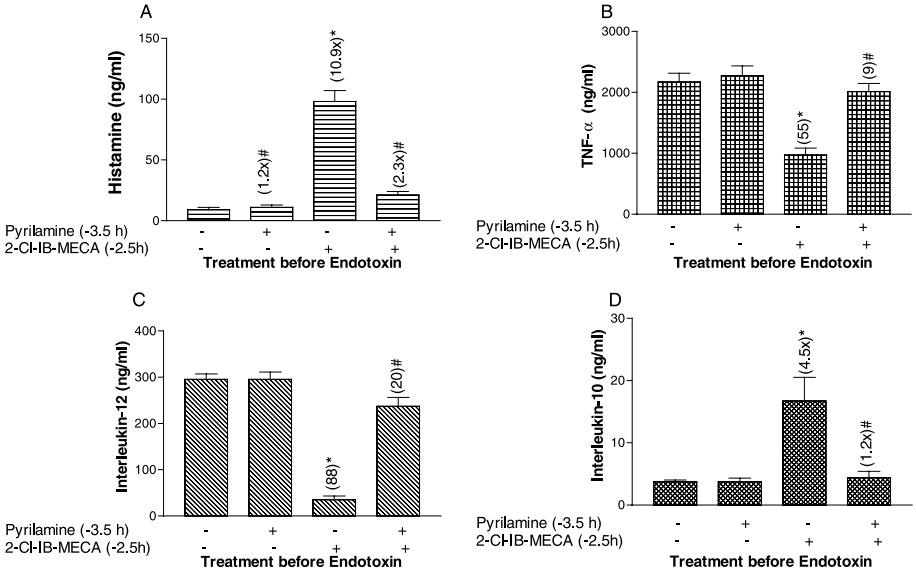

Fig. 8. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in *C. parvum* primed BDF1 mice that were treated with 2-Cl-IB-MECA (200 μg/kg, i.v.) 2.5 h before an endotoxin challenge. Some mice received pyrilamine (20 mg/kg, i.p.) 1 h before treatment with 2-Cl-IB-MECA. Data were pooled from two independent experiments and the values shown represent the mean ± S.E.M. of 12 mice per treatment group. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $^{*}P<0.01$. Significantly different from the levels in mice treated with 2-Cl-IB-MECA alone, $^{\#}P<0.01$.

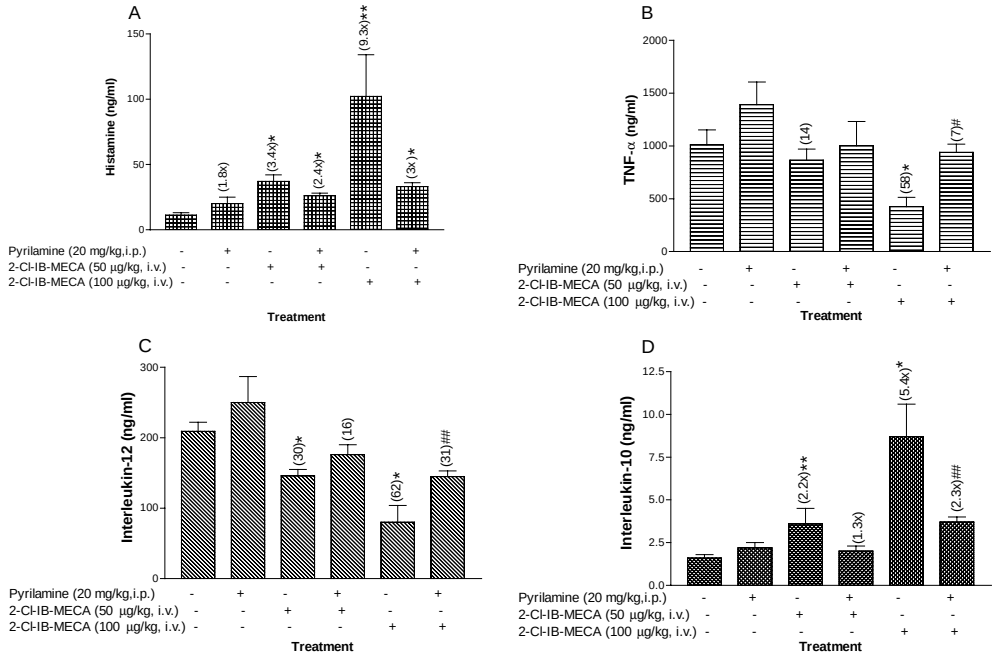

Fig. 9. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in *C. parvum* primed BDF1 mice that were treated with 2-Cl-IB-MECA (50 or 100 μg/kg, i.v.) at the time of an endotoxin challenge. Some mice were given pyrilamine (50 mg/kg, i.p.) 1 h before treatment with 2-Cl-IB-MECA. The values shown represent the mean ± S.E.M. of six mice per treatment group and the results presented are representative of those obtained in two independent experiments. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $*P<0.01$ and $**P<0.05$. Significantly different from the levels in mice that received 100 μg/kg 2-Cl-IB-MECA alone, $^{\#}P<0.01$ and $^{\#\#}P<0.05$.

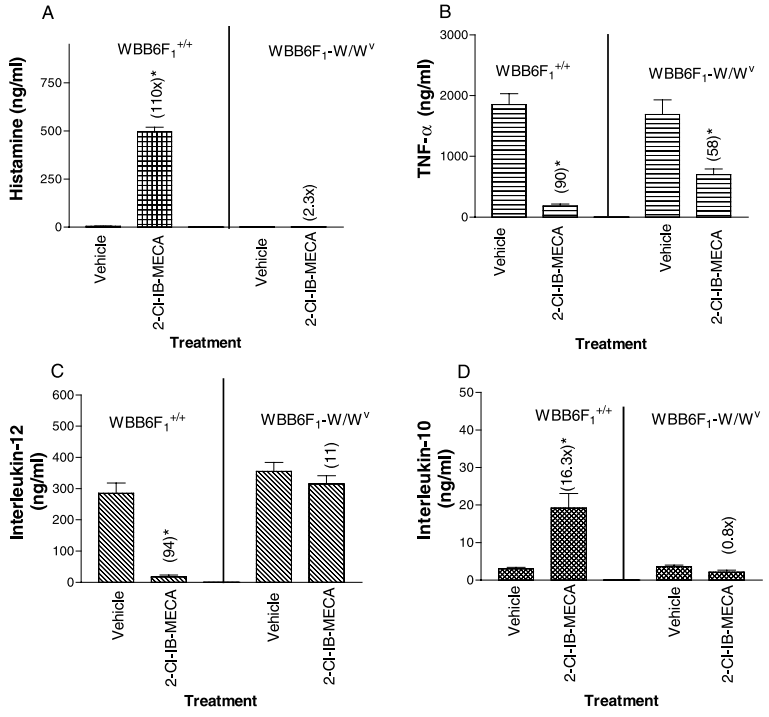

Fig. 10. Serum histamine (A), tumor necrosis factor α (B), interleukin-12 (C) and interleukin-10 (D) responses in WBB6F1$^{+/+}$ and WBB6F1-W/W^{V} mast cell deficient mice that were treated with 2-Cl-IB-MECA (200 μg/kg, i.v.) at the time of an endotoxin challenge. The values shown represent the mean ± S.E.M. of six mice per treatment group and the results presented are representative of those obtained in two independent experiments. The percent decrease or fold increase in the response as compared to that of vehicle-treated control mice is shown in the parentheses. Significantly different from the levels in vehicle-treated control mice, $*P<0.01$.

appeared to at least partially counter the changes caused by a 100 μg/kg dose of the adenosine A_3 receptor antagonist. Thus, in the absence of the antihistamine, 2-Cl-IB-MECA decreased the TNF-α (Fig. 9B) and interleukin-12 (Fig. 9C) responses by 58% and 62%, respectively and increased that of interleukin-10 five-fold (Fig. 9D). In pyrilamine-treated mice, the same dose of the adenosine A_3 receptor agonist reduced the TNF-α and interleukin-12 responses by only 7% and 31%, respectively, and increased interleukin-10 levels twofold. Thus, in this experiment, where conditions were employed to minimize the differences in the histamine levels obtained with 2-Cl-IB-MECA in the presence and absence of pyrilamine, there was additional evidence of the capacity of the antihistamine to antagonize cytokine modulation by the adenosine A3 receptor agonist.

3.5. Cytokine modulation by 2-Cl-IB-MECA in mast cell deficient WBB6F1-W/W^V mice

The role of histamine in cytokine modulation by 2-Cl-IB-MECA was further examined in genetically mast cell-deficient W/W^V mice. An experiment in which *C. parvum* primed mast cell deficient W/W^V and wild-type control (WBB6F$_1^{+/+}$) mice were treated with 2-Cl-IB-MECA (200 μg/kg, i.v.) at the time of an endotoxin challenge is presented in Fig. 10. 2-Cl-IB-MECA produced an increase in circulating histamine in WBB6F$_1^{+/+}$ control mice, but not in mast cell deficient W/W^V mice (Fig. 10A). The TNF-α response (Fig. 10B) was not as strongly modulated by 2-Cl-IB-MECA in W/W^V mice as in WBB6F$_1^{+/+}$ controls and the drug had no effect on the production of interleukin-12 (Fig. 10C) and interleukin-10 (Fig. 10D) in mast cell deficient mice.

4. Discussion

In the present study, we show that 2-Cl-IB-MECA, a highly selective adenosine A_3 receptor agonist has the capacity to modulate endotoxin-induced cytokine responses in mice. This phenomenon was observed in D-galactosamine-sensitized mice where endotoxin lethality results from TNF-α-induced caspase-3-dependent liver injury (Mignon et al., 1999) as well as in *C. parvum* primed mice where death is preceded by the release of several proinflammatory cytokines into the circulation. TNF-α and interferon-γ have been shown to be the major mediators of lethality in the latter model and are among several cytokines that are produced at relatively high levels following an endotoxin challenge to primed mice (Smith et al., 1994). Because 2-Cl-IB-MECA has been shown to increase plasma histamine when administered to rats by the i.v. route (Van Schaick et al., 1996), it became of interest to determine whether this mast cell-derived mediator had a role in cytokine modulation by the adenosine A_3 receptor agonist. The ability of histamine to modulate cytokine responses has been demonstrated in vitro with cells from both human and animal subjects. For example, histamine was reported to inhibit the secretion of interleukin-12 and to increase the production of interleukin-10 in endotoxin-stimulated cultures of human whole blood or peripheral mononuclear cells (Elenkov et al., 1998). In a subsequent study, histamine was shown to stimulate the release of interleukin-18 and interferon-γ, but inhibit the production of interleukin-2 when added to cultures of human PBMCs (Kohka et al., 2000). These investigators also found histamine to block the production of interleukin-10 in contrast to previous findings indicating the ability of histamine to stimulate the production of this cytokine (Elenkov et al., 1998). More recently, histamine was shown to inhibit lipopolysaccharide-stimulated tumor necrosis factor α and interleukin-12 responses and to block chemotaxis, phagocytosis and superoxide anion production by rat peritoneal macrophages (Azuma et al., 2001). In each of the foregoing in vitro studies, the histamine-induced changes in cytokine responses were blocked by selective histamine H_2 receptor antagonists.

There are several lines of evidence in the present study which point to histamine as a mediator of cytokine modulation by 2-Cl-IB-MECA. First, the in vivo effects of 2-Cl-IB-MECA on endotoxin-stimulated cytokine responses (inhibition of TNF-α and interleukin-12 and stimulation of interleukin-10) were identical to those obtained when histamine was added to cultures of human PBMC and rat peritoneal macrophages (Elenkov et al., 1998; Azuma et al., 2001). Furthermore, the changes in cytokine responses elicited by 2-Cl-IB-MECA were always preceded by a large increase in circulating histamine. Although histamine reached maximum levels in the circulation shortly after the injection of 2-Cl-IB-MECA, the levels were still markedly elevated at the time cytokines were being generated in response to endotoxin. This was especially true when 2-Cl-IB-MECA was given to mice at the time of the endotoxin challenge. Second, the mast cell degranulating agent, compound 48/80 exhibited the same cytokine modulating profile as 2-Cl-IB-MECA and was only effective in modulating cytokine responses at doses that caused histamine to be released into the circulation. Third, when 2-Cl-IB-MECA-treated mice were given a second dose of the drug 2.5 h later, there was no additional release of histamine into the circulation or further modulation of cytokine responses. Together, these observations strongly suggest a link between the histamine released into the circulation and cytokine modulation by 2-Cl-IB-MECA.

As discussed above, a substantial body of evidence has been obtained from in vitro studies indicating that histamine has the capacity to modulate cytokine responses through the histamine H_2 receptor. However, in the present study, cimetidine a highly selective histamine H_2 receptor antagonist, was found to have no effect on cytokine modulation by 2-Cl-IB-MECA when administered to mice at doses ranging from 50 to 150 mg/kg. Therefore, it was clearly important to focus on the possible role of other histamine receptors in cytokine modulation. Because all of the histamine H_1 receptor antag-

onists employed in this study exhibited a capacity to block the release of histamine from mast cells, it was difficult to determine whether histamine produced any of its effects through the histamine H_1 receptor. Although pyrilamine was employed as an histamine H_1 receptor antagonist in the majority of experiments presented here, other histamine H_1 receptor antagonists were also found to block the increase in serum histamine that was elicited by 2-Cl-IB-MECA or compound 48/80 including diphenhydramine, terfenadine, cetirizine, chlorpheniramine and nor-astemizole. There have been several studies with cells and tissues from both animal and human sources indicating that histamine H_1 receptor antagonists have the capacity to block mediator release (Sekardi and Friedberg, 1989; Cuss, 1999; Baroody and Naclerio, 2000). However, it was somewhat surprising that all of the commercially available histamine H_1 receptor antagonists that were evaluated for this study were found to inhibit histamine release.

Despite its capacity to dramatically reduce serum histamine levels in 2-Cl-IB-MECA-treated mice, pyrilamine was not always observed to block the changes in cytokine responses that were caused by the adenosine A_3 receptor agonist. This finding led to early speculation that factors other than histamine might be involved in the modulatory effects of 2-Cl-IB-MECA on cytokine responses. However, upon further reflection, it became apparent that mice pretreated with pyrilamine were exposed to significantly lower levels of circulating histamine than mice that had been given 2-Cl-IB-MECA in the absence of the antihistamine. Assuming a functional role for histamine in cytokine modulation by 2-Cl-IB-MECA, it was likely that such responses would be more strongly modulated in those mice with higher circulating levels of the mast cell-derived mediator. This disparity in the circulating levels of histamine that were induced by 2-Cl-IB-MECA in the presence and absence of pyrilamine created some initial difficulties in determining whether there might also be some antagonism of cytokine modulation by the antihistamine at the level of the histamine H_1 receptor. Therefore, strategies were devised to minimize the differences between the two groups in the levels of histamine that were present in the circulation as cytokines were being generated. This was accomplished by (1) extending the interval between the time of treatment with 2-Cl-IB-MECA and the endotoxin challenge to 2.5 h and (2) reducing the dose of 2-Cl-IB-MECA administered to mice simultaneously with endotoxin. With these approaches, there was more parity between pyrilamine -treated mice and their corresponding untreated controls in the levels of circulating histamine that were produced in response to 2-CL-IB-MECA. It then became apparent that cytokine responses were being modulated to a lesser extent in the presence of pyrilamine. These findings were considered as evidence of the capacity of pyrilamine to antagonize cytokine modulation by 2-Cl-IB-MECA and suggested that histamine, as the direct mediator of such effects, was acting through the histamine H_1 receptor. Theoretically, since mice were being exposed to similar levels of histamine in the presence and absence of pyrilamine, the cytokine responses should have been modulated to a similar extent in the two groups of mice if there had been no antagonism at the level of the histamine H_1 receptor. It should also be stressed that the circulating levels of histamine in pyrilamine-treated mice were extremely low whether or not there was evidence of antagonism of cytokine modulation. Therefore, the antagonism was unrelated to the reduced circulating levels of histamine.

Studies in rats have shown that connective tissue mast cells have a higher content of histamine and are more susceptible than mucosal mast cells to degranulation by compound 48/80 (Jarrett and Haig, 1984). The connective tissue mast cell was likely to be the major target of 2-Cl-IB-MECA as well, since extremely high levels of histamine were detected in the circulation shortly after exposure to the drug. Mast cell degranulation in rats and mice also leads to the release of serotonin, which is a second preformed mediator present at relatively high levels in secretory granules. However, serotonin appeared to have no role in cytokine modulation by 2-Cl-IB-MECA or compound 48/80 because modulated responses were not altered by methysergide, a non-selective serotonin receptor antagonist. Other mast cell products such as lipid mediators and cytokines are generated upon mast cell activation and would be expected to be released at relatively low levels by mast cell degranulating agents.

With the exception of TNF-α, there was no evidence of cytokine modulation by 2-Cl-IB-MECA in mast cell deficient mice. The modulating effects of 2-Cl-IB-MECA on TNF-α production were greatly reduced although not completely abolished in these mice. Therefore, it is possible that the modulating effects of 2-Cl-IB-MECA on the production of this cytokine may not be wholly mediated by histamine. The drug IB-MECA is also a highly selective adenosine A_3 receptor agonist and was shown to suppress TNF-α and interleukin-12 production by lipopolysaccharide-stimulated peritoneal macrophages from wild-type and adenosine A_{2A} deficient mice (Hasko et al., 2000). Interestingly, IB-MECA lost some of its efficacy in adenosine A_{2A} receptor deficient mice suggesting that its activity was in part produced through that receptor. Although 2-Cl-IB-MECA has been reported to exhibit a higher degree of selectivity for the adenosine A_3 receptor than IB-MECA (Ho et al., 1994), it is possible that it also has the ability to alter TNF-α production through the adenosine A_{2A} receptor.

In summary, both selective adenosine A_{2A} (CGS 21680) and adenosine A_3 receptor (2-Cl-IB-MECA) agonists were found to have the ability to modulate endotoxin-induced cytokine responses in mice. We have presented a substantial body of evidence suggesting that histamine is the direct mediator of cytokine modulation by 2-Cl-IB-MECA, at least in rodent species. In contrast to results obtained in vitro, the histamine H_2 receptor appears to have no role in the modulating effects of histamine on endotoxin-induced cytokine responses in vivo. Instead, the results presented herein

suggest that in vivo, the modulatory effects of histamine on cytokine responses are produced largely through the histamine H_1 receptor. Thus, this study provides new insights into the capacity of histamine to modulate cytokine responses and as a consequence to produce anti-inflammatory effects in vivo.

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