EFFECT OF N-(2-CARBOXYPHENYL)-4-CHLOROANTHRANILIC ACID DISODIUM SALT (CCA) ON THE INDUCTION OF HELPER AND SUPPRESSOR T CELLS IN VITRO AND IN VIVO

Itaru YAMAMOTO, Hitoshi OHMORI and Minoru SASANO

Department of Immunochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-naka 1-1-1, Okayama 700, Japan

Accepted April 2, 1983

Abstract—Keyhole lympet hemocyanin (KLH)-specific suppressor T (Ts) cells that suppress the in vitro secondary anti-trinitrophenyl (TNP) PFC response to TNP-KLH could be induced when murine spleen cells were precultured with KLH. N-(2-carboxyphenyl)-4-chloroanthranilic acid disodium salt (CCA) at 1–100 μg/ml augmented the in vitro induction of Ts cells when the cells were precultured with a suboptimal dose of KLH (10 μg/ml). Ts cell-induction was, however, rather slightly inhibited by the same concentrations of CCA when the lymphocytes were precultured with an optimal amount of KLH (100 μg/ml). In the in vivo experiments, the daily administration of 10 mg/kg CCA for 4 weeks augmented or inhibited Ts cell-induction when mice were immunized with a suboptimal (30 μg/body) or an optimal (100 μg/body) amount of KLH, respectively. However, CCA had no effect on the induction of Ts cells by concanavalin A in vivo. On the other hand, CCA augmented the induction of helper T (Th) cells both in vitro and in vivo when Th cells were induced with a suboptimal amount of antigens. In contrast, the augmentative effect was no longer observed when Th cells were induced by an optimal amount of antigens. These results suggest that CCA is a compound showing immunomodulating properties that affect Ts and Th cell-induction depending on immunological conditions. These immunopharmacological profiles are discussed in connection with its clinical application to an autoimmune disease like rheumatoid arthritis.

N-(2-carboxyphenyl)-4-chloroanthranilic acid disodium salt (CCA) is a compound that has been shown to be clinically effective as an anti-rheumatic drug (1). Although the precise mechanism of action of this drug still remains unknown, it appears to exert its therapeutic effects by modulating immunological processes (2–8). We have reported that CCA showed suppressive effects on the enhanced antibody response to sheep erythrocytes (SRBC), but was augmentative on the lowered response (5, 6). Namely, CCA showed immunopotentiating effects when antibody responses were lowered under the following conditions: a) Decreasing the amount of antigen to a suboptimal level (4–6), b) Administration of an immunosuppressive agent like hydrocortisone (7). The drug, on the contrary, had immunosuppressive effects when it was applied to the enhanced immune responses as follows: a) The spontaneously enhanced antibody response in NZB/NZW F₁ mice (3, 5, 6), b) The secondary antibody response to SRBC, c) The enhanced response to trinitrophenyl (TNP)-human gammaglobulin (HGG) induced by colchicine injection (5, 6). On the other hand, Ohsugi et al. have reported that CCA enhanced blastformation of thymocytes induced by concanavalin A (Con A) in NZB/NZW F₁ mice (3). We have also reported that CCA had almost no effects...
on the antibody response to a thymus-independent antigen, DNP-Ficoll, or the proliferative response to lipopolysaccharide (LPS), a B cell mitogen in normal mice (5, 6). These results suggest that CCA might be a compound possessing a profile as an immunomodulator that would modify T cell functions. In the present paper, we examined the effect of CCA on the induction of suppressor T (Ts) cells or helper T (Th) cells both in vivo and in vitro.

Materials and Methods

Materials

Female BALB/c mice were purchased from Japan Charles River (Tokyo, Japan) and used during 8–12 weeks of age. CCA was supplied from Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Other materials were obtained from the following sources: fetal calf serum (FCS) (GIBCO, Gland Island, NY and Nishi-Nippon Sheep Farm, Fukuyama, Japan), 2-mercaptoethanol (2-ME) (Tokyo Chemical Industry, Tokyo, Japan), RPMI-1640 medium (Nissui Seiyaku, Tokyo, Japan), SRBC (Nishinippon Sheep Farm, Fukuyama, Japan), Con A (E.Y. Laboratories, San Mateo, CA), HGG (Sigma Chemical Company, St. Louis, MO), Keyhole lymypt hemocyanin (KLH) (Calbiochem-Behring Corp., La Jolla, CA). TNP-HGG or TNP-KLH was prepared as described by Haba and Hamaoka (9) or Rittenberg and Amkraut (10), respectively. Antigen doses were expressed as μg protein/ml or μg protein/body for in vitro or in vivo experiments, respectively.

Antibody response in vitro

By using a NUNC multi-dish culture plate with 24 wells (NUNC, Kamstrup, Denmark), BALB/c spleen cells (0.6–1.0×10⁶) were cultured with 2×10⁶ SRBC or 0.1–1.0 μg/ml TNP-KLH in 2 ml of RPMI-1640 medium supplemented with 10% FCS, 1×10⁻⁵ M 2-ME, 50 μg/ml streptomycin and 50 μg/ml penicillin G at 37°C for 4–5 days under 5% CO₂ and 95% air. Plaque-forming cells (PFC) were enumerated according to the method of Jerne and Nordin (11). The number of indirect PFC was calculated by subtracting the number of the direct PFC from the number of plaques detected in the presence of rabbit anti-mouse IgG serum (1:10,000). Lightly conjugated TNP-SRBC were prepared by the modified method of Rittenberg and Pratt (12) and used for assaying anti-TNP PFC.

Induction of antigen-specific Ts cell

In vitro: For the induction of KLH-specific Ts cells, spleen cells (6×10⁶) from BALB/c mice were cultured with 10–100 μg/ml KLH for 4 days. The cells were collected, washed and counted for the number of viable cells. The viable cells (2–8×10⁵) were added to the freshly prepared spleen cells (6×10⁶) from TNP-KLH-primed mice and were cultured with TNP-KLH (0.1 μg/ml) for 5 days. The control culture received the same number of viable cells which were precultured in the absence of KLH for 4 days. The activity of Ts cells was estimated as the degree of suppression of secondary anti-TNP immune response to TNP-KLH. Mice were primed with TNP-KLH by the intraperitoneal injection of TNP-KLH (100 μg) absorbed on 4 mg of aluminum hydroxide gel (alum) 1–2 months prior to use.

In vivo: Mice were injected intraperitoneally with 100 μg KLH dissolved in saline twice at 2 weeks of interval as reported by Tada and Takemori (13). Two weeks after the second injection, these mice were sacrificed, and their spleens were removed. The viable spleen cells (5×10³–5×10⁵) were added to the freshly prepared spleen cells (6×10⁶) from TNP-KLH-primed mice as described above. Then, the Ts cells activity was determined in the same way as above.

In vivo induction of Ts cells by Con A

Ts cells were induced by the injection of
150 μg/body Con A intravenously 24 hr before immunization with SRBC (2×10⁸) according to the procedure of Rich and Pierce (14). Ts cell activity was estimated as the suppression of anti-SRBC PFC response.

**Induction of antigen-specific Th cells**

**In vitro:** For the induction of HGG-specific Th cells, spleen cells (1×10⁷) were cultured with 0.01–0.1 μg/ml HGG for 4 days as described by Kontiainen and Feldmann (15). The cells were collected, washed, and counted for the number of viable cells. The educated viable cells (2×10⁵) were added to the freshly prepared spleen cells (1×10⁶) and cultured with TNP-HGG (0.1 μg/ml) for 4 days. The control culture received the same number of viable cells that were precultured in the absence of HGG. In this experiment, all cultures were performed according to Marbrook's method (15).

**In vivo:** KLH-specific Th cells were induced in vivo by the intraperitoneal injection of KLH (30–100 μg) absorbed on 4 mg of alum. Four weeks after KLH injection, these mice were sacrificed, and their spleens were removed. The viable spleen cells (1×10⁷) were cultured with TNP-KLH (1 μg/ml) for 4 days. Th cell activity was estimated by enumerating the number of anti-TNP direct PFC.

**Results**

**Induction of KLH-specific Ts cells in vitro:** Spleen cells that were precultured with 10–100 μg/ml KLH for 4 days suppressed the secondary anti-TNP PFC response to TNP-KLH in TNP-KLH-primed spleen cells in a dose-dependent manner (Fig. 1A). According to our preliminary experiments, 100 μg/ml KLH was found to induce Ts cell activity maximally. It has been pointed out that the culture of lymphocytes in the absence of added antigen often resulted in the generation of non-specific suppressor activity (16). We also confirmed that this was the case. Some components in FCS appears to be involved in this phenomenon (16). Thus, it is difficult to observe antigen-specific Ts cell activity.

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**Fig. 1.** A) In vitro induction and characterization of KLH-specific suppressor cells. Spleen cells were cultured with 10–100 μg/ml KLH for 4 days. Recovered KLH-educated cells (10⁵–10⁶) were added to TNP-KLH-primed spleen cells (6×10⁶) and cultured with TNP-KLH (0.1 μg/ml) or 2×10⁶ SRBC for 4 days. B) KLH-educated cells were treated with antisera or Sephadex G-10 column prior to the culture with TNP-KLH-primed cells. Cultures were performed in duplicate (panel A) or triplicate (panel B).
under these experimental conditions. We tested a variety of lots of FCS and found a lot (NSF 107) that did not induce such a non-specific suppressor activity. By using this lot of FCS, we succeeded in the in vitro induction and assay of antigen-specific Ts cells.

The induced suppressor activity was specifically abrogated by the treatment with anti-Thy-1.2 plus complement (13), thus indicating that antigen-induced Ts cells were involved in the suppression as shown in Fig. 1B. Ts cell activity appeared to be specific for the carrier protein of the antigen because no inhibition was observed with anti-SRBC response. These properties resemble those of KLH-specific Ts cells reported by Kontiainen and Feldmann (17).

Effect of CCA on the induction of KLH-specific Ts cells in vitro and in vivo: Under our experimental conditions, the maximal Ts cell induction was attained with 100 μg/ml KLH in vitro. CCA at 1–100 µg/ml augmented the induction of Ts cells when a suboptimal dose of KLH (10 μg/ml) was used for the induction. However, the drug showed opposite effects when Ts cells were induced with an optimal dose of KLH (100 μg/ml) as shown in Fig. 2A.

On the other hand, it has been reported that antigen-specific Ts cells can be induced in vivo by injecting KLH without adjuvant twice at 2 weeks of interval (13). We also confirmed that optimal Ts cell activity was generated with 100 μg/body KLH. Ts cells thus induced were assayed for their activity in vitro using TNP-KLH-primed cells as described in Fig. 2A. Almost similar results to those of in vitro experiments were obtained when mice received 10 mg/kg CCA daily for 4 weeks after the first injection of KLH. Namely, CCA inhibited the induction of Ts cells when an optimal dose of KLH was used for the induction. As shown in Fig. 2B, Ts cell induction, however, was not inhibited but rather slightly augmented when Ts cells were induced with 30 μg/body KLH that was

![Fig. 2. A) Effect of CCA on the in vitro induction of KLH-specific Ts cells. Spleen cells were cultured with 10 or 100 μg/ml KLH for 4 days in the presence or absence of CCA. The induced Ts cell activity was assayed by culturing in duplicate with TNP-KLH-primed spleen cells as described in Methods. B) Effect of CCA on the in vivo induction of KLH-specific Ts cells. Mice (n=3) were injected with 30 or 100 μg/body KLH dissolved in saline twice at 2 weeks of interval. Where indicated, CCA was administered p.o. daily for 4 weeks after the first immunization with KLH. Ts cell activities of spleen cells in each mouse were assayed by culturing in duplicate with TNP-KLH-primed spleen cells as described in Methods. Each point represents the mean value from three mice.](image-url)
found to be a suboptimal dose.

**Effect of CCA on the induction of Ts cells by Con A in vivo:** Primary anti-SRBC PFC response in mice were reported to be suppressed by the injection of Con A 24 hr prior to immunization (14). This suppression was found to be mediated by Con A-induced non-specific Ts cells (14). In our experiments, Con A injection resulted in the suppression of the antibody response to approx. 3% of the control level. Daily administration of 10-50 mg/kg CCA for 7 days starting 2 days before Con A injection did not reverse the suppressed response as shown in Table 1.

**Effect of CCA on the induction of Th cell activity in vitro:** Kontiainen and Feldmann have reported that carrier-specific Th cells that augment the anti-hapten antibody response to hapten-carrier antigen can be induced when spleen lymphocytes were cultured with the carrier protein by Marbrook’s method (15). We also confirmed their observation. Spleen cells were cultured with 0.01-0.1 µg/ml HGG for 4 days, and recovered viable cells were cultured with fresh spleen cells using TNP-HGG as an

<table>
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<th>Treatment</th>
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<tr>
<td>Control</td>
<td>204000±12500</td>
<td>1433±108</td>
</tr>
<tr>
<td>Con A</td>
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Four BALB/c mice per group were used.

Table 1. Effect of CCA administration on the Con A-induced suppression of primary PFC response to SRBC in mice

![Graph](image_url)

**Fig. 3.** Effect of CCA on the induction of HGG-specific helper cell activity in vitro. Spleen cells (1×10⁷) were precultured with HGG for 4 days in the presence or absence of 1 µg/ml CCA. Then, these cells (2×10⁶) were cultured in triplicate with fresh unprimed spleen cells (1×10⁷) for 4 days in the presence of 0.1 µg/ml TNP-HGG. All cultures in this experiment were performed by Marbrook’s method.
Fig. 4. Effect of CCA administration on the induction of KLH-specific Th cell activities in vivo. Mice (n=3) were primed with 30 or 100 μg/body KLH in alum 4 weeks before sacrifice. During these periods, CCA was administered p.o. according to the schedule indicated in the figure. Th cell activities in the spleen cells from these mice were assayed by culturing the cells in triplicate with TNP-KLH as described in Methods.

antigen. As illustrated in Fig. 3, addition of carrier-primed lymphocytes resulted in the augmentation of anti-TNP response to TNP-HGG, suggesting that HGG induced Th cell activity. Thus, CCA was examined for its effect on Th cell induction. As depicted in Fig. 3, CCA (1 μg/ml) augmented the induction of Th cells when Th cells were induced by a suboptimal dose of HGG (0.01 μg/ml) (15). On the other hand, CCA did not affect Th cell activity when an optimal dose of HGG (0.1 μg/ml) (15) was employed for the induction.

Effect of CCA on the induction of Th cell activity in vivo: Th cells can also be induced in vivo. Unprimed spleen cells could not elicit primary anti-TNP PFC response to TNP-KLH in vitro. However, a significant number of anti-TNP PFC were observed when KLH-primed spleen cells were cultured with TNP-KLH, thus indicating that Th cell activity was induced by the immunization with KLH (Data not shown). Administration of 10 mg/kg CCA every other day for 4 weeks after priming with KLH augmented the induction of Th cells when a low dose of KLH (30 μg/body) was employed for the induction (Fig. 4). Moreover, much higher enhancement was obtained by CCA administration daily for only the initial 4 days after KLH-priming. The augmentative effect, however, was not observed when Th cells were induced by a high dose of KLH (100 μg/body) as shown in Fig. 4.

Discussion

Our data and those of other authors obtained from in vitro and in vivo experiments demonstrated that CCA exerts its immunomodulating activities by acting on Ts and Th cells. In summary, CCA could a) restore the activity of Ts cells suppressed by colchicine pretreatment (5, 6), b) suppress spontaneously enhanced antibody response in NZB/NZW F1 mice (5, 6), c) enhance blastformation of thymocytes induced by Con A in NZB/NZW F1 mice in which Ts cell activity is known to decline with age (3), d)
IMMUNOMODULATION BY CCA 865

augment the induction of antigen-specific Ts cells when suboptimal doses of antigen were used for the induction (Fig. 2), and e) suppress Ts cell induction by an optimal amount of antigen (Fig. 2). Furthermore, it was shown in this paper that the drug also augmented the induction of antigen-specific Th cells when the cells were induced by suboptimal doses of antigen (Figs. 3, 4). These data demonstrated that CCA would exert either enhancing or suppressive effects depending on the magnitude of the immune responses. Therefore, CCA would fall into the category of an immunomodulator.

Previous reports from our laboratory on the immunomodulating properties of CCA showed that CCA suppresses the antibody response to T cell-dependent antigens when an optimal amount of antigen was present in the culture, while the drug did not suppress but rather enhanced the response when spleen cells were cultured with a suboptimal dose of the antigen (5, 6). Such a modulating activity was not observed with the response to T cell-independent antigen, suggesting that CCA modulates the functions of immunocompetent cells at the T cell level. Tamura and Ishizaka have reported that antibody response is highly dependent on the dose of immunogen in IgE antibody responses (18), i.e., antigen-specific Th cells were induced by the immunization with a lower dose of antigen, and Ts cells by a higher dose of antigen. Antibody response in vitro was found to be dependent on the cell density in the culture; a higher response was obtained at a higher cell-density and a lower response at a lower density. It has been reported that an antibody response is limited by Ts cells in the culture of higher cell-density, but by Th cells in the lower cell-density culture (19). Similar observations were made by Eardley et al. (20). They have reported that SRBC-educated spleen cells suppressed the antibody response of a Mishell-Dutton culture challenged with optimal doses of SRBC. But, the same educated cells helped the response when the assay cultures were performed with suboptimal doses of the antigen. By employing these culture conditions, changing the magnitude of the immune response, we have found that CCA suppressed the enhanced antibody responses, but showed augmenting effects on the lowered responses (5, 6). In the present paper, we showed that CCA is able to enhance or suppress the activities of both Ts and Th cells depending on the dose of antigens. It is likely that the drug would stimulate a T cell population that is more weakly activated, thus resulting in the modulation of the immune response to a normal level. On the other hand, it seems interesting to consider these T cell-activating effects of CCA in relation to its enhancing effect on the intrathymic level of both cyclic GMP and thymosin (8).

In conclusion, although the precise mechanism of action of CCA still remains unknown, these results suggest that the drug possesses an immunopharmacological profile as an immunomodulator that would modify Ts or Th cell functions. It has been reported that the occurrence of autoantibodies including rheumatoid factors and the deletion of Ts cell functions are usually observed in rheumatoid arthritis (21). CCA is expected to augment Ts cell activity and to suppress the immune responses directed to autologous components. Therefore, the present results would explain, at least in part, the clinical therapeutic effects of CCA (1) in the treatment of rheumatoid arthritis.

References
2) Ohsugi, Y., Hata, S., Tanemura, M., Nakano, T., Matsuno, T., Takagaki, T., Nishii, Y. and


