REFERENCES

HAGGENDAL, J. & JOHNSSON, G. (1967). *Acta pharmac. tox.*, **25**, 461–464.
HAGGENDAL, J. & SVEDMYR, N. (1967). *Ibid.*, **25**, 364–368.
WEST, G. B. (1952). *J. Pharm. Pharmac.*, **4**, 560–565.

N-(2-Carboxyphenyl)-4-chloroanthranilic acid disodium salt: prevention of autoimmune kidney disease in NZB/NZW F_1 hybrid mice

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NZB × NZW F_1 hybrid (B/W) mice spontaneously develop antinucleic acid antibodies and immune complex glomerulonephritis which resembles human systemic lupus erythematosus (Helyer & Howie, 1963a; Lambert & Dixon, 1968). Cell-mediated immunity is deficient with age in B/W mice, e.g. the spleen cells of old mice lack the ability to induce a graft-*vs*-host reaction (Gerber, Hardin & others, 1974), and to respond to mitogens (Leventhal & Talal, 1970), and the rejection process of these mice against skin allografts (Gelfand & Steinberg, 1973) and some tumors (Gazdar, Beitzel & Talal, 1971), is impaired. Evidence has accumulated suggesting that the deficiency of suppressor T-cell activity allows the formation of autoantibody and the development of autoimmune diseases in NZB and B/W mice (Allison, Denman & Barnes, 1971; Chused, Steinberg & Parker, 1973; Barthold, Kysela & Steinberg, 1974; Steinberg & Talal, 1975; Krakauer, Waldmann & Strober, 1976; Talal & Steinberg, 1976). In addition, neonatal thymectomy results in a high incidence of disorders in B/W mice (Helyer & Howie, 1963b).

Gershwin & Steinberg (1975a) reported that biweekly injections of thymocytes from young NZB mice, which include suppressor T lymphocytes, inhibited the pathogenesis of autoimmune haemolytic anaemia in syngeneic mice. Very recently a similar prophylactic effect was observed in B/W mice treated with the soluble factor from suppressor T lymphocytes (Krakauer, Strober & others, 1977). Further, it has been reported that injections of concanavalin A (Gershwin & Steinberg, 1975b) or thymosin (Gershwin, Ahmed & others, 1974; Talal & Steinberg, 1976) depressed immunologic abnormality and delayed the onset of, or prevented development of, autoimmune diseases in NZB or B/W mice.

From the above evidence, we considered that agents activating the T-cell function might prevent the appearance of autoantibody and the development of autoimmune desease in B/W mice.

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N-(2-Carboxyphenyl)-4-chloroanthranilic acid disodium salt (CCA), a newly synthesized drug, has immunostimulating activities, i.e. it enhances antibody formation against sheep erythrocytes (Ohsugi, Nakano & others, 1977a) and transformation of spleen cells by concanavalin A both in mice and rats (in preparation). We also reported that CCA inhibited adjuvant arthritis in rats and we thought that its activity might be based upon its stimulation of thymus regulatory function (Ohsugi, Hata & others, 1977b). We therefore examined the effect of CCA on the development of autoimmune desease in female B/W mice. These were bred in our laboratories, and at 9 weeks old, were divided into 3 groups. The first group (controls) consisted of 13 mice given water. The second and third groups had 8 mice each and these were given CCA by mouth daily doses of 5 or 50 mg kg^{-1}, respectively. The drug was administered every day except Sundays until the end of the experiment.

As shown in Figs 1 and 2, in the control group, proteinuria began to appear at age 28 weeks. it then rapidly increased, 9 of 13 mice dying of renal failure at 45 weeks while intense proteinuria was observed in the surviving mice. In contrast, 3 of 16 mice treated with CCA died at the same age as the control group, and proteinuria was markedly depressed. The mean survival times of control and CCA-treated groups at a dose of 5 or 50 mg kg^{-1} was 38, 60 and 62 weeks, respectively.

In the next experiment, B/W mice aged 13 weeks were treated with CCA. At 38 weeks, 6–7 mice in each group, were autopsied, and the kidneys were examined histopathologically. Judgement was made in a blind fashion. In the controls, severe lesions such as hypercellularity, hyaline thickening of capillary walls resembling 'wire loop lesions', and fibrinoid necrosis were observed in glomeruli of most of animals. Mice, treated with CCA, 5 or 50 mg kg^{-1}, showed less glomerular lesions than controls and had almost normal glomeruli.

In the third experiment, administration of CCA was begun at 9-weeks old. Mice were killed at 28 weeks.

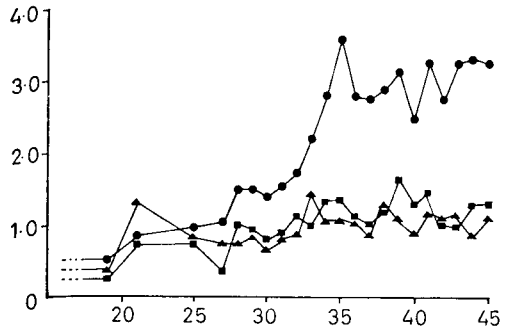

FIG. 1. Effect of oral administration of CCA on proteinuria in B/W mice. Administration of CCA was begun at 9 weeks of age. Proteinuria was tested for once a week using Combistix (papers integrated with tetrabromophenol-blue [pH 3·0]). A grading of 0 to 4 was made depending on the protein concentration in urine, each score shows 0, 0–30, 30–100, and 300–1000 mg ml^{-1}, respectively. The average of score was calculated in each group. ●—Control, ■—CCA (5 mg kg^{-1}), ▲—CCA (50 mg kg^{-1}). Ordinate: Proteinuria. Abscissa: Age (weeks).

Kidneys were removed, frozen at −20°, sectioned at 6 μm in a cryostat, and stained with FITC-labelled rabbit anti-mouse IgG serum (Miles Lab.) for 60 min at 24°. Semiquantitative estimation, depending on the intensity of fluorescence, showed decreased amounts of IgG deposited in glomeruli in CCA-treated animals compared with controls (data not shown).

To investigate the immunological function of the thymuses obtained from these mice, transformation of thymocytes by concanavalin A was examined. Responsiveness of the cells from CCA-treated mice was 2 to 7-fold higher than that of controls (Table 1).

At the age of 28 weeks, anti-DNA antibody in serum was detected by a test kit (The Radiochemical Centre, Amersham). We found that CCA treatment inhibited the antibody production to double stranded native DNA.

As the deficiency of suppressor T-cell function is considered to be relevant to the appearance of autoantibody, an experiment was designed to assess whether

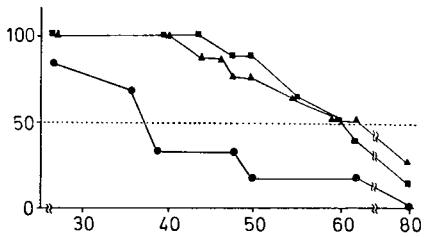

FIG. 2. Effect of oral administration of CCA on survival of B/W mice. CCA was orally administered starting from 9 weeks of age to death of each animal. ●—Control, ■—CCA (5 mg kg^{-1}), ▲—CCA (50 mg kg^{-1}). Ordinate: % Survival. Abscissa: Age (weeks).

CCA activates suppressor T-cell function which is deficient with age. Female B/W mice 12 weeks old were injected with 5 μg of lipopolysaccharide from *Escherichia coli* 055: B5 (Difco, Detroit, Mich.) (LPS) intraperitoneally. Four days later, numbers of splenic plaque forming cells (PFC) were counted by the method of Cunningham & Szenberg (1968). LPS-coated sheep erythrocytes were prepared according to the chromium chloride coupling method (Baker, Stashak & Prescott, 1969). When a dose of 10 or 50 mg kg^{-1} of CCA was given orally 24 h after antigen injection, PFC numbers decreased compared with the corresponding control group (Table 2). These results suggested that CCA had an enhancing effect on suppressor T-cell function. A cell transfer experiment supported this possibility. B/W mice, 8 to 12 weeks old, were used as recipient or donor animals. PFC production in mice that had received 5 × 10^7 thymus cells collected 24 h after oral administration of 10 mg kg^{-1} of CCA, was suppressed more than that of control mice given thymus cells from non-treated donor mice. From these results, CCA seemed to potentiate the suppressor T-cell activity of the thymus.

It has been previously reported that immunosuppressive or anti-inflammatory agents such as cyclophosphamide (Russell, Hicks & Burnet, 1966; Walker & Bole, 1973), glucocorticoids (Casey, 1968), and flufenamic acid (Shiokawa, 1972) showed a prophylactic effect on the incidence of renal diseases in B/W mice. However, CCA shows no anti-inflammatory activity (Ohsugi & others, 1977b), and has an immuno-

Table 1. *Responsiveness of thymocytes to concanavalin A in CCA-treated B/W mice.* B/W F_1 mice were treated with CCA daily from 9 to 28 weeks old. Mice were killed 24 h after the last dose. Thymuses were removed and teased in RPMI 1640 medium supplemented with kanamycin (60 mg $litre^{-1}$), 2 mM L-glutamine and 10% foetal calf serum inactivated at 56° for 30 min. Cell suspensions (1 × 10^6 cells ml^{-1}), pooled from 4 mice per group with or without concanavalin A were maintained in 5% CO_2 in air for 48 h at 37°. [^{3}H]Thymidine (1 μCi, spec. act. 20 mCi μmol^{-1}) was added for 3 h before cell harvest. The radioactivity incorporated into TCA-insoluble DNA was measured using a Beckman liquid scintillation counter. Values are means of duplicate experiments. The stimulation index (S.I.) was expressed as the ratio of counts min^{-1} of concanavalin A-added culture to that of non-stimulated culture.

Concn of concanavalin A ($\mu g\ ml^{-1}$)	^{3}H-Thymidine incorporation					
	Control counts min^{-1}	S.I.	CCA 5 mg kg^{-1} counts min^{-1}	S.I.	CCA 50 mg kg^{-1} counts min^{-1}	S.I.
0	516	1·0	238	1·0	281	1·0
2·5	3937	7·6	3528	14·8	7110	25·3
5	5720	11·1	7232	30·4	20221	72·0
10	3077	6·0	5955	25·0	8755	31·2

Table 2. *Effect of CCA on primary immune response to LPS in NZB/W mice.* Mice were intraperitoneally injected with 5 μg of LPS. Administration of CCA and the plaque-forming cells (PFC) assay was performed 24 h and 4 days after immunization, respectively.

Dose of CCA (mg kg^{-1})	No. of mice	Antibody response to LPS	
		PFC/spleen ± s.e.	PFC/10^6 spleen cells ± s.e.
0	5	24080 ± 5896	310 ± 79
10	5	8880 ± 3363	107 ± 30
50	5	10752 ± 4052	114 ± 42

potentiating rather than immunosuppressive effect (Ohsugi & others, 1977a). Thus, it is obvious that the effect of CCA is based upon a mechanism distinct from that of earlier drugs. Gershwin & Steinberg (1975a) reported that injections of thymus cells, including suppressor cells, from young B/W mice depressed the autoantibody formation and prevented the development of autoimmunity. Recently Krakauer & others (1977) reported that the administration of the soluble factor derived from suppressor T-cells showed a similar prophylactic effect towards autoimmune kidney disease in B/W mice. Moreover, injections of thymosin into young NZB mice preserve suppressor T-cell function (Bach & Niaudet, 1976) and delay the onset of autoimmune disease (Talal & Steinberg, 1976). Administration of Freund's complete adjuvant (Morton & Siegel, 1970) or concanavalin A (Gershwin & Steinberg, 1975b) prevents the development of autoimmune disease in these mice. These findings suggest that non-specific activation of the thymus and/or T-cells prevents autoimmunity.

Our present work suggested that CCA, a non-specific immunostimulant, might exert its activating effect on suppressor T-cells (as shown by suppression of antibody formation against LPS), possibly as a result of stimulation of thymic function (as shown by responsiveness to concanavalin A), and that a mode of action for CCA in the prevention of autoimmune kidney disease might be based on these effects. Moreover, other possible mechanisms of action for CCA such as an antiviral effect, an inhibitory effect on nephritis, or an enhancing effect on the elimination of circulating immune-complex cannot be ruled out.

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REFERENCES

ALLISON, A. C., DENMAN, A. M. & BARNES, R. D. (1971). *Lancet*, **2**, 135–140.

BACH, M. & NIAUDET, P. (1976). *J. Immun.*, **117**, 760–764.

BAKER, P. J., STASHAK, P. W. & PRESCOTT, B. (1969). *Appl. Microbiol.*, **17**, 422–426.

BARTHOLD, D. R., KYSELA, S. & STEINBERG, A. D. (1974). *J. Immun.*, **112**, 9–16.

CASEY, T. P. (1968). *J. Lab. clin. Med.*, **71**, 390–399.

CHUSED, T. M., STEINBERG, A. D. & PARKER, L. M. (1973). *J. Immun.*, **111**, 52–57.

CUNNINGHAM, A. J. & SZENBERG, A. (1968). *Immunology*, **14**, 599–600.

GAZDAR, A. F., BEITZEL, W. & TALAL, N. (1971). *Clin. exp. Immun.*, **8**, 501–509.

GELFAND, M. C. & STEINBERG, A. D. (1973). *J. Immun.*, **110**, 1652–1662.

GERBER, N. L., HARDIN, J. A., CHUSED, T. M. & STEINBERG, A. D. (1974). *Ibid.*, **113**, 1618–1625.

GERSHWIN, M. E., AHMED, A., STEINBERG, A. D., THUMAN, G. B. & GOLDSTEIN, A. L. (1974). *Ibid.*, **113**, 1068–1071.

GERSHWIN, M. E. & STEINBERG, A. D. (1975a). *Clin. Immun. Immunopath.*, **4**, 38–45.

GERSHWIN, M. E. & STEINBERG, A. D. (1975b). *Int. Archs Allergy appl. Immun.*, **48**, 220–224.

HELYER, B. J. & HOWIE, J. B. (1963a). *Nature*, **197**, 197.

HELYER, B. J. & HOWIE, J. B. (1963b). *Lancet*, **2**, 1026-1029.

KRAKAUER, R. S., WALDMANN, T. A. & STROBER, W. (1976). *J. exp. Med.*, **144**, 662–673.

KRAKAUER, R. S., STROBER, W., RIPPEON, D. L. & WALDMANN, T. A. (1977). *Science*, **196**, 56–59.

LAMBERT, P. H. & DIXON, F. J. (1968). *J. exp. Med.*, **127**, 507–523.

LEVENTHAL, B. G. & TALAL, N. (1970). *J. Immun.*, **104**, 918–923.

MORTON, J. I. & SIEGEL, B. V. (1970). *Immunology*, **18**, 379–386.

OHSUGI, Y., NAKANO, T., HATA, S., MATSUNO, T., NISHII, Y. & TAKAGAKI, Y. (1977a). *Chem. Pharm. Bull. Tokyo*, **25**, 2143–2145.

OHSUGI, Y., HATA, S., TANEMURA, M., NAKANO, T., MATSUNO, T., TAKAGAKI, Y., NISHII, Y. & SHINDO, M. (1977b). *J. Pharm. Pharmac.*, **29**, 636–637.

RUSSELL, P. J., HICKS, J. D. & BURNET, F. M. (1966). *Lancet*, **1**, 1281–1284.

SHIOKAWA, Y. (1972). *Diagnosis and Treatment*, **60**, 1646–1651.

STEINBERG, A. D. & TALAL, N. (1975). *Science*, **188**, 245–247.

TALAL, N. & STEINBERG, A. D. (1976). *Ibid.*, **192**, 1089–1091.

WALKER, S. E. & BOLE, G. G. (1973). *J. Lab. clin. Med.*, **82**, 619–633.