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Suppressive Effect of Cycloheximide-*p*-chlorocinnamate on Immune Responses in Mice[1]

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Effects of cycloheximide-*p*-chlorocinnamate on humoral and cellular immune response in mice were investigated. The compound i) suppressed primary and secondary hemagglutinin response, ii) inhibited preferentially the production of IgG type antibodies, iii) reduced the number of hemolytic plaque forming cells in the spleen to nearly zero, and iv) suppressed the induction of delayed-type skin hypersensitivity and foot pad reaction.

Keywords——cycloheximide-*p*-chlorocinnamate; cyclophosphamide; azathioprine; indomethacin; immunosuppression; humoral immunity; cellular immunity; antibody-forming cell; immunoglobulin class; mouse

In the preceding report,[1] it was shown that some cinnamic acid esters of cycloheximide (CHI) were more potent inhibitors of antibody production than CHI in mice. In the present study, we investigated the immunosuppressive action of *p*-chlorocinnamic ester of CHI (CHI-*p*-Cl-cin) in comparison with those of cyclophosphamide and azathioprine. CHI-*p*-Cl-cin inhibited both humoral and cellular responses. Evidence suggested that one of the suppression mechanisms may be inhibition of protein synthesis.

Materials and Methods

Chemicals——CHI-*p*-Cl-cin was generously donated by Mr. Y. Sugawara, Drug Metabolism Research Laboratory of Tanabe Seiyaku Co., Ltd. Cyclophosphamide (Asta-Werke A.G., Chemische Fabrik) and azathioprine (Burroughs Wellcome and Co. (U.S.A.), Inc.) were commercial products. Indomethacin was prepared by extraction and recrystallization of a commercial preparation (Merck and Co., Inc.).

Mice——Male albino dd mice, four weeks of age, were purchased from Shizuoka Agricult. Coop. Assoc. Lab. Animals.

Immunization of Mice with rat erythrocytes (RBC), **Measurement of Hemagglutinin Titer** and **Treatment with Test Compounds** were described previously.[1] One fifth milliliter of blood was taken from the tail tip under ether anesthesia for determination of the hemagglutinin titer when the mouse was to be kept alive for further observations. All the control mice were given the vehicle (5 ml/kg) only.

Immunoglobulin (Ig) Class——To titrate hemagglutinins of the IgG type, serum samples were treated with 0.1 M 2-mercaptoethanol (2-ME) at 37° for 30 min[3] before titration.

Plaque Forming Cells were counted by Jerne's method[4] using 2×10^8 rat RBC per plate.

Delayed Skin Hypersensitivity——Mice were sensitized according to the method of Crowle[5] on day 0 and 7 by injection *s.c.* at the inguinal region with 0.1 ml of a water-in-oil emulsion containing 0.25 mg of ovalbumin (Nutritional Biochem. Corp.; five times crystallized) and 0.3 mg of tubercle bacilli. For the skin test, mice were challenged *i.c.* on day 22 and 28 on the clipped flank with 0.03 ml of 1% ovalbumin dissolved in physiological saline. After three (immediate) and 24 (delayed hypersensitivity) hr, skin reactions were examined for induration size, skin thickness, and presence of central necrosis.

Foot Pad Swelling——Tamura's method[6] was employed with some modifications as follows: mice were sensitized on day 0 by *s.c.* injection at the inguinal region with 2×10^9 rat RBC, and challenged on day 6 by

1) Previous paper in this series of investigation: K. Tsuzurahara, *Chem. Pharm. Bull.* (Tokyo), **25**, 102 (1977).
2) Location: *2-2-50, Kawagishi, Toda-shi, Saitama.*
3) K. Sahiar and R.S. Schwartz, *Science*, **145**, 395 (1964).
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6) S. Tamura and Y. Egashira, Proceedings of the IVth Annual Meeting of the Japanese Society of Immunology, Fukuoka, December, 1974, p. 153.

injecting 2×10^8 RBC in the right hind paw. Foot pad thickness was measured by a dial guage 24 hr after the challenge, and compared with that of the intact left paw.

Results and Discussion

Effect of Administration Route

CHI-*p*-Cl-cin was administered to mice through three different routes, *i.e.*, *s.c.*, *i.p.*, and *p.o.* As shown in Fig. 1, CHI-*p*-Cl-cin suppressed hemagglutinin production most effectively when it was administered *s.c.*. The oral route was without effect. The drugs were therefore administered *s.c.* in the following experiments unless otherwise mentioned.

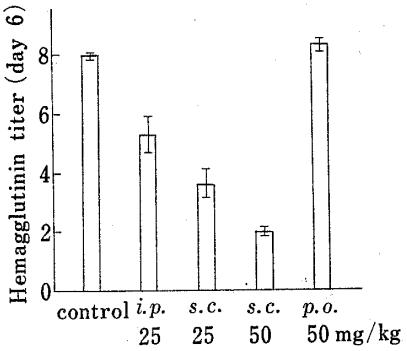

Fig. 1. Effect of Administration Route on Immunosuppressive Activity of CHI-*p*-Cl-cin

Mice were immunized on day 0 and treated with CHI-*p*-Cl-cin on day 0 to 3 and bled on day 6 for assay of hemagglutinin.
mean value with standard error (S.E.) ($n=5$)

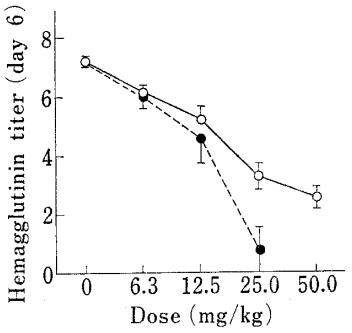

Fig. 2. Dose-response Relations of CHI-*p*-Cl-cin and Cyclophosphamide

Mice, immunized on day 0, were treated *s.c.* with CHI-*p*-Cl-cin (○) or cyclophosphamide (●) on day to 3.
mean value with S.E. ($n=5$)

Dose Response

CHI-*p*-Cl-cin was administered *s.c.* to mice in various doses, and the suppressive effect of hemagglutinin production was compared with that of cyclophosphamide. Dose-dependent suppression of the immune response by the two compounds is obvious as shown in Fig. 2. The suppressive activity of CHI-*p*-Cl-cin was somewhat less than that of cyclophosphamide.

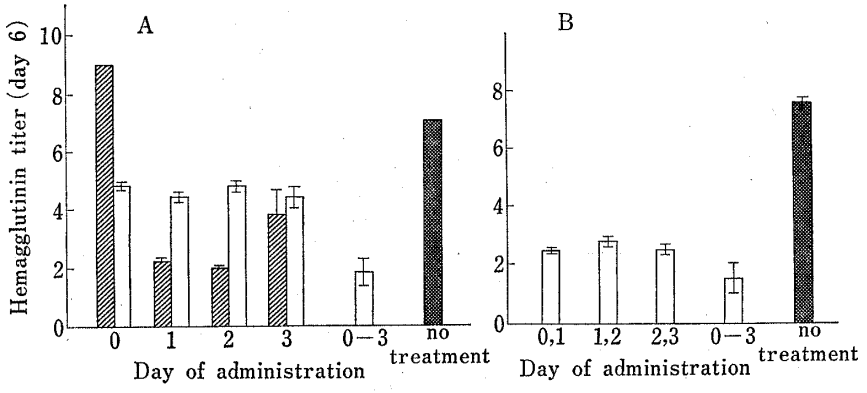

Fig. 3. Effect of Single and Repeated Administration of CHI-*p*-Cl-cin and Cyclophosphamide on Antibody Response

Mice, immunized on day 0, were treated *s.c.* with 50 mg/kg of CHI-*p*-Cl-cin (□) or cyclophosphamide (▨) on the day indicated.
mean value with S.E. ($n=5$)

Effect of Single and Repeated Administrations

CHI-*p*-Cl-cin or cyclophosphamide was administered once (day 0, 1, 2 or 3), twice (day 0 and 1; 1 and 2; or 2 and 3) or four times (day 0 to 3) in the inductive phase of hemagglutinin synthesis. Hemagglutinin titers on day 6 are shown in Fig. 3. Repeated administration of CHI-*p*-Cl-cin was more effective than single administration. There was no difference in response among the different timings of administration with CHI-*p*-Cl-cin, while the effect of a single injection of cyclophosphamide on the hemagglutinin titer varied depending on the day of administration.

From the results with cyclophosphamide the inductive phase of hemagglutinin production could be divided into at least three phases: i) day 0 (insensitive to cyclophosphamide), ii) day 1 to 2 (sensitive), and iii) day 3 (less sensitive). CHI-*p*-Cl-cin may have partially suppressed all of these three phases nondiscriminately. The greater suppression produced by repeated administration might be explained by its possible interference with two or all of these three phases.

Effect on the Later Period of Primary Response

In one group of mice CHI-*p*-Cl-cin was administered on day 0 to 3 and hemagglutinin titer was measured on day 6 and 13. Another group of mice was treated with CHi-*p*-Cl-cin on day 6 to 9 and hemagglutinins were assayed on day 6 and 10.

Treatment on Day 0 to 3——As shown in Fig. 4A the suppressive effect of CHI-*p*-Cl-cin was long-lasting. If it is assumed that the drug has disappeared from the body by day 13, the depressed level of antibody production reflects a suppressive effect of CHI-*p*-Cl-cin on the immune response at an early stage.

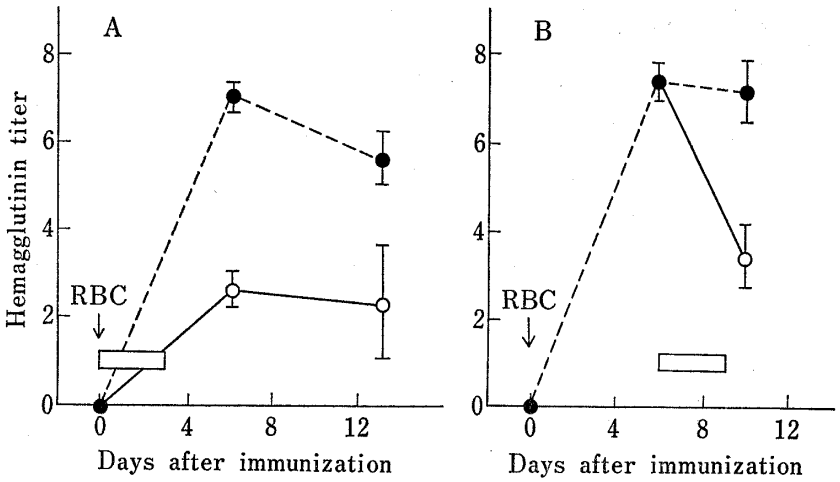

Fig. 4. Effect of CHI-*p*-Cl-cin on Primary Antibody Response

Mice, immunized on day 0, were treated *s.c.* with CHI-*p*-Cl-cin (50 mg/kg per day, ○) or with vehicle (●) on (A) day 0 to 3 or (B) day 6 to 9.
mean value with S.E. ($n=5$)

Treatment on Day 6 to 9——As shown in Fig. 4B, the titer on day 10 was already suppressed markedly by the tratment. This rapid suppressive effect is probably due to an inhibition of hemagglutinin synthesis, since CHI-*p*-Cl-cin is likely to liberate CHI *in vivo*, which is a potent inhibitor of protein synthesis.[1)]

Effect on Secondary Immune Response

Mice were immunized on day 0 and 7 and treated with CHI-*p*-Cl-cin on day 0 to 3 in one group and on day 6 to 9 in another. The titer was assayed on day 6 for the primary response and on day 13 (first group) and 10 (second group) for the secondary response.

Treatment on Day 0 to 3 (Fig. 5A)——The titer on day 6 was naturally suppressed by the treatment on day 0 to 3, but after the second antigenic stimulation the titer on day 13 increased to 5.0, which was, however, lower than the primary response in control mice. This may mean that the drug affects the development of immunological memory, or that the treated animals have not fully recovered from the effect of the drug at the time of second stimulation.

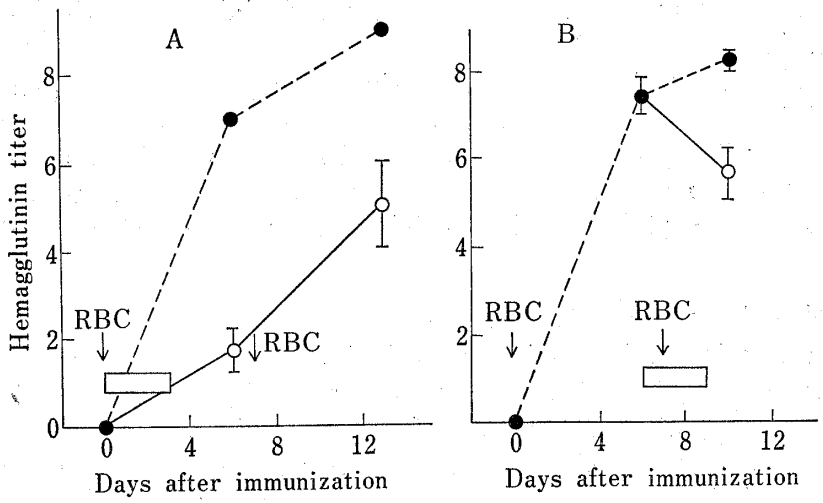

Fig. 5. Effect of CHI-*p*-Cl-cin on Secondary Antibody Response

Mice, immunized on day 0 and 7, were treated *s.c.* with CHI-*p*-Cl-cin (50 mg/kg per day, ○) or with vehicle (●) on (A) day 0 to 3 or (B) day 6 to 9. mean value with S.E. ($n=5$)

Treatment on Day 6 to 9 (Fig. 5B)——The titer on day 10 (one day after the last treatment) of the treated mice was lower than not only the secondary response (on day 10) but also the primary response (on day 6) of control mice, suggesting again the inhibition of antibody protein synthesis.

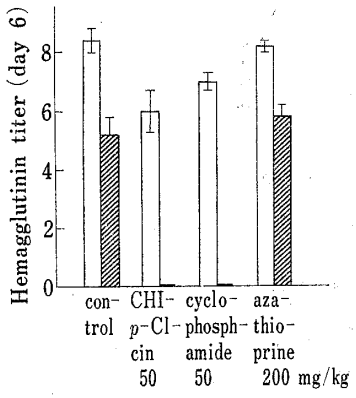

Fig. 6. Effect of CHI-*p*-Cl-cin, Cyclophosphamide and Azathioprine on 2-ME-sensitive and -resistant Hemagglutinin Response

Mice were treated *s.c.* with CHI-*p*-Cl-cin (50 mg/kg), cyclophosphamide (50 mg/kg) or azathioprine (200 mg/kg), 2 hr after immunization. Hemagglutinin titer (day 6) was assayed with intact (□) or 2-ME treated (▨) sera. mean value with S.E. ($n=5$)

On the Immunoglobulin (Ig) Class Affected

In order to test which class of Ig is preferentially affected, the serum samples from the treated mice were treated with 2-ME before assay. When the titer on day 6 with untreated serum was partially suppressed by single administration on day 0 of CHI-*p*-Cl-cin (50 mg/kg), 2-ME-resistant antibodies (IgG) were totally absent (Fig. 6). Although the effect of cyclophosphamide on the level of total hemagglutinins was insignificant (also see Fig. 3A), the production of 2-ME-resistant antibodies were almost completely inhibited by this alkylating agent. Under the same conditions, 200 mg/kg of azathioprine did not modify the production of both 2-ME-sensitive and -resistant classes of hemagglutinins.

Effect on Plaque Forming Cells

Hemolytic plaque forming cells in the spleen of treated mice (50 mg/kg per day from day 0 to 3) were counted by Jerne's direct method. The results shown in Table I indicate that the suppression of hemagglutinin production was also attributable to the reduction of antibody forming cells.

Table I. Effect of CHI-*p*-Cl-cin on Hemolytic Plaque Forming Cells (PFC) in the Spleen

Experiment	Treatment	No. of mice	Spleen Wt. (mg)	PFC (per spleen)
1	CHI-*p*-Cl-cin	5	60.4± 9.1	0
	Control	4	150.0±16.2	14700±3300
2	CHI-*p*-Cl-cin	5	94.5±13.3	30± 10
	Control	4	152.8±14.3	8700±1200

Mice, immunized on day 0, were treated *s.c.* with 50 mg/kg per day of CHI-*p*-Cl-cin on day 0 to 3, and killed on day 4 for counting PFC in the spleen by Jerne's direct method. No PFC were observed in the spleen of non-immunized mice.
mean value with S.E. ($n=5$)

Effect of Cellular Immunity

Delayed Skin Hypersensitivity——Mice sensitized with ovalbumin were treated with CHI-*p*-Cl-cin (25 mg/kg per day), cyclophosphamide (25 mg/kg per day) or azathioprine (50 mg/kg per day) three times (every two or three days) a week for two or three weeks. On day 22 and 28, the mice were challenged with the antigen and the skin reactions were examined. The results (Table II) show that the three compounds inhibited the delayed skin hypersensitivity in mice and that the effects of CHI-*p*-Cl-cin and cyclophosphamide were more long-lasting than that of azathioprine.

Table II. Effect of CHI-*p*-Cl-cin, Cyclophosphamide and Azathioprine on Delayed Skin Hypersensitivity

Treatment	Period (wk)	No. of mice	No. of mice positive at day 22		No. of mice positive at day 28
			3 hr	24 hr	24 hr
CHI-*p*-Cl-cin	3	5	0	0	0
	2	4	0	1	0
Cyclophosphamide	3	5	0	0	0
	2	3	0	0	1
Azathioprine	3	5	0	1	2
	2	4	0	1	4
Control	3	9	0	8	8

Mice were sensitized on day 0 and 7 by injecting *s.c.* into the inguinal region 0.25 mg of ovalbumin in complete Freund adjuvant, and treated *s.c.* with CHI-*p*-Cl-cin (25 mg/kg per time), cyclophosphamide (25 mg/kg per time) or azathioprine (50 mg/kg per time), three times a week for two or three weeks. On day 22 and 28, mice were challenged by injecting *i.c.* on the clipped flank 0.03 ml of 1% ovalbumin solution. Skin reactions were examined 3 (immediate) and 24 (delayed hyper sensitivity) hours after challenge.

Foot Pad Response——Mice sensitized with rat RBC were treated *s.c.* or *i.p.* on day 0 to 2 (inductive phase) or day 5 to 6 (symptomatic phase) with 12.5 or 25.0 mg/kg per day of CHI-*p*-Cl-cin, and foot pad swelling was measured on day 7 (Table III). CHI-*p*-Cl-cin inhibited the reaction whether it was given during the inductive or symptomatic phase. The inhibition by the treatment on day 5 and 6 was complete and comparable with that produced by 6 mg/kg per day of indomethacin, while cyclophosphamide showed no inhibition under the conditions used.

In conclusion, CHI-*p*-Cl-cin seems to inhibit various phases and types of immune responses in mice. The immunosuppressive activity of the drug might be attributed to the cytotoxic effect of CHI on immune lymphocytes by virtue of its capacity as an inhibitor of protein synthesis, since CHI would easily produced by hydrolysis of the ester bond in the body.[1)]

Table III. Effect of CHI-*p*-Cl-cin, Cyclophosphamide and Indomethacin on Foot Pad Reaction

Compound	Treated on day	Dose (mg/kg per day)	Route	Δ Thickness ($\times 10^{-2}$ mm)
CHI-*p*-Cl-cin	0—2	25.0	*i.p.*	24.2±4.4[a]
CHI-*p*-Cl-cin	0—2	12.5	*s.c.*	25.6±3.4[b]
Cyclophosphamide	0—2	25.0	*i.p.*	49.6±9.2[c]
CHI-*p*-Cl-cin	5—6	25.0	*i.p.*	17.0±4.7[b]
CHI-*p*-Cl-cin	5—6	12.5	*s.c.*	17.3±2.9[d]
Indomethacin	6[e]	6.0[e]	*i.p.*	16.0±1.4[d]
Control	0—2		*i.p.*	39.0±1.8
Unsensitized control				12.2±1.7[d]

Mice were sensitized on day 0 by injecting *s.c.* 2×10^9 rat RBC, treated with CHI-*p*-Cl-cin, cyclophosphamide or indomethacin, and challenged on day 6 by injecting 2×10^8 RBC in the right rear paw. The foot pad swelling was measured 24 hr later.

a) $p<0.02$
b) $p<0.01$
c) not significant
d) $p<0.001$
e) divided into two injections (2 hr before and 2 hr after the challenge)
mean value with S.E. ($n=5$)

The reason for the greater activity of CHI-*p*-Cl-cin compared with free CHI[1] should probably be sought in differences in their pharmacokinetic behavior, such as the rate of metabolic inactivation and cell membrane permeability.

The well-known inhibitors of protein synthesis, puromycin and chloramphenicol, have been shown to alter the immune response in experimental animals.[7] Amiel and Dore[7b,7c,8] reported a selective effect of puromycin on immune responses in mice, showing that treatment with the antibiotic inhibited the production of antibodies to polio virus, but did not prolong the survival of allogeneic skin grafts. On the other hand, chloramphenicol inhibited both humoral and cellular immunity in rabbits.[9] The reason why puromycin was inert in allograft immunity is not clear, but it should be noted that the high toxicity of the antibiotic is inconvenient for thorough studies.

Butler and Coons[10] investigated the effect of chloramphenicol on the secondary immune response in mice. They showed that chloramphenicol administered at the priming of mice with the antigen (diphtheria toxoid) almost completely inhibited the secondary response, but the effect was slight or negligible when given during the secondary response. In the present study, however, the suppressive effect of CHI-*p*-Cl-cin on the secondary response was only partial (Fig. 5A), and the suppression of ongoing antibody production, which may be considered to reflect the inhibition of protein synthesis, was significant (Fig. 4B and Fig. 5B for the primary and secondary immune responses, respectively). The inhibition of antibody protein synthesis has also been observed by Ambrose and Coons[11] with chloramphenicol in *in vitro* secondary immune response. Discrepancy between these results and that obtained by Butler and Coons[10] may be only of quantitative nature, and might be explained by differences in antigen and duration of drug administration.

7) *a*) A.E. Gabrielsen and R.A. Good, "Advances in Immunology," Vol. 6, ed. by F.J. Dixon, Jr., and J.H. Humphrey, Academic Press, New York, 1967, p. 91; *b*) G.W. Camiener and W.J. Wechter, "Progress in Drug Research," Vol. 16, ed. by E. Jucker, Birkhäuser Verlag, Basel and Stuttgart, 1972, p. 67; *c*) T. Makinodan, G.W. Santos, and R.P. Quinn, *Pharmacol. Rev.*, **22**, 189 (1970).
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