THE IMMUNOSUPPRESSIVE EFFECTS OF TRICHOHECENES AND CYCLOCHLOROTINE ON THE ANTIBODY RESPONSES IN GUINEA PIGS

HIROMICHI ITO, KYOKO WATANABE, AND JIRO KOYAMA

Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita-ku, Sapporo, 060, Japan

(Received January 9, 1982)

Immunosuppressive effects of trichothecenes of *Fusarium solani* and *Fusarium nivale*, T-2 toxin and fusarenon-X, and also of a mycotoxin of *Penicillium islandicum*, cyclochlorotine, were studied by measuring the anti-2,4-dinitrophenyl (DNP) antibody responses in guinea pigs immunized with DNP-bovine serum albumin. Among these mycotoxins, T-2 toxin alone suppressed strongly the anti-DNP antibody responses at a certain sublethal dose. With other mycotoxins, no effect was observed at any sublethal doses tested. All of the mycotoxins, on the other hand, inhibited the *in vitro* blast transformation of guinea pig splenic cells stimulated with lipopolysaccharide of *Escherichia coli* (B cell mitogen) or concanavalin A (T cell mitogen), when measured by incorporation of ³H-thymidine into DNA. Their inhibitory activities were independent of the sort of mitogen used. As in the case of the antibody responses, T-2 toxin was most potent in reducing the DNA synthesis, and exhibited about 10 and 1000 times as potent inhibitory activity as fusarenon-X and cyclochlorotine, respectively.

Keywords—T-2 toxin; fusarenon-X; cyclochlorotine; antibody response; guinea pig

Many trichothecenes have been isolated as causative toxicants in etiological studies of naturally occurring intoxications associated with moldy foods and feeds.^{1,2)} The common symptoms of acute intoxications by these mycotoxins in animals are diarrhea, nausea, vomiting, and decreases in spontaneous movement and body temperature.³⁾ The histopathological changes caused are characterized by marked damage to actively dividing cells of the gastrointestinal tract, spleen, thymus, etc., as seen in so-called “radiomimetic injury”.⁴⁻⁹⁾ It seems, therefore, of interest to investigate the immunosuppressive effects of trichothecene mycotoxins on the antibody responses in animals. For this purpose, we attempted to investigate the effects of T-2 toxin and fusarenon-X isolated from culture filtrates of *Fusarium solani* and *Fusarium nivale* on the anti-2,4-dinitrophenyl (DNP) antibody responses in guinea pigs

immunized with DNP-bovine serum albumin (DNP-BSA). For comparison, the effect of cyclochlorotine, a mycotoxin isolated from culture filtrate of *Penicillium islandicum*, also was studied, since the mycotoxin was found to cause hepatic damage by disturbing carbohydrate metabolism.¹⁰⁾

In this paper, the effects of these mycotoxins on the *in vivo* anti-DNP antibody responses as well as the *in vitro* responses of guinea pig splenic cells toward lipopolysaccharide of *Escherichia coli* (LPS) and concanavalin A (Con A) are reported.

MATERIALS AND METHODS

Mycotoxins—T-2 toxin, fusarenon-X and cyclochlorotine purified from culture filtrates of *Fusarium solani*, *Fusarium nivale* and *Penicillium islandicum*, respectively, were generously donated by Dr. T.Tatsuno, The Institute of Physical and Chemical Research, Wako, Saitama, Japan.^{7,11-13)}

Antigens — The DNP-BSA and DNP-hen ovalbumin (DNP-OA) used as antigens were prepared, as described previously.¹⁴⁾ The DNP-BSA and DNP-OA contained 9.0 and 15 mol of DNP residue per 70000 and 45000 g of protein, respectively.

Guinea Pigs — Randomly bred Hartley strain albino guinea pigs, weighing 250–300 g, were obtained from the Hokkaido Experimental Animal Center, Co.

Administration of Mycotoxins and Immunization of Guinea Pigs — Several groups of guinea pigs, each consisting of ten animals, were treated with mycotoxins, as shown in Fig. 1. For this purpose, various amounts of T-2 toxin, fusarenon-X and cyclochlorotine in 1.0 ml of saline were intraperitoneally injected on each occasion. The doses of these mycotoxins were expressed by the amounts administered on each occasion (mg per kg of body weight).

The animals thus treated were immunized two times by intraperitoneal injections of 0.1 mg of DNP-BSA at an interval of three weeks. For immunization, 0.05 ml of an antigen solution was emulsified with an equal volume of Freund's complete adjuvant (FCA) and injected.

Determination of Amounts of Antibodies — The serum levels of anti-DNP antibodies produced were determined by passive hemagglutination and precipitin reaction. Passive hemagglutination was performed using DNP-OA-coupled sheep

erythrocytes prepared with bis-diazotized benzidine.¹⁵⁾ Serial two-fold dilutions of anti-DNP antibodies (0.025 ml) were reacted with 0.025 ml of a 2 % suspension of the sensitized erythrocytes in 0.11 M phosphate buffer supplemented with 0.02 % gelatin, pH 7.4, using microtitrators.¹⁶⁾ After incubation for 16 h at room temperature, the hemagglutination patterns were observed, and the activity (AU) was expressed by maximal dilution which still gave clear agglutination of erythrocytes.

Quantitative precipitin reaction was performed by adding various amounts of DNP-OA in 10 mM phosphate buffered saline, pH 7.5 (PBS) to a constant amount of anti-DNP antibody.¹⁶⁾ The volumes of reaction mixtures were adjusted to 1.0 ml by adding PBS. After incubation for 1 h at 37°C and then for 48 h at 4°C, the precipitates formed were determined by measuring the absorbance at 280 nm after washing three times with PBS and dissolving in 3.0 ml of 1 % sodium carbonate. The amounts of anti-DNP antibody were calculated from both the amounts of precipitates at an equivalence point and antigen added.

Determination of Amounts of IgG1 and IgG2 Antibodies — Fractionation of IgG antibodies to IgG1 and IgG2 subclasses was performed by DEAE-cellulose column chromatography, as described in a previous paper.¹⁶⁾ The IgG2 antibody was recovered in the unadsorbed fraction in 5 mM phosphate buffer, pH 7.6, and the IgG1 antibody was eluted at a phosphate concentration of 20 mM. Each amount of IgG1 and IgG2 anti-DNP antibodies was determined by measuring passive hemagglutinating activity and expressed by AU per ml of antiserum.

Preparation of Splenic Cell Suspension — A splenic cell suspension was prepared, as described by Braciale and Parish,¹⁷⁾ with some modifications. The organ was isolated from a single guinea pig. Using Eagle's minimal essential medium (MEM) supplemented with 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), the spleen, free from adjacent tissues, was gently pressed and passed through a cloth net.

FIG. 1. The Schedule of Administration of Mycotoxins and Immunization
 ←←, immunization; ←, injection of mycotoxin; —, bleeding.

The cell suspension thus obtained were centrifuged down at 1000 rpm at 4°C for 7 min, and was washed with MEM-HEPES. The number of live cells was determined by a trypan blue exclusion test,¹⁸⁾ and the cells were suspended in an appropriate volume of MEM.

Incorporation of ³H-Thymidine by Splenic Cells stimulated with Mitogens—Uptake of ³H-thymidine by splenic cells was determined, as described by Blomgren and Svedmyr.¹⁹⁾ MEM medium containing 10 % fetal calf serum heated at 56°C for 30 min was used as reaction medium. Splenic cells (1×10^6) were distributed into sterile plastic dishes (Falcon Plastics, U.S.A., 35×10 mm) and incubated with 20 μg of LPS of *E. coli* (Difco Laboratories, U.S.A.) or 4 μg of Con A (a gift from Dr. S.Ishii) in 1.0 ml of the reaction medium. After 24 h of incubation in a humidified atmosphere of 5 % CO₂ and 95 % air at 37°C, 1 μCi of ³H-thymidine (³H-TdR, 21.8 Ci/mmol, New England Nuclear Corp., U.S.A.) was added to each dish and incubated further for 24 h. An equal volume of 20 % trichloroacetic acid (TCA) was added to each dish, and the reaction mixtures were filtered on glass filters (Whatman CF/C, 2.4 cm, Whatman Ltd., U.S.A.). The precipitates thus collected were washed with cold 10 % TCA and then dried. Radioactivity of the precipitates was determined in a liquid scintillation counter, using 10 ml of scintillation fluid (Econofluor, New England Nuclear Corp., U.S.A.).

The arithmetic mean of the triplicate samples was determined, and results were expressed as cpm or as the difference in cpm between stimulated and control cultures (Δ_{cpm}). The effects of mycotoxins were determined by measuring Δ_{cpm} in the presence of mycotoxins and expressed by percentage against control Δ_{cpm} .

RESULTS

The Effects of Mycotoxins on the Antibody Responses

To study the immunosuppressive effects of T-2 toxin, fusarenon-X and cyclochlorotine on the antibody responses in guinea pigs, three groups of animals received intraperitoneal injections of sub-

lethal doses of each mycotoxin during two periods of five days around primary and secondary immunization by injections of DNP-BSA (Fig. 1). The doses of T-2 toxin, fusarenon-X and cyclochlorotine were 0.5, 0.75 and 0.15 mg/kg, respectively. Through the entire course of experiment, these doses did not cause any appreciable decrease in body weight of the animals.

When the amounts of anti-DNP antibodies of pooled sera of each group were determined by passive hemagglutination, T-2 toxin was found to suppress markedly the anti-DNP antibody response through the entire course of immunization (Fig. 2). Fusarenon-X and cyclochlorotine, on the other hand, did not appreciably affect the antibody response.

The effects of these mycotoxins were confirmed more definitely by quantitative precipitin reaction, which generally gives a more accurate value for quantitative estimation of antibody content than does the passive hemagglutination reaction (Table I). The precipitin analysis revealed

FIG. 2. Effects of Mycotoxins on the Anti-DNP Antibody Response

Each group consisting of ten guinea pigs was treated with each mycotoxin and immunized, as shown in Fig. 1. Control group treated with saline (○); A, T2-toxin (0.5 mg, ●); B, fusarenon-X (0.5 mg, ●); C, cyclochlorotine (0.15 mg, ●).

that administration of T-2 toxin reduced the antibody response in 32 % of control animals, as estimated after the secondary immunization. With fusarenon-X and cyclochlorotine, no appreciable effect again was observed.

The incapacibilities of fusarenon-X and cyclochlorotine in suppressing the antibody response was further confirmed by measuring IgG1 and IgG2 anti-DNP antibodies (Table II). T-2 toxin, on the other hand, suppressed both the IgG1 and IgG2 anti-DNP antibody responses. In addition, no significant difference in the susceptibility existed between these two responses.

The Effects of Mycotoxins on the in Vitro Responses of Splenic Cells toward Mitogens

To study the effects of mycotoxins on B and T cells, splenic cells isolated from a single guinea pig were tested for their reactivities toward LPS and Con A in the absence or presence of mycotoxins,

since LPS and Con A are presumably a B cell- and T cell-mitogen, respectively.^{20,21)} As evidenced by the incorporation of ³H-thymidine into the cells, the reactivities of splenic cells toward LPS and Con A were inhibited, in a dose-dependent manner, by three mycotoxins used (Fig. 3).

Complete inhibition of the DNA synthesis occurred at 10, 100 and 10000 ng/ml of T-2 toxin, fusarenon-X and cyclochlorotine, respectively; the toxic activity of T-2 toxin was 10 and 1000 times higher than those of fusarenon-X and cyclochlorotine, respectively. The susceptibilities of splenic cells to the toxic effects of these three mycotoxins did not seem to be dependent on the nature of mitogen used, though an enhancing effect was observed with the cells stimulated with Con A in the presence of a low concentration of cyclochlorotine (1 μ g/ml). These results may indicate that the B and T cells of guinea pigs are

TABLE I. *Effects of Mycotoxins on the Anti-DNP Antibody Response estimated by Precipitin Analysis*

Mycotoxin (dose)	Antibody content	
	mg/ml	(%)
Control	1.38	(100)
T2-toxin (0.5 mg)	0.44	(32)
Fusarenon-X (0.75 mg)	1.21	(88)
Cyclochlorotine (0.15 mg)	1.05	(76)

The antibody contents were estimated by precipitin analysis, using pooled sera obtained 1 week after secondary immunization.

TABLE II. *Effects of Mycotoxins on the IgG1 and IgG2 Anti-DNP Antibody Responses*

Mycotoxin (dose)	2 Weeks		4 Weeks	
	IgG1	IgG2	IgG1	IgG2
	AU/ml (%)	AU/ml (%)	AU/ml (%)	AU/ml (%)
Control	200 (100)	69 (100)	1100 (100)	670 (100)
T2-toxin (0.5 mg)	100 (50)	29 (42)	390 (35)	370 (55)
Fusarenon-X (0.75 mg)	160 (80)	55 (80)	1100 (100)	760 (113)
Cyclochlorotine (0.15 mg)	220 (110)	56 (81)	1300 (118)	660 (99)

Pooled sera used were the same as those in Fig. 1, and amounts of IgG1 and IgG2 anti-DNP antibodies were estimated by passive hemagglutination reaction after fractionation by DEAE-cellulose chromatography.

not different in the susceptibilities to these mycotoxins.

To confirm the incapacibilities of fusarenon-X and cyclochlorotine in suppressing the *in vivo* antibody responses, guinea pigs were treated with increased doses of 1.0 mg/kg of fusarenon-X and 0.5 mg/kg of cyclochlorotine, respectively. All the animals, however, died of acute intoxication by two administrations of these doses before primary immunization. These results may indicate that certain types of target cells other than the immunocompetent cells involved in the antibody responses are more susceptible to the toxicities of these mycotoxins.

DISCUSSION

Many studies on trichothecene mycotoxins indicate that the effects of these mycotoxins on the thymus, spleen and lymph nodes, and the development of infective foci in various organs of mice strongly suggest an immunosuppressive

action.⁴⁻⁹⁾ Histological examination also reveals marked cytotoxic changes in the tissues with actively dividing cells, such as the mucosa of the small intestine, germinal center of the lymph node, and other lymph apparatus. The results reported in this paper, in fact, demonstrated that T-2 toxin suppressed, at a sublethal dose, the antibody response in guinea pigs. Another trichothecene, fusarenon-X, however, was not found to exhibit any immunosuppressive activity at the sublethal doses tested. Though fusarenon-X inhibited the *in vitro* responses of splenic cells toward mitogenic stimulation with LPS and Con A, its toxicity was less than that of T-2 toxin. The failure of fusarenon-X for suppressing selectively the *in vivo* antibody response, therefore, may be explained by either the less toxicity on the B and T cells or the less selective toxicity on these cells.

The inhibitory effects of T-2 toxin and fusarenon-X on protein and DNA syntheses were investigated by Ueno *et al.*²²⁾ using whole cell and cell-free systems of reticulocytes, Ehrlich ascites tumor cells and liver cells. For instance, they reported that T-2 toxin as well as fusarenon-X inhibited protein synthesis in guinea pig reticulocytes, and also that the cells were markedly more sensitive to T-2 toxin than fusarenon-X; the ID₅₀ values causing 50 % inhibition were 7 and 200 ng/ml for T-2 toxin and fusarenon-X. These values are of the same order of magnitude as the ID₁₀₀ values for the DNA synthesis in splenic cells obtained in this study.

As in the case of fusarenon-X, cyclochlorotine also did not exhibit any selective toxicity on the antibody response. Its toxicity on the DNA synthesis in splenic cells stimulated with LPS and Con A also was found to be markedly less than those of T-2 toxin and fusarenon-X. When rats and mice are injected with cyclochlorotine, the organ most striking by and rapidly involved is the liver, and as for other organs no remarkable change was found.^{10,12,23)} This nature of cyclochlorotine may explain the failure of suppressing selectively the antibody response.

It is well known that the IgG antibody populations produced in guinea pigs vary markedly in

FIG. 3. Effects of Mycotoxins on the *in Vitro* Responses of Splenic Cells toward LPS and Con A. Splenic cells were stimulated with LPS (●) or Con A (○) in the absence or presence of various concentrations of mycotoxins, and uptake of ³H-thymidine into DNA was measured, as described in "METHODS". A, T2-toxin; B, fusarenon-X; C, cyclochlorotine.

respect to the ratio of IgG1 and IgG2 subclasses, depending on the nature and dose of antigen.^{16,24-26)} In addition, the IgG1 antibody response is found to be more sensitive to certain immunosuppressive agents such as Methotrexate, Bredinin and azathiopurine, compared with the IgG2 antibody response.^{16,24)} These properties seem to provide critical information for the mechanism causing different behaviors of the IgG1 and IgG2 antibody responses. The results with T-2 toxin, however, did not reveal any definite difference in the susceptibility to this mycotoxin between the IgG1 and IgG2 antibody responses. In this respect, the immunosuppressive activity of T-2 toxin seems to be similar to those of cyclophosphamide and hydrocortisone, which were found to inhibit the IgG1 and IgG2 antibody responses in the same manner.¹⁶⁾

Acknowledgement This work was supported in part by a grant-in-aid from Science and Technology Agency, Japan. The authors wish to thank Dr. T.Tatsuno, The Institute of Physical and Chemical Research, Wako, Saitama, Japan for providing the mycotoxins used in this work, and also thank to Prof. S.Ishii, Faculty of Pharmaceutical Sciences, Hokkaido University for supplying Con A.

REFERENCES

- 1) M.Saito and K.Ohtsubo: Trichothecene toxins of *Fusarium* species. In I.F.H.Purchase (ed.). Mycotoxins. Elsevier, Amsterdam, 1974, pp.263-282.
- 2) E.B.Smalley and F.M.Strong: Toxic trichothecenes. In I.F.H.Purchase (ed.). Mycotoxins. Elsevier, Amsterdam, 1974, pp.199-228.
- 3) N.Sato and Y.Ueno: Comparative Toxicities of trichothecenes. In I.F.H.Purchase (ed.). Mycotoxins in human health, Pathotox Publishers, Inc., 1977, pp.295-307.
- 4) M.Saito, M.Enomoto and T.Tatsuno: Radiomimetic biological properties of the new scirpene metabolites of *Fusarium nivale*, *Gann* **60**, 599-603 (1960).
- 5) A.Z.Joffe: Toxicity of *Fusarium poae* and *F. sporotrichioides* and its relation to alimentary toxic aleukia. In I.F.H.Purchase (ed.). Mycotoxins. Elsevier, Amsterdam, 1974, pp.229-262.
- 6) Y.Ueno, I.Ueno, Y.Itoi, H.Tsunoda, M.Enomoto and K.Ohtsubo: Toxicological approaches to the metabolites of *Fusaria* III. Acute toxicity of fusarenon-X, *Japan J. exp. Med.*, **41**, 521-539 (1971).
- 7) Y.Ueno, K.Ishii, K.Sakai, S.Kanaeda, H.Tsunoda, T.Tanaka and M.Enomoto: Toxicological approaches to the metabolites of *Fusaria* IV. Microbial survey on "Bean-hulls poisoning of horses" with the isolation of toxic trichothecenes, neosolaniol and T-2 toxin of *Fusarium solani* M-1-1, *Japan J. exp. Med.*, **42**, 187-203 (1972).
- 8) Y.Ueno, N.Sato, K.Ishii, K.Sakai and M.Enomoto: Toxicological approaches to the metabolites of *Fusaria* V. Neosolaniol, T-2 toxin and butenolide, toxic metabolites of *Fusarium sporotrichioides* NRRL 3510 and *Fusarium poae* 3287, *Japan J. exp. Med.*, **42**, 461-472 (1972).
- 9) K.Terao, K.Kera and T.Yazima: The effects of trichothecene toxins on the bursa of Fabricius in day-old chicks, *Virchows Arch. B Cell Path.*, **27**, 359-370 (1978).
- 10) M.Saito, M.Enomoto and T.Tatsuno: Yellow rice toxins, luteoskyrin and related compounds, chlorine-containing compounds, and citrinin. In A. Ciegler, S.Kadis and S.J.Ajl (ed.), Microbial toxins. Academic Press, New York and London, 1971, pp.299-380.
- 11) Y.Fujimoto, Y.Morita et T.Tatsuno: Recherches toxicologiques sur les substances toxiques de *Fusarium nivale*: Étude chimique des toxins principales, Nivalenol, Fusarenon-X Nivalenol-4,15-di-o-acétate, *Chem. Pharm. Bull.*, **20**, 1194-1203 (1972).
- 12) K.Uraguchi, T.Tatsuno, M.Tsukioka, Y.Sakai, F.Sakai, O.Yonemitsu, H.Ito, M.Miyake, M.Saito, M.Enomoto, T.Shikata and T.Ishiko: Isolation of two toxic agents, luteoskyrin and chlorine-containing peptide, from the metabolites of *Penicillium islandicum* Sopp. with some properties thereof. *Japan J. exp. Med.*, **31**, 19-35 (1961).
- 13) M.Sato and T.Tatsuno: Chemical studies on chlorine-containing peptide - one of the toxic metabolites of *Penicillium islandicum* Sopp. I. Structure and synthesis of dehydrochlorinated peptide amide, *Chem. Pharm. Bull.*, **16**, 2182-2185 (1968).
- 14) K.Furuichi and J.Koyama: The effect of antigen dose on the amounts and affinities of guinea pig IgG1 and IgG2 antihapten antibodies, *Immunochemistry*, **13**, 807-812 (1976).
- 15) E.Hyslop and E.M.Roeder: Passive immune hemolysis: titration of hemolytic anti-protein antibody using bis-diazotized benzidine to couple antigen to erythrocytes, *Proc. Soc. exp. Biol. Med.*, **121**, 514-522 (1966).
- 16) H.Ito, T.Nakamura and J.Koyama: The effects of various immunosuppressive agents on antibody populations produced in guinea pigs, *J. Pharm. Dyn.*, **2**, 157-163 (1979).
- 17) V.L.Braciale and C.R.Parish: Inhibition of *in vitro* antibody synthesis by cyclophosphamide-induced sup-

- pressor cells, *Cell. Immunol.*, **51**, 1–12 (1980).
- 18) R.R.Rich and C.W.Pierce: Biological expressions of lymphocyte activation I. Effects of phytomitogens on antibody synthesis *in vitro*, *J. exp. Med.*, **137**, 205–223 (1973).
 - 19) H.Blomgren and E.Svedmyr: Evidence for thymic dependence of PHA-reactive cells in spleen and lymph nodes and independence in bone marrow, *J. Immunol.*, **106**, 835–841 (1971).
 - 20) I.Grey, J.Kruger and S.Z.Spiesel: Stimulation of B-lymphocytes by endotoxin. Reactions of thymus-deprived mice and karyotypic analysis of dividing cells in mice bearing T_6T_6 thymus grafts, *J. Immunol.*, **108**, 1088–1091 (1972).
 - 21) J.D.Stobo, A.S.Rosenthal and W.E.Paul: Functional heterogeneity of murine lymphoid cells I. Responsiveness to and surface binding of concanavalin A and phytohemagglutinin, *J. Immunol.*, **108**, 1–17 (1972).
 - 22) Y.Ueno, M.Nakajima, K.Sakai, K.Ishii, N.Sato and N.Shimada: Comparative toxicology of trichothec mycotoxins: Inhibition of protein synthesis in animal cells, *J. Biochem.*, **74**, 285–296 (1973).
 - 23) M.Umeda: Cytotoxic effects of the mycotoxins of *Penicillium islandicum* Sopp, luteoskyrin and chlorine-containing peptide on Chang's liver cells and Hela cells, *Acta Pathol. Japan*, **14**, 373–394 (1964).
 - 24) S.Harel, H.P.Liacopoulos and S.Ben-Efaim: Inhibition of immunological components by antigenic competition and other immuno-depressing procedures, *Immunology*, **22**, 515–524 (1972).
 - 25) T.Nakamura, T.Ohkawa and J.Koyama: Effect of varying the immunizing dose of ovalbumin on the production of nonprecipitating IgG1 and IgG2 antibodies in guinea pigs, *J. Biochem.*, **75**, 23–32 (1973).
 - 26) K.Furuichi, T.Nakamura and J.Koyama: Sequential IgM and IgG2 anti-DNP antibody responses against DNP-*E. coli* and DNP-lipopolysaccharides in guinea pigs, *Immunology*, **32**, 435–443 (1977).