THE EFFECTS OF VARIOUS IMMUNOSUPPRESSIVE AGENTS ON ANTIBODY POPULATIONS PRODUCED IN GUINEA PIGS

HIROMICHI ITO, TOHORU NAKAMURA AND JIRO KOYAMA

Department of Hygienic Chemistry, Faculty of Pharmaceutical Sciences, Hokkaido University, Kitaku, Sapporo, 060, Japan

(Received November 18, 1978)

Effects of immunosuppressive agents on immunochemical properties of antibody populations produced in guinea pigs were studied with Methotrexate, cyclophosphamide, hydrocortisone, Bredinin (4-carbamoyl-1- β -D-ribofuranosyl imidazolium-5-olate), azathioprine and 6-mercaptopurine. Among these agents, Methotrexate and cyclophosphamide alone suppressed strongly the antibody responses, not depending on the degree of the T cell-dependency of antigens used. Hydrocortisone, Bredinin and azathioprine also suppressed the antibody responses slightly, and no effect was observed with 6-mercaptopurine. With cyclophosphamide and hydrocortisone, no substantial difference in the susceptibility was observed between the IgG1 and IgG2 antibody responses. On the other hand, the IgG1 antibody response appeared to be slightly more sensitive to the immunosuppressive activities of Bredinin and azathioprine, compared with the IgG2 antibody response. With Methotrexate, the difference in the susceptibility between these two responses seemed to vary depending on the kind of antigens used. In addition, the antigen-binding abilities of antibodies produced were not influenced by the treatment with Methotrexate and cyclophosphamide.

Keywords —Methotrexate; cyclophosphamide; hydrocortisone; Bredinin; azathioprine; 6-mercaptopurine; antibody response; guinea pig

It is well known that the antibody populations produced in guinea pigs vary in respect to the ratio of IgG1 and IgG2 antibody and the affinity for antigen, depending on the nature and dose of antigen and the sort of adjuvant used.¹⁻⁴⁾ On the other hand, Harel *et al.*²⁾ reported that the treatment of guinea pigs with Methotrexate diminished extensively the formation of IgG1 anti-2,4-dinitrophenyl (DNP) antibody with DNP-bovine serum albumin (DNP-BSA) but the formation of IgG2 anti-DNP antibody was not reduced as much. Apart from clinical purpose, such study with immunosuppressive agents seems to provide critical information for the mechanism causing different behaviors of the IgG1 and IgG2 antibody responses on antigen stimulus.

Then, the effects of various immunosuppressive

agents were studied by measuring immunochemical properties of antibody populations produced by immunization with T cell-dependent antigens [ovalbumin (OA) and DNP-BSA] and a T cell-independent antigen [DNP-lipopolysaccharide of *Escherichia coli* (DNP-LPS)].⁴⁾ The effects of Methotrexate, cyclophosphamide, hydrocortisone, Bredinin (4-carbamoyl-1- β -D-ribofuranosyl imidazolium-5-olate),^{5,6)} azathioprine and 6-mercaptopurine are reported in this paper.

MATERIALS AND METHODS

Chemicals —Methotrexate, cyclophosphamide and Bredinin were kindly supplied by Japan Lederle Co., Ltd., Shionogi & Co., Ltd., and Toyo Jozo Co., Ltd., respectively. Hydrocortisone monoacetate, azathioprine and 6-mercaptopurine were

obtained from Nakarai Chemicals, Ltd., the Wellcome Foundation Ltd., and Sigma Chemical Co., respectively.

Antigens—The hen ovalbumin (OA), DNP-OA, DNP-BSA and DNP-LPS used as antigens were prepared, as described in previous papers from our laboratory.^{3,4)} The DNP-OA and DNP-BSA contained 15 and 9.0 mol of DNP residue per 45000 and 70000 g of protein, respectively. The DNP-LPS contained 2.0 μ mol of DNP residue per mg nitrogen.

Guinea Pig—Female randomly bred Hartley strain albino guinea pigs, weighing 250–300 g, were obtained from the Hokkaido Experimental Animal Center, Co. and used for experiments.

Administration of Immunosuppressive Agents and Immunization of Guinea Pigs—Several groups of guinea pigs, each consisting of ten animals, were treated with immunosuppressive agents, as shown in Fig. 1. For this purpose, various amounts of Methotrexate, cyclophosphamide and Bredinin in 1–2 ml of saline were intraperitoneally injected on each occasion. In the case of hydrocortisone, azathioprine and 6-mercaptopurine, each agent was suspended in 1–2 ml of 0.1% methylcellulose in saline and similarly injected. The doses of these agents were expressed by the amounts administered on each occasion (mg per kg of body weight).

The animals thus treated were immunized two times by either intramuscular injections of 0.1 mg of OA or intraperitoneal injections of 0.1 mg of DNP-LPS or DNP-BSA at an interval of three weeks. For immunization, 0.05 ml of antigen solutions was emulsified with 0.05 ml of Freund's

complete adjuvant (FCA) and injected.

The animals were bled by cardiac puncture at intervals of seven days after primary immunization, and the antibody responses were followed by measuring the serum level of antibody produced.

Determination of Amounts of Antibodies—The serum levels of antibodies produced were determined by passive hemagglutination and precipitin reaction.

Passive hemagglutination was performed using OA- or DNP-OA-coupled sheep erythrocytes prepared with *bis*-diazotized benzidine.⁷⁾ Serial two-fold dilutions of anti-OA or anti-DNP antibody (0.025 ml) were reacted with 0.025 ml of a 2% suspension of OA- or DNP- OA-erythrocytes in 0.11 M phosphate buffer supplemented with 0.02% gelatin, pH 7.4, using microtitrators (Tominaga Co.). After incubation for 16 hr at room temperature, the hemagglutination patterns were observed. Hemmagglutinating activity of antibody (AU) was expressed by maximal dilution which still gave clear agglutination of erythrocytes.

In the case of precipitin reaction, precipitation was made by adding varying amounts of OA or DNP-OA in 10 mM phosphate-buffered saline, pH 7.5 (PBS) to a constant amount of anti-OA or anti-DNP antibody in PBS. The volumes of reaction mixtures were adjusted to 1.0 ml by adding PBS. After incubation for 1 hr at 37° and then for 48 hr at 4°, the precipitates formed were centrifuged and washed three times with PBS. The amount of antibody was determined by measuring the absorbance at 280 nm after dissolving in 3.0 ml of 1% sodium carbonate and calculated from both the amounts of precipitate at an equivalence point and antigen added.⁸⁾

Determination of Amounts of IgM, IgG1 and IgG2 Antibodies—IgM antibody was separated from IgG antibodies by gel filtration on Sepharose 6B using PBS.⁴⁾ Fractionation of IgG antibodies to IgG1 and IgG2 subclasses was performed by DEAE-cellulose column chromatography. Antisera were dialyzed extensively against 5 mM phosphate buffer, pH 7.6, and chromatographed on DEAE-cellulose columns equilibrated with the same buffer. The columns were washed until the

FIG. 1. *The Schedule of Administration of Various Immunosuppressive Agents and Immunization*

protein concentration of eluate became trace as estimated by measuring the absorbance at 280 nm, and then the bound proteins were eluted by a linearly increasing phosphate concentration gradient. The IgG2 antibody was recovered in the unadsorbed fraction and the IgG1 antibody was eluted at a phosphate concentration of 20 mM.³⁾ Each amount of IgM, IgG1 and IgG2 antibodies was determined by measuring passive hemagglutinating activity and expressed by AU per ml of antiserum.

Estimation of Affinity of Anti-OA Antibody to OA — The affinity of anti-OA antibody for homologous antigen was measured by the Farr technique,⁹⁾ using ¹²⁵I-OA which was prepared by the ICI method.¹⁰⁾ A series of test tubes was prepared, containing varying amounts of ¹²⁵I-OA in 0.5 ml of borate buffer, $\mu = 0.1$, pH 8.4 (BB), and 0.1 ml of antibody (2.0×10^{-7} mmol) in BB and 0.4 ml of normal guinea pig serum diluted ten-fold with BB. After incubation for 1 hr at 37° and then 16 hr at 4°, 1.0 ml of saturated ammonium sulfate was added, and the reaction mixtures were centrifuged in the cold. Radioactivities of the supernatants and precipitates washed three times with 50% saturated ammonium sulfate were measured to determine concentrations of free and bound ¹²⁵I-OA. The reaction mixtures consisting of normal guinea pig serum and ¹²⁵I-OA were also processed as controls.

The binding data obtained were plotted by the Scatchard plots¹¹⁾ based on the amounts of anti-OA antibody estimated by precipitin analysis.

RESULTS

The Effects of Immunosuppressive Agents on the Antibody Response to OA

Six groups of guinea pigs received intraperitoneal injections of Methotrexate, cyclophosphamide, hydrocortisone, Bredinin, azathioprine or 6-mercaptopurine, respectively, during two periods of five days around primary and secondary immunization by intramuscular injections of OA in FCA. The measurement of anti-OA antibody of pooled sera of each group by passive hemagglutination indicated that Methotrexate and cyclophosphamide suppressed markedly the anti-

FIG. 2. Effects of Various Agents on the Antibody Response to OA

Each group consisting of ten guinea pigs was treated with each agent and immunized, as described in "METHODS". Control group treated with saline ($\circ$ — $\circ$): A, Methotrexate (2 mg, $\times$ — $\times$; 5 mg, $\bullet$ — $\bullet$); B, cyclophosphamide (13 mg, $\times$ — $\times$; 38 mg, $\bullet$ — $\bullet$); C, hydrocortisone (100 mg, $\bullet$ — $\bullet$); D, Bredinin (200 mg, $\bullet$ — $\bullet$); E, azathioprine (100 mg, $\bullet$ — $\bullet$); F, 6-mercaptopurine (50 mg, $\bullet$ — $\bullet$).

OA antibody response through the entire course of immunization, depending on the dosage administered (Fig. 2). On the other hand, the treatment with hydrocortisone and Bredinin also reduced the antibody response though slightly, and no definite effect was observed with azathioprine and 6-mercaptopurine.

The effects of these agents were confirmed more definitely by precipitin analysis of anti-OA antibody produced after the secondary immunization, excepting the case of azathioprine (Table I). Since the precipitin reaction generally gives a more accurate

TABLE I. *Effects of Immunosuppressive Agents on the Anti-OA Antibody Response*

Agent (Dose)	Antibody content	
	mg/ml	%
Control	2.81	100
Methotrexate (5 mg)	0.075	2.8
Cyclophosphamide (13 mg)	0.87	31
Hydrocortisone (100 mg)	0.65	23
Bredinin (200 mg)	0.64	23
Azathioprine (100 mg)	1.70	61
6-Mercaptopurine (50 mg)	3.24	115

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

value for quantitative estimation of antibody content than does the passive hemagglutination reaction, the result by precipitin analysis indicates that the treatment with azathioprine reduces the antibody response in 61% of control animals.

The suppressive effects of these agents were further analyzed after fractionation of the IgG antibodies to IgG1 and IgG2 subclasses by DEAE-cellulose column chromatography. Methotrexate, cyclophosphamide and hydrocortisone suppressed both the IgG1 and IgG2 antibody responses (Table II). No significant difference in the susceptibility existed between these two responses. On the other hand, the IgG1 antibody response appeared slightly

TABLE II. *Suppression of the IgG1 and IgG2 Anti-OA Antibody Responses*

Agent (Dose)	2 Weeks		4 Weeks	
	IgG1	IgG2	IgG1	IgG2
	AU/ml(%)	AU/ml(%)	AU/ml(%)	AU/ml(%)
Control	120 (100)	50 (100)	4300 (100)	2000 (100)
Methotrexate (5 mg)	1 (0.8)	3 (5.4)	52 (1.2)	21 (1.1)
Cyclophosphamide (13 mg)	20 (17)	7 (14)	1400 (33)	450 (23)
Hydrocortisone (100 mg)	22 (18)	19 (39)	750 (17)	570 (29)
Bredinin (200 mg)	50 (42)	45 (90)	850 (20)	1000 (50)
Azathioprine (100 mg)	61 (51)	54 (110)	1900 (44)	2100 (110)

Pooled sera used were the same as those in Fig. 1.

TABLE III. *Effects of Immunosuppressive Agents on the IgM and IgG Anti-DNP Antibody Responses to DNP-LPS and DNP-BSA*

Antigen	Agent (Dose)	Primary immunization ^{a)}		Secondary immunization ^{a)}
		IgG	IgM	IgG
DNP-LPS		AU/ml(%)	AU/ml(%)	AU/ml(%)
	Control	110 (100) ^{b)}	140 (100)	1600 (100) ^{b)}
	Methotrexate (2 mg)	64 (58)	66 (47)	240 (15)
	Cyclophosphamide (13 mg)	85 (77)	53 (38)	290 (18)
	Bredinin (200 mg)	46 (42)	59 (42)	410 (26)
DNP-BSA				
	Control	160 (100)	16	1500 (100)
	Methotrexate (2 mg)	71 (44)		510 (34)
	Cyclophosphamide (13 mg)	68 (43)		460 (31)
	Bredinin (200 mg)	23 (14)		230 (15)

a) Pooled sera of each group of animals used were obtained 2 weeks after primary immunization and 1 week after secondary immunization, respectively.

b) More than 85% of the IgG anti-DNP antibodies was of IgG2 subclass, when estimated by fractionation by DEAE-cellulose chromatography.

FIG. 3. Comparison of Antigen-Binding Abilities of Anti-OA Antibodies Produced in Either the Presence or Absence of Methotrexate or Cyclophosphamide

Antigen-binding abilities of anti-OA antibodies produced in guinea pigs treated with Methotrexate and cyclophosphamide were determined using pooled sera 1 week after secondary immunization. The procedures are described in "METHODS". A, control group treated with saline; B, Methotrexate (5 mg); C, cyclophosphamide (13 mg). C = concentration of free antigen; r = mol of antigen bound per mol of antibody.

more sensitive to the suppressive activities of Bredinin and azathioprine than the IgG2 antibody response.

The Influence of T Cell-Independency of Antigen on Immunosuppressive Activities of Various Agents

A previous paper⁴⁾ from our laboratory reported that DNP-LPS induced a unique antibody response in guinea pigs; it elicited sequential production of IgM and IgG2 anti-DNP antibodies, both of which were substantially T cell-independent.

Then, the immunosuppressive effects of Methotrexate, cyclophosphamide and Bredinin were examined using DNP-LPS as an antigen and compared with those on the T cell-dependent antibody response to DNP-BSA. In this case, pooled sera on the early phase of immunization (two weeks after primary immunization) were gel filtered on columns of Sepharose 6B for separate measurements of IgM and IgG antibodies since DNP-LPS elicited a markedly higher IgM antibody response, relative to OA and DNP-BSA, after primary immunization.⁴⁾

The treatment with these three agents again suppressed both the IgM and IgG antibody responses (Table III). The degrees of suppression with each agent were essentially the same as those on the responses to OA and DNP-BSA, indicating that the susceptibility of the antibody responses did not appreciably depend on degree of the T cell-dependency of antigens used. The separate measurements of IgG1 and IgG2 antibodies after fractionation by DEAE-cellulose column chromatography indicate that the IgG1 antibody response to DNP-BSA appeared slightly more sensitive to the suppressive activities of Methotrexate and Bredinin (Table IV).

Antigen-Binding Abilities of Anti-OA Antibodies Produced under the Influence of Immunosuppressive Agents

Antigen-binding abilities of anti-OA antibodies produced under the marked immunosuppression with Methotrexate and cyclophosphamide were measured by the Farr technique.⁹⁾ The results obtained did not reveal any definite difference in the ability between the anti-OA antibodies produced in

TABLE IV. Suppression of the IgG1 and IgG2 Anti-DNP Antibody Responses to DNP-BSA

Agent (Dose)	Primary immunization		Secondary immunization	
	IgG1	IgG2	IgG1	IgG2
	AU/ml(%)	AU/ml(%)	AU/ml(%)	AU/ml(%)
Control	150 (100)	54 (100)	660 (100)	360 (100)
Methotrexate (2 mg)	35 (23)	54 (100)	190 (29)	180 (50)
Cyclophosphamide (13 mg)	46 (31)	33 (61)	110 (17)	66 (18)
Bredinin (200 mg)	11 (7)	6 (11)	43 (7)	290 (81)

Pooled sera of each group of animals used were the same as those in Table III.

either the presence or absence of the agents (Fig. 3). In all the cases, extrapolation of the binding curves plotted by the Scatchard plots gave a value of 2 for r (mol of bound antigen per mol of antibody).

The binding curves, however, were not linear, being made up of two parts having different slopes, as observed by Tamoto *et al.*¹²⁾ This indicates two major groups of combining sites with respect to affinity for OA, the one of high affinity with an average binding constant of the order of 10^7 M^{-1} and the other of low affinity with an average constant of the order of 10^5 M^{-1} . No significant difference in these average binding constants of high or low affinity sites may be explained as follows; the treatment with Methotrexate and cyclophosphamide reduces the amounts of anti-OA antibodies produced without any influence on antigen-binding abilities of the antibodies.

DISCUSSION

Many investigations have demonstrated that the immune responses in guinea pigs are reduced strongly by administration of Methotrexate,²⁾ cyclophosphamide,¹³⁻¹⁷⁾ and hydrocortisone,¹⁸⁾ but azathioprine¹⁵⁾ and 6-mercaptopurine^{14,17,19)} are less and not effective, respectively. The results presented in this paper not only support previous observations but also reveal that the suppressive effects of these agents do not appear to depend on the degree of the T cell-dependency of antigens used. In addition, Bredinin was found also to reduce the antibody responses in guinea pigs as in the case of mice.⁵⁾

Recently, Harel *et al.*²⁾ reported the results suggesting that some Methotrexate-sensitive factor might be involved in the IgG1 antibody response but not in the IgG2 antibody response. Our results appear to confirm those by Harel *et al.*²⁾ when DNP-BSA was used as an antigen. The difference in the susceptibility to the agent between the IgG1 and IgG2 antibody responses, however, was not so marked as expected from the data by Harel *et al.* Furthermore, the difference in the susceptibility disappeared in the animals immunized with OA and DNP-LPS. On the contrary, Bredinin and azathioprine seemed to reduce more strongly the

IgG1 antibody response than the IgG2 antibody response, though the suppression was not high.

At present, we have no evidence demonstrating the involvement of any mechanism causing the different sensitivities of the IgG1 and IgG2 antibody responses. It might be possible that these different sensitivities reflect different dependencies of the IgG1 and IgG2 antibody responses on T cell function. Previous study⁴⁾ in our laboratory on guinea pigs with depleted thymus cells by neonatal thymectomy and treatment with anti-thymus cell antibody did not show any definite difference in T cell-dependency between the IgG1 and IgG2 antibody responses.

It is well known that antigen-binding abilities of antibodies vary depending on the dosage of antigen. Furthermore, a number of investigations¹⁾ using various methods for measuring antigen-binding abilities have noted a progressive change in the binding characteristics of antibodies with increase in time after immunization. In particular, the increase of affinity of anti-hapten antibodies with time after immunization has been shown to be an important feature of the antibody response. This phenomenon, referred to by the term "maturation of the antibody response" was found to occur also in guinea pigs.^{3,20)} The antigen-binding abilities of anti-OA antibodies produced under the influence of Methotrexate and cyclophosphamide were not found to differ from those of antibodies produced in control animals. Therefore, the administration of these agents may not affect the maturation of antibody response.

Acknowledgement The authors are indebted to Japan Lederle Co., Ltd., Shionogi & Co., Ltd., and Toyo Jozo Co., Ltd., for kindly supplying Methotrexate, cyclophosphamide and Bredinin.

REFERENCES

- 1) G.M.Siskind and B.Benacerraf: Cell selection by antigen in the immune response, *Adv. Immunol.*, **10**, 1—50 (1968).
- 2) S.Harel, P.Liacopoulos and S.Ben-Efraim: Inhibition of immunological components by antigenic competition and other immunodepressing procedures, *Immunology*, **22**, 515—524 (1972).
- 3) K.Furuichi and J.Koyama: The effect of antigen dose on

- the amounts and affinities of guinea pig IgG1 and IgG2 anti-hapten antibodies, *Immunochemistry*, **13**, 807—812 (1976).
- 4) 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).
 - 5) K.Mizuno, M.Tsujino, M.Takada, M.Hayashi, K.Atsumi, K.Asano and T.Matsuda: Studies on Bredinin I, Isolation, characterization and biological properties, *J. Antibiotics*, **27**, 775—782 (1974).
 - 6) H.Yoshioka, K.Nakatsu, M.Hayashi and K.Mizuno: Studies on Bredinin II, The molecular structure of Bredinin, *Tetrahedron Lett.*, **46**, 4031—4034 (1975).
 - 7) 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).
 - 8) T.Nakamura, K.Ofune, K.Tamoto and J.Koyama: Immunochemical studies on guinea pig non-precipitating antibodies to ovalbumin, *J. Biochem.*, **71**, 245—256 (1972).
 - 9) R.S.Farr: A quantitative immunochemical measure of the primary interaction between I°BSA and antibody, *J. Infect. Dis.*, **103**, 239—262 (1958).
 - 10) A.L.Grossberg, G.Radzimski and D.Pressman: Effect of iodination on the active sites of several anti-hapten antibodies, *Biochemistry*, **1**, 391—401 (1962).
 - 11) F.Karush: Immunologic specificity and molecular structure, *Adv. Immunol.*, **2**, 1—40 (1960).
 - 12) K.Tamoto, T.Nakamura and J.Koyama: Non-precipitating guinea pig antibodies produced by administration of excessive doses of ovalbumin, *Immunology*, **26**, 455—463 (1974).
 - 13) H.C.Maguire and E.Steers: Inhibition of precipitin formation in the guinea-pig with cyclophosphamide, *Experientia*, **19**, 591—592 (1963).
 - 14) H.I.Maibach and H.C.Maguire: Role of species specificity in failure of inhibition of guinea pig anaphylactic sensitization with cancer chemotherapy drugs, *Nature* (London), **197**, 82—83 (1963).
 - 15) H.I.Maibach and H.C.Maguire: Studies on the inhibition of antibody formation in guinea-pig with cyclophosphamide, azathioprine, 5-fluorouracil and urethane, *Int. Arch. Allergy*, **29**, 209—212 (1966).
 - 16) J.L.Turk, D.Parker and L.W.Poulter: Functional aspects of the selective depletion of lymphoid tissue by cyclophosphamide, *J. Immunol.*, **23**, 493—501 (1972).
 - 17) A.Winkelstein: Differential effects of immunosuppressants, *J. Clin. Invest.*, **52**, 2293—2299 (1973).
 - 18) P.M.Seebohm, M.M.Tremaine and W.S.Jeter: The effect of cortisone and adrenocorticotrophic hormone on passively transferred delayed hypersensitivity to 2,4-dinitrochlorobenzene in guinea pigs, *J. Immunol.*, **73**, 44—48 (1954).
 - 19) D.S.Genghof and J.R.Battisto: Antibody production in guinea pigs receiving 6-mercaptopurine, *Proc. Soc. Exp. Biol. Med.*, **107**, 933—936 (1961).
 - 20) E.A.Goidel, W.E.Paul, G.W.Siskind and B.Benacerraf: The effect of antigen dose and time after immunization on the amount and affinity of anti-hapten antibody, *J. Immunol.*, **100**, 371—375 (1968).