ANTIGEN-INDUCED ELEVATION OF CYCLIC GMP LEVEL IN PLASMA AND ITS ABOLITION IN EXPERIMENTALLY IMMUNOSUPPRESSIVE MICE

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Intravenous injection of antigenic dose of sheep erythrocytes (SRBC)* into mice caused a 150–200 % increase in plasma cyclic GMP level within 5 min which continued for 60 min thereafter. Immunization by soluble antigens such as dextran sulfate and bovine serum albumin also elevated cyclic GMP level in plasma. The plasma cyclic GMP increased by antigen stimulation might be derived from immunocompetent cells. This assumption was supported by several lines of evidence as follows: (1) rat erythrocytes which are less antigenic for mice caused a relatively low response in terms of increase in cyclic GMP compared with SRBC, (2) this cyclic GMP response was abrogated in animals under immunosuppressive states where X-irradiated, azathioprine-treated or tumor-bearing mice were used. Mice pretreated with agents which block autonomic nervous system functions develop normal plasma cyclic GMP responses upon SRBC injection. Our observation in this report and many *in vitro* studies by other investigators suggested that plasma cyclic GMP elevated by antigen stimulation may be mainly derived from lymphoid cells as a consequence of the triggering of immune response.

Keywords—SRBC; plasma cyclic GMP; immunosuppression; PFC; immune response

INTRODUCTION

It has been widely documented in recent years that cyclic nucleotides (cyclic AMP and cyclic GMP) are possibly involved in immune responses.¹⁾ It was originally reported by Hadden *et al.*²⁾ that optimal mitogenic concentration of PHA or Con A produced 10- to 50-fold elevation in lymphocyte cyclic GMP level within 20 min of exposure to these mitogens. Watson³⁾ reported that the addition of LPS to mouse spleen cell culture induced rapid and transient increase in the intracellular cyclic GMP level, but not cyclic AMP. Furthermore, Weinstein *et al.*⁴⁾ demonstrated that exogenous cyclic GMP and its deriva-

tives could stimulate resting mouse spleen lymphocytes in culture to synthesize RNA and DNA, with a time course similar to that caused by Con A. Based on these results, it has been proposed that endogenous cyclic GMP acts as an intracellular mediator of the proliferative response to mitogens. In relation to these *in vitro* observation, Yamamoto and Webb⁵⁾ previously showed that mice injected intravenously with SRBC demonstrated a transient increase in splenic cyclic AMP level (4-fold) which peaked at 2 min, and continuous increase in splenic cyclic GMP level (1.5-fold) which occurred beginning 5–10 min after injection.

* Abbreviations used in this paper: cyclic AMP, adenosine 3',5'-cyclic monophosphate; cyclic GMP, guanosine 3',5'-cyclic monophosphate; SRBC, sheep erythrocytes; RRBC, rat erythrocytes; BSA, bovine serum albumin; PFCs, plaque forming cells; EDTA, ethylenediamine tetraacetic acid; PHA, phytohemagglutinin; Con A, concanavalin A; PWM, pokeweed mitogen.

There are several reports that the administration of hormones or neurotransmitters to experimental animals and human subjects results in a sharp increase in plasma level of cyclic nucleotide.⁶⁾ Since the cyclic nucleotide level in plasma is in a dynamic steady-state relation with its intracellular pools and is usually kept low, a slight increase of these nucleotides in tissue would be expected to result in a significant increase in extracellular fluids.^{6a,b,7)}

Based on these findings, we decided to investigate the effects of *in vivo* antigenic stimulation on plasma cyclic GMP levels.

MATERIALS AND METHODS

Animals — ddY male mice (6–8 weeks of age) and Sprague-Dawley strain of male rats (6–8 weeks of age) were used in these studies.

Antigens — Sheep erythrocytes were purchased from Nishinohon Sheep Farm (Hiroshima, Japan). The SRBC were washed three times in normal saline prior to injection. Bovine serum albumin was obtained from Sigma (St. Louis, Mo). Dextran sulfate (MDS Kowa Injection) was purchased from Kowa Co., Ltd. (Nagoya, Japan). Rat erythrocytes were purified from heparinized blood by Ficoll-Conray sedimentation and washed three times in normal saline prior to use. All antigens were dissolved or suspended in normal saline and intravenously injected into the tail vein of mice.

Reagents — Azathioprine and propranolol hydrochloride (Inderal Injectable) were kindly supplied from Tanabe Seiyaku Co., Ltd. (Osaka, Japan) and from Sumitomo Chemical Industry (Osaka), respectively. Phenoxybenzamine was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Atropine was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan) and hexamethonium chloride from Wako Chemical Industries, Ltd. (Osaka). Ficoll 400 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and Conray 400 was purchased from Daiichi Seiyaku Co., Ltd. (Tokyo). Heparin sodium (Injectable) was obtained from Shimizu Seiyaku Co., Ltd. (Osaka). Cyclic GMP radioim-

munoassay kits were generously donated by Yamasa Shoyu Co., Ltd. (Chiba, Japan). Other reagents were of analytical grade from the commercial sources.

Treatment of Animals — Sarcoma-180 was kindly donated from Chugai Seiyaku Co., Ltd. (Tokyo). Experimental mice were sensitized against sarcoma-180 cells from ascites of donor mice by intraperitoneal inoculation with 5×10^6 cells 10 d prior to the experiment. Irradiated mice were obtained by the systemic X-ray irradiation (600 or 300 R) 12 h prior to the experiment.

Antigen Administration and Sample Collection — Mice were intravenously injected with an appropriate antigen in 0.2 ml solution or suspen-

FIG. 1. Effect of Intravenous Injection of SRBC on Plasma Cyclic GMP Levels in Mice

Groups of mice were given intravenously either 3×10^8 SRBC in 0.2 ml normal saline (●) or normal saline alone (○) and sacrificed at indicated times. The plasma samples were obtained and the concentrations of cyclic GMP were determined in duplicate. Each point represents the mean $\pm$ SE of 4 mice.

sion and sacrificed by decapitation at the indicated time. Whole blood from mouse was mixed quickly with 0.01 ml of normal saline containing 500 mM EDTA. After centrifugation, an aliquote

(50–100 μ l) of the supernatant was directly assayed for cyclic GMP.

Measurement of Plasma Cyclic GMP — The assay of plasma cyclic GMP was carried out using commercially available radioimmunoassay kits.⁸⁾

Measurement of Anti-SRBC Antibody Forming Cells — Animals were sacrificed 4 d after SRBC immunization (i.v.) and splenic direct plaque forming cells (PFC) were determined according to the method of Jerne and Nordin with modification.⁹⁾

TABLE I. *Effects of Different Types of Antigens on Plasma Cyclic GMP Level in Mice*

Antigens	Plasma cyclic GMP (pmol per ml)
Saline control	62.8 $\pm$ 5.8
SRBC (3×10^8)	98.5 $\pm$ 2.0
Dextran sulfate (50 mg/kg)	96.0 $\pm$ 5.5
BSA (0.5 mg/kg)	71.0 $\pm$ 1.4
Saline control	43.0 $\pm$ 5.4
SRBC (3×10^8)	80.3 $\pm$ 5.0
RRBC (3×10^8)	60.0 $\pm$ 9.3

Groups of mice were immunized intravenously with different types of in 0.2 ml of saline or saline alone. The mice sacrificed 5 min after the immunizations and the plasma concentrations of cyclic GMP were determined. Each value represents the mean $\pm$ SE of 4 mice.

RESULTS

Effects of Antigens on the Plasma Concentration of Cyclic GMP

The concentration of plasma cyclic GMP was increased by 150–200 % in 5 min after the intravenous injection of antigenic doses of SRBC into mice (Fig. 1). This elevation was continued until 60 min post-injection. No significant changes in plasma cyclic GMP level were observed during these periods in control mice which received normal saline. Since we previously established that the increase of splenic cyclic nucleotides are caused by SRBC, a particulate anti-

TABLE II. *Effects of Intravenous Injection of SRBC on Plasma Cyclic GMP Levels in Normal and Irradiated Mice*

Expt. No.	Irradiation	SRBC	Plasma cyclic GMP (pmol/ml)
I	—	—	48 $\pm$ 10.0
		+	97 $\pm$ 8.0
	300 R	—	46 $\pm$ 4.5
		+	45 $\pm$ 3.8
	600 R	—	52 $\pm$ 9.5
		+	63 $\pm$ 13.7
II	—	—	52 $\pm$ 6.9
		+	73 $\pm$ 4.9
	300 R	—	57 $\pm$ 6.6
		+	55 $\pm$ 9.3
	600 R	—	56 $\pm$ 5.9
		+	58 $\pm$ 7.6

Irradiation was performed 24 h before immunization. Groups of mice were immunized intravenously with 3×10^8 SRBC or saline. The mice were sacrificed 5 min after the immunization and the plasma concentrations of cyclic GMP were determined. Each value represents the mean $\pm$ SE of 4 mice.

gen, but not caused by soluble antigens such as BSA,⁵⁾ experiments were performed using soluble antigens such as dextran sulfate and BSA. As shown in Table I, both dextran sulfate and BSA induced a significant elevation of plasma cyclic GMP concentration at 5 min after the intravenous injection. To determine whether anti-

FIG. 2. Inhibiting Effect of Azathioprine Treatment on the Increase of Plasma Cyclic GMP Levels induced by SRBC in Mice

Mice were daily administered intraperitoneally with azathioprine (10 or 100 mg/kg) for three days. On the next day after the last administration, SRBC (3×10^8) were injected intravenously into those mice and sacrificed 5 min later.

genicity might be essential for this plasma cyclic GMP response, groups of mice were given rat erythrocytes (RRBC) which are less antigenic to mice. RRBC (3×10^8 cells) caused, in fact, an elevation of plasma cyclic GMP level, but it was quite low (1.4-fold) in response as compared to that by the same dose of SRBC (1.7-fold) (see Table I).

SRBC-induced Change in Plasma Cyclic GMP in immunologically Defected Mice

If cyclic GMP elevated in plasma was derived from immunocompetent cells, it would be expected that the slight or no augmentation of plasma cyclic GMP by antigen stimulation should occur in immunosuppressive states. As shown in Table II, no elevation of cyclic GMP in plasma was observed, when SRBC was injected into mice which received a lethal (600 R) or a sublethal (300 R) dose of X-ray irradiation.

Next, the effect of treatment with an immunosuppressive drug, azathioprine on the SRBC-induced elevation of plasma cyclic GMP was examined. Mice were daily administered intraperitoneally with azathioprine suspended in 0.3 % arabic gum at the dose of 10 or 100 mg/kg for 3 d before SRBC injection. On the next day after the last administration, SRBC (3×10^8 cells) were injected intravenously into those mice and sacrificed 5 min later. Control mice were treated with 0.3 % arabic gum for 3 d. As depicted in Fig.

TABLE III. Correlation between PFC Formation and SRBC-induced Increase in Plasma Cyclic GMP Levels in Normal and Sarcoma-180-bearing Mice

Condition	PFC/ 10^6 cells	PFC($\times 10^3$)/spleen	SRBC	Plasma cyclic GMP (pmol per ml)
Normal (intact) mice	4261 ± 336	2253 ± 778	-	49 ± 4.2
			+	75 ± 27.1
Sarcoma-180-bearing mice	96 ± 43	175 ± 77	-	53 ± 6.2
			+	53 ± 6.8

Both normal and tumor-bearing mice were separated into two groups for the PFC responses and plasma cyclic GMP assays. In the former experiment, mice were immunized intravenously with 2×10^8 SRBC and sacrificed 4 d after the immunization and then splenic PFCs were determined. In the latter experiment, mice were injected intravenously with 3×10^8 SRBC or saline and sacrificed 5 min after the injection and the plasma cyclic GMP concentrations were determined. Each value represents the mean $\pm$ SE of 4 animals.

2, a suppression of plasma cyclic GMP increase was observed in the azathioprine-treated mice in a dose-dependent manner.

The third experiment of this type was performed to compare the antigen-induced cyclic GMP response in normal and tumor-bearing mice. It has been well recognized that mice bearing various transplantable tumors show a suppressed immune response following antigenic stimulation.¹⁰⁾ We employed sarcoma-180 (ascites form) which was maintained by serial intraperitoneal transplantation into ddY mice. Tumor-bearing group of mice were intraperitoneally inoculated with 5×10^6 sarcoma-180 cells 10 d prior to the experiment. On the day of the experiment, both normal and tumor-bearing

mice were challenged intravenously with 3×10^8 SRBC and the plasma cyclic GMP concentration was determined at 0 or 5 min after antigen administration. Direct splenic PFC formation of each group of mice was also assessed as follows: Mice were immunized with 2×10^8 SRBC 6 d after sensitization with 5×10^6 sarcoma-180 cells. On day 10 (4 d after SRBC immunization), splenic anti-SRBC IgM antibody forming cells were measured by the hemolytic plaque assay. As summarized in Table III, the SRBC-induced plasma cyclic GMP response was completely inhibited in tumor-bearing mice where a substantial decrease in number of PFC was observed.

The Effect of Agents which block Autonomic Nervous System on Antigen Induced Plasma Cyclic GMP

FIG. 3. *Effects of Adrenergic, Cholinergic and Ganglionic Blockers on Antigen-induced Increases of Plasma Cyclic GMP Levels in Mice*

Phenoxybenzamine, propranolol and atropine were intraperitoneally injected 2 h before and hexamethonium 1 h before antigens at the dose indicated. Mice were sacrificed 5 min after antigen treatments. Each bar delineates mean $\pm$ SE.

Increase

Our previous report has shown that the biosynthesis of cyclic nucleotides in certain tissue may be regulated by the autonomic nervous system,¹¹⁾ thus it seemed relevant to examine whether this antigen-induced increase in plasma cyclic GMP may be also under the influence of autonomic nervous system. Therefore, we investigated the effect of α - and β -adrenergic, cholinergic and ganglionic blocking agents on the plasma cyclic GMP response. Groups of mice were intraperitoneally treated 2 h prior to antigen injection with either phenoxybenzamine (2 mg/kg), propranolol (10 mg/kg) or atropine (1 mg/kg), and 1 h prior to antigen with hexamethonium (0.5 mg/kg). The results are presented in Fig. 3. These drugs showed no significant effect on antigen-induced increase in plasma cyclic GMP. In this study the influences of these blockers themselves on plasma levels of cyclic GMP were examined and it was confirmed that none of them significantly changed the nucleotide levels in mice (data not shown).

DISCUSSION

We have demonstrated in this report that both particulate and soluble antigens caused significant increases (1.5–2.0-fold) in plasma cyclic GMP level in mice. However, this antigen-induced elevation of plasma cyclic GMP was abolished in animals under various immunosuppressive states where X-irradiated, azathioprine-treated or sarcoma-180-bearing mice were used.

Over the last 10 years, many investigators have presented evidence that endogenous cyclic GMP might act as an intracellular mediator of the proliferative response to mitogens *in vitro*.¹⁾ On the other hand, there are two major difficulties in studying the *in vivo* role of endogenous cyclic GMP in the induction of immune response after antigenic stimulation although there is a report by Yamamoto and Webb⁵⁾ demonstrating that both cyclic AMP and cyclic GMP were increased in spleen cells after intravenous injection of SRBC into mice: (1) Antigen-reactive lymphocytes are a minority of the population, so that it is difficult to

detect a change of cyclic GMP in lymphoid cells, (2) it would be difficult to identify which tissues or cell populations contribute to such cyclic GMP elevation. Since the plasma levels of cyclic nucleotides which is usually low is in a dynamic steady-state relation with its intracellular pools, a slight elevation of these nucleotides in tissues would be expected to cause a significant increase in plasma.^{6a,b,7)} In fact, it has been proposed that assay of plasma cyclic nucleotides following exposure to the hormones, neurotransmitters or other agents was very promising as a test for various bodily functions in experimental and clinical studies.¹²⁾ We have found that antigens cause an increase in plasma cyclic GMP level without any apparent influence of cholinergic regulation. The assumption that this plasma cyclic GMP responses induced by various antigens are derived largely from immunocompetent cells is supported by several lines of evidence as follows: (1) Less antigenic PRBC caused a lower elevation of plasma cyclic GMP level, (2) SRBC-induced plasma cyclic GMP increase was completely inhibited in irradiated, azathioprine-treated or sarcoma-180 tumor-bearing mice where immunes are suppressed. Ohara *et al.*¹³⁾ suggested that peripheral blood lymphocytes from patients with common variable immunodeficiency did not show any increase or cyclic GMP by PWM-stimulation. This *in vitro* observation strongly correlates with our *in vivo* findings presented here.

We have demonstrated that the intravenous injection of SRBC into mice produced the plasma levels of both cyclic GMP and cyclic AMP. The increase of cyclic GMP was observed not only in normal (intact) mice, but also in adrenalectomized ones. However, the significant change in cyclic AMP was observed only in normal mice (manuscript in preparation). These results and other findings that exogenous administration of catecholamine(s) increase the plasma levels of cyclic AMP in rats^{12a)} would suggest that the release of catecholamine(s) plays an important role in the elevation of cyclic AMP by antigen stimulation.

In this experiment, we could detect the change

in plasma cyclic GMP after the injection of soluble antigen such as BSA and dextran sulfate as well as particulate antigens, into mice. In our earlier report,⁵⁾ we had not been able to demonstrate any changes in splenic cyclic nucleotides in mice following injection of a soluble antigen, dextran sulfate. It is possible to suggest that this is due to different kinetics of antigen processing or slower and more gradual interaction with relevant antigen-sensitive T- and B-cells.

At present, we do not have any direct evidence as to which population of lymphoid cells (macrophage, T or B cells) produces the cyclic GMP. Our data, however, are compatible with the hypothesis that increased plasma cyclic GMP levels induced by antigen may be derived from lymphoid cells and represent a positive signal in the development of *in vivo* immune responses. In conclusion, the present experiments showed that there might be a good correlation between the plasma cyclic GMP elevation and immunological states *in vivo*. Therefore, it would be expected that the determination of plasma cyclic GMP after an appropriate antigen injection would serve as a diagnostic tool for testing immunological condition in subjects with immunological defects.

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