Immunology, 1970, **19**, 291.

Studies on the Induction of Immunological Paralysis to Bovine γ-Globulin in Adult Mice

II. THE EFFECT OF CYCLOPHOSPHAMIDE

SUSUMU KAWAGUCHI

Laboratory of Radiation Biology, Department of Zoology, Faculty of Science, Kyoto University, Kyoto, Japan

(*Received 20th November* 1969)

Summary. The immune response elicited by immunogenic forms of bovine γ-globulin (BGG), such as heat aggregated BGG (aBGG), BGG in Freund's incomplete adjuvant (FIA) or BGG plus endotoxin (ET), was interrupted by a single injection of cyclophosphamide. The amount of soluble BGG (sBGG) required to induce paralysis did not differ significantly between cyclophosphamide-treated mice and untreated mice. The injection of 1 mg sBGG together with 100 μg aBGG or 10 μg ET caused an immune response in normal mice but induced paralysis in cyclophosphamide-treated mice. However, without sBGG, the administration of aBGG suspension or aBGG in FIA could not induce paralysis, even with the aid of cyclophosphamide.

INTRODUCTION

It has been reported previously (Muramatsu and Kawaguchi, 1968; Kawaguchi, 1970), that X-irradiation hardly influences the paralysis-inducing action of deaggregated soluble bovine γ-globulin (sBGG), but suppresses the immune response to aggregated BGG (aBGG). Thus, X-irradiation facilitates induction of paralysis in mice injected simultaneously with sBGG and aBGG, although the simultaneous injection of these two forms of BGG elicits an immune response in unirradiated mice.

Besides X-irradiation, treatment with immunosuppressive drugs is also known to facilitate the induction of paralysis (Schwartz, 1965). Among these, cyclophosphamide (CY) is known to be very effective in inducing paralysis in adult animals injected with even a potent antigen such as sheep red blood cells (Aisenberg, 1967). A single dose of CY suppresses the immune response only transiently, however (Frisch and Davis, 1965).

The present experiment was designed to explore how CY facilitates the induction of paralysis in adult animals. The action of immunogen as well as paralytogen and the interaction of these two forms of antigen in CY-treated mice were studied by employing sBGG as a paralytogen and either aBGG, sBGG plus endotoxin, or BGG in Freund's incomplete adjuvant as immunogens. The results indicate that administration of CY interrupts the action of immunogen but does not effect the action of paralytogen.

MATERIALS AND METHODS

Animals

Male and female ddD mice, 10–14 weeks old and weighing about 25 g, were used. These mice were supplied from the Central Animal Laboratory at the School of Medicine, Kyoto University.

Antigen

Bovine γ-globulin (BGG) (Cohn Fraction II, Armour Pharm. Co.) was used.

Soluble BGG (sBGG). Two per cent of BGG dissolved in physiological saline was centrifuged at 105,000 ***g*** for 120 minutes to sediment the molecular aggregates. The supernatant was diluted to desired concentrations with saline and injected intravenously (i.v.).

Aggregated BGG (aBGG). Aggregated BGG denotes the heat-denatured (63°, 20 minutes) and homogenized preparation, which was prepared according to the method described in the preceding paper (Kawaguchi, 1970). Aggregated BGG was also injected i.v.

FIA-BGG. BGG in saline was treated at 63° for 20 minutes and the whole suspension was incorporated into Freund's incomplete adjuvant (FIA). FIA-BGG was injected subcutaneously (s.c.) at a single site in a volume of 0·3 ml containing 1 mg of BGG.

*I*BGG.* Soluble BGG was labelled with ^{131}I by the method of Helmkamp, Goodland, Bale, Spar and Mutschler (1960).

Endotoxin (ET)

Bacterial endotoxin (lipopolysacchride extracted from *Escherichia coli*, strain 0 : B6, Difco Lab.) was dissolved in saline and was injected i.v.

Cyclophosphamide (CY)

Cyclophosphamide (Endoxan, Shionogi Pharm. Co., Osaka, Japan) was dissolved in saline immediately before use and was injected i.v.

Test for immune status

The immune status was assessed by the elimination test described in the previous paper (Kawaguchi, 1970).

RESULTS

THE EFFECT OF CY ON THE PRIMARY IMMUNE RESPONSE TO FIA-BGG

Mice were divided into six groups of five. Four groups were given single doses of CY 1·25, 2·5, 5·0, and 7·5 mg, respectively—followed by primary immunization with FIA-BGG 3 hours later. One of the remaining groups served as the immune control receiving FIA-BGG alone, the other as the non-immune control receiving neither CY nor FIA-BGG. Three days later, all the test animals were injected with I*BGG to examine the degree of their immune response to FIA-BGG. The results are shown in Fig. 1(a). As expected, suppression of the immune response by CY was found to be dose-dependent; 5 and 7·5 mg of CY suppressed the primary response almost completely so that the elimination rates of I*BGG were not significantly different from those of non-immune controls. Partial suppression occurred in mice treated with 2·5 mg of CY. The immune response was not affected by 1·25 mg of CY. To investigate the time relationship between immunization and suppression of primary immune response by CY, mice were given 2·5 mg of CY at

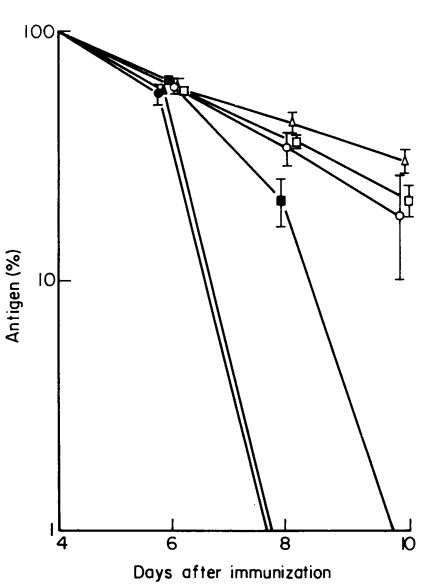

FIG. 1(a).

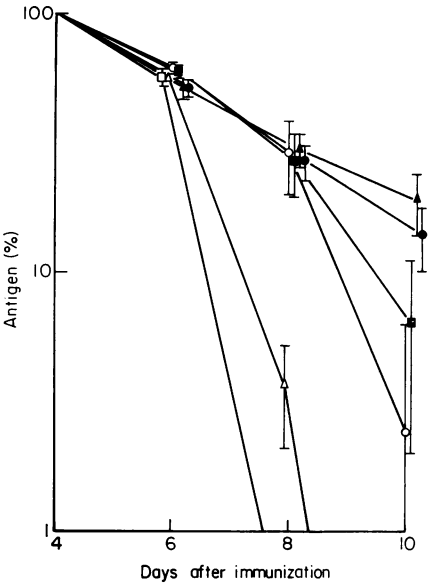

FIG. 1(b).

FIG. 1(a). Mean elimination curves of I*BGG from the blood of mice given varying doses of cyclophosphamide 3 hours before immunization with FIA-BGG. Doses of CY: 1·25 mg (▲), 2·5 mg (■), 5·0 mg (○), 7·5 mg (△), 0 mg (immune control) (●), non-immune control (□).

FIG. 1(b). Mean elimination curves of I*BGG from the blood of mice given 2·5 mg of cyclophosphamide at different times before or after immunization with FIA-BGG. Time of CY administration in relation to antigen injection: 2 days before (□), 1 day before (△), 4 hours after (○), 1 day after (■), 2 days after (▲), 4 days after (●).

varying times before or after the injection of FIA-BGG. The results shown in Fig. 1(b) indicate that suppression was most conspicuous when CY was given 2 or 4 days after FIA-BGG. The injection of CY 4 hours or 1 day after FIA-BGG still caused considerable suppression. CY was ineffective when given 1 or 2 days before FIA-BGG. It can be concluded from this experiment that CY acts more immunosuppressively when administered after antigen injection than before antigen injection.

THE EFFECT OF CY ON PARALYSIS INDUCTION BY sBGG

This experiment aimed at answering the question whether CY might reduce the amount of sBGG required for paralysis induction. Three groups of mice were given single injection of 10, 100, or 1000 μg of sBGG, respectively, which was followed immediately by an injection of 5 mg of CY (Fig. 2b). The other three groups of mice were injected only with sBGG at the same doses without CY-treatment (Fig. 2a)*. They were challenged with FIA-BGG 20 days later.

It can be seen from Fig. 2(b), when compared with Fig. 2(a), that CY-treatment scarcely interfered with paralysis induction by sBGG. In both CY-treated and untreated mice, 1000 μg and 100 μg of aBGG induced complete and partial paralysis, respectively, and 10 μg caused only a slight immunosuppression. The mice given 5 mg of CY without sBGG showed normal primary response against FIA-BGG given 20 days later. This indicates that these mice recovered from the non-specific suppression of immune response by CY-treatment within 20 days.

* This graph is Fig. 1 in the preceding paper (Kawaguchi, 1970).

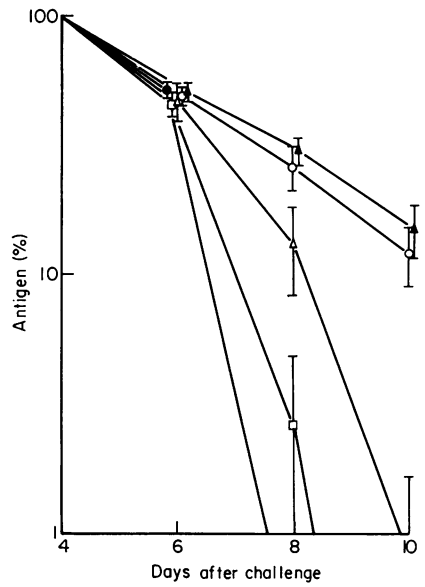

FIG. 2(a).

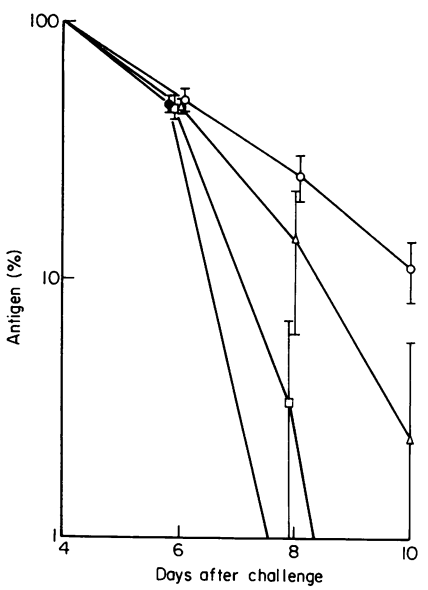

FIG. 2(b).

FIG. 2(a). Mean elimination curves of I*BGG from the blood of mice given varying doses of sBGG 20 days before challenge. Doses of sBGG: 1000 μg (○), 100 μg (△), 10 μg (□), 0 μg (immune control) (●), non-immune control (▼).

FIG. 2(b). Mean elimination curves of I*BGG from the blood of mice given varying doses of sBGG and 5 mg of CY 20 days before challenge. Doses of sBGG: 1000 μg (○), 100 μg (△), 10 μg (□), CY-treated immune control (●).

THE EFFECT OF CY ON THE IMMUNE RESPONSE TO EITHER sBGG+aBGG OR sBGG+ET

In view of the fact that both aBGG and ET disturb induction of paralysis by sBGG (Kawaguchi, 1970), it was interesting to test the effect of CY on the paralysis-preventing action of aBGG and ET. Forty-five mice were divided into nine groups of five. Three groups (A, B and C) were given 1 mg sBGG, 1 mg sBGG+100 μg aBGG, and 1 mg sBGG+10 μg ET, respectively (*plus* indicates simultaneous injection). Three other groups (D, E and F) were treated as groups A, B and C, respectively, but then immediately received 5 mg CY. Group G was injected with 5 mg of CY, group H with 1 mg aBGG+5 mg CY, and group I with 100 μg sBGG+1 mg aBGG+5 mg CY. All groups were challenged with FIA-BGG 20 days later. The results are shown in Table 1. As expected from earlier studies, a secondary response was observed in groups B and C, indicating that the injection of 1 mg sBGG+100 μg aBGG, or 1 mg sBGG+10 μg ET elicited a primary immune response in otherwise untreated mice. In contrast, paralysis was induced in CY-treated mice (groups E and F), and was ascribable solely to the action of sBGG and not to the action of aBGG, since even 1 mg aBGG could not induce paralysis in the presence of CY (group H). Moreover, the additional injection of 1 mg aBGG prevented rather than facilitated the immunosuppressive action of 100 μg sBGG in CY-treated mice (group I). These results show the effect of CY in facilitating paralysis when both immunogen and paralytogen (sBGG+aBGG or sBGG+ET) are injected simultaneously.

TABLE 1

THE EFFECT OF aBGG AND ENDOTOXIN ON THE INDUCTION OF PARALYSIS BY sBGG IN CY-TREATED MICE[a]

Group	Treatment 20 days before challenge	Immune status			
		UR[b]	HR[c]	PR[d]	SR[e]
A	1 mg sBGG	5/5[f]			
B	1 mg sBGG+0·1 mg aBGG				5/5
C	1 mg sBGG+10 μg ET				5/5
D	1 mg sBGG+5 mg CY	5/5			
E	1 mg sBGG+0·1 mg aBGG+5 mg CY	5/5			
F	1 mg sBGG+10 μg ET+5 mg CY	4/5	1/5		
G	5 mg CY			5/5	
H	1 mg aBGG+5 mg CY				5/5
I	0·1 mg sBGG+1 mg aBGG+5 mg CY		1/5	1/5	3/5

[a]: Mice were challenged with FIA-BGG.
[b]: The unresponsiveness to the challenge.
[c]: The hypo-responsiveness to the challenge.
[d]: The primary response to the challenge.
[e]: The secondary response atttributable to the primary antigen injection and the challenge.
[f]: Number of mice per total number in each experimental group.

THE EFFECT OF CY INJECTED AT VARYING TIMES BEFORE OR AFTER THE INJECTION OF sBGG +aBGG

Mice were given 7·5 mg of CY at varying times before or after the injection of 1 mg sBGG+100 μg aBGG. This amount of CY was shown to be highly immunosuppressive (Fig. 1a). The challenge injection with FIA-BGG was performed 20 days after treatment with CY. The results shown in Table 2 indicate that the injection of CY after antigen injection was more effective than before antigen injection in facilitating induction of paralysis. The paralysis-preventing action of aBGG was nullified by CY given on the day of or 4 days after the antigen injection, none of the mice so treated responding to the challenge injection. When CY was given 8 days after antigen, complete paralysis was seen in two of five mice and in three mice hypo-responsiveness was observed. Cyclophosphamide-treatment given 2 days before antigen rendered two of five mice completely paralysed, but the three other mice gave a secondary response to the challenge injection. CY given 4 days before antigen was ineffective in nullifying the action of aBGG and a secondary response to the challenge injection was observed.

TABLE 2

THE EFFECT OF VARYING THE INTERVAL BETWEEN INJECTIONS OF CYCLOPHOSPHAMIDE AND sBGG+aBGG[a]

Time of injection (days before challenge)		Immune status			
1 mg sBGG+0·1 mg aBGG	7·5 mg CY	UR[b]	HR[c]	PR[d]	SR[e]
20					5/5[f]
	20			5/5	
28	20	2/5	3/5		
24	20	5/5			
20	20	5/5			
18	20	2/5			3/5
16	20				5/5

[a]: Mice were challenged with FIA–BGG.
[b,c,d,e]: Categories of the responsiveness to the challenge injection (see Table 1).
[f]: Number of mice per total number in each experimental group.

THE EFFECT OF CY ON THE ACTION OF ANTIGEN INCORPORATED INTO FREUND'S INCOMPLETE ADJUVANT

It seems probable from the results given in Fig. 1(b) and Table 2 that cells activated specifically by immunogen are very susceptible to the action of CY. Some investigators propose that it is by ablation of such specifically activated cells that immunosuppressive drugs produce the specifically unresponsive state (Schwartz, 1965; Aisenberg, 1967). The experiment described below was undertaken to make clear whether immunogenic antigen can induce paralysis with the aid of CY treatment.

Mice were given subcutaneous injection of FIA-sBGG or FIA-aBGG. Cyclophosphamide (2·5 mg) was injected 2 days later, when it was expected to be most effective in suppressing the immune response (refer to Fig. 1b). Following CY-treatment, FIA-BGG was injected to assess the degree of ablation of the anti-BGG response. This was done 1 day after CY treatment, on the assumption that this was too short an interval for immunological capacity to recover after any antigen-induced damage. The results are shown in Table 3. Pretreatment with CY 1 day before FIA-BGG did not cause any disturbance in the primary response (group G). FIA-sBGG produced an immune response in the absence of CY

TABLE 3

THE EFFECT OF CYCLOPHOSPHAMIDE ON THE ACTION OF ANTIGEN IN FIA[a]

Group	Treatment		Immune status			
	3 days before challenge	1 day before challenge	UR[b]	HR[c]	PR[d]	SR[e]
A	FIA-sBGG (2 mg s.c.)					4/4[f]
B	FIA-sBGG (2 mg s.c.)	2·5 mg CY		5/5		
C	FIA-aBGG (2 mg s.c.)	2·5 mg CY			5/5	
D	FIA-aBGG (2 mg s.c.) and sBGG (2 mg i.v.)	2·5 mg CY	5/5			
E	FIA-aBGG (0·2 mg s.c.)	2·5 mg CY			5/5	
F	FIA–EA[g] (2 mg s.c.)	2·5 mg CY			4/4	
G		2·5 mg CY			4/4	
H	FIA-aBGG (2 mg s.c.) and sBGG (1 mg i.v.)					4/4

[a]: Mice were challenged with FIA-BGG.
[b,c,d,e]: Categories of the responsiveness to the challenge injection (see Table 1).
[f]: Number of mice per total number in each experimental group.
[g]: Egg-albumin.

(group A), but a hypo-responsive state in the presence of CY (group B). FIA-aBGG failed to induce a hypo-responsive state in CY treated mice, which showed typical primary responses to the challenge injection (groups C and E). When 1 mg of sBGG was given i.v. simultaneously with FIA-aBGG, mice were rendered unresponsive to the challenge in the presence of CY but were sensitized in the absence of CY giving secondary responses to the challenge injection (groups D and H). The injection of FIA–egg albumin (an unrelated antigen) followed by CY treatment did not interfere with the primary response to FIA-BGG (group F).

In order to find out whether the hypo-responsive state in group B in Table 3 was attributable to the action of paralytogen released from FIA-sBGG emulsion, the following experiment was carried out. One group of three mice was injected i.v. with 0·1 mg of I*BGG in solution and another group of three mice was injected s.c. with 1 mg of I*BGG incorporated into Freund's incomplete adjuvant. The amount of antigen in the circulation

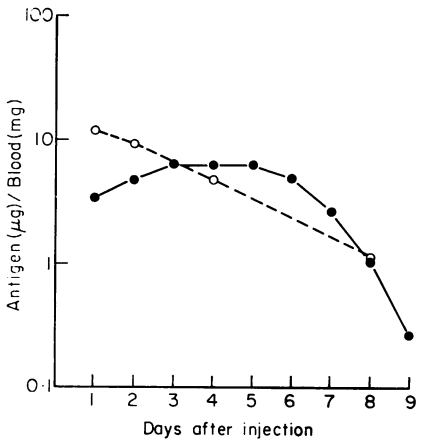

FIG. 3. The amount of antigen in the blood: ●, 1 mg of I*BGG in FIA given s.c.; ○, 0·1 mg of I*BGG given i.v.

was traced by serial bleedings from retro-orbital plexus. The results are shown in Fig. 3. Two days after antigen injection, the level of antigen in the blood of mice injected with FIA-I*BGG was about one half of that in the blood of mice injected with I*BGG i.v. Thus, taking into account the fact that the amount of sBGG in FIA injected into group B in Table 3 was 2 mg, i.e. twice as much as in this experiment, the amount of free sBGG in the circulation of the mice belonging to group B in Table 3 may be approximately equal on day two to that in mice given an intraveneous injection of 100 μg sBGG. This amount of sBGG contained in the blood may be enough to cause a hyporesponsive state, as shown in Fig. 2(a). These results therefore suggest that immunogenic antigen even in the presence of CY cannot cause the induction of paralysis.

DISCUSSION

Cyclophosphamide, a cytotoxic alkylating agent, has been used as an effective immunosuppressive drug in the mouse (Berenbaum and Brown, 1964; Frisch and Davis, 1965). Administration of CY following antigen injection is much more effective than before antigenic stimulation (Fig. 1b). This suggests that damage to the antigen-processing mechanism may not be as important a factor in the immunosuppressive effect of CY as in the immunosuppressive effect of X-irradiation (Gallily and Feldman, 1967; Mitchison, 1969); and that antigen sensitive cells activated to multiply may be especially susceptible to CY. The resting precursor cells seem less sensitive to CY than activated cells, because the administration of CY (2·5 mg) simultaneously with antigen did not suppress the primary response completely and administration of CY 1 day before antigen showed only a slight immunosuppressive effect (Fig. 1b).

Immunosuppressive drugs have been shown to facilitate the induction of paralysis (Schwartz, 1965). To explain this phenomenon, the hypothesis has been proposed that a high dose of antigen per antigen sensitive cell, exceeding the optimum dose to elicit immune response, would cause paralysis (Schwartz and Dameshek, 1963). If this were the case, a decrease in the number of antigen sensitive cells through the action of chemical agents should result in the reduction of the threshold dose of antigen required to induce

paralysis. It seems probable from the experiment of Aisenberg (1967) that the administration of CY causes a reduction in the number of antigen sensitive cells. However, the present experiment indicates that the paralytogenic efficiency of sBGG is unchanged in mice given 5 mg of CY (Figs 2a and 2b). These observations concur with the view that the important factor involved in induction of paralysis is not the amount of injected antigen per number of antigen sensitive cells but the concentration of free antigen in the intra- and extra-vascular spaces (Muramatsu and Kawaguchi, 1968), and they also support the hypothesis that direct contact of antigen with antigen sensitive cells causes paralysis (Dresser and Mitchison, 1968; Lescowitz, 1967; Paul, Siskind and Benacerraf, 1967).

In the preceding paper, it was shown that there is competition between paralytogen and immunogen (Kawaguchi, 1970). The immunogenic information which triggers antigen sensitive cells to multiply and differentiate into antibody-forming cells seems to differ from paralytogenic information. The administration of CY suppresses the immune response to immunogen (Figs 1a and 1b), but does not influence the paralysing effect of paralytogen (Figs 2a and 2b). This raises the possibility that CY may give some advantage to paralytogen in the competition between paralytogen and immunogen. Indeed, the injection of either 1 mg sBGG+100 μg aBGG, or 1 mg sBGG+10 μg ET caused an immune response in untreated mice, but induced paralysis in CY-treated mice (Table 1).

It has been proposed that the immunogenic action of antigen may play an active role in inducing paralysis in drug-treated animals (Aisenberg, 1967; Schwartz, 1965). This implies that when all the antigen sensitive cells are stimulated by an immunogen in the presence of an immunosuppressive drug such as CY, the specific clone is ablated and paralysis is established. This view is based on the clonal selection theory (Burnet, 1959) and on the assumption that immunogenic stimulation in the presence of immunosuppressive drugs inactivates proliferating antigen sensitive cells. In the present experiment, however, the injection of an immunogenic form of antigen, such as 1 mg of aBGG (Table 1) or 2 mg of aBGG in FIA (Table 3), did not induce a hypo-responsive state when CY was given as well. Moreover, although the administration of 2·5 mg CY 2 days after FIA-BGG seemed to inactivate almost all the stimulated cells (Fig. 1b), pre-treatment of mice with FIA-aBGG followed by 2·5 mg CY administered 2 days later, did not suppress primary type reactivity in response to subsequent injection of FIA-BGG. This indicated that the immunogenic form of antigen did not induce paralysis in the presence of CY.

The present work shows that the paralysis-facilitating effect of CY can be seen only when immunogen is injected concomitantly with paralytogen. It seems that under these conditions some of the antigen sensitive cells will be activated by immunogen and the rest will fall into a paralytic state through the action of paralytogen. The administration of CY seems simply to eliminate the immunogen-activated cells so that only paralysis results.

ACKNOWLEDGMENTS

The advice so kindly extended by Dr S. Muramatsu throughout the course of these investigations and in the preparation of the manuscript is most gratefully acknowledged. I wish to thank Dr T. Sado of the Natural Institute of Radiation Sciences for his critical reading of the manuscript. Thanks are also due to the Research Laboratories of Shionogi Pharmacentical Co., Osaka, Japan, for the generosity in providing the preparation of cyclophosphamide (Endoxan).

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