IMMUNOSUPPRESSIVE EFFECT OF MYCOPHENOLIC ACID

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The immune response to sheep red blood cells of the mice treated with mycophenolic acid was studied from both humoral and cellular aspects. Mycophenolic acid showed markedly immunosuppressive effect on the antibody formation when mice were treated with daily doses of more than 60 mg/kg for 5 consecutive days starting on the day of antigen injection. Late treatment resulted in a weaker immunosuppressive effect, while pretreatment was ineffective on antibody formation. The pattern of antibody formation between control and mice treated with mycophenolic acid was different. In the primary response of mice immunized with sheep red blood cells, the administration of mycophenolic acid prolonged the induction period, suppressed the total titer, and the formation of 2-mercaptoethanol resistant antibody (7S) was not detected throughout experimental period. Compared with other agents, mycophenolic acid is approximately equipotent to 6-mercaptopurine in immunosuppressive effect, determined by the decreases in the number of plaque forming cells and hemolysin titer.

In the screening tests described in the former papers for antiviral, antitumor and antifungal activities, it was found that mycophenolic acid is a potential antiviral agent[1]. Antifungal and antitumor activities of this agent were reported by our coworkers[2,3] and also by other workers[4]. This paper deals with immunosuppressive activity of mycophenolic acid on antibody formation.

Materials and Methods

Animals. Albino mice of 4CS strain were supplied from the breeding department of our laboratory. The mice were initially 5 weeks of age and weighed approximately 25 g. They were housed in groups of 6 in plastic cages and fed on commercial mouse pellets and water *ad libitum*.

Antigen. Sheep red blood cells (S-RBC) were obtained commercially (Toshiba Kagaku Co.). The erythrocytes were washed three times with physiological saline solution (PSS) and resuspended in PSS at a concentration of 10 % v/v (4×10^8 S-RBC/ml).

Immunization. Mice were immunized by intravenous injection of 0.2 ml of the washed S-RBC suspension.

Hemolysin titration. At appropriate intervals after the antigen injection, the mice were bled by cardiac puncture. Serum was obtained by centrifugation and used for titration. The specimens were stored at $-20°C$ and were titrated all together when each series of experiments was completed. Hemolysin activities were determined by two-fold serial dilution of individual serum specimens in microtiter plates according to SEVER's method[5], using 0.025 ml calibrated spiral wire loops. All dilutions were performed with PSS in 0.025 ml volumes, ranging usually from 1 : 2~1 : 4096. An equal volume of a 1.0 % suspension of freshly washed S-RBC and a 1 : 15 dilution of guinea pig serum were added to

each dilution cup and the plates were agitated and incubated at 37°C for 1 hour. The degree of hemolysis was ranked from 4 (complete hemolysis) to 0 (no hemolysis). Titers were expressed as $\log_2$ of the reciprocal of the highest dilution which showed hemolysis of more than degree 3. Mercaptoethanol (2-ME) sensitivity of the hemolysins was determined by incubation of serum with an equal quantity of 0.2 M 2-ME at room temperature for 1 hour before dilution. The hemolysin titer remaining after this treatment was considered to be due to 2-ME resistant antibody of the 7S variety (IgG). That portion of the titer inactivated by 2-ME treatment was considered to be 19S antibody (IgM). The sera of individual mice were used for each determination.

Hemolytic plaque. After serum specimens were obtained, the spleens were removed and weighed. The cells were then teased out of the whole spleen into a culure medium by the aid of dissecting forceps, filtered through stainless steel mesh, washed by cold EAGLE's medium and counted. The total number of spleen cells was calculated from the number of nucleated cells per ml.

Plaque-forming cells (PFC) were counted with semi-micro modification of JERNE's method[6]. A freshly prepared spleen cell suspension (0.3 ml) was mixed with 2.7 ml of EAGLE's medium, 0.3 ml of 35 % washed S-RBC and 0.3 ml of 1 : 5 dilution of guinea pig serum in the cold, and the mixture was poured into 14 mm diameter chambers, which consisted of a slide glass, a cover glass and a sheet of suitably punched Parafilm. The micro-plates were incubated at 37°C for 20 minutes and the number of the resulting hemolytic plaques was counted. The kinetics of the antibody-plaque response was represented by both the number of PFC per spleen and the number of PFC per million nucleated splenic leukocytes.

Agents. Mycophenolic acid in a purified form was prepared in our laboratory. It was brought into phosphate buffer solution (PBS) with the minimal amount of 0.1 N NaOH necessary to dissolve the drug at a concentration of 30 mg/ml. 6-Mercaptopurine (Leukerin, Takeda Pharmaceutical Co.) was suspended in PBS at a concentration of 100 mg/ml. Azathioprine (Imuran) was kindly supplied from Dr. HASHIMOTO of Tokyo Biochemical Research Institute and suspended in PBS at a concentration of 150 mg/ml. ε-Aminocaproic acid (Tokyo Kasei Co.) was dissolved in PBS at a concentration of 200 mg/ml. Mitomycin C (Kyowa Hakko Co.) was dissolved in PBS at a concentration of 0.05 mg/ml.

Results

Dose-response of Immunosuppressive Effect of Mycophenolic Acid

The number of nucleated lymphoid cells producing hemolytic antibody to sheep erythrocytes and hemolysin titer were determined in mice receiving various doses of mycophenolic acid after antigenic stimulation.

For each experiment a group of 6 mice, 5 weeks of age, were immunized with washed sheep red blood cells and then injected with 0.2 ml of various doses of mycophenolic acid intraperitoneally once daily for 5 consecutive days. A similar number of mice were injected only with S-RBC in PBS as control. On the 5th day after the immunization, mice were killed for the hemolysin titration, and the enumerations of PFC in the spleen. This interval was chosen from earlier observations that the peak in the number of PFC appeared on the 5th day after immunization in the control mice.

As shown in Table 1, the body weight gain and spleen weight were suppressed slightly in mycophenolic acid groups. Marked suppression was observed in the mice treated with mycophenolic acid at doses of more than 120 mg/kg per day both in the

number of PFC and in hemolysin titer. Although the inhibition of PFC at a dose of 60 mg/kg per day was of the same magnitude as observed at the dose level of 120 mg/kg per day, the suppression of hemolysin titer was weaker than at the latter dose.

Table 1. Dose response of immunosuppressive effect of mycophenolic acid

(a) Average body weight gain, spleen weight and hemolytic titer

Daily dose (mg/kg)	Body wt. gain (g)	Spleen wt. (mg)	Hemolysin titer (log_2)
240	0.3	146	5.0
120	0.5	132	4.5
60	1.4	175	6.3
Control	2.5	167	8.0

(b) Number of hemolytic plaque forming cells

Daily dose (mg/kg)	PFC/10^6 cells*	Inhibition (%)	PFC/spleen	Inhibition (%)
240	120	59	27,911	62
120	186	37	36,147	50
60	138	53	47,465	48
Control	293	0	72,718	0

* Plaque forming cells per 10^6 spleen cells.

Time Course of Administration of Mycophenolic Acid and its Effect on Antibody Formation

Four groups of 6 mice each were immunized with 0.2 ml of 10% S-RBC intravenously. The mice were then treated with mycophenolic acid at a dose of 240 mg/kg per day, using the following schedules. 1) The mice were treated with mycophenolic acid from 3 to 1 day before S-RBC immunization. 2) The mice were treated for 5 consecutive days starting on the day of S-RBC immunization. 3) The mice were treated for 2 days starting on the third day after S-RBC immunization. 4) The mice were injected with PBS instead of mycophenolic acid following the schedule for group 2. Five days after the immunization, mice were killed for the hemolysin titration, and the enumeration of PFC in the spleen.

Table 2. Time course of antibody formation following the administration of mycophenolic acid

(a) Average body weight gain, spleen weight and hemolysin titer

Dosing schedule*	Body wt. gain (g)	Spleen wt. (mg)	Hemolysin titer (log_2)
−3～−1	3.8	165	7.7
0～4	2.5	100	4.7
3～4	3.0	92	7.7
Control	3.6	125	7.5

(b) Number of hemolytic plaque forming cells

Dosing schedule*	PFC/10^6 cells	Inhibition (%)	PFC/spleen	Inhibition (%)
−3～−1	100	23	16,871	−8
0～4	34	74	3,319	79
3～4	68	48	4,314	72
Control	130	0	15,579	0

* −3～−1 : Injected 3 to 1 day before S-RBC immunization.
0～4 : Injected daily for 5 days starting on the day of S-RBC immunization.
3～4 : Injected daily for 2 days, starting 3 days after S-RBC immunization.

As shown in Table 2, mycophenolic acid showed immunosuppressive activity both in hemolytic titer and in the number of PFC when treatment was made for 5 consecutive days starting on the day of antigen injection. When the treatment was initiated 3 days later than the antigen injection, mycophenolic acid had no effect on serum antibody titer, but the number of PFC in the spleen decreased markedly (79%). Pretreatment was ineffective on antibody formation.

Effect of Mycophenolic Acid on the Primary Response

Two groups of mice were immunized with 0.2 ml of S-RBC intravenously. A

group of mice were treated intraperitoneally with a dose of 240 mg/kg of mycophenolic acid once every day starting on the day of antigen injection. The control mice were treated with PBS instead of mycophenolic acid. At appropriate intervals after the immunization, the mice were killed for the hemolysin titration and for the determination of the number of PFC in the spleen. The results are shown in Table 3.

Table 3. The effect of mycophenolic acid on the primary response

Mice	Days after antigen injection	Spleen wt. (mg)	Hemolysin titer PFC per			
			Total	7S*	10^6 cells	Spleen
Control	0	125	<2	<2		
	1	147	<2	<2		
	2	155	<2	<2		
	3	151	3	<2		
	4	151	7	<2		
	5	139	7	3	152.4	39,065
	6	143	6	4	74.0	18,161
	7	129	5	4	1.4	4,236
	8	102	5	4		
Mycophenolic acid treated	1	90	<2	<2		
	2	120	<2	<2		
	3	98	<2	<2		
	4	108	<2	<2	0.4	847
	5	107	4	<2	20.6	3,671
	6	124	5	<2	0.3	941
	7	158	4	<2		
	8	140	4	<2		

* Hemolysin titers of 2-ME resistant antibody.

The pattern of antibody formation in normal mice immunized with S-RBC was similar to those reported by others[7,8]. The 2-ME sensitive antibody (19S) appeared earlier on the 3rd day after immunization while the 2-ME resistant antibody (7S) appeared later on the 5th day and reached a peak on the 6th day after the immunization.

Table 4. Immunosupressive effect of mycophenolic acid compared to various agents

(a) Average body weight gain, spleen weight and hemolysin titer

Agents	Daily dose (mg/kg)	Body wt. gain (mg)	Spleen wt. (mg)	HL* (log_2)
Mycophenolic acid	240	2.3	92	<2
6-Mercaptopurine	80	−0.5	71	<2
Azathioprine	120	0.2	64	6.7
ε-Amino caproic acid	1,600	1.5	140	5.7
Mitomycin C	0.4	1.8	91	5.0
Control		2.6	149	7.0

(b) Number of hemolytic plaque forming cells

Agents	Dose (mg/kg)	PFC/10^6**	Inhibition (%)	PFC/spleen	Inhibition (%)
Mycophenolic acid	240	46	86	6,119	90
9-Mercaptopurine	80	55	83	4,471	93
Azathioprine	120	11	33	685	99
ε-Amino caproic acid	1,600	323	1	64,811	−3
Mitomycin C	0.4	65	80	8,237	87
Control		325	0	62,887	0

* Average total hemolysin titer.
** Plaque forming cells per 10^6 spleen cells.

The administration of mycophenolic acid 240 mg/kg starting on the day of antigen injection, resulted in an abnormal pattern of antibody synthesis as shown in Table 3. Four aspects are noteworthy: a) the prolonged induction period (5 days after antigen immunization in contrast to 3 days in the control group); b) the decrease in the total antibody titers more than 1 log_2 unit below control group; c) the undetectable formation of 2-ME resistant antibody (7S) throughout the experimental period; d) the sustained level of 2-ME sensitive antibody in contrast to the marked decline in the control group after the peak.

Immunosuppressive Effect of Mycophenolic Acid Compared to Various Agents

Six groups of 6 mice were immunized with 0.2 ml of 10 % S-RBC intravenously. The mice were then treated with various agents for 5 consecutive days once daily. The mice were killed on the 5th day after immunization for titration of hemolysin titer and enumeration of PFC in the spleen. The results obtained were shown in Table 4.

The mice treated with mycophenolic acid showed a body weight gain comparable to the controls while the other agents suppressed it markedly. The administration of all the agents including mycophenolic acid resulted in a decrease in the weight of the spleen. Only exception for this was ε-amino caproic acid. Control mice had 6.2×10^4 PFC in their spleens and a hemolysin titer of 7 on the 5th day. When compared to these values, in the mycophenolic acid and 6-mercaptopurine groups, there was no detectable serum hemolysin, and the suppression of serum hemolysin titer was more marked than with the other agents. In all but ε-amino caproic acid, the depression of the number of PFC was approximately 90 %. This was also the case for mycophenolic acid.

Discussion

The immunosuppressive effects of mycophenolic acid in mice have been demonstrated at the cellular and humoral levels of antibody formation. The data presented here indicate that this agent depresses the immune response to sheep erythrocytes, as determined by the number of the individual antibody-producing cells in lymphoid tissues and by the level of serum antibodies (Table 1).

The time of drug treatment in relation to the immunization was found to be critical in determining the mycophenolic acid inhibition of the immune response. When mycophenolic acid was administrated simultaneously with the antigenic stimulus, both the appearance of antibody-forming cells in the spleen and production of hemolysin in the serum were inhibited severely. When mycophenolic acid was given 3 days after the antigen injection, the number of PFC per spleen decreased, but hemolysin titer in serum remained similar to the control. On the other hand, when the agent was given 3 days before the antigen injection, there was no detectable inhibition. It appears therefore reasonable to assume that mycophenolic acid acts during the induction period of primary immuno-response.

The study of the effect of mycophenolic acid, administrated to S-RBC immunized mice, on the kinetics of appearance of circulating hemolysins and PFC yielded results indicating a prolongation of the induction period, the decrease of total antibody and the absence of 2-ME resistant titer (7S). A significant effect of mycophenolic acid was the synthesis of 19S antibody continued long after 19S had disappeared from the sera of control mice. As SAHIAR and SCHWARTZ have demonstrated, 7S antibody inhibits the synthesis of 19S antibody[9]. The continued synthesis of 19S antibody observed in this study may therefore be due to the absence of 7S antibody, the appearance of which in ordinary cases signals the decline of 19S.

When the immunosuppressive effect of mycophenolic acid was compared to other various agents, mycophenolic acid had marked immunosuppressive effect similar to 6-mercaptopurine both in the number of PFC and in hemolysin titer. From these results, mycophenolic acid might be a useful immunosuppressive agent.

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