SpA-Induced Immunoglobulin Production in Human Peripheral Blood Lymphocytes.

I. Conditions of SpA-Induced Immunoglobulin Production.

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(Received, January 14, 1983)

INTRODUCTION

Protein A (SpA) is a component of the cell wall of *Staphylococcus aureus*. SpA is unique in that it combines with the Fc portion of immunolgobulins from many species[1,2] and has been used in immunochemical studies[3,4]. Another distinctive feature of SpA is its potent mitogenic activity on lymphocytes from several species, including humans[5,10]. SpA also exerts a mitogenic activity on both T and B cells [6,8,10]. Moreover, SpA is a striking contrast to other polyclonal B cell activators (PBAs) because it combines with the Fc portion of immunoglobulins. It is thus likely that SpA reacts with cell surface immunoglobulin receptors and in turn delivers signals to B cells. Recently it was shown that in human and murine spleen lymphocytes, both soluble SpA and insoluble SpA in the bacterial cell wall could successfully induce Ig productions[7,8]. On the contrary in peripheral blood lymphocytes (PBL), although insoluble SpA in the bacterial cell wall was able to induce Ig productions[7,8,11], soluble SpA failed to do so[8]. Thus the capacity of soluble SpA to induce Ig production in PBL remains controversial. In the study of the mechanisms of human antibody responses, PBAs of which pokeweed mitogen (PWM) is representative one, have been proved to be useful probes[12,14]. In this context, SpA is expected to be another useful probe for the study of the induction and regulatory mechanisms of human antibody responses. Hence we attempted to induce Ig production in PBL using soluble SpA and to determine the optimal necessary conditions and we compared the characteristics of SpA induced Ig production with PWM induced one.

MATERIALS AND METHODS

1. *Preparation of peripheral blood lymphocytes.* Venous blood from healthy adult volunteers was withdrawn into heparinized tubes and purified PBL suspensions were obtained by standard Ficoll-Hypaque gradient centrifugation[15].

2. *Reagents.* Protein A from *Staphyloccus aureus* (SpA) was purchased from Pharmacia Fine Chemicals Co. (Upsala, Sweden). PWM was purchased from Grand Island Biological Co.

(Grand Island, N.Y.).

3. *Cell culture.* Cells were cultured in RPMI 1640 (GIBCO) buffered with HEPES and supplemented with 2 mM L-glutamine, 10 μg/ml gentamycin (Schering Co., Kenilworth, N.J.), and 10% FCS (GIBCO). Cultures were set up in round-bottomed microtestplate (A/S Nunc, Roskilde, Denmark). 2×10^5 cells in 0.2 ml of culture medium were placed into wells and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 6 days. 10 μg/ml SpA was used as the standard stimulation dose, since the maximal response was obtained at concentrations between 10 μg/ml and 25 μg/ml. For reasons described in the result, cells were routinely washed 4 times with MEM to remove soluble SpA from the culture on day 2.

4. *Assay for Ig secreting cells.* Ig secreting cells were enumerated by the hemolytic plaque assay method of Gronowicz et al [16]. Details were as described in our previous report [13].
Briefly, SpA was coupled to SRBC by the chromium chloride method. One volume of packed SRBC, one volume of SPA solution in saline (0.5 mg/ml), and ten volumes of chromium chloride solution in saline (67 μg/ml) were mixed and allowed to react at 30°C for 60 min. SpA coupled SRBC (SpA SRBC) were then washed 3 times and resuspended to 25% (v/v) in MEM. 0.05 ml of SpA SRBC suspension, 0.1 ml of cultured cells suspension, 0.025 ml of rabbit anti human immunoglobulin serum (Medico-Biological Laboratory, Nagoya, Japan), and fresh guinea pig serum were mixed and placed in a Cunningham chamber. The chambers were sealed with hot paraffin, incubated at 37°C for 3 to 6 hrs and the number of plaques were counted.

RESULTS

Induction of Ig production by soluble SpA in PBL.

To evaluate whether soluble SpA has the capacity to induce Ig production in PBL, 2×10^5 PBL were cultured in the presence of soluble SpA (10 μg/ml) and after 6 days assayed for Ig producing cells. Significant increase in PFC responses was obtained, thus proving that SpA has the capacity to induce Ig production in PBL. However, the magnitude of PFC responses was lower and more variable, in comparison with the PWM-induced responses. In the

Table 1. Soluble SpA-induced PFC responses of PBL

	Donor	Not stimulated		SpA (10 μg/ml)		PWM (5 μl/ml)	
		IgM[a]	IgG[b]	IgM	IgG	IgM	IgG
Exp. 1	Y.G.	40	44	304	76	3920	2260
	O.J.	28	8	200	36	2520	2270
	F.K.	8	16	8	12	1200	1480
	T.K.	144	76	736	264	4400	3240
	K.T.	68	28	720	236	1560	1660
Exp. 2	T.H.	42	138	24	0	1190	2080
	M.T.	38	24	488	480	1150	1540
	Y.N.	88	204	916	1168	980	1500
	S.B.	0	28	1088	428	1096	944

a) Mean number of IgM PFC/well of duplicate cultures.
b) Mean number of IgG PFC/well of duplicate cultures.

Table 2. Effect of stimulation periods on SpA-induced PFC responses

Day[a]	IgM PFC/well		
	Y.Y.	T.H.	M.I.
1 day	496[b]	1168	164
2 day	432	1952	648
2 day (not stimulated by SpA)[c]	4	18	4
2 day+SpA on day 2[d]	0	0	304
2 day+SpA on day 5[e]	244	664	324
3 day	394	880	928
4 day	180	236	936
5 day	72	100	952
6 day	4	32	788
6 day (not stimulated by SpA)[f]	140	444	32

a) Day when cultures were washed to remove free soluble SpA. 2×10^5 PBL were cultured for a total 6 days.
b) Mean PFC numbers of duplicate cultures.
c) PBL were cultured in the absence of SpA and washed on day 2.
d) PBL were cultured in the presence of SpA (10 μg/ml) and washed on day 2. Thereafter PBL were again cultured in the presence of freshly added SpA for another 4 days.
e) PBL were cultured in the presence of SpA (10 μg/ml) and washed on day 2. Thereafter cultures were continued in the absence of SpA except last 24 hrs.
f) PBL were cultured for 6 days in the absence of SpA.

25 subjects examined, the number exhibiting over 1000 PFC per well was 6 in IgM and 4 in IgG response, respectively. Representative results of such experiments are shown in Table 1.

Effect of the period of the soluble SpA stimulation on Ig production of PBL.

We attempted to determine the length of time the soluble SpA must be present in order to induce Ig production of PBL (Table 2). 2×10^5 PBL were cultured in the presence of soluble SpA (10 μg/ml). After initiation of the culture, PBL were washed on various days and assayed for Ig producing cells on day 6. We found that the presence of soluble SpA up to day 3 was adequate for induction of the PFC responses (Donor M.I.). It was also noted that presence of SpA for even 2 days induced 70% of the maximum PFC responses. However, more frequently, the presence of soluble SpA beyond day 2 was rather suppressive in the PFC responses (Donor T.H. and Y.Y.). The mechanisms involved in the suppression of PFC responses by continuous presence of soluble SpA are unclear. Neither accumulation of toxic metabolites nor depletion of nutrients could explain this suppression since when the culture was washed on day 2 and again incubated in the presence of freshly added soluble SpA (10 μg/ml) for another 4 days, the magnitude of PFC responses still remained at low levels.

Effect of the culture washing on day **2** *and removal of free soluble SpA on Ig production of PBL.*

Thus, soluble SpA appeared to have dual activity on the induction of Ig production in PBL, depending on length of the stimulation, the one ability to induce Ig production and the another ability to inhibit it. As described above, soluble SpA induced PFC responses were lower and variable among subjects, in comparison with PWM-induced responses. It is conceivable that these characteristics of soluble SpA-induced Ig production might be due to this dual

Table 3. Effect of the removal of free soluble SpA in the early time of the culture on the SpA-induced PFC responses.

Donor		Not stimulated		SpA (10 μg/ml)			
				Not washed[a]		Washed[b]	
		IgM[c]	IgG[d]	IgM	IgG	IgM	IgG
Low responders[e]	Y.H.	0	18	72	24	312*	592*
(IgG PFC<300)	A.R.	8	56	240	276	2080*	3536*
	I.K.	52	20	68	60	276*	600*
	K.T.	8	24	172	180	792*	1776*
	T.H.	100	340	190	60	1470*	1050*
	M.Y.	0	20	180	120	170	310*
	F.J.	92	140	640	284	316	228
	O.Y.	6	20	136	80	24	20
	I.Z.	12	48	128	36	116	112*
High responders[f]	S.N.	276	688	1944	3824	1688	3104
(IgG PFC>300)	H.I.	0	44	1888	1304	1680	1872
	I.N.	0	80	1190	640	220	940
	T.K.	28	44	336	440	180	356
	K.J.	80	400	1184	3232	1227	4224
	S.G.	56	280	616	928	304	632
	M.T.	140	1030	1008	976	2728*	4575*
	I.D.	16	4	430	340	430	310

a) 2×10^5 PBL were cultured in the continuous presence of SpA (10 μg/ml).
b) 2×10^5 PBL were stimulated with SpA (10 μg/ml) for 48 hrs. Thereafter PBL were washed to remove free soluble SpA and cultured in the absence of SpA for another 4 days.
c) Mean IgM PFC number of duplicate cultures.
d) Mean IgG PFC number of duplicate cultures.
e), f) Subjects were devided arbitrary into high and low responders according to the magnitude of IgG responses.

activity of soluble SpA, and in subjects expressing a low level of PFC responses, the generation of PFC was inhibited by the continuous presence of soluble SpA. In an attempt to verify this possibility, we carried out the following experiment. PBL (2×10^5) were cultured in the presence of soluble SpA (10 μg/ml). One group of cultures was extensively washed on day 2 and free soluble SpA was removed from the cultures. Another group of cultures was incubated without washing, namely in the continuous presence of soluble SpA. After 6 days, the cultures were assayed for Ig producing cells in each group of cultures (Table 3). In this experiment it became evident that in a considerable number of subjects, PFC responses were markedly increased by washing cultures on day 2. Moreover, this tendency was more remarkable in low responders when subjects were arbitrarily divided into high (IgG PFC $\geq$ 300) and low responders (IgG PFC $<$ 300). In low responders, the PFC responses were increased in 7 out of 9 in the IgG class and 5 of 9 in the IgM class, respectively. On the contrary, in high responders, increase in PFC responses was observed in only one of 8 subjects. Thus, in the majority of low responders, low levels of PFC responses proved to be due to the inhibitory effect of continuous presence of soluble SpA. However, it should be noted that in a few subjects, only a low level of responses

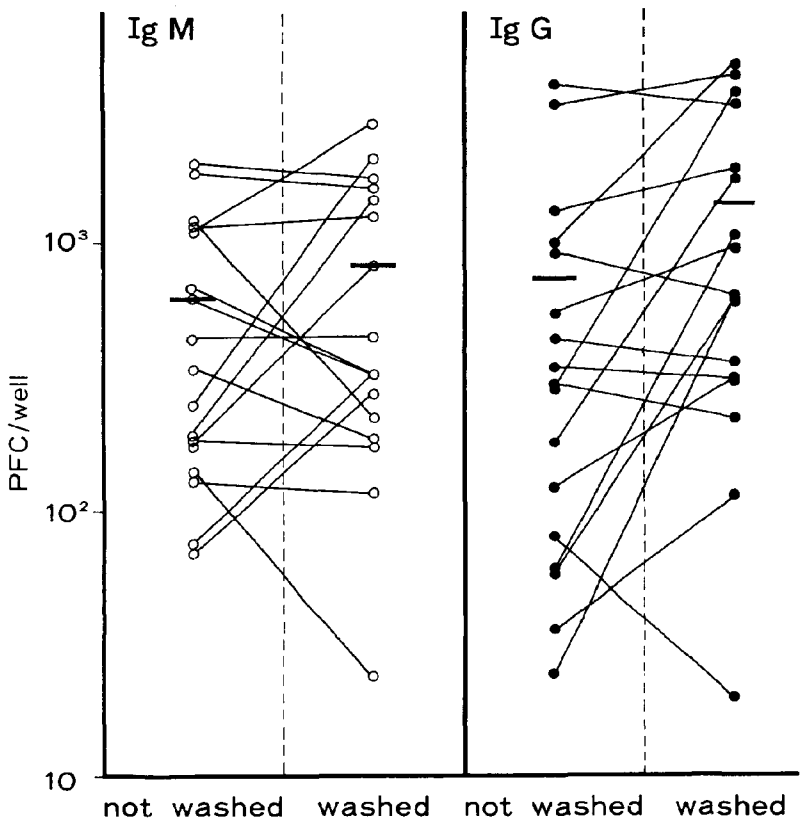

Figure 1. Effect of the washing of the culture on day 2 on soluble SpA-induced Ig production by PBL. 2×10^5 PBL were cultured in the presence of SpA (10 μg/ml). In one group, cultures were washed extensively on day 2. In another group, PBL were cultured without washing. PBL were cultured for total 6 days and assayed for Ig producing cells by SpA PFC assay. In left panel are shown IgM PFC responses (○) and in right panel are shown IgG PFC responses (●). Left side and right side of the each panel express the PFC responses of nonwashed and washed group, respectively. Corresponding responses of washed and nonwashed culture in the same individual are connected by a line. The solid horizontal bar in each panel indicates the mean PFC responses of 17 subjects. Ordinate indicates PFC number in logarithmic scale.

was obtained, even in the washed group of the cultures. These results are shown in Figure 1. Accordingly, because of this apparent inhibitory effect of the continuous presence of soluble SpA on Ig production of PBL in low responders, cultures were routinely washed on day 2 in order to obtain the optimal response.

Figure 2 shows SpA stimulated PFC responses in PBL from 26 healthy subjects. As a comparison, PWM-induced PFC responses were also plotted. SpA were able to induce both IgM and IgG PFC responses, the latter being slightly higher than the former. Mean IgM and IgG PFC responses of 26 individuals were 604/well and 1014/well respectively, however, the magnitude was approximately one third less than the PWM-induced PFC responses. Moreover the magnitude of PFC responses and the ratio of IgM to IgG PFC did not necessarily parallel findings in the PWM-induced responses.

The optimum dose of soluble SpA for Ig production.

To determine the optimal concentration of SpA, PBL were cultured in the presence of varing doses of SpA (Figure 3). In a concentration of 0.5 μg/ml, a significant increase in the number

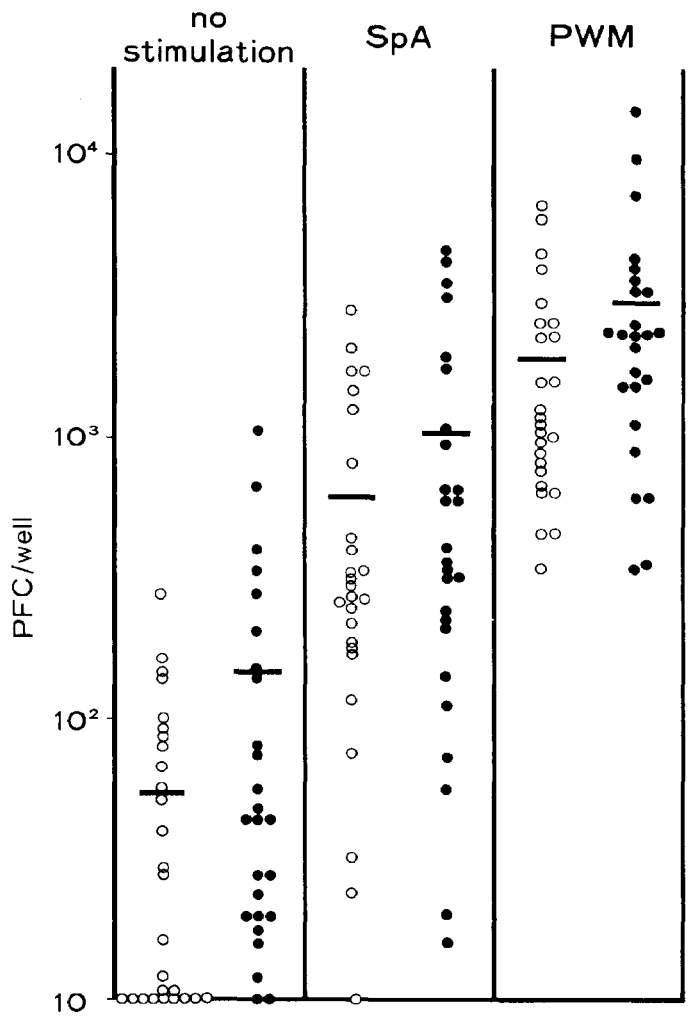

Figure 2. SpA of PWM induced Ig production by human PBL. 2×10^5 PBL from 26 healthy subjects were cultured in the presence of SpA (10 μg/ml) or PWM (5 μl/ml). After 6 days, cultures were assayed for Ig producing cells by SpA PFC assay. Cultures containing SpA were washed on day 2 and incubated in the absence of SpA for another 4 days. In the left panel, PFC responses of cultures were not sitmulated by any mitogens, in the middle panel, PFC responses of SpA stimulated cultures, and in right panel PFC responses of PWM stimulated cultures are shown, respectively. Left side and right side of each panel represent IgM (○) and IgG (●) responses. Solid horizontal bar in each panel represents the mean PFC responses of 26 subjects.

of PFC was obtained. Up to a dose of 10 μg/ml, the number of PFC responses increased, dose dependently, and the maximum PFC responses were obtained at concentrations between 10 and 25 μg/ml. PFC responses diminished with larger concentrations. Therefore, 10 μg/ml was used as the standard dose in further experiments.

Kinetics of SpA induced PFC responses.

The time course of PFC generation by SpA stimulated PBL was compared to that of the PWM-induced PFC responses (Figure 4). On day 3, low but significant PFC responses were evident. Thereafter, the number of PFC rapidly increased and reached a peak on day 5. At this point the IgM PFC gradually decreased. However, in IgG PFC responses, the peak level was maintained up to day 12. In PWM-induced PFC responses, the peak responses were obtained on day 7, 2 days later than in SpA-induced responses. Thereafter the PFC responses declined

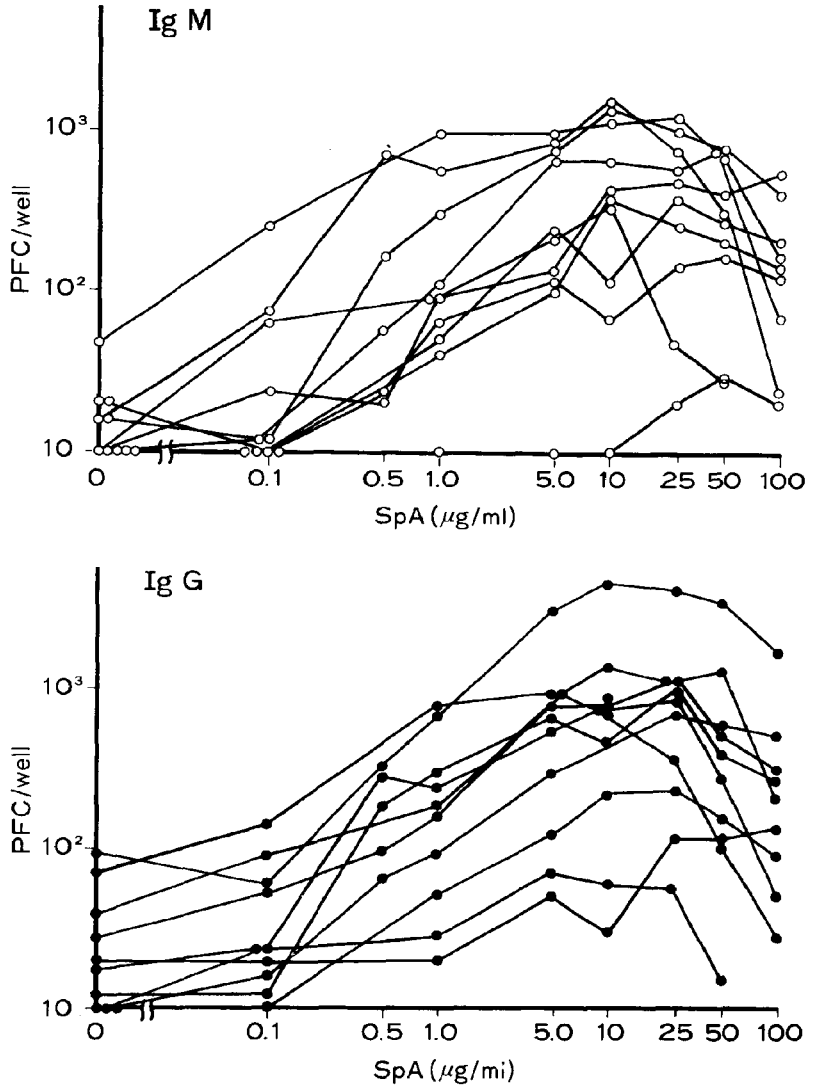

Figure 3. Dose-response relationships of SpA-induced PFC responses. 2×10^5 PBL were cultured in the presence of various doses of SpA. After 6 days, the cultures were assayed for Ig producing cells. Upper panel shows IgM responses (○) and lower panel the IgG responses (●). SpA concentrations are indicated on ordinate, in logarithmic scale, respectively.

rapidly. This tendency was more evident in IgM PFC responses. Thus, the pattern of kinetics diferred in several respects between SpA-and PWM-induced Ig production.

DISCUSSION

We have demonstrated that soluble SpA had the capacity to elicite Ig production of PBL. Both IgM and IgG class of immunoglobulin were produced. The SpA-induced Ig production was polyclonal in nature since anti SRBC antibody was also produced (50 to 300/10^6 recovered cells). However, the magnitude of PFC responses was lower and more variable in comparison with PWM-induced responses. To obtain the optimal responses, a special culture condition was necessary, namely the culture had to be washed on day 2 to remove free soluble SpA since the presence of free soluble SpA for more than 3 days suppressed the PFC responses. This was

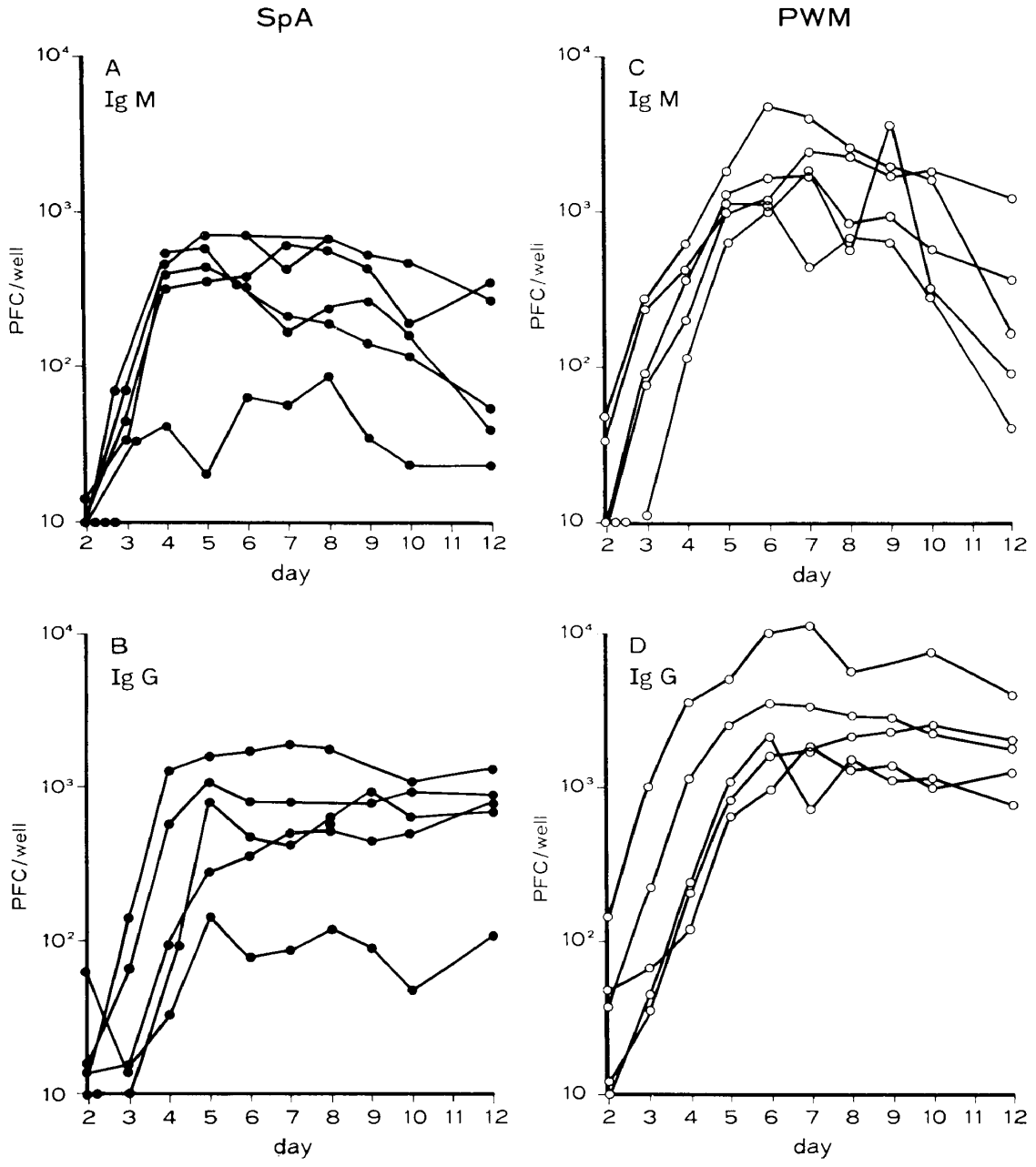

Figure 4. Kinetic study of SpA or PWM-induced PFC responses by PBL. 2×10^5 PBL from 5 subjects were cultured in the presence of SpA (10 μg/ml) or PWM (5 μl/ml). Cultures were assayed for Ig producing cells daily from days 2 to 12. Time course of SpA-induced IgM PFC response (panel A), SpA induced IgG PFC response (panel B), PWM induced IgM PFC response (panel C), and PWM induced IgG (panel D) are shown. Closed circles (●) and open circles (○) represent SpA and PWM induced PFC responses, respectively.

especially the case in low responders. Our findings differ from those of Ringden and Rynnel-Dagoo who found only few PFC responses in PBL when soluble SpA was used[8]. These discrepancies may be due to technical differences such as culture conditions as described above or/and the assay method used for Ig producing cells.

Concerning the suppressive effect of the continuous presence of soluble SpA, the precise mechanisms are unclear. Competitive inhibition of SpA plaque formation by being carried over

soluble SpA in cultured PBL, the change in the culture conditions by consumption of nutrients or accumulation of toxic metabolites can be excluded because cultured PBL were extensively washed at the termination of the culture and addition of concentrations of SpA as high as 1.0 μg/ml during PFC assay decreased little in the size and number of hemolytic plaques (data not shown). In addition, when the cultures were washed on day 2 and again incubated in the presence of SpA for 6 days, PFC responses remained at low levels.

Several other possible mechanisms such as blockade of Ig secretion of plasma cells or inhibition of full B cell differentitaion by the continuous presence of soluble SpA should be taken into consideration. Evidence suggesting the latter possibility is now being accumulated. Moreover, this suppressive effect of the continuous presence of soluble SpA on B cell maturation seems to be exerted indirectly on B cells via SpA activated T cells.

In the preliminary experiment which was designed to evaluate the T cell dependency of SpA induced Ig production, it was revealed that prior irradiation of T cells partially recovered SpA induced PFC responses which were remarkably depressed in the control culture containing nonirradiated T cells and B cells in the continuous presence of free soluble SpA. It is known that in PWM induced Ig production, suppressor T cells of which activity was abolished by prior irradiation were simultaneously induced as well as helper T cells and net Ig production was determined by the balance of the opposing activities of these two functional T cell subsets[17 19]. Accordingly, it is probable that the continuous presence of soluble SpA in the culture might favor the generation of suppressors over the activation of helper T cells or promote the action of such suppressors thereby inhibiting B cell differentiation.

Another important problem is whether SpA induced Ig production is T cell dependent or not. Preliminary data suggested that this was the case (Now a manuscript in preparation). Thus soluble SpA proved to be a PBA as is the case with PWM or PPD. However, even though SpA-induced Ig production shares certain characteristics in common, it has several different aspects such as the magnitude of PFC responses, kinetics profiles. There is increasing evidence suggesting B cells are composed of heterogeneous populations and various mitogens activate distinct subsets[20,21]. So is the case with helper T cells[22 24]. Therefore SpA reactive B cells and/or helper T cells may differ from PWM or other PBAs reactive ones. In this sense, SpA is expected to become a useful probe for the analysis of the intricate and sophisticated human antibody responses. Now the related studies are currently in progress.

ACKNOWLEDGMENTS

The author wishes to thank Dr. Takateru Izumi and Dr. Shunsaku Ôshima for their kind supports.

SUMMARY

The capacity of soluble Protein A (SpA) to elicite immunoglobulin production of human peripheral blood lymphocytes (PBL) was investigated. Both IgM and IgG class of immunogolbulin (Ig) were produced. Presence of soluble SpA for 2 days in the culture was sufficient

for the induction of Ig production of PBL. However, in some subjects, presence of soluble SpA beyond day 2 suppressed Ig production of PBL. Accordingly, in order to obtain optimal responses, cultures were routinely washed on day 2 to remove the free soluble SpA. The magnitude of PFC responses was about one third lower than the Pokeweed mitogen (PWM) induced responses. The magnitude of PFC responses depended upon the concentration of SpA and the highest responses were obtained with concentrations between 10 and 25 μg/ml. In the kinetics study, a significant increase in the number of PFC was observed on day 3. The PFC numbers reached a peak on day 5. On the contrary, in PWM-induced responses, the PFC responses reached a peak on day 7. Thus SpA was proved to be have the capacity to induce polyclonal Ig production by PBL as is the case with PWM and to be a useful tool for the study of the mechanisms of human antibody responses.

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