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Comparative studies on the actions of antigen and polyclonal B-cell activator in differentiation and proliferation of B-cells and B memory cells

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Summary. Using the capsular polysaccharide of *Klebsiella pneumoniae* (CPS-K) as a polyclonal B-cell activator (PBA) and sheep red blood cells (SRBC) as a T cell-dependent antigen, we compared the ability of PBA and antigen to differentiate (generate antibody-forming cells, AFC) and proliferate (generate immunological memory) virgin B cells and B memory cells. *In vitro* CPS-K induced the differentiation of IgM virgin B cells, IgM B memory cells and IgG B memory cells to AFC, as well as or better than SRBC. The differentiation of B memory cells to AFC by CPS-K did not require the participation of macrophages or T cells, whereas the action of SRBC depended strictly upon the helper actions of these cells. The responsiveness to CPS-K and SRBC of normal and antigen-primed spleen cells as judged by anti-SRBC PFC responses *in vitro* was markedly decreased after stimulation of virgin B cells and B memory cells *in vivo* by CPS-K injection into normal or primed mice but greatly increased after the injection of SRBC. The decrease in the responsiveness to CPS-K of spleen cells from mice treated with CPS-K appeared principally due to exhaustion of the functions of B cells and B memory cells. From the present data it has been concluded that the signals required for the differentiation and proliferation of B cells or B memory cells are different from each other, the signal for differentiation being provided by either antigen (SRBC) or PBA (CPS-K), while the signal for proliferation is delivered only by antigen.

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INTRODUCTION

There are a number of so-called polyclonal B-cell activators (PBA) capable of stimulating B lymphocytes to generate IgM-secreting cells (Andersson, Sjöberg & Möller, 1972; Nilsson, Sultzer & Bullock, 1973; Melchers, Braun & Galanos, 1975; Nakashima & Kato, 1974; Nakashima, Kojima & Kato, 1976; Greaves, Owen & Raff, 1973; Coutinho & Möller, 1975). Many studies have been made of the mechanism of B-cell activation by PBA (Melchers & Andersson, 1974; Greaves, Owen & Raff, 1973; Coutinho & Möller, 1975). Recently, Coutinho, Möller and their colleagues (Coutinho, Gronowicz, Bullock & Möller, 1974; Coutinho & Gronowicz, 1975; Coutinho & Möller, 1975) proposed the 'one non-specific signal' hypothesis for B-cell activations. They showed that there were no fundamental differences in the mode of B-cell activation between PBA and T cell-independent antigens, suggesting that the roles of antigen and cell-co-operative mechanisms were to give a suitable amount of non-specific stimulus to B cells. It appears, however, that there are clear differences in the mode of B-cell

activation by PBA and T-cell-dependent antigen. First, B-cell activation by PBA is usually restricted to the process of IgM synthesis (Andersson *et al.*, 1972; Nilsson *et al.*, 1973; Melchers *et al.*, 1975; Nakashima *et al.*, 1976; Greaves *et al.*, 1973) although some evidence has been presented against this conclusion (Kreisler & Möller, 1974; Kolb, DiPauli & Weiler, 1974; Nakashima & Kato, 1974). Secondly, PBA do not act to induce immunological memory (Nakashima & Kato, 1974; Nakashima *et al.*, 1976; Nakano, Uchiyama, Tanabe & Saito, 1975; Coutinho & Möller, 1975). In this study, by the use of the capsular polysaccharide of *Klebsiella pneumoniae* (CPS-K) as a PBA and sheep blood cells (SRBC) as a T-cell-dependent antigen, we tried to see: (1) if and how PBA and T-cell-dependent antigens would activate B cells and B-memory cells of either IgM or IgG type *in vitro* and (2) if and how PBA and antigen would change the responsiveness of B cells and B memory cells to restimulation *in vitro*.

MATERIALS AND METHODS

Animals

Female SMA mice approximately 6 weeks of age were used.

Antigen and PBA

SRBC were used as a T-cell-dependent antigen. CPS-K was prepared from the culture supernatant of *Klebsiella pneumoniae* type 1 Kasuya strain as described previously (Nakashima, Kobayashi & Kato, 1971) and used as a PBA (Nakashima & Kato, 1974; Nakashima *et al.*, 1976).

Preparation of cell suspensions

Mouse spleens were dissected to prepare single cell suspensions in Eagle's minimum essential medium (MEM). They were washed once with MEM by centrifugation at 800 r.p.m. for 7 min.

Cultures

Spleen cells were cultured according to the method of Mishell & Dutton (1967) using as culture medium MEM supplemented with 5 per cent decomplemented foetal calf serum, 2 mM L-glutamine and 5×10^{-5} M mercaptoethanol. Two millilitres of spleen-cell suspension (10^7 cells) were seeded into 45-mm glass Petri dishes and incubated at 37° in a 5 per cent CO_2 atmosphere without rocking. SRBC (1×10^6) or various amounts of CPS-K were added into the cultures at the start of incubation. Triplicate cultures were made for each experimental group.

Removal of adherent cells

For removal of adherent cells, 10 ml of spleen cell suspension in MEM supplemented with 5 per cent foetal calf serum containing 5×10^6 cells per millilitre were placed into 90-mm glass Petri dishes and incubated at 37° for 30 min. The non-adherent cells were collected by washing gently the dishes and treated again in the same way using other dishes to remove the residual adherent cells.

Treatment with anti-thymocyte serum (ATS)

ATS was prepared as follows. Thymocytes of SMA mice (10^9 cells) were injected intravenously twice into rabbits at an interval of 2 weeks, and the rabbits were bled 7 days after the last injection. The serum was absorbed with an equal volume of packed mouse erythrocytes. ATS treatment of cells was performed by incubating cells together with ATS (1:20) and complement at 37° for 30 min. When incubated together with ATS and complement, 97 per cent of thymocytes, 36 per cent of spleen cells and 4 per cent of bone marrow cells were killed (determined by trypan blue exclusion test (Boyse, Old & Chouroulinkov, 1974). With 1:40 dilution of ATS, the values were 77, 18, and 0 per cent respectively. This indicated that ATS used in this study was specific for thymocytes and T cells.

Assay for anti-SRBC plaque-forming cells (PFC)

PFC which were developed in the cultures of spleen cells were assayed by Cunningham & Szenberg's technique (1968), as described previously (Nakashima *et al.*, 1976). Indirect (IgG) PFC were estimated as the total number of PFC developed after the addition of antiserum to mouse IgG into the reaction mixture, minus the number of direct (IgM) PFC. For each group, the geometric mean of three dishes with its standard deviation was estimated.

RESULTS

Anti-SRBC PFC responses *in vitro* of normal and SRBC-primed spleen cells to SRBC and CPS-K

A study was made to see if and how SRBC and CPS-K

Table 1. Anti-SRBC PFC responses *in vitro* of normal and SRBC-primed spleen cells to SRBC and CPS-K

Experiment number	Type of* cells	Materials added†	Direct PFC numbers/dish‡	Indirect PFC numbers/dish‡
1	Normal	None	23±20	<10
		SRBC	33±33	<10
		CPS-K	273±135	<10
2	Normal	None	10	
		SRBC	<10	
		CPS-K	133±42	
	Primed	None	80±42	
		SRBC	510±470	410
		CPS-K	2840±2250	2060
3	Primed	None	50±40	
		SRBC	390±70	
		CPS-K	495±25	

* Groups of mice (each group contained three mice) were injected i.p. with 2×10^8 SRBC 10 days (expt 2) or 13 days (expt 3) before excision of spleens.
† The concentration of CPS-K was 5 μg/ml (expts 2 and 3) or 50 μg/ml (expt 1).
‡ PFC were assayed after 3 days' incubation.

would stimulate anti-SRBC PFC responses in *in vitro* cultures of spleen cells from normal mice (normal spleen cells) and spleen cells from mice primed with SRBC (primed spleen cells). The results are shown in Table 1. By addition of SRBC to cultures of normal spleen cells, no significant primary PFC responses were induced. This seemed due to the lack of some factors in the foetal calf serum used in this study to support the primary PFC responses to SRBC (Watson & Epstein, 1973). In contrast, definite direct (but not indirect) anti-SRBC PFC responses were induced by addition of CPS-K to cultures of normal spleen cells. On the other hand, high direct and indirect PFC responses were induced by addition of either SRBC or CPS-K to cultures of primed spleen cells. It should be emphasized that CPS-K could elicit both direct and indirect PFC responses of primed spleen cells as markedly as or more markedly than SRBC. These results indicate that in an *in vitro* culture system CPS-K can stimulate either normal or primed spleen cells to generate anti-SRBC PFC and the intensity of its action is rather more potent than SRBC.

Relationship of anti-SRBC PFC responses *in vitro* by normal and primed spleen cells to dose of CPS-K

The relationship between the dose of CPS-K and anti-SRBC PFC responses in cultures of normal primed spleen cells was explored. The results are shown in Fig. 1. The level of direct PFC responses

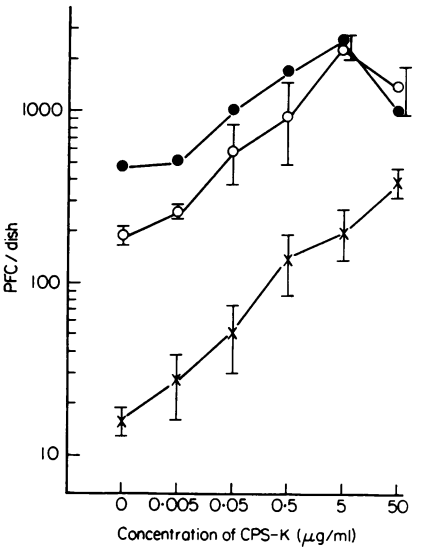

Figure 1. Relationship of anti-SRBC PFC responses *in vitro* by normal and SRBC-primed spleen cells to dose of CPS-K. Spleen cells from normal mice (×) and mice injected i.p. with 2×10^8 SRBC 10 days before excision of spleens (○ and ●) were cultured. Direct (○ and ×) and indirect (●) PFC were assayed after 3 days' incubation.

of normal spleen cells was dependent on the dose of CPS-K between 0·005 and 50 μg/ml. The dose-response relationship for both direct and indirect PFC responses of primed spleen cells was similar to direct PFC responses of normal spleen cells except that 5 μg/ml was the optimum concentration for primed cells, with a slight fall at 50 μg/ml.

Kinetics of anti-SRBC PFC responses *in vitro* of normal and primed spleen cells to CPS-K

The kinetics of increase in anti-SRBC PFC in cultures of normal and primed spleen cells after stimulation by CPS-K were analysed (Fig. 2). Even in normal spleen cell cultures to which CPS-K was not added, direct PFC numbers increased after incubation of longer than 2 days. This background increase in direct PFC may be due to the non-specific B-cell activating action of foetal calf serum (Coutinho & Möller, 1973). The increase in direct PFC numbers on stimulation by CPS-K was already detectable 1 day after the start of incubation and was augmented thereafter. On the other hand, in primed spleen cells, there were a large number of direct and indirect PFC at the beginning of incubation, and in the absence of CPS-K they decreased during incubation, slowly during the first 2 days and rapidly during the succeeding 2 days. This indicates that antibody-forming cells (AFC) matured *in vivo* are difficult to maintain for a long time *in vitro*. The increase in both direct and indirect PFC numbers based on stimulation by CPS-K in primed spleen cells was clearly detectable after 1 day and reached a peak at 2 days, after which both direct and indirect PFC numbers decreased. The rate of increase during the first 2 days in indirect PFC was very similar to that in direct PFC, but the rate of decrease during the succeeding 2 days in the former was more rapid than that in the latter.

Dependency of the activation of primed spleen cells by SRBC and CPS-K *in vitro* on accessory cells

A study was made to see if there might be qualitative differences between SRBC and CPS-K in the mechanisms of induction of direct and indirect anti-SRBC PFC responses of primed spleen cells *in vitro* with regard to their dependency on accessory cells such as macrophages and T cells. As shown in Table 2, removal of adherent cells from primed spleen cells reduced greatly the level of direct and indirect PFC responses to SRBC. In contrast, such a treatment of primed cells did not significantly alter their responsiveness to CPS-K. As shown in Table 3, preincubation *in vitro* of primed spleen cells with ATS and complement resulted in almost complete loss of their responsiveness to SRBC. The same treatment of primed cells diminished only slightly their responsiveness to CPS-K. On the contrary, the increase in both direct and indirect

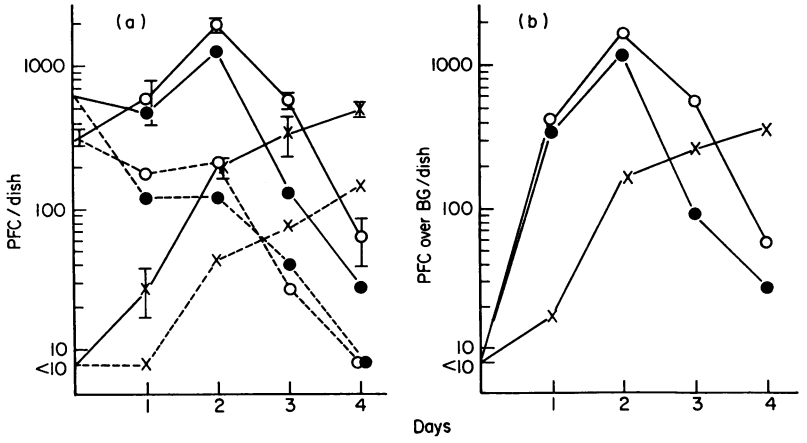

Figure 2. Kinetics of anti-SRBC PFC responses *in vitro* of normal and SRBC-primed spleen cells to CPS-K. Spleen cells from normal mice (×) and mice injected i.p. with 2×10^8 SRBC 10 days before excision of spleens (○ and ●) were cultured with (——) or without (– – –) 25 μg/ml of CPS-K. Direct (○ and ×) and indirect (●) PFC were assayed. PFC numbers in CPS-K-treated cultures, from which PFC numbers in untreated cultures (background) were subtracted, are presented in (b).

Table 2. Effect of removal of adherent cells from SRBC-primed spleen cells on their anti-SRBC PFC responses to SRBC and CPS-K

Kind of cells*	Materials added†	Direct PFC numbers/dish‡	Indirect PFC numbers/dish‡
Original	SRBC	1870±410	900
Adherent cell-removed	SRBC	320±43 (−83%)	450 (−50%)
Original	CPS-K	470±77	300
Adherent cell-removed	CPS-K	500±128 (+6%)	290 (−2%)

* Mice were injected i.p. with 2×10^8 SRBC 10 days before excision of spleens. For the method of removal of adherent cells, see the Materials and Methods section.
† The concentration of CPS-K was 50 μg/ml.
‡ PFC were assayed after 3 days' incubation.

Table 3. Effect of ATS treatment of SRBC-primed spleen cells on their anti-SRBC responses *in vitro* to SRBC and CPS-K

Kind of cells*	Materials added†	Recovered viable cell numbers	Direct PFC numbers/dish‡	Indirect PFC numbers/dish‡
Original	SRBC	840,000	733±288	64
ATS-treated	SRBC	260,000 (−69%)	27±17 (−96%)	< 10 (< −84%)
Original	CPS-K	850,000	683±94	455
ATS-treated	CPS-K	300,000 (−65%)	440±192 (−36%)	344 (−24%)

* Mice were injected i.p. with 2×10^8 SRBC 10 days before excision of spleens. For the method of ATS treatment, see the Materials and Methods section.
† The concentration of CPS-K was 5 μg/ml.
‡ PFC were assayed after 3 days' incubation.

PFC numbers after stimulation by CPS-K was rather augmented by ATS treatment when expressed as PFC per constant number of viable spleen cells. These results indicate that direct and indirect PFC responses *in vitro* of primed spleen cells to CPS-K do not require participation of accessory cells such as macrophages and T cells, whereas their responses to SRBC are strictly dependent on the helper actions of these cells.

Time course of changes in the responsiveness of spleen cells to CPS-K and SRBC after injection of SRBC into normal mice

A kinetic study was made to see how the responsiveness of spleen cells to CPS-K and SRBC would change after injection of SRBC into normal mice. Spleens were excised at various times after SRBC injection, and direct and indirect anti-SRBC PFC responses of spleen cells to CPS-K and SRBC were assessed *in vitro*. The results are shown in Fig. 3. The ability of spleen cells to respond to CPS-K *in vitro* by generating direct PFC was increased 1 day after SRBC injection, reached the peak by 6 or 9 days, and decreased thereafter. The generation of indirect PFC *in vitro* was first detectable 6 days after SRBC injection and decreased there after. These results indicate that SRBC injection into mice induces a rapid increase in B-cell function through possible proliferation of B-memory cells. The *in vitro* response of spleen cells to SRBC (direct PFC) increased after SRBC injection in a similar but not identical way to the response to CPS-K. The slight differences in the patterns of increase in the responsiveness to CPS-K and SRBC may possibly be due to participation of accessory cells in the responses to SRBC as the helpers for B-cell activation.

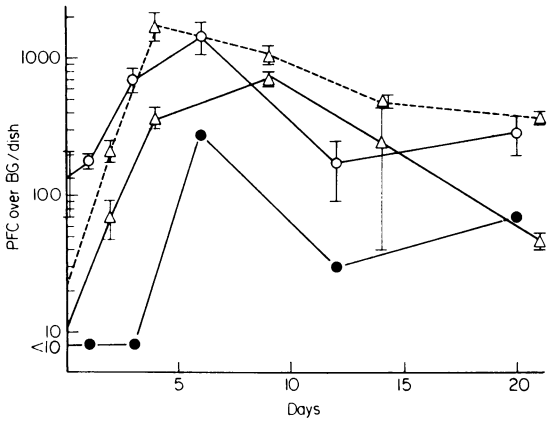

Figure 3. Kinetics of changes in the responsiveness of spleen cells to CPS-K and SRBC after injection of SRBC into normal mice. Mice were injected i.p. with 2×10^8 SRBC, at various times before excision of spleens. Spleen cells were cultured with or without 5 μg/ml of CPS-K (——) or 5×10^6/ml of SRBC (– – –). Anti-SRBC direct (○ and △) and indirect (●) PFC were assayed after 2 days' incubation (△——△) or after 3 days' incubation (△ – – – △, ○——○ and ●——●). The values shown are PFC numbers in CPS-K-treated cultures and in SRBC-treated cultures, from which PFC numbers in untreated cultures (background) have been subtracted.

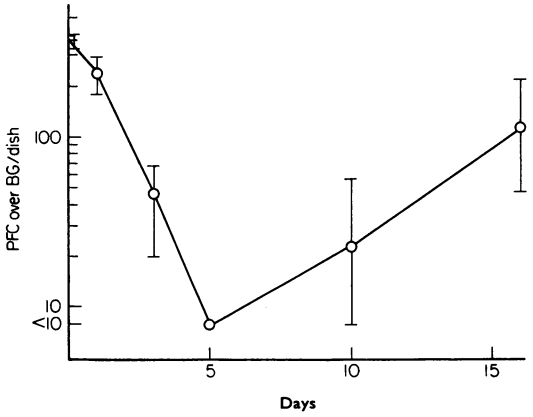

Figure 4. Kinetics of changes in the responsiveness of spleen cells to CPS-K after injection of CPS-K into normal mice. Mice were injected i.p. with 200 μg of CPS-K at various times before excision of spleens. Spleen cells were cultured with or without 25 μg/ml of CPS-K. Anti-SRBC direct PFC were assayed after 3 days' incubation. PFC numbers in CPS-K-treated cultures, from which PFC numbers in untreated cultures were subtracted, are presented.

Time course of changes in the responsiveness of spleen cells to CPS-K after injection of CPS-K into normal mice

A kinetic study was made to see how the responsiveness of spleen cells to CPS-K would change after injection of CPS-K into normal mice. Spleens were excised at various times after CPS-K injection, and direct anti-SRBC PFC responses to spleen cells to CPS-K were assessed *in vitro*. The results are shown in Fig. 4. The direct *in vitro* PFC response of spleen cells to CPS-K was rapidly decreased after CPS-K injection, reached the bottom level at 5 days, after which it recovered nearly to the original level by 15 days. As reported previously (Nakashima *et al.*, 1976), injection of more than 100 μg of CPS-K into normal mice induces a marked increase in direct anti-SRBC PFC in spleens. It appears therefore that CPS-K stimulates B cells to differentiate to AFC and, as a result, the B cells become unable to respond to the subsequent stimuli.

Changes in the responsiveness of spleen cells to CPS-K and SRBC after injection of SRBC and CPS-K into mice primed with SRBC

A study was made to see how the responsiveness of spleen cells to SRBC and CPS-K would change after injection of SRBC or CPS-K into mice already primed with SRBC. The results are shown in Table 4. The responsiveness *in vitro* of primed spleen cells to SRBC and CPS-K was markedly decreased after injection of CPS-K into primed mice, whereas it was greatly increased after a secondary injection of SRBC. These results suggest that B-cell memory to SRBC can be depressed by CPS-K *in vivo*, whereas it is further amplified by SRBC.

Effect of mixture of normal, primed, and primed and CPS-K-treated spleen cells on their responsiveness to CPS-K

It was possible that the unresponsiveness of spleen cells to subsequent stimulation by CPS-K or SRBC which is induced by CPS-K injection into normal and primed mice (Fig. 4 and Table 4) resulted from the exhaustion of the functions of B cells or B-memory cells themselves, from the depletion of some other factors essential for B-cell activation, or from the stimulation of suppressor cells. Therefore, the effect of combining normal spleen cells, primed spleen cells, and spleen cells from mice primed with

Table 4. Effect of injection of CPS-K and SRBC into SRBC-primed mice on anti-SRBC PFC responses *in vitro* of their spleen cells to CPS-K and SRBC

Expt no.	Type of cells*	Materials added†	Direct PFC numbers/dish‡	Indirect PFC numbers/dish‡
1	Normal	CPS-K	133±42	
	SRBC-primed	CPS-K	2840±2250	2060
	SRBC-primed & CPS-K-treated	CPS-K	110±85	197
	Normal	SRBC	<10	
	SRBC-primed	SRBC	510±470	410
	SRBC-primed & CPS-K-treated	SRBC	50±21	67
2	SRBC-primed	CPS-K	52±5	
	SRBC-primed & CPS-K-treated	CPS-K	<10	
	SRBC-primed & SRBC-boosted	CPS-K	920±524	
	SRBC-primed	SRBC	57±45	
	SRBC-primed & CPS-K-treated	SRBC	10	
	SRBC-primed & SRBC-boosted	SRBC	633±176	

* Groups of mice (each group contained three mice) were injected i.p. with 2×10^8 SRBC 10 days (expt 1) or 14 days (expt 2) before excision of spleens. Some of them were injected secondarily i.p. with 200 μg of CPS-K 3 days (expt 1) or 7 days (expt 2) before excision of spleens or with 2×10^8 SRBC 7 days before.

† The concentration of CPS-K was 5 μg/ml.

‡ PFC were assayed after 2 days' incubation (CPS-K groups in expt 2) or after 3 days' incubation (other groups).

SRBC and given CPS-K (primed and CPS-K-treated spleen cells) on their responsiveness to CPS-K was investigated *in vitro*. The results are shown in Table 5.

The direct PFC response to CPS-K of a mixture of primed spleen cells and normal spleen cells was lower than that of primed spleen cells alone. It appears therefore that the responsiveness is reduced non-specifically by increasing the cell concentration from 1×10^7 per dish to 2×10^7 per dish. The responsiveness of a mixture of primed spleen cells and primed-and-CPS-K-treated spleen cells was very similar to that of a mixture of primed spleen cells and normal spleen cells. This fact suggests that the low responsiveness of primed-and-CPS-K-treated spleen cells is not principally due to an increase in the activity of suppressor cells. On the other hand, the responsiveness of primed-and-CPS-K-treated spleen cells was not enhanced by adding normal spleen cells. Moreover, the responsiveness of a mixture of primed and CPS-K-treated spleen cells and primed spleen cells was lower than that of the same cell numbers of primed spleen cells alone. This may rule out the possibility that the decrease in the responsiveness to CPS-K induced by a preceding injection of CPS-K is due to exhaustion of some accessory factors which are essential for activation of B cells and B memory cells, since if this were the case, the PFC responses of spleen cells from CPS-K-treated mice would have been enhanced by addition of normal spleen cells or primed spleen cells. All of these experimental results support the idea that when injected into normal mice or primed mice CPS-K induces ultimate

Table 5. Effect of mixture of various kinds of spleen cells on their anti-SRBC PFC responses *in vitro* to CPS-K

Cells and *in vivo* treatment*	Direct PFC numbers/dish†	
	No CPS-K *in vitro*	CPS-K *in vitro*‡
Normal	<10	40±22 (80±35)
Primed	188±47	2037±350 (2640±2120)
Normal+primed		1210±1180
Primed-CPS-K	105±16	291±130 (300±223)
Primed-CPS-K+normal		154±145
Primed-CPS-K+primed		1023±640

* Groups of mice (each group contained three mice) were injected i.p. with 2×10^8 SRBC 12 days before excision of spleens (primed). Some of them were injected secondarily i.p. with 200 μg of CPS-K 3 days before excision of spleens (primed-CPS-K).

† PFC were assayed after 2 days' incubation of 1×10^7 or 2×10^7 (numbers in parentheses) cells, or the mixture of 1×10^7 cells of each kind.

‡ The concentration of CPS-K was 5 μg/ml.

exhaustion of the functions of B cells and B-memory cells themselves after activation of these cells to differentiate to AFC.

DISCUSSION

We have shown that CPS-K, a potent PBA, stimulated IgM anti-SRBC PFC responses in cultures of normal spleen cells and both IgM and IgG anti-SRBC PFC responses in cultures of primed spleen cells as markedly as or more markedly than did SRBC, a T cell-dependent antigen. IgM PFC responses of primed spleen cells to CPS-K were generally higher than those of normal spleen cells. CPS-K could directly stimulate IgM and IgG B memory cells to generate AFC without the help of accessory cells such as macrophages and T cells (Tables 2 and 3), just as it stimulated virgin IgM B cells (Nakashima *et al.*, 1976). Previously, Kreisler & Möller (1974) showed that IgG anti-SRBC AFC were generated by addition of PPD to cultures of spleen cells from mice hyperimmunized with SRBC, although they did not compare IgM PFC responses to PPD of primed spleen cells with those of normal spleen cells. They showed also that the optimum concentration of PPD to activate IgG precursor cells was 100 times lower than that necessary to activate IgM precursor cells (Kreisler & Möller, 1974). In our study, it was shown that there was no difference in the optimum concentration of CPS-K to activate IgG B-memory cells and IgM B-memory cells, although this optimum concentration of CPS-K was lower than that necessary to activate IgM virgin B cells (Fig. 1). Moreover, we found that there were great differences in the kinetics of increase in anti-SRBC PFC after stimulation by CPS-K between normal and primed spleen cell cultures (Fig. 2). These differences may be mainly due to differences in numbers of antibody-forming cell precursors (AFCP). Anyhow, the present findings support strongly the idea that the signal mediated by antigen can be replaced by the signal mediated by PBA to induce the differentiation of both virgin and memory B cells of either IgM or IgG type to AFC.

We found that the responsiveness of normal and primed spleen cells to CPS-K and SRBC, as judged by anti-SRBC PFC responses *in vitro*, was decreased after treatment of the cells *in vivo* with CPS-K, whereas it was increased after treatment with SRBC *in vivo*. Similarly, a previous injection of CPS-K decreased the responsiveness of mice to subsequent antigenic stimuli *in vivo* (Nakashima *et al.*, 1971; Nakashima & Kato, 1975). Gronowicz & Coutinho (1974) observed that the responsiveness *in vitro* to LPS of mouse spleen cells was decreased by the previous treatment of the spleen cells with LPS *in vivo*. In their studies, however, how the responsiveness to LPS and antigen of spleen cells from antigen-primed mice was changed by injection of LPS into antigen-primed mice was not tested, and the possibility was not completely excluded that accessory cells other than B cells contributed in the phenomenon. In the present study, we could confirm that the decrease in the *in vitro* response to CPS-K of normal and primed spleen cells was principally due to the exhaustion of the functions of B cells and B-memory cells. Previously, we showed that AFC were generated by the action of CPS-K in spleens of normal mice possibly through one or a few cell divisions of a large number of AFCP (B cells), whereas they were generated by the action of SRBC through frequent divisions of a small number of AFCP (Nakashima *et al.*, 1976). The most likely explanation for the decrease in the functions of B cells and B memory cells after injection of CPS-K may be that a majority of available AFCP are differentiated to AFC as end cells which are no longer responsive to restimulation, without the AFCP themselves being significantly stimulated to proliferate, either because CPS-K does not generate new AFCP, or because it stimulates the generation of AFCP which are too immature to respond to restimulation. In the previous study (Nakashima *et al.*, 1976), it was concluded that although nearly all of the mature rosette-forming cells (RFC) (probably AFCP) in spleens were differentiated to PFC (AFC) by the action of CPS-K, almost equal numbers of new RFC were generated from the precursors of RFC through one or a few cell divisions, suggesting that the new AFCP generated after stimulation by CPS-K are unresponsive to restimulation.

The lack of proliferation of B cells after stimulation by CPS-K is not due to overstimulation of B cells nor to non-specific suppression of individual B-cell clones through overall stimulation of spleen cells, since injection of antigen together with CPS-K has been shown to increase immunological memory (Nakashima *et al.*, 1971; Nakashima, 1972; Nakashima & Kato, 1975) which is accompanied by

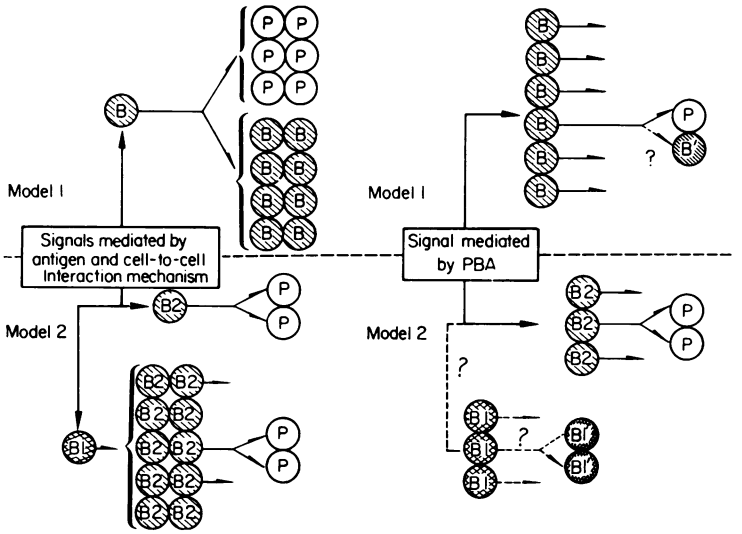

Figure 5. Two models proposed to explain the cytokinetics occurring after stimulation by antigen (SRBC) and PBA (CPS-K), applicable to both virgin B cells and B-memory cells of either IgM or IgG type. In model 1 (above), the same B cells respond to antigen and PBA in different ways. In model 2 (below), it is postulated that there are two subpopulations of B cells; one responds to both antigen and PBA and another to antigen only. B = B cells in spleens of normal and primed mice. P = antibody-forming plasma cells as end cells. B1 and B2 = subpopulations of B cells in spleens of normal and primed mice. B′ and B1′ = modified B cells or B1 cells which are not ready to respond to further stimuli.

proliferation of B cells specific for the antigen (unpublished results). It appears therefore that although the non-specific signal mediated by CPS-K is enough to stimulate the differentiation of both virgin B cells and B memory cells to AFC, signals mediated by antigen and cell co-operative mechanisms are required to stimulate their proliferation. Two models are presented to explain the possible cytokinetics occurring after stimulation by antigen and CPS-K (Fig. 5). In the first model, the same B cells or B-memory cells of either IgM or IgG type respond to antigen and CPS-K in different ways. Both proliferation and differentiation to AFC can be induced through the signal mediated by antigen, whereas the signal mediated by CPS-K induces differentiation with very little proliferation. In the second model, it is postulated that there are mature and immature subpopulations of B cells in spleens of normal and primed mice. Mature B cells (B2) respond to either CPS-K or to antigen by differentiating to AFC, whereas immature B cells (B1) respond to antigen by proliferation and development into mature B cells, while they hardly respond to CPS-K. It might, of course, be possible that CPS-K and SRBC act on quite different subpopulations of B cells. However, since CPS-K injected into mice primed with SRBC decreased the responsiveness of primed spleen cells to both CPS-K and SRBC, it seems likely therefore that CPS-K acts on the majority, if not all, of the antigen-reactive B-memory cells. From the present data, it may be concluded that qualitatively (not merely quantitatively) different signals are necessary for the differentiation and proliferation of both virgin B cells and B-memory cells, and the signal for the proliferation is not provided by the action of PBA such as CPS-K.

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