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Polyclonal activation of murine B cells by a membrane proteoglycan of *Klebsiella pneumoniae*

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SUMMARY

The lymphocyte activating properties of a membrane proteoglycan (MPG) extracted from a mutant non-encapsulated strain of *Klebsiella pneumoniae* (Kp) (biotype a I-145) were investigated. Kp MPG induced a strong proliferative response of BALB/c spleen cells and Peyer's patches cells. Thymidine incorporation was dose-related (from 1 to 100 μg Kp MPG/ml) and reached a maximum at day 3. It was not reduced by removal of most adherent cells, nor by depletion of Thy1-2 positive cells, but it was abrogated by removal of surface immunoglobulin bearing cells. Spleen cells from nude mice and those from C3H/Hej mice were strongly stimulated by Kp MPG. Conversely Kp MPG did not induce interleukin 2 production and did not trigger the proliferation of thymocytes but stimulated interleukin 1 production by adherent spleen cells. Finally, unfractionated or B-enriched spleen cells cultured with Kp MPG synthesized IgM and, to a lesser extent, IgG and IgA. It is concluded that Kp MPG is a T-independent polyclonal B cell activator and an inducer of interleukin 1 production.

Keywords *Klebsiella pneumoniae* polyclonal B cell activator membrane proteoglycan interleukin 1

INTRODUCTION

A great number of bacterial components which possess adjuvant activity can stimulate macrophages and B or T lymphocytes. However, these properties were shown to vary, depending on bacterial species and strains, the type of bacterial component (cell wall, polysaccharide, peptidoglycan, teichoic acid, cytoplasmic fractions, etc) and the animal species (Räsänen & Arvilommi, 1981; Takada *et al.*, 1979; Damais *et al.*, 1975; Barot-Ciorbaru *et al.*, 1985). Lipopolysaccharide (LPS), the major constituent of the outer membrane of Gram-negative bacteria, was extensively investigated with respect to its polyclonal B cell activating properties which may involve the triggering of a B cell receptor for lipid A and the stimulation of macrophages by polysaccharide (Williamson *et al.*, 1984).

The immunostimulating properties of several components of encapsulated *Klebsiella pneumoniae* (Kp) have been reported. Thus, the capsular polysaccharide was shown to stimulate polyclonal Ig synthesis when administered to mice (Nakashima & Kato, 1974). More recently, glycoproteins extracted from encapsulated Kp cell walls (strain K_2O_1) were reported to induce murine polyclonal B cell activation (Wood & Möller, 1984; Guenounou *et al.*, 1984). The membrane proteoglycans (MPG) of a peculiar non-encapsulated, non-virulent strain of Kp (biotype a, I-145) have been prepared and used clinically in association with bacterial ribosomes (Dussourd d'Hinterland,

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Normier & Durand, 1980). This composite preparation (trade name Ribomunyl, Pierre Fabre SA, Castres, France) has been extensively used in the prevention of recurrences of respiratory tract infections (Michel *et al.*, 1978; Bousquet & Dussourd d'Hinterland, 1982). Since this Kp extract differs in its origin and mode of preparation from other Kp components with immunostimulatory properties, we investigated its capacity to induce macrophages to produce interleukin 1 (IL-1) and lymphocytes to proliferate and differentiate in the mouse.

MATERIALS AND METHODS

Animals. Adult male and female mice were used. BALB/c were bred in our laboratory. C3H/HeJ, CBA/J, and Nude (nu^+/nu^+) were purchased from IFFA-CREDO (L'Arbresle, France).

Preparation of Kp MPG. The MPG from a mutant non encapsulated strain of Kp were obtained according to a method described by Dussourd d'Hinterland *et al.* (1980). The lyophilized preparation was shown to contain: proteins (31·8%), galactose (21%), glucose (1·8%), hexosamin (5·5%), uronic acids (3·1%) and fatty acids (3·9%). Kp MPG contains less than 1% of LPS as determined by the Limulus assay. RNA and DNA were undetectable. Kp MPG (batch GN 415 Pierre Fabre SA, Castres, France) were kept lyophilized at room temperature.

Culture conditions. Spleen cells, Peyer's patches cells or thymocytes were cultured in flat bottom microtitres plates (Greiner 655 180) with 4×10^5 cells in 0·2 ml or in plastic tubes (2003F Falcon Plastics, Oxnard, CA) with 2×10^6 cells in 1 ml. The culture medium (CM) was made of RPMI 1640 (Biomérieux, Lyon, France) supplemented with 1% glutamine, antibiotics (Vancomycin 20 μg/ml, Getamycin 20 μg/ml) and 2% serum-free medium (ADCM 8503, Centre de Transfusion Sanguine, Lyon, France). Cultures were incubated at 37°C in a humidified atmosphere of 7% CO_2 for various periods of time.

^{3}H-thymidine assay. Twenty-four hours before the end of the culture, ^{3}H-thymidine (THM 79A, CEA, Saclay, France) was added (2 μCi/well, sp. act. 1 Ci/mM). Cells were collected on glass fibre filters using a Mash II harvester. Radioactivity was measured in a Packard scintillation counter. Data were expressed as arithmetical means of triplicate cultures.

Depletion of adherent cells. Spleen cells were incubated in RPMI 1640 supplemented with 20% heat-inactivated fetal calf serum (FCS, Seromed, GmbH, Munich, FRG) for 90 min, at 37°C, in 7% CO_2 in a Falcon plastic dish.

After washing, the cells were incubated once more for 60 min at 37°C. Non-adherent cells were then recovered. Such suspensions contained less than 1% macrophages as assessed by May-Grünwald-Giemsa staining.

B cell enrichment. The 'panning' method of Wysocki & Sato (1978) was used. Spleen cells were incubated in plastic Petri dishes coated with a mixture of goat anti-mouse IgG, IgA, IgM (Zymed Laboratories, San Francisco, CA) at 1 μg/ml in 2·5 mM Veronal buffer, pH 8·6. After removal of non-adherent cells, adherent cells were recovered with a rubber policeman after addition of RPMI containing 5% FCS. These adherent cells comprised at least 80% S-Ig positive cells and less than 1% Thy1.2 positive cells. In other experiments spleen cells were depleted of Thy1.2 positive cells by complement dependent lymphocytotoxicity. Spleen cells (10^7) were incubated with a 1:300 dilution of anti-Thy 1.2 monoclonal antibody (Cedarlane Laboratories, Hornby, Ontario) for 60 min at 4°C. The cells were treated with 1:6 low tox M rabbit complement (Cedarlane Laboratories) for 60 min at 37°C, then spun over Lympholyte M cell separation medium (Cedarlane Laboratories) to eliminate dead cells. Those cell suspensions contained an average of 68% S-Ig positive and less than 3% Thy1.2 positive cells. Absence of T cell contamination was checked by ^{3}H-thymidine incorporation after phytohaemagglutinin (PHA) stimulation (PHA, HA 15, 90 μg/ml Wellcome Laboratories, Dartford, England).

Quantification of immunoglobulins (Ig). The amount of Ig in 5 day culture supernatants was quantified by a sandwich type ELISA. Briefly, microtitre plates (Immunoplates, Nunc, Copenhagen, Denmark) were coated overnight at 4°C with 100 μl of 1:500 dilutions of polyclonal goat anti-mouse IgG or anti-IgA or anti-IgM (Zymed Laboratories), in pH 8·6, 25 mM Veronal buffer. After three washings, 100 μl serial dilutions of mouse Ig in Veronal-buffered saline, Tween 0·1%,

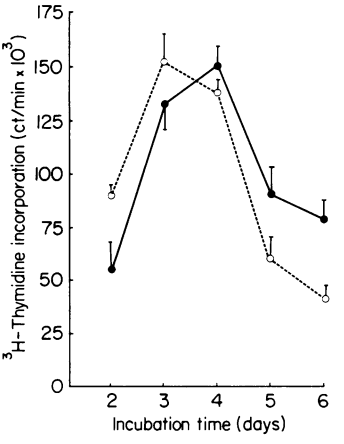

Fig. 1. Mitogenic effects of Kp MPG at 100 μg/ml on BALB/c spleen cells (○) and Peyer's patches cells (●): comparative kinetic studies.

bovine serum albumin 1%, were added to the plates to build the calibration curve. Dilutions of culture supernatants were added to triplicate wells. After 1 h at 37°C, and three washings, 100 μl of diluted specific alkaline phosphatase conjugated anti-IgA, anti-IgM and anti-IgG (Zymed Laboratories) were added. To avoid cross-reactions, normal goat serum (1%) (Cappel, Cochranville, PA) was added to the conjugated anti-serum. After 1 h at 37°C, the plates were washed five times and 100 μl of *p*-nitrophenyl phosphate (PNPP-Sigma, Saint-Louis, MO) at 2 mg/ml, in AMP buffer (Sigma) were added to each well. Microplates were incubated 1 h at 37°C and optical densities were measured at 405 nm, using a Kontron SLT 210 reader. Optical densities from diluted culture supernatants were converted into ng/ml. The mean variation between triplicates was less than 3% of the optical densities. The interassay coefficient of variation for IgA, IgG, IgM was less than 8%. The same method was used to determine IgG subclass concentration using IgG3, IgG2a and IgG2b subclass specific antisera (Nordic Immunological Laboratories, Tilburg, Netherlands).

Cytofluorometry analysis of Kp MPG stimulated cells. Lymphocyte suspensions (1×10^6 by aliquots) were incubated for 30 min at 0°C with fluorescent isothiocyanate conjugated (FITC) goat anti-mouse Ig or FITC anti-Thy1.2. After final washings, the cell suspension was run through a Cytofluorograf 50H (Ortho Instruments, Westwood, MA). Blast cells were identified by their light scattering properties on the cytogram (right and forward angles scatter) and the percentage of fluorescent cells was determined.

Interleukin 2 production and assay. BALB/c spleen cells (5×10^6/ml) were incubated in CM supplemented with 2% heat-inactivated FCS at 37°C, 7% CO_2, for 24 h with Kp MPG (1 to 100 μg/ml), with or without Concanavalin A (Con A, 5 μg/ml, IBF, Villeneuve La Garenne, France). The

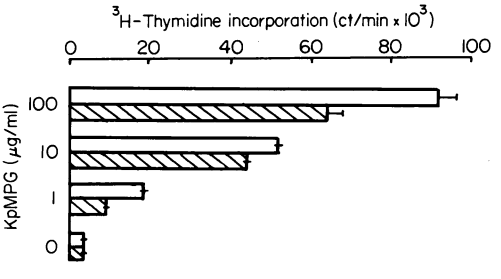

Fig. 2. Mitogenic effects of various concentrations of Kp MPG on BALB/c mice spleen cells evaluated by ^{3}H-thymidine incorporation (□) 4 days; (■) 5 days.

Table 1. Ig production by nude and BALB/c spleen cells stimulated with Kp MPG or LPS

Strains		Polyclonal activators	No expts	IgM*	IgG	IgA
Nude	Unfractionated	None	2	2,400 (1,800–3,000)	190 (118–262)	31 (30–33)
	spleen cells	Kp MPG 100 μg/ml	2	8,538 (4,076–13,000)	608 (294–921)	40 (30–50)
BALB/c	Unfractionated	None	5	724 (126–1,700)	275 (87–600)	1,021 (90–2,000)
	spleen cells	Kp MPG 100 μg/ml	7	10,411 (1,200–26,880)	4,184 (389–23,789)	2,879 (450–6,741)
		Kp MPG 10 μg/ml	3	21,655 (2,578–50,000)	12,365 (643–33,700)	2,409 (465–4,919)
		Kp MPG 1 μg/ml	3	16,466 (2,555–29,184)	6,172 (657–17,180)	1,979 (480–4,243)
		LPS 100 μg/ml	5	32,970 (12,414–50,000)	5,416 (637–20,617)	2,716 (390–3,651)
		LPS 10 μg/ml	2	11,880 (11,520–28,320)	1,000 (960–1,040)	1,998 (1,788–2,208)
	B enriched	None	1	340	209	1,029
	cells†	Kp MPG 100 μg/ml	1	16,000	401	4,702
	Thy 1.2-depleted	None	2	140 (109–170)	127 (118–136)	1,359 (1,018–1,700)
	cells‡	Kp MPG 100 μg/ml	2	3,807 (2,605–5,009)	572 (412–732)	5,123 (4,395–5,851)
	Adherent-depleted	None	1	479	267	306
	cells§	Kp MPG 100 μg/ml	1	22,325	1,134	3,168

* Arithmetical mean of Ig concentration in ng/ml as measured by ELISA in culture supernatants (range in parentheses).

† B-enriched cells obtained after panning on anti-Ig coated plastic dish.

‡ B cells obtained after depletion of Thy 1.2 positive cells by complement dependent lysis.

§ Spleen cells were depleted of adherent cells after two cycles of adherence on plastic dish.

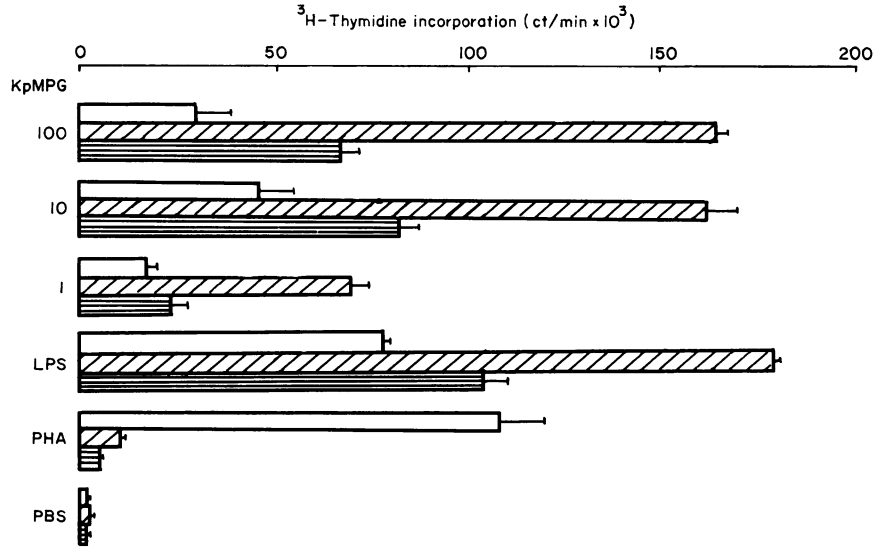

Fig. 3. Mitogenic effects of Kp-MPG at 1 to 100 μg/ml after a 72 h culture of crude BALB/c spleen cells (□), purified B spleen cells after panning with anti-Ig (■) or Thy1.2 depleted spleen cells (■).

interleukin 2 (IL-2) activity of supernatants was determined by using the IL-2-dependent CTLL2 cell line according to the method of Gillis *et al.* (1978).

Interleukin 1 production and assay. BALB/c spleen cells (5×10^6/ml) were incubated on plastic surfaces in flat-bottomed 24-well plates (Falcon, Oxnard, CA) for 2 h at 37°C in a 7% CO_2 humidified atmosphere. After four washings with RPMI 1640, adherent cells were incubated with Kp MPG (1-100 μg/ml) or *E. coli* $O_{111}B_4$ LPS (100 μg/ml, Difco, Detroit, MI) in RPMI 5% FCS medium for 24 h to 48 h at 37°C in a 7% CO_2 humidified atmosphere. The assay for IL-1 activity was performed according to the method of Mizel & Mizel (1981). One million thymocytes from 3–6-week-old C3H/HeJ mice were put in flat-bottomed microplate wells. Supernatants to be assayed for IL-1 activity were added in CM with 5% FCS. The final volume of 200 μl contained Con A 0·5 μg/ml. Thymocyte proliferation was determined by ^{3}H-thymidine uptake during the last 12 h of a 72 h culture period.

RESULTS

Polyclonal B cell activation by Kp MPG. Spleen cells from BALB/c, C3H/HeJ and CBA/J mice incubated for 1 to 6 days with Kp MPG 100 μg/ml, were stimulated to undergo blastogenesis. In BALB/c mice ^{3}H-thymidine incorporation was maximal at day 3 (Fig. 1). It was dose-dependent, as shown with doses from 1 to 100 μg/ml (Fig. 2). The same dose response curve was observed with CBA/J spleen cells (data not shown). Spleen cells from LPS unresponsive C3H/HeJ mice were strongly stimulated by Kp MPG at 100 μg/ml (data not shown). Peyer's patches cells from BALB/c mice were also stimulated by Kp MPG at optimal concentration but the kinetics of response was slightly delayed (Fig. 1).

BALB/c spleen cells produced IgM, IgG and IgA when stimulated with Kp MPG. As measured after 5 days of culture, Ig concentrations were elevated in supernatants of cultures stimulated with 1, 10 or 100 μg/ml (Table 1). IgM was the predominant class. Of note is that IgG3 was found in the same proportion after Kp MPG stimulation as in cultures stimulated with *E. coli* LPS (data not shown).

Flow cytometry analysis showed that S-Ig positive B cell blasts accounted for 7% of all splenocytes in unstimulated 5-day-cultures and 24% in culture with 100 μg/ml Kp MPG.

Conversely, T cell blasts (Thy1.2 positive) did not increase under Kp MPG stimulation (Table 2 top).

Lack of T cell stimulation and IL-2 production under Kp MPG activation. Incubation of thymocytes from BALB/c mice with Kp MPG at various concentrations (1 to 100 μg/ml) did not induce an increase in ^{3}H-thymidine uptake, although a good response to Con A (1 μg/ml) was obtained with the same cells (data not shown). Whatever its concentration, from 1 to 100 μg/ml, Kp MPG did not stimulate IL-2 production by spleen cells. Furthermore, IL-2 production by BALB/c spleen cells stimulated by Con A at optimal concentrations (5 μg/ml) was not altered by Kp MPG. Finally, Kp MPG had no IL-2 like effects and did not interfere with the IL-2 dependent proliferation of CTLL2 at 1 to 100 μg/ml (data not shown).

T-independent B cell activation by Kp MPG. The possible role of mature T cells in the activation of B cells by Kp MPG was investigated using adult nu$^+$/nu$^+$ spleen cell suspensions. Nu$^+$/nu$^+$ spleen cells showed a strong proliferative response to Kp MPG at 100, 10 and 1 μg/ml (99855 ± 8868 ct/min, 140626 ± 2193 ct/min, 82157 ± 6934 ct/min, respectively). Thymidine incorporation by nu$^+$/nu$^+$ spleen cells was higher than that observed with BALB/c spleen cells (71071 ± 2742 ct/min and 23881 ± 2361 ct/min at 10 and 1 μg/ml, respectively). At 100 μg/ml, the level of IgM production by nu$^+$/nu$^+$ cells was comparable to that of BALB/c cells, whereas IgG was lower and IgA undetectable (Table 1). In order to ascertain whether polyclonal B cell activation by Kp MPG required the cooperation of T cells, B cell suspensions were prepared according to two different methods: 'panning' with anti-Ig or Thy1·2 positive cell depletion. In both cases, B cells were stimulated by Kp MPG as shown by ^{3}H-thymidine incorporation (Fig. 3) and Ig synthesis (Table 1). However, positively selected B cells obtained by 'panning' had a much higher proliferative response to Kp MPG than unfractionated or T cell depleted spleen cell suspensions (Fig. 3) but their production of Ig was not greater than that of unfractionated spleen cells. ^{3}H-Thymidine uptake by residual T cells under stimulation by PHA at optimal concentration was negligible (Fig. 3). Furthermore the few residual T cells present in B-enriched cell suspensions were not stimulated by Kp MPG as shown by cytofluorometric analysis of Thy 1.2 positive blast cells (Table 2 bottom). Depletion of adherent cells did not decrease the proliferative response of spleen cells to Kp MPG (47,000 ± 4,000 versus 40,000 ± 10,000), 72 h culture, at 100 μg Kp MPG/ml. With respect to Ig production, removal of adherent cells resulted in an enhanced IgM response (Table 1).

Induction of IL-1 production by Kp MPG. Kp MPG was able to induce splenic adherent cells to produce IL-1 activity. The effect was maximal at 100 μg/ml, but still demonstrable at 10 or 1 μg/ml (Fig. 4). The same magnitude of IL-1 activity was induced with Kp MPG or with *E. coli* LPS at 100 μg/ml.

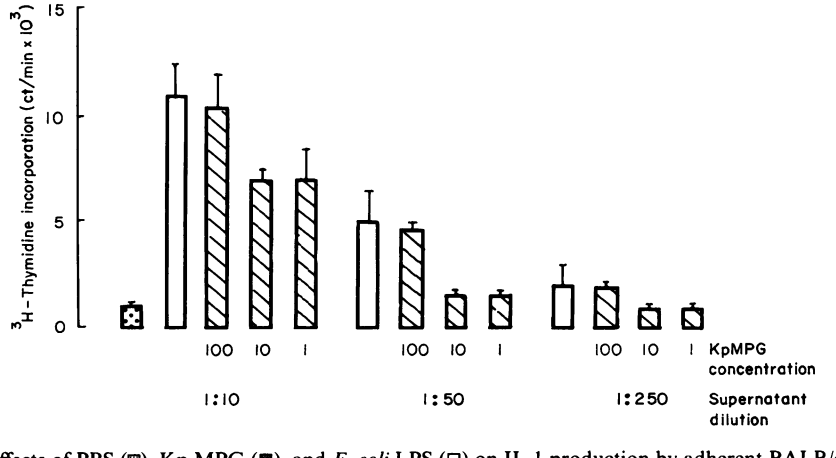

Fig. 4. Effects of PBS (▨), Kp MPG (■), and *E. coli* LPS (□) on IL-1 production by adherent BALB/c spleen cells. IL-1 activity was assayed by ^{3}H-thymidine incorporation into Con A (0·5 μg/ml) co-stimulated C3H/HeJ thymocytes.

Table 2. Flow cytometry analysis of blast cells induced by Kp MPG, LPS and Con A

Cells	Polyclonal activator	Blasts*		Thy1.2 positive blast cells†		S-Ig positive blast cells†	
		day 3	day 5	day 3	day 5	day 3	day 5
Unfractionated spleen cells	None	13	17	7	7	8	7
	Kp MPG (100 μg/ml)	26	50	8	6	21	24
	LPS (100 μg/ml)	20	39	10	6	16	34
	Con A (5 μg/ml)	36	24	10	15	29	21
B cells‡	None	7	6	3	2	7	6
	Kp MPG (100 μg/ml)	35	25	3	3	35	25
	LPS (100 μg/ml)	22	21	3	2	22	21
	Con A (2 μg/ml)	15	ND	5	ND	14	ND

* Percentage of blast cells, as defined by their light scattering properties, among all cells recovered at day 3 or 5 of culture.

† Percentage of stained blast cells, stained with anti-Thy1.2 antibody or with anti-mouse Ig antibody, among all cells recovered at day 3 or 5 of culture.

‡ B cells purified by 'panning' on anti-Ig coated plastic dish.

ND not done.

DISCUSSION

The present study shows that Kp MPG induces blastogenesis and proliferation of murine spleen cells and Peyer's patches cells, with a clearcut dose response effect from 1 to 100 μg/ml. Kp MPG does not seem to activate T cells because of the following evidence: (i) it did not stimulate ^{3}H-thymidine incorporation into thymocytes; (ii) as defined by their light scattering properties, all Kp MPG-induced blast cells were Thy1.2 negative, and (iii) Kp MPG did not stimulate IL-2 production and did not potentiate Con A-induced IL-2 production.

Using a sensitive ELISA technique, we checked that sera from BALB/c mice did not contain detectable antibodies against Kp MPG. Thus Kp MPG is unlikely to trigger a secondary response *in vitro* but should be regarded as a potent non specific polyclonal B cell activator in view of its capacity to stimulate the generation of S-Ig positive blast cells and the secretion of Ig. These activities seem to require the cooperation of very few or no mature T cells since nude spleen cells and T-depleted BALB/c spleen cells were induced to proliferate better than unfractionated spleen cell suspensions. Further indirect evidence for the T-independent nature of polyclonal B cell activation by Kp MPG was shown by the distribution of Ig classes and IgG subclasses in BALB/c culture supernatants. The predominance of IgM and the relatively low levels of IgA and IgG, as well as the production of relatively high amounts of IgG3, are typical of T-independent polyclonal B cell activators (McKearn *et al.*, 1982). Furthermore Kp MPG-induced IgG synthesis was still lower with nude than with BALB/c spleen cells and that of IgA was not detectable. Since nude mice lack IgA B cells whose differentiation is T-dependent (Elson, 1985), the relative concentrations of each of the three major Ig classes in Kp MPG stimulated cultures are likely to reflect the distribution of S-Ig isotypes among spleen B cells. Kp MPG seems to stimulate mostly resting S-IgM positive B cells. It is unlikely to trigger isotype switching *in vitro*.

The T-independent B cell activating properties of Kp MPG are similar to those of *E. coli* LPS (McKearn *et al.*, 1982). However they cannot be accounted for by LPS contamination of the Kp MPG, as shown by the dose response curves of ^{3}H-thymidine incorporation (data not shown). Moreover, C3H/HeJ mice which bear a defective *lps* gene and show a low response to *E. coli* LPS, were strongly responsive to Kp MPG. Therefore Kp MPG and LPS should be regarded as two distinct polyclonal B cell activators.

A remarkable property of Kp MPG, shared with LPS and several other bacterial components, is its capacity to provide competence and progression signals for cell division as well as signals for Ig secretion, without the need for T helper cells. The present data show that the positive B cell selection which involved transient cross-linking of S-Ig resulted in greater proliferation upon further culture with Kp MPG than did removal of T cells. Since anti-IgM antibodies only provide a competence signal (Defranco *et al.*, 1982), the data suggest that activation by Kp MPG could induce the release of mediators which allow anti-Ig primed B cells to progress into cell division. A likely candidate for this activity is IL-1 (Howard & Paul, 1983). Indeed, Kp MPG triggered IL-1 production by adherent cells as strongly as did LPS. However, removal of most adherent cells did not reduce but rather enhanced B cell activation by Kp MPG. These data may suggest that fewer than 1% macrophages can provide sufficient IL-1 activity or, alternatively, that Kp MPG triggers a macrophage independent B cell activation pathway. Further studies now in progress, using fractions of Kp MPG, will attempt to correlate these biological properties with defined molecular and submolecular structures.

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