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Polyclonal B-cell activation by a synthetic analogue of bacterial lipoprotein is functionally different from activation by bacterial lipopolysaccharide

B. KLEINE,*§ R. SPRENGER,† C. MARTINEZ-ALONSO‡ & W. G. BESSLER*† **Institut für Immunbiologie, University of Freiburg, Freiburg, †Arbeitsbereich Mikrobiologie und Immunologie, University of Tübingen, Tübingen, West Germany, ‡Clinica Puerta de Hierro, Madrid, Spain, and § Unité d'Immunobiologie, Institut Pasteur, Paris, France*

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SUMMARY

The reactivity of 38 murine strains to a synthetic analogue of bacterial lipoprotein, tripalmitoyl-pentapeptide (TPP), was tested and compared with the reactivity to lipopolysaccharide (LPS). These strains include common laboratory mice and H-2 recombinant inbred lines, as well as some newly bred lines originating from animals recently captured in different regions of Europe. All animals analysed were reactive to TPP and polyclonally activated to proliferation and immunoglobulin synthesis. Large differences in mitogen reactivities of various H-2 recombinant inbred strains suggest that MHC or closely linked gene products influence the reactivity to the LPS and TPP mitogens. By analysing the frequences of precursor cells reactive to TPP or LPS and the isotype patterns obtained after stimulation, we demonstrated that both mitogens activate individual B cells in different ways.

INTRODUCTION

Mitogens from plant or bacterial origin have facilitated the analysis of lymphocyte activation as they induce large subpopulations of either B or T cells (for review see Möller, 1972; Oppenheim & Rosenstreich, 1976). The interaction of these mitogens with eukaryotic cells could occur via common determinants on clonally distributed specific receptors, as has been shown for T cells and the corresponding mitogen concanavalin A (Larsson & Coutinho, 1979; Larsson *et al.*, 1982). In the case of B cells triggered by bacterial cell wall components like lipopolysaccharide (LPS) or lipoprotein, activation is not mediated by the immunoglobulin receptor (Coutinho & Möller, 1974) but by other surface structures yet to be identified.

Our attempts to analyse B-cell activation by lipoprotein and the relevant structures in the B-lymphocyte membrane, e.g. specific receptors for the mitogen, are hampered by the lack of lipoprotein non-responder mice. Since genetic variants have been very helpful in elucidating the activation by LPS (Watson & Riblet, 1974; Coutinho & Meo, 1978; Forni & Coutinho, 1978), we started a survey on the qualitative and quantitative response of a great number of inbred murine strains to lipoprotein. This is justified, too, by the availability of a newly synthesized analogue of bacterial lipoprotein, tripalmitoyl-pentapeptide (TPP) (Wiesmüller, Bessler & Jung, 1983), which acts indistinguishably from the native lipoprotein (Bessler *et al.*, 1982). This synthetic product is free of any contaminating LPS, which might have influenced previous biological experiments with native lipoprotein.

The present article describes the TPP and LPS reaction patterns of 38 inbred murine strains. Splenic lymphocytes were tested for proliferation after mitogenic activation by TPP and LPS. We also describe the induction of terminal maturation of B cells into plasma cells from several strains as a result of TPP action, tested in bulk cultures as well as under limiting dilution conditions. The results suggest that reactivity to lipoprotein is independent of reactivity to LPS, and that genes of the MHC locus play a role in determining the magnitude of the responses.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline sulphonic acid); FCS, fetal calf serum; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Ig, immunoglobulin; LPS, lipopolysaccharide; MEM, minimal essential medium; PFC, plaque-forming cells; TdR, thymidine; TPP, tripalmitoyl-pentapeptide = S-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-*N*-palmitoyl-(R)-cysteinyl-(S)-seryl-(S)-seryl-(S)asparaginyl-(S)-alanine.

Correspondence: Dr B. Kleine, Institut für Immunbiologie, Stefan-Meier Str. 8, D-7800 Freiburg, West Germany.

MATERIALS AND METHODS

Animals

Mice of both sexes were kindly provided by B. Kindred and J. Klein, Max-Planck Institut für Biologie, Tübingen, and by J.-L. Guenet, Institut Pasteur, Paris. They were used between 6 weeks and 15 weeks of age. Four-week-old Sprague–Dawley rats were provided by Ivanovas, Kisslegg, FRG.

Mitogens

Tripalmitoyl-pentapeptide (TPP), synthetically prepared (Wies-

müller *et al.*, 1983), was suspended in minimal essential medium (MEM, Flow Labs, Irvine, Ayrshire, U.K.). Triton X-100 (Merck, Darmstadt, FRG) was added from a 10% stock solution in distilled water to a molar ratio of 100 mols TPP/1 mol Triton X-100. After heating to 70°, the solution was sonicated, passed through a 0·22 μm sterile Millipore filter and stored at −20°. LPS from *S. abortus equi* (Difco, Detroit, MI) was suspended in MEM, sonicated, filtered, and stored at −20°.

Culture conditions

Splenic cell suspensions, aseptically prepared and washed twice with MEM, were cultured in RPMI-1640 medium supplemented with 3 mM glutamine, 25 mM HEPES, 0·05 mM 2-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. Fetal calf serum (FCS) was added to 5%. The FCS batch (Flow, 29063101) was tested for low background activity and optimal support of mitogenic activation by LPS or concanavalin A. Proliferation was measured by the uptake of 23 KBq (0·6 μCi) [^{3}H]thymidine during the last 4 hr of culture. Isotypes of the secreted immunoglobulins were determined in a modified protein A plaque assay with rabbit anti-mouse Ig subclass-specific antisera as described elsewhere (Bernabé *et al.*, 1981; Coutinho *et al.*, 1982).

Limiting dilution analysis

Graded numbers of spleen cells were added to 600 mGy (60 rads) irradiated rat thymus cells (660×10^3 per 0·2 ml culture). For each determination 48 replicate cultures were seeded. The background control without murine spleen cells was performed with 96 replicates. The mitogens were added at 25 μg/ml LPS and 6 μg/ml TPP. The IgM content in the supernatant was evaluated in a solid-phase ELISA assay using rabbit anti-mouse Ig (Behring, Marburg, FRG) to coat polystyrene microtitre plates and peroxidase-coupled rabbit anti-mouse IgM (Jackson, Bar Harbour, ME) to develop the binding by the reaction with the ABTS substrate. Positives were scored when the extinction was above the average value of the control cultures plus three times the standard deviation.

RESULTS

Induction of proliferation

In Table 1 the data on TdR incorporation after stimulation with TPP and LPS for 38 different murine strains are listed. Data from conventional laboratory mice are shown in the first group. The second group consists of recombinant inbred strains carrying H-2 alleles of established origin. The third group of mice includes recombinant inbred lines with H-2 alleles from newly captured wild mice bred into a common laboratory background. We also tested mice that, after having been captured in different parts of Europe, could be successfully maintained (fourth group). Some of these latter mice are already inbred by normal criteria (Potter, 1986).

The dose–response curve showed broad peak responses between 0·5 μg and 20 μg TPP/ml in DBA/2 and BALB/c and between 1 μg and 50 μg LPS/ml (data not shown). Thymidine incorporation was determined for at least two time-points, usually Day 2 and Day 3 of culture. For the sake of clarity we depict only the Day 2 response, which was always characteristic for the reactivity of the individual strain.

The experiments were repeated for several strains: C57BL/6, C57BL/10.Sc.Cr., A.CA, B10.RIII, A.SW, B10.T7WF, B10.WSLP and TO2. These duplicate experiments demonstrated reproducibility of the results within the limits of the TdR-uptake measurements.

High background incorporation in the fourth group of mice (nos. 33–38) may be due to the fact that these mice are not yet kept under pathogen-free conditions and could therefore reflect some degree of preactivation of the immune system in these animals.

All the mice analysed were responsive to TPP. With the exception of the known LPS non-responder strain C57BL/10.Sc.Cr (Coutinho & Meo, 1978), they were also stimulated by LPS. We found at least a three- to four-fold activation over background with a few exceptions: in CBA/J and in PWK background incorporations were already high, and B10.WSLP was poorly responsive to TPP (1·7-fold of background), being the lowest LPS responder as well. The proliferative responses to LPS and TPP differed in most of the strains analysed: in all strains of A and C57BL background, except B10.GAA20, LPS responses were higher than TPP responses. Of the other strains, four showed a similar activation by LPS and TPP, five were better stimulated by LPS, and four strains were more efficiently activated by TPP.

Comparing the congenic mice with different MHC haplotypes on the same C57BL/10 (B10) background, we observed large variations in stimulation indices (SI) ranging from four-fold (no. 8) to 48-fold (no. 20) for the LPS response and from 1·7- to 20-fold (no. 21 resp. no. 20) for the TPP responses. The differences between the highest and the lowest responses were five-fold for the LPS and ten-fold for the TPP-induced stimulation. The lowest TdR uptake after TPP activation amounted to $5·1 \times 10^3$ c.p.m., and after LPS activation to $21·2 \times 10^3$ c.p.m. In r, z, w3, w17, w21 and w26 haplotypes the LPS induced TdR uptake was more than three times higher than the TPP-induced uptake. As the only reported differences for these mice are MHC or closely linked gene products, these seem to influence the reactivity to the two mitogens.

Induction of immunoglobulin synthesis

TPP was also tested for the induction of Ig synthesis. Earlier experiments from our group had demonstrated induction of anti-TNP and anti-SRBC antibodies by synthetic analogues of lipoprotein (Johnson *et al.*, 1983). In the studies presented here we tested Ig synthesis in wild mice (Table 2). The activation by TPP of spleen cells from the newly derived lines revealed a strong (LPS-like) induction of IgM synthesis after 3 days of culture for four out of six strains tested. More IgM plaques were induced with TPP than with LPS in SPE, MAI, MYL and MBT. We had previously titrated TPP in an anti-TNP plaque assay to determine the optimal TPP concentration (Bessler *et al.*, 1985). Peak responses, obtained with 10 μg/ml TPP, were not as broad as in the proliferation assay. The concentrations used in Table 2 span the range of the optimal concentration. The strong PFC induction in four out of six strains parallels a equally strong proliferative response. PWK, with the lowest stimulation of TdR uptake, also showed the lowest PFC induction. The low numbers of IgM PFC in this strain were confirmed in a replicate experiment. Low numbers of IgM PFC in SPE in contrast to a normal TdR uptake are consistent with other reported variations of immunoglobulin synthesis in this strain (Amor *et al.*, 1984).

Table 1. Proliferative responses to LPS and TPP

Strain	H-2 haplotype*	Stimulation index LPS	Stimulation index TPP	Background (c.p.m. × 10^{-3})			
1. A	a	11·0†	8.0	(2·8)	LPS > TPP		
2. BALB/c	d	4·0	7·3	(7·6)		TPP > LPS	
3. C57BL/6	b	8·8	6·1	(9·6)	LPS > TPP		
		10·7	6·0	(4·8)	LPS > TPP		
4. C57BL/10Sc. Cr	b	1·2	6·8	(1·8)		TPP»LPS‡	
		1·5	35·0	(0·4)			
5. CBA/J	k	-2·8	1·7	(22·0)	LPS > TPP		
6. DBA/2	d	12·5	13·0	(2·2)			TPP ≥ LPS
7. A.CA	f	12·2	8·4	(5·1)	LPS > TPP		
8. B10.WB	j	8·3	4·1	(6·5)	LPS > TPP		
9. B10.P	p	9·4	6·4	(9·1)	LPS > TPP		
10. BQ2	q	18·0	10·9	(3·1)	LPS > TPP		
11. B10.RIII	r	20·1	3·2	(4·1)	LPS»TPP		
12. A.SW	s	9·6	4·8	(4·8)	LPS > TPP		
13. B10.PL	u	18·9	6·5	(2·0)	LPS > TPP		
14. B10.SM	v	9·7	7·2	(2·1)	LPS > TPP		
15. B10.NSW	z	11·8	4·1	(5·5)	LPS»TPP		
16. B10.KPA42	w1	18·7	8·3	(2·6)	LPS > TPP		
17. B10.SAA48	w3	14·1	3·4	(1·5)	LPS»TPP		
18. B10.GAA20	w4	9·5	9·2	(4·3)			LPS = TPP
19. B10.KEA5	w5	13·8	10·8	(3·3)	LPS > TPP		
20. B10.T7WF	w6	48·0	20·7	(1·3)	LPS > TPP		
21. B10.WSLP	w7	4·6	1·7	(6·3)	LPS > TPP		
		6·2	3·8	(4·4)			
22. B10.STC90	w15	20·8	13·8	(2·7)	LPS > TPP		
23. B10.BUA1	w16	11·1	5·0	(1·9)	LPS > TPP		
24. B10.CAS2	w17	23·3	3·3	(2·9)	LPS»TPP		
25. B10.KPB128	w19	28.3	7·5	(3·6)	LPS > TPP		
26. B10.GAA37	w21	18·7	4·4	(1·5)	LPS»TPP		
27. B10.CAS1	w23	9·6	5·8	(5·6)	LPS > TPP		
28. B10.CHA2	w26	15·0	3·8	(6·7)	LPS»TPP		
29. B10.STA62	w27	10·1	6·8	(7·5)	LPS > TPP		
30. STU	w34	10·4	12·8	(2·8)		TPP > LPS	
31. TO2	w35	4·4	4·0	(6·6)			LPS ≥ TPP
32. TO1	w39	11·8	3·4	(2·6)	LPS > TPP		
33. WLA76/Pas§	?	10·1	6·5	(17·3)	LPS > TPP		
34. SPE/Pas	?	4·3	5·8	(14·8)		TPP > LPS	
35. MAI/Pas	?	4·6	4·8	(23·5)			TPP ≥ LPS
36. MYL/Pas	?	7·9	9·3	(13·7)		TPP > LPS	
37. MBT/Pas	?	10·4	7·4	(13·5)	LPS > TPP		
38. PWK/Pas	?	3·7	2·5	(10·2)	LPS > TPP		

* Haplotype designation due to Klein, Figueroa & Klein (1983).

† 10^5 spleen cells were incubated in the presence of 5 μg/ml lipopolysaccharide or 0·8 μg/ml TPP in 0·2 ml triplicate cultures for 2 days. 0·625 μCi [^{3}H]thymidine (TdR) was added for the last 4 hr of culture. Stimulation indices were calculated by dividing the counts incorporated in the presence of mitogen by those of control cultures without mitogen (background c.p.m., given in parentheses).

‡ LPS non-responder strain.

§ These mice belong to different subgroups of the species *Mus*: WLA/76 is of the *Mus* 1 group (*Mus musculus domesticus*), MAI, PWK, MYL, and MBT are of the *Mus* 2 group (*Mus musculus musculus*), and SPE is of the *Mus* 3 group (*Mus spretus*) (Potter, 1986). Their MHC haplotype has not yet been established.

Table 2. Immunoglobulin synthesis of wild mice-derived new strains

Strain	Mitogen	Conc. (μg/ml)	IgM PFC* ($\times 10^{-3}$) on Day 3	On Day 6 IgG3	IgG1	IgA
33.† WLA76/Pas	LPS	100	25·3	132	183	900
		10	13·0	78	664	582
	TPP	25	13·6	69	63	318
		6·2	21·1	42	48	165
	None		0·45	18	12	33
34. SPE/Pas	LPS	100	0·80	105	178	16
		10	0·56	107	210	ND‡
	TPP	25	0·60	61	105	63
		6·2	1·92	146	140	54
	None		0·17	11	54	10
35. MAI/Pas	LPS	100	5·25	188	92	4
		10	8·24	878	264	4
	TPP	12·5	20·7	250	8	24
		2·5	9·14	1081	6	8
	None		1·05	2	1	3
36. MYL/Pas	LPS	100	19·1	448	244	2
		10	14·4	185	94	6
	TPP	12·5	23·7	142	10	4
		2·5	29·9	326	0	0
	None		0·45	20	4	4
37. MBT/Pas	LPS	50	18·7		ND	
		5	14·7			
	TPP	12·5	24·9			
		1·2	13·0			
	None	1·33				
38. PWK/Pas	LPS	50	3·57 (3·40)§		ND	
		5	5·65 (8·99)			
	TPP	12·5	4·03 (0·94)			
		1·2	1·74 (0·22)			
	None		0·77 (0·12)			

* Isotype-specific PFC were determined as described in the Materials and Methods.

† Strain numbers as in Table 1.

‡ ND, not determined.

§ Data in parentheses are from a second independent experiment.

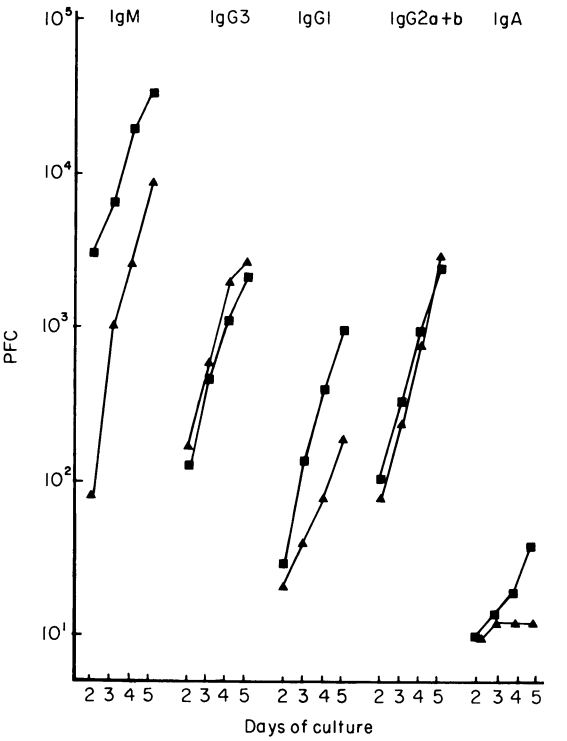

Figure 1. 10^5 spleen cells from C57BL/6 were cultured in the presence of 25 μg/ml LPS (■) or 10 μg/ml TPP (▲). Ig-secreting plaque-forming cells (PFC) were determined at the indicated days by the protein A plaques assay.

We now tested the isotypic pattern induced by TPP and LPS in C57BL/6 (B6) mice at the predetermined optimal dose of TPP (10 μg/ml) (Fig. 1). After 5 days of culture in B6, TPP induced strong IgM (9000 PFC/10^5 cells on Day 5 compared to 80 PFC on day 2), IgG3 (2600–170), and IgG2a + b (3000–85) responses, whereas the Ig1 induction was about ten-fold lower (200–20 PFC). IgA PFC numbers were very low, only 10–20 PFC per culture. Compared to LPS, the numbers of IgG2a + b and IgG3 were similar with TPP; however, IgM plaques were about four times, IgG1 PFC five times, and IgA PFC three times lower with TPP than with LPS.

The analysis of class switching in wild mice revealed some differences compared to B6. Differences in IgM responses between LPS and TPP could not be observed, the TPP response being higher in four out of six strains. However, in contrast to IgM induction, in the four strains analysed TPP led to only a marginal IgG1 induction, whereas the LPS-induced IgG1 plaques were at least two-fold, in WLA76 10-fold and in MYL even 24-fold higher than with TPP. Compared to the poor IgG1 induction, IgG3 plaque counts were as high or even slightly higher in MAI for TPP than for LPS. IgA responses could be found in two strains, WLA76 and SPE, and in WLA 76 this IgA response was higher than the IgG3 or IgG1 responses. This is in contrast to B6, where only very few IgA PFC were obtained, and to the other strains.

Limiting dilution analysis of TPP-reactive precursor frequencies

It has already been shown for some strains that the reactivity to lipoprotein as well as to LPS is specifically expressed on the level of individual cells (Andersson, Coutinho & Melchers, 1977). Therefore the reactivity to LPS and to TPP can be quantified as the frequency of reactive cells in a given population by limiting dilution analysis. We were particularly interested in the role of I-E class II antigens during the activation by TPP, because class II molecules have been shown to bind to TPP affinity columns (Scheuer, Biesert & Bessler, 1986). Therefore, we chose two combinations of congenic mice where, due to a defective Eα gene, no I-E molecule is expressed in one strain, while in the corresponding strain the Eα gene is functional and the I-E protein is expressed on the plasma membrane (Jones, Murphy & McDevitt, 1981).

Table 3 demonstrates the result of one of these experiments to determine TPP-reactive precursor frequencies. Spleen cells from B10.S(7R), B10.S(9R), A.TH, and A.TL were titrated to slightly (600 mGy = 60 rads) irradiated rat thymocytes. After 10 days of culture in the presence of TPP, we tested the superna-

Table 3. Frequency of TPP-reactive B-cell precursor cell

	Precursor frequencies with:		
	TPP	LPS	Expression of I-E
1. B10.S(9R)	1/84	1/4·3	+
2. B10.S(7R)	1/146	1/8·9	−
3. A.TL	1/74	1/86	+
4. A.TH	1/68	1/50	−

tants for the presence of murine IgM and determined the fraction of negatives from 48 replicate cultures. LPS cultures were performed in parallel. No significant differences due to the expression of I-E could be found for any mitogen. However, reactivity to LPS and TPP was differentially expressed on the single cell level: 15–25% of spleen cells reactive to LPS in the B10.S mice, whereas only about 1% showed reactivity to TPP. In the same experiment frequencies between 1·2% and 2% for LPS- and TPP-reactive cells in A.TH and A.TL mice were obtained. We conclude from these experiments that the reactivities to LPS and TPP are distinct characteristics of individual cells and not influenced by the I-E-histocompatibility antigen.

DISCUSSION

The analysis described in the present article was started to obtain tools to study moleculer events during the activation of B lymphocytes by the well-characterized synthetic mitogen TPP. The finding of a TPP non-responder mouse would have enabled us to use classical genetic studies to determine loci responsible for reactivity towards the mitogen as it has been successfully performed for LPS. However, among the 38 strains analysed here and the animals tested before (Andersson *et al.*, 1977; A. Coutinho, personal communication) no lipoprotein non-responder has been found. When tested for proliferation or final maturation induced by TPP, each strain was reactive and mounted a response that was at least two- to three-fold above the mitogen-free control. In most assays the stimulation was much higher, up to 20-fold over background.

During this study we compared 20 congenic strains of C57BL background bearing most of the identified MHC alleles. Due to the large variations in the mitogenic responses, our experiments strongly suggest influences of MHC or closely linked genes on the response to LPS and lipoprotein. The reasons for the variations are still unknown. On the one hand, the polymorphic differences of MHC antigens themselves could differentially increase affinity or avidity of mitogen-binding complexes. Such influences of MHC class I antigens on other membrane proteins have been suggested by Simonsen *et al.* (1985). Or, on the other hand, MHC-linked genes might control reactivity to LPS and TPP. An analysis of these two alternatives using congenic mice with mixed haplotypes is in progress.

A further conclusion from our experiments is that the reactivities to LPS and lipoprotein/TPP are linked neither functionally nor genetically. This evidence comes from limiting dilution studies on the LPS and TPP precursor frequencies: LPS frequencies were high in two B10.S recombinant inbred mice but low in A mice, whereas TPP frequencies were low in both combinations showing the independence of TPP and LPS reactivity on the level of individual cells.

TPP as well as lipoprotein and LPS are reported to activate B cells in a thymus-independent (TI) way (Melchers, Braun & Galanos, 1975; Bessler *et al.*, 1985). The Ig isotypes following such TI activation are described to be preferentially IgM, IgG3 and IgG2b (Coutinho *et al.*, 1982). on contrast, thymus-dependent activation induces predominantly the IgG1 and IgG2a subclasses. TPP could induce class switches to various isotypes; however, compared with LPS, it induced only low numbers of IgG1 PFC and only marginal amounts of IgA PFC. The different isotype patterns suggest that TPP acts on a different cell population or, acting on the same cell (Andersson, Coutinho & Melchers, 1979), triggers the activation and maturation in a different way. The differences might also suggest specific interactions of the two mitogens with proteins or protein complexes responsible for the late steps of B-cell activation, especially for isotype class switching. Since the B-cell stimulatory factor 1 (BSF-1) constitutes a IgG1 switching factor (Noma *et al.*, 1986), the hypothesis that LPS and TPP also function as triggering, growth and maturation signals (Forsgren *et al.*, 1984) could be extended. Thus, LPS could be able to mimic BSF-1 to induce a marked IgG1 production. Lipoprotein, on the other hand, could preferentially induce other isotypes.

Differences in the responses to LPS and lipoprotein have also been observed when other sources of lymphocytes were analysed (Freitas & Coutinho, 1980). Thoracic duct lymphocytes from C3H/Tif were twice as reactive to lipoprotein than to LPS under limiting dilution conditions, suggesting that reactivity to LPS or to lipoprotein is differentially expressed during the ontogeny of B lymphocytes. Our finding that TPP induces one-fifth of the IgG1 PFC of the LPS response and almost no IgA PFC might be interpreted in the same context. It has been shown that the LPS- and the lipoprotein-reactive splenic B-lymphocyte populations are overlapping (Andersson *et al.*, 1979), but no information is so far available for other sources of B lymphocytes. One could therefore imagine functional LPS or lipoprotein receptor molecules expressed differentially on B cells depending on the stage of differentiation and on the organ where the B cell is homing.

In a recent publication we have shown that WEHI 279.1 tumour cells, which die when cultured with LPS, TPP, or dextran sulphate, lose the reactivity to one mitogen independent of the reactivity to the other two mitogens (Kleine, Bessler & Coutinho, 1985). As with this paper, these results support the view of functional responsiveness to LPS and TPP as distinguishable entities of B lymphocytes. We are currently using the hypothesis of independence of functional TPP and LPS reactivity to analyse specific events during the activation of B lymphocytes by both mitogens.

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