INFECTION AND IMMUNITY, Mar. 1983, p. 1481–1484
0019-9567/83/031481-04$02.00/0

Vol. 39, No. 3

Polyclonal Activation of B-Lymphocytes In Vivo by *Salmonella typhimurium* Lipoprotein

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Received 2 November 1982/Accepted 1 December 1982

Lipoprotein prepared from the outer membrane of *Salmonella typhimurium* is a polyclonal activator of murine B-lymphocytes. It was shown to be mitogenic for splenic cultures, stimulating increased incorporation of [^{3}H]thymidine into DNA. When injected intravenously into mice, the lipoprotein induced splenomegaly and polyclonal B-cell activation. The latter was evident from an increase in the number of plaque-forming cells against trinitrophenylated sheep erythrocytes. Similar results were obtained with *Escherichia coli* lipoprotein.

Lipoprotein from the outer membrane of *Escherichia coli* was characterized and sequenced by Braun (8). Its polypeptide chain is composed of 58 amino acids and contains, at the N-terminal end, one amide-linked and two ester-linked fatty acids bound to glycerylcysteine. Over recent years, work in this and other laboratories has established that *E. coli* lipoprotein is mitogenic, stimulating cultures of murine B-lymphocytes into both proliferation and immunoglobulin secretion (1, 5, 13). These polyclonal responses have been observed with splenic cultures from several strains of mice, including both nude and lipopolysaccharide (LPS) nonresponder strains (5, 6).

In addition to lipoprotein, LPS (2) and proteins I and II* (3) are *E. coli* cell wall components known to possess mitogenic activity in vitro. Although LPS has been shown to induce polyclonal responses in vivo (9, 15), the biological significance of bacterial mitogens is currently unknown. However, it is tempting to suggest that at least some of these potent mitogenic bacterial surface components may influence the host-parasite relationship. Supporting this conclusion, von Jeney et al. (20) provided evidence that the resistance of several strains of mice to intraperitoneal infection with *Salmonella typhimurium* may correlate with the capacity of their spleen cells to respond to the mitogenic effects of LPS. More recently, genetic studies have suggested that the innate susceptibility of C3H/HeJ mice to murine typhoid is due to the defective allele of the endotoxin response gene locus (Lps^d) (14).

We therefore decided to determine whether lipoprotein extracted from *S. typhimurium* was mitogenic and to investigate the in vivo effects of this bacterial membrane protein on the immune system. In this paper, we report the results of studies which demonstrate that *S. typhimurium* lipoprotein is a potent polyclonal activator of murine B-lymphocytes both in vitro and in vivo.

The female mice used in this study were purchased from the following sources: BALB/c from Ivanovas, Kisslegg, Germany; C3H/HeJ from Jackson Laboratories, Bar Harbor, Me.; and C3H/Tif/Bom-nunu from Bomholtgard, Ry, Denmark. Spleen cell suspensions were prepared as described previously (6). Tissue remnants were removed by filtering the cells through cotton wool.

Lipoprotein was prepared from both *E. coli* 0111K58 and *S. typhimurium* SL761 according to the method of Inouye et al. (11). *E. coli* B/r LPS was a gift from W. Scheuer, Universität, Tübingen, Germany, and *E. coli* O55:B5 LPS was obtained from Difco Laboratories, Detroit, Mich. Concanavalin A was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The mitogens were suspended in 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered Eagle minimal essential medium and, where necessary, with the aid of sonication (60 s, 100 W; Braun Labsonic 1510 sonifier; Braun, Melsungen, Federal Republic of Germany). Mice were injected intravenously with 35 μg of mitogen in a volume of 0.2 ml of medium. Control mice were uninjected, as preliminary experiments had indicated that the medium had no effect on the mice (unpublished observation).

Lymphocyte proliferation was measured by [^{3}H]thymidine uptake, as described previously (4). Polyclonal antibody synthesis was assessed with a hemolytic plaque-forming cell (PFC) as-

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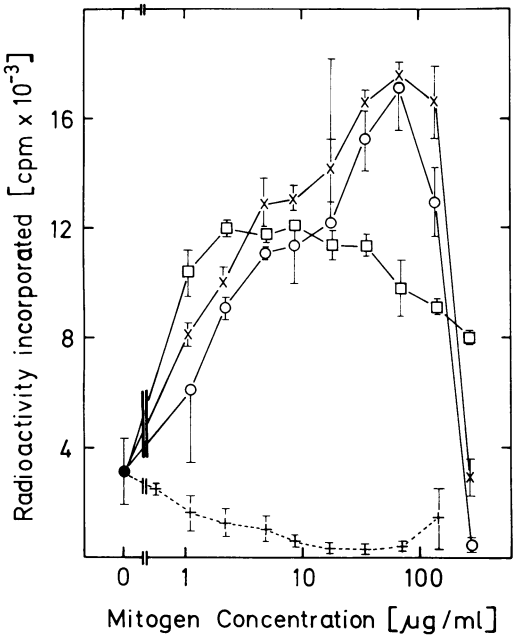

FIG. 1. Dose-response plots of [^{3}H]thymidine uptake (counts per minute per 4.2 × 10^5 cells) by C3H/Tif/Bom-nunu splenocytes after 48 h of incubation with *S. typhimurium* lipoprotein (○), *E. coli* lipoprotein (X), *E. coli* B/r LPS (□), or concanavalin A (+). Each point represents the mean ± standard error of triplicate assays.

say against trinitrophenylated sheep erythrocytes (SRBC) (4). The lymphocytes (10^7 cells per ml) were suspended in 20 mM HEPES-buffered Eagle minimal essential medium containing 0.45% glucose (glucose medium). SRBC were coated with the hapten trinitrophenol (TNP) by the method described by Rittenberg and Pratt (16). The number of PFC against TNP-SRBC or SRBC was measured by mixing 100 μl of the lymphocyte suspension, 20 μl of guinea pig serum (Serva, Heidelberg, Germany), 20 μl of a 30% suspension of the TNP-SRBC, and 300 μl of glucose medium containing 0.5% agarose at 45°C. The suspensions were spread on disposable plastic petri dishes (Greiner, Nürtingen, Federal Republic of Germany) and incubated at 37°C in a humidified atmosphere for 3 h. Plaques were scored with a plaque viewer (Tecnomara, Zürich, Switzerland).

The mitogenic effects of *S. typhimurium* lipoprotein on spleen cells from C3H/Tif/Bom-nunu mice can be seen from the dose-response plots of Fig. 1. The uptake of [^{3}H]thymidine by cultures of these cells was greatly enhanced in the presence of lipoprotein, particularly at concentrations around 50 to 180 μg/ml. However, at a concentration of 300 μg/ml, its mitogenicity had decreased noticeably. Lipoprotein and LPS prepared from *E. coli* induced similar proliferative responses. In contrast, the T-lymphocyte mitogen concanavalin A appeared to be slightly inhibitory, as the incorporation of labeled nucleotides into DNA was reduced. Thus, these data, obtained with splenocytes from congenitally athymic mice, confirmed that *S. typhimurium* lipoprotein was mitogenic for lymphocytes of the B-cell lineage. Both preparations of bacterial lipoprotein were also able to stimulate spleen cells from C3H/HeJ mice (unpublished observation). As this strain is genetically nonresponsive to LPS (17), it is unlikely that contaminating endotoxin is responsible for these proliferative effects. Interestingly, the *S. typhimurium* lipoprotein is only weakly mitogenic toward human peripheral blood lymphocytes (unpublished observation). Although similar species-restricted responses have also been reported for *E. coli* lipoprotein (5) and LPS (7), the basis for this variation is poorly understood.

Earlier studies have shown that LPS, when administered to mice, induces splenomegaly and cellular proliferation (15, 18, 19). The injection of *S. typhimurium* lipoprotein into BALB/c mice produced similar effects, as the spleens of the lipoprotein-treated mice had, by day 3, almost doubled in weight (Table 1). This gross morphological change was accompanied by an enhanced recovery of nucleated cells (unpublished results) and is presumably also due to increased lymphocyte proliferation within this organ. In addition to splenomegaly, lipoprotein was found to stimulate the splenic B-lymphocytes into immunoglobulin secretion (Table 1). The polyclonal nature of this activation was evident from a 10-fold increase in the number of PFC against TNP-SRBC. The number of PFC against unsensitized SRBC increased about fourfold.

TABLE 1. Comparison of the properties of spleen cells excised from normal or *S. typhimurium* lipoprotein-treated BALB/c mice

Mice	Spleen wt[a] (g)	No. of TNP-SRBC PFC per 10^6 cells[b]	No. of SRBC PFC per 10^6 cells[b]
Normal[c]	0.115 ± 0.001	9.2 ± 3.5	5.3 ± 3.4
Lipoprotein treated[d]	0.220 ± 0.024	102.6 ± 12.6	21.7 ± 4.8

[a] The mean weight ± standard error of the three spleens in each group.
[b] The mean PFC response ± standard error from quadruplicate assays.
[c] The three normal mice were uninjected, age-matched controls.
[d] The three lipoprotein-treated mice had each been injected intravenously with 35 μg of *S. typhimurium* lipoprotein 3 days previously.

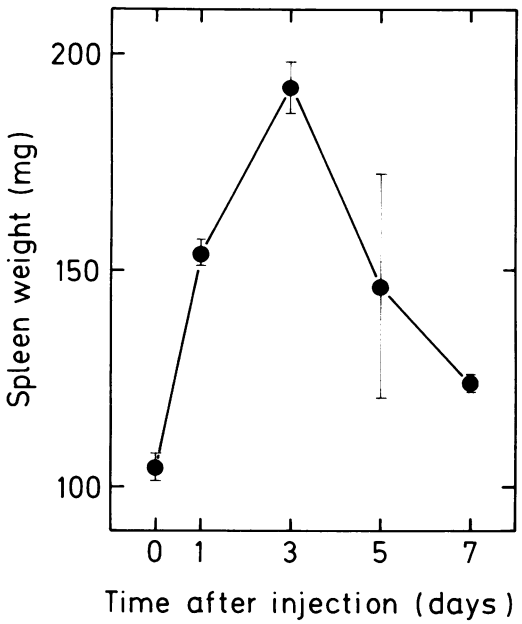

FIG. 2. Kinetics of splenic enlargement induced by *S. typhimurium* lipoprotein. Pairs of BALB/c mice were injected intravenously with 35 μg of lipoprotein 1, 3, 5, or 7 days before assay. Uninjected age-matched mice were used as controls. Each point represents the mean splenic weight ± standard error.

The development of this splenic activation was investigated in groups of mice which had been injected intravenously with 35 μg of *S. typhimurium* lipoprotein 1, 3, 5, or 7 days before assay. The resultant increases in splenic weight (Fig. 2) and PFC numbers (Fig. 3) were similar to those in the previous experiment. Interestingly, both responses developed quickly, peaked on day 3, and then decayed rapidly.

Finally, the in vivo polyclonal B-cell activation induced by *S. typhimurium* lipoprotein was compared to that induced by *E. coli* lipoprotein and by *E. coli* LPS. Separate groups of BALB/c and C3H/HeJ mice were each injected intravenously with 35 μg of one of the three mitogens, and their spleens were assayed for the number of PFC against TNP-SRBC. It is apparent that the two preparations of bacterial lipoprotein were able to induce polyclonal responses in both strains of mice, whereas *E. coli* LPS had such an effect only in BALB/c mice (Table 2). Recently, a synthetic analog of the N-terminal end of lipoprotein, *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl]-*N*-palmitoyl-(*R*)-cysteinyl-seryl-seryl-asparaginyl-alanine (4) was also found to stimulate murine spleen cells in vivo (unpublished observation). These results, like their in vitro counterparts, indicate that any contribution by LPS to the activity of lipoprotein must only be minimal.

Throughout these experiments, lipoprotein prepared from *S. typhimurium* had an activity comparable to that of lipoprotein prepared from *E. coli*, suggesting that the mitogenic moiety may be conserved amongst the *Enterobacteriaceae*. From the practical point of view, *S. typhimurium* SL761 has proved to be a particularly useful source of lipoprotein. First, this temperature-sensitive mutant is unable to synthesize the entire LPS molecule when grown at 42°C (12). Second, the yield of lipoprotein is consistently greater than that obtained with *E. coli* (unpublished observation).

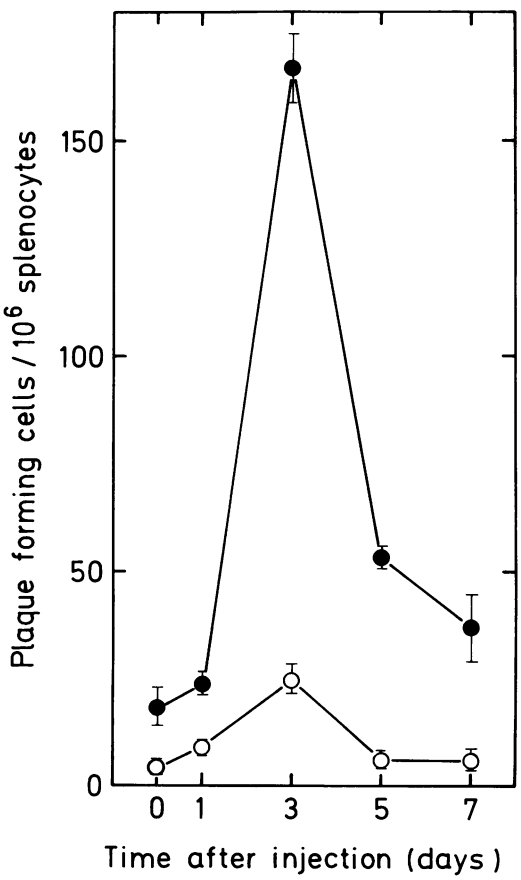

FIG. 3. Kinetics of the polyclonal B-cell response induced in the spleen by *S. typhimurium* lipoprotein. Pairs of BALB/c mice were injected intravenously with 35 μg of lipoprotein 1, 3, 5, or 7 days before assay. Uninjected age-matched mice were used as controls. The spleen cells within each group were pooled, and the number of direct PFC against either TNP-SRBC (●) or SRBC (○) was determined. Each point represents the mean number of PFC ± standard error from quadruplicate assays.

TABLE 2. Comparison of the TNP-SRBC PFC response in spleens of BALB/c and C3H/HeJ mice to in vivo stimulation with various mitogens

Mitogen[a]	TNP-SRBC PFC per 10^6 spleen cells[b]	
	BALB/c	C3H/HeJ
Control	7.5 ± 1.2	8.7 ± 1.7
S. typhimurium lipoprotein	106.7 ± 6.0	87.7 ± 4.3
E. coli lipoprotein	58.7 ± 7.8	85.7 ± 7.1
E. coli O55:B5 LPS	62.7 ± 5.8	10.0 ± 2.9

[a] Pairs of mice were injected intravenously with 35 μg of mitogen.

[b] The spleen cells within each group were pooled, and the average number of direct PFC against TNP-SRBC ± standard error was determined from quadruplicate assays.

In conclusion, these studies have shown that *S. typhimurium* lipoprotein is also able to polyclonally activate murine B-lymphocytes and that its effects in vivo complement those observed in vitro. They also suggest that these potent bacterial mitogens may have a significant influence on the host-parasite relationship, particularly before the establishment of specific humoral and cellular immune responses. A rapid elevation in the level of serum immunoglobulin, found when specific-pathogen-free mice are exposed to *S. typhimurium* organisms (10), supports this hypothesis. Hence, during the initial phase of a bacterial infection, some "protective" antibody, generated as a result of these polyclonal B cell responses, may contribute to host resistance.

The excellent technical assistance of Marianne Cox and Margret Pfeifer is gratefully acknowledged. We also thank Gudrun Eck and Patricia Johnson for their help in preparing the manuscript.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG 76).

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