Vol. 97, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 28, 1980 Pages 493-499

BACTERIAL LIPOPOLYSACCHARIDES AND MYCOPLASMAL LIPOGLYCANS: A COMPARISON BETWEEN THEIR ABILITIES TO INDUCE MACROPHAGE-MEDIATED TUMOR CELL KILLING AND LIMULUS AMEBOCYTE LYSATE CLOTTING

J. Brice Weinberg, Paul F. Smith, Itzhak Kahane

Division of Hematology-Oncology
VA Medical Center and Duke University Medical Center
Durham, NC 27705

and

Department of Microbiology
University of South Dakota
Vermillion, SD 57069

and

Department of Membranes and Ultrastructure
Hebrew University, Hadassah Medical School
Jerusalem, Israel

Received October 13,1980

Summary: The ability of various bacterial lipopolysaccharides and mycoplasmal lipopolysaccharides (lipoglycans) to induce macrophage-mediated tumor cell killing and Limulus amebocyte lysate clotting was determined. Lipoglycans from the mycoplasma Acholeplasma axantum or Acholeplasma granularum had no activity or 10^4 to 10^5 less activity than lipopolysaccharides from Escherichia coli 0128:B12, Escherichia coli K235, or Salmonella minnesota R595 in causing Limulus lysate clotting and tumor cell killing by peritoneal macrophages from normal or bacillus Calmette-Guérin-infected mice. Previous studies have shown that the lipid A portion of bacterial lipopolysaccharide is responsible for the effects on macrophage-mediated tumor cell killing and Limulus lysate clotting. The known differences in the lipid structures of bacterial lipopolysaccharides and mycoplasmal lipopolysaccharides (lipoglycans) may account for the noted differences in the biologic potencies observed here.

Introduction: Bacterial endotoxin is capable of rendering mouse peritoneal macrophages nonspecifically cytotoxic and cytocidal for tumorigenic target cells in vitro (1,6,12,23). The activating effects of the bacterial lipopolysaccharide (LPS) reside in the lipid A portion of the molecule; its effect is reproduced by polysaccharide-free lipid A, is negated by the lipid A-binding antibiotic polymyxin B, and is less potent when using macrophages from the lipid A-nonresponder mouse strain C_3H/HeJ (1,23). Lipopolysaccha-

Abbreviations: LG, lipoglycan; LPS, lipopolysaccharide; BCG, bacillus Calmette-Guérin; LAL, Limulus amebocyte lysate; [^{3}H]TdR, tritiated thymidine; KDO, ketodeoxyoctanates.

0006-291X/80/220493-07$01.00/0

rides extracted from mycoplasma [termed lipoglycans (LG) to distinguish from bacterial LPS] are structurally distinct from LPS of gram negative bacteria with differences in the lipid and polysaccharide portions (20-22). The mycoplasmal lipoglycan represents a microbial lipopolysaccharide which, although differing from bacterial LPS, might have comparable biologic effects. The purpose of this study was to compare the effectiveness of LPS from gram negative bacteria and LG from mycoplasma to induce macrophage-mediated tumor cell killing. The mycoplasmal LG were found to be dramatically less capable than bacterial LPS of inducing macrophage-mediated tumor cell killing.

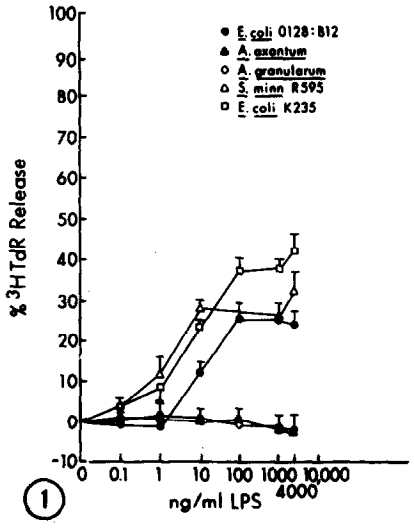
Materials and Methods: Female C_3H/HeN mice, age 8-15 weeks were obtained from Charles Rivers (Wilmington, Massachusetts). Bacillus Calmette-Guérin (BCG) infection was established by injecting $1x10^6$ colony forming units of Phipps Strain BCG in saline (Trudeau Mycobacterial Collection 1029, Trudeau Institute, Saranac Lake, New York). BCG infected mice were used 2-4 weeks after initial infection (23).

LG from Acholeplasma granularum strain BTS-39 and Acholeplasma axantum strain 743 were prepared as previously described (22). Escherichia coli K235 LPS extracted by hot phenol (13) was supplied by Dr. David Morrison. The glycolipid extracted from the polysaccharide deficient Salmonella minnesota R595 (16) was obtained from Dr. Edgar Ribi. Hot phenol-extracted LPS from E. coli 0128:B12 was obtained from Sigma Chemical Company. The LG and LPS were dissolved in tissue culture medium prior to use in cytotoxicity assays and Limulus amebocyte lysate clotting assays. Sonifying the solutions did not alter the results.

Dulbecco's modified Eagle medium (Grand Island Biological Company, Grand Island, New York) was supplemented with 1mgm/ml dextrose, 100 mg/ml Streptomycin, 100 M/ml penicillin, and 20mM HEPES. The powdered medium was formulated with endotoxin-free water, and the supplemented medium was negative in the Limulus amebocyte lysate assay (23). Endotoxin-free adult bovine serum was obtained from Sterile Systems, Inc., Logan, Utah. The tumorigenic 3T12 fibrosarcoma cell line (of Balb/c origin) is maintained in this lab in serial culture (23). It is free of mycoplasma (4).

The Limulus amebocyte lysate (LAL) assay was performed as previously described (5,12,23). It was capable of detecting 0.1 to 1.0 ng/ml of gram negative bacterial LPS (E. coli 0128:B12). The LAL was purchased from Panmed, Inc. of New Buffalo, Michigan (15).

The tumor cell killing assay was done as previously described (12,23,24). Briefly, macrophage monolayers were established in 6mm chambers of Microtitre II plates (Falcon #3040) using peritoneal macrophages from normal or BCG-infected mice that had received 1 ml of 10% peptone (Difco, Detroit, Michigan) 3 days prior to sacrifice. After adherence of $4x10^5$ peritoneal cells per chamber at 37°C for 60 minutes, the nonadherent cells were removed by repetitive washing with saline. This resulted in monolayers of cells consisting of >90% macrophages as determined by phagocytic capability and the presence of napthyl butyrate esterase activity (25). One x 10^4 3T12 cells, that were previously labeled with tritiated thymidine ([^{3}H]TdR) as previously described (23), were added to the chambers with or without macrophage monolayers. Desired amounts of LPS or LG were added and left throughout the culture which was done in media containing 10% adult bovine serum. LPS and LP dilutions were made carefully using fresh pipettes between dilutions to avoid "carry



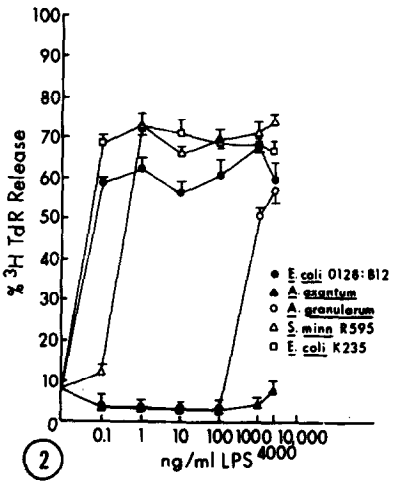


Figure 1. Killing of 3T12 tumor cells (%[^{3}H]TdR release) by macrophages from normal mice in the presence of different amounts of LPS or LG. Points represent means of triplicate samples with vertical bars showing one standard error of the mean.

Figure 2. Killing of 3T12 tumor cells (%[^{3}H]TdR release) by macrophages from BCG-infected mice in the presence of different amounts of LPS or LG. Points represent means of triplicate samples with vertical bars showing one standard error of the mean.

over" inaccuracies. After 60 hours of culture, supernatants were counted for radioactivity and %[^{3}H]TdR release was calculated by the formula $\frac{Sx - Sc}{Total} \times 100$, where Sx is cpm in experimental well, Sc is cpm in control (normal macrophage with no added LPS or LG), and Total is cpm in tumor cell alone well determined by lysing with 0.2% sodium dodecylsulfate. Spontaneous release over the 60 hour period was less than 20% of total. Total count was approximately 2000 cpm. Cultures were monitored by inverted phase microscopy and by Giemsa-stained fixed plates; cytocidal activity as determined by the [^{3}H]TdR release assay corresponded to that determined by the visual cell counting assay (24).

Results: Macrophages from normal mice that had received intraperitoneal peptone became mildly tumoricidal *in vitro* in the presence of 10 to 100 ng/ml *E. coli* K235, *E. coli* 0128:B12, or *S. minnesota* R595 LPS (Figure 1). The LG from *A. axantum* and *A. granularum* did not enhance tumor cell killing in doses as high as 4000 ng/ml. Neither the LG from mycoplasma nor LPS from bacteria was toxic to the tumor cells in the absence of macrophages (data not shown). Macrophages from BCG-infected animals have heightened sensitivity to bacterial endotoxin (12,18,23). Figure 2 shows that 0.1 to 1.0 ng/ml of *E. coli* K235, *E. coli* 0128:B12, or *S. minnesota* R595 LPS induced dramatic cytolysis by the

Table 1. Comparison of macrophage-mediated tumor cell killing and Limulus amebocyte lysate clotting induced by bacterial lipopolysaccharide or mycoplasmal lipoglycan.

	Normal Macrophage			
LPS or LG present	E. coli 0128:B12		A. granularum	
(ng/ml)	%[^{3}H]TdR Release[1]	LAL[2]	%[^{3}H]TdR Release	LAL
0	-0.8 ± 2.5	-	-0.8 ± 2.4	-
1	3.4 ± 1.6	+	-0.7 ± 0.8	-
10	4.2 ± 4.4	+	1.2 ± 2.6	-
100	30.9 ± 3.9	+	0.8 ± 2.9	-
1000	52.4 ± 5.0	+	-1.6 ± 0.2	+
10000	44.0 ± 8.1	+	-3.6 ± 3.7	+
	BCG Macrophage			
LPS or LG present	E. coli 0128:B12		A. granularum	
(ng/ml)	%[^{3}H]TdR Release	LAL	%[^{3}H]TdR Release	LAL
0	9.4 ± 7.6	-	9.4 ± 4.6	-
1	46.9 ± 0.4	+	0.2 ± 2.4	-
10	52.4 ± 0.7	+	9.9 ± 4.3	-
100	52.8 ± 0.8	+	14.5 ± 9.4	-
1000	51.7 ± 0.8	+	36.4 ± 4.4	+
10000	33.2 ± 2.1	+	45.2 ± 2.2	+

[1] %[^{3}H]TdR release from labeled 3T12 fibrosarcoma cells expressed as mean ± S.E.M. of triplicate samples (see Methods section).

[2] Clotting of Limulus amebocyte lysate (LAL): - means no clot and + means solid clot (see Methods section).

macrophages whereas LG from the mycoplasma were much less effective, being 10^4 to 10^5 less active. The magnitude of the cytolysis mediated by normal macrophages in the presence bacterial LPS was consistently less than that by BCG macrophages.

To determine the relationship between the abilities of LG and LPS to cause LAL clotting and to induce macrophage-mediated tumor cytolysis, we did

clotting assays in conjunction with cytolysis assays. Table 1 shows that A. granularum LG had much less ability to clot LAL than did E. coli LPS; the ability to initiate clotting in general paralleled the ability to initiate macrophage-mediated tumor cell killing. Not shown in the table is that A. axantum LG, which was the least potent of all preparations in inducing macrophage-mediated tumor cytolysis (Figure 1 and Figure 2), caused LAL clotting only with $\geq$10,000 ng/ml.

Discussion: Bacterial LPS causes differentiation of macrophages to the tumoricidal state (1,6,12,18,19,23,24). The macrophages' responsiveness to LPS parallels their degree of activation toward the cidal state. Peritoneal macrophages from BCG-infected mice are extremely sensitive to LPS. Indeed, the ability to detect LPS by a standard in vitro tumor cytotoxicity assay using BCG macrophages as effector cells is as sensitive or more sensitive than other LPS bio-assays in current use (5,12,23).

This study demonstrated that LG of mycoplasma were 10^4 to 10^5 less active than LPS of gram negative bacteria in causing in vitro tumor cell killing by macrophages from normal or BCG-infected mice. Also, LG, as compared to LPS, were much less potent in causing LAL clotting; LAL clotting was caused by high amounts of LG comparable to those necessary to induce tumor cytotoxicity by BCG macrophages.

The ability of the LG of A. axantum and A. granularum to cause tumor cell killing by BCG macrophages or to clot LAL at high amounts could conceivably be due to contamination with bacterial LPS incurred during isolation and processing of the LG. Contamination with amounts of bacterial LPS capable of influencing assays like the two we use here is very common (3,7,8,11,12,17, 23). Dissociating the effects of various agents from those of LPS is difficult, and complete removal of contaminating LPS from some agents is extraordinarily trying (23).

The reasons for the dramatic functional differences between LG and LPS are not known. The LG of mycoplasma differ structurally from the LPS of

gram negative bacteria (20-22). LPS generally consist of a lipid portion (lipid A) made of a diglucosamine backbone with fatty acids attached through ester or amide bonds, a core sugar portion with ketodeoxyoctanates (KDO) and heptoses, and a long polysaccharide chain attached to the core (9,14). LG have oligosaccharide chains covalently attached to a diglyceride, and they lack heptoses and KDO (20-22). Some mycoplasma do not contain LG (20). The lipid A portion of LPS is responsible for its ability to induce macrophage-mediated tumor cytolysis (1,23), its mitogenic activity for B lymphocytes (2), its pyrogenic activity in rabbits (10), its LAL clotting activity (26), and several of its other activities (9,14). Our results demonstrate that LG is not capable of triggering the macrophages properly to induce tumor cytolysis or of initiating the sequence of coagulative proteases in LAL. We postulate that the difference between LG and LPS in these respects is due to differences in the lipid portions of the molecules.

Acknowledgments: The work was supported by the Veterans Administration, and by grant number 27070-2 awarded by the National Cancer Institute, DHEW. We thank M. Naramore, P. Terrell, S. Kupit, J. Henderson, and D. Powell for technical assistance.

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