Mitogenic Response of C3H/HeJ Mouse Lymphocytes to Polyanionic Polysaccharides Obtained from * Bordetella pertussis* Endotoxin and from Other Bacterial Species

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Lipopolysaccharide extracted from * Bordetella pertussis* was mitogenic for spleen cells of endotoxin-resistant C3H/HeJ mice. Although endotoxic lipid A was inactive, mitogenic activity of lipopolysaccharide was exhibited by purified preparations of polysaccharides I and II, which constitute the carbohydrate moiety of the macromolecule. These low-molecular-weight (2,800 and 3,600) polysaccharides, containing carboxyl groups, were not mitogenic for thymocytes and splenic T-cells of C3H/HeJ mice, but did show mitogenic activity for splenic B-cells of C3H/HeJ mice and for spleen cells of C57BL/6 athymic nude mice. The mitogenic activities of polysaccharides I and II were also compared with those of other polyanionic polysaccharides, and the results indicate that high molecular weight is not necessary, and negative charges are not sufficient, for mitogenicity.

Lipopolysaccharides (LPS) extracted from gram-negative bacteria are known to be mitogenic for B-lymphocytes of normal mice, but are generally not mitogenic for spleen cells of C3H/HeJ mice. However, an LPS preparation from * Brucella abortus*, mitogenic for the spleen cells of this endotoxin-resistant mouse strain, has recently been described (17). At that time we had found that other LPS such as those extracted from * Bordetella pertussis* and * Proteus mirabilis* were also mitogenic for the spleen cells of C3H/HeJ mice. The purpose of this study was to explore the possibility that this activity might be due to a polyanionic structure different from that of lipid A, particularly to the carbohydrate moiety of these endotoxins.

**MATERIALS AND METHODS**

**Mice.** Male mice from inbred strains, 4 to 6 weeks of age (unless otherwise specified), were obtained from the following sources: C57BL/6 and congenitally athymic C57BL/6 nu/nu mice, Centre National de la Recherche Scientifique, Orsay, France; C3H/CRL mice, Charles River, Saint-Aubin les Elbeuf, France; and C3H/HeJ mice, Institut Pasteur Production, Renemoulin, France.

**Reagents and culture media.** 2-Mercaptoethanol was obtained from Eastman Organic Chemicals (Rochester, N.Y.) and [3H]thymidine was from the Commissariat à l’Energie Atomique (Saclay, France). Concanavalin A was obtained from GIBCO Laboratories (Glasgow, Scotland) and RPMI 1640 was from Flow Laboratories (Irvine, U.K.). Guinea pig complement and calf serum were purchased from Institut Pasteur Production, and monoclonal anti-Thy 1.2 antisem was obtained from Olac Cie (Bicester, U.K.).

**Source and characteristics of the LPS and polysaccharides.** Cellulose phosphate and cellulose sulfate were obtained from Serva (Heidelberg, F.R.G.), and carboxymethyl cellulose CM11 was from Whatman (Springfield Mill, U.K.). Heparin sulfate was purchased from Choay (Paris, France). Dextran sulfate was from Pharmacia Fine Chemicals (Uppsala, Sweden). *Shigella sonnei* phase II LPS, *Salmonella typhi* Vi polysaccharide, and pneumococcal S-II capsular polysaccharide were obtained from A. M. Staub’s collection. *Klebsiella pneumoniae* type 1 and 2 capsular polysaccharides were gifts from J. M. Fournier (Institut Pasteur, Paris, France). *Salmonella senftenberg* bacteria were cultured at the Institut Pasteur (2), and both * P. mirabilis* and * B. pertussis* (strain 1414, phase I) microorganisms were cultured at the Institut Médecin J. M. Fournier and P. Senftenberg (strain 1414, phase II) microorganisms were cultured at the Institut Pasteur (2), and both * P. mirabilis* and * B. pertussis* (strain 1414, phase I) microorganisms were cultured at the Institut Médecin J. M. Fournier and P. Senftenberg (strain 1414, phase II) microorganisms were cultured at the Institut Pasteur (2), and both * P. mirabilis* and * B. pertussis* (strain 1414, phase I) microorganisms were cultured at the Institut Médecin J. M. Fournier and P. Senftenberg (strain 1414, phase II) microorganisms were cultured at the Institut Pasteur (2), and both * P. mirabilis* and * B. pertussis* (strain 1414, phase I) microorganisms were cultured at the Institut Médecin
myristic acid after hydrolysis and analysis by gas-liquid chromatography. Furthermore, both PS-I and PS-II were devoid of endotoxic activities since they were neither toxic nor pyrogenic (1) and were negative in the Limulus amoebocyte lysate test.

Cell preparations. Mice were killed by cervical dislocation. Spleens or thymuses (or both) were removed aseptically from mice and placed in sterile Hanks 199 medium (7.5 ml). Cell suspensions were prepared by teasing the tissue with mouse tooth forceps. Cell clumps and debris were removed by gravity sedimentation for 5 min, after which the supernatants were centrifuged at 400 × g for 10 min. Cell pellets were suspended in 1 ml of Hanks 199 medium and diluted with the supplemented RPMI medium to a final density of 5 × 10^6 cells per ml.

Splenic T-cell-enriched populations were prepared by the technique of Julius et al. (13) by elution of the cells after incubation at 37°C for 45 min on a nylon wool column. To obtain B-cell-enriched populations, splenic cells (2.7 ml, 10^8 cells per ml in balanced salt solution) were incubated at 37°C for 1 h with anti-Thy 1.2 antiserum (100 µl, diluted 1:200) and complement (0.7 ml, diluted 1:2). The cell suspension was then layered on top of 9 ml of calf serum in glass conical centrifuge tubes which were centrifuged for 10 min at 400 × g in a swinging-bucket centrifuge head. Dead cells were removed with the supernatant. The cell pellet was then depleted of residual T-cells by a nylon wool column, and the absorbed cells (B cell enriched) were squeezed out from the nylon wool by vigorous washing with Hanks 199 medium.

Assay for mitogenic activity. The culture medium was RPMI 1640 supplemented with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES buffer), 2 mM L-glutamine, 100 µg of streptomycin per ml, 100 IU of penicillin per ml, and 5 × 10^-5 M 2-mercaptoethanol. Agents used in mitogenesis studies were sterilized either by exposure to ultraviolet light or by heating at 110°C for 40 min. LPS and lipid A were suspended in the culture medium by sonication for 2 min at a concentration of 1 µg/ml. A volume of 0.1 ml of the cell suspension (5 × 10^6 splenocytes or 10^6 thymocytes) was placed in triplicate wells of round-bottom microtiter plates (Nunc lon Delta; Poly-Labo, Paris, France). Mitogens diluted in medium were added to wells to give a final total volume of 0.2 ml. For each cell population studied, 12 control wells had medium without mitogen added. The plates were incubated at 37°C in an atmosphere of 5% CO_2 in air for 45 h. [3H]thymidine (0.5 µCi, 25 Ci/mmol) was added to the wells for 18 h before harvesting (unless otherwise specified). The cells were harvested with a multimanifold sample harvester on glass fiber filter paper and washed with distilled water. The disks were allowed to dry. To each disk was added 2 ml of PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazolyl)benzene] solution in toluene. The vials were placed at 4°C in the dark for 10 min and then counted in a liquid scintillation spectrometer (Intertechnique SL30) for uptake of [3H]thymidine. Results were expressed as net counts per minute per culture (5 × 10^6 cells) and calculated as follows: net counts per minute = average counts in test wells – average counts in control wells.

RESULTS

Responses of C3H/CRL and C3H/HeJ spleen cells to different LPS preparations. A comparison of the mitogenic responses of spleen cells from C3H/CRL and C3H/HeJ mice to different preparations of LPS is shown in Fig. 1. Mitogenic responses of C57BL/6 spleen cells (data not shown) were similar to those obtained with C3H/CRL cells.

![Fig. 1. Mitogen responsiveness of C3H/CRL and C3H/HeJ spleen cells for different LPS preparations. Thymidine incorporation by spleen cells from C3H/CRL (A) and C3H/HeJ (B) mice, stimulated with varying doses of LPS extracted from S. senftenberg (△), Shigella sonnei (●), P. mirabilis (○), and B. pertussis (■).](http://iai.asm.org/)
Although the four LPS preparations were mitogenic for the spleen cells of C3H/CRL mice (Fig. 1A) there was, as expected, no response of C3H/HeJ spleen cells to S. senftenberg and Shigella sonnei LPS. However, the spleen cells of the endotoxin-resistant C3H/HeJ mice exhibited a mitogenic response to both B. pertussis and P. mirabilis LPS (Fig. 1B).

To determine the optimal conditions of the mitogenic response of C3H/HeJ spleen cells to B. pertussis LPS, the influence of four parameters was investigated: (i) age of mice, (ii) spleen cell concentration, (iii) incubation period with the mitogen, and (iv) incubation period with [3H]thymidine. Contrary to the reported influence of the age of spleen donors on the mitogenic response to LPS (8) in the strains we studied, we observed no significant influence of the age of C3H/HeJ mice on the response of their spleen cells to B. pertussis LPS. With regard to the other parameters studied, a concentration of $5 \times 10^6$ spleen cells per culture (Fig. 2A) and incubation periods of 48 h with the mitogen (data not shown) and 18 h with [3H]thymidine (Fig. 2B) were found to be optimal.

**Difference in responsiveness of B- and T-cells from C3H/HeJ mice to B. pertussis LPS.** The target cells from C3H/HeJ mice, which incorporate thymidine after stimulation with B. pertussis LPS, were characterized by exposing purified B- and T-cells to this mitogen. The purity of these cell subpopulations was estimated by their responsiveness to dextran sulfate (a B-cell mitogen; 10) and to concanavalin A (a T-cell mitogen). By these criteria, the prepared subpopulations of splenic T- and B-cells appeared to be reasonably pure (Table 1), since the preparation of splenic B-cells was not stimulable by concanavalin A and the mitogenic response of the preparation of splenic T-cells to dextran sulfate was low compared with that of the unfractionated splenic cells.

After incubation with B. pertussis LPS, thymidine incorporation by purified splenic B-cells from C3H/HeJ mice was greater than that by unfractionated spleen cells (Table 1). Furthermore, neither thymocytes nor purified splenic T-cells were stimulated by the same mitogen. These results indicate that B. pertussis LPS is a B-cell mitogen for C3H/HeJ mice.

Cell responses to the polysaccharide and lipid A moieties obtained from B. pertussis LPS. To determine whether the mitogenic response of C3H/HeJ spleen cells to B. pertussis LPS was due to the polysaccharide or the lipidic moiety of the macromolecule, increasing concentrations of PS-I, PS-II, and lipid A were tested, and the responses of spleen cells from C3H/CRL and C3H/HeJ mice were compared with those obtained with the intact LPS. The results are shown in Fig. 3. Contrary to the reported absence of mitogenic activity of the O polysaccharides obtained from the enterobacteria usually used (22), the carbohydrate moieties of B. pertussis LPS (PS-I and PS-II) were at least as mitogenic as its lipidic moiety (lipid A) for both C3H/CRL (Fig. 3A) and C57BL/6 spleen cells (data not shown). B. pertussis lipid A, however, appeared, to be completely inactive for the spleen cells of C3H/HeJ mice (Fig. 3B), the mitogenic response of these cells to the intact endotoxin being exclusively due to PS-I and PS-II. Similar amounts of these polysaccharides, however, induced quantitatively different mitogenic responses in C3H/HeJ cells, PS-I being nearly twofold more mitogenic than PS-II.

**Mitogenicity of B. pertussis LPS and polysaccharides for spleen cells of athymic nude mice.** The results described above, that B. pertussis LPS is a B-cell mitogen for C3H/HeJ mice and that the polysaccharide moieties...
of the macromolecule are responsible for that mitogenicity, were indirectly confirmed by the fact that *B. pertussis* PS-I and II, which were effective in stimulating thymidine uptake by C57BL/6 normal mice spleen cells, stimulated the spleen cells of athymic nude C57BL/6 (nu/nu) mice to the same extent (Table 2). Furthermore, the stimulating activities of the polysaccharides were as effective as that obtained with the intact LPS, which carries a second B-cell mitogen, namely, lipid A.

**Mitogenicity of different anionic polysaccharides for C3H/HeJ mice spleen cells.** Two polyanionic polysaccharides, dextran sulfate (10) and pneumococcal polysaccharide S-III (5), are known to be very effective in stimulating thymidine uptake by murine B-cells. On the

<table>
<thead>
<tr>
<th>Mitogen (µg/ml)</th>
<th>B. pertussis LPS (25)</th>
<th>Dextran sulfate (25)</th>
<th>Concanavalin A (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymocytes</td>
<td>87 ± 132</td>
<td>207 ± 51</td>
<td>17,300 ± 6,671</td>
</tr>
<tr>
<td>Unfractionated</td>
<td>3,330 ± 226</td>
<td>8,709 ± 2,163</td>
<td>15,872 ± 8,414</td>
</tr>
<tr>
<td>Splenic B-cells</td>
<td>4,028 ± 75</td>
<td>11,566 ± 1,793</td>
<td>290 ± 445</td>
</tr>
<tr>
<td>Splenic T-cells</td>
<td>906 ± 62</td>
<td>1,250 ± 222</td>
<td>18,747 ± 1,493</td>
</tr>
</tbody>
</table>

**Table 1. Comparison of thymidine incorporation by different lymphoid cells from C3H/HeJ mice, after incubation with B. pertussis LPS**

**Table 2. Comparison of thymidine incorporation by normal or nude C57BL/6 mice spleen cells, after incubation with B. pertussis LPS and polysaccharides**

<table>
<thead>
<tr>
<th>Mitogen (µg/ml)</th>
<th>Normal mice</th>
<th>Athymic nude mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pertussis LPS (50)</td>
<td>31,431 ± 4,023</td>
<td>15,929 ± 1,144</td>
</tr>
<tr>
<td>B. pertussis PS-I (50)</td>
<td>20,886 ± 2,737</td>
<td>23,852 ± 2,956</td>
</tr>
<tr>
<td>B. pertussis PS-II (50)</td>
<td>18,902 ± 2,831</td>
<td>18,479 ± 1,313</td>
</tr>
<tr>
<td>Concanavalin A (1.2)</td>
<td>42,144 ± 3,918</td>
<td>-1,368 ± 481</td>
</tr>
</tbody>
</table>

*Responsiveness to concanavalin A served as the control for the absence of the T-cell subpopulation in nude mouse spleen cells. SD, Standard deviation.
other hand, neutral polysaccharides, or polysaccharides with a very low charge density such as Salmonella or Escherichia coli O antigens, are known to be devoid of mitogenic activity for murine spleen cells, the mitogenicity of the intact LPS of such microorganisms being exclusively due to the lipid A moiety (23).

As B. pertussis PS-I and PS-II, which are anionic polysaccharides containing glucuronic acid (3) and 3-deoxy-2-octulosonic acid (4), appeared to be mitogenic for murine B-cells, we wished to determine whether the presence of anionic charges on polysaccharides always confers a mitogenic activity on these substances. For this purpose, the mitogenicities of different anionic polysaccharides were compared, using spleen cells of C3H/HeJ mice to avoid having to take into account the influence of a putative contamination of these different polysaccharide preparations with endotoxin.

The established chemical structures of the different bacterial polysaccharides used are given in Table 3. Although all of the polysaccharides known until now to be mitogenic are polyanions, this characteristic is certainly not sufficient to confer on any given polysaccharide a mitogenic activity, since polyanions derived from cellulose appeared to be completely unable to stimulate thymidine uptake by spleen cells from C3H/HeJ mice (Table 4).

**DISCUSSION**

Sultzer and Nilsson (20) first reported that spleen cells from C3H/HeJ mice do not divide in response to LPS. It was shown subsequently that C3H/HeJ mice do not respond to LPS in a number of other immunological tests, whereas other C3H substrains do (21). Genetic analysis indicates that this unresponsiveness is the consequence of a single gene defect (21). However, the inability of this strain of mice to respond to endotoxins was demonstrated only with a small number of LPS usually extracted from Enterobacteriaceae. Moreno and Berman (17) recently reported that Brucella abortus LPS is mitogenic for spleen cells of C3H/HeJ mice. In the present work we demonstrated that two other LPS, extracted respectively from P. mirabilis and B. pertussis, are also mitogenic for spleen cells of C3H/HeJ mice (Fig. 1).

We decided to focus our study on B. pertussis endotoxin because of the availability of highly purified polysaccharide fractions (see Materials and Methods; 15) devoid of contaminants such as proteins (amino acid analysis) or lipid A (absence of β-hydroxy myristic acid). The data obtained (Table 1) clearly indicated that B. pertussis LPS is a B-cell mitogen for C3H/HeJ mice. The polysaccharide and lipid A moieties of this LPS are both mitogenic for the spleen cells of the endotoxin-sensitive C3H/CRL mice (Fig. 3A). However, as with lipid A preparations obtained from other gram-negative bacteria, and contrary to the postulated activity of the lipid moiety of Brucella LPS (17), B. pertussis lipid A was completely devoid of mitogenic activity for the spleen cells of C3H/HeJ mice. From the data obtained (Fig. 3B) it appears that the mi-

**Table 3. Identified structures of different anionic polysaccharides**

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Compound</th>
<th>Partial structure identified*</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bordetella pertussis</td>
<td>PS-I</td>
<td>β-GlcUA-1.2-Hept</td>
<td>3, 15</td>
</tr>
<tr>
<td>B. pertussis</td>
<td>PS-II</td>
<td>KDO</td>
<td>3, 15</td>
</tr>
<tr>
<td>L. mesenteroides</td>
<td>Dextran sulfate*</td>
<td>6-α-Glc-sulfate-1</td>
<td>4, 15</td>
</tr>
<tr>
<td>K. pneumoniae</td>
<td>Type 1 capsular polysaccharide</td>
<td>3-β-Glc-1.4-β-Man-1.4-α-Glc-1</td>
<td>9</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>S-II capsular polysaccharide</td>
<td>3-α-Rha-1.3-α-Rha-1.3-β-Rha-1.4-α-Glc-1</td>
<td>11</td>
</tr>
<tr>
<td>S. mirabilis</td>
<td>O polysaccharide</td>
<td>4-β-Glc-UA-1.3-β-Glc-UA-1.3-β-Glc-1</td>
<td>11</td>
</tr>
<tr>
<td>C. freundii</td>
<td>Vi polysaccharide</td>
<td>4-α-GalNAcUA-1</td>
<td>12</td>
</tr>
</tbody>
</table>

* Abbreviations: Fuc, fucose; GalNAcUA, N-acetyl-amino-lacturonic acid; GalUA, galacturonic acid; Glc, glucose; GlcN, glucosamine; GlcUA, glucuronic acid; Hept, heptose; KDO, 3-deoxy-2-octulosonic acid; Man, mannose; Rha, rhamnose.

* Bacterial dextran esterified with chlorosulfonic acid in pyridine.
The mitogenic activity of B. pertussis LPS for the spleen cells of C3H/HeJ mice is due to the polysaccharide moieties (PS-I and PS-II) of the macromolecule. PS-I and PS-II also appeared to be very effective mitogens for spleen cells of normal and athymic nude C57BL/6 mice (Table 2).

As PS-I and PS-II obtained from B. pertussis endotoxin both contain negatively charged carbohydrates such as 3-deoxy-2-octulosonic acid (4, 15) and D-glucuronic acid (3), their mitogenic activity was reminiscent of the well-known B-mitogenicity of other polyanions such as double-stranded homopolyribonucleic acids (polynucleosinic acid-polycytidylic acid (6), pneumococcal polysaccharide S-III (5), and dextran sulfate (10). Furthermore, other polyanions such as heparin and poly-L-glutamic acid were even reported to increase the net synthesis of dexyribonucleic acid in isolated nuclei and chromatin (18). The mitogenicities of B. pertussis PS-I and PS-II were compared with those of other polyanionic polysaccharides. For this purpose we used spleen cells of C3H/HeJ mice to eliminate the influence of possible endotoxin contamination in these preparations. The results obtained (Table 4) lead us to conclude that the presence of negative charges is not sufficient to confer mitogenic activity on polysaccharides since sulfite, phosphate, and carboxymethyl derivatives of cellulose, as well as heparin sulfate, were unable to stimulate dexyribonucleic acid synthesis by spleen cells of C3H/HeJ mice, whereas other anionic polysaccharides such as P. mirabilis O polysaccharide, dextran sulfate, and S. typhi Vi polysaccharide behaved as very effective mitogens on the same cells.

Some authors have stressed the importance of a minimum molecular weight of the polyanionic mitogens in the ability of these mitogens to activate B-cells. Thus, Strong et al. (19) observed that, although dextran sulfate of molecular weight 500,000 produced activation, the lower-molecular-weight (75,000) dextran sulfate did not. They suggested that the molecular size of dextran sulfate and of the other B-cell mitogens is an important determinant of ability to initiate B-cell mitogenesis. Our data indicate, however, that a high molecular weight is not required for B-cell activation since the low-molecular-weight polysaccharides (PS-I, 2,800; PS-II, 3,600) obtained from B. pertussis LPS were as effective in stimulating C3H/CRL spleen cells (thymidine uptake: 47,261 and 44,257 net cpm, respectively) as the high-molecular-weight dextran sulfate (thymidine uptake: 47,643 net cpm).

Our data, therefore, suggest that structural characteristics other than repeating negative charges or high molecular weight are necessary for the observed activation of B-cells by some polyanions. The mechanism of action of these substances remains unknown and requires further studies.

ACKNOWLEDGMENTS

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LITERATURE CITED