Spiralin, a Mycoplasma Membrane Lipoprotein, Induces T-Cell-Independent B-Cell Blastogenesis and Secretion of Proinflammatory Cytokines

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Mycoplasmas are bacteria which can cause respiratory, arthritic, and urogenital diseases. During the early phase of infection, mycoplasmas usually induce an inflammatory response and a humoral response preferentially directed against their membrane-bound, surface-exposed lipoproteins. In this report, we describe the effects on immune cells of spiralin, a well-characterized mycoplasmal lipoprotein. Purified spiralin stimulated the in vitro proliferation of human peripheral blood mononuclear cells and murine splenocytes. The stimulation pathway was probably different from that followed by Escherichia coli lipopolysaccharide because the effect of spiralin was not abolished by polymyxin B. Comparison of the effects of whole, native spiralin with those induced by proteinase K-digested spiralin or by the C-terminal half of spiralin (peptide p[13.5]y) revealed that the first half of the protein, which contains the lipoylated N terminus, is responsible for the mitogenic activity. In contrast to whole spiralin, proteinase K-digested spiralin did not trigger murine B-cell differentiation and immunoglobulin G and M secretion. Stimulation of human or murine immune cells led to early secretion of proinflammatory cytokines (human tumor necrosis factor alpha and murine interleukin 1 or 6). Spiralin induced the T-cell-independent blastogenesis of murine B cells but did not stimulate T cells. Altogether, our data demonstrate that spiralin possesses potent immunostimulating activity, similar to that reported for lipoproteins of pathogenic graciulites (gram-negative eubacteria; e.g., Borrelia burgdorferi OspA and E. coli Braun lipoprotein), and are consistent with the fact that lipoproteins are major antigens during mycoplasma infections.
trigger the secretion of proinflammatory cytokines? (v) What type of immune cell response is stimulated by spiralin?

**MATERIALS AND METHODS**

**Reagents and suppliers.** N-4-pyridyl-l-phenylalanyl chloromethyl ketone-trypsin, proteinase K, RPMI 1640, trypsin blue, phytohemagglutinin A (PHA), concanavalin A (ConA), bovine serum albumin (BSA), and formalin were purchased from Sigma Chemical Co., St. Louis, Mo. Penicillin, streptomycin, and L-glutamine were from Gibco BRL, Egry, France. Fetal calf serum was from TechGen, Les Ulis, France. Tritiated thymidine (dThd) and HRP (horseradish peroxidase)-streptavidin were from Amersham, Les Ulis, France. Escherichia coli O5:B5 LPS and polymyxin B were from Difco Laboratories, Detroit, Mich. B. burgdorferi OspA was kindly provided by Y. L. Erddie, Pasteur Institute, Paris, France. Serum and Vaccines, Marcy l’Etoile, France. HRP-conjugated anti-immunoglobulin G (IgG) and anti-IgM and alkaline phosphatase-labeled anti-rabbit IgG were from Bio-Rad, Ivry sur Seine, France. PamCysSer(Lys)3-OH was obtained from Boehringer Mannheim, Meylan, France.

**Bacterial culture and preparation of cell membranes.** S. melliferum BFC3 was grown as previously described (41). Cells were harvested by centrifugation at 15,000 x g for 1 h at 4°C and washed once in 50 mM phosphate-buffered saline (PBS; pH 7.4) containing 0.15 M NaCl and 8% sorbitol. The cell pellets were then dispersed into 50 mM Tris-HCl buffer (pH 7.5) and disrupted by ultrasonication at 20 kHz twice for 1 min each time at 0°C. The membranes were centrifuged three times at 38,000 x g for 1 h at 4°C in 50 mM Tris-HCl buffer (pH 8.0) and finally diluted to the bulk of extrinsic proteins as described earlier (41).

**Purification of spiralin and the p[13,5] fragment.** Spiralin was purified by agarose suspension electrophoresis after selective extraction with sodium deoxycholate (40, 41). Detergent-free spiralin micelles were obtained by 15% dialysis against Veronal buffer (pH 7.5) for 12 h at room temperature (41). Spiralin micelles were digested with trypsin as described previously (41). Briefly, 1 mg of detergent-free spiralin micelles was dispersed in 1 ml of 100 mM Tris-HCl (pH 8.0) and incubated for 24 h at 37°C in the presence of 3 mM Na2CtCl4–40 µg of N-4-pyridyl-l-phenylalanyl chloromethyl ketone-trypsin. The preparation was then centrifuged at 260,000 x g for 15 min at 4°C to separate the water-soluble fraction from the insoluble one. The water-soluble fragment [13,5] of spiralin was further purified by size exclusion high-performance liquid chromatography with a Waters Protein-Pack 200SW column. The purity of spiralin and p[13,5] was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (17) and silver staining of protein bands. Spiralin and p[13,5] were kept at –80°C in PBS (pH 7.5) after irradiation (**15.5** Cs, 25,000 rads).

**N-terminal sequencing of spiralin peptide.** Purified p[13,5] (50 µg) was subjected to SDS-PAGE (15% to 20% polyacrylamide linear gradient) (17) and electroblotted onto a polyvinylidene difluoride membrane in 50 mM Tris-HCl, 50 mM NaCl and 8% sorbitol. The membrane was then dispersed into 50 mM Tris-HCl buffer (pH 7.5) and disrupted by ultrasonication at 20 kHz twice for 1 min each time at 0°C. The membranes were centrifuged three times at 38,000 x g for 1 h at 4°C in 50 mM Tris-HCl buffer (pH 8.0) and finally diluted to the bulk of extrinsic proteins as described earlier (41).

**Proteinase K digestion.** Spiralin and p[13,5] were totally digested with proteinase K for 2 h at 56°C (1 µg of proteinase K/2.5 µg of protein), and the protein fragments were inactivated by heating for 10 min at 95°C. The efficacy of the digestion was checked by SDS-PAGE and silver staining.

**Protein determination.** Protein concentrations were determined by using the bichromic acid method (Pierce, Rockford, Ill.) with BSA as the standard.

**Purification and culture of PBMC and mouse spleen cells.** Human peripheral blood mononuclear cells PBMC were obtained from healthy donors who had never been involved with work in spiralin or spiroplasmas. PBMC were isolated from fresh blood by centrifugation over Ficoll-Hypaque and cultured in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, l-glutamine (2 mM), penicillin G (50 U/ml), and streptomycin (50 µg/ml) at 37°C in the presence of 3 mM Na2CtCl4–40 µg of N-4-pyridyl-l-phenylalanyl chloromethyl ketone-trypsin. The lack of endotoxin in spiralin preparations was checked by using limulus amoebocyte lysate assay. The lack of endotoxin in spiralin preparations and in cell culture media was monitored by using the Limulus amoebocyte lysate assay as recommended by the manufacturer (BioWhittaker, Walkersville, Md.).

**Purification and culture of PBMC and mouse spleen cells.** Human peripheral blood mononuclear cells PBMC were obtained from healthy donors who had never been involved with work in spiralin or spiroplasmas. PBMC were isolated from fresh blood by centrifugation over Ficoll-Hypaque and cultured in RPMI 1640 supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, l-glutamine (2 mM), penicillin G (50 U/ml), and streptomycin (50 µg/ml) at 37°C under 5% CO2 in a humidified atmosphere.

**Murine lymphocyte depletion.** Depletion of the lymphocyte subset from freshly isolated murine spleen cells was performed by using immunomagnetic beads coated with Tyl.2-specific monoclonal antibodies (MABS) (Dynabeads mouse pan B, and Dynabeads mouse pan B, Dynal), mouse spleen, mouse mesenteric lymph nodes, mouse peritoneal macrophages, or B. burgdorferi OspA mouse spleen, mouse peritoneum, mouse mesenteric lymph nodes, mouse peritoneal macrophages, or B. burgdorferi OspA mouse spleen. The spleen cells were incubated for 20 min at 4°C with beads at a bead-to-cell ratio of 4:1. The efficacy of and -T-cell depletion was checked by flow cytometry and reached 98.5 and 99.4%, respectively.

**Flow cytometry response to spiralin.** Human PBMC (0.75 x 107/ml) or mouse splenocytes (107/ml) were cultured in 96-well microtiter plates (Costar, Cambridge, Mass.) in 0.2 ml volumes with different concentrations of spiralin and [13,5] for various periods of time. Cell proliferation was measured after pulsing with 1 µCi of [3H]Tthd per well of the culture for 8 or 16 h. PBA (1 µg/ml), E. coli LPS (2.5 µg/ml), ConA (1 µg/ml), and B. burgdorferi OspA (0.5 µg/ml) were used as positive controls. Cell viability was checked by trypan blue exclusion. Each treatment was performed in triplicate. The results were expressed as average [3H]Tthd uptake (counts per minute in thousands). A Fisher test of variance comparison was performed to estimate whether the use of the t test for mean analysis was justified. In some cases, as indicated in the test, the modified t test was also used.

**TNF-α, IL-1, and IL-6 titration.** Human PBMC and BALB/c splenocytes (106/ml) were cultured in 24-well microtiter plates in 1-ml volumes with different concentrations of spiralin. After 1, 3, or 5 days, the culture supernatants were collected. TNF-α, IL-1, and IL-6 concentrations were determined by enzyme-linked immunosorbent assay (ELISA) in the supernatant (Quantikine, R&D SYSTEMS, Oxton, United Kingdom).

**Type of immune cell response is stimulated by spiralin? (v) What type of immune cell response is stimulated by spiralin?**

**RESULTS**

**Purity of spiralin and [13,5].** Spiralin was extracted from S. melliferum isolated membranes with two surfactants, Sarkosyl and sodium deoxycholate, and purified by preparative column electrophoresis. The purity of the protein was checked by SDSPAGE and silver staining (Fig. 1A). Surfactant removal by extensive dialysis led to the formation of spiralin protein micelles displaying an average diameter of about 10 nm (Fig. 1B). The absence of contaminating exogenous bacterial endotoxin in spiralin preparations was monitored by using the Limulus amoebocyte lysate assay and was found, as expected, to be lower than 0.1 endotoxin unit (EU)/ml, i.e., <0.01 ng/ml or <0.01 mg/ml of spiralin.

The [13,5] fragment was purified by high-performance size exclusion chromatography from a tryptic hydrolysate of spiralin, as described in Materials and Methods. Its purity was verified by using SD-PAGE (Fig. 1A). The N-terminal sequence of this fragment, HGVEKT, determined by the Edman technique, shows that its N terminus corresponds to histidine110 of the 219-residue spiralin polypeptide. As a molecular mass of 13.5 kDa corresponds to approximately 117 residues,
This is the second (i.e., C-terminal) half of spiralin. However, we cannot rule out the possibility that the terminal octapeptide VTATAPTE following lysine 211 was chopped off by trypsin.

**Spiralin micelle-induced human PBMC proliferation.** Spiralin micelles induced the proliferation of PBMC from two of three human blood donors in a dose-dependent manner. After a 3.5-day stimulation, spiralin induced no detectable effect, but after 4.5 days, cell proliferation was detected (data not shown). At optimal concentrations, i.e., 3 to 6 μg/ml, spiralin induced a proliferation corresponding to 20% of that induced by PHA. When the protein was used at higher concentrations, it exhibited a toxic effect on the cultured cells, as evidenced by a decrease in dThd uptake.

**Spiralin-stimulated TNF-α secretion by human PBMC.** TNF-α secretion by human PBMC was determined in cell culture supernatant upon stimulation with various concentrations of spiralin (Table 1). Since the first day, TNF-α secretion was maximal and proportional to the spiralin concentration. In the presence of 6 μg of spiralin per ml, the secretion reached almost 70% of that induced by 1 μg of PHA per ml.

**Spiralin-stimulated murine splenocyte proliferation.** The PBMC response to spiralin stimulation varied from one donor to another. Consequently, the subsequent investigations were performed by using murine splenocytes to obtain more reproducible results. In this in vitro system, spiralin was mitogenic for BALB/c mouse splenocytes in a dose-dependent manner (Fig. 2). After 3 days of culture, cell proliferation was detected with 0.01 μg of spiralin per ml (by using the modified t test, P < 0.01) and the maximal dThd incorporation was observed with 5 μg of spiralin per ml. At this concentration, the incorporation levels induced by spiralin were 51, 31, and 184% of those induced by *E. coli* LPS, ConA, and *B. burgdorferi* lipoprotein OspA, respectively (Table 2). At concentrations higher than 12.5 μg/ml, spiralin was toxic for the splenocytes, as reflected by a marked decrease in the dThd incorporation level.

Spiralin also efficiently stimulated C3H/HeOuJ and C57BL/6 murine splenocytes (*P* < 0.001 for C57BL/6 mice and *P* < 0.001 for C3H/HeOuJ mice), but the proliferative response of the cells from BALB/c mice was the highest (Table 2).

To check the possibility that spiralin uses the same pathway of activation as *E. coli* LPS, spiralin and control molecules were pretreated before their addition to BALB/c splenocytes and incubation for 1 h with 10 μg of polymyxin B per ml, a concentration sufficient to neutralize the effect of 20 ng of LPS per ml. As previously shown (21), polymyxin B treatment abolished LPS activity but not that of *B. burgdorferi* OspA. Similar

![Spiralin SDS-PAGE and Transmission Electron Microscopy](image-url)

**FIG. 1.** SDS-PAGE analysis of purified spiralin and p[13.5]T (A) and transmission electron microscopy of spiralin protein micelles (B). (A) Lanes: 1, *S. melliferum* membrane proteins (15 μg) solubilized with 1% SDS; 2, membrane proteins (10 μg) selectively extracted with 100 mM sodium deoxycholate from the fraction insoluble in 20 mM Sarkosyl; 3, spiralin (1 μg); 4, p[13.5]T (1 μg). SDS-PAGE was performed with the discontinuous system buffer of Laemmli using a stacking gel (T, 4.8%; C, 2.6%) and a separating gel (T, 12.5%; C, 2.6%). Protein bands were revealed by silver staining. S, spiralin. (B) Micelles were stained with 2% uranyl acetate (pH 4.5) before observation. The arrowheads point out some micelle particles (diameter, 10 nm).

| TABLE 1. Induction of cytokine secretion by spiralin*

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Medium</th>
<th>Spirelin</th>
<th>PHA</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 5</td>
<td>Day 1</td>
<td>Day 5</td>
</tr>
<tr>
<td>H-TNF-α</td>
<td>180</td>
<td>250</td>
<td>1,405</td>
<td>1,550</td>
</tr>
<tr>
<td>M-IL-1β</td>
<td>8</td>
<td>13</td>
<td>58</td>
<td>60</td>
</tr>
<tr>
<td>M-IL-6</td>
<td>15</td>
<td>31</td>
<td>190</td>
<td>234</td>
</tr>
</tbody>
</table>

*H-TNF-α, TNF-α secreted by human PBMC. M-IL-1β and M-IL-6, IL-1β and IL-6, respectively, secreted by murine splenocytes. PHA and LPS were used at 1 μg/ml of culture medium. The spiralin concentrations were 6 μg/ml of human PBMC culture and 1 μg/ml of murine splenocyte culture. In experiments with human PBMC, an increase in the spiralin concentration from 1 to 6 μg/ml induced a proportional increase in TNF-α secretion, but in experiments with murine splenocytes, 1 μg of spiralin per ml induced maximal secretion. The cytokine concentrations shown are means of duplicate assays and are representative of two independent experiments.

**ND, not determined.**
to the result obtained with OspA, the spiralin-induced proliferation was not significantly diminished ($P > 0.05$) (Table 2).

Proteinase K split spiralin into peptides which proved too small to be detected by SDS-PAGE (data not shown). This treatment reduced the mitogenic activity of spiralin on murine splenocytes by only 11% (Table 2). No activity was detected when heat-inactivated proteinase K was used.

**p[13.5]$_T$-induced proliferation of preactivated murine splenocytes.** Under the same conditions as those used to study the proliferation induced by spiralin, p[13.5]$_T$ was not mitogenic for BALB/c murine splenocytes (Fig. 3A). In contrast, when some batches of fetal calf serum were used for culture medium preparation, 0.1 and 0.5 $\mu$g of p[13.5]$_T$ per ml induced a low but significant level of $[^3]$HdThd uptake ($P < 0.05$). This weak activity was probably due to preactivation of the cells by the serum of the culture medium, as suggested by a high background level of dThd incorporation in the control samples (Fig. 3A).

However, even under optimal conditions, the mitogenic effect of p[13.5]$_T$ remained much weaker than that obtained with whole spiralin. The splenocyte proliferation was observed for concentrations of $\geq 0.05$ $\mu$g of p[13.5]$_T$ per ml of culture medium ($P < 0.05$) and was maximal for 0.5 $\mu$g of p[13.5]$_T$ per ml. This activity was specific because it totally disappeared after the digestion of p[13.5]$_T$ with proteinase K ($P < 0.05$) (Fig. 3B).

**Spiralin-triggered IL-1 and IL-6 secretion by murine cells.** The ability of spiralin to stimulate the secretion of cytokines from splenocytes in the culture supernatants was tested with BALB/c mice because of their sensitivity to spiralin stimulation. Spiralin induced the secretion of the two proinflammatory cytokines tested, IL-1$\beta$ and IL-6, by mouse splenocytes (Table 1). The level of cytokine secretion was independent of the spiralin concentration in the range of 1 to 6 $\mu$g/ml. The level of murine IL-1$\beta$ secretion was maximal on the first day of stimulation and remained stable for 5 days. Murine IL-6 was also secreted on the first day of culture in the presence of spiralin.

### Table 2. Spiralin effect on murine splenocyte proliferation

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>LPS</th>
<th>ConA</th>
<th>OspA</th>
<th>Spiralin</th>
<th>Spi/pK</th>
<th>Spi/pB</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c</td>
<td>60.0 ± 3.2 (2.5)</td>
<td>97.0 ± 4.1 (1.0)</td>
<td>16.5 ± 0.9 (0.5)</td>
<td>30.4 ± 2.1 (5.0)$^d$</td>
<td>27.1 ± 1.6 (5.0)$^d$</td>
<td>28.4 ± 1.7 (5.0)$^d$</td>
</tr>
<tr>
<td>C57BL/6</td>
<td>70.5 ± 5.7 (2.5)</td>
<td>58.0 ± 2.8 (1.0)</td>
<td>38.5 ± 2.3 (0.5)</td>
<td>26.5 ± 1.9 (1.0)$^d$</td>
<td>ND$^e$</td>
<td>ND</td>
</tr>
<tr>
<td>C3H/HeOuJ</td>
<td>87.8 ± 3.9 (2.5)</td>
<td>89.0 ± 3.9 (1.0)</td>
<td>15.5 ± 1.1 (0.5)</td>
<td>16.0 ± 0.9 (1.0)$^d$</td>
<td>ND$^e$</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ $[^3]$HdThd uptake is expressed as counts per minute (10$^3$), and the values are means of triplicate cultures. For untreated cells, the $[^3]$HdThd incorporation measured was about 1.5 $\times$ 10$^3$ cpm.

$^b$ Spi/pK, spiralin digested with proteinase K.

$^c$ Spi/pB, spiralin pretreated with polymyxin B.

$^d$ Spiralin concentration that induced the greatest $[^3]$HdThd incorporation.

$^e$ ND, not done.
but increased by about 23% after 5 days of culture. In the two cases, the amount of secreted cytokines was higher than that obtained with E. coli LPS.

To determine if the spiralin stimulation pathway involves macrophages and to compare the activity of spiralin with those of various stimuli in a homogeneous cell system, the mouse BALB/c J774 cell line was used and IL-1β production was determined after stimulation (Table 3). After a 24-h treatment, the concentrations of IL-1β induced by E. coli LPS, Pam3CysSer(Lys)4-OH, and B. burgdorferi OspA were 90, 57, and 66%, respectively, of that induced by spiralin. Under the same conditions, the production of IL-1β by cells stimulated by proteinase K-digested spiralin was decreased by 60% whereas p[13.5]-SPI/pK did not induce detectable IL-1β production (concentration, <5 pg/ml).

**Spiralin-induced IgG and IgM secretion by BALB/c splenocytes.** To determine the ability of spiralin to induce the differentiation of murine B cells into Ig-secretory cells, IgG and IgM in the cell culture supernatant were measured by ELISA after 1 and 5 days (Fig. 4A and B). In the presence of 1 μg of spiralin per ml, the levels of IgG and IgM production were similar and the kinetics were the same as those obtained with E. coli LPS treatment, i.e., no or very low secretion after 1 day and significant secretion of both Igs after 5 days of stimulation. The proteinase K-digested protein triggered neither IgG nor IgM secretion (5 pg/ml) after 5 days of culture, P < 0.001 and P < 0.01, respectively.

**Spiralin-induced blastogenesis of BALB/c spleen B cells.** To characterize the responsive cells, BALB/c splenocytes were cultured after adding spiralin, E. coli LPS, or ConA. After 3 days, cell morphology was analyzed by flow cytometry. Cells were labeled with CD40-RPE and CD3-fluorescein isothiocyanate MAbs and analyzed by gating size and granularity. Activated cells were identified by their increased size and granularity. Results indicated that 46% of the cells cultivated in medium alone died by apoptosis and only a fraction (14%) was activated (Fig. 5A). A majority of B cells were activated by LPS (89.5%) and spiralin (79%), whereas ConA stimulated mainly T cells (76%).

**T-cell-independent B-cell response of BALB/c spleen cells to spiralin.** To determine the type of spleen cells activated by spiralin, BALB/c splenocytes were depleted of B cells (Fig. 5C) or T cells (Fig. 5B) as described in Materials and Methods. By using this method, 98.5% of B cells and 99.4% of T cells were eliminated as determined by flow cytometry. These B-cell- or T-cell-depleted populations were then cultured for 3 days, and cell morphology was determined as described above.

**DISCUSSION**

Lipoproteins constitute a class of membrane-bound proteins of particular interest in the study of bacterial pathogenicity. Indeed, they induce polyclonal activation of immune cells such as T-cell-dependent B cells.
as monocytes-macrophage, B lymphocytes, platelets, and NK cells and their stimulating effects are evidenced by cell activation, cell proliferation, cytokine and nitric oxide production, induction of major histocompatibility complex expression, and immunoglobulin secretion (1, 28, 34, 35). Due to their immunogenicity and adjuvant potency, lipoproteins are currently being evaluated in vaccine preparations as purified antigens (16), synthetic lipopeptides (2), and recombinant lipoproteins (32) and produced by naked DNA vaccine (42). However, a major problem in studying the biological activities of mycoplasmal lipoproteins comes from the difficulty in obtaining sufficient amounts of these compounds with a high degree of purity under nondenaturing conditions. This difficulty is overcome with spiralin, a very well-characterized mycoplasmal lipoprotein which is available in sufficient amounts in highly purified and native forms (Fig. 1).

Spiralin micelles devoid of detergent and lipids induced human PBMC and murine splenocyte proliferation, which is the first demonstration of the stimulating properties of a pure mycoplasmal lipoprotein for immune cells. Because the response of human PBMC varied from one donor to another, the murine system was chosen to obtain more homogeneous and reproducible data. Our results are consistent with previous studies which showed that bacterial lipoproteins from *E. coli*, *B. burgdorferi*, and *Treponema pallidum* activate immune cells (21, 22, 28). The kinetics of the induction of murine splenocyte proliferation by spiralin were the same as those of *B. burgdorferi* OspA or OspB (21) or *E. coli* Braun lipoprotein (22). Spiralin stimulated the proliferation of splenocytes at a lower level than LPS or ConA but similar to that induced by *B. burgdorferi* OspA (Table 2). Indeed, the range of spiralin activity was similar to that reported for other lipoproteins (from the least active to the most active): *E. coli* Braun lipoprotein (5 or 20 μg/ml, depending on experimental conditions) (22), spiralin (1 to 5 μg/ml, depending on the genotype of the mice) (Table 2), and *B. burgdorferi* OspB and OspA (0.5 to 1 μg/ml) (21). These different ranges of activity may be due to sequence or conformation diversity, in particular within the region in the

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**FIG. 5.** Blast formation of lymphocytes induced by spiralin. Splenocytes stimulated with spiralin for 3 days were analyzed by flow cytometry by using dot plot analysis. Three-day cultured BALB/c splenocytes were labeled with anti-CD40–R-phycocyanin (red dots) and anti-CD3–fluorescein isothiocyanate (green dots) and analyzed for size (forward scatter [FSC]) and granularity (side scatter [SSC]). ConA, LPS, and spiralin were added at concentrations of 1, 2.5, and 5 μg/ml. A, total splenocytes; B, T-cell-depleted splenocytes; C, B-cell-depleted splenocytes. R1, quiescent cells; R2, dead cells; R3, activated cells.
vicinity of the N-terminal glyceride cysteine. The fatty acyl composition of these lipoproteins may also be responsible for the observed differences. The latter interpretation is supported by the fact that synthetic lipotetrapeptides differing only in their fatty acyl chains display different mitogenic activities (34).

In addition, an M. fermentans lipoprotein named MALP-2 has recently been described as being capable of activating macrophages in the picomolar range (25), compared to the 0.4 nM spiralin (or 0.01 μg/ml) necessary for induction of cell proliferation (Fig. 2). Determination of the acylated structure and the length of the N-terminal sequence allowing the highest activity is thus of the utmost importance to the understanding of the adjuvancy of lipoproteins and for the development of lipoprotein- or lipoprotein-based vaccines.

Polymyxin B is a cationic cyclic lipopeptide known to inhibit LPS activity. This inhibition results from the binding of polymyxin B to the lipid A of LPS (24). As previously shown for B. burgdorferi OspA and OspB (21), polymyxin B treatment had no effect on the spiralin mitogenic effect, which was expected because of the very high purity of spiralin. This result also reinforces the conclusions of a study of the signalling events in immune cell activation by lipoproteins of T. pallidum and B. burgdorferi, lipoproteins and LPS (26). The authors demonstrated a difference in the cell surface initiating events and a convergence of the cellular responses of human monocytic cells stimulated by these various compounds, such as induction of the transcriptional activator NF-κB and cytokine production, which lead to an inflammatory response.

To locate the most active moiety of spiralin, we tested its properties after digestion with proteinase K. Interestingly, the mitogenic activity was only slightly reduced by proteolysis, which clearly demonstrates that the spiralin structural integrity is not crucial for the cell activation process in vitro. In accordance with this result, recombinant OspA with its N-terminal acylated end deleted loses its immunogenicity in mice, even in the presence of alum adjuvant (9), whereas synthetic analogs of the N termini of the E. coli, B. burgdorferi, and T. pallidum lipoproteins retain the stimulating properties of the whole lipoproteins, depending on the presence of the five or six amino acids next to the N-terminal acylated cysteine (28). Although the proteic part of spiralin is not sufficient to trigger cell activation, p[13.5] is not totally devoid of immunological activity since it is immunogenic in rabbits (unpublished data) and contains, at least, a B-cell epitope within a 20-residue amphipathic segment (4) which is conserved in spiralins from different spiroplasmas species (13). On the other hand, spiralin, but not proteinase K-digested spiralin, was capable of triggering the production by murine spleen cells of IgG and IgM as efficiently as E. coli LPS, indicating that spiralin was able to induce the differentiation of B cells (Fig. 4). Since T cells were not activated by spiralin (Fig. 5), this Ig secretion, like spiralin-induced blastogenesis, was T cell independent. Altogether, these results suggest a dual activity in spiralin: a nonspecific activation of immune cells by the lipoylated N end and a specific role of the polypeptide antigenic determinants in B-cell differentiation and Ig production. The nonspecific activity of the N-terminal part of spiralin is similar to the adjuvant potency of synthetic lipopeptides. This property is consistent with the higher level of rabbit humoral response against spiralin in comparison with other mycoplasma membrane antigens produced by immunized rabbits (40).

Mycoplasma pathogenesis has been associated with the early production of proinflammatory cytokines by their hosts, e.g., in M. pneumoniae and M. pulmonis infections of mice (10, 27). These multifunctional cytokines (TNF-α, IL-1, and IL-6) have a broad host-protective role in combating bacterial infection. In M. pulmonis infection, the ability of mice to resist disease has been correlated to the amount of TNF-α and IL-6 produced but not to a particular H-2 or Bcg genotype, revealing the genetic complexity of the resistance to this mycoplasmal disease (6). These cytokines have also been implicated in the pathogenesis of syphilis and Lyme disease (21, 28). In addition, it has been shown in vitro that partially purified lipoproteins from M. arginini (14) and LAMPS from M. penetrans and M. fermentans (29) induce the production of these cytokines from human monocytes. Spiralin also triggered rapid in vitro secretion of human TNF-α, murine IL-1β, and murine IL-6 (Table 1), probably with a major contribution of monocytes-macrophages, as suggested by the experiment using the murine J774 cell line (Table 3). This activity was contained in the N-terminal part of spiralin but not in the C-terminal half, as evidenced by the lack of activity of p[13.5] and the ability of proteinase K-digested spiralin to induce the production of IL-1β by J774 cells. This suggests that, among mycoplasmal membrane components, lipoproteins are likely the key inflammatory mediators during infection.

By using murine spleen cells depleted of B or T cells, we also found that, like E. coli LPS, spiralin induces T-cell-independent blastogenesis of B cells (Fig. 5) and is consequently a T-cell-independent antigen according to the nomenclature reviewed by Mond et al. (23). Under our experimental conditions, spiralin activated a larger population of quiescent cells than did LPS. Snapper et al. (35) demonstrated that bacterial lipoproteins and the lipopeptide PamCysSer(Lys)4-OH are not able to activate purified, resting murine B cells and require a costimulating molecule, such as a polysaccharide antigen, to induce cell proliferation and Ig secretion. Our study shows that T cells are not necessary for the cell response to spiralin stimulation, i.e., polyclonal activation and differentiation of B cells. Other cells, such as monocytes-macrophages and NK cells, could be involved in this process, but further studies are required to elucidate their specific contributions.

Our results obtained with spiralin support the assumption that natural mycoplasmal lipoproteins containing an N-terminal glyceride cysteine are potent B-cell polyclonal activators. This correlates with the strong and early humoral response directed against lipoproteins in some human or animal mycoplasmal infections (15, 20) and validates the interest in their use as antigens for serodiagnosis or for vaccine development.

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