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*J Immunol 2002; 168:5240-5251; *
doi: 10.4049/jimmunol.168.10.5240
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Two msbB Genes Encoding Maximal Acylation of Lipid A Are Required for Invasive Shigella flexneri to Mediate Inflammatory Rupture and Destruction of the Intestinal Epithelium

Hélène d’Hauteville,* Shahid Khan,‡ Duncan J. Maskell,§ Andreas Kussak,§ Andrej Weintraub,§ John Mathison,‖ Richard J. Ulevitch,* Nicole Wuscher,† Claude Parsot,* and Philippe J. Sansonetti²*

Shigella flexneri is a Gram-negative pathogen that invades and causes inflammatory destruction of the human colonic epithelium, thus leading to bloody diarrhea and dysentery. A type III secretion system that delivers effector proteins into target eukaryotic cells is largely responsible for cell and tissue invasion. However, the respective role of this invasive phenotype and of lipid A, the endotoxin of the Shigella LPS, in eliciting the inflammatory cascade that leads to rupture and destruction of the epithelial barrier, was unknown. We investigated whether genetic detoxification of lipid A would cause significant alteration in pathogenicity. We showed that S. flexneri has two functional msbB genes, one carried by the chromosome (msbB1) and the other by the virulence plasmid (msbB2), the products of which act in complement to produce full acyl-oxy-acylation of the myristate at the 3’ position of the lipid A glucosamine disaccharide. A mutant in which both the msbB1 and msbB2 genes have been inactivated was impaired in its capacity to cause TNF-α production by human monocytes and to cause rupture and inflammatory destruction of the epithelial barrier in the rabbit ligated intestinal loop model of shigellosis, indicating that lipid A plays a significant role in aggravating inflammation that eventually destroys the intestinal barrier. In addition, neutralization of TNF-α during invasion by the wild-type strain strongly impaired its ability to cause rupture and inflammatory destruction of the epithelial lining, thus indicating that TNF-α is a major effector of epithelial destruction by Shigella. The Journal of Immunology, 2002, 168: 5240–5251.

Shigelllosis is an acute recto-colitis caused by Shigella, a Gram-negative species that disrupts, invades, and elicits inflammatory destruction of the intestinal epithelium (1). Bowel inflammation accounts for complications that can be lethal in infants, the principal victims of this infection in the developing world (2). The Shigella invasive phenotype plays a major role in promoting rupture, invasion, and inflammatory destruction of the intestinal mucosa. Proteins required for invasion are encoded on a 184-kb virulence plasmid (3, 4). A type III secretion apparatus and its cognate effector proteins (Ipa proteins) account for entry into epithelial cells, escape into the cell cytoplasm, and apoptotic killing of infected macrophages (reviewed in Ref. 5). IcsA, a 120-kDa outer membrane protein, causes the nucleation of actin filaments that assemble in a polar comet tail, allowing actin-driven motility of intracellular bacteria and their passage into adjacent cells (reviewed in Ref. 5). Several other potential virulence genes are present on this plasmid, whose possible role in modulating inflammation remains to be studied (4).

The current physiopathological scheme of shigellosis proposes that luminal bacteria initially cross the epithelial barrier, predominantly via M cells of the follicle-associated epithelium, and quickly invade resident macrophages that undergo apoptosis due to IpaB-mediated activation of caspase-1 (6–8). This leads to the release of IL-1β, which, in conjunction with a dramatic drop in IL-1R antagonist, is likely to play a major role at the early stage of Shigella pathogenesis by causing inflammation-mediated disruption of epithelial permeability that facilitates extension of bacterial translocation (9–11). Bacteria that have reached subepithelial tissues and survived macrophage killing may efficiently invade epithelial cells basolaterally and proceed to cell-to-cell spread. LPS delivered by Shigella in the cytoplasmic compartment of infected epithelial cells activates the translocation of NF-κB (12) via a Nod1-dependent pathway (13) and causes these cells to produce proinflammatory cytokines and chemokines such as IL-8 (12, 14, 15). This places epithelial cells at the front line (16) in the elicitation of the inflammatory cascade that leads to destruction of the epithelial barrier. IL-8 plays a major role by inducing massive influx of polymorphonuclear leukocytes (PMN)³ that participate in mucosal destruction (17).
As the signals leading to acute intestinal inflammation in shigellosis are being deciphered, it appears that the actual impact of the bacterial endotoxin (i.e., the lipid A moiety of LPS) has remained largely unexplored. It may participate in triggering or enhancing inflammation upon interaction with Toll-like receptor 4 expressed, along with CD14, by monocyte-macrophages (18) and upon invasion of epithelial cells through Nod1 (19). In an attempt to define this role, we previously conducted experimental Shigella infection in ligated intestinal loops of rabbits injected with a neutralizing anti-CD14 mAb (20). Compared with animals receiving a control Ab, rabbits in which CD14 was neutralized showed a dramatic increase in the quantity of bacteria invading the intestinal mucosa and more severe tissue destruction. Considering that these paradoxical results did not shed light on the actual role of lipid A in the development of intestinal inflammation, we decided to take a more straightforward approach, relying on the recent possibility to genetically attenuate the endotoxin activity of lipid A by constructing mutants in the genes responsible for the last two steps of its biosynthesis (21, 22), during which the 12-carbon fatty acid laurate and the 14-carbon fatty acid myristate are acyl-oxyacyl-linked to two of the four 3-OH-myristic acids available on keto-doxy-octenate 2 lipid A (Fig. 1). The htrB gene encodes the transferase that catalyzes the acyl-oxyacyl linkage of laurate to the 3' hydroxymyristate that is itself linked to the 2' position of the glucosamine. Inactivation of this gene results in a conditionally lethal phenotype (21). The msbB gene encodes the transferase that catalyzes the acyl-oxyacyl linkage of myristate on the hydroxy-myristate that is itself linked to the 3' position of the glucosamine disaccharide. The msbB mutations are not lethal (23). The acyl-oxyacyl-linked secondary myristate chain is essential for full host recognition of lipid A of a living microorganism (22), because an Escherichia coli msbB mutant shows dramatic decrease in proinflammatory potential as monitored in vitro by induction of selectin expression by endothelial cells and production of TNF-α by monocytes.

Following identification of a msbB homolog on the 214-kb plasmid of Shigella flexneri 5a, designated msbB2 (4), in addition to the chromosomal copy (designated msbB1), this work demonstrates that msbB2 is functional and completes acyl-oxyacylation on all 3' sites. Strains in which either the msbB1 or the msbB2 genes, or both genes, had been inactivated showed either partial or complete loss of lipid A synthesis, or both genes, had been inactivated showed either partial or complete loss of lipid A synthesis. Inactivation of this gene results in a conditionally lethal phenotype (4), in addition to two of the four 3-OH-myristic acids available on keto-doxy-octenate 2 lipid A (Fig. 1). The htrB gene encodes the transferase that catalyzes the acyl-oxyacyl linkage of laurate to the 3' hydroxymyristate that is itself linked to the 2' position of the glucosamine. Inactivation of this gene results in a conditionally lethal phenotype (21). The msbB gene encodes the transferase that catalyzes the acyl-oxyacyl linkage of myristate on the hydroxy-myristate that is itself linked to the 3' position of the glucosamine disaccharide. The msbB mutations are not lethal (23). The acyl-oxyacyl-linked secondary myristate chain is essential for full host recognition of lipid A of a living microorganism (22), because an Escherichia coli msbB mutant shows dramatic decrease in proinflammatory potential as monitored in vitro by induction of selectin expression by endothelial cells and production of TNF-α by monocytes.

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Materials and Methods

Bacterial strains and growth media

S. flexneri strains used in this study derived from the wild-type strain M90T (serotype 5a), including the streptomycin-resistant strain M90T-Sm, and the virulence plasmid-cured strain BS176 (3, 24). E. coli strains were derivatives of E. coli K-12: DH5α (25) was used for plasmid constructions, DH5α Apir, SM10 Apir (26), and B2155.88-A (pir RP4 DAP) were used to construct derivatives of the suicide vector pLAC2 (24) and to transfer these plasmids to S. flexneri, and CC118 Apir and S17-1 (pN35000) were used to construct derivatives of the suicide vector pCDV442 (27) and to transfer these plasmids to S. flexneri. Bacteria were grown in Luria-Bertani medium or tryptic soy broth. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; kanamycin (Km), 40 μg/ml; streptomycin, 100 μg/ml; and chloramphenicol, 30 μg/ml. When indicated, the growth medium was supplemented with Congo red (0.01%) and dianimopinolic acid (0.3 mM).

Inactivation of the chromosomal msbB1 gene

We used PCR to amplify a DNA fragment corresponding to the 5' portion of the msbB1 gene, the aphA gene from pUC4K (28), and the 3' portion and downstream region of the msbB1 gene. PCR fragments were digested by appropriate restriction enzymes, the sites for which had been incorporated within the PCR primers, mixed, and ligated together with SphI- and Xhol-digested pUC19 DNA (Fig. 2). In the pUC19 msbB1-Km recombinant plasmid thus constructed, the 3.4-kb SphI-Xhol insert corresponded to the aphA gene flanked by 1) the 5' region of msbB1, from nucleotide +859 (coordinates from the ATG of msbB1) to nucleotide +145, and 2) the 3' region of msbB1, from nucleotide +866 to nucleotide +2016 (coordinates from the ATG of msbB1). This insert was then cloned between the SphI and Xhol sites of the suicide vector pCDV442 (27) to construct pCDV442 msbB1-Km. The E. coli CC118 Apir derivative harboring pCDV442 msbB1-Km was then mated with S. flexneri MS17-1 (pN35000), which was used to mobilize the suicide plasmid into S. flexneri M90T. Transconjugants were selected by plating onto plates that contained sucrose (10%) and Km. The allelic exchange between the mutated msbB1 gene carried by the suicide plasmid and the wild-type msbB1 gene carried by the chromosone was confirmed by PCR using appropriate primers. The msbB1 mutant thus constructed was designated SfmsbB1-Km. It appeared that strain SfmsbB1-Km had lost the capacities to bind Congo red and invade HeLa cells, probably as a consequence of rearrangements within the virulence plasmid. Therefore, the msbB1-Km mutation was then transduced from strain SfmsbB1-Km to the wild-type strain M90T to construct the strain SC574 (msbB1). Similarly, the MsbB1-Km mutation was transduced to the virulence plasmid-cured strain BS176 to construct strain SC575 (VP-msbB1). Both recombinant strains were then tested for the
presence of the mutated msbB1 gene using PCR. In these msbB1 mutants, the msbB1 gene was interrupted after codon 48 (CGT).

Inactivation of the plasmid msbB2 gene

A 0.46-kb fragment extending from nucleotide +146 to nucleotide +606 of the msbB2 gene was amplified by PCR from the virulence plasmid pWR100 and cloned between the BamHI and Stpl sites of the vector pK19 (29). The clone was introduced into strains M90T and pHS50. The insert carried by pHS50 was then cloned between the Stpl and XbaI sites of the suicide vector plasmid pLAC2 (24) to construct the plasmid pHS53. In this later plasmid, the msbB2 internal fragment was cloned upstream from and in the same orientation as the promoterless lacZ gene carried by the plasmid. Plasmid pHS53 was then introduced by transformation into E. coli strains Sm10apir or G21558-8e (AprR44). and transformants were used to transfer the plasmid to S. flexneri strains M90T-Sm (msbB1) and SC574 (msbB2), respectively, by conjugation. Transconjugants were selected on plates containing ampicillin and either streptomycin or Km. The structure of the derivatives of pWR100 carrying the integrated pH53 plasmid was confirmed by PCR, and the resulting strains were designated SC576 (msbB2) and SC577 (msbB1 msbB2). In the msbB2 mutants, the msbB2 gene was interrupted after codon 202 (GGG). To complement the msbB1 msbB2 mutant, we constructed a derivative of the vector pSU2718 carrying a complete msbB2 gene under the control of a lac promoter. Briefly, a DNA fragment encompassing the region from nucleotide −37 to nucleotide +950 of msbB2 was amplified and cloned between the BamHI and HindIII sites of pSU2718 to construct the plasmid pH54. The sequence of the insert cloned into pH54 was verified by sequencing, and the plasmid was introduced by transformation into the strain SC573 to construct strain SC578 (msbB1 msbB2 pH54/MsbB2+).

Analysis of lipid A by MS

Preparation of LPS. The strains were grown in submerged culture to late exponential phase in 22 L of Luria broth containing 1% glucose, using a 30-L fermenter (Belach, Stockholm, Sweden) under constant aeration at 37°C (pH 7). Depending on the antibiotic cassette used to select the mutations, either Km (40 μg/ml) or ampicillin (100 μg/ml) was added to the medium. Strain M90T was grown in batch culture of 3 L. A preculture in the same medium was used to inoculate the fermenter. The medium was inoculated with a loop directly from a Congo red-positive clone on Congo red-agar plates. All cultures were checked for purity at the end of the growth cycle. Bacteria were then killed with 1% (mass/volume) formaldehyde. After incubation overnight at 4°C, cells were separated from the medium by continuous-flow centrifugation using a Cepa model LE centrifuge (C. Padberg, Centrifugenbau, Lahr, Germany) at a cylinder speed of 35,000 rpm and a cylinder temperature of 185°C and the temperature of the heated capillary was set at 185°C, and the temperature of the heated capillary was set at 20 kV. For MALDI-MS, lipid A was dissolved in chloroform-flower distilled water and the solution was dried using a stream of nitrogen.

Electrospray ionization (ESI) mass spectrometry (MS) in negative mode was performed using an LCQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Lipid A was dissolved in 0.5 ml chloroform/methanol (1/1) to a concentration of ~0.2–0.4 mg/ml. This dilution of lipid A was introduced into the mass spectrometer at a flow of 5 μl/min. Nitrogen was used as sheath gas, the needle voltage was set to −4 kV, and the temperature of the heated capillary was set at 185°C. Matrix-assisted laser desorption ionization (MALDI-MS) in the negative mode was performed using a Lasermat 2000 time-of-flight mass spectrometer (Thermo Finnigan). For MALDI-MS, lipid A was dissolved in chloroform at a concentration of 50–500 μg/ml and a saturated solution of n-octanamide (Sigma-Aldrich, St. Louis, MO) in chloroform was used as matrix. An equal volume of sample and matrix was mixed and 1 μl was loaded onto the sample plate. The plate was then dried at room temperature and introduced into the mass spectrometer. Spectra are the average from 10 pulses from a nitrogen laser (337 nm), and the extraction potential used was 20 kV.

Virulence assays

Virulence of the various strains was evaluated by testing their ability to invade HeLa cells, to form plaques on confluent Caco2 cells (33, 34), and to induce keratoconjunctivitis in Guinea pigs (35).

In addition, a total of 16 New Zealand White rabbits weighing 2.5–3 kg (Charles River Breeding Laboratories, Wilmington, MA) were used for experimental infections. From each of these animals, nine intestinal ligated loops, each of 5 cm, were prepared, as previously described (36, 37). Within each loop, 105 bacteria were injected in 0.5 ml of isotonic saline. Following 8 h of infection, animals were sacrificed. In the eight rabbits that were used to test the msbB mutants in comparison to the wild-type M90T strain and the noninvasive BS176 strain, the exudates contained within the loops were aspirated, measured, and frozen at −80°C before assay for TNF-α activity. The loops were dissected, longitudinally opened, and fixed in 4% buffered formalin or zinc sulfate buffer before histopathological analysis. According to this protocol, each strain was tested an average of 12 times (i.e., 9 loops × 8 rabbits/6 different strains).

Among the eight rabbits that were used to evaluate the effect of TNF-α neutralization, 30 min before the laparotomy was started four animals were injected i.v. with a murine IgG1 mAb (23H1) that neutralizes rabbit TNF-α activity at a concentration of 5 mg/kg. When injected at this concentration in rabbits, 23H1 completely neutralizes TNF-α cytotoxic activity appearing in serum 1–2 h following injection of 10 mg of LPS from Salmonella enterica Minnesota Re595 (J. Mathison and R. J. Ulevitch, unpublished data). The four control rabbits received 5 mg/kg i.v. of a nonrelated, anti-γ-aminobutyric acid receptor monoclonal antibody (Oriental Yeast, Aizuwa, Japan). Following sacrifice of the animals after an 8-h infection, collection and measurement of intestinal fluid and of tissue samples were conducted as above. According to this protocol, the wild-type strain M90T was tested to be an average of 25 times in each of the two situations (i.e., TNF-α neutralization and control), because the noninvasive strain BS176 was introduced in at least two loops per rabbit as a negative control.

Histopathological analysis of tissue samples

Intestinal biopsies fixed in 4% buffered formalin were dehydrated, embedded in paraffin, and sectioned in 5-μm slices. As previously described (20), sections were used for H&E staining or for LPS immunostaining. Bacterial LPS was labeled by using a primary murine mAb anti-S. flexneri 5a LPS (6 μg/ml; A. Phalipon, Institut Pasteur, Paris, France). Intestinal biopsies were incubated in zinc sulfate buffer at 4°C for 5 days were embedded in low-temperature paraffin (melting point, 37°C) from which 4-μm sections were prepared and labeled with primary murine mAb anti-rabbit CD14 clone CD14 clone 1116, 1106 (8 μg/ml) (38). Slides were washed three times in PBS, then the secondary biotinylated goat-anti mouse Ig (A0433, DAKO, Glostrup, Denmark) diluted 1/400 was added for 1 h. Slides were washed in PBS and incubated in the presence of streptavidin-HRP conjugate (DAKO) for 45 min. For anti-CD14 immunohistochemistry, a pyridine signal amplification step was added (NEN Life Sciences, Boston, MA). The color reaction was developed by addition of H2O2 and aminoethylcarbazole (Sigma-Aldrich). Sections were counterstained with hematoxylin.

TNF-α production by stimulated human adherent monocytes

Human PBMCs were isolated by density gradient centrifugation with low-calcium Ficoll-Hypaque (Amersham Pharmacia Biotech, Orsay, France). The mononuclear cells were resuspended at 2 × 106 cells/ml in 0.5% FCS-RPMI 1640 medium and 10 ml of the cell suspension were added to petri dishes and left to adhere for 1 h at 37°C. The nonadherent cells were washed out with RPMI 1640, and 10 ml of 5% FCS-RPMI medium were added to the adherent cells. These cells were then incubated for 18 h at 37°C. Adherent monocytes were scraped using EDTA (10 mM), centrifuged, and resuspended in fresh RPMI 1640. After counting, cells were incubated at 37°C for 4 h with 10% or 100% of the suspension of this suspension was added to each well of 24-well tissue culture plates coated with 100 μl of human normal serum type AB (Sigma-Aldrich). Cells were allowed to adhere for 2 h at 37°C. Bacteria were grown in tryptic soy broth to stationary phase and adjusted to 108 CFU/ml in 2% human normal serum-RPMI medium, and dilutions of the bacterial suspensions were used to infect adherent monocytes. After 4 h of infection at 37°C, the culture media were harvested and centrifuged, and the supernatants were assayed for the presence of TNF-α using a specific ELISA (Amersham Pharmacia Biotech).
TNF-α bioassay

Supernatants recovered from centrifuged intestinal exudates were filtered and assayed for bioactivity using the TNF-α-sensitive murine fibrosarcoma cell line WEHI 164, clone 13, as previously described (39, 40). The cytotoxic activity of the tested supernatants could be inhibited by coincubation with a polyclonal Ab directed against rabbit TNF-α (38800; BD PharMingen, Le Pont de Claix, France).

Statistical analyses

The nonparametric Mann-Whitney test was used for two-way comparison and determination of the statistical significance of the differences in absolute values. Values of p < 0.05 were considered statistically significant and are marked on figures as * or **.

Results

Construction of S. flexneri msbB mutants and characterization of their lipid A

To investigate the role of the lipid A modifications on the intensity of the inflammatory response induced by S. flexneri upon infection of an epithelium, we decided to use lipid A mutants in which the hydroxy-myristate linked to the 3′ position of the glucosamine disaccharide would not be modified by acyl-oxyacycl linkage of a myristate molecule. Analysis of the complete sequence of the virulence plasmid pWR100 had revealed the presence of an open reading frame, the product of which exhibited 69 and 30% sequence identity with the MsbB and HrB proteins of E. coli, respectively (4). This suggested that the virulence plasmid encodes a protein endowed with an MsbB-like activity. This plasmidic gene was designated msbB2 and the chromosomal gene was designated msbB1. To analyze the respective contributions of each of these two genes in the modification of lipid A, we constructed strains in which either msbB1 or msbB2, or both genes, had been inactivated and used the following strains: M90T, the wild-type strain; BS176, a derivative of M90T that had been cured of pWR100 and therefore lacked the msbB2 gene; SC575, a derivative of BS176 in which the msbB1 chromosomal gene was inactivated; SC574 and SC576, two derivatives of M90T in which either the chromosomal msbB1 or the plasmidic msbB2 genes, respectively, were inactivated; SC577, a derivative of M90T in which both msbB1 and msbB2 were inactivated; and SC578, a derivative of SC577 carrying a plasmid expressing a wild-type copy of msbB2 from a constitutive promoter. The lipid A obtained by mild acidic hydrolysis of the LPS prepared from the different strains was analyzed by ESI-MS and MALDI-MS. The wild-type strain M90T had a dominant (M−H−) ion at m/z 1716 that corresponds to a monophosphorylated lipid A containing six acyl groups linked to the disaccharide. The structure of this ion was assumed to contain hydroxymyristic acid at C2′, C3′, C2, and C3, which was further esterified with myristic acid at C3′ and lauric acid at C2′ (Fig. 1), as previously proposed (41). In contrast to that of the wild-type strain, the lipid A of strain SC575 (msbB1 pWR100−) showed a dominant (M−H−) ion at m/z 1506 and a smaller ion at m/z 1279. The ion at m/z 1506 corresponds to a lipid A containing five acyl groups, with hydroxymyristic acids at C2′, C3′, C2, and C3, where one hydroxymyristic acid is further esterified with lauric acid. The ion at m/z 1279 corresponds to a lipid A containing four acyl groups. As indicated in Table I, strains SC574 (msbB1), SC576 (msbB2), and BS176 (pWR100−) had a dominant (M−H−) ion at m/z 1716 and ions with lower relative intensity at m/z 1506 and 1279, whereas SC577 (msbB1 msbB2) had a dominant (M−H−) ion at m/z 1506. The absence of N-acetyl-glucosamine (GlcN2HPO3(14:O3-0H)(2)(14:O3-O(14:O))(14:O3-O(12:O))) in the lipid A of SC575 (msbB1 pWR100−) reflected the lack of acyl-oxy-acylation by myristic acid in C3′ of lipid A. This indicated that there was a single copy of the msbB gene on the chromosome of S. flexneri. The relative proportions of (GlcN2HPO3(14:O3-O(14:O))(14:O3-O(12:O))) among the different fatty-acyl groups produced by M90T (wild-type) and BS176 (pWR100−) were 93 and 63%, respectively, which indicated that saturation of the lipid A was almost complete in the presence of the virulence plasmid and only partial in its absence. This suggested that the msbB2 gene carried by the virulence plasmid was expressed and encoded a protein endowed with acyl-oxy-acycl-transferase activity. Indeed, the lipid A of the msbB1 derivative of M90T (SC574) still contained 59% of (GlcN2HPO3(14:O3-OH)(2)(14:O3-O(14:O))(14:O3-O(12:O))), a product that was longer present in the lipid A produced by SC575 (msbB1 msbB2). Furthermore, SC576 (msbB1) exhibited a significant decrease in the percentage of (GlcN2HPO3(14:O3-OH)(2)(14:O3-O(14:O))(14:O3-O(12:O))) as compared with the wild-type strain. The presence of the ion (M−H−) at m/z 1716 in the lipid A extracted from strains SC574 (msbB1), SC576 (msbB2), and BS176 (pWR100−) and its absence in the lipid A extracted from strains SC577 (msbB1 msbB2) and SC575 (msbB1 pWR100−) indicated that both MsbB1 and MsbB2 are endowed with some activity carrying out acylation by (14:O3-O(14:O)) in position 3′. In conclusion, the lipid A of SC577 (msbB1 and msbB2) and SC575 (msbB1 pWR100−) showed no acyl-oxy-acylation of the myristate in 3′, whereas that of SC574 (msbB1), SC576 (msbB2), and BS176 (pWR100−) had partial acyl-oxy-acylation and that of the wild-type strain M90T was fully modified.

In parallel, 5 μg of LPS extracted from strains M90T, BS176, SC574, SC575, SC576, SC577, and SC578 were run in a standard 12% SDS-PAGE followed by silver staining. This allowed us to verify that neither qualitative nor quantitative alteration had occurred in LPS O chains in the course of the mutagenesis experiments (data not shown).

Effect of msbB mutations on S. flexneri interaction with epithelial cells and monocytes

To investigate the effect of the lipid A modification on the efficiency of entry of S. flexneri, the various mutants were tested in the plaque assay on Caco-2 cells (34). Derivatives of M90T in which either msbB1 or msbB2, or both genes, were inactivated induced the formation of the same number of plaques as compared with the wild-type strain, which indicated that the mutants had no defect in their ability to enter into epithelial cells. Likewise, the size of the plaques produced by the mutants was similar to the size of those produced by the wild-type strain, indicating that the mutants were not impaired for cell-to-cell spread (data not shown). The production of TNF-α by human monocytes from peripheral blood was used as a read-out for expression of endotoxicity. Monocytes were incubated in the presence of bacteria at various multiplicities of infection (MOI) for 4 h, and the amount of TNF-α released in the

Table I. The relative amounts of lipid A from S. flexneri containing different fatty acyl groups as determined by negative ESI-MS

<table>
<thead>
<tr>
<th>Strain</th>
<th>m/z 1716 (%)</th>
<th>m/z 1506 (%)</th>
<th>m/z 1279 (%)</th>
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<tr>
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<td>93</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>SC574</td>
<td>59</td>
<td>34</td>
<td>7</td>
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<td>85</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>BS176</td>
<td>63</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>SC575</td>
<td>0</td>
<td>87</td>
<td>13</td>
</tr>
</tbody>
</table>

*a* (GlcN2HPO3(14:O3-OH)(2)(14:O3-O(14:O))(14:O3-O(12:O)))

*b* (GlcN2HPO3(14:O3-OH)(3)(14:O3-O(12:O)))

*c* (GlcN2HPO3(14:O3-OH)(2)(14:O3-O(12:O)))
medium was measured by ELISA as a read-out for stimulation. The dose-response curves obtained with the invasive strains M90T (wild-type) and SC577 (msbB1 msbB2) and the noninvasive strains BS176 (pWR100−) and SC575 (msbB1 pWR100−) are shown in Fig. 3. At MOI 2 (10⁴ CFU), all the strains induced the release of similar amounts of TNF-α by the infected monocytes; however, at lower MOI, differences appeared between the strains that produced a modified lipid A, such as M90T (wild-type) and BS176 (pWR100−), and those that produced unsaturated lipid A, such as SC575 (msbB1 pWR100−) and SC577 (msbB1 msb2), and reached statistical significances for MOI 0.02 (10⁴ CFU) and 0.002 (10³ CFU). This indicated that differences in modification of the lipid A rather than in the invasive abilities of the mutants were responsible for the decreased stimulation. For each strain, the stimulating dose 50 (SD₅₀), i.e., the number of bacteria that was required to cause 50% of the maximal release of TNF-α, was calculated (see insert in Fig. 3). The various strains could thus be grouped into three classes: 1) M90T (wild-type), BS176 (pWR100−), and SC578 (msb1 msbB2 pMsbB2), with SD₅₀ values of 4−7 × 10⁴; 2) SC574 (msbB1) and SC576 (msbB2), with SD₅₀ values of 20−30 × 10⁴; and 3) SC575 (msbB1 pWR100−) and SC577 (msbB1 msbB2), with SD₅₀ values of 80 × 10⁴.

**Evaluation of the effect of msbB mutations on Shigella pathogenicity in vivo**

Before proceeding to rabbit infections, msbB mutants were tested in the classical keratoconjunctivitis assay in Guinea pigs (Sereny test, Ref. 35). Although detailed quantification of inflammation is difficult in this test, compared with the wild-type strain M90T, which caused severe keratoconjunctivitis in 48 h, the msbB mutants, particularly SC577 (msbB1 msbB2), caused delayed (i.e., 72−96 h) and significantly weaker tissue reaction. This indicated that endotoxin affects the degree of inflammation in this test. However, compared with the negative reaction observed with the noninvasive strain BS176, it was clear that these mutants were still able to cause significant damage to the invaded conjunctival and corneal epithelium, thus the need for further analysis in the rabbit ligated intestinal loop model of experimental shigellosis.

In this assay, severity of tissue inflammation can be evaluated by recording qualitative and quantitative alterations of the intestinal mucosa. Infections of ligated loops by each of the strains described above were conducted for 8 h. The amount of fluid exudate in infected loops, shown as volume/length (V/L) and the concentrations of TNF-α in these samples were recorded. Histopathological alterations were quantified and recorded as the average ratio between the length and the width of the villi that measures villus atrophy on hematoxylin-eosin-safranin (HES)-stained tissue sections (9) and the average number of abscesses disrupting the epithelial lining on tissue sections stained with an anti-LPS serum (9). These data are shown in Fig. 4. The wild-type strain M90T caused maximum fluid accumulation (0.59 ± 0.2 ml/cm), whereas the noninvasive strain BS176 barely caused fluid production (0.05 ± 0.01 ml/cm), and the amount of fluid elicited by SC577 (msbB1 msbB2) was one-third of that elicited by M90T (both values were significantly different from M90T), while the mutants SC574 (msbB1) and SC576 (msbB2) and the complemented strain SC578 (msbB1 msbB2 pMsbB2) elicited intermediate values. The acute villus atrophy caused by M90T (wild type) was reflected by a length-width (L:W) ratio of 1.7, in contrast to the absence of atrophy caused by BS176 (pWR100−), which was reflected by a L:W ratio of 8. SC577 (msbB1 msbB2) appeared strongly attenuated, as reflected by a L:W value of 5.8, and the degrees of atrophy caused by SC574 (msbB1), SC576 (msbB2), and SC578 (msbB1 msbB2 pMsbB2) were intermediate between those caused by M90T and SC577 (msbB1 msbB2). Compared with M90T, L:W ratios reached significant difference for BS176, SC576, SC577, and SC578. Similarly, the density of foci of epithelial rupture by abscesses was highest (80%) following infection by M90T, whereas no significant lesions were observed with BS176. SC577 (msbB1 msbB2) showed low density of lesions, and the single mutants, as well as SC578 (msbB1 msbB2 pMsbB2), again caused intermediate values. All values appeared significantly different from M90T, with SC577 itself being significantly lower than single mutants. Qualitatively, as detailed below, lesions caused by SC577 (msbB1 msbB2), SC574 (msbB1), and SC576 (msbB2) corresponded essentially to limited areas of rupture of the epithelial lining and not to extensively destructive mucosal abscesses, as observed with M90T. Finally, M90T caused the highest concentration of TNF-α in the collected fluid samples and SC577 caused the lowest concentration (one-third of M90T, 80% statistically significant difference), while the single mutants and SC578 caused intermediate concentrations.

Histopathological analysis supports the quantitative data provided above, HES staining showing alterations to villi as illustrated in Fig. 5. In the characteristic lesions observed after infection by the wild-type strain M90T (Fig. 5A), the villi were shortened and enlarged in cases with tissue necrosis, leaving crypts as the only identifiable epithelial structure. Often, the lamina propria appeared enlarged with the lamina propria swollen by a significant edema. In addition, submucosal tissues were strongly edematous, with a large area infiltrated by inflammatory cells between the residual muscularis mucosae and the muscular layer. The most salient feature, in all circumstances, was the rupture of the epithelial lining. In contrast, intestinal villi of loops infected with the noninvasive strain BS176 appeared long and narrow, with no evidence of rupture of the epithelial lining, no significant infiltrate of the lamina propria, and no submucosal edema (Fig. 5B). The msbB1 and msbB2 mutants caused an intermediate phenotype with regard to tissue alterations. In intestinal loops infected by the msbB1 mutant (SC574, Fig. 5C) or the msbB2 mutant (SC576, Fig. 5D), the villi appeared shortened and enlarged with the lamina propria swollen by a significant inflammatory infiltrate. The epithelial lining was rarely disrupted; however, it was very often indented, leading to multiple zones of constriction of the villi. Variable submucosal edema was observed. The msbB1 msbB2 mutant (SC577, Fig. 5E) showed a dramatic decrease in the pattern of pathology. Villi remained in general of a length and a width very similar to those observed following infection with the noninvasive strain BS176. A limited edema was observed with few cells infiltrating the lamina propria and, most

![Figure 3](http://www.jimmunol.org/)  
**FIGURE 3.** Production of TNF-α by adherent human monocytes infected at increasing MOI by wild-type S. flexneri and mutants. For the 10³ and 10⁴ inocula, differences in average levels of TNF-α production reached significance when M90T and BS176 were compared with SC575 and SC577 (marked by *).
importantly, no rupture of the epithelial lining was ever observed. Conversely, the complemented strain SC578 recovered its capacity to cause major alteration in the villi characterized by areas of rupture of the epithelial lining (Fig. 5F), although these lesions were rather scarce.

Immunostaining to CD14 reveals cells of myeloid lineage, particularly resident macrophages, recruited monocytes, and PMNs (42). Used in this study, this illustrated a major aspect of Shigella pathogenesis, namely the recruitment of inflammatory cells, their crossing of the epithelial lining, and, in many places, the rupture and destruction of the epithelium. Areas of epithelial rupture and destruction caused by the wild-type strain M90T were characterized by a massive influx of CD14-positive cells including PMNs (arrowheads) and mononuclear cells, most probably recruited monocytes (Fig. 6, A and B). In contrast, as shown in Fig. 6, G and H, infection by the noninvasive strain BS176 did not elicit detectable recruitment of CD14-positive cells. Minor staining was associated with vessel walls in the lamina propria, but the intact epithelial lining was devoid of any inflammatory infiltrate. Interestingly, as shown in Fig. 6, C and D, in intestinal tissues infected by SC577 (msbB1 msbB2), CD14-positive cells were recruited in the lamina propria and immediate subepithelial areas of infected villi. However, two salient features characterized the lesions: 1) the almost total lack of recruited PMNs and 2) the lack of epithelial rupture. This attenuated phenotype was not observed with strain SC578 (msbB1 msbB2 pMsbB2) and, although the epithelial and mucosal destruction did not reach the level observed with M90T, the two characteristic features were reacquired (Fig. 6, E and F): 1) epithelial rupture and, in some villi, large areas of epithelial necrosis, and 2) presence of PMNs in the inflammatory infiltrate (arrowheads) associated with epithelial rupture or destruction.
Bacteria were detected by LPS staining. As shown in Fig. 7, A and B, a massive amount of bacteria and bacterial LPS was associated with the lamina propria and the epithelium, particularly in areas of rupture/destruction of the epithelial lining following infection with M90T. Under similar conditions, BS176 showed no invasion at all (data not shown). In intestinal loops infected either by SC574 (msbB1) (Fig. 7, C and D) or by SC576 (msbB2) (Fig. 7, E and F), an interesting pattern of staining was observed that matched the previous observations and provided better characterization of the impact of lipid A toxicity on the development of the infectious process. Single msbB1 or msbB2 mutants caused less tissue damage, as reported above, but the numbers of invasive bacteria appeared similar, compared with the wild-type strain M90T. Bacteria were seen in the lamina propria but also in large amounts inside epithelial cells. Indentation of the epithelium that was reported above (i.e., HES staining) corresponded to the development of infectious foci. This indicates that infected tissues, both the epithelial layer and the lamina propria, are more tolerant to the presence of invasive microorganisms, thus limiting the rupture/destruction of the intestinal barrier. Moreover, following invasion by SC577 (msbB1 msbB2), an even more striking pattern appeared (Fig. 7, G and H) in which bacteria were observed both in the lamina propria and in epithelial cells, in the absence of significant lesion, indicating dramatic tolerance of the infected intestinal barrier to invasive pathogens. As shown in Fig. 7, I and J, this phenotype of tolerance was reversed when the trans-complemented strain SC578 (msbB1 msbB2 pMsbB2) was used. In conclusion, histopathological analysis of infected rabbit intestinal tissues indicates that genetic attenuation of lipid A endotoxicity causes dramatic attenuation of pathogenicity that is particularly characterized by the lack of rupture/destruction of the epithelial lining.

**TNF-α as a major effector of endotoxin-mediated rupture of the epithelial barrier**

To evaluate the importance of TNF-α in the rupture and destruction of the epithelial barrier, we compared the parameters of fluid production (V/L), acute villi atrophy (L/W), average number of areas of epithelial rupture/destruction per villus unit, and histopathological lesions in control rabbits infected for 8 h with M90T and rabbits also infected with M90T, in which TNF-α was neutralized. Fig. 8 shows data from four control rabbits and an equivalent number of rabbits in which TNF-α was neutralized. In both groups, BS176 induced neither fluid in the loops nor significant acute villi atrophy or epithelial alteration. Control animals infected with M90T showed V/L values ranging between 1.14 ± 0.14 and 0.38 ± 0.09 ml/cm, L/W values ranging between 1 and 2.3, and the average number of epithelial rupture/destruction per individual villus ranging between 40 and 100%. In contrast, animals in which TNF-α was neutralized showed dramatic attenuation of the symptoms of mucosal injury with V/L values ranging between 0.058 ± 0.04 and 0.29 ± 0.13, L/W values ranging between 6.5 and 7.2, and the average number of epithelial rupture/destruction per individual villus ranging between 15 and 3%.
In agreement with the above quantitative data, histopathological studies demonstrated that, following neutralization of TNF-α/H9251, intestinal lesions were limited compared with control conditions. Interestingly, the pattern of lesions observed was very similar to that observed following infection with strain SC577. HES staining confirmed that, in the absence of TNF-α neutralization, villi were shortened and enlarged, with massive cellular infiltrate of the lamina propria and many areas of epithelial rupture or destruction (Fig. 9, A and B, arrowheads). In addition, the submucosal tissue appeared massively edematous with numerous mono- and polymorphonuclear cells adhering to the vessel wall on their way to diapedesis (Fig. 9A, arrow). In contrast, following infection with M90T in animals treated with the anti-TNF-α-neutralizing mAb, villi showed the typical appearance observed with mshB mutants, i.e., the epithelial lining was rarely disrupted, although often indented, leading to multiple constrictions of the villi (Fig. 9, C and D, arrowheads). The lamina propria in villi was consistently edematous and variable submucosal edema was observed.

**FIGURE 8.** Alterations observed in the rabbit intestinal ligated loop model following 8 h of infection by *S. flexneri*. A, Average volume of inflammatory fluid in infected loops (milliliters per centimeter). B, Intensity of acute villus atrophy (average values of the ratio between the length and width of 100 villi counted on each among at least five different loops). C, Density of abscesses disrupting or destroying the epithelial lining (percentage of individual lesions per 100 villi counted on each among at least five different loops). Rabbits 33, 37, 39, and 44 are controls injected with a nonrelevant mAb. Rabbits 32, 36, 38, and 43 are injected with the TNF-α-neutralizing mAb.
Immunostaining of bacteria with anti-LPS serum, again, showed striking similarities between the pattern of tissue invasion observed with M90T following neutralization of TNF-α/H9251 and with msbB mutants. As seen in Fig. 10, A and B, bacterial invasion was associated with rupture/destruction of the epithelial lining (arrowheads), whereas in animals in which TNF-α has been neutralized the continuity of the epithelial lining was preserved and, again, both the lamina propria and infected epithelial cells appear to tolerate the bacteria without eliciting destructive inflammation.

Discussion

LPS, the amphiphilic molecule that participates in and extends out of the outer membrane of Gram-negative bacteria (43), contributes to the pathogenesis of Shigella both through its hydrophobic end, the lipid A that bears the endotoxin activity, and its hydrophilic end, the core and O-antigenic polysaccharide that provides the bacteria with resistance to host defense factors such as complement lysis and complement-mediated opsono-phagocytosis (44, 45). E. coli-S. flexneri hybrids require full expression of O side chains to express a fully invasive phenotype in vivo, both in Guinea pig keratoconjunctivitis and in the rabbit ligated ileal loop assays (37), and bacterial mutants that are affected in the core and O side chain (i.e., rfa, rfb, and rol) are severely impaired in invasion in the keratoconjunctivitis assay (46, 47). In contrast, little is known about the role of lipid A in the Shigella disease process, particularly how its endotoxin activity affects the inflammatory destruction of the intestinal epithelial barrier that characterizes bacillary dysentery. Neutralization of CD14, a component of the LPS-sensing complex (20), in the course of experimental shigellosis in the rabbit ligated intestinal loop model provided a paradoxical result because, instead of a decrease in tissue damage, we observed increased numbers of bacteria invading mucosal tissues and more severe tissue damage. These data suggested that endotoxin recognition via CD14 acts as a warning that indicates intrusion of a Gram-negative microbe into host tissues. Interference with this sensory signaling process may retard the innate immune response, thereby accounting for uncontrolled bacterial growth. However, parallel increase in tissue lesions remained a puzzling observation that may reflect the Shigella invasive phenotype by a greater number of bacteria facing the monocytes/macrophages of the intestinal barrier, thereby causing the release of IL-1β upon caspase-1 activation (11, 48) and early drop in production of IL-1R antagonist (10). Endotoxin may also signal through pathways independent of CD14, particularly once the increased, unchecked mass of shigellae has reached the cytoplasmic compartment of...
epithelial cells (12, 13). This result emphasized the complexity of the signaling cascades that support the innate response and was a strong incentive to directly alter the endotoxic level of the lipid A. This has been made possible following the identification of the genes and enzymes that carry out lipid A synthesis (21, 22).

However, despite several contributions comparing the effect of purified LPS or synthetic lipid A preparations of variable endotoxic level in in vitro assays or in various animal models of septic shock or organ damage (49–51), still few studies have reported the effect of genetically attenuating lipid A endotoxic activity on the pathogenicity of the mutant bacteria in animal models. A mutation in the waaM (hrbB) gene of *Salmonella typhimurium* severely altered the capacity to cause systemic dissemination and to colonize organs in mice. However, the conditional, temperature-dependent lethality of this mutation may have accounted for impairment (52). More recently, following discovery of the msbB gene function (23), a *S. typhimurium* waaN (msbB) mutant administered systemically to mice showed higher LD<sub>50</sub> and reached higher numbers in organs, confirming that death in the murine typhoid fever model depends greatly on the endotoxin activity of lipid A (53). TNF-α, IL-1β, and inducible NO synthase are likely mediators of lipid A-dependent lethality. Similarly, a msbB mutant of *E. coli* was tested systemically in mice. In an *E. coli* K-12 background the attenuating effect of the mutation was limited. When transduced into a wild-type, encapsulated isolate, the msbB mutation considerably increased the LD<sub>50</sub>, showing that the msbB gene product is an important virulence factor. However, it should be emphasized that the msbB mutation also resulted in filamentation at 37°C and reduction in expression of the capsule, indicating that this mutation has a pleiotropic effect that may contribute to virulence attenuation (54). In contrast, another msbB mutant of *E. coli* showed no alteration in membrane permeability barrier (55). Similarly, none of our *Shigella* msbB mutants expressed significant defect in growth or division and no filamentous phenotype was observed. Furthermore, the mutation did not affect their pathogenic behavior in an in vitro assay system, unlike mutations in the LPS core and O side chains, which severely affect actin-dependent intracellular motility and cell-to-cell spread (56). In vitro, LPS from an *E. coli* hrbB msbB mutant induces a high level of macrophage-inflammatory protein-1α/Q, without induction of TNF-α and IL-1β (57), indicating that, in addition to attenuating the endotoxicity, these attenuated LPS may also modulate the innate response.

Only one study has evaluated the impact of a msbB mutation on the capacity of *S. typhimurium* to cause enteritis in the rabbit ligated ileal loop model (58). This study showed that introduction of the msbB mutation had no significant effect on the mutant’s capacity to cause enteritis because neither the amount of fluid secretion nor the severity of villus alterations was affected, compared with the wild-type control. Similarly, a sstA mutation (i.e., SPI2 mutation) did not significantly affect the severity of enteritis. In contrast, an invA mutation (i.e., SPI1 mutation) caused a dramatic decrease in the severity of enteritis. This indicated that, in *Salmonella*, SPI1, but not SPI2, is essential to cause enteritis, and that there is a very little role for the lipid A. These results are inconsistent with the results observed with *Shigella*, because we have shown a clear attenuating effect of the msbB mutation on the major parameters of enteritis: fluid production, acute villus atrophy, and inflammatory rupture/destruction of the epithelium. This difference may reflect the different kinetics of infection used in the respective studies, because *Salmonella* infection was conducted for 18 h, whereas *Shigella* infection was conducted for only 8 h. It is likely that an 18-h infection course introduces a time effect that minimizes the effect of mutations that only partially affect enteritis.

Alternatively, the discrepancy may point to intrinsic differences between *Shigella* and *Salmonella*. In the course of *Salmonella* infection, flagella that can be recognized through Toll-like receptor 5, thereby activating NF-kB (59), may be a front-line effector inducing enteritis, whereas in the course of infection by *Shigella* which do not produce flagella, lipid A may be a front line effector (13, 60). However, other options may be envisioned: 1) the recent demonstration that Nod1 in epithelial cells (13, 16) recognizes intracellular LPS suggests that, because it remains inside a vacuolar compartment (unlike *Shigella* that quickly escape into the cytoplasm) (34), *Salmonella* may present LPS more slowly to Nod; and 2) macrophage apoptosis is also a proinflammatory event caused by *Shigella* infection (11) following the activation of caspase-1 that leads to the release of mature IL-1β. Pretreatment of macrophage with LPS before challenge with invasive *Shigella* considerably increases the release of mature IL-1β (48).

*Shigella* possesses two msbB genes, msbB1 located on the chromosome and msbB2 located on the 214-kb virulence plasmid. This is also observed in enterohemorrhagic *E. coli* (EHEC), where a second msbB gene is present on the virulence plasmid of serotype O-157 (61, 62). No function has been attributed so far to this extra copy of the msbB gene, which is not present on the enteropathogenic *E. coli* virulence plasmid (63). EHEC are extracellular microorganisms that express their pathogenic potential by binding intimately to the apical brush border of intestinal epithelial cells and secreting Shiga-like toxins (64). Therefore, the presence of an additional msbB gene is not related to an intracellular mode of life. In contrast, both *Shigella* and EHEC, unlike enteropathogenic *E. coli*, express their pathogenic potential in the colon and cause infection at very low inoculum. Future research will tell whether this particular ecosystem represents a selective pressure for maintenance of an extra copy of this gene and whether its regulation responds to particular environmental conditions similarly to *Salmonella*, in which msbB expression is regulated by phoPQ (65). The tolerance of important amounts of invasive shigelae harboring the two msbB1 and msbB2 mutations (SC577) without major inflammatory rupture/destruction of the epithelial lining suggested a significant role for lipid A endotoxicity in the disease process and raised the question of which factor produced in the presence of wild-type *Shigella* may account primarily for intestinal destruction. There is growing evidence in murine models (66) as well as in new therapeutic approaches of inflammatory bowel diseases such as Crohn’s disease, using receptor or neutralizing mAb therapy (67), that TNF-α plays a central role in causing the destructive inflammatory lesions. In addition, high concentrations of TNF-α have been found in the stools of children with *Shigella* infection (68). Therefore, we studied whether inhibition of TNF-α, in the course of experimental *Shigella* infection in the rabbit, could control the lesions. We observed that neutralization led to a decrease in the lesions that mimicked the situation observed when infection was conducted with the msbB1 msbB2 mutant of *S. flexneri*, essentially the loss of epithelial rupture/destruction, despite the presence of numerous foci of infection, thus indicating that TNF-α is a major mediator of lipid A-induced epithelial rupture in the course of experimental shigelliosis.

These observations have several implications. Anti-TNF therapy may be considered an option to control the severe forms of acute bacterial colitis such as shigellosis. This is a theoretical concept, as shigellosis occurs essentially in the poorest areas of the world where costly immuno-interventions are not available. In contrast, we are currently introducing msbB mutations in live attenuated vaccine candidates against *Shigella*, as phase 1 trials have shown 9% reactivity in naïve western volunteers, essentially short-term fever and intestinal discomfort, that are likely to reflect residual
proinflammatory capacity of these strains (69). The phenotype of virulence attenuation that has been observed in this study following introduction of mutations in the two msbB genes may respond to the need of a less reactivogenic oral vaccine.

Acknowledgments
We thank Catherine Fittig (Institut Pasteur) for help in the titration of TNF-α bioactivity, Josette Arondel for expert technical help, Michel Huere for permanent interest in this work, and Colette Jacquemin for editing this manuscript.

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