INFECTION AND IMMUNITY, Aug. 1996, p. 3385–3393 Vol. 64, No. 8
0019-9567/96/$04.00+0

The *Salmonella* Virulence Plasmid Enhances *Salmonella*-Induced Lysis of Macrophages and Influences Inflammatory Responses

LAURENCE A. GUILLOTEAU,[1]† TIMOTHY S. WALLIS,[2] ANNE V. GAUTIER,[1]‡
SHEILA MACINTYRE,[1] DAVID J. PLATT,[3] AND ALISTAIR J. LAX[2]*

School of Animal and Microbial Sciences, University of Reading, Reading,[1] and Institute for Animal Health, Compton, Nr Newbury,[2] Berkshire, and Department of Bacteriology, Glasgow Royal Infirmary, Glasgow,[3] United Kingdom

Received 29 January 1996/Returned for modification 4 April 1996/Accepted 23 May 1996

The *Salmonella dublin* virulence plasmid mediates systemic infection in mice and cattle. Here, we analyze the interaction between wild-type and plasmid-cured *Salmonella* strains with phagocytes in vitro and in vivo. The intracellular recovery of *S. dublin* from murine peritoneal and bovine alveolar macrophages cultured in the presence of gentamicin in vitro was not related to virulence plasmid carriage. However, the virulence plasmid increased the lytic activity of *S. dublin*, *Salmonella typhimurium*, and *Salmonella choleraesuis* for resident or activated mouse peritoneal macrophages. Lysis was not mediated by *spv* genes and was abolished by cytochalasin D treatment. Peritoneal and splenic macrophages were isolated from mice 4 days after intraperitoneal infection with wild-type or plasmid-cured *S. dublin* strains. The wild-type strain was recovered in significantly higher numbers than the plasmid-cured strain. However, the intracellular killing rates of such cells cultured in vitro for both *S. dublin* strains were not significantly different. Four days after infection, there was a lower increase of phagocyte numbers in the peritoneal cavities and spleens of mice infected with the wild-type strain compared with the plasmid-cured strain. The virulence plasmid influenced the survival of macrophages in vitro following infection in vivo as assessed by microscopy. Cells from mice infected with the plasmid-cured strain survived better than those from mice infected with the wild-type strain. This is the first report demonstrating an effect of the virulence plasmid on the interaction of *Salmonella* strains with macrophages. Plasmid-mediated macrophage dysfunction could influence the recruitment and/or the activation of phagocytic cells and consequently the net growth of *Salmonella* strains during infection.

Salmonella enterica is the etiological agent of different clinical forms of infection designated salmonellosis (24). The virulence of *Salmonella* strains is highly complex, requiring the expression of numerous genes, some of which are located on large plasmids, which are generally serotype associated (3, 19). An 8-kb region common to plasmids from different serotypes is required for the expression of virulence in vivo (4, 38, 39), and this system contains five open reading frames designated *spv* genes (*Salmonella* virulence plasmid) (16).

The role of the virulence plasmid in the pathogenesis of salmonellosis is still not understood. In mice, the virulence plasmid is not required for translocation of salmonellas through the intestinal mucosa but is necessary in the systemic phase of the disease (18, 25, 26). In cattle, the virulence plasmid is necessary for the persistence of *Salmonella dublin* in systemic sites but is not required during the enteric phase of infection (37). Virulence plasmid functions are not involved in the regulation of lipopolysaccharide (LPS) biosynthesis (23, 33), expression of major outer membrane proteins (37), resistance to serum killing (32), or invasion and growth in nonprofessional phagocytic cell lines (15). It has been suggested that plasmid genes promote intracellular growth of *Salmonella* strains (17). Although the intracellular environment of phagocytic and nonprofessional phagocytic cells induces the expression of *spv* genes (12, 28), in vitro studies have not shown any relationship between carriage of the virulence plasmid by *Salmonella* strains and survival within macrophages (10, 15, 29).

There is no direct evidence that virulence plasmid functions are directly related to survival of salmonellas within phagocytes in vivo. In mice, wild-type salmonellas induce a strong inflammatory response as shown by marked splenomegaly and hepatic abscess formation (21), whereas plasmid-cured strains induce a mononuclear response in the liver, mediated by radio-

TABLE 1. *Salmonella* strains used in this study

Strain	Properties and/or derivation	Reference
SD2229	Wild-type *S. dublin* strain from an outbreak of bovine salmonellosis	25
SDM173c	Virulence plasmid-cured derivative of SD2229	25
SDM51	Tn*A* insertion mutant of SD2229 in *spv* fragment	25
SDM173(pCRV4)	8-kb *Sal*I-*Xho*I fragment	39
ST12/75	Wild-type *S. typhimurium* strain from an outbreak of bovine salmonellosis	3
STM8c	Virulence plasmid-cured derivative of ST12/75	39
SCS14/74	Wild-type *S. choleraesuis* strain from an outbreak of porcine salmonellosis	36
SCS14/74(Tp231)	Virulence plasmid-cured derivative of SCS14/74	This study

* Correspondent footnote. Mailing address: Institute for Animal Health, Compton, Nr Newbury, Berkshire RG20 7NN, United Kingdom. Phone: (44) 1635 578411. Fax: (44) 1635 577263. Electronic mail address: alistair.lax@bbsrc.ac.uk.

† Present address: Laboratoire de Pathologie Infectieuse et Immunologie, Institut National de la Recherche Agronomique, 37380 Nouzilly, France.

‡ Present address: Institute for Animal Health, Compton, Nr Newbury, Berkshire, United Kingdom.

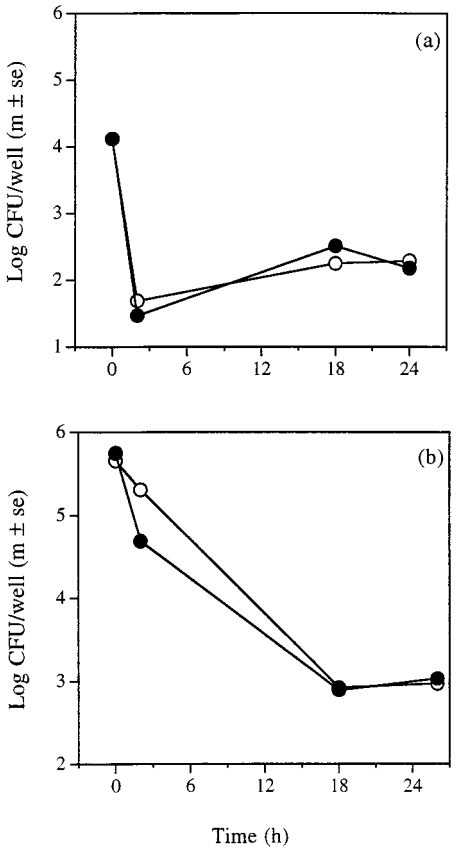


FIG. 1. Survival of *S. dublin* in murine peritoneal (a) and bovine alveolar (b) macrophages infected in vitro. Macrophages (5×10^5 per well) were infected with SD2229 (●) or SDM173c (○). Experiments were performed on 10 (a) and 2 (b) separate occasions, and representative data are expressed as the mean ± standard error of three wells per time point.

sensitive effectors (14, 18). The virulence plasmid has been implicated in immunomodulation of the host and in reducing the early expansion of γδ T cells in *Salmonella choleraesuis*-infected mice (9). However, this effect may be serotype specific since the virulence plasmid did not modulate the early T-cell response in *S. dublin*-infected mice (14).

In this study, we have analyzed the interaction of wild-type and derivative plasmid-cured strains of *S. dublin*, *Salmonella typhimurium*, and *S. choleraesuis* in murine and bovine macrophages after in vitro or in vivo infection.

MATERIALS AND METHODS

Bacterial strains. Strains of *Salmonella* used in this study are listed in Table 1. Following overnight culture in Luria-Bertani (LB) broth, bacteria were subcultured 1/100 and grown in LB broth at 37°C for 6 h with shaking to stationary phase (optical density at 550 nm of 1.6 = 10^9 CFU/ml). Ampicillin (100 μg/ml) and kanamycin (75 μg/ml) were added to media as appropriate for plasmid maintenance. Bacteria were enumerated by serial dilution and plating on MacConkey or LB agar.

Escherichia coli K-12 strain J53-2 Tp231 (NCTC 50087) was used in the plasmid curing experiment.

Plasmid curing. The *Salmonella* virulence plasmids are very stable and nonconjugative and largely belong to a single undesignated incompatibility group. pOG669 is a 147-kb natural plasmid cointegrate between the *S. typhimurium* virulence plasmid and an IncX, conjugative plasmid (pOG670) that specifies resistance to ampicillin and kanamycin (27). It exhibits dual incompatibility, and its conjugative introduction into *Salmonella* serotypes that harbor virulence plasmids results in displacement of the indigenous plasmids if selection for either of the pOG669 resistance markers is maintained (5). Accordingly, pOG669 was introduced by conjugation from *E. coli* K-12 strain J53-2 into *S. choleraesuis* under kanamycin selection (10 μg/ml). Transconjugants were purified and then maintained under this selection for three successive subcultures. The loss of the 50-kb plasmid was confirmed by agarose gel electrophoresis. Subsequent transfer of the IncX plasmid Tp231 (*E. coli* K-12 strain J53-2 Tp231) by chloramphenicol (20 μg/ml) or tetracycline (10 μg/ml) selection led to displacement of pOG669 by IncX incompatibility and establishment of Tp231. The resultant *S. choleraesuis* Tp231 lacked *spv* genes and was resistant to ampicillin, chloramphenicol, and tetracycline.

Animals. C57BL/6 male and female mice aged 7 to 8 weeks were bred in our animal facilities and used for the infection studies and as a source of macrophages. Outbred, adult Friesian cows were used as a source of alveolar macrophages.

Preparation of macrophages. Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity with 3 ml of Iscove's modified Dulbecco's medium (IMDM) (Gibco Life Technologies Ltd., Paisley, United Kingdom) containing heparin (5 U/ml). PEC were centrifuged at 600 × *g* for 10 min and suspended in IMDM containing 10% fetal calf serum (FCS) (Gibco) and 100 μg of gentamicin per ml or in phenol red-free Dulbecco's modified Eagle's medium nutrient mixture F12 Ham (DME/F12) (Sigma, Poole, United Kingdom) containing 4% FCS and 100 μg of gentamicin per ml for the cytotoxicity assay. The number and the viability of cells in the suspension were determined by trypan blue exclusion. Viability was always greater than 95%. Cells were allowed to adhere in 24-well plates (Greiner Labortechnik, Stuttgart, Germany) for 2 h to yield between 2×10^5 and 5×10^5 macrophages per well, which represented about 30% of harvested PEC. The medium was exchanged for fresh gentamicin-free medium, and cells were incubated overnight at 37°C in a 5% CO_2 atmosphere.

Resident and elicited macrophages were used for lysis experiments. Macrophages were elicited by injecting 1.5 ml of 10% (wt/vol) Proteose Peptone into the peritoneal cavities of mice and were harvested 3 days later as described above; macrophages represented about 80% of PEC. Resident macrophages were activated in vitro with *S. typhimurium* LPS (Sigma) (10 ng/ml) and gamma interferon (IFN-γ) (Sigma) (50 IU/ml) for 2 h before infection and during the assay. In some experiments, macrophages were treated with cytochalasin D (Sigma) at 1 μg/ml in culture medium, for 30 min before and during infection.

Alveolar bovine macrophages were prepared by a method modified from that described by Van Leengoed et al. (35). Lungs were removed from cattle and washed by introducing 600 ml of phosphate-buffered saline (PBS) via the trachea. The washings were collected after gentle massage, and the process was repeated twice more. Washings were filtered through muslin, and cells were harvested by centrifugation (10 min, 150 × *g*, room temperature) and washed with IMDM containing 10% FCS and 5 U of heparin per ml. Cells were resuspended in IMDM containing 10% FCS and 100 μg of gentamicin per ml at a concentration of 5×10^5 cells per ml.

Infection of murine macrophages with *S. dublin* in vitro. Resident peritoneal macrophages were used to test for intracellular persistence of *S. dublin*. Three hours before infection with *S. dublin*, macrophages were washed and overlaid with gentamicin-free medium. Macrophages were infected with *S. dublin* suspended in IMDM with 10% FCS at a ratio of five bacteria per macrophage for 1 h at 37°C in a 5% CO_2 atmosphere. Monolayers were washed three times with gentamicin-free medium, overlaid with IMDM with 10% FCS and 100 μg of gentamicin per ml to inhibit extracellular proliferation of *S. dublin*, and incubated at 37°C in a 5% CO_2 atmosphere. At different time intervals, cells were washed three times with gentamicin-free medium and three wells per bacterial strain were overlaid with 1 ml of 0.5% (wt/vol) sodium deoxycholate to lyse macrophages. The released viable *S. dublin* cells were enumerated on LB agar.

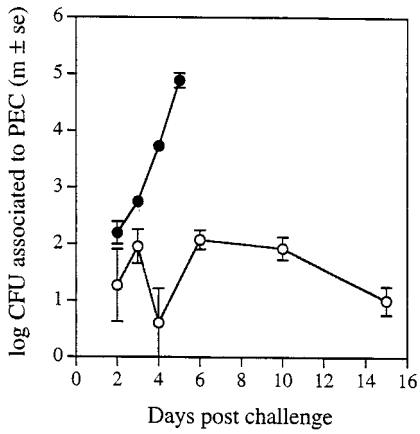


FIG. 2. In vivo persistence of *S. dublin* associated with murine PEC. Mice were infected i.p. with 10^2 CFU of SD2229 (●) or 10^3 CFU of SDM173c (○), and PEC were harvested at different times. Each point represents the mean ± standard error of data from three mice.

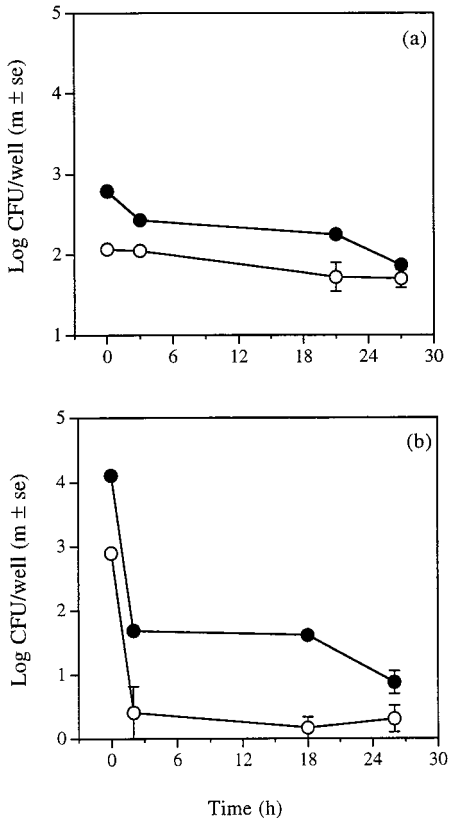


FIG. 3. In vitro persistence of *S. dublin* in murine peritoneal (a) and splenic (b) macrophages after in vivo infection. Peritoneal macrophages were isolated 4 days after i.p. infection with either 6×10^2 CFU of SD2229 (●) or 3×10^4 CFU of SDM173c (○). Splenic macrophages were isolated 4 days after i.p. infection of mice with either 5×10^2 CFU of SD2229 (●) or 6×10^5 CFU of SDM173c (○) and maintained in culture in vitro. Experiments were performed on at least three separate occasions, and representative data are expressed as the means ± standard errors of three wells per time point.

The 0-h sample was taken directly following the 1-h infection period and designated the total cell-associated recovery.

In vivo infection of mice with *S. dublin* and isolation of macrophages. Mice were infected intraperitoneally (i.p.) with *S. dublin* and killed by CO_2 asphyxiation at different time intervals after infection. Intracellular survival of *S. dublin* after in vivo infection was analyzed on peritoneal and splenic macrophages isolated from mice 4 days after infection. PEC were collected as described above and washed twice in gentamicin-free medium. PEC pellets were lysed with 1 ml of 0.5% (wt/vol) sodium deoxycholate, and the viable counts of cell-associated *S. dublin* per peritoneal cavity were determined. Peritoneal macrophages were isolated and resuspended at 5×10^5 macrophages per ml in IMDM with 10% FCS, which represented about 60% of PEC. Splenic macrophages were selected by isolating adherent cells from a single-cell suspension of spleen cells (6). They were obtained by pressing spleens through nylon gauze and lysing erythrocytes by adding NH_4Cl to the homogenate to a final concentration of 0.155 M. Spleen cells were resuspended at 5×10^6 cells per ml in IMDM with 10% FCS. For both peritoneal and splenic macrophages, 1 ml of cell suspension was removed before seeding and centrifuged at $600 \times g$ for 10 min, and the pellet was resuspended in 1 ml of 0.5% (wt/vol) sodium deoxycholate. *Salmonella* bacteria were enumerated on LB agar, and this represented the count at time zero. Remaining macrophages were resuspended in IMDM with 10% FCS and 100 μg of gentamicin per ml. Macrophages were seeded in 24-well plates, at a concentration of 5×10^5 macrophages per well for PEC and 5×10^6 cells per well for spleen cells, and incubated at 37°C with 5% CO_2. The nonadherent cells were removed after 4 or 2 h of incubation for peritoneal or splenic macrophages, respectively, by vigorous repeated washing and incubated at 37°C with 5% CO_2 in medium containing gentamicin. At different time intervals, the viable counts of intracellular *S. dublin* were determined after washing with gentamicin-free medium before lysis of macrophages and were expressed as log CFU per well.

Calculation of the rate constant of intracellular killing (K_k). Killing of *S. dublin* was calculated as a rate constant (K_k; $hour^{-1}$) according to the following equation: $K_k = [\log n(t = 0) - \log n(t)]/t$, in which $n(t)$ is the number of viable intracellular bacteria after time t (hours) has elapsed and $n(t = 0)$ represents the number of viable bacteria at the beginning of the assay (34). Values of K_k shown are means of rates determined from three wells per time point.

Macrophage lysis assay. Peritoneal macrophages suspended in DME/F12 containing 4% FCS and 100 μg of gentamicin per ml were seeded into 96-well plates (5×10^4 cells per well) and incubated overnight. Before use, macrophages were washed in gentamicin-free DME/F12 with FCS and cultured with fresh medium for 2 h. Monolayers were infected at a ratio of five bacteria per macrophage with *S. dublin*, *S. typhimurium*, or *S. choleraesuis*. Supernatants from the infected macrophages were sampled and assayed for lactate dehydrogenase activity (41) with the cytotoxic 96 kit (Promega, Southampton, United Kingdom) according to the manufacturer's instructions. Percentage of cytotoxicity was calculated as a fraction (percent):

$$\frac{A_{490}\,(\text{assay}) - A_{490}\,(\text{medium}) - A_{490}\,(\text{spontaneous release})}{A_{490}\,(\text{total lysis}) - A_{490}\,(\text{medium + lysis solution}) - A_{490}\,(\text{spontaneous release})} \times 100$$

Phenotypic analysis. (i) Microscopic observations. Macrophages were seeded onto 13-mm glass coverslips within wells of a 24-well plate. Macrophages were infected in vitro with *S. dublin* or were directly isolated from infected mice as described above. Coverslips were air dried, fixed with methanol, stained by a modified May-Grünwald-Giemsa stain (Diff Quik; Baxter Dade AG, Düdingen, Switzerland), and examined by light microscopy.

(ii) Flow cytometry (FCM) analysis. Fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 monoclonal antibody (MAb) (complement receptor type 3 [CD11b/CD18], M1/70 clone), used at a working dilution of 1:20, was obtained from Boehringer Mannheim (Lewes, United Kingdom). Anti-F4/80 MAb (working dilution, 1:10) and anti-7/4 MAb (working dilution, 1:20) were obtained from Serotec (Kidlington, United Kingdom). These MAbs were produced by rat cells; the anti-Mac-1 and anti-F4/80 MAbs were of the immunoglobulin G2b (IgG2b) isotype, and the anti-7/4 MAb was of the IgG2a isotype. An FITC rabbit anti-rat Ig [$F(ab')_2$, STAR 49] was used as secondary antibody (working dilution, 1:50) for unconjugated F4/80 and 7/4 MAbs and was obtained from Serotec. All antibodies were diluted in PBS containing 0.2% (wt/vol) bovine serum albumin and 0.01% (wt/vol) NaN_3 (PBS-B) alone or with 10% mouse serum for FITC anti-rat Ig.

For labelling, cells (10^6) were incubated at 4°C for 20 min with 50 μl of MAb at the appropriate concentration, washed twice with PBS-B, and fixed with 0.5% paraformaldehyde in PBS. Cells were single stained by FITC anti-Mac-1 MAb or anti-F4/80 MAb or by anti-7/4 MAb followed by FITC anti-rat Ig. Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, Calif.) with forward- and side-scatter gating to select leukocyte populations. The proportion of single-stained cells in total gated cells was expressed in a histogram. Binding of PBS-B or secondary antibody was used to determine nonspecific immunofluorescence for each set of staining and was deduced from the proportion of positive cells. An irrelevant FITC rat IgG2b isotypic control (V.H. Bio Ltd., Newcastle-upon-Tyne, United Kingdom) used at a working dilution of 1:20 was used as a negative control. Data were expressed as the proportion of positive cells in the total leukocyte population per organ. The total number of leukocytes per organ was calculated from the total number of cells in PEC and spleen per mouse, and the proportion of leukocytes was determined by FCM.

Mac-1 (31) and F4/80 (2) markers were used to analyze newly recruited phagocytes and mature macrophages, respectively (13), and 7/4 was used to label neutrophils (20).

Statistics. The results were expressed as the means ± standard errors of values and analyzed by one-way analysis of variance followed by an unpaired Student-Newman-Keuls multiple comparison test to calculate significant differences ($P < 0.05$) between two groups (InStat for MacIntosh; GraphPad, San Diego, Calif.).

TABLE 2. Intracellular killing rate of *S. dublin* by murine peritoneal and splenic macrophages infected in vivo and cultured ex vivo

Time (h)	Rate of intracellular killing (h^{-1}) (mean ± SE)[a]			
	Peritoneal macrophages		Splenic macrophages	
	SDM173c	SD2229	SDM173c	SD2229
4	−0.020 (0.092)	0.013 (0.123)	0.733 (0.39)	1.225 (0.022)
21	0.018 (0.001)	0.016 (0.01)	0.138 (0.018)	0.148 (0.014)
28	0.016 (0.004)	0.019 (0.015)	0.102 (0.003)	0.126 (0.003)

[a] Rate of intracellular killing of *S. dublin* strains within resident peritoneal and splenic macrophages at different times during ex vivo cultures. Macrophages were isolated from mice infected i.p. with 5×10^2 CFU of SD2229 or with 5×10^5 CFU of SDM173c. Data are expressed as the means ± standard errors from three different experiments. The negative value represents the absence of killing.

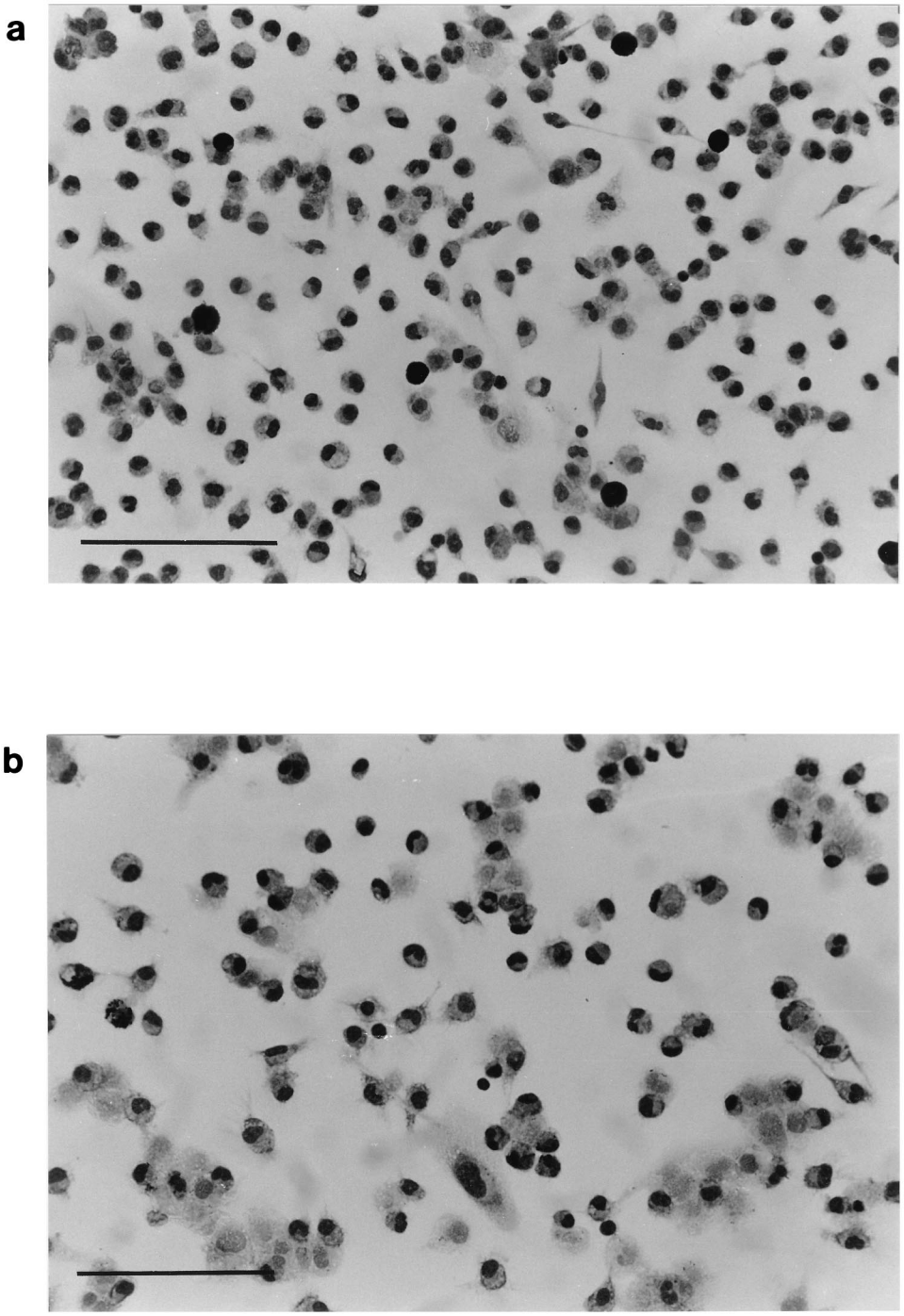


FIG. 4. May-Grünwald-Giemsa staining (Diff Quik) of resident peritoneal macrophages infected in vitro with SDM173c (a) or SD2229 (b) for 1 h and cultured in the presence of gentamicin for 3.5 h. Bar = 0.1 mm.

RESULTS

(i) In vitro survival of *S. dublin* in macrophages. Within 2 h of infection of mouse resident peritoneal macrophages, there was a 2-log reduction in both the wild-type strain SD2229 and the plasmid-cured derivative strain SDM173c of *S. dublin*. Thereafter, the number of *S. dublin* bacteria increased slightly. No significant differences in survival were observed between the two strains ($P > 0.05$) (Fig. 1a). The recovery of these strains from bovine alveolar macrophages, under the same conditions, was not significantly different ($P > 0.05$), although higher numbers of bacteria were recovered at all time points (Fig. 1b).

(ii) In vitro persistence of *S. dublin* in mouse macrophages after in vivo infection. The numbers of *S. dublin* bacteria associated with PEC following i.p. infection with either wild-type or plasmid-cured strains were compared in a gentamicin protection assay. Following i.p. infection with 10^2 CFU of the wild-type strain and with 10^3 CFU of the plasmid-cured strain,

SD2229 increased in number exponentially over the first 5 days, at which point the mice had died; in contrast, the number of SDM173c remained relatively constant over the 15 days following infection (Fig. 2).

We analyzed the persistence of *S. dublin* in peritoneal and splenic macrophages isolated 4 days after i.p. infection of mice and cultured in vitro (termed ex vivo). The i.p. infection dose of strain SDM173c was 3×10^4 CFU compared with 6×10^2 CFU for strain SD2229. These infection doses were used in an attempt to obtain comparable numbers of both strains of *S. dublin* associated with PEC at day 4. In vitro bactericidal activities of peritoneal macrophages for *S. dublin* were not significantly different for both strains (Fig. 3a; Table 2). An even higher infection dose of strain SDM173c (6×10^5 CFU) was used for ex vivo analysis of splenic macrophages. The bactericidal activities of splenic macrophages for both strains of *S. dublin* were not significantly different (Fig. 3b; Table 2) but were considerably higher than those seen with PEC.

(iii) Lysis of macrophages by *Salmonella* strains. Analysis of resident peritoneal macrophages stained with Diff Quik suggested that *S. dublin* infection induced cell damage. Wild-type *S. dublin* caused substantially more damage than plasmid-cured *S. dublin* as early as 3.5 h after infection in a gentamicin protection assay (Fig. 4). Cells incubated with wild-type *S. dublin* were present in lower numbers, and the remaining cells were larger and more frequently lysed. This was not related to a large intracellular proliferation of *S. dublin*. Lysis of macrophages was quantified by measuring the lactate dehydrogenase activity released from lysed macrophages into the medium for up to 3 h after infection. *Salmonella* strains do not produce measurable lactate dehydrogenase activity when grown aerobically (data not shown). A significant cytotoxic effect ($P < 0.001$) was observed after 1 h of incubation with *S. dublin*. It was significantly higher for strain SD2229 than for strain SDM173c at up to 3 h of interaction ($P < 0.001$) (Fig. 5a). Both strains were isolated from the culture medium in comparable numbers at all time points (data not shown) and increased by 1 log between 0 and 3 h. This plasmid-associated cytotoxicity was also found when wild-type and cured strains of *S. typhimurium* ($P < 0.001$) were compared (Fig. 5a). The role of the virulence plasmid in *S. choleraesuis*-induced macrophage lysis was assessed at 3 h after in vitro infection of resident peritoneal macrophages. The wild-type strain caused 77.3% lysis, which was significantly greater than the 43.3% lysis caused by the plasmid-cured strain ($P < 0.01$). The final levels of lysis reached varied slightly in each experiment, but the differences between wild-type and plasmid-cured strains were always highly reproducible.

The reintroduction of the *spv* region into the cured strain of *S. dublin* [SDM173(pCRV4)] did not affect cytotoxicity. Furthermore, a mutant deficient in functional *spv* genes (SDM51) was as cytotoxic as strain SD2229 (Fig. 5b). These results show that the cytotoxic effect of *S. dublin* on macrophages was mediated by the presence of the virulence plasmid but not the *spv* genes.

S. dublin induced a similar cytotoxic effect on peritoneal adherent cells incubated with or without nonadherent cells (data not shown). *S. dublin* induced comparable cytotoxicity on both resident macrophages and macrophages activated with IFN-γ and LPS ($P > 0.05$) but significantly greater cytotoxicity on Proteose Peptone-elicited macrophages ($P < 0.001$). Under all conditions, SD2229 remained more cytotoxic than SDM173c ($P < 0.01$) (Fig. 5c). Cytochalasin D treatment abolished the difference between *S. dublin* wild-type and plasmid-cured strains in both resident and Proteose Peptone-elicited perito-

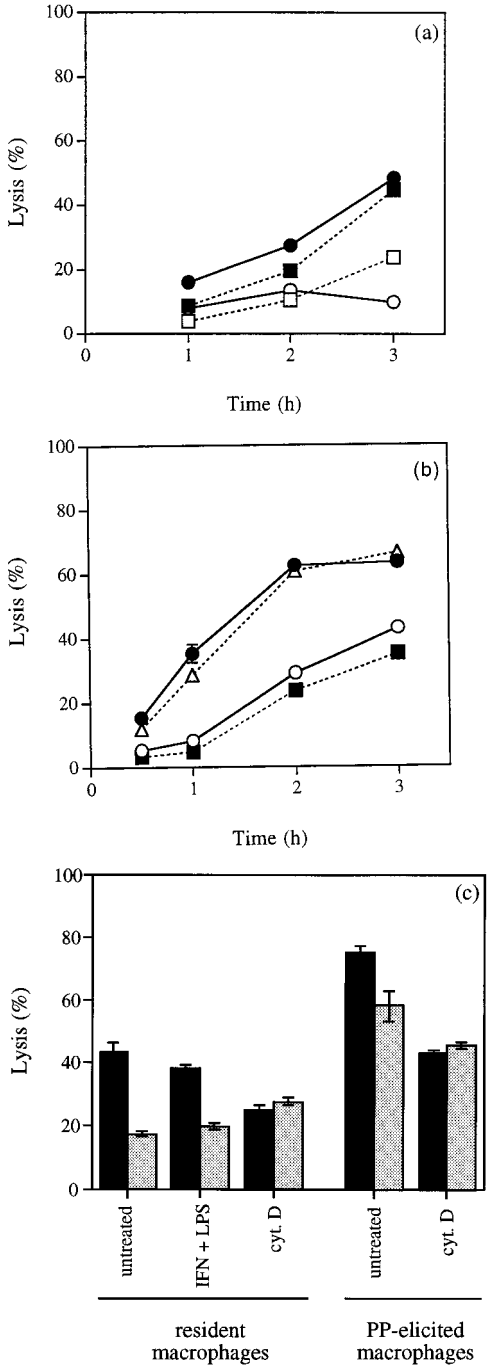


FIG. 5. *Salmonella*-induced lysis of murine peritoneal macrophages. (a) Macrophages were infected with SD2229 (●), SDM173c (○), ST12/75 (■), or STM8c (□). (b) Macrophages were infected with SD2229 (●), SDM173c (○), SDM173 (pCRV4) (■), or SDM51 (△). (c) *S. dublin*-induced lysis of macrophages at different states of activation. Macrophages were resident or Proteose Peptone (PP) elicited and either LPS- and IFN-γ-activated, cytochalasin D-treated, or untreated. Macrophages were then infected with SD2229 (■) or SDM173c (▨). Lysis was measured 3 h after infection. Experiments were performed on at least three separate occasions, and representative data are expressed as the means ± standard errors of three wells per time point.

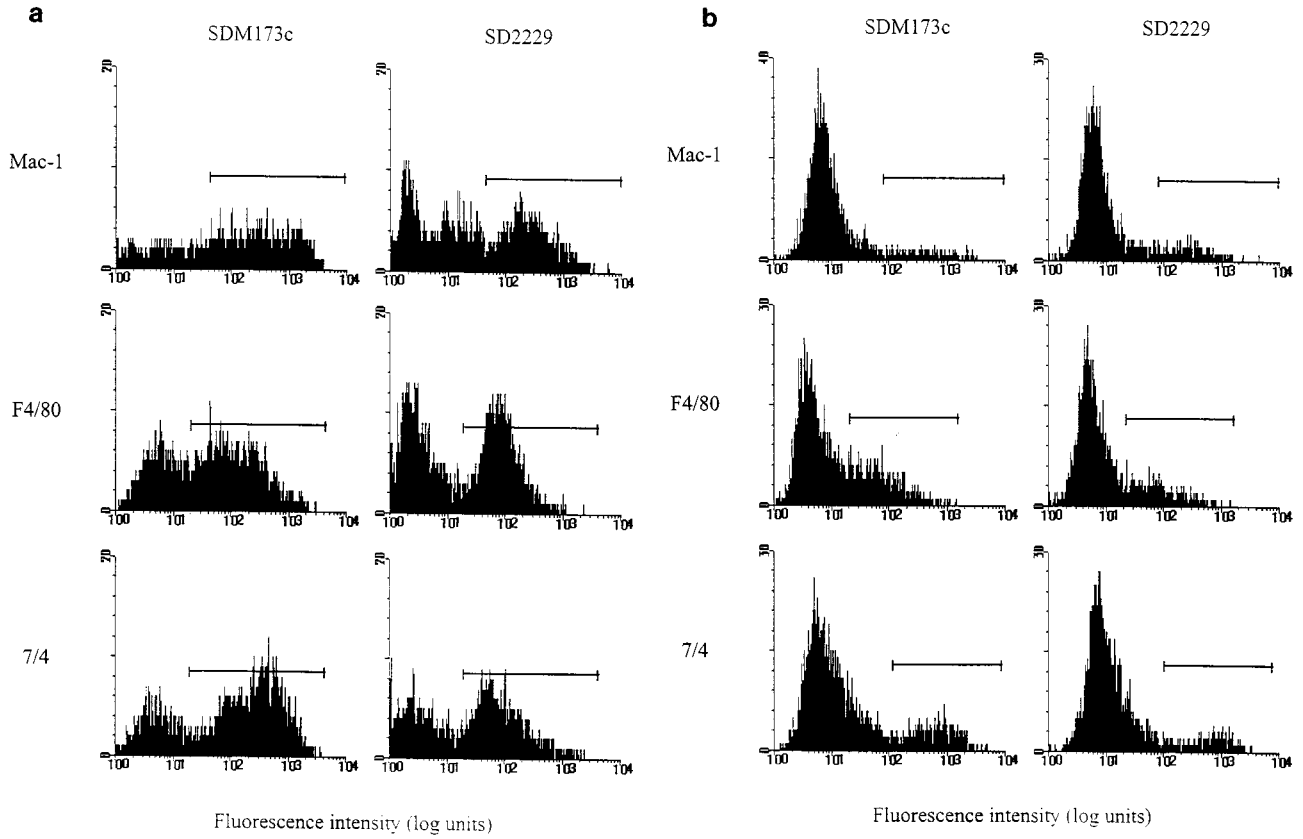
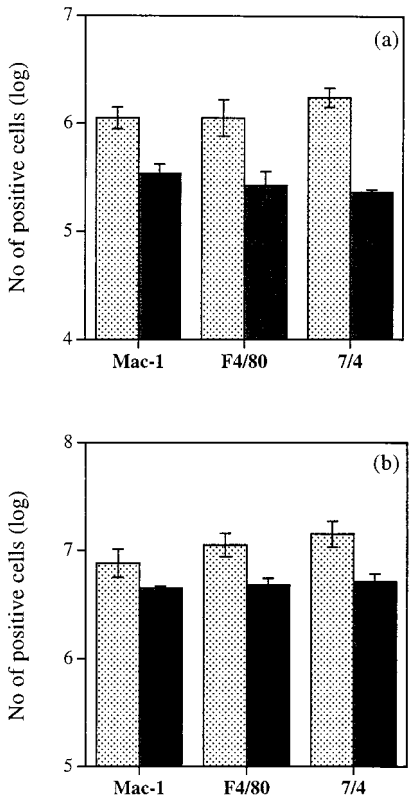


FIG. 6. Scatter plots depicting the proportion of Mac-1$^+$, F4/80$^+$, or 7/4$^+$ cells in the peritoneal cavity (a) and spleen (b) on day 4 after an i.p. infection with strain SD2229 or SDM173c. Cells were stained with FITC-conjugated anti-Mac-1 MAb or with anti-F4/80 MAb or anti-7/4 MAb followed by FITC-conjugated anti-rat F(ab')$_2$ antibody.



neal macrophages ($P > 0.05$) (Fig. 5c). A similar pattern of responses was seen with wild-type and plasmid-cured strains of *S. typhimurium* on resident and Proteose Peptone-elicited macrophages treated with IFN-γ and LPS or with cytochalasin D (data not shown).

(iv) Phenotypic characterization of *S. dublin*-induced inflammatory cells. The phenotypes of phagocytes from the peritoneal cavity and spleen were analyzed 4 days after i.p. infection of mice with either strain SD2229 (5×10^2 CFU) or strain SDM173c (5×10^5 CFU). The proportion of phagocytic cells was quantified by FCM on PEC and spleen with Mac-1, F4/80, and 7/4 markers (Fig. 6). A general hypercellularity was observed in PEC from mice infected with strain SDM173c compared with PEC from mice infected with strain SD2229. This corresponded to significantly higher numbers of Mac-1$^+$, F4/80$^+$, and 7/4$^+$ cells at this site ($P < 0.01$) (Fig. 7a). Likewise in the spleen, higher numbers of Mac-1$^+$ and F4/80$^+$ cells and significantly higher numbers of 7/4$^+$ cells ($P < 0.05$) were observed in mice infected with SDM173c compared with mice infected with SD2229 (Fig. 7b).

Splenic macrophages isolated from mice 4 days after in-

FIG. 7. Phenotypic analysis of PEC (a) and splenic cells (b) from mice infected i.p. with 5×10^2 CFU of SD2229 (■) or 5×10^5 CFU of SDM173c (▨). Cells were single stained for the indicated markers and analyzed by FCM. Cells were gated on leukocyte population, and the proportion of positive cells was determined. The number of positive cells was calculated from the total number of leukocytes per organ and expressed as the log of the number of positive cells. Data are expressed as the means ± standard errors of values from three different experiments.

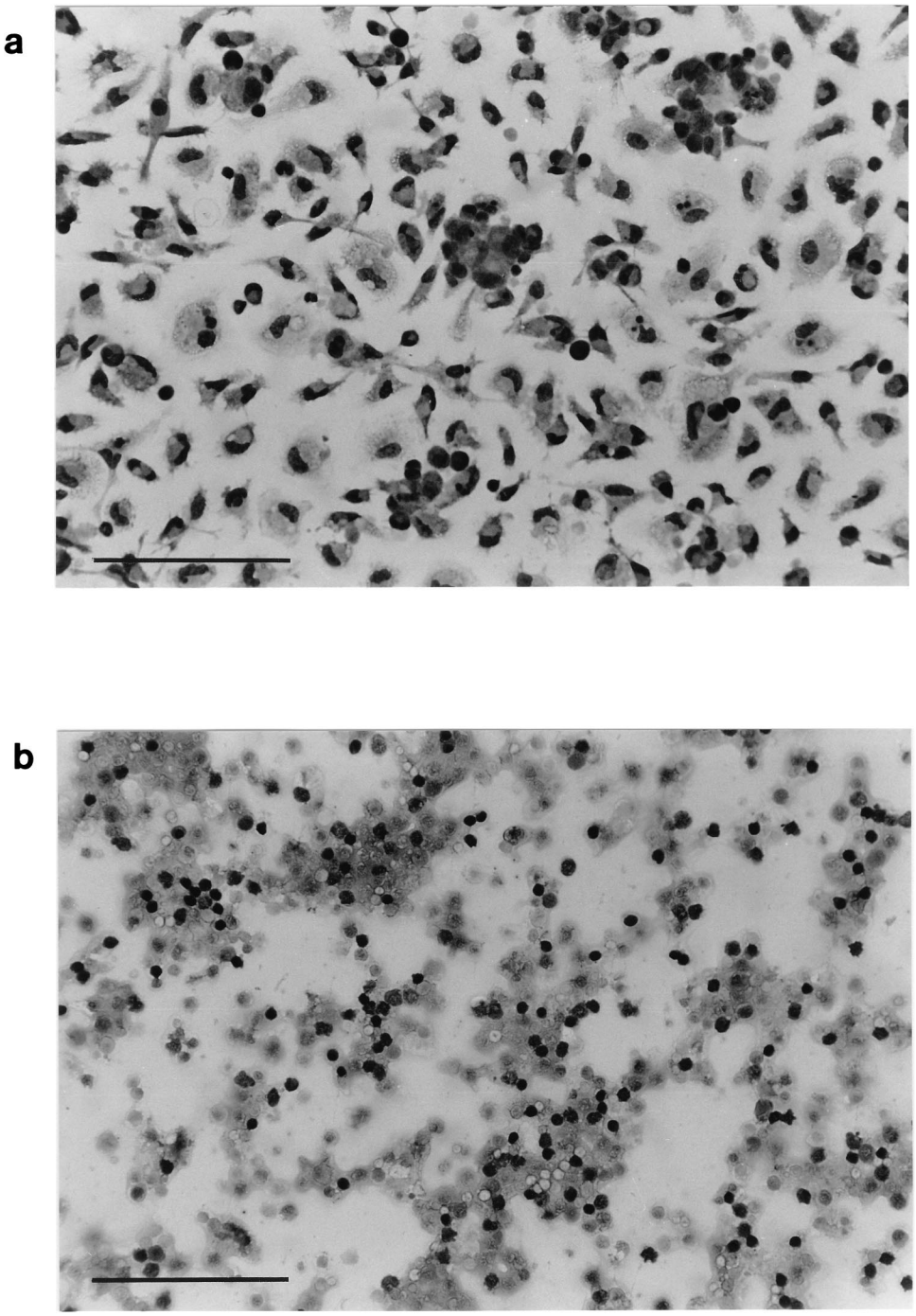


FIG. 8. Splenic macrophages in culture for 42 h after isolation from mice infected i.p. either with 5×10^5 CFU of SDM173c (a) or with 5×10^2 CFU of SD2229 (b). Cells were stained with May-Grünwald-Giemsa stain (Diff Quik). Bar = 0.1 mm.

fection with SD2229 or SDM173c were checked for viability, and comparable numbers of cells were seeded into wells. Splenic macrophages isolated from mice infected with strain SDM173c remained healthy in vitro whereas those isolated from mice infected with strain SD2229 were mainly lysed (Fig. 8).

DISCUSSION

Several groups have shown that the *Salmonella* virulence plasmid is involved in the systemic phase of infection in a variety of host species (7, 15, 18, 25, 26, 37). However, the role(s) of the plasmid in the pathogenesis of salmonellosis is still unknown. Persistence within macrophages is considered an essential step in the pathogenicity of *Salmonella* strains (11). Studies assessing the intracellular survival of *S. dublin*, *S. typhimurium*, or *Salmonella enteritidis* in resident and activated macrophages from *Ity*^{s} or *Ity*^{r} mice (29) or chickens (10) cultured in vitro have not shown any relationship between the survival of *Salmonella* strains and carriage of the virulence plasmid. We have confirmed and extended these observations

with in vitro- and in vivo-infected peritoneal macrophages from C57BL/6 mice (*Ity*^{s}). Previously, it has been shown that wild-type and cured strains of *S. typhimurium* were phagocytosed and killed in vivo to an equal extent by peritoneal macrophages (15). We have performed similar experiments with *S. dublin* over a longer time period but also with macrophages from the spleen, a target organ of systemic infection and therefore a possible site of virulence plasmid expression. The tissue source of the macrophages used is potentially very important, since not all macrophages would be expected to be involved in the control of infection (6). Although the number of *S. dublin* bacteria associated with splenic macrophages was higher in mice infected with wild-type strain SD2229 than in those infected with the cured strain SDM173c, the bacterial persistence in our assay was the same. These results are consistent with previous reports that the virulence plasmid is apparently not directly related to intracellular survival in macrophages in vivo. However, the plasmid-associated lysis of macrophages that we have observed makes the *Salmonella* survival data difficult to interpret. Gentamicin present in the assay does not affect intracellular *Salmonella* bacteria in viable macrophages, but *Salmonella* bacteria which lyse macrophages will be exposed to gentamicin, resulting in potentially misleading bacterial recoveries. Thus, the virulence plasmid may influence survival within macrophages, although any such activity would not be detected in this assay.

We observed a higher expansion of phagocyte numbers in the peritoneal cavity and spleen from mice infected with strain SDM173c than in those from mice infected with strain SD2229. This inflammatory response was characterized by the presence of numerous Mac-1$^+$ cells, a marker of polymorphonuclear leukocytes, newly recruited macrophages, and natural killer cells, and the presence of F4/80$^+$ cells, a marker for mature macrophages. The Mac-1 marker is a type 3 complement receptor involved in the recruitment of myelomonocytic cells to inflammatory sites (30). Our results showed that strain SD2229 induced a lower expansion of inflammatory cells in mice than did strain SDM173c, suggesting that the carriage of the virulence plasmid allows a down regulation of phagocyte recruitment to the site of infection. However, this activity must be either site or host specific as the virulence plasmid did not influence the magnitude of the inflammatory response in bovine ileal mucosa (37). In vitro, splenic macrophages survived better when they were isolated from mice infected with strain SDM173c than when isolated from mice infected with strain SD2229. This supports the role of the virulence plasmid of *S. dublin* in immunomodulation of the early immune response, possibly by the induction of macrophage dysfunction.

We also showed that *Salmonella* strains lysed murine peritoneal macrophages in vitro and that this cytotoxic effect was amplified by the presence of the virulence plasmid in *S. dublin*, *S. typhimurium*, and *S. choleraesuis* strains but not by the *spv* genes. This would suggest the existence of uncharacterized plasmid genes involved in macrophage lysis. Lysis was observed on resident, activated, and elicited macrophages, the last apparently being more susceptible. The reduction of cytotoxicity for the wild-type strains by cytochalasin D treatment suggests that microfilament reorganization (40) and internalization of *Salmonella* bacteria are necessary for this effect. This rapid-onset lysis of macrophages is both serotype and host specific. *S. dublin* and *S. typhimurium* but not *S. choleraesuis* will lyse bovine macrophages; none of these serotypes will lyse porcine macrophages (36).

The *Salmonella* bacterium is considered a facultative intracellular organism which survives intracellularly in vitro (1, 6, 11) and in vivo (8); there is also evidence for an extracellular phase of infection (22). The plasmid-cured strain is able to colonize and to persist in spleen and liver, but the systemic phase of the infection does not develop and the net growth is controlled. If one property of the virulence plasmid is to mediate macrophage lysis in vivo, then clearly wild-type *Salmonella* strains will at some stage during the disease process occupy a different niche from plasmid-cured strains. The virulence plasmid did not influence the rapid extracellular growth rate of *Salmonella* strains in peritoneal chamber implants in mice (17) or cattle (37). However, escape from macrophages may influence growth rate, and this is consistent with data showing that the virulence plasmid increases the net growth rate of *Salmonella* strains (17). The differences in inflammatory reactions in mice infected with either wild-type or plasmid-cured strains are further evidence that the virulence plasmid influences *Salmonella*-macrophage interactions. The mechanisms involved in macrophage lysis and the genes involved in this process are the subject of ongoing studies.

ACKNOWLEDGMENTS

This work was supported by a BBSRC Link grant.

We thank S. Paulin for preparations of alveolar bovine macrophages; P. Sopp for FCM analysis; R. Barclay for help in initiating this work; and M. Gardiner, P. Plank, and P. Prior for animal husbandry.

REFERENCES

1. **Abshire, K. Z., and F. C. Neidhardt.** 1993. Growth rate paradox of *Salmonella typhimurium* within host macrophages. J. Bacteriol. **175:**3744–3748.
2. **Austyn, J. M., and S. Gordon.** 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur. J. Immunol. **11:**805–815.
3. **Baird, G. D., E. J. Manning, and P. W. Jones.** 1985. Evidence for related virulence sequences in plasmids of *Salmonella dublin* and *Salmonella typhimurium*. J. Gen. Microbiol. **131:**1815–1823.
4. **Benninger, P. R., G. Chikami, K. Tanabe, C. Roudier, J. Fierer, and D. G. Guiney.** 1988. Physical and genetic-mapping of the *Salmonella dublin* virulence plasmid pSDL2—relationship to plasmids from other *Salmonella* strains. J. Clin. Invest. **81:**1341–1347.
5. **Brown, D. J., J. E. Olsen, and D. J. Platt.** 1992. The effect of the introduction of *Salmonella typhimurium* virulence genes into other serotypes of *Salmonella enterica*, p. 123–129. *In* Salmonella and salmonellosis. Proceedings of the World Veterinary Poultry Association, CNEVA/INRA, Ploufragan/Saint Brieuc, France.
6. **Buchmeier, N. A., and F. Heffron.** 1989. Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. Infect. Immun. **57:**1–7.
7. **Danbara, H., R. Moriguchi, S. Suzuki, Y. Tamura, M. Kijima, K. Oishi, H. Matsui, A. Abe, and M. Nakamura.** 1992. Effect of 50 kilobase plasmid, pKDSC50, of *Salmonella choleraesuis* RF-1 strain on pig septicemia. J. Vet. Med. Science. **54:**1175–1178.
8. **Dunlap, N. E., W. H. Benjamin, Jr., R. D. McCall, Jr., A. B. Tilden, and D. E. Briles.** 1991. A "safe site" for *Salmonella typhimurium* is within splenic cells during the early phase of infection in mice. Microb. Pathog. **10:**297–310.
9. **Emoto, M., H. Danbara, and Y. Yoshikai.** 1992. Induction of γ/δ cells in murine salmonellosis by an avirulent but not by a virulent strain of *Salmonella choleraesuis*. J. Exp. Med. **176:**363–372.
10. **Ermel, G., J. Protais, and P. Colin.** 1995. Survie intracellulaire de souches de salmonelles dans les macrophages péritonéaux de poulet, abstr. JE-33. *In* report of the Fourth National Congress of the Société Française de Microbiologie, Tours, France.
11. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
12. **Fierer, J., L. Eckmann, F. Fang, C. Pfeifer, B. B. Finlay, and D. Guiney.** 1993. Expression of the *Salmonella* virulence plasmid gene *spvB* in cultured macrophages and nonphagocytic cells. Infect. Immun. **61:**5231–5236.
13. **Gordon, S., I. Fraser, D. Nath, D. Hughes, and S. Clarke.** 1992. Macrophages in tissues and *in vitro*. Curr. Opin. Immunol. **4:**25–32.
14. **Guilloteau, L. A., A. J. Lax, S. MacIntyre, and T. S. Wallis.** 1996. The *Salmonella dublin* virulence plasmid does not modulate early T-cell responses in mice. Infect. Immun. **64:**222–229.
15. **Gulig, P. A., and R. Curtiss III.** 1987. Plasmid-associated virulence of *Salmonella typhimurium*. Infect. Immun. **55:**2891–2901.
16. **Gulig, P. A., H. Danbara, D. G. Guiney, A. J. Lax, F. Norel, and M. Rhen.** 1993. Molecular analysis of *spv* virulence genes of the salmonella virulence plasmids. Mol. Microbiol. **7:**825–830.
17. **Gulig, P. A., and T. J. Doyle.** 1993. The *Salmonella typhimurium* virulence

plasmid increases the growth rate of salmonellae in mice. Infect. Immun. **61:**504–511.
18. **Heffernan, E. J., J. Fierer, G. Chikami, and D. Guiney.** 1987. Natural history of oral *Salmonella dublin* infection in BALB/c mice: effect of an 80-kilobase-pair plasmid on virulence. J. Infect. Dis. **155:**1254–1259.
19. **Helmuth, R., R. Stephan, C. Bunge, B. Hoog, A. Steinbeck, and E. Bulling.** 1985. Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common *Salmonella* serotypes. Infect. Immun. **48:**175–182.
20. **Hirsch, S., and S. Gordon.** 1983. Polymorphic expression of a neutrophil differentiation antigen revealed by monoclonal antibody 7/4. Immunogenetics **18:**229–239.
21. **Hoertt, B. E., J. Ou, D. J. Kopecko, L. S. Baron, and R. L. Warren.** 1989. Novel virulence properties of the *Salmonella typhimurium* virulence-associated plasmid: immune suppression and stimulation of splenomegaly. Plasmid **21:**48–58.
22. **Hsu, H. S.** 1989. Pathogenesis and immunity in murine salmonellosis. Microbiol. Rev. **53:**390–409.
23. **Kawahara, K., T. Hamaoka, S. Suzuki, M. Nakamura, S. Y. Murayama, T. Arai, N. Terakado, and H. Danbara.** 1989. Lipopolysaccharide alteration mediated by the virulence plasmid of *Salmonella*. Microb. Pathog. **7:**195–202.
24. **Lax, A. J., P. A. Barrow, P. W. Jones, and T. S. Wallis.** 1995. Current perspectives in salmonellosis. Br. Vet. J. **151:**351–377.
25. **Manning, E. J., G. D. Baird, and P. W. Jones.** 1986. The role of plasmid genes in the pathogenicity of *Salmonella dublin*. J. Med. Microbiol. **21:**239–243.
26. **Pardon, P., M. Y. Popoff, C. Coynault, J. Marly, and I. Miras.** 1986. Virulence-associated plasmids of *Salmonella* serotype typhimurium in experimental murine infection. Ann. Inst. Pasteur. Microbiol. **137:**47–60.
27. **Platt, D. J., J. Taggart, and K. A. Heraghty.** 1988. Molecular divergence of the serotype specific plasmid (pSLT) among strains of *Salmonella typhimurium* of human and veterinary origin and comparison of pSLT with the serotype specific plasmids of *S. enteritidis* and *S. dublin*. J. Med. Microbiol. **27:**277–284.
28. **Rhen, M., P. Riikonen, and S. Taira.** 1993. Transcriptional regulation of *Salmonella enterica* virulence plasmid genes in cultured macrophages. Mol. Microbiol. **10:**45–56.
29. **Riikonen, P., P. H. Mäkelä, H. Saarilahti, and S. Sukupolvi.** 1992. The virulence plasmid does not contribute to growth of *Salmonella* in cultured murine macrophages. Microb. Pathog. **13:**281–291.
30. **Rosen, H., and S. Gordon.** 1990. The role of the type 3 complement receptor in the induced recruitment of myelomonocytic cells to inflammatory sites in the mouse. Am. J. Respir. Cell Mol. Biol. **3:**3–10.
31. **Springer, T., G. Galfré, D. S. Secher, and C. Milstein.** 1979. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. **9:**301–306.
32. **Sukupolvi, S., P. Riikonen, S. Taira, H. Saarilahti, and M. Rhen.** 1992. Plasmid-mediated serum resistance in *Salmonella enterica*. Microb. Pathog. **12:**219–225.
33. **Vandenbosch, J. L., D. R. Kurlandsky, R. Urdangaray, and G. W. Jones.** 1989. Evidence of coordinate regulation of virulence in *Salmonella typhimurium* involving the *rsk* element of the 95-kilobase plasmid. Infect. Immun. **57:**2566–2568.
34. **Van Dissel, J. T., J. J. M. Stikkelbroeck, B. C. Michel, P. C. J. Leijh, and R. Van Furth.** 1987. *Salmonella typhimurium*-specific difference in rate of intracellular killing by resident peritoneal macrophages from *Salmonella*-resistant CBA and *Salmonella*-susceptible C57BL/10 mice. J. Immunol. **138:**4428–4434.
35. **Van Leengoed, L. A., E. M. Kamp, and J. M. A. Pol.** 1989. Toxicity of *Haemophilus pleuropneumoniae* to porcine lung macrophages. Vet. Microb. **19:**337–349.
36. **Wallis, T. S., S. Paulin, and P. W. Jones.** Submitted for publication.
37. **Wallis, T. S., S. Paulin, J. Plested, P. R. Watson, and P. W. Jones.** 1995. The *Salmonella dublin* virulence plasmid mediates systemic but not enteric phases of salmonellosis in cattle. Infect. Immun. **63:**2755–2761.
38. **Williamson, C. M., G. D. Baird, and E. J. Manning.** 1988. A common virulence region on plasmids from eleven serotypes of *Salmonella*. J. Gen. Microbiol. **134:**975–982.
39. **Williamson, C. M., G. D. Pullinger, and A. J. Lax.** 1988. Identification of an essential virulence region on *Salmonella* plasmids. Microb. Pathog. **5:**469–473.
40. **Yahara, I., F. Harada, S. Sekita, K. Yoshihira, and S. Natori.** 1982. Correlation between effects of 24 different cytochalasins on cellular structures and cellular events and those on actin *in vitro*. J. Cell Biol. **92:**69–78.
41. **Zychlinsky, A., C. Fitting, J. M. Cavaillon, and P. J. Sansonetti.** 1994. Interleukin 1 is released by murine macrophages during apoptosis induced by *Shigella flexneri*. J. Clin. Invest. **94:**1328–1332.

Editor: P. J. Sansonetti