

Oral Immunization of Mice with Attenuated *Salmonella typhimurium* Containing a Recombinant Plasmid Which Codes for Production of a 31-Kilodalton Protein of *Brucella abortus*

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Salmonella typhimurium χ 4064, an attenuated Δ *cya* Δ *crp* mutant of *S. typhimurium* SR-11, was used as a carrier for the plasmid pBA31-R7. This plasmid codes for the expression of a 31-kilodalton (kDa) protein from *Brucella abortus* (BCSP31). Recombinant *S. typhimurium* χ 4064(pBA31-R7) expressed BCSP31 in vitro as shown by Western blot (immunoblot) analysis. The plasmid was stable in vitro and in vivo and did not affect the ability of the mutant to invade and colonize the small intestine, mesenteric lymph nodes, liver, or spleen of BALB/cByJ mice. Animals orally immunized with *S. typhimurium* χ 4064(pBA31-R7) developed serum and intestinal antibody responses to the *B. abortus* 31-kDa protein and to salmonella endotoxin as measured by enzyme-linked immunosorbent assay. Mice orally immunized with *S. typhimurium* χ 4064pBA31-R7 did not develop a delayed-type hypersensitivity following a footpad injection with recombinant BCSP31. Antigen-specific blastogenic data also support these in vivo results. All data indicate that this route of antigen delivery is effective for stimulating antibody-mediated immunity but that the *B. abortus* 31-kDa protein is a poor immunogen for inducing a cell-mediated immune response in BALB/cByJ mice.

The majority of infectious agents which affect man and animals gain access to the host through a mucosal surface. Many of these pathogens are invasive, whereas others produce disease only at mucosal membranes (27). An effective oral vaccine may need to target specific tissues and induce antibody or cell-mediated immunity. Currently, the majority of effective vaccines are administered parenterally, with the exception of a few live viral vaccines. Much of the difficulty in developing protocols for oral immunization has involved the delivery of sufficient antigen to effectively immunize the host without development of oral tolerance (24). Since *Salmonella typhimurium* naturally invades and persists in gut-associated lymphoid tissue (4), this organism delivers foreign antigen directly to the mucosal surface (7, 21, 22, 32) and stimulates secretory, humoral, and cellular immune responses (3, 7, 26). With the use of recombinant DNA technology, the potential exists to effectively combine antigens within a single microorganism and to produce a vaccine for several different diseases simultaneously. This strategy is particularly attractive for the development of vaccines against enteric pathogens.

The present study examines the capabilities of an attenuated strain of *S. typhimurium* χ 4064 (Δ *cya* Δ *crp*) to serve as a vehicle for delivery of a cloned antigen to the murine immune system. This strain has retained its ability to colonize and invade murine Peyer's patches and has been shown to induce protection against a lethal challenge of parent strain *S. typhimurium* SR-11 (9). Mayfield et al. (23) recently cloned the gene for a *Brucella abortus* 31-kilodalton (kDa) protein (BCSP31) into *Escherichia coli* K-12. As a marker antigen, the plasmid (pBA31-R7) expressing BCSP31 in *E. coli* K-12 was transduced into *S. typhimurium* χ 4064. The efficacy of *S. typhimurium* χ 4064(pBA31-R7) to colonize the murine gut-associated lymphoid tissue, liver, and spleen was

correlated with the induction of mucosal and systemic immunity to the heterologous BCSP31 marker antigen.

MATERIALS AND METHODS

Bacterial strains and plasmid. pBA31-R7, containing a 4.2-kilobase *Eco*RI fragment coding for BCSP31 (a 31-kDa *Brucella* protein), was propagated in *E. coli* HB101. *S. typhimurium* SL5283 (a restriction-negative, modification-positive strain) was used as an intermediate transformation recipient of the plasmid DNA. An avirulent *S. typhimurium* SR-11 double mutant (Δ *cya* Δ *crp*) χ 4064 (kindly provided by R. Curtiss III, Washington University, St. Louis, Mo.) was used for delivery of antigen in vivo. A P22 (HT, *int*⁻) transducing phage lysate was routinely produced from cultures of *S. typhimurium* χ 3000 (kindly provided by R. Curtiss III).

Genetic transformation and transduction. Because of restriction barriers, it was necessary to first transform pBA31-R7 into a restriction-negative, modification-positive rough salmonella strain (SL5283) (kindly provided by R. W. Griffith, Iowa State University, Ames, Iowa). This was accomplished by using the procedure of Lederberg and Cohen (18) with DNA isolated by using the method of Birnboim and Doly (1) and purified over a cesium chloride gradient (20). The presence of the plasmid was confirmed by using a rapid screening technique (29), and then the plasmid was transduced to *S. typhimurium* χ 4064 (Δ *cya* Δ *crp*) by using P22 (HT, *int*⁻) as previously described (10). Transductants were screened for expression of BCSP31 as described below.

Electrophoresis and immunoblot analysis. Horizontal gel electrophoresis of DNA was performed on 0.75% agarose slab gels in 89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA (TBE buffer). *Hind*III-digested bacteriophage λ DNA fragments were used as molecular weight standards. Restriction endonucleases were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

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Vertical discontinuous polyacrylamide gel electrophoresis containing sodium dodecyl sulfate was performed with a 4% stacking gel over a 12.5% separating gel by the technique of Laemmli (17). To detect protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gels were stained with Coomassie blue (R250) by the method of Fairbanks et al. (13).

To detect BCSP31 in *S. typhimurium* χ 4064(pBA31-R7), proteins from sodium dodecyl sulfate-polyacrylamide gels were electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Inc., Keene, N.H.) by using a modification of the procedure described by Towbin et al. (33). Membranes were treated with either rabbit anti-*B. abortus* strain 19 whole cell lysate sera or various sera from mice immunized with either purified BCSP31 or *S. typhimurium* χ 4064(pBA31-R7). Immunoblots were then incubated with either horseradish peroxidase or alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Fisher Scientific Co., Orangeburg, N.Y.). Conjugated antibodies were visualized with either TMB membrane peroxidase substrate (Kirkegaard and Perry, Gaithersburg, Md.) or with a substrate solution of 1 mg of naphthol AS-MX phosphate per ml and 2 mg of Fast Red TR salt per ml (Sigma Chemical Co., St. Louis, Mo.) as previously described (31).

Antigen preparations. Purified BCSP31 was obtained from *E. coli* by using a modified procedure of Bricker et al. (2) and was designated rBCSP31. *E. coli* was grown in 2 liters of Luria-Bertani (LB) broth (20) containing ampicillin (100 μ g/ml) for 36 h with shaking (260 rpm at 37°C). Cultures were centrifuged (8,000 \times *g* for 15 min), supernatants were discarded, and the pellets were weighed. The pellets were suspended in 2 ml of extraction buffer (10 mM phosphate buffer [pH 7.5] containing 0.1% Triton X-100) per g of packed cells and incubated at 37°C overnight with shaking (260 rpm). The cell suspension was transferred to glass Corex tubes and centrifuged (8,000 \times *g* for 15 min), and the pellet was washed once with extraction buffer. Pooled supernatants were preserved by the addition of 0.01% Merthiolate, incubated for 1 h at room temperature, and dialyzed extensively against 10 mM Tris-5 mM NaCl (pH 7.5). The dialysate was then loaded onto a DEAE Sepharose CL6B column (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) (bed volume, 280 ml) equilibrated in 10 mM Tris-5 mM NaCl (pH 7.5) (column buffer). After washing the column with 1.5 bed volumes of column buffer, selective elution of bound material was achieved with an NaCl gradient (5 to 250 mM) in 10 mM Tris (pH 7.5). An elution profile was determined spectrophotometrically by monitoring fractions at 280 nm. Protein concentrations were determined by the method of Lowry et al. (19). For parenteral immunizations, rBCSP31 was emulsified in Freund incomplete adjuvant (FIA).

S. typhimurium χ 4064(pBA31-R7) soluble antigen for foot-pad injection and in vitro blastogenesis assays was prepared by the procedure of Shaible et al. (28). Briefly, *S. typhimurium* χ 4064(pBA31-R7) was grown for 24 h in LB broth containing ampicillin (100 μ g/ml) with shaking (260 rpm at 37°C). The cells were washed three times by centrifugation in 0.01 M phosphate-buffered saline (PBS) (pH 7.2). Suspended cells were sonicated with a cell disrupter (Braunsonic 1510; B. Braun Instruments, South San Francisco, Calif.) eight times for 15 s in an ice bath. Sonic extracts were centrifuged (10,000 \times *g* for 15 min), and supernatants were filtered through a Millex GV Membrane (low protein binding; pore size, 0.22 μ m) (Millipore Corp., Bedford, Mass.).

Protein concentrations were determined by the method of Lowry et al. (19), and samples were frozen at -70°C.

S. typhimurium χ 4064 endotoxin was extracted by a butanol-water method (25).

Animals. Female and male BALB/cByJ mice (Jackson Laboratory, Bar Harbor, Me.) were bred and maintained in the Laboratory Animal Resource Facility at the College of Veterinary Medicine at Iowa State University, Ames, Iowa. Mice were housed in sterile cages and given sterile food (no. 5010; Purina Mills Inc., St. Louis, Mo.) and water ad libitum. All mice used in these experiments were 8 to 10 weeks of age.

Immunization of mice with *S. typhimurium* χ 4064. Inocula for oral immunizations were prepared from log-phase cultures of *S. typhimurium* χ 4064(pBA31-R7) or *S. typhimurium* χ 4064. LB broth cultures (5 ml per tube) were inoculated with cells from frozen stocks (-70°C) and incubated overnight at 37°C without shaking. These cultures were diluted 1/20 in prewarmed LB broth, and incubated for 4 h with shaking (260 rpm at 37°C) to obtain log-phase growth. Cultures were then put on ice to inhibit further replication. The cell suspension was pelleted (8,000 \times *g* for 10 min, 4°C) and then suspended in 0.01 M PBS (pH 7.2) to 1/50 the original volume.

Food and water were removed from each cage of mice 4 h prior to oral infection and returned 30 min postinfection. Stomach acidity was neutralized with 30 μ l of 10% sodium bicarbonate administered orally 5 min prior to a 20- μ l oral dose of *S. typhimurium* (4×10^8 cells/ml). Oral doses were given with a 1-in. (2.54-cm), 21-gauge intubation needle (Popper and Sons, Inc., New Hyde Park, N.Y.).

Secondary parenteral immunization consisted of 25 μ g of rBCSP31 per mouse administered subcutaneously (s.c.) or intraperitoneally (i.p.), whereas secondary oral doses were identical to primary oral doses.

Groups of seven mice were killed on days 2, 7, 14, 21, 28, 35, and 42 post-oral inoculation with *S. typhimurium* χ 4064(pBA31-R7) (2×10^8 to 4×10^8 CFU per mouse). The spleen, liver, mesenteric lymph nodes, and small intestine were removed from each animal and placed in individual sterile Whirl-Pak bags (Nasco, Fort Atkinson, Wis.). After being weighed, the tissues were homogenized for 1 min in 10 ml of PBS (pH 7.2) by using a Stomacher 80 laboratory blender (A. J. Seward, London). Homogenates were plated on MacConkey agar containing 100 μ g of ampicillin per ml and incubated for 24 h at 37°C. Ampicillin-resistant colonies were then biochemically identified as *S. typhimurium* (9, 12).

Collection of saliva. To determine the isotype and quantity of secretory antibody produced, saliva was collected from each mouse with a capillary pipette (5, 26). Mice were anesthetized with 0.15 ml i.p. of ketamine hydrochloride diluted 1/35 (4%; Aveco Co., Inc., Fort Dodge, Iowa) prior to an i.p. injection of 160 μ g of pilocarpine hydrochloride (Alcon Inc., Puerto Rico). Salivary samples were centrifuged at 16,000 \times *g* for 10 min to remove debris and were stored at -20°C.

Intestinal antibody secretion. Local intestinal secretory immunity was measured in tissue homogenates prepared by the procedure of Clements et al. (7). Groups of three mice were killed at various intervals, and the small intestine was removed and homogenized in a solution containing 50 mM EDTA and 0.1 mg of soybean trypsin inhibitor (Sigma) per ml. After being homogenized, samples were clarified by centrifugation at 8,000 \times *g* for 20 min (4°C), lyophilized, suspended in 1 ml of TEAN buffer (0.05 M Tris, 0.001 M EDTA, 0.003 M NaN₃, 0.2 M NaCl [pH 7.5]), dialyzed

against TEAN buffer (4°C), and stored at -20°C until assayed by enzyme-linked immunosorbent assay (ELISA) for the presence of antibody to either the recombinant BCSP31 or to *S. typhimurium* χ 4064 endotoxin.

ELISA. All antibody titers were determined by ELISA by use of 96-well Immulon 1 microtiter plates (Dynatech, Chantilly, Va.) incubated overnight at 4°C with 2 μ g of either rBCSP31 or *S. typhimurium* χ 4064 endotoxin diluted in 0.01 M PBS (pH 7.2). All sera were diluted 1/10 with saline containing 0.05% Tween-20 (TS); intestinal homogenates and saliva were used undiluted. Total immunoglobulin, immunoglobulin G (IgG), IgM, or IgA were determined by using alkaline phosphatase-conjugated goat anti-mouse antibodies to immunoglobulin (Fisher), IgG (gamma-chain specific), IgM (mu-chain specific), and IgA (alpha-chain specific) (Kirkegaard and Perry) at a 1/2,000 dilution in TS. Enzyme-substrate reactions were initiated by the addition of no. 104 phosphatase substrate (Sigma). Absorbance was measured at 405 nm, and the results were expressed as the mean plus or minus standard error of the mean of triplicate samples.

Delayed type hypersensitivity (DTH) reaction. Mice were sensitized orally with 20 μ l of 10^7 to 10^9 recombinant *S. typhimurium* χ 4064 or immunized s.c. with 50 μ g of rBCSP31 emulsified in FIA (total injection volume, 200 μ l). At 21 days postimmunization, the right hind footpad of each mouse was injected s.c. with either 20 μ g of soluble *S. typhimurium* χ 4064 extract or with 20 μ g of rBCSP31. The left foot was injected with saline as a negative control. Under double-blind conditions, footpad swelling was measured with a sliding-scale caliper (Helios, Federal Republic of Germany) at 24, 48, and 72 h post s.c. injection. Reported values are the differences in swelling between saline-injected and antigen-injected footpads.

Blastogenesis assay. Groups of mice were either orally infected with *S. typhimurium* χ 4064(pBA31-R7), injected i.p. with 50 μ g of rBCSP31 in FIA, or injected i.p. with 50 μ g of *S. typhimurium* χ 4064(pBA31-R7) soluble protein in FIA. In addition, one group of naive mice was used as a negative control. Mice were killed and spleens were removed 21 days post-primary immunization. Individual spleen cell homogenates were inoculated into wells of 96-well microtiter plates (2.5×10^5 cells per well) containing one of the following: RPMI 1640 (Sigma), 0.5 μ g of concanavalin A (Sigma) per ml, 5 μ g of pokeweed mitogen (Sigma) per ml, rBCSP31 (0.5, 5, or 50 μ g/ml), or *S. typhimurium* χ 4064(pBA31-R7) soluble protein (0.5, 5, or 50 μ g/ml). All cells were incubated in RPMI 1640 containing 1% fetal calf serum (J. R. Scientific, Woodland, Calif.) for 5 days. Cells were pulsed with [3 H]thymidine (Amersham Corp., Arlington Heights, Ill.) 18 h prior to harvest. Counts were quantitated with a model 1500 liquid scintillation analyzer (Packard Instrument Co., Inc., Downers Grove, Ill.).

RESULTS

Construction of a recombinant *S. typhimurium* χ 4064 BCSP31 expression vector. Construction of the pBA31-R7 plasmid containing the gene for the production of BCSP31, a *B. abortus* cell surface protein, has already been reported (23). Cesium chloride-purified pBA31-R7 was transformed into a restriction-negative, modification-positive intermediate host *S. typhimurium* SL 5283 prior to transduction into *S. typhimurium* χ 4064 by using P22 bacteriophage (HT, *int*⁻). The presence of the plasmid in *S. typhimurium* χ 4064 was confirmed by plasmid analysis of cell lysates (Fig. 1). West-

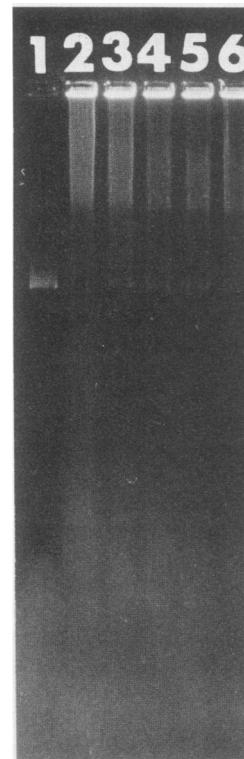


FIG. 1. Plasmid analysis of recombinant *S. typhimurium* χ 4064. Recombinant *S. typhimurium* χ 4064(pBA31-R7) were lysed in the well of a 0.8% agarose gel, and DNA was separated electrophoretically and stained with ethidium bromide. Lanes: 1, positive control (purified pBA31-R7); 2, negative control (*S. typhimurium* χ 4064); 3 and 4, transduced *S. typhimurium* χ 4064(pBA31-R7); 5 and 6, *S. typhimurium* χ 4064(pBA31-R7) isolated from BALB/cByJ mice 10 weeks postinfection.

ern blot (immunoblot) analysis showed expression of the rBCSP31 protein by transduced *S. typhimurium* χ 4064 cells (Fig. 2). Osmotic shock experiments suggested that the protein was periplasmic in *S. typhimurium* (data not shown). Cell lysates from recombinant *S. typhimurium* χ 4064 recovered 21 days postinfection showed in vitro expression of rBCSP31 as determined by immunoblot analysis (data not shown). Recombinant colonies isolated between 2 and 70 days post-oral immunization of mice were shown to contain pBA31-R7 by restriction digest mapping (Fig. 3). Colonies recovered 70 days postinoculation were shown to express cloned BCSP31 by Western blot analysis (Fig. 1).

Colonization of mice with recombinant *S. typhimurium* χ 4064. The insertion of the plasmid and expression of rBCSP31 protein in *S. typhimurium* χ 4064(pBA31-R7) did not affect colonization characteristics of the strain (data not shown). *S. typhimurium* χ 4064(pBA31-R7) colonization of the small intestine and mesenteric lymph nodes occurred rapidly within the first week after oral immunization, with peak colonization between days 7 and 10 (1×10^4 and 2.7×10^4 cells per g of tissue, respectively) (Fig. 4). Colonization of the spleen and liver occurred more gradually and at much lower cell numbers (3×10^3 and 1×10^3 cells per g of tissue, respectively). Peak colonization of both the spleen and the liver occurred at day 14 postinoculation (Fig. 4). By day 21, salmonella CFU in all tissues were virtually undetectable. However, some *S. typhimurium* χ 4064(pBA31-R7) could still

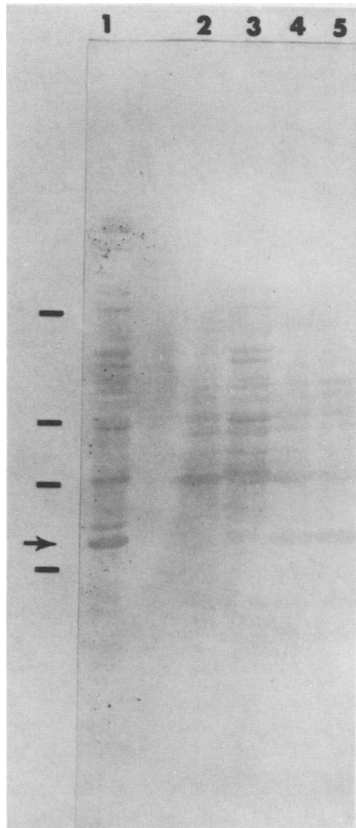


FIG. 2. Western blot analysis of rabbit anti-BCSP31 sera reacted against *S. typhimurium* whole-cell lysates. Lanes: 1, positive control (pBA31-R7-transformed *S. typhimurium* SL5283 expressing BCSP31 [arrow]); 2 and 3, negative controls (without pBA31-R7) (*S. typhimurium* χ 4064 [lane 2] and *S. typhimurium* χ 3385 [lane 3]); 4 and 5, transduced *S. typhimurium* χ 4064 expressing BCSP31. Apparent molecular mass markers for 66, 45, 36, and 29 kDa (top to bottom) are indicated to the left of the gel.

be recovered from the spleen and liver 10 weeks after oral immunization (data not shown).

Serum titers. Mice orally immunized once with *S. typhimurium* χ 4064(pBA31-R7) developed a serum immunoglobulin anti-rBCSP31 titer equal to that developed in mice parenterally immunized with purified rBCSP31 in FIA (Fig. 5). Mice orally immunized with *S. typhimurium* χ 4064(pBA31-R7) were slower to develop an anti-rBCSP31 titer than mice parenterally immunized with rBCSP31 in FIA (27 versus 21 days, respectively). These sera were shown by Western blot analysis to be highly reactive with a 31-kDa protein, rBCSP31 (Fig. 6A). *S. typhimurium* χ 4064(pBA31-R7)-immunized mice also developed a strong immunoglobulin serum response to purified χ 4064 endotoxin (Fig. 6B). The predominant antibody isotype in all cases was IgG, with a small amount of serum IgA and no IgM (data not shown). A second oral dose of *S. typhimurium* χ 4064(pBA31-R7) given at either 14 or 21 days post-primary immunization had no effect on existing BALB/cByJ serum titers to rBCSP31 or *S. typhimurium* χ 4064 endotoxin (data not shown).

Saliva titers. As late as 42 days post-primary immunization, mice immunized with either rBCSP31 in FIA or *S. typhimurium* χ 4064(pBA31-R7) showed no detectable saliva antibody titer to rBCSP31. Secondary immunizations with rBCSP31 or *S. typhimurium* χ 4064(pBA31-R7) or both were

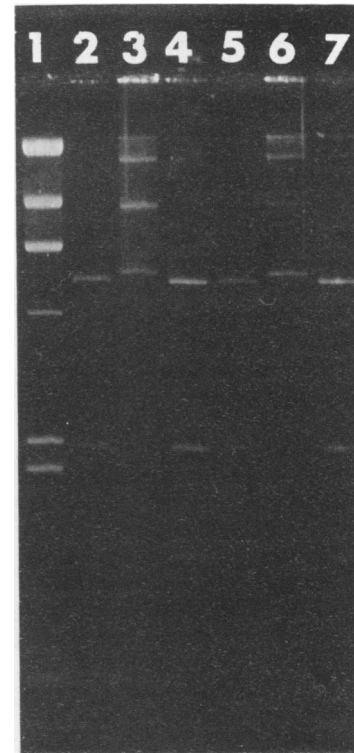


FIG. 3. Restriction enzyme analysis of plasmid DNA from *S. typhimurium* χ 4064(pBA31-R7) recovered from BALB/cByJ mice 10 weeks postinfection. Lanes: 1, molecular size markers (*Hind*III-digested λ DNA); 2 through 4, *S. typhimurium* χ 4064(pBA31-R7) plasmid DNA (positive control) (*Hind*III + *Kpn*I digest [lane 2], *Kpn*I digest [lane 3], *Hind*III digest [lane 4]); 5 through 7, *S. typhimurium* χ 4064(pBA31-R7) plasmid DNA recovered from infected mice (*Hind*III + *Kpn*I digest [lane 5], *Kpn*I digest [lane 6], *Hind*III digest [lane 7]).

also unsuccessful at stimulating a specific salivary antibody response to rBCSP31 (data not shown).

Intestinal homogenate titers. Mice orally infected with *S. typhimurium* χ 4064(pBA31-R7) showed peak intestinal IgA and IgG responses to rBCSP31 and *S. typhimurium* χ 4064 endotoxin on days 23 and 25 of infection, respectively. No specific IgM titers were detected against either antigen (Fig. 7). Under the given conditions, the intestinal IgA response to endotoxin was much stronger than the IgA response to rBCSP31, whereas the intestinal IgG response to each antigen was more nearly equal. A second oral dose of *S. typhimurium* χ 4064(pBA31-R7) (2×10^8 cells) given 21 days after the primary immunization failed to enhance intestinal antibody titers to rBCSP31 or *S. typhimurium* χ 4064 endotoxin (data not shown).

DTH and blastogenic responses following immunization. Mice orally sensitized with *S. typhimurium* χ 4064(pBA31-R7) did not respond to a s.c. footpad DTH stimulatory dose of rBCSP31 protein. This same treatment group did respond to *S. typhimurium* χ 4064(pBA31-R7) soluble antigen, indicating that a cell-mediated DTH response was elicited by vector antigens (i.e., the *S. typhimurium* χ 4064) (Fig. 8). Moreover, mice sensitized s.c. with rBCSP31 protein in FIA did not respond to a homologous footpad s.c. DTH stimulatory dose, or to *S. typhimurium* χ 4064(pBA31-R7) soluble antigen.

Neither oral immunization with *S. typhimurium*

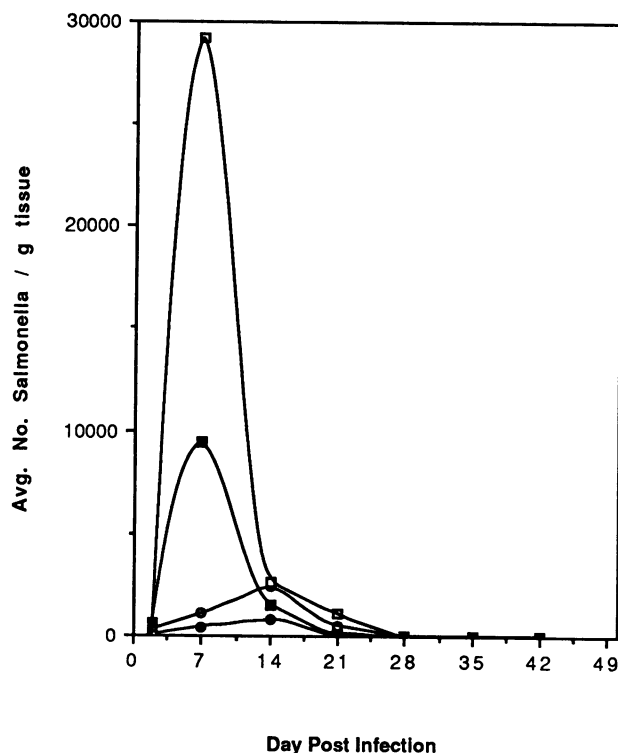


FIG. 4. Determination of the CFU in mice infected with *S. typhimurium* χ 4064(pBA31-R7). BALB/cByJ mice were orally infected with 2×10^8 to 4×10^8 *S. typhimurium* χ 4064(pBA31-R7). Mice (seven per day) were killed on days 2, 7, 14, 21, 28, 35, and 42 postinfection. The spleen (○), liver (●), mesenteric lymph nodes (□), and small intestine (■) were removed from each animal and homogenized. The numbers of CFU per gram of tissue were determined by inoculating serial dilutions of the respective homogenates onto MacConkey agar plates containing ampicillin. Levels of colonization often varied considerably among the mice. Reported values are the mean of seven different mice.

χ 4064(pBA31-R7) nor i.p. injection with rBCSP31 in FIA was capable of priming spleen cells to be stimulated in vitro with BCSP31 as indicated by the lack of [3 H]thymidine uptake over a 5-day incubation period. However, spleen cells from mice injected with *S. typhimurium* χ 4064(pBA31-R7) soluble protein in FIA were primed to in vitro stimulation with *S. typhimurium* χ 4064(pBA31-R7) soluble protein (data not shown).

DISCUSSION

The pBA31-R7 plasmid containing the gene for *B. abortus* 31-kDa protein (BCSP31) was transduced into *S. typhimurium* χ 4064 (Δ *cya* Δ *crp*). The plasmid was found to be stable in both *S. typhimurium* SL5283 and χ 4064. *S. typhimurium* isolated from tissue homogenates of BALB/cByJ mice were still capable of in vitro expression of the cloned 31-kDa periplasmic protein (rBCSP31).

Colonization studies determined that between 7 and 14 days postinfection, *S. typhimurium* χ 4064(pBA31-R7) effectively invades and colonizes the small intestine, mesenteric lymph nodes, liver, and spleen. The progression of events after infection is in agreement with that noted by Carter and Collins (4) when mice were orally fed *S. typhimurium*. Except for a few resident salmonella, the majority of *S. typhimurium* χ 4064(pBA31-R7) cells were cleared by day 21.

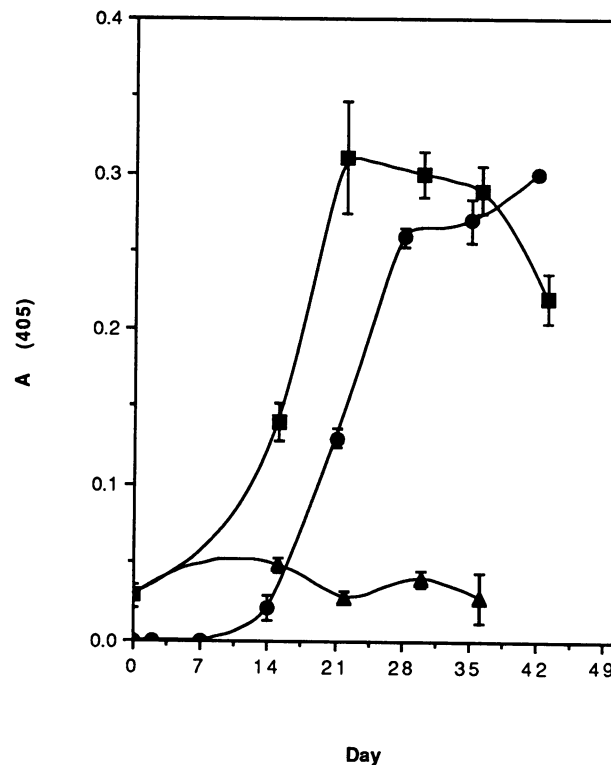


FIG. 5. Serum antibody response of mice immunized against a brucella protein (BCSP31). BALB/cByJ mice (seven mice per group) were immunized on day 0 as follows: orally with 2×10^8 to 4×10^8 *S. typhimurium* χ 4064(pBA31-R7) per mouse (●), s.c. with 25 μ g of purified protein (rBCSP31) in FIA (■), or s.c. with 0.2 ml of PBS in FIA (▲). Those mice receiving rBCSP31 or PBS on day 0 were reimmunized with the same antigen 15 days later. Serum was collected and assayed by ELISA for the total antibody response against rBCSP31. Reported values are the mean of seven mice plus or minus the standard error of the mean.

However, recombinant *S. typhimurium* χ 4064(pBA31-R7) has been isolated from the small intestines and spleens of infected mice 10 weeks post-primary oral immunization.

Oral delivery of rBCSP31 in an inherently invasive enteric bacteria, such as *S. typhimurium*, was an effective method for stimulation of humoral immunity. The strong humoral antibody response of orally infected mice against the rBCSP31 protein was equivalent to titers produced by mice parenterally immunized with rBCSP31 in FIA. In mice orally immunized with *S. typhimurium* χ 4064(pBA31-R7), serum immunoglobulin to rBCSP31 and salmonella endotoxin peaked at day 35 (Fig. 5). Antigen-specific IgG was largely responsible for the increase in titer, although a small serum IgA response to rBCSP31 and endotoxin occurred on days 2 and 25, respectively (data not shown). Interestingly, the oral route of inoculation did not elicit a detectable serum IgM response to either the rBCSP31 or salmonella endotoxin.

The oral route of infection with live recombinant bacteria was also intended to stimulate a localized secretory antibody response to rBCSP31. Intestinal homogenates contained high-titer IgA directed against *S. typhimurium* χ 4064 endotoxin and a low-titer IgA response to rBCSP31. Each antigen also stimulated a low-level IgG response. In both cases, no IgM titers were apparent (Fig. 7). Secretory responses to rBCSP31 and *S. typhimurium* χ 4064 endotoxin showed peak IgA titers at days 23 and 25 post-oral immunization, respec-

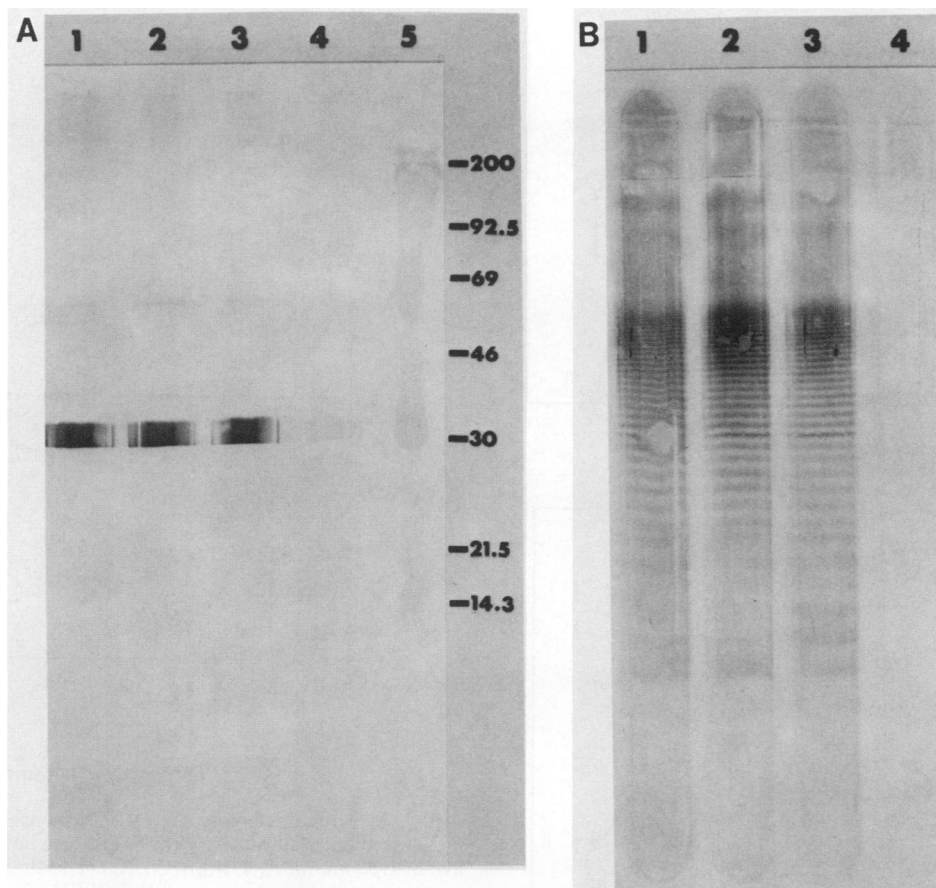


FIG. 6. Immunoblot analysis of serum collected from immunized mice. rBCSP31 (A) or butanol-water-extracted *S. typhimurium* χ 4064 endotoxin (B) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred to nylon membranes, immunoblotted with various mouse sera (1/100 dilution), and incubated with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin. Lanes: 1 and 2, sera collected from mice 35 (lane 1) and 42 (lane 2) days postinfection; 3, serum on day 7 following secondary oral immunization with *S. typhimurium* χ 4064(pBA31-R7); 4, preimmune serum; 5 (panel A), Amersham rainbow molecular mass markers (in kilodaltons).

tively. In mice, 38% of the serum-derived, polymeric IgA has been shown to be transported via the liver into the bile (11). This mechanism may have contributed to the level of antigen-specific IgA detected in the intestinal homogenates. However, it is unlikely that hepatobiliary transport of IgA was responsible for the low level of antigen-specific IgA detected in the serum. It has been shown in rats that as much as 90% of the serum-derived IgA is transported into the bile and that this mechanism contributes to the low level of serum IgA detected in rats (11). To enhance mucosal immunity, Keren et al. (15) have reported that a parenteral dose of antigen 1 day prior to oral inoculation enhanced intestinal IgA secretion. A parenteral dose of specific antigen may be necessary to optimize the secretion of intestinal IgA prior to oral stimulation with a live-vector vaccine.

Attempts to monitor secretory antibody to rBCSP31 in saliva were unsuccessful. Others have detected a salivary IgA response to antigen cloned into *S. typhimurium* SR-11 mutants (8, 14). Data supporting transport of serum polymeric IgA into glandular secretions other than the hepatobiliary system provide conflicting results. For example, serum IgA has been detected in saliva, colostrum, and milk samples, but its presence may vary from species to species depending on the availability of secretory component (30). Antigen-specific IgA has been detected in murine saliva (5,

8, 14). However, the serum titers of antigen-specific IgA demonstrated in the current studies may have been too low to facilitate detection of salivary IgA with our assay system. The data agree with observations made by LaBrooy et al. (16) who found that detection of salivary IgA is not a reliable indicator of specific intestinal secretory antibody.

The DTH and blastogenic responses indicate that the purified 31-kDa protein administered alone or via *S. typhimurium* χ 4064 does not stimulate cellular immunity in BALB/cByJ mice. Multiple oral doses of *S. typhimurium* χ 4064(pBA31-R7) or multiple parenteral doses of rBCSP31 were also unsuccessful in eliciting an antigen-specific DTH response to rBCSP31. The lack of a cell-mediated response in contrast to the presence of good humoral antibody responses may indicate the development of suppressor T-cell mechanisms. A similar observation was noted in mice that developed oral tolerance when fed sheep erythrocytes (M. J. Wannemuehler, unpublished observation). In addition, T-cell epitopes necessary for recognition of the major histocompatibility complex (MHC) class II-BCSP31 antigen complex may not be present in BALB/cByJ mice or C3H/HeN mice (unpublished observation). These data may also indicate that the DTH response is a poor indicator of cell-mediated immunity (6). Chen-Woan et al. (6) demonstrated T-cell-mediated immunity to *Listeria* infections in the ab-

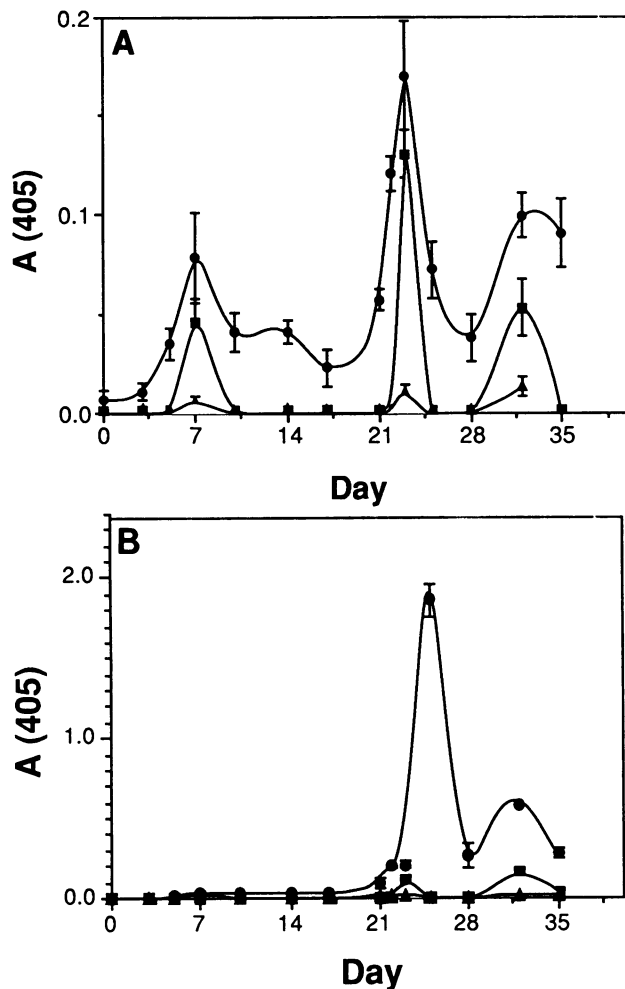


FIG. 7. Intestinal antibody response of mice immunized with live recombinant *S. typhimurium* χ 4064(pBA31-R7). Pooled intestinal homogenates of *S. typhimurium* χ 4064(pBA31-R7)-infected mice (three mice per group) were assayed by ELISA for specific antibody to rBCSP31 (A) or *S. typhimurium* χ 4064 endotoxin (B). Symbols: ●, IgA; ■, IgG; ▲, IgM. Data represent the mean plus or minus the standard error of the mean.

sence of detectable DTH responses. The detection of low numbers of antigen-specific T cells, generated by live-vector vaccines, may require *in vitro* amplification in the presence of interleukin 2. It is also possible that the T-cell-mediated response to rBCSP31 was restricted to peripheral lymphoid tissues and was not present in the spleen. This is unlikely since immunized mice failed to respond to a footpad injection of the rBCSP31. The ability of salmonella to induce cell-mediated immunity to heterologous antigens has been demonstrated by others. Brown et al. (3) have successfully elicited a DTH response to β -galactosidase expressed in an *aroA* mutant of *S. typhimurium*. However, they were using a different *Salmonella* strain and a different cloned protein and their route of immunization was intravenous and was followed by an intravenous boost 35 days later. This immunization protocol may be required to induce a DTH response, but the parenteral route of immunization may not induce significant secretory immune responses.

BALB/cByJ serum and intestinal antibody titers were not enhanced by a second oral dose of *S. typhimurium*

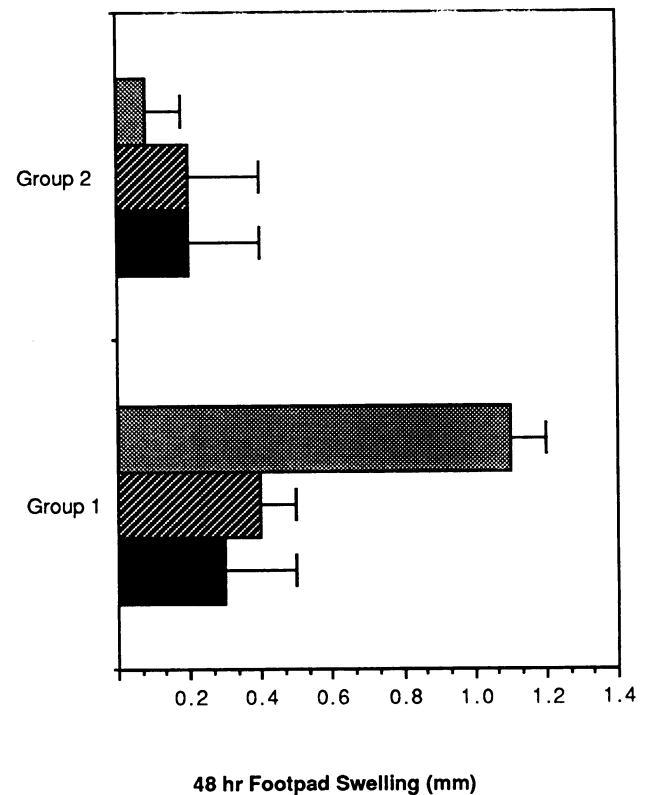


FIG. 8. Measurement of the DTH response in the footpads of immunized mice. Mice (five per group) were immunized orally with live *S. typhimurium* χ 4064(pBA31-R7) (2×10^8 to 4×10^8 CFU per mouse) (stippled bars) or s.c. with rBCSP31 (50 μ g) in FIA (hatched bars). Control mice received no primary immunization (solid bars). At 21 days, mice received a footpad injection s.c. of either a soluble extract of *S. typhimurium* χ 4064(pBA31-R7) (group 1) or rBCSP31 (group 2). Footpads were measured at 10, 24, 48, and 72 h. Results represent the mean plus or minus the standard error of the mean of footpad thickness at 48 h postinfection.

χ 4064(pBA31-R7) given on day 14 or 21 post primary immunization. This lack of responsiveness may be due to IgA bound to the cell surface causing interference with intestinal absorption and invasion (27). Others have proposed that the induction of regulatory T cells following oral immunization causes suppression (24, 34) of a local anamnestic response.

Even though *S. typhimurium* χ 4064(pBA31-R7) proved to be highly invasive, this alone was insufficient for production of an all-encompassing immune response to rBCSP31 (i.e., cell-mediated, humoral, and secretory responses). However, results indicated that the vector itself was adequately presented to the host immune system and elicited a cellular immune response. In both the DTH and blastogenesis assays, a cell-mediated response was elicited by primary s.c. immunization with an *S. typhimurium* χ 4064(pBA31-R7) whole-cell sonic extract. It has been shown that *S. typhimurium* χ 4064 provided good protective immunity against virulent *S. typhimurium* infections (9). Our data suggest that protection against salmonellosis may be due in part to excellent antibody responses toward lipopolysaccharide or endotoxin.

Identical studies in swine with rBCSP31 protein and the *S. typhimurium* χ 4064 vector are in progress to determine if the results obtained from mice are generally applicable to other species. Preliminary data indicate that, unlike mice, swine

develop strong DTH cell-mediated immunity in response to parenteral immunization with rBCSP31 in FIA. In addition, other mutant vectors are also being considered for development of swine immunity to BCSP31. It appears that *S. typhimurium* χ 4064 will provide an effective means of presenting many different antigens to the host mucosal immune system of various animal species.

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LITERATURE CITED

- Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* 7:1513-1523.
- Bricker, B. J., L. B. Tabatabai, B. L. Deyoe, and J. E. Mayfield. 1988. Conservation of antigenicity in a 31-kDa *Brucella* protein. *Vet. Microbiol.* 18:313-325.
- Brown, A., C. E. Hormaeche, R. Demarco de Hormaeche, M. Winther, G. Dougan, D. J. Maskell, and B. A. D. Stocker. 1987. An attenuated *aroA* *Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned β -galactosidase in mice. *J. Infect. Dis.* 155:86-92.
- Carter, P. B., and F. M. Collins. 1974. The route of enteric infection in normal mice. *J. Exp. Med.* 139:1189-1203.
- Challacombe, S. J. 1983. Salivary antibodies and systemic tolerance in mice after oral immunization with bacterial antigens. *Ann. N.Y. Acad. Sci.* 409:177-193.
- Chen-Woan, M., D. H. Sajewski, and D. D. McGregor. 1985. T-cell co-operation in the mediation of acquired resistance to *Listeria monocytogenes*. *Immunology* 56:33-42.
- Clements, J. D., F. L. Lyon, K. L. Lowe, A. L. Farrand, and S. El-Morshidy. 1986. Oral immunization of mice with attenuated *Salmonella enteritidis* containing a recombinant plasmid which codes for production of the B subunit of heat-labile *Escherichia coli* enterotoxin. *Infect. Immun.* 53:685-692.
- Curtiss III, R., R. Goldschmidt, S. Kelly, M. Lyons, S. M. Michalek, R. Pastian, and S. Stein. 1987. Recombinant avirulent *Salmonella* for oral immunization to induce mucosal immunity to bacterial pathogens. *In* H. Kohler and P. T. LoVerde (ed.), *Vaccines: new concepts and developments*. Proceedings of the Tenth International Convocation on Immunology. Longman Scientific and Technical Harlow, Essex, Great Britain.
- Curtiss III, R., and S. M. Kelly. 1987. *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. *Infect. Immun.* 55:3035-3043.
- Davis, R. W., D. Botstein, and J. R. Roth (ed.). 1980. A manual for genetic engineering: advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Delacroix, D. L., G. N. Malburny, and J. P. Vaerman. 1985. Hepatobiliary transport of plasma IgA in the mouse: contribution to the clearance of intravascular IgA. *Eur. J. Immunol.* 15:893-899.
- Difco Laboratories. 1984. Difco manual, 10th ed. Difco Laboratories, Detroit.
- Fairbanks, G., T. L. Steck, and D. F. Wallach. 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* 10:2606-2617.
- Katz, J., S. M. Michalek, R. Curtiss III, C. Harmon, G. Richardson, and J. Mestecky. 1987. Novel oral vaccine: the effectiveness of cloned gene products on inducing secretory immune responses. *Adv. Exp. Med. Biol.* 216B:1741-1747.
- Keren, D. F., R. A. McDonald, and J. L. Carey. 1988. Combined parenteral and oral immunization results in an enhanced mucosal immunoglobulin A response to *Shigella flexneri*. *Infect. Immun.* 56:910-915.
- LaBrooy, J. T., G. P. Davidson, D. J. C. Shearman, and D. Rowley. 1980. The antibody response to bacterial gastroenteritis in serum and secretions. *Clin. Exp. Immunol.* 41:290-296.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227:680-685.
- Lederberg, E. M., and S. N. Cohen. 1974. Transformation of *Salmonella typhimurium* by plasmid deoxyribonucleic acid. *J. Bacteriol.* 119:1072-1074.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maskell, D., F. Y. Liew, K. Sweeney, G. Dougan, and C. Hormaeche. 1986. Attenuated *Salmonella typhimurium* as live oral vaccines and carriers for delivering antigens to the secretory immune system, p. 213-217. *In* F. Brown (ed.), *Vaccines 86: new approaches to immunization—developing vaccines against parasitic, bacterial and viral diseases*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Maskell, D. J., K. J. Sweeney, D. O'Callaghan, C. E. Hormaeche, F. Y. Liew, and G. Dougan. 1987. *Salmonella typhimurium* *aroA* mutants as carriers of the *Escherichia coli* heat-labile enterotoxin B subunit to the murine secretory and systemic immune systems. *Microb. Pathog.* 2:211-221.
- Mayfield, J. E., B. J. Bricker, H. Godfrey, R. M. Crosby, D. J. Knight, S. M. Halling, D. Balinsky, and L. B. Tabatabai. 1988. The cloning, expression, and nucleotide sequence of a gene coding for an immunogenic *Brucella abortus* protein. *Gene* 63:1-9.
- Michalek, S. M., H. Kiyono, M. J. Wannemuehler, L. M. Mosteller, and J. R. McGhee. 1982. Lipopolysaccharide (LPS) regulation of the immune response: LPS influence on oral tolerance induction. *J. Immunol.* 128:1992-1998.
- Morrison, D. C., and L. Leive. 1975. Fractions of lipopolysaccharide from *Escherichia coli*:O111:B4 prepared by two extraction procedures. *J. Biol. Chem.* 250:2911-2919.
- Poirier, T. P., M. A. Kehoe, and E. H. Beachey. 1988. Protective immunity evoked by oral administration of attenuated *aroA* *Salmonella typhimurium* expressing cloned streptococcal M protein. *J. Exp. Med.* 168:25-32.
- Rowley, D. 1983. Immune responses to enterobacteria presented by various routes. *Prog. Allergy* 33:159-174.
- Schaible, U. E., M. D. Kramer, C. W. E. Justus, C. Museteanu, and M. M. Simon. 1989. Demonstration of antigen-specific T cells and histopathological alterations in mice experimentally inoculated with *Borrelia burgdorferi*. *Infect. Immun.* 57:41-47.
- Sekar, V. 1987. A rapid screening procedure for the identification of recombinant bacterial clones. *Biotechniques* 5:11-13.
- Sheldrake, R. F., A. J. Husband, D. L. Watson, and A. W. Cripps. 1984. Selective transport of serum IgA into mucosal secretions. *J. Immunol.* 132:363-368.
- Sidberry, H., B. Kaufman, D. C. Wright, and J. Sadoff. 1985. Immunoenzymatic analysis by monoclonal antibodies of bacterial lipopolysaccharides after transfer to nitrocellulose. *J. Immunol.* 76:299-305.
- Stevenson, G., and P. A. Manning. 1985. Galactose epimeraseless (gal E) mutant G30 of *Salmonella typhimurium* is a good potential live oral vaccine carrier for fimbrial antigens. *FEMS Microbiol. Lett.* 28:317-321.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* 76:4350-4353.
- Wannemuehler, M. J., H. Kiyono, J. L. Babb, S. M. Michalek, and J. R. McGhee. 1982. Lipopolysaccharide (LPS) regulation of the immune response: LPS converts germfree mice to sensitivity to oral tolerance induction. *J. Immunol.* 129:959-965.