Secretory immunity in Balb/c mice against Salmonella typhimurium
by Steven Richard Shope

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University

Abstract:
Experiments were designed to test the ability of avirulent mutants of Salmonella typhimurium to induce secretory immune responses in mice and demonstrate the passive transfer of protection resulting from these immune responses to foster pups. S. typhimurium Aro- mutant SL3235 and cya crp mutant X4064 retained the ability to adhere to and invade intestinal epithelial cells in tissue culture assays. Findings demonstrated that plasmid loss did not alter the ability of S. typhimurium to adhere to and invade epithelial cells in vitro. Subcutaneous priming using live avirulent S. typhimurium with adjuvant followed by oral exposure to the same organism was used to immunize two groups of mice. One group of mice was immunized using 3 oral exposures through drinking-water to live avirulent S. typhimurium. Using an immunoassay to measure specific antibody in the feces of immunized mice, it was determined that subcutaneous priming was not an effective method for inducing reproducible intestinal immune responses. In contrast, mice immunized using multiple oral exposures demonstrated strong intestinal responses characterized by specific IgA production and a memory response. The production of protective antibody resulting from the induction of a secretory response was demonstrated by the passive transfer of protection to foster pups. The survival time for 50% of foster pups placed with immunized mothers was at least two times greater than controls consisting of foster pups placed with unimmunized mothers.

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A thesis submitted in partial fulfillment
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of

Master of Science

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Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana

July 1990

APPROVAL

of a thesis submitted by

Steven Richard Shope

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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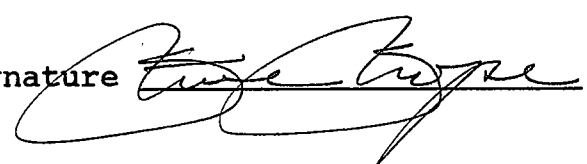
Signature 

Date 7/24/90

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ABSTRACT

Experiments were designed to test the ability of avirulent mutants of *Salmonella typhimurium* to induce secretory immune responses in mice and demonstrate the passive transfer of protection resulting from these immune responses to foster pups. *S. typhimurium Aro-* mutant SL3235 and *cya crp* mutant X4064 retained the ability to adhere to and invade intestinal epithelial cells in tissue culture assays. Findings demonstrated that plasmid loss did not alter the ability of *S. typhimurium* to adhere to and invade epithelial cells *in vitro*. Subcutaneous priming using live avirulent *S. typhimurium* with adjuvant followed by oral exposure to the same organism was used to immunize two groups of mice. One group of mice was immunized using 3 oral exposures through drinking-water to live avirulent *S. typhimurium*. Using an immunoassay to measure specific antibody in the feces of immunized mice, it was determined that subcutaneous priming was not an effective method for inducing reproducible intestinal immune responses. In contrast, mice immunized using multiple oral exposures demonstrated strong intestinal responses characterized by specific IgA production and a memory response. The production of protective antibody resulting from the induction of a secretory response was demonstrated by the passive transfer of protection to foster pups. The survival time for 50% of foster pups placed with immunized mothers was at least two times greater than controls consisting of foster pups placed with unimmunized mothers.

INTRODUCTION

Salmonella typhimurium

S. typhimurium is a member of a group of bacteria consisting of more than 2,000 serotypes as described by the serotyping scheme of Kauffmann and White (21). This organism as a member of the genus *Salmonella* in the family Enterobateriaceae is a gram-negative, facultative anaerobic, peritrichously flagellated rod which produces gas from glucose. It is a facultative intracellular enteric pathogen which does not ferment lactose.

Salmonellosis, a human enteric disease caused by representatives of the *Salmonella* serotype group, is considered an important medical problem in the United States (13). The number of cases reported annually to the CDC is approximately 40,000 (13), but these cases probably represent only a small fraction of the number of cases which occur in the United States (13,63). Estimates have indicated that as many as 800,000 to 5,300,000 cases may occur annually in this country (13,63), with as many 9,000 deaths resulting from these cases. CDC reports from 1983 cite *S. typhimurium, S. enteritidis, S. heidelburg,* and *S. newport* as the four most common serotypes isolated from human sources, *S. typhimurium* being the most common of these four with 13,172

isolates (12). Most human cases of salmonellosis come from foodborne sources (58). Poultry, meat, and dairy products are the most important vehicles in the transmission of the disease (13). *S. typhimurium* infections in certain inbred strains of mice mimic a more serious human disease. Murine salmonellosis in mice homozygous for the chromosome 1 allele *Ity*^{s} caused by this organism is commonly regarded as an analogous disease to human typhoid fever and offers a good model for the study of this disease (38).

Hoiseth *et al.* (37) and Dougan *et al.* (18) described the construction and preliminary testing of several avirulent auxotropic mutants of *S. typhimurium*. Curtiss *et al.* (16) constructed a mutant lacking adenylate cyclase and the cyclic AMP receptor protein which exhibited reduced virulence in mice. These mutants as well as others, such as the mutants deficient in the enzyme UDP-glucose-4-epimerase (*galE* mutants) (14) have been the subject of testing with regard to their ability to effectively elicit an immune response protective against challenge from their virulent counter-parts (14,16,18,32,37,46,47,56,59,65).

The Pathogenesis of S. typhimurium

The pathogenesis of *S. typhimurium infections* in a murine host can be divided into two steps: (i) The invasion of epithelial cells of the gastrointestinal mucosa and migration

of the organism through the epithelial cell layer to the underlying lymphoid tissue. (ii) The survival, replication and migration of the organism within the hosts lymphoid and reticuloendothelial system (RES).

The Invasion of Host Epithelial Cells

Once *Salmonella* have been ingested, the first interaction with the host is attachment to the gastrointestinal mucosa. In its simplest form, this attachment requires the participation of two factors: (i) receptors present on the surface of the eukaryotic cells; and (ii) adhesin located on the surface of the bacterial cell (24). The bacterial adhesin(s) of *S. typhimurium* have yet to be clearly defined. Fimbriae and a mannose-resistant hemagglutinin have been proposed as possible adhesins, but non-fimbriated strains of *S.typhimurium* are as virulent as fimbriated strains when fed orally to mice (24,40). During the process of attachment, the invading organism proceeds through two phases: an unstable reversible form of attachment involving hydrophobic interactions, and a more stable, irreversible form of attachment involving adhesin-receptor interactions (41). It was proposed by Finlay *et al.* (25,27) that during the initial interactions between cell and bacteria several proteins are induced on the surface of the invading organism by eukaryotic proteins leading to the more stable irreversible form of attachment and eventual internalization.

Internalization of the organism into host cells is a process that requires the synthesis of several bacterial proteins. It has been proposed that these proteins are also induced by cell-bacteria interactions but that they are not the same proteins which mediate stable attachment (29). Galán *et al.* (29) isolated and described a genetic locus, *inv,* which was necessary for wild-type *S. typhimurium* strains to penetrate epithelial cells *in vitro.* This group of workers also found that while the proteins encoded by this locus were necessary for the internalization of the organism they were not necessary for the initiation of stable attachment. The invading organism is enclosed in a membrane bound vacuole as it enters the cell through a process similar to phagocytosis that actively involves cellular microfilaments but not acidification of the vacuole which is an important part of endocytic pathways (23). Each invading organism enters the cell in a separate vacuole which later coalesce into one large intracellular vacuole (24). *Salmonella* replicate within these vacuoles and appear to remain vacuolized following this replication (23). Finlay *et al.* (25,26) used polarized Madin Darby canine kidney epithelial cell cultures to demonstrate the transcytosis of *Salmonella.* It was shown that *Salmonella* do not use any pre-existing pathway for intracellular transport through the epithelial cell. Instead, most of the invading organisms remain within the cell, with only a few exiting to either the apical or basolateral epithelial cell

surface. *In vivo,* the successful penetration of a few microorganisms through the epithelium is probably sufficient to cause disease (25,26).

Survival in the Host

Once through the intestinal barrier, the invading organism passes through the lamina propria and into the gut associated lymphoid tissue (GALT). *S. typhimurium* colonizes the Peyer's patches, invades into draining mesenteric lymph nodes, and, due to *S. typhimurium's* resistance to killing by phagocytic cells, spreads through the lymphatic system to the liver and spleen (30). *S. typhimurium's* resistance to the bactericidal activity of serum complement allows a disseminated systemic infection to follow.

Plasmid Associated Virulence. It appears that a 60 megadalton (Mdal) "cryptic" plasmid [100 kilobases (kb)] is responsible for *S. typhimurium's* antiphagocytic properties (33,36) and resistance to the activity of serum complement as determined by the structure of the organisms lipopolysaccharide (LPS) (33,34,70), although there has been some argument concerning whether or not resistance to phagocytosis and the activity of serum complement can be attributed to this organism (30,31). Colonization of Peyer's patches was found not to be mediated by the cryptic plasmid (30,33) as proposed by Jones *et al.* (39), but the plasmid was found to be involved in the infection of the spleen and

mesenteric lymph nodes following peroral (po) inoculation of mice (30).

Other Virulence Associated Properties of S. typhimurium. The survival and replication of *S. typhimurium* in the cells of the RES (polymorphs and macrophages) are attributes which have been associated with the virulence of this organism. While there is some evidence demonstrating the possibility of a mechanism involving the survival of *S. typhimurium* within murine macrophages (11,71), data supporting replication of *S. typhimurium* within murine macrophages is limited (38). Hsu (38) has indicated that observations of the replication of *S. typhimurium* within murine macrophages is based on the artifacts in crude experimental procedures and the erroneous assumption that because the liver and spleen are organs of the RES, organisms are trapped by and replicate within resident macrophages (Kupffer cells). It has been proposed by Hsu that *S. typhimurium* proliferates in the sinusoids and intercellular spaces found in the liver and spleen, not in the resident macrophages.

Host Defenses Against Colonization by Salmonella typhimurium

The gastrointestinal mucosa is the first barrier which an enteroinvasive bacteria such as *S. typhimurium* must adhere to and cross in order to effectively colonize its host. This barrier is defended by the cooperative action of specific

immunoglobulins derived from the mucosal immune system and innate nonspecific factors such as the mucous layer coating the mucosa (55). Together, the specific action of secretory IgA (SIgA) and IgM binding an antigen, the nonspecific interactions which trap SIgA coated antigens in the mucus layer of the intestine, and the shedding of the trapped antigen from the gastrointestinal tract form a protective process termed immune or antigen exclusion (8,50,52).

Mucosal Immunity

The existence of a protective local immune system that seemed to function independently of systemic immunity was initially proposed by Besredka early in this century (7). It was not until 1965 that the molecular basis for local immunity was established when Tomasi *et al.* confirmed the external secretions contained a unique immunoglobulin subsequently called SIgA (7). It is now known that a functional local immune system which is mediated by secretory tissue and is independent of the humoral immune system exists (7,8,69).

The exocrine secretory tissues constitute the most important mediator of specific immunity with the intestinal mucosa being the greatest contributor (7). Estimates indicate that roughly 70-80% of all immunoglobulin producing cells in the human are located in the gastrointestinal mucosa (7).

The Uptake of Antigen from the Gut. The defense system of the gastrointestinal mucosa is based on the continuous surveillance of lumenal antigens by specialized antigen transport mechanisms in the Peyer's patches and the epithelium (55). Antigen present in the gut lumen is taken up and transported into the Peyer's patches either by specialized microfold (M) cells located in the intestinal epithelium in close association with intraepithelial lymphocytes or by MHC class II-positive epithelial cells (7,55). The antigen is then either processed by a macrophage and presented to a T cell by a MHC class II-positive dendritic cell, presented directly to a T cell by a MHC class II-positive dendritic cell, or presented directly to a T cell by a MHC class II-positive epithelial cell (7).

B-cell Differentiation and Migration. T-cells activated by the presentation of the antigen provide various regulatory "first signals" (gamma-interferon and possibly interleukins 2, 5, and 6) to the B-cell which induce B-cell differentiation (IgM or IgA expression) (7,8,53,67). Early studies showed that clonal immaturity, as evidenced by the potential for J-chain production, combined with prominent IgA expression characterize the B-cells that migrate to secretory tissue from the Peyer's patches (7). But more recent studies indicate that the majority of Peyer's patch derived cells arriving in the intestinal mucosa are not IgA expressing cells but belong

to a population of IgM and IgD bearing memory cells (8). Such lymphocytes were probably exposed to stimulatory "first signals" and require "second signals" in order to further differentiate and divide into IgA-producing immunocytes (8,67). These "second signals" are most likely encountered as the B-cell migrates through the mesenteric lymph nodes and spleen and in the gut mucosa or secretory tissue where they end up (8,67).

Several hypotheses have been put forward to explain the migration of B-cells to secretory tissue after their initial differentiation: (i) The stimulated B-cells have an affinity for secretory component (SC) which directs them to the glandular epithelium where SC is expressed. (ii) Epithelial class II MHC molecules act to direct the B-cells to secretory epithelial tissue. (iii) Unidentified epithelial factor(s) expressed in secretory tissue may selectively attract certain lymphoid cell populations such as the stimulated B-cells (7,8).

Secretory IgA (SIgA). SIgA is the major species of immunoglobulin synthesized and secreted as the result of the induction of a mucosal immune response, with IgM playing a minor role (7,8,55,67). Complete SIgA is characterized by two immunoglobulin molecules (molecular weight of approximately 190,000) (alpha heavy chains) cross-linked by a J-chain (molecular weight of approximately 15,000) and complexed with

one SC molecule (molecular weight of approximately 80,000) (8). The IgA dimer becomes complexed with an SC molecule as it interacts with a glandular epithelial cell. It has been proposed by Brandtzaeg (6,7) that the SC molecule acts as a glandular cell membrane receptor for J-chain containing immunoglobulins, and that the receptor-substrate complexes formed on the cell surface are taken up by absorptive pinocytosis for secretion into the intestinal lumen. SIgA has a broad specificity which allows it to recognize antigens which are slightly altered due to antigenic drift (69). The structure of the complete SIgA molecule also confers an inherent resistance to enzymatic digestion and low pH to the molecule which is necessary for the survival and activity of SIgA in the gut lumen (2,8,55).

SIgA's mode of action differs from that of IgG and IgM in that it does not exert its effectiveness through complement activation or opsonization, rather as an efficient cross-linking agent and possibly by mediating the inhibition of the ability of organisms to adhere to the mucosa (50,72). It has been shown that antigen bound by SIgA is not absorbed by the gut but remains in the lumen (1). Magnusson *et al.* (50) described two protective properties which SIgA offers to the gastrointestinal mucosa: (i) The binding of antigens by SIgA increases their affinity for the mucus lining of the intestine promoting immune exclusion at the mucosal membranes.

(ii) SIgA modifies IgG-mediated phagocytosis as well as the general surface properties of bacteria, thereby possibly depressing local inflammatory responses at the mucosa. Many studies have shown that bacteria bound to SIgA are prevented from associating with mucosal membranes by mechanisms of immune exclusion or by inhibition of adherence. The adherence of *Campylobacter jejuni* to epithelial cells *in vitro* was inhibited by SIgA-containing mucus (54). *In vitro* studies have also demonstrated that colostral SIgA had the ability to decrease the association of *Salmonella* with tissue culture cells (49), and that the penetration of HeLa cells by invasive *Escherichia coli* could be inhibited by the presence of colostrum containing IgM (57). It has also been demonstrated *in vivo* that the presence of passively-provided immunoglobulins could inhibit the colonization of rabbits and humans by enteroinvasive or adherent organisms (4,9,10,19,57,68). Cantey (9) demonstrated that SIgA given passively could prevent the colonization of rabbits by non-piliated *E. coli* and that in the absence of IgA diarrhea would occur (10). Low-birth-weight infants fed human serum preparations containing 73% IgA were protected against enterocolitis (19). Tacket *et al.* (68) demonstrated that lyophilized immunoglobulins prepared from the colostrum of immunized cows could protect against challenge with enterotoxigenic *E. coli* when fed to human volunteers. Oral immunization with live avirulent bacteria has been shown to

induce an immune response protective against oral challenge by virulent organisms (22,28). Fierer *et al.* (22) demonstrated that a plasmid-cured strain of *Salmonella dublin* given orally to mice provided protection against a subsequent challenge with group B and D virulent *Salmonella* which left the intestinal walls, Peyer's patches, and mesenteric lymph nodes free of the challenge organism. Oral immunization with an *E. coli* strain carrying the invasion-mediating plasmid of *Shigella* provided protection to monkeys challenged by *Shigella* (28).

SIgA has also been shown to enhance the bacteriostatic effects of lactoferrin by inhibiting bacterial production of iron-chelating agents which may interfere with its activity, and it may also enhance the effect of the peroxidase defense system by some unknown mechanism (8).

Stimulation of Mucosal Immunity. Mucosal immunity can be stimulated by a variety of antigens as long as they possess one of two general characteristics: (i) special binding characteristics; or (ii) the ability to promote delivery into the GALT (17). Keren *et al.* (44,45) demonstrated the induction of a strong specific mucosal IgA memory response in parenterally primed rabbits orally immunized with live *Shigella*, where rabbits immunized with killed *Shigella* did not show such a response. Curtiss *et al.* (15) described the induction of a mucosal response in mice immunized orally with

an avirulent strain of *S. typhimurium* carrying the *Spa*A gene of *Streptococcus mutans* specifity to both the organism and the *Spa*A protein. Curtiss also indicated that oral immunization of mice with live avirulent aromatic mutants of *S. typhimurium* offered protection against challenge by a virulent strain of the same organism. Cholera toxin has been shown to stimulate a strong mucosal IgA memory response due to its ability to effectively enter the GALT (48).

The effectiveness of different immunization protocols in eliciting a strong mucosal memory response is still under investigation. The standard protocol for inducing a mucosal memory response has been to administer multiple oral doses of the antigen over a period of several weeks (43-45,48), but Keren *et al.* have demonstrated an effective method involving the use of a parenteral priming dose of the antigen (intravenous or subcutaneous) prior to the administration of an oral dose (43,44).

Statement of Research Problem

This study was undertaken to investigate the ability of mutant strains of *S. typhimurium* to elicit a specific protective secretory immune response. This study had three objectives: (i) to characterize these mutants with respect to their ability to adhere to and invade intestinal epithelial cells *in vitro,* and their plasmid profile relative to the virulent *S. typhimurium* strain SL3201; (ii) to induce a measurable and specific secretory immune response in Balb/cBy mice using these mutant strains; and (iii) to demonstrate protection provided by this response through the use of passive protection experiments with mouse foster pups.

MATERIALS AND METHODS

Bacterial Strains

The two strains of *S. typhimurium* of primary interest in this study were SL3235 and X4064. Both of these strains contain insertion mutations which are reflected in their inability to effectively colonize host tissue (16,37). This characteristic has generated interest in their use as live vaccines against virulent *S. typhimurium* (16,37,65)

Strain SL3235 was provided by B.A.D. Stocker, Stanford University School of Medicine. The mutation was constructed by the introduction of the transposon *aro*A554::*Tn*10 into gene *aro*A of the virulent *S. typhimurium* strain SL3201 (37). This strain is a stable auxotrophic transductant (*aro*A-) which exhibits a requirement for both p-aminobenzoic acid (PABA) and 2,3-dihydroxybenzoic acid (DHB). The lack of these two metabolites in vertebrate tissues renders SL3235 unable to grow *in vivo*, and, therefore, is avirulent (LD_{50} >$3x10^6$ ip in Balb/cBy mice).

S. typhimurium strain X4064 was received from Roy Curtiss III, Washington University, St. Louis, Missouri. Strain X4064 contains stable insertion mutations (Δcya,Δcrp) which block the synthesis of adenylate cyclase and cAMP receptor protein (CRP) (16). The mutant was constructed by the introduction

of transposon *cya*::*Tn*10 and *crp*::*Tn*10 into genes *cya* and *crp* of the virulent *S. typhimurium* SR-11 strain X3306, respectively. Without adenylate cyclase this organism cannot synthesize cyclic AMP (cAMP) which, along with CRP, is necessary for the transcription of many genes and operons associated with catabolic pathways (16). Cyclic AMP exerts positive control on systems used for the transport of fuel and carbon sources and on several amino acid permeases (16). Lysogeny of temperate phages, the synthesis pili, fimbriae, and flagella are also influenced by the concentration of cAMP within the organism (16). Exogenous sources of cAMP can be utilized by this strain, however, its activity is greatly reduced by the absence of CRP. X4064 exhibits a reduced ability to grow and a reduction in virulence (LD_{50} >1x10^6, ip in Balb/cBy mice) relative to its wild-type parent strain (16).

Other strains of *S. typhimurium* that were used in this study are SL3201, SL1306, and X3337. Strain SL3201 (obtained from B.A.D. Stocker) is a highly virulent (LD_{50} <20 cells, ip in C57BL and Balb/cBy mice) example of the FIRN biotype (unable to produce fimbriae or to ferment inositol and rhamnose) (37). Strain 1306 (obtained from B.A.D. Stocker) carries a mutation in gene *galE* and is unable to synthesize UDP-galactose-4-epimerase, an enzyme important in the formation of LPS. Without this enzyme, strain SL1306 must synthesize LPS by an alternate pathway which leads to the

accumulation of the toxic precusors galactose-1-phosphate and UDP-galactose within the organism. Accompanying the build-up of these compounds is a reduction in the organisms ability to grow and its virulence (14,56). Strain X3337 (obtained from Roy Curtiss III) was previously cured of the 60 Mdal (100 Kb) plasmid which has been associated with the survival of *S. typhimurium* within animal hosts (30).

Several strains of *S. typhimurium* and *Escherichia coli* containing plasmids of known size were used to produce standards for the sizing of plasmids in experimental strains. These include the *S. typhimurium* strain X3306 (obtained from Roy Curtiss III) containing a 60 Mdal plasmid and *E. coli* strains C114 (obtained from Diane Taylor, University of Alberta), C153 (National Collection for Type Cultures #50005), C154 (National Collection for Type Cultures #50012), and C155 (National Collection for Type Cultures #50047) containing 86, 26, 72, and 46 Mdal plasmids, respectively.

Storage of Cultures

Stock cultures were stored at -20°C (primary stock) and -70°C (backup or long-term stock) in a solution of 1.0% peptone and 40% glycerol. All experimental cultures were subcultured from the -20°C stock to avoid any changes in the strain due to repeated subculturing. The -70°C stock culture was used to replace depleted -20°C stock cultures.

Preparation and Handling of Bacteria

Bacterial cultures were prepared by inoculating 4 ml of Difco Brain Heart Infusion broth (BHI) with a small amount of the -20°C stock culture. The broth was incubated (PSYCROTherm, New Brunswick Scientific) static overnight at 35°C and then subcultured to a growth medium. The growth conditions and medium used for subcultures are identified in experimental methods.

In most cases, bacteria were washed prior to use in experiments to remove the growth medium. Washing required pelleting the bacteria by centrifugation (Sorvall model RC-5B highspeed centrifuge, SS-34 rotor), (5000xg, 20 min, 4°C) and resuspending the pellet in either sterile saline (0.85% NaCl) or sterile glass distilled H_2O. The resuspended bacteria were then pelleted again by centrifugation and resuspended in the appropriate diluent. This procedure constituted one wash.

A non-selective plating medium, Trypticase Soy Agar with 0.6% Yeast Extract (TSY), was used for the enumeration of bacteria and the screening of cultures for contamination. Selective plating media (Hektoen and XLD agars, Difco) were used for the isolation of *S. typhimurium* from samples, such as feces and infected animal tissues, containing high numbers of background organisms. Plating of dilutions for counting was achieved by the spread plate method. All inoculated plates were incubated overnight at 35°C.

Development of Standard Curves for Bacterial Strains

Standard curves of absorbance at 540 nm (A_{540}) vs concentration of bacteria (bacteria/ml) were developed for each of the bacterial strains (Appendix H). Bacteria from an 18 h BHI broth culture (10 ml) was washed 1x and diluted 1:10 in saline. This suspension was diluted further by a series of three serial dilutions (1:2), resulting in 1:10, 1:20, 1:40, 1:80 dilutions of the culture. The A_{540} of each dilution (4) was read (DMS 80 Spectrophotometer, Varian). The original 1:10 dilution was then diluted further in saline for enumeration by duplicate plating on TSY agar. The plate counts were then divided by their respective dilution factor in order to determine the concentration of bacteria in the original dilution. A curve plotting the A_{540} vs the concentration of bacteria for each of the four original dilutions (determined mathematically from the concentration of bacteria in the original 1:10 dilution) was constructed and its linear regression formula determined. The linear regression formula was used to determine the concentration of bacteria in washed cultures in order to adjust the concentration of bacteria to a desired value.

Tissue Culture Attachment and Invasion Assay

An *in vitro* method for quantitative description of the interactions between epithelial cells and invasive

enteropathogenic bacteria has been previously described (60,62). A modification of this method was developed during this study.

Preparation and Maintenance of Epithelial Cell Monolayers

Henle 407 intestinal epithelial cells (ATCC CCL-6) were maintained in Eagle's basal medium (BME) with Hanks' balanced salts, 15 mM N-2-Hydroxyethyl-N-2-ethanesulfonic acid (HEPES), and 16 mM sodium bicarbonate (BME-1) containing 10% fetal bovine serum (FBS), penicillin (50 IU/ml) and sterptomycin (50 ug/ml). The Henle cells were incubated (water-jacketed Incubator, Forma Scientific) at 37°C under 5% CO_2 in 75 cm^2 tissue culture flasks until they reached a confluent monolayer at which point they were used to make individual monolayers and/or passed to another tissue culture flask. Cells were never passed more than eight times to avoid the possibility of any change in cell characteristics.

Cell monolayers were prepared 48 h prior to use in an assay. A monolayer in a 75 cm^2 tissue culture flask was washed with 25 ml of Hanks' balanced salt solution without calcium or magnesium (HBSS-A) and treated with 5 ml of Sigma Cell Dissociation Solution (SCDS). The cells were incubated with SCDS for 30 sec and then 4.5 ml of the solution were removed from the flask. The remaining SCDS was allowed to stay on the cells for an additional 5 min to completely detach the cells from the plastic. Once free from the plastic, the

cells were suspended in 5 ml BME with 10% FBS and transfered to a 15 ml tissue culture tube. The cells were then gently pelleted by centrifugation (Sorvall model T6000B) (409xg, 5 min). The cell pellet was resuspended in 5 ml BME with 10% FBS. Trypan Blue exclusion was used to determine the number of viable cells by microscopically counting. The cell density was adjusted to $2.5x10^5$ cells/ml by dilution in BME with 10% FBS. The adjusted cell suspension (0.5 ml) was added to the appropriate wells of a 24 well tissue culture plate in and the plate was incubated for 48 h at 37°C under 5% CO_2. The cell density was approximately $5x10^5$ after 48 h.

Preparation of Bacteria for Tissue Culture Assays

Bacteria for tissue culture assays were prepared by transferring 10 ul of an 18-h BHI broth culture into a 125 ml baffle flask containing 10 ml BHI and incubating the inoculated broth for 18 hr at 35 or 25°C under atmospheric conditions suitable to the assay being run. Anaerobic and aerobic conditions as well as temperature were investigated relative to their effect on the ability of the bacteria to adhere to and invade tissue culture cells in vitro. Anaerobic conditions were produced by placing an inoculated flask of BHI broth into an anaerobe jar (Oxoid) containing an activated gas generation envelope (BBL). The sealed jar was held static at 35°C. Anaerobic conditions were monitored by the presence of a methylene blue indicator strip within the jar. Aerobic

conditions were achieved by shaking an inoculated flask of BHI broth at 240 rpm. Incubation for 18 h produced a stationary phase culture which was pelleted by centrifugation and then washed 1x with saline. The washed bacteria were diluted 1:10 in saline and the absorbance of the suspension at 540 nm was determined spectrophotometrically. Using a standard curve of absorbance at 540 nm vs concentration of bacteria, the density of the bacteria in suspension was determined and adjusted to $1x10^7$ bacteria/ml by dilution in BME with 20mM HEPES (BME-S). The concentration of bacteria was confirmed by duplicate plate counts.

Tissue Culture Attachment and Invasion Assay

A 24-well tissue culture plate organized into 4 rows of 3 wells containing monolayers (~$5x10^5$ cells/monolayer) and a row of 3 wells containing only BME-1 (control wells) was prepared 48 h prior to the start of each assay. The wells (monolayers and control wells) were washed 3x with 0.5 ml of Dulbecco's Phosphate Buffered Saline (DPBS) with 5% FBS. The wells in rows 1 and 2 were treated with 3% paraformaldehyde in DPBS, and the wells in rows 3, 4, and 5 were treated with DPBS-5% FBS for 5 min at 25°C. All wells were washed once with DPBS-5% FBS and treated with a blocking solution consisting of BME containing 20mM HEPES and 0.1% reagent grade gelatin (BME-G) for 1 h at 37°C under 5% CO_2. The blocking solution was removed and 0.5 ml of a prepared dilution ($1x10^7$

bacteria/ml in BME-S) added to each well, resulting in an estimated ratio of bacteria to cells of 10:1. The plate was centrifuged (500×g, 10 min) (Sorvall T6000B) to increase bacterial contact with the monolayers. The inoculated plate was incubated for 2 h at 37°C under 5% CO_2. Following this incubation the wells were washed 5x with DPBS-5% FBS (chilled to 4°C) with gentle rocking for 5 min at 4°C during each wash. The wells in rows 2, 4, and 5 were treated with 0.5 ml 0.5% N-lauroylsarcosine (sarcosine) in saline (warmed to 35°C) and the wells in rows 1 and 3 treated with 100 ug/ml gentamicin in DPBS-5% FBS (warmed to 35°C) for 10 min at 37°C under 5% CO_2. Sarcosine is a detergent which lyses the cells releasing any bacteria which are intracellular and strips attached bacteria from the surface of the wells. The sarcosine washes from the individual wells in each row were collected and combined. Two subsequent washes with sarcosine (without the 10 min incubation) followed for rows 2, 4, and 5. These washes were also collected and combined with the contents of the first sarcosine wash. Dilutions were prepared in saline and plated on TSY agar to enumerate bacteria. The remaining wells in rows 1 and 2 treated with gentamicin were incubated an additional 50 min at 37°C under 5% CO_2. Wells in rows 1 and 3 were washed 2x with DPBS-5% FBS (chilled to 4°C), each wash being rocked 5 min at 4°C, and then washed 3x with sarcosine in saline following the same procedure as above. These washes were also counted by plating on TSY agar.

Preparation of Infected Cells for Electron Microscopy

A method for the preparation of cells infected with bacteria for electron microscopy was developed which combined protocols for the interaction of cells and bacteria in liquid a medium (61) with basic methods for the preparation of samples for electron microscopy (73). Bacteria for this protocol were prepared in a manner identical to that of the attachment and invasion assays. Cells in monolayers were detached from 75 cm^2 tissue culture flasks, resuspended in BME -10% FBS and diluted to a density of $1.11x10^6$ cells/ml. The Henle 407 cells were infected by combining 0.2 ml of a prepared dilution of bacteria in BME-10% FBS ($1x10^9$ bacteria/ml) with 1.8 ml of a cell suspension also diluted in BME-10% FBS in a 15-ml tissue culture tube resulting in a bacteria to cell ratio of 100:1. The bacteria/cell suspension was then incubated for 3 hr at 35°C on a test tube roller. Karnofsky's fixative (2 ml) was added to the tube and held at 4°C for 1 h. Cells and bacteria were pelleted by centrifugation (Sorvall model T6000B) (100×g, 5 min) and washed 3x with 2 ml cacodylate buffer (0.1M NaCacodylate in PBS, pH 7.2) chilled to 4°C. The washed bacteria-cell pellet was then resuspended in 1 ml cacodylate buffer and warmed to 37° in a waterbath. Following the addition of 1 ml 4% agarose (SEAPREP ultra-low gelling temperature agarose) to the bacteria-cell suspension the mixture was transfered to BEEM

capsules (0.5 ml/capsule) and the bacteria\cells pelleted in the agarose by gentle centrifugation (Sorvall model RC-5B, HB-4 rotor) (100×g, 10 min). Gelling of the agarose was facilitated by chilling the BEEM capsules on ice for 10 min. The gelled agarose pellet was then removed from the BEEM capsule and the tip of this pellet containing the bacteria-cells removed with a razor blade. From this point the sample was processed following standard methods outlined in Appendix A.

Plasmid Preparation and Analysis

Plasmids were prepared from each of the experimental strains of *S. typhimurium*. The plasmid preparations were compared and analyzed using agarose gel electrophoresis and the results used to generate a comparative plasmid profile of each strain.

Preparation of Plasmids from Bacterial Strains

Most of the plasmid preparations were made from experimental and standard strains using CIRCLEPREP (BIO 101, Inc.), a rapid plasmid DNA purification kit. In several cases it was necessary, due to the lack of commercial reagents to use a method similar to that of CIRCLEPREP described by Marko *et al.* (51). The reagents for this method were produced in our lab and used following the CIRCLEPREP protocol as outlined in Appendix B.

Analysis of Plasmids Using Gel Electrophoresis

A 0.45% agarose gel (Sigma Type 5 Agarose in 1x Tris-Borate buffer) was poured on the gel platform of a submerged gel apparatus (Bio-Rad). The buffer tanks of the gel apparatus were filled with TBE buffer until the gel was submerged under approximately 2 mm of liquid. Ficoll loading buffer (0.25% Bromphenol Blue, 0.25% Xylene Cyanol, 15% Ficoll 400 in distilled H2O, 6x) was diluted 1:6 with the plasmid preparations before loading. Samples were loaded (15 ul/well) onto the gel and run at 90 volts (LKB Broma 2197 power-supply) for 3 h. The buffer was circulated to avoid uneven heat distribution in the system. The gel was stained for 2 h in a solution of 1 ug/ml ethidium bromide in sterile distilled H_2O. The gel was then destained for 1.5 h in sterile distilled H_2O with changes of fresh H_2O at 15, 30, and 60 min. Photographs of the gel were taken using a UV light-table Polaroid C55 film and a Polaroid camera at F4.5 for 40 sec.

Sizing Plasmids

Plasmids were sized using a standard curve of the log of the migration distance for plasmids of known size vs the actual size of these plasmids. The curve was generated seperately for each gel by including standard plasmid preparations. The size of the plasmid(s) in each experimental preparation was determined by measuring the distance migrated

on the gel and correlating this distance with its appropriate size relative to the standard curve prepared for the gel.

Animal Experimentation and Immunological Assays

Mouse models were used to demonstrate the effectiveness of *S. typhimurium* strains SL3235 and X4064 in eliciting secretory immunity against virulent strains of *S. typhimurium*. Serum and feces samples were collected from immunized mice and analyzed for isotype-specific antibodies against whole bacteria of the *S. typhimurium* strain SL3201 using an enzyme-linked immunosorbent assay (ELISA).

Immunization of Balb/cBy Mice

Balb/cBy mice were immunized using two different protocols. The first used an initial sub-cutaneous (sc) injection of live avirulent *S. typhimurium* followed later by an oral exposure to the same organism to boost the primary immune responses. The second protocol used an initial oral exposure to live avirulent *S. typhimurium* followed by two oral booster doses with the same organism.

Sub-cutaneous immunization. Bacteria from a 10-ml BHI broth culture grown overnight at 35°C on a shaker at 100 rpm were washed 2x with cold saline (4°C) and resuspended to its original volume in cold saline. The washed bacteria were diluted to $5x10^6$ bacteria/ml in saline warmed to 35°C and 2 ml were transferred to a vial containing Ribi monophosphoryl

lipid A (MPL) + trehalose dimycolate (TDM) adjuvant (Ribi Adjuvant System, Ribi Immunochemicals) also warmed to 35°C. The vial was vortexed for 3 min to form an emulsion containing bacteria and adjuvant. The mice were immunized by sc injection of 0.1 ml of the emulsion into 2 sites located in the neck region. Each mouse received 0.2 ml of the emulsion which provided a dose of $1x10^6$ bacteria.

Manual Oral Immunization. Bacteria from a 10-ml BHI broth culture grown overnight at 35°C on a shaker at 100 rpm were washed 2x with cold saline, recovered by centrifugation and resuspended in 10 ml cold saline. The resuspended bacteria were diluted to $5x10^8$/ml in 0.1% gelatin in saline. The mice were prepared for immunization by removing their drinking water 6 h prior to the inoculation. At the time of immunization each mouse received 30 ul of a 10% sodium bicarbonate solution to neutralize stomach acid and 20 ul of the diluted culture. The inoculation dose by this method was $1x10^7$ bacteria/mouse.

Drinking-Water Immunization. Bacteria from a 10-ml BHI broth culture grown over-night at 35°C on a shaker at 100 rpm were pelleted by centrifugation (5000×g, 20 min). The pellet was resuspended in 10 ml of 0.1% peptone (Difco) and then diluted to $1x10^8$/ml in 0.1% peptone. Drinking water was removed from the mice for 6 h and then replaced with the diluted bacteria in 0.1% peptone. The bacterial suspension

was held for 24 h with the mice. This method did not allow determination of the dose.

Feces Collection

A method previously described for the preparation and storage of intestinal secretions for the analysis of antibody by Elson *et al.* (20) was used in this study. Fecal pellets were collected from the mice by placing them on a wire screen covering the bottom half of a Petri plate for 30 min. The fecal material was weighed and then suspended in 3 ml of 0.1 mg/ml trypsin inhibitor (Sigma, II-S) in 50 mM EDTA (pH 7.2). The pellets were broken up using a glass rod and vigorous vortexing in a 15 ml conical disposable centrifuge tube. Phosphate-buffered saline (PBS) was used to bring the volume up to 6 ml before the suspension was centrifuged (Sorvall model T6000B) (850xg, 10 min). Four ml of the supernatant was transferred to a 50 ml centrifuge tube and 100 ul of a 100 mM solution of phenylmethylsulfonylfluoride (PMSF) in 95% ethanol was added to each tube. Centrifugation (22,530xg, 20 min) was used to further clear the supernatant of any suspended particles. For storage, 3 ml of the cleared supernatant was transferred to a vial with 30 ul 100 mM PMSF, 30 ul of a 1% solution of sodium azide, and 150 ul of FBS to guard against the loss of antibody activity. Fecal preparations were stored frozen at -20°C. These preparations were analyzed for antibody activity using ELISAs. The concentration of the

feces in each sample was adjusted to 30 mg/ml and 100 ul applied to each test well which resulted in 3 mg of feces/well. The antibody activity was measured relative to positive and negative feces samples.

Serum collection

Serum was collected from the test mice by peri-orbital bleeding from the orbital venous sinus. Each mouse was anaesthetized using methoxyflurane (Metafane, Pittman-Moore) and its orbital venous sinus ruptured using a Pasteur pipet. Blood was drawn from the sinus into the Pasteur pipet and transferred to a microcentrifuge tube for storage. The collected blood was allowed to stand at 35°C for 1 h and then held at 4°C overnight. This treatment promoted clot formation and the separation of the serum. Centrifugation (Eppendorf microcentrifuge) for 10 min was used to pellet the red blood cells. The cleared serum was then transferred to a new microcentrifuge tube for storage at -20°C.

ELISA

An ELISA was used to analyze serum and feces samples for the presence of antibody specific for *S. typhimurium* strain SL3201. The method described below was developed in our lab using the guidance of an extensive publication on the subject (42).

Preparation of the Antigen. The antigen which served as the solid phase of this assay was comprised of whole viable

bacteria (*S. typhimurium* strain SL3201). To prepare the antigen, strain SL3201 was subcultured to 2 flasks each containing 25 ml of BHI broth and allowed to grow static overnight at 35°C. The cultures were combined, the bacteria washed 2x with cold (4°C) sterile distilled H_2O, and then resuspended in 5 ml cold sterile distilled H_2O. The dry weight of the bacteria/ml of suspension of bacteria was determined by drying 0.5 ml on each of three small aluminum planchets of known weight in a dessicator. The bacteria were dried to a point where their weight stablized and this weight was recorded. The dry weight of the bacteria in each ml of the culture was then determined and the culture diluted to 20 ug of bacteria/ml in carbonate coating buffer (0.04 M Na_2CO_3, 0.06 M $NaHCO_3$, 0.02% NaN_3, in sterile distilled H_2O, pH 9.6) for application to 96 well Nunc-Immuno plates (Maxisorb, Nunc).

Preparation of ELISA Plates. Plates were prepared by adding 100 ul of prepared antigen to each of the wells of 96 well Nunc-Immuno plates excluding the 36 outside wells. The coated plates were then incubated overnight at 4°C to insure binding of the antigen the polystyrene plate material. The plates were washed (each wash included 5 min of rocking) 3x with PBS using a manual ELISA washer (Corning model 26300) and treated with 200 ul/well of a blocking solution (2% chicken

egg albumin in PBS) for 4 h at 25°C. The blocking solution was removed and the plates sealed for storage at -20°C.

The ELISA Procedure. A coated and blocked plate was washed 3x with PBS. Samples (serum or feces) were diluted in sample buffer (0.1% bovine serum albumin (BSA) in PBS) and applied at a volume of 100 ul/well to the plate. The plate was incubated overnight at 4°C. Following 5 washes with PBST (PBS with 0.05% Tween 20), the plate was treated to a second blocking step with 200 ul/well of 1.0% BSA in PBS for 1 h at 25°C. The plate was washed 2x with PBST and treated with 100 ul/well of goat anti-mouse IgA, IgM, or IgG (Kirkegaard and Perry), depending on the antibody assay, for 2 h at room temperature. Working dilutions for ELISA were prepared with 0.1% BSA in PBST as follows: anti-mouse IgA, 1:4000, anti-mouse IgM, 1:2000, anti-mouse IgG, 1:2000. PBST was used to wash the plate 5 additional times before adding 100 ul/well of rabbit anti-goat IgG conjugated with alkaline phosphatase (Cappel) for 1.5 h at room temperature. The conjugate was diluted in 0.1% BSA-PBST to different concentrations depending upon the anti-mouse isotype; anti-mouse IgA, 1:6000, anti-mouse IgM, 1:4000, anti-mouse IgG, 1:4000. Following 5 washes with PBST, 200 ul of phosphatase substrate (Sigma 104 phosphatase substrate) diluted to a concentration of 1 mg/ml in diethanolamine buffer (50 mM $MgCl_2$, 9.7% diethanolamine in sterile distilled H_2O, pH 9.8) was dispensed in each reaction

well of the plate. The plate was covered with an adhesive cover (Corning) and incubated by floating in a 37°C circulating waterbath (Precision) for 2 h. The enzyme reaction was stopped by the addition of 100 ul of 3.0 M NaOH. The color produced by the phosphatase indicator system was analyzed by measuring the absorbance at 405 nm with an automated ELISA reader (Bio-Rad).

Foster Pup Studies

The presence of a protective secretory immune response in immunized Balb/cBy mice was investigated through the passive transfer of this immunity to unimmunized Balb/cBy foster pups. A method was developed which employed immunized lactating mothers as the source of the *S. typhimurium* specific antibody and unimmunized foster pups as the recipient of this immunity. Immunized female Balb/cBy mice were held with Balb/cBy males for 4 to 7 days after which time the males were removed. Any mouse showing signs of pregnancy was given an oral booster of the vaccine strain through its drinking water. The mother was left alone for 24 h following the birth of her pups at which time the natural pups were removed and replaced with 8 "foster" pups (pups born to an unimmunized mother) of approximately the same age. At this time the mothers and pups were moved to cages with wire bottoms to prevent continuous infection through coprophagy. Cotton bedding was used to prevent the pups from falling through the wire mesh. The

foster pups were then challenged orally with virulent *S. typhimurium* strain SL3201. A set of eight pups with an unimmunized mother were also challenged at this time to provide a control group. The challenge dose was prepared from a 10 ml BHI broth culture grown overnight at 35°C on a shaker at 100 rpm. The culture was washed 2x with cold saline (4°C), resuspended to its original volume in saline and diluted to $5x10^4$ bacteria/ml with 0.1% gelatin in saline. Each pup was given 20 ul of the bacterial suspension orally using an Eppendorf pippetor and a small pipet tip. Challenge by this method resulted in a dose of $1x10^3$ bacteria/pup. The pups in the test and control groups were then monitored for death over a 21 day period.

Feeding Studies for Measuring Passive Antibody Protection

Passive antibody protection provided by samples containing antibody specific for *S. typhimurium* strain SL3201 was evaluated by determining the implantation dose for strain SL3201 in mice. A test group of 17-day-old male and female Balb/cBy mice consisting of 4 sets of 5 mice were fed 3 doses of the sample at 24 h intervals. A dose consisted of 100 ul of the sample per mouse per feeding. A control group of 17-day-old male and female Balb/cBy mice also consisting of 4 sets of 5 mice were fed 3 doses of a negative sample which was either negative ascites or PBS depending on the test sample being evaluated, at 24 h intervals. A dose of the negative

sample also consisted of 100 ul per mouse per feeding. The mice were fed by gavage tube (intragastrically). The final feeding included the challenge bacteria. Graded doses of the challenge strain SL3201 were from a 10 ml BHI broth culture broth culture grown overnight (35°C, 100 rpm) by washing the bacteria 1x with cold saline and resuspending them in 10 ml of 0.1% gelatin in saline. The resuspended bacteria were diluted serially in the test sample (diluted 1:5 in 0.1% gelatin in saline) to provide 4 graded doses. A set of test mice and a set of control mice were challenged with 100 ul of each of the graded doses by intragrastric feeding. The mice were held for 8 days and then killed by cervical dislocation. Rectal swabs (CalgiSwab) and spleens were taken from each of the mice. The rectal swabs were transferred to tubes containing 5 ml of tetrathionate broth (Difco) and the spleens placed in vials containing 4 ml BHI broth. The tubes were incubated at 35°C for 48 h. The selective and non-selective broths were streaked onto XLD agar, which was incubated for 24 h at 35°C. Growth of *S. typhimurium* strain SL3201 was confirmed by colony morphology and by serology using Difco poly A antiserum.

Abbreviations

Abbreviations used in this thesis are as follows; subcutaneous, sc; intraperitoneally, ip; peroral, po; megadalton, Mdal; kilobase, Kb; milliliter, ml; microliter,

ul; milligram, mg; microgram, ug; hour, h; second, sec; molar, M; millimolar, mM; brain-heart infusion, BHI; Trypticase Soy agar with 0.6% yeast extract, TSY; tris-borate-EDTA buffer, TBE; phosphate buffered saline, PBS; phosphate buffered saline with tween 20, PBST; Dulbecco's phosphate buffered saline, DPBS; Hanks' balanced salt solution, HBSS; fetal bovine serum, FBS; bovine serum albumin, BSA; chicken egg albumin, CEA; phenylmethylsulfonylfluoride, PMSF; N-2-hydroxyethyl-N-2-ethane sulfonic acid, HEPES; lipopolysaccharide, LPS; enzyme-linked immunosorbent assay, ELISA.

RESULTS

Plasmid Analysis

Plasmid preparations from each of the *S. typhimurium* strains used in this study were analyzed using agarose gel electrophoresis. The results are presented in Figures 1 and 2 and summarized in Table 1. Analysis of the plasmid profile for each strain demonstrated the presence of a plasmid of approximately 60 Mdal in size in *S. typhimurium* strains SL3201, SL3235, and SL1306 (Figure 2). Analysis of the migration distances of these plasmids using a standard curve generated on the same gel (Appendix E) confirmed their size at 60 Mdal (Figure 2). This plasmid was absent from the plasmid profiles of the plasmid-cured strain X3337 and the avirulent strain X4064 (Figure 1). A small plasmid of approximately 5 Mdal was present in strain SL3201 and strain SL3235 (Figure 2).

Table 1. Plasmids of the *S. typhimurium* strains used in this study. Small plasmid size is an approximation.

	Plasmids	
Strain	Distance Migrated	Size (Mdal)
SL3201	4.40 cm	60
	13.60 cm	5
SL3235	4.40 cm	60
	13.60 cm	5
SL1306	4.39 cm	60
X4064	-	-
X3337	-	-

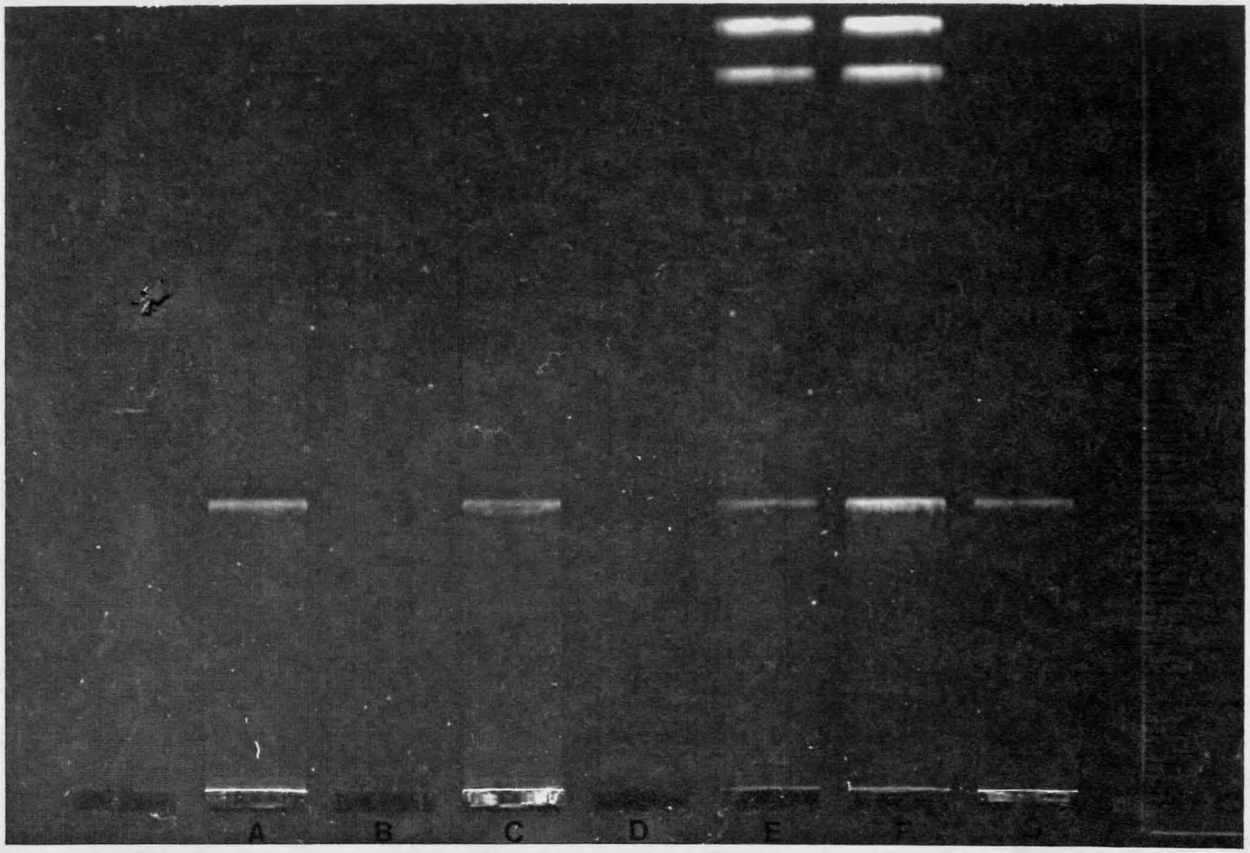

Figure 1. Agarose gel electrophoresis of plasmids obtained from S. typhimurium strains. (A & G) The 60 mdal plasmid present in the S. typhimurium strain X3306. (B & D) Lanes demonstrating the absence of plasmids in the plasmid-cured strain X3337 (Lane B) and the avirulent strain X4064 (Lane D). (C, E, & F) Plasmids of unknown size present in strain SL3201 (Lane F), strain SL3235 (Lane E), and in strain SL1306 (Lane C).

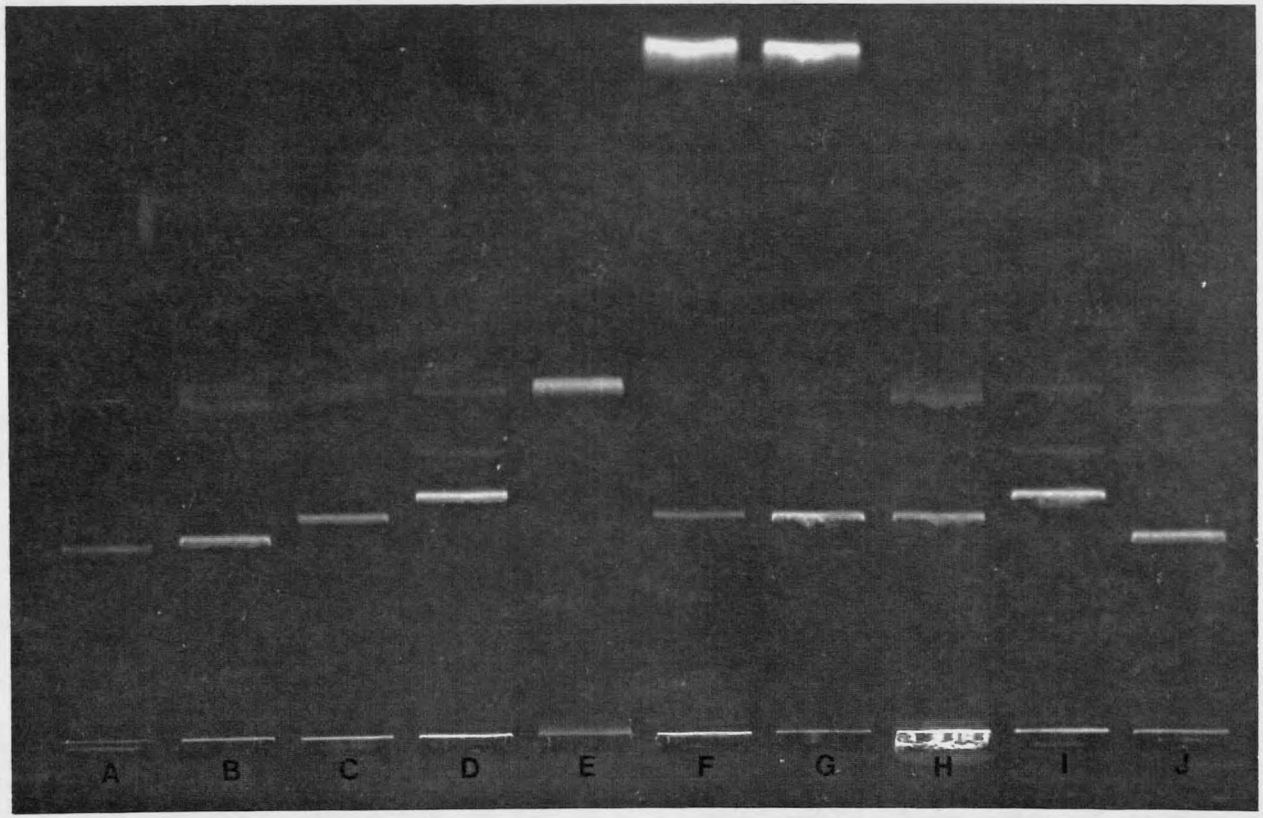

Figure 2. Agarose gel electrophoresis of plasmid standards and plasmids obtained from S. typhimurium strains. (A) An 86 mdal plasmid obtained from E. coli strain C114. (B & J) A 72 mdal plasmid obtained from E. coli strain C154. (C) A 60 mdal plasmid obtained from S. typhimurium strain X3306. (D & I) A 46 mdal plasmid obtained from E. coli strain C155. (E) A 26 mdal plasmid obtained from E. coli strain C153. (F) Plasmid profile of S. typhimurium strain SL3235. (G) Plasmid profile of S. typhimurium strain SL3201. (H) Plasmid profile of S. typhimurium strain SL1306.

Attachment and Invasion Assay

The ability of each strain of *S. typhimurium* used in this study to attach to and invade intestinal epithelial cells was quantified using *in vitro* tissue culture assays. The results of attachment and invasion assays for each of these strains are summarized in Tables 2-6. Each count represents an average of the number of bacteria associated with monolayers in three individual wells of a tissue culture plate which received identical treatment. The counts represent the following: "Added" represents the number of bacteria which were added to each monolayer. "Associated" refers to the total number of bacteria which were associated (attached and intracellular) with the monolayer. "Intracellular" refers to the number of survivors following treatment with gentamicin which are presumably intracellular. "Plastic" represents the number of bacteria which adhered to polystyrene tissue culture wells which lacked monolayers but received the same treatment as wells containing monolayers. "Fixed-Associated" refers to the total number of bacteria associated with monolayers treated with paraformaldehyde (Fixed). "Fixed-Gentamicin" represents the total number of bacteria associated with fixed monolayers following treament with gentamicin. The values represented by "Fixed-Associated" and "Fixed-Gentamicin" were used to determine the effectiveness of the gentamicin treatment in killing bacteria *in situ*. Because gentamicin

will kill only extracellular bacteria, leaving intracellular bacteria viable, the effectiveness of the gentamicin kill (% kill) represented the accuracy with which the "Intracellular" counts could be made. These counts represented the total number of bacteria, both intracellular and extracellular survivors of the gentamicin treatment associated with the fixed monolayers. It was noted during the course of this study that *S. typhimurium* strain SL3201 grown under anaerobic conditions demonstrated a ten-fold increase in association and a two-fold increase in invasion relative to the same strain grown aerobically. An increased affinity for polystyrene and fixed monolayers did not accompany the increase in over-all association (Table 2). Growth temperature was also evaluated for its influence on the attachment and invasion properties of *S. typhimurium* strain SL3201. It was demonstrated that bacteria grown at 25°C exhibited a significant increase in association and invasion relative to the same organism grown at 35°C (Table 3) SL3201 grown at 25°C did not demonstrate an accompanying increase in adhesion to the polystyrene or to the fixed monolayers when compared to the organism grown at 35°C.

The avirulent and plasmid-cured strains of *S. typhimurium* (SL3235, X4064, and X3337) were grown under anaerobic conditions and assayed for their ability to attach to and invade intestinal epithelial cells *in vitro* relative to that of the virulent *S. typhimurium* strain SL3201.

Table 2. A comparison between the *in vitro* interactions of *S. typhimurium* strain SL3201 grown under aerobic and anaerobic conditions with Henle 407 intestinal epithelial cells.

	Number of Bacteria per Monolayer					
	Aerobic			Anaerobic		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
Added	7.10×10^6	7.83×10^6	7.23×10^6	2.85×10^6	2.55×10^6	2.55×10^6
Associated (% of added)	1.76×10^5 (2.5)	3.57×10^6 (4.6)	3.99×10^5 (5.5)	1.05×10^6 (36.5)	9.30×10^5 (36.5)	1.39×10^6 (54.5)
Intracellular (% of associated)	2.48×10^5 (14.1)	2.85×10^5 (79.8)	1.16×10^5 (29.1)	9.98×10^5 (96.0)	5.70×10^5 (61.3)	9.60×10^5 (69.1)
Plastic (% of added)	1.70×10^5 (2.4)	9.00×10^4 (1.1)	3.87×10^5 (5.4)	1.93×10^5 (6.8)	1.08×10^5 (4.2)	4.07×10^5 (16.0)
Fixed-Associated (% of added)	2.00×10^5 (2.8)	1.88×10^5 (2.4)	4.79×10^5 (6.6)	2.50×10^5 (8.8)	3.47×10^5 (13.6)	9.15×10^5 (35.9)
Fixed-Gentamicin (% kill)	7.50×10^0 (99.99)	6.45×10^2 (99.66)	3.08×10^2 (99.95)	1.20×10^2 (99.95)	6.30×10^2 (99.82)	3.00×10^2 (99.97)

Table 3. A comparision between the *in vitro* interactions of *S. typhimurium* grown at 25°C and 35°C with Henle 407 intestinal epithelial cells.

	Number of Bacteria per Monolayer					
	25°C			35°C		
	Exp 1	Exp 2	Exp 3	Exp 1	Exp 2	Exp 3
Added	5.88×10^6	5.95×10^6	5.78×10^6	5.05×10^6	4.43×10^6	4.73×10^6
Associated (% of added)	1.64×10^6 (27.9)	1.75×10^6 (29.4)	2.38×10^6 (41.2)	3.24×10^5 (6.4)	2.96×10^5 (6.7)	5.57×10^6 (11.8)
Intracellular (% of associated)	2.23×10^6 (136)	2.10×10^6 (120)	2.57×10^6 (108)	2.60×10^5 (80.2)	3.98×10^4 (13.4)	2.78×10^5 (49.9)
Plastic (% of added)	5.40×10^4 (0.9)	1.20×10^5 (2.0)	2.37×10^5 (4.1)	6.98×10^4 (1.4)	1.64×10^5 (3.7)	2.45×10^5 (5.2)
Fixed-Associated (% of added)	3.76×10^5 (6.4)	6.08×10^5 (10.2)	1.19×10^6 (20.6)	6.98×10^4 (1.3)	1.34×10^5 (3.0)	2.24×10^5 (4.7)
Fixed-Gentamicin (% kill)	1.28×10^2 (99.97)	2.02×10^2 (99.97)	3.98×10^2 (99.97)	1.50×10^1 (99.98)	10 (99.99)	3.75×10^1 (99.98)

The aromatic mutant SL3235 demonstrated slightly reduced over-all association but the percentage of associated bacteria which were intracellular remained comparable to its parent strain SL3201 (Table 4). SL3235 also demonstrated a considerable reduction in adhesion to polystyrene and to fixed cells. The adenylate cyclase mutant X4064 showed a substantial increase in over-all association and similar invasion characteristics when compared to both SL3201 and SL3235 (Table 5). X4064 exhibited a slightly higher ability to adhere to polystyrene and to fixed cells than was demonstrated by SL3235. Plasmid-cured strain X3337 demonstrated a substantial increase in over-all association but also exhibited reduced invasion characteristics when compared to SL3201 (Table 6).

Table 4. Interactions of the avirulent *S. typhimurium* mutant SL3235 grown under anaerobic conditions with Henle 407 intestinal epithelial cells *in vitro*.

	Number of Bacteria per Monolayer		
	Exp 1	Exp 2	Exp 3
Added	4.75×10^6	5.32×10^6	8.23×10^6
Associated	5.18×10^5	5.10×10^5	1.28×10^6
(% of added)	(10.9)	(9.6)	(15.6)
Intracellular	2.55×10^5	1.99×10^5	8.18×10^5
(% of associated)	(49.2)	(39.0)	(63.9)
Plastic	2.10×10^4	1.75×10^4	1.82×10^4
(% of added)	(0.44)	(0.33)	(0.22)
Fixed-Associated	2.15×10^4	2.62×10^4	3.83×10^4
(% of added)	(0.45)	(0.49)	(0.47)
Fixed-Gentamicin	7.50×10^1	1.35×10^2	4.50×10^1
(% kill)	(99.65)	(99.48)	(99.88)

Table 5. Interaction of the avirulent *S. typhimurium* mutant X4064 grown under anaerobic conditions with Henle 407 intestinal epithelial cells *in vitro*.

	Number of Bacteria per Monolayer		
	Exp 1	Exp 2	Exp 3
Added	5.32×10^6	5.25×10^6	5.50×10^6
Associated (% Of added)	3.56×10^6 (66.9)	3.58×10^6 (73.3)	3.59×10^6 (65.3)
Intracellular (% of associated)	2.10×10^6 (59.0)	1.92×10^6 (49.9)	1.34×10^6 (37.3)
Plastic (% of added)	5.63×10^4 (1.06)	4.05×10^4 (0.77)	6.38×10^4 (1.16)
Fixed-Associated (% of added)	3.44×10^5 (6.47)	4.43×10^5 (8.44)	9.45×10^4 (1.72)
Fixed-Gentamicin (% kill)	1.80×10^2 (99.95)	9.00×10^1 (99.98)	2.25×10^2 (99.76)

Table 6. Interaction of plasmid-cured *S. typhimurium* strain X3337 grown under anaerobic conditions with Henle 407 intestinal epithelial cells *in vitro*.

	Number of Bacteria per Monolayer		
	Exp 1	Exp 2	Exp 3
Added	3.60×10^6	4.08×10^6	4.18×10^6
Associated (% of added)	7.73×10^6 (215)	9.83×10^6 (241)	1.04×10^7 (249)
Intracellular (% of associated)	1.99×10^6 (25.5)	2.51×10^6 (25.5)	2.65×10^6 (25.5)
Plastic (% of added)	7.43×10^4 (2.06)	7.95×10^4 (1.95)	5.03×10^4 (1.20)
Fixed-Associated (% of added)	1.69×10^5 (4.69)	2.71×10^5 (6.64)	3.24×10^5 (7.75)
Fixed-Gentamicin (% kill)	<10 (>99.99)	4.50×10^1 (99.98)	8.25×10^1 (99.97)

Microscopic Evaluation of Attachment and Invasion

The process of *S. typhimurium* strain SL3201 attaching to and entering Henle 407 intestinal epithelial cells (Henle cells) was observed by transmission electron microscopy. Attached bacteria were found in depressions in the cytoplasmic membrane and the absence of microvilli was noted around the area of attachment (Figure 3 & 4). Invaginations of the Henle cell's cytoplasmic membrane formed vacuoles which enclosed the bacteria (Figure 3 & 4). Intracellular vacuoles containing bacteria were observed as well as many intracellular bacteria which were not found in vacuoles (Figures 3, 4, & 5). There was some evidence of bacteria multiplying within the cytoplasm of Henle cells (Figure 5).

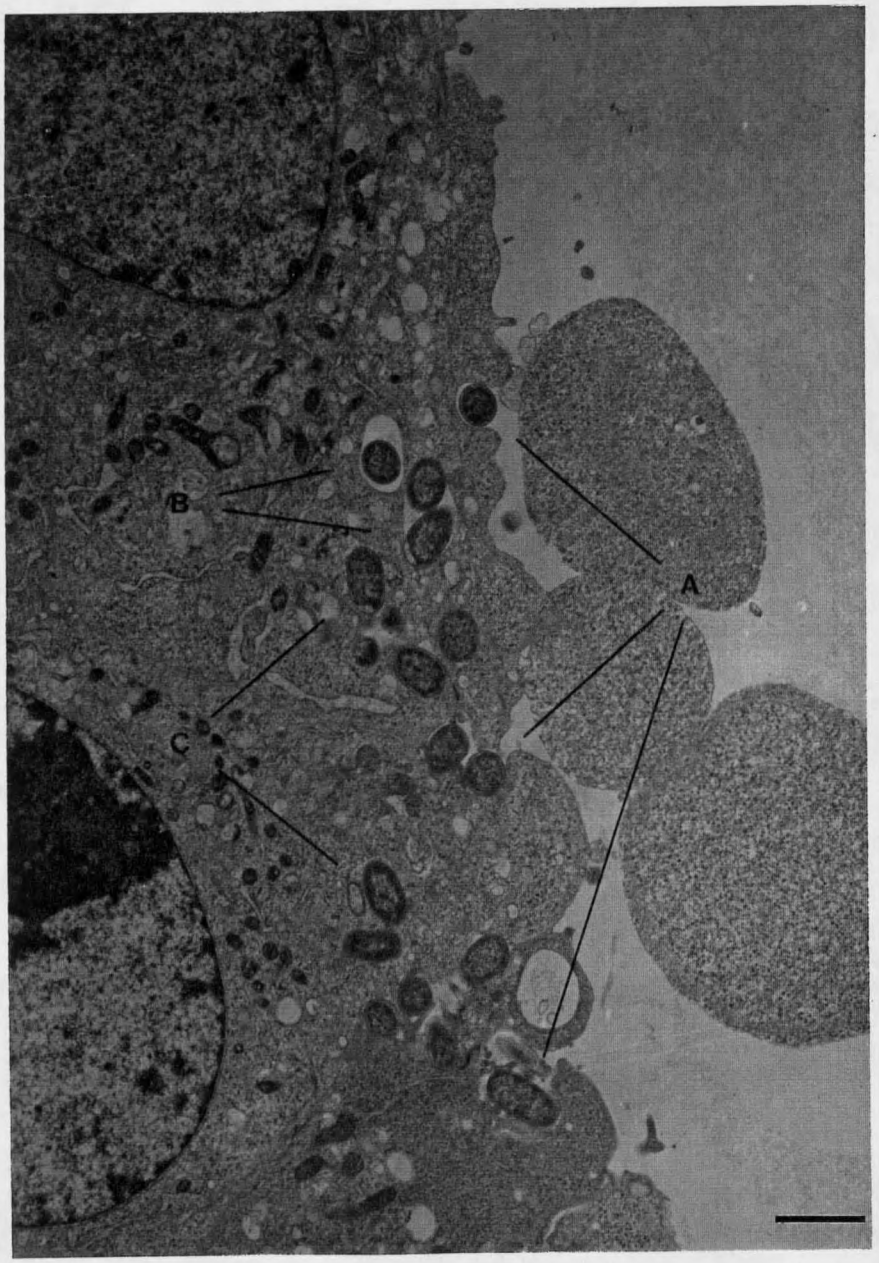

Figure 3. Transmission electron micrograph of Henle cells infected with *S. typhimurium*. (A) Attached *S. typhimurium* being taken up by Henle cells. (B) Intracellular *S. typhimurium* residing within vacuoles. (C) Intracellular *S. typhimurium* free in the cytoplasm. Bar indicates 2 um.

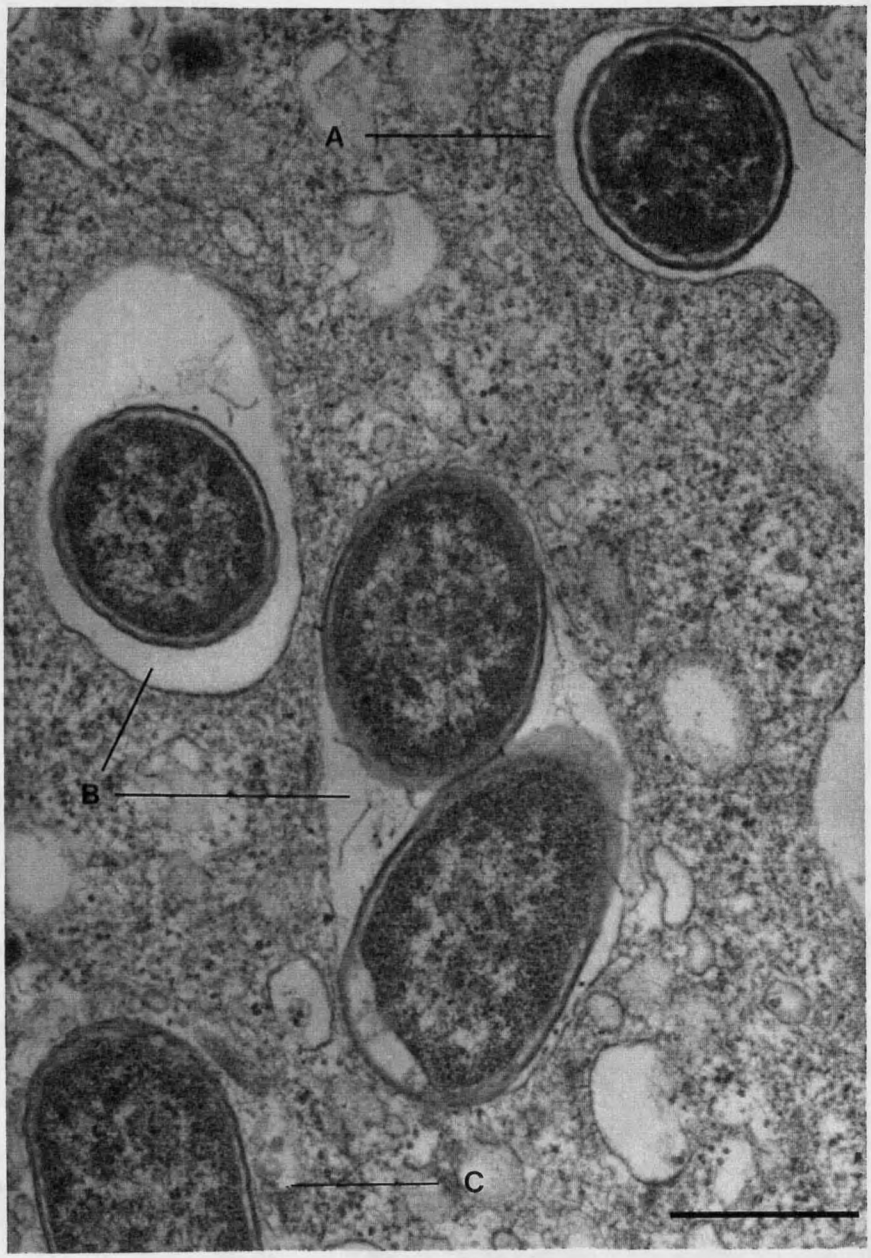

Figure 4. Transmission electron micrograph of a Henle cell infected with *S. typhimurium*. (A) Attached *S. typhimurium* being taken up by a Henle cell. (B) Intracellular *S. typhimurium* residing within vacuoles. (C) Intracellular *S. typhimurium* free in the cytoplasm of a Henle cell. Bar indicates 1 um.

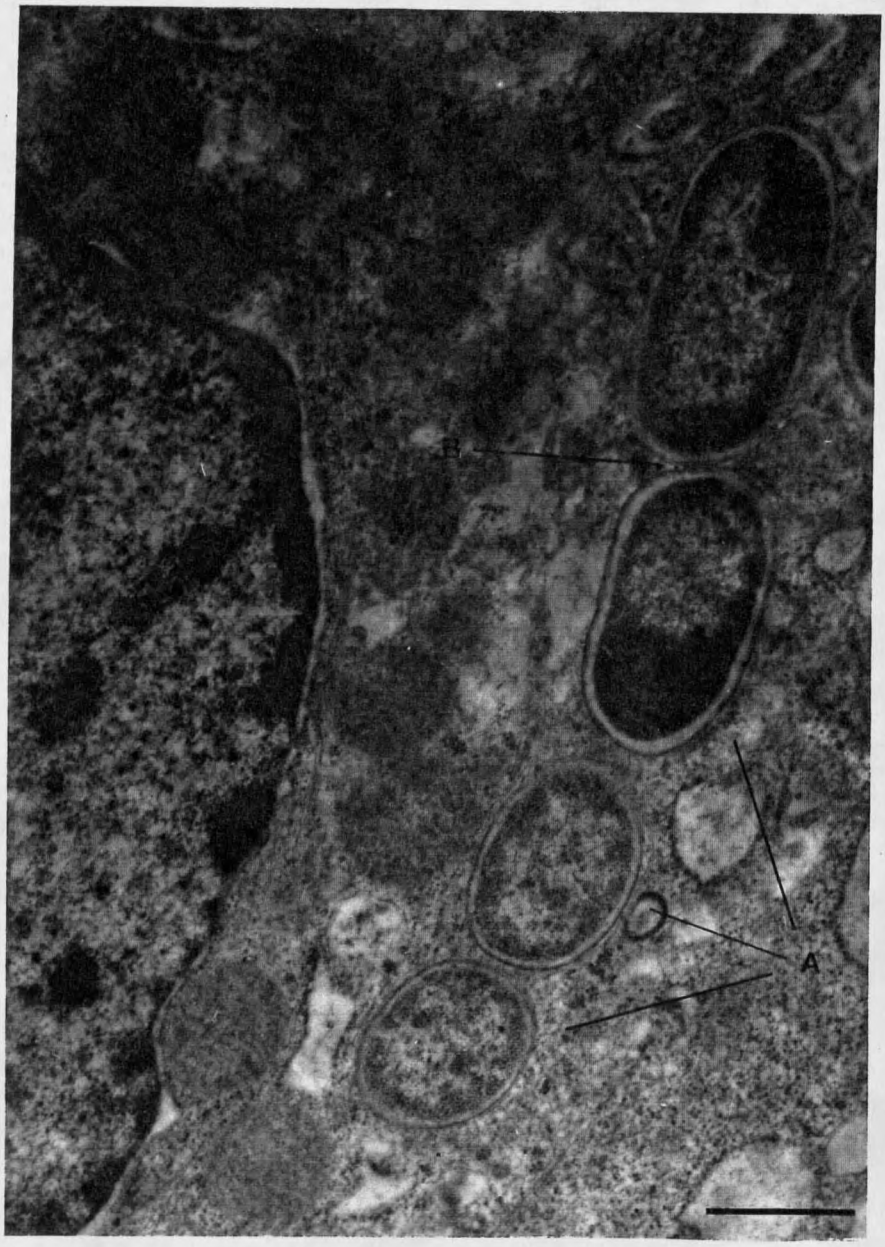

Figure 5. Transmission electron micrograph of a Henle cell infected with *S. typhimurium*. (A) Intracellular *S. typhimurium* free in the cytoplasm of a Henle cell. (B) Proximity of these two bacteria may indicate division. Bar indicates 1 um.

Foster Pup Studies

Protective secretory immune responses were measured using enzyme linked immunosorbant assays (ELISAs) and demonstrated by the passive transfer of protection from immunized lactating female mice to unimmunized foster pups.

Induced Specific Secretory Immune Responses in Immunized Mice

The immune status of the immunized mice was monitored through the antibody content of their feces. Fecal extracts prepared from the feces immunized mice were analyzed using an ELISA. Each ELISA measurement was repeated 3 times on the same ELISA plate and the mean of the 3 measurements calculated. The mean test sample measurement was divided by the mean of 3 negative sample (feces from unimmunized mice) measurements, included on the same ELISA plate, which generated a positive:negative (P/N) ratio. These ratios were plotted versus time to generate a curve demonstrating fluctuations in antibody response. Serum reponses to immunizations were measured using ELISA and the result reported as titer. The titer being defined as the inverse of the last positive dilution of serum in a serial dilution series as determined by a 99% confidence interval of the mean of three negative sample ELISA measurements.

Five immunized mice were selected at random from each experimental group of mice. These mice were not bred and served as immunized controls, providing a way to monitor

the immune status of their respective experimental groups without handling the immunized bred females. Information concerning the immune status of each group was gathered using these mice.

SL3235 Experimental Group #1. These mice were primed with a sc dose of strain SL3235 which was followed at three months with a manual-oral immunization and at four months with a drinking-water immunization. Drinking water immunizations were administered after breeding was completed and before the mice littered. The data presented in Figure 6 indicate a small, sustained, rise in the amount of SL3235 specific IgA present in the feces following the drinking water immunization which was not accompanied by a significant rise in either specific IgM or IgG. It should be noted that prior to the drinking-water immunization there was a measurable amount of specific IgA already present in the feces of the primed mouse (Figure 6). Results for immunized control mice S2-5 are presented in Appendix F. Rises in the immune responses of these mice following the drinking-water immunization could not be documented due to the poor nature of these results.

X4064 Experimental Group #1. These mice received an sc priming dose of strain X4064 followed at four months by a drinking-water immunization with the same strain. Erratic measurements and high initial values of the specific IgA,

IgM, and IgG present in the feces (Figure 7) interfere with the documentation of any rise in specific immunoglobulins following the drinking-water immunization. Results for mice S7-10 are presented in Appendix G. These results were also very erratic and could not be interpreted.

SL3235 Experimental Group #2. These mice were given multiple (3) drinking-water immunizations with strain SL3235. The first two immunizations were given within a week of each other. The final immunization was given 19 days later following an 8 day period . The antibody responses induced by this immunization protocol are presented in Figures 8-12. Each mouse demonstrated a significant rise in specific IgA following the final immunization which was not accompanied by a corresponding rise in specific IgG. Several mice did, however, demonstrate a significant rise in specific IgM (Figures 9 & 10). Small rises, mainly in specific IgA were seen in all mice following the second immunization.

Serum samples were collected from the immunized control mice prior to and following the immunization protocol. Specific IgA, IgM, and IgG serum titers are reported in Table 6. The mean serum titer for each class was used solely as a way to compare gross changes occurring between collections. There was approximately a three-fold increase in average serum IgA and IgG titers in the immunized control mice following oral immunization. The pre-immune sera demonstrated an average

IgM serum titer of >7,360 which fell by a factor of 4 in the immune sera. Unimmunized control mice did not demonstrate any significant rise in IgA, IgM, or IgG serum titers during the experiment.

Table 7. Titers of specific IgA, IgM, and IgG present in the serum of immunized and unimmunized control mice prior to and following multiple drinking-water immunizations with *S. typhimurium* strain SL3235.

Immunized Control Mice						
	Pre-immune Sera Titers			Immune Sera Titers		
Mouse	IgA	IgM	IgG	IgA	IgM	IgG
13	<400	>12,800	1,600	3,200	1,600	3,200
14	<400	12,800	800	1,600	1,600	3,200
15	<400	3,200	400	400	1,600	3,200
16	<400	1,600	400	400	3,200	3,200
17	<400	6,400	400	400	1,600	800
x	<400	>7,360	720	1,200	1,920	2,720
Unimmunized Control Mice						
	Serum Collection 1			Serum Collection 2		
Mouse	IgA	IgM	IgG	IgA	IgM	IgG
11	<100	>100	>100	<400	400	3,200
12	<100	>100	>100	<400	1,600	1,600

Passive Transfer of Protection to Foster Pups

Protection provided by induced secretory immune responses against challenge by a virulent strain of *S. typhimurium* was evaluated by challenging unimmunized 4-day old mice, receiving secretory antibody passively through the colostrum from immunized female mice, with virulent *S.typhimurium* strain SL3201. Protection was expressed in terms of survival time.

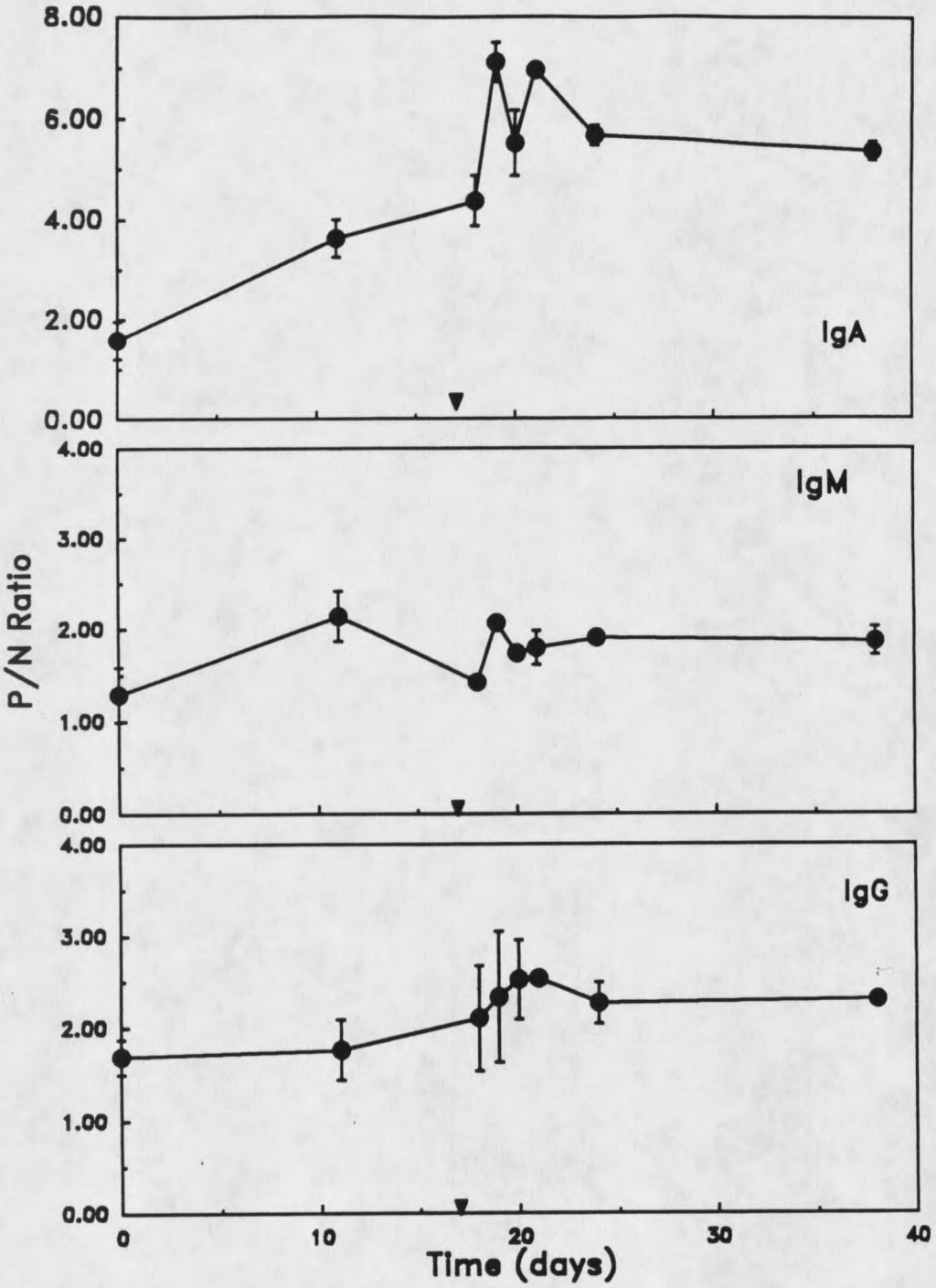

Figure 6. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S1 prior to and following drinking-water immunization with *S. typhimurium* strain SL3235. The time of drinking-water immunization is indicated by an arrow.

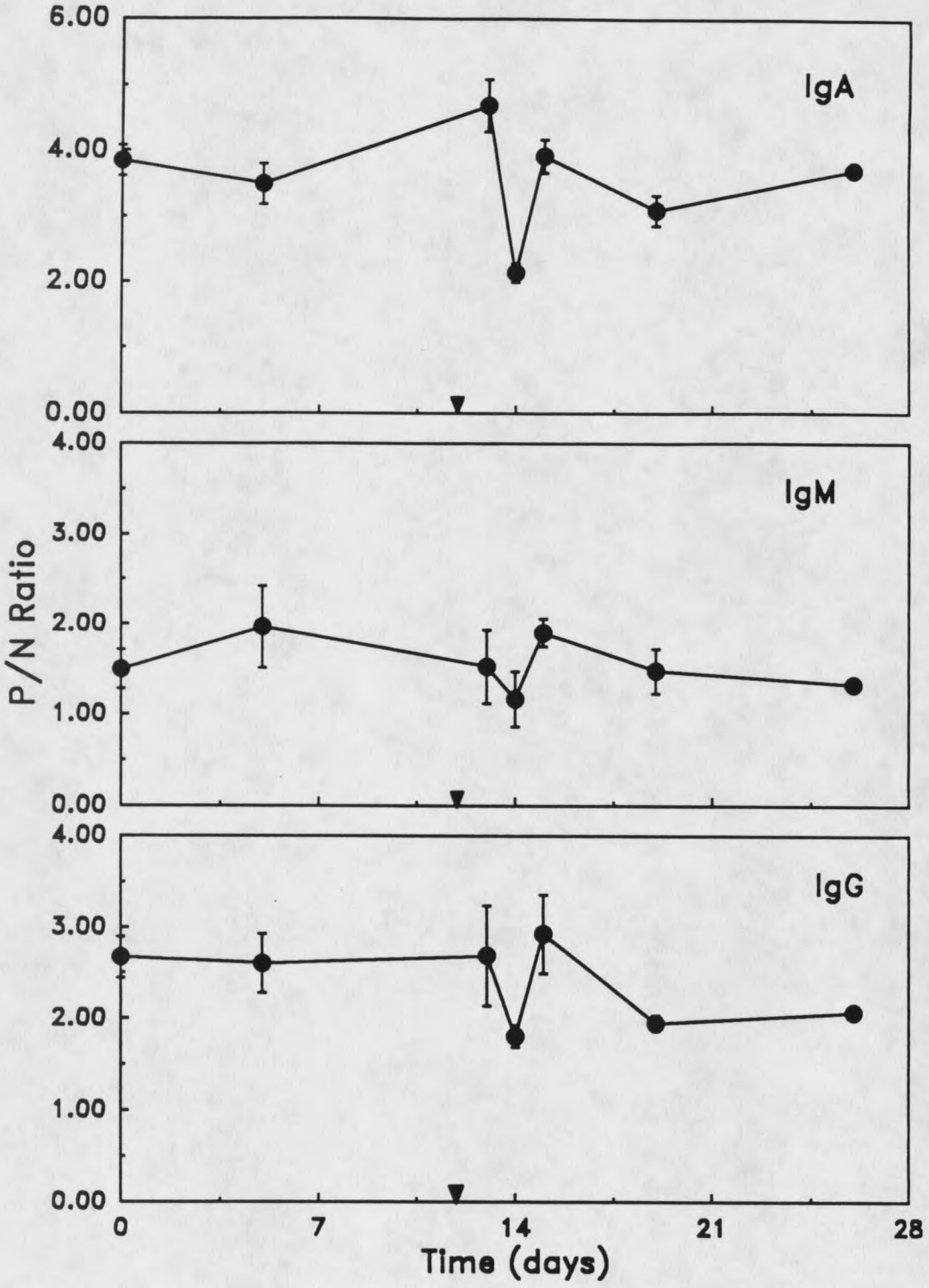

Figure 7. Relative amounts of specific IgA, IgM, and IgG present in the feces of immunized control mouse S6 prior to and following drinking-water immunization with *S. typhimurium* strain X4064. The time of drinking-water immunization is indicated by an arrow.

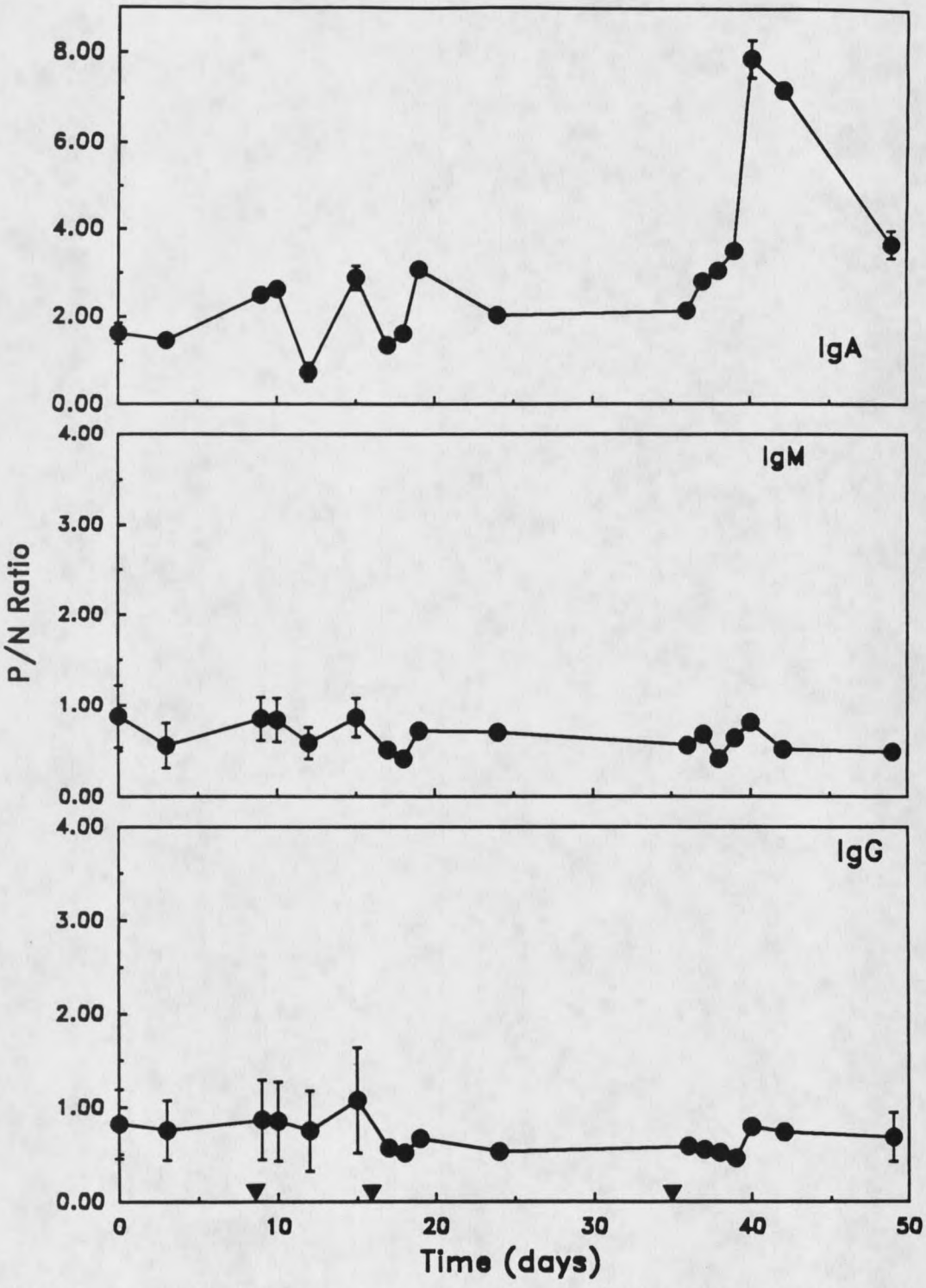

Figure 8. Relative amount of specific IgA, IgM, and IgG present in the feces of immunized control mouse 13 prior to, during, and following the administration of multiple drinking-water immunizations with *S. typhimurium* strain SL3235. Immunizations are indicated by arrows located at the bottom of the figure.

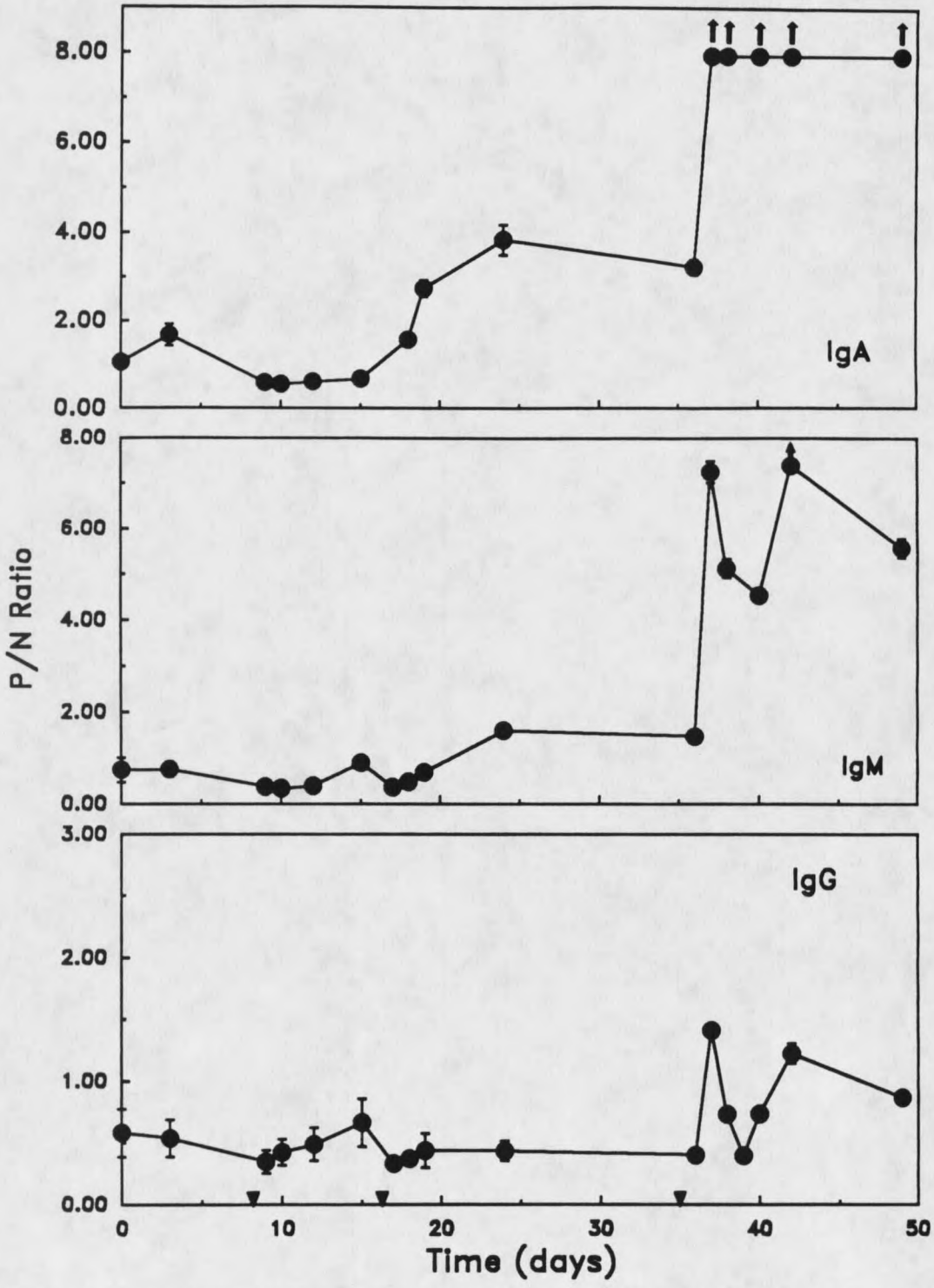

Figure 9. Relative amounts of specific IgA, IgM, and IgG present in the feces of immunized control mouse 14 prior to, during, and following the administration of multiple drinking-water immunizations with *S. typhimurium* strain SL3235. Immunizations are indicated by arrows at the bottom of the figure.

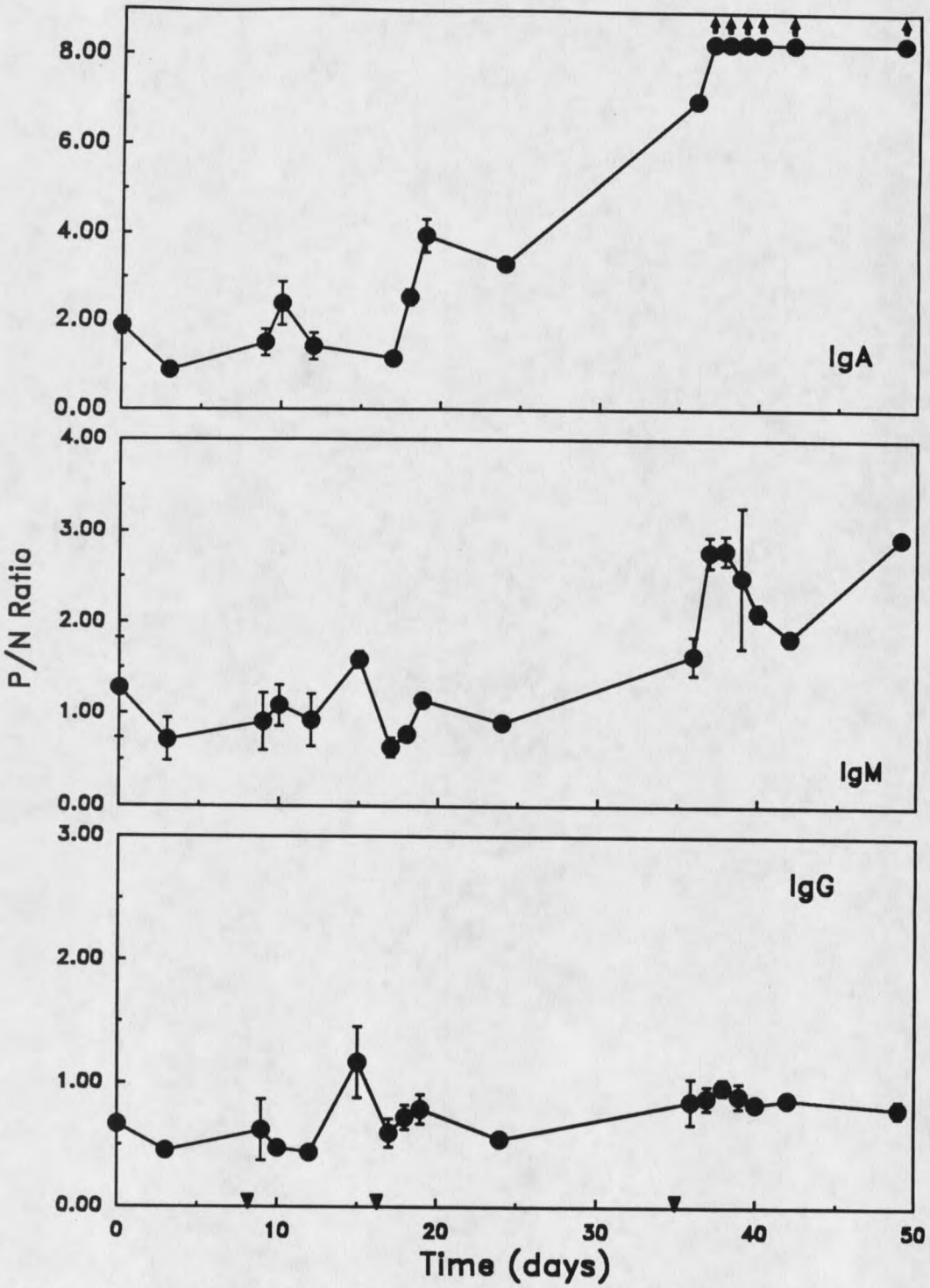

Figure 10. Relative amounts of specific IgA, IgM, and IgG present in the feces of immunized control mouse 15 prior to, during, and following the administration of multiple drinking-water immunizations with *S. typhimurium* strain SL3235. Immunizations are indicated by arrows at the bottom of the figure.

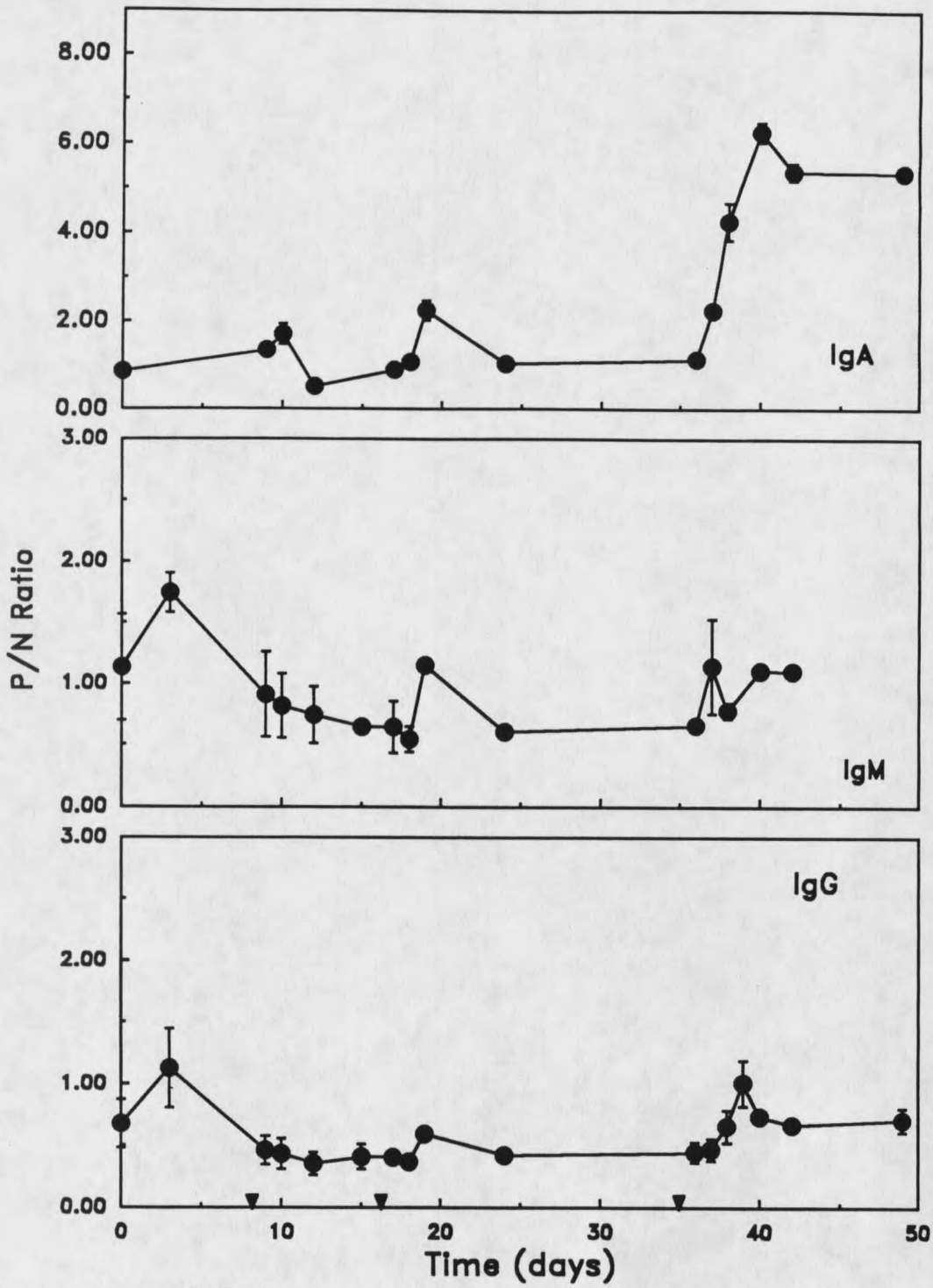

Figure 11. Relative amounts of specific IgA, IgM, and IgG present in the feces of immunized control mouse 16 prior to, during and following the administration of multiple drinking-water immunizations with *S. typhimurium* strain SL3235. Immunizations are indicated by arrows located at the bottom of the figure.

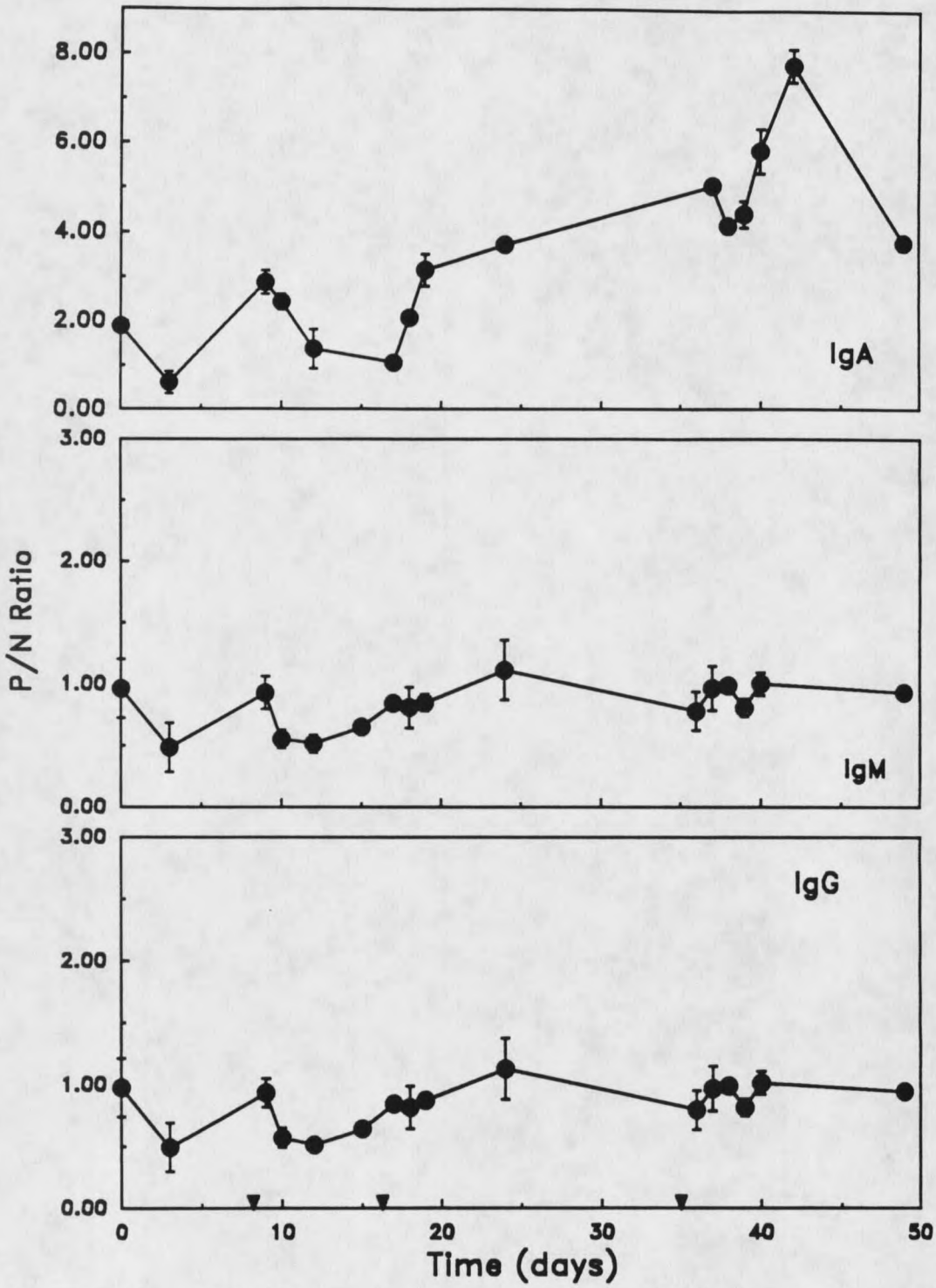

Figure 12. Relative amounts of specific IgA, IgM, and IgG present in the feces of immunized control mouse 17 prior to, during and following the administration of multiple drinking-water immunizations of *S. typhimurium* strain SL3235. Immunizations are indicated by arrows located at the bottom of the figure.

SL3235 Experimental Group #1. The results of the first challenge study are presented in Figure 13. Fifty percent of the test group of mice receiving antibody passively were alive at 11 days (ST_{50} of 11 days) as opposed to an ST_{50} of 7 days in the unprotected control group. There were no survivors in the test group at 15 days. None of the mice in the control group survived past 8 days.

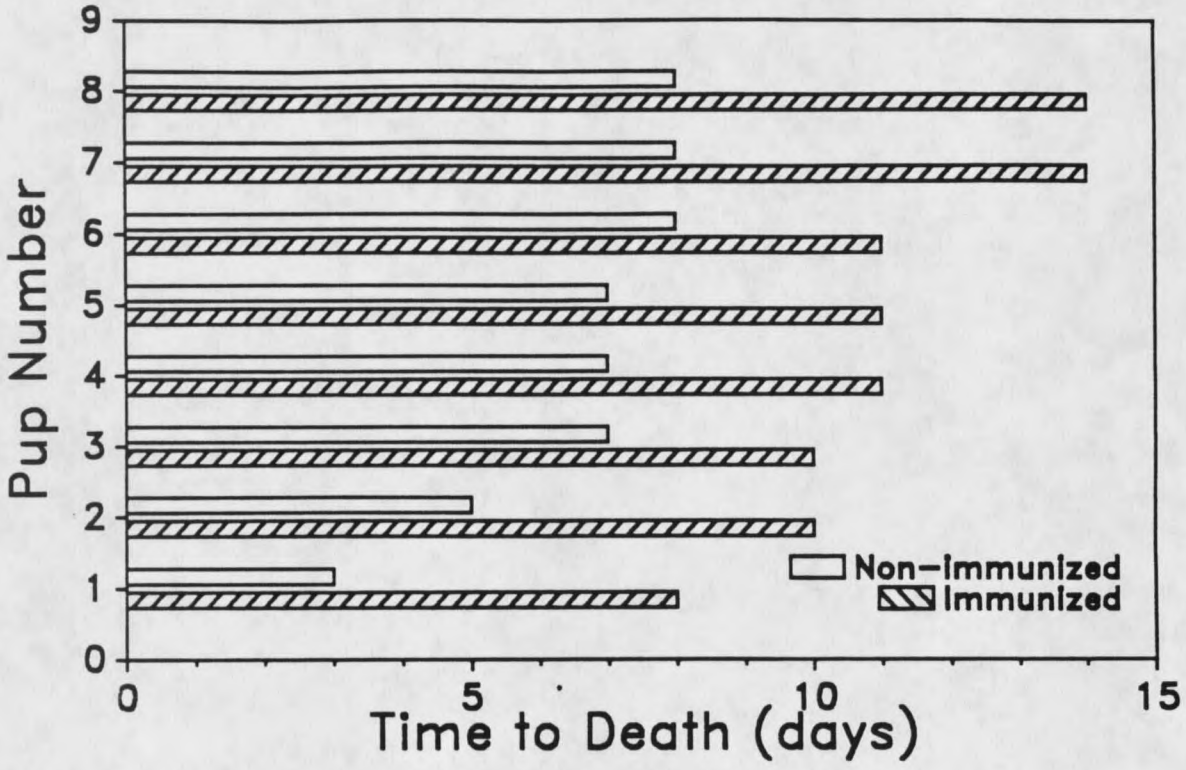

Figure 13. Results of the first SL3235 foster pup experiment demonstrating the survival time of individual passively immunized and non-immunized mice which were challenged with virulent *S. typhimurium* strain SL3201.

X4064 Experimental Group #1. The results of the second challenge study are presented in Figure 15. The passively immunized test group demonstrated an ST_{50} of 17 days for 15 mice which was more than 2x the ST_{50} of 8 days exhibited by the unimmunized control group. Five out of 15 passively-immunized mice survived the duration of the experiment (21 days), whereas none of the control mice survived past 11 days.

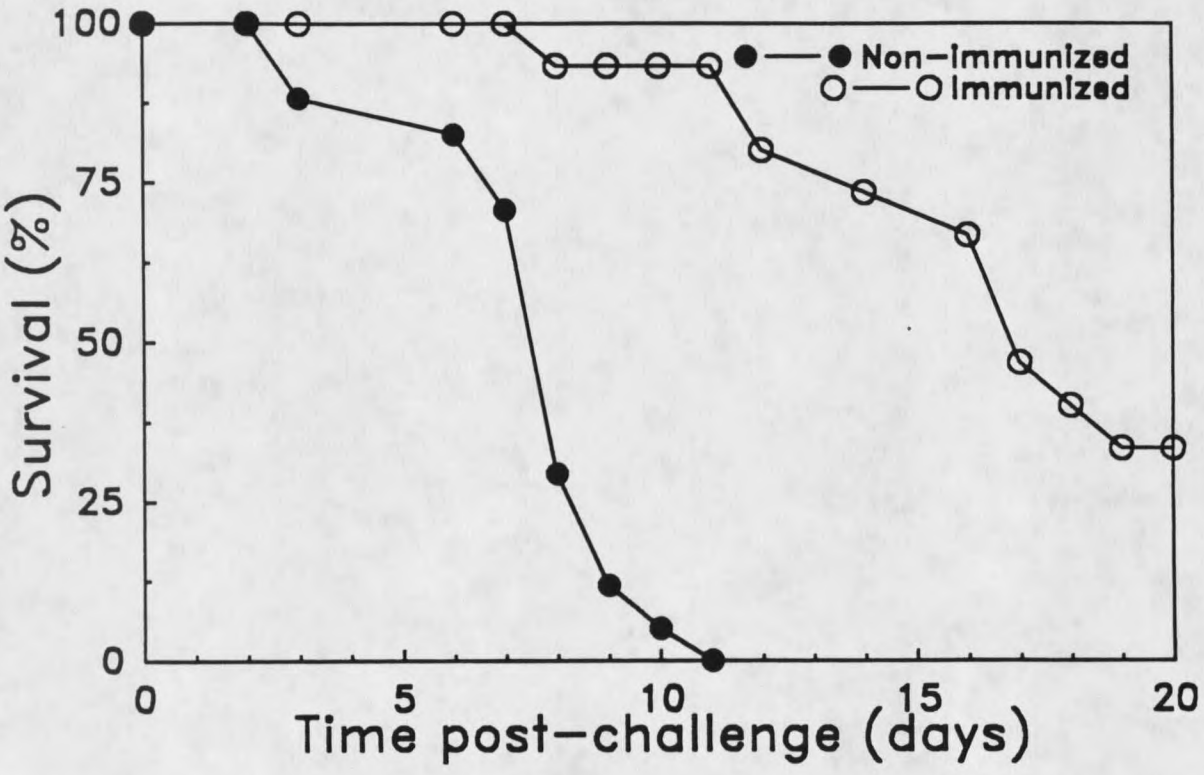

Figure 14. Results of the X4064 foster-pup experiment comparing the percent survival of passively-immunized mice (n=15) with the percent suvival in a group of unimmunized control mice (n=16) following challenge with virulent *S. typhimurium* strain SL3201 over time.

SL3235 Experimental Group #2. The results of the final challenge study are presented in Figure 16. An ST_{50} of 16 days for 24 mice was found in the passively immunized test group. The unimmunized control group demonstrated an ST_{50} of only 8 days for 24 mice. While none of the control mice survived past day 9, the test group exhibited 12.5% survival at the conclusion of the experiment (day 21).

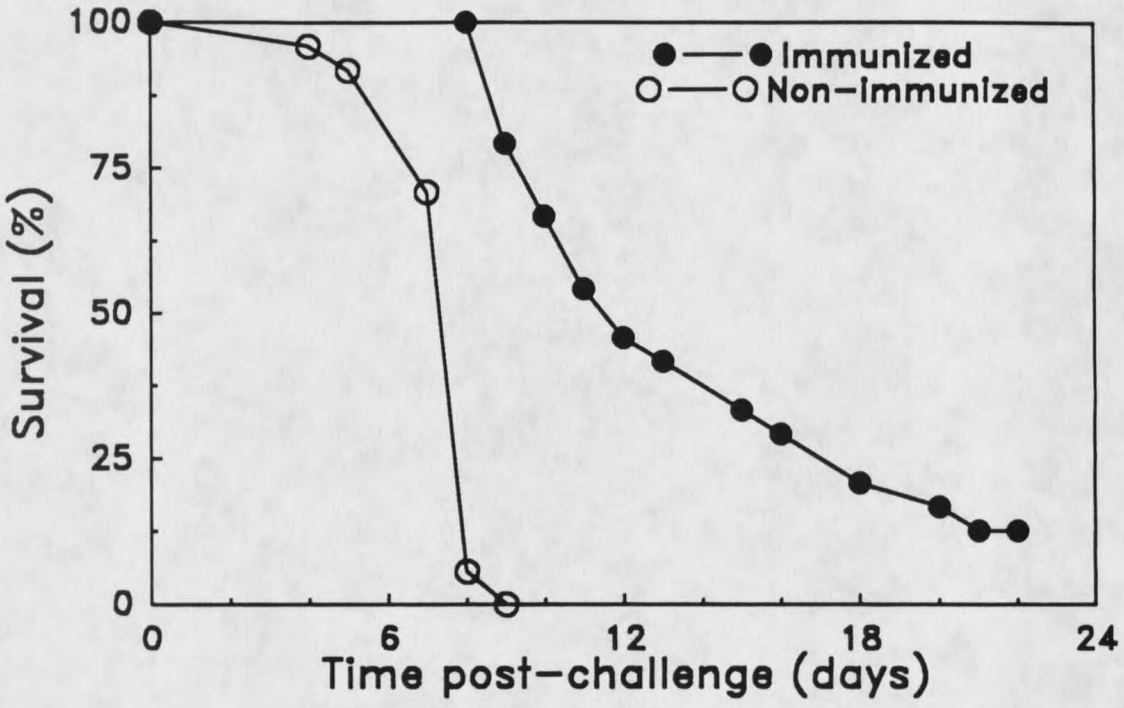

Figure 15. Results of the second SL3235 foster-pup experiment comparing the percent survival of passively-immunized mice (n=24) with the percent survival in a group non-immunized controls (n=24) following challenge with *S.typhimurium* strain 3201 over time.

Passive Feeding Studies

Attempts to provide passive protection against challenge with virulent *S. typhimurium* by feeding weaned mice antibody containing samples were unsucessful. Two attempts were made: (1) one involving the feeding wean mice ascites containing non-agglutinating IgM specific for *S. typhimurium* produced by the hybridoma M-F10 (obtained from D.A. Schiemann); and (2) another involving the feeding of a concentrated preparation of *S. typhimurium* specific IgA (titer = 6,400) prepared from the feces of SL3235 immunized mice. Results of the later experiment are presented in Table 7. While there is not any indication of antibody induced protection the precision of the experimental method was demonstrated by the almost identical results seen in both the control and test groups.

Table 8. Feeding study results demonstrating the frequency of isolation of Group A *S. typhimurium* from the spleens and rectums of challenged mice. Unimmunized mice received PBS.

	Mice Positive Salmonella[a]			
	Immunized (IgA)		Unimmunized	
Challenge Dose (bacteria/mouse)	Spleens	Rectal Swabs	Spleens	Rectal Swabs
$1x10^4$	0/5	0/5	0/5	0/5
$1x10^5$	0/5	0/5	0/5	0/5
$1x10^6$	0/5	0/5	0/5	0/5
$1x10^7$	4/5	3/5	5/5	3/5
$1x10^8$	5/5	5/5	5/5	5/5

a) Identification of isolates confirmed with Difco Group A antiserum

DISCUSSION

Parenteral immunization has been the main vaccination route for a wide variety of infectious diseases (45). Immunization by this method has been used successfully in establishing protection against diseases of a systemic nature, such as rubella, rubeolla, and tetanus. Parenteral immunization has not, however, been an effective means of inducing protective immune responses at the mucosal level as a way of defending against enteropathogens. As an example, the use of parenteral immunization against bacillary dysentery has proven so ineffective in preventing the disease that it is no longer recommended by the World Health Organization (45).

Attention has now turned to the use of oral immunization as a way to induce a localized secretory immune response for the purpose of defending the host against colonization by enteropathogens. The gastrointestinal mucosa is the primary barrier responsible for the prevention of host colonization by these organisms (8,55) and inducing a "mucosal" immune response characterized by the production of SIgA is the logical approach for defending it. Numerous studies have demonstrated the protective potential of mucosally-derived antibody, (9,49,54,57,68) and have indicated that these antibodies can be stimulated by oral immunization (17,44,45,48).

This study was undertaken to demonstrate the presence of protective secretory antibody induced following oral immunization with mutant strains of avirulent *Salmonella*. The use of recombinant avirulent *Salmonella* as live vaccines has been proposed and studied in several animal models (14,16,18,32,37,46,47,56,59,65). The majority of these studies have focused on the stimulation and demonstration of protective humoral responses, leaving questions regarding the effectiveness of these mutants in raising protective secretory immune responses relatively unanswered. Do these mutants have the ability to induce a secretory immune response when administered as live oral vaccines? If induced, will this secretory immune response exhibit memory and will it protect against oral challenge with virulent organisms of the same genus and species?

Characterization of Experimental Strains

Plasmid Analysis

A 60 Mdal "cryptic" plasmid which is found in some strains of *S. typhimurium* (30) has been associated with survival related virulence characteristics of this organism (30,31,33,39,70). The results of gel electrophoresis (Figures 1 & 2) summarized in Table 1 indicate that a plasmid equivalent in size to the cryptic plasmid is present in the virulent strain SL3201 and in the avirulent strains SL3235, and SL1306. Plasmids of this size were not found in the

plasmid-cured strain X3337 or the avirulent strain X4064 in five attempts at isolating it.

The absence of a 60 Mdal plasmid in X4064 is contrary to the published description of this strain (16) and raises questions about the ability of this strain to survive in the reticuloendothelial system. Because of this plasmid's importance to the persistence of strain X4064 *in vivo*, which has already been limited through mutation, it can be assumed that its absence might limit strain X4064's ability to establish itself in great enough numbers, following oral inoculation of a host, to elicit a strong immune response. There is, however, evidence indicating that plasmid-cured strains of *S. typhimurium* are immunogenic (16,33).

In vitro Adhesion and Invasion

In vitro adhesion and invasion assays were used to determine whether or not the presence of mutations which inhibit the survival of strains X4064 and SL3235 *in vivo* and the absence of the 60 Mdal plasmid in strain X3337 would influence the ability of these organisms to adhere to and invade intestinal epithelial cells. The results (Tables 4-6) demonstrated that the ability of these strains to adhere to and invade cells *in vitro* was not significantly diminished by the presence of mutations or the lack of the cryptic plasmid and in several instances increased relative to the "wild-type" strain SL3201 (Table 2). The abililty of these

organisms to exhibit the invasive phenotype associated with the virulent "wild-type" strains indicates that these organisms could possibly elicit immune responses to the moieties which mediate attachment and invasion. The ability of immune responses, directed at the mediators of attachment and invasion, to limit invasion has been documented in several studies (49,54,57).

The plasmid-cured strain X3337 (Table 6) and the plasmid negative strain X4064 (Table 5) demonstrated a higher degree of association invasion of epithelial cells *in vitro* than did strain SL3201. These results are in contrast to those reported. Jones *et al.* (39) reported that the elimination of the naturally-occurring 60 Mdal plasmid from a virulent *S. typhimurium* host resulted in a significant decrease in adhesion and invasion of HeLa cells. These results confirm the findings of two more recent studies discounting the relationship between the cryptic plasmid and the invasive phenotype (30,33).

A dramatic increase in the level of association and invasion was observed when SL3201 was grown under anaerobic conditions relative to that of SL3201 grown aerobically (Table 2). Approximately 30% of the added bacteria grown anaerobically were found to be intracellular as opposed to the 3% intracellular bacteria exhibited in tests using aerobically grown bacteria. These results demonstrate levels of invasion significantly higher than those described in the literature.

Small *et al.* (64) conducted experiments indicating that approximately 16% of the bacteria added were found to be intracellular in HEp-2 cell assays and only 0.4% of the added bacteria were intracellular in Vero cell assays described by Barrow and Lovell (3). To compare the differences in the degree of invasiveness found in experiments using different cell lines and dissimilar protocols is difficult. There does, however, appear to be a significant increase in the degree of invasiveness accompanying growth under anaerobic conditions and the level of invasiveness found in these studies, which used bacteria grown aerobically, provides a basis for comparision. Similar increases in association and invasiveness were demonstrated by bacteria grown at 25°C as opposed to 35°C (Table 3). These results contradict the results of Small *et al.* (64) which indicate a reduction in the degree of invasiveness accompanying growth at 30°C relative to the invasiveness of organisms grown at 37°C. The mechanism or mechanisms involved in the increase of invasiveness demonstrated by SL3201 grown under anaerobic condition and at 25°C are unknown. Comparative polyacrylamide gel electrophoresis of the outer-membrane proteins of strain SL3201 grown aerobically, anaerobically, and aerobically at 25°C have indicated that a reduction in several proteins occurs when the organism is grown anaerobically and aerobically at 25°C (data not reported). *In vitro* assays for the uptake of strain SL3201 by peritoneal macrophages indicate

that the organism grown aerobically is less susceptible to phagocytosis than Strain SL3201 grown anaerobically (data not reported). These results have been related to the suppression of an outer membrane protein of about 24 Kdal during anaerobic growth which may have anti-phagocytic properties (D.A. Schiemann, unpublished data). Further investigation of these observations could yeild information helpful in the characterization of the mechanisms involved in the adhesion and invasion properties associated with *S. typhimurium*.

Microscopic Evaluation of Attachment and Invasion

Transmission electron microscopy (TEM) is one method by which the presence of intracellular bacteria can be demonstrated. The electron micrographs presented in Figures 3, 4, & 5 show the engulfment and the location of intracellular *S. typhimurium* strain SL3201 in infected Henle cells. A few microvilli are present on the cell surface surrounding the area of attachment (Figure 3) which is in agreement with previously published findings reporting the degeneration of microvilli following the attachment of *S.typhimurium* to a host cell (24,26). Several intracellular bacteria were contained within vacuoles but the majority were found free in the cytoplasm. This is contrary to the literature (24) and the absence of vacuoles probably represents an artifact of the treatment necessary for the preparation of samples for the electron microscope. Figure

5 shows two bacteria in the final stages of division. Whether or not this is an example of intracellular replication cannot be determined due to the possibility that replication may have been initiated prior to engulfment by the epithelial cell.

Responses to Oral Immunization

Subcutaneous priming was applied prior to oral immunization in two separate test groups of mice. The results presented in Figure 6 indicate that a rise in the amount of IgA present in the feces of a mouse primed with live SL3235 occurred following oral immunization with live organisms of the same strain. These results may indicate that a mucosal IgA response did occur as a result of the immunization protocol. These data, however, are not directly supported by the results found in other mice immunized with the same strain and followed by the same protocol (Appendix F). These results are not consistent with the data presented in Figure 6 but in some cases lend support to the demonstration of a mucosal immune response (Figure 18), but they are inconclusive. Erratic results and in some cases high initial amounts of specific IgA present in the feces prior to the oral immunization confounded the interpretation of these data. Figure 7 and Figures 21-24 (Appendix G) represent the results for mice primed and orally immunized using live organisms of the strain X4064. The problems of erratic measurements and high initial amounts of specific IgA were also exhibited in

these assays. Therefore, it could not be conclusively determined whether or not a mucosal immune response had occurred as the result of the oral immunization. The erratic nature of the measurements cannot be explained except by the fact that a mucosal memory responses were not induced by the oral immunization. The method of priming the animals may be responsible for the presence of high amounts of specific IgA in the feces prior to the oral immunization and may also be partially responsible for the absence of a rise in specific IgA. The live bacteria (SL3235 and X4064) were introduced into the animal by sc immunization in the presence of an adjuvant. The adjuvant is used to promote the immunogenicity of the antigen and to prolong the release of the antigen to the lymphoid tissues. The persistence of the antigen due to the presence of an adjuvant may explain the presence of specific IgA in the feces prior to the oral immunization. The data (Figures 6 & 7) indicates that the sc priming with both strains X4064 and SL3235 did produce immune responses which were apparent prior to oral immunization but they do not indicate the induction of strong memory responses following oral immunization. These results confirm reports of Keren *et al.* (45) describing the failure of sc priming (without adjuvant) followed by oral immunization in producing a memory response.

Subcutaneous priming was abandoned in favor of multiple (3) oral immunizations due to the problems encountered with

the first two groups of mice for use with the final group of mice. A more rigorous schedule of feces collection was also used to develop a more accurate picture of the fluctuations in the amounts of immunoglobulins present in the feces prior to and following each oral immunization. The protocol modifications instituted for the final group of mice were successful in correcting the problems encountered with the first two groups. Results indicating induction of a mucosal immune response, characterized by intestinal IgA production and shown by fecal analysis, following three oral immunizations with SL3235 are presented in Figures 8-12. The rises in fecal IgA were not accompanied by significant rises in fecal IgG and there were only two cases where a significant rises in fecal IgM occurred (Figures 9 & 10). The presence of a small rise in intestinal IgA following the second immunization and a rapid, more vigorous rise following the third immunization demonstrated clearly the presence of a mucosal memory reponse. These results substantiate recent reports of the presence of mucosal memory (43,44) but they also contradict findings which demonstrated greater increases in the mucosal production of IgA resulting from oral immunization when an animal was primed parenterally than in an unprimed animal receiving only oral immunizations (43). Serum responses were measured prior to and following the entire immunization protocol. The serum titers (Table 7) demonstrated small rises in both specific serum IgA and IgG

indicating a limited systemic response to oral immunization. The induction of a muscosal immune response by an oral immunogen is accompanied by a weak systemic response (8). These results would tend to indicate that the rise in specific intestinal IgA was not due to a rise in serum antibody. The most striking result of the serum analysis was the high specific IgM titer present in the pre-immune serum which declined in the serum following immunization. IgM is synthesized by the near-full term rodent (rats and mice) fetus. This serum component is present in the serum of the new-born rodent but begins to disappear at three weeks (5). These facts explain the presence of IgM in the serum of the young mice (approximately 17 days), but they do not explain the specificity for *S. typhimurium* exhibited by the IgM. The specificity exhibited could be explained as a cross reaction with other bacteria to antigens present on *S. typhimurium*, but a definitive answer would require further investigation.

The Passive Transfer of Immune Protection to Foster Infant Mice

Protective antibody while stimulated at the level of the gastrointestinal mucosa, is not only confined to the gastrointestinal tract but can be demonstrated in the secretions of other exocrine secretory tissues such as the salivary and mammary glands (8,53,55). The ability of colostral antibody, induced as part of the immune responses described in the previous section, to provide protection

against colonization by a virulent strain of *S. typhimurium* was demonstrated by the foster pup model of passive transfer developed for this study. The use of passively-provided immunoglobulins to inhibit host colonization by enteropathogenic organisms has been demonstrated in other types of models (4,9,10,19,57,68).

The results (Figure 13) demonstrated a small degree of protection associated with the transfer of colostral antibodies from mice primed sc with SL3235 and then orally immunized with the same organism (Figure 6) to foster suckling mice. These results indicated that efforts to elicit a protective immune response were partially successful, but due to the lack of documentation on the presence of an induced specific immune response, conclusions as to the source, systemic or otherwise, of the protective antibodies could not be drawn. This was also the case in the passive transfer experiment involving mice sc primed strain X4064 and then orally immunized with the same organism. The results of this experiment (Figure 14) indicated that a high degree of protection was conferred on the foster suckling mice by passively acquired antibody, but the antibodies responsible for this protection could not be directly attributed to a secretory (mucosal) immune response induced by oral immunization due to the inability to document any such response (Figure 7). Results of the final passive transfer experiment (Figure 15) showed a high degree of protection in

passively immunized foster mouse pups. Strong specific intestinal IgA responses, indicating the induction of a mucosal immune response, were documented for this group of mice (Figures 8-12). While these data are not conclusive they do suggest that protective antibody in the colostrum was produced as a result of this intestinal immune response. It is known that mouse colostrum contains a higher percentage of IgG than the other classes of immunoglobulins, therefore, it cannot be assumed that this protection was part of "classic" mucosal immune response, characterized by IgA production, but belonged to a more general "secretory" immune response found in exocrine tissue. Systemic responses to the immunizations were probably not significant in the protection of these mice. Serum responses (Table 7) to the immunizations demonstrated only a slight rise in serum IgG and IgA titers and a decrease in IgM titers. It has been shown that the transfer of antibody through the yolk-sac to the fetus during gestation is only a minor route compared to colostral transmission of antibody to the neonate (66). Therefore, the protection demonstrated could not be attributed to pre-existing maternal antibody in the foster pups.

A problem confronted in these passive transfer experiments centered around the possibility of cross-contamination between the mother and the pups. Wire bottom cages were employed to limit fecal contamination and dead pups were removed as soon after death as possible to limit

cannibalism. The main concern was in reducing the repeated inoculation of the mother by contaminated feces and the infected tissue of dead pups as a way of saying that the protection resulted from the immunizations and not from incidental exposure to organisms throughout the duration of the experiment.

Feeding Studies

The failure of a feeding study conducted to demonstrate the ability of *S. typhimurium* specific fecal immunoglobulins, mostly IgA isolated from immunized mice, to protect mice from challenge (Table 8) left questions concerning the protective potential of these antibodies in the foster pup model. Further investigation is necessary to demonstrate whether or not mucosal antibodies alone produced by oral immunizations can in fact inhibit colonization of the young mice by *S. typhimurium*.

Conclusions

The following conclusions can be made from the data presented in this report:

1. The presence of mutations did not significantly diminish the ability of *avirulent S. typhimurium* strains SL3235 and X4064 to adhere and invade intestinal epithelial cells *in vitro*.

2. The ability of *S. typhimurium* to adhere to and invade intestinal epithelial cells *in vitro* is not mediated by the 60 mdal cryptic plasmid.

3. A specific intestinal memory response can be induced by oral immunization with avirulent strains of *S. typhimurium* which can be reliably monitored by coproantibodies.

4. Secretory antibodies produced against *S. typhimurium* by oral immunization can provide passive immune protection for foster mouse pups.

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APPENDICES

APPENDIX A

Preparation of Samples for Electron Microscopy

Samples were treated with Karnofsky's fixative (3% glutaraldehyde, 3% formaldehyde, in 0.2 M cacodylate buffer containing 0.1% $CaCl_2$, pH 7.2) for 1 h at 4°C. Following primary fixation, the samples received a second fixation in a solution of 2.0% osmium tetroxide diluted in 0.1 M potassium-sodium phosphate buffer (PSPB) for 4 h at 25°C. The samples were rinsed 2x with PSPB and dehydrated using 15 min exposures to each concentration in a graded concentration series of ethanol (50%, 50%, 70%, 95%, 100%, 100%, and 100%) through 7 changes. Dehydration was continued by treating the samples to 2 washes (15 min/wash) in propylene oxide. The samples were infiltrated with the embedment resin (Spurr's resin) by first treating them with a 2:1 dilution of propylene oxide in Spurr's resin (30 min on a shaker at 25°C) and then with a 1:2 dilution of propylene oxide in Spurr's resin (60 min on a shaker at 25°C). The infiltration was completed by an overnight exposure of the samples to 100% Spurr's resin at 4°C. Each sample was embedded individually in 100% Spurr's resin hardened by heat treatment (70°C for 8 h). Thick (2 um) and thin sections (60-100 nm) were taken from each of the samples using an ultramicrotome (Reichert OM-2 Ultramicrotome). Thin sections were collected on copper grids and stained using a combination of uranyl acetate and lead citrate for viewing on the electron microscope (Zeiss EM 10C).

APPENDIX B

Preparation of Plasmids

Plasmids were prepared from bacterial strains either using the CIRCLEPREP plasmid DNA purification kit (BIO 101 Inc.) or the original method on which CIRCLEPREP is based (51). The protocol which follows was taken from the CIRCLEPREP instructional literature. Bacteria were grown overnight in 50 ml of CIRCLEGROW growth medium (35°C, 240 rpm) and pelleted by centrifugation (5000xg, 20 min). The pellet was resuspended in 4 ml sterile pre-lysis buffer (25 mM Tris-HCl, 50 mM glucose, 10 mM CDTA, pH 8.0). Alkaline lysis reagent (4 ml) (0.2 N NaOH with 1% sodium dodecyl sulfate) was added to the suspension and the mixture was mixed gently for 5 min. The addition of 4 ml neutralizing solution (3 M potassium/ 5 M acetate) to the mixture was used to stop the lysis reaction. The mixture was centrifuged (10,500xg, 30 min) to separate cellular material from the suspended DNA. The supernatant was removed and its volume measured. An equal volume of isopropanol was added to the supernatant to precipitate the suspended DNA. The precipitated DNA was pelleted by centrifugation (8,000xg, 5 min), resuspended in 0.5 ml of sterile distilled H_2O and transferred to a 2 ml microcentrifuge tube. The resuspended pellet was treated with 250 ul of the alkaline lysis reagent which was followed by the addition of 250 ul of the neutralizing reagent. The resulting precipitate was removed by centrifugation (2 min) on a microcentrifuge (Eppendorf). The supernatant was transferred

to a new microcentrifuge tube and treated with an equal volume of isopropanol. The precipitated DNA was pelleted by centrifugation and resuspended in 0.5 ml of sterile distilled H_2O. The small volume alkaline treatment was repeated 2x to remove most of the cellular DNA and RNA. Any remaining ssDNA and RNA was removed using LiCl precipitation. The resuspended plasmid DNA was treated with 300 ul of LiCl solution (15 mM LiCl, 50 mM EDTA, pH 8.0) for 5 min at 25°C. The resulting precipitate was removed by pelleting on a microcentrifuge (2 min). The supernatant was transferred to a new 2 ml microcentrifuge tube and treated with an equal volume of isopropanol. The precipitated plasmid DNA was pelleted on a microcentrifuge (2 min) and resuspended in 0.2 ml Tris-EDTA buffer (pH 8.0). The resuspended plasmid DNA was heated to 70°C in a water-bath for 5 min to reduce nuclease activity before storage at -70°C.

APPENDIX C

Preparation of Hybridomas

Monoclonal antibodies and the protocol used for their production were obtained from D.A. Schiemann, Montana State University.

Immunization Protocol

Balb/cBy mice were immunized by sc injection of *S. typhimurium* strain SL3235 ($1x10^6$ bacteria/mouse) mixed with Ribi adjuvant [TDM (trehalose dimycolate) + MPL (monophosphoryl lipid A), Ribi ImmunoChem Research, Hamilton, MT] according to the manufacturer's instructions. Three months later the mice received an oral booster dose ($1x10^7$ bacteria/mouse), followed by a second oral booster ($5x10^7$ bacteria/mouse) a month later and one day before sacrifice and recovery of spllen cells. The bacteria in the oral bosters were suspended in 0.1% gelatin-saline and administered following oral feeding of 10 ul 10% $NaHCO_3$.

Preparation of Spleen Cells

The spleen from one mouse was removed, washed in 10 ml of IMDM tissue culture medium (Sigma), and the cells ejected by repeated injection of IMDM medium with an 18G needle. The cells were recovered by centrifugation (400xg, 5 min) and suspended in 1 ml of erythrocyte lysis buffer (NH_4Cl, 8.3 g/l; Tris-HCl, 0.01 M; pH 7.5) for 1 min. The cells were recovered by centrifugation, and resuspended in IMDM medium. The viable cell density was determined by microscopic count of a dilution

prepared in 0.4% trypan blue-DPBS.

Preparation of Myeloma Cells

Myeloma cell P3X63Ag8.653 grown in IMDM medium with 10% FBS, penicillin (50 U/ml), and streptomycin (50 ug/ml) were recovered in fresh IMDM medium, washed once and resuspended in IMDM medium. The viable cell density was determined by microscopic count on a dilution prepared in trypan blue.

Fusion Protocol

Spleen cells and myeloma cells were mixed at a ratio of 10:1, centrifuged, and suspended in the small amount of medium remaining with the pellet. The cell mixture was held for 1 min in a 37°C water bath. Fusogen (0.3) [IMDM pH 8.0, 4.5 ml; dimethyl sulfoxide, 0.5 ml; polyethylene glycol (Sigma, MW = 3,000-3,700), 5g] warmed to 37°C was added dropwise over 45 sec while agitating the mixture. Fifteen ml of warm IMDM were then added over 90 sec with continued agitation of the suspension. The cell mixture was held for 8 min at 25°C and then 2 min at 37°C. The cells were recovered by centrifugation and the pellet was suspended to a density of $2x10^6$ spleen cells/ml of complete IMDM medium [IMDM, 50%; myeloma conditioned IMDM, 10%; mouse peritoneal cell-conditioned DME, 10%; FBS, 20%; penicillin, 100 U/ml; streptomycin, 100 ug/ml; amphotericin B, 2.5 ug/ml; endothelial cell growth supplement (Sigma EO760), 50 ug/ml]. Aliquots (100 ul) were placed in the

wells of a 96-well tissue culture plate conditioned with IMDM-10% FBS. The next day, 100 ul of complete IMDM containing hypoxathine-aminopterin-thyimidine (HAT) (2x concentration) was added to each well. The medium was changed 4 days later by removing 100 ul and replacing it with the same volume of fresh complete IMDM containing HAT (1x). The medium was changed in a similar manner about every 3 days.

Antibody Screening Assay

Hybridoma supernatants were examined for the presence of antibody by enzyme immunoassay using whole bacteria of *S. typhimurium* strain SL3201 as the antigen. The washed bacteria were suspended in bicarbonate coating buffer at a concentration of 20 ug (dry weight)/ml, and 100 ul were added to each well of a 96-well immunoassay plate (Nunc Immunoplate). The plates were held overnight at 4°C, washed 3x with PBS and treated with a blocking buffer (BSA, 1.0%; NaN_3, 0.02%; PBS) for 3 h at room temperature. The blocker was removed and the plates stored dry at -20°C. Hybridoma supernatants (50 or 100 ul) were added to the antigen plates after washing 2x with PBS, and the plates were held overnight at 4°C. The following morning, the plates were washed 5x with PBST (0.05% Tween 20 in PBS), and then blocked again for 1 h. After 2 washes with PBST, 100 ul of anti-mouse IgA/IgM/IgG-biotin conjugate (Kirkegaard & Perry Laboratories, Inc.)

diluted 1:500 in 0.1% BSA-PBST were added and the plates held at room temperature for 2 h. The plates were washed 5 times with PBST, followed by the addition of 100 ul of streptavidin peroxidase conjugate (Kirkegaard & Perry) diluted 1:1,000 in 0.1% BSA-PBST. The plates were at room temperature for 90 min, and then washed 5x with PBST. The enzyme reaction was developed by adding 100 ul of substrate [TMB peroxidase substrate + hydrogen peroxide, 1:1 (Kirkegaard & Perry) to each well. The reaction was stopped after 10 min at room temperature by adding 1 M H_3PO_4 (100 ul), or letting color development proceed for about 30 min. The yellow or blue-purple color, respectively, was compared visually against negative controls prepared with fresh tissue culture medium.

Cloning and Expansion

Antibody-producing hybridomas were cloned by removing the cells from the wells of the tissue culture plate, preparing dilutions in complete IMDM medium, and inoculating 8 or 12 wells per dilution in a medium-conditioned plate. Feeder cells prepared either from the spleen or peritoneal washes of non-immunized mice were included at the same time. Clones that appeared to originate from single cells were again examined for antibody production by EIA and cloned again by limiting dilution.

Antibody-positive clones of hybridomas were expanded by passage through 24-well plates, a 6-well plate, a 25 cm^2 tissue culture flask and finally a 75 cm^2 flask. Aminopterin was omitted from the medium at the 25 cm^2 flask stage. Cell cultures were removed from the last flask by scraping, and either used for the preparation of ascites or suspended in 3 ml of preservation medium (IMDM, 70%; FBS, 20%; DMSO, 10%) and stored in liquid nitrogen. Fifteen hybridomas were preserved, all producing a non-agglutinating IgM antibody against *S. typhimurium*.

Ascites Production

Balb/cBy mice received 0.2 ml of pristane (Sigma) ip about 4 to 9 days before ip injection of about $5x10^6$ hybridoma cells. After abdominal distention was apparent, ascites fluid was collected by tapping the abdominal cavity with an 18G needle. The ascites was held at 35°C for 1 h, refrigerated overnight, centrifuged (3,300xg, 4°C, 10 min), and stored at -70°C.

APPENDIX D

Ammonium Sulfate Precipitation of Immunoglobulins from Feces Samples

A method for removing the immunoglobulins present in wide range of samples using ammonium sulfate precipitation has been previously described (35). A measured volume of prepared feces sample was cleared by centrifugation (3,000xg, 30 min). An equal volume of saturated ammonium sulfate [$(NH_4)_2SO_4$, 800 g/l] was added to the cleared feces sample dropwise over 90 min at 4°C with constant stirring. The sample was stirred overnight at 50% of the saturation point of ammonium sulfate. The precipitated immunoglobulins were pelleted by centrifugation (3,000xg, 30 min) and resuspended in 0.1 of the original volume of PBS. To remove any ammonium sulfate from the precipitated sample it was dialyzed (09777 dialysis tubing) against 4 changes of 1.5 l each of PBS overnight at 4°C. The sample was centrifuged (3,000xg, 30 min) to remove any material that did not go into solution during the dialysis. The sample was concentrated to 0.1 of its volume by dialysis against Aquacide II (Union Carbide) at 4°C.

APPENDIX E

Standard Curve for
Plasmid Sizing

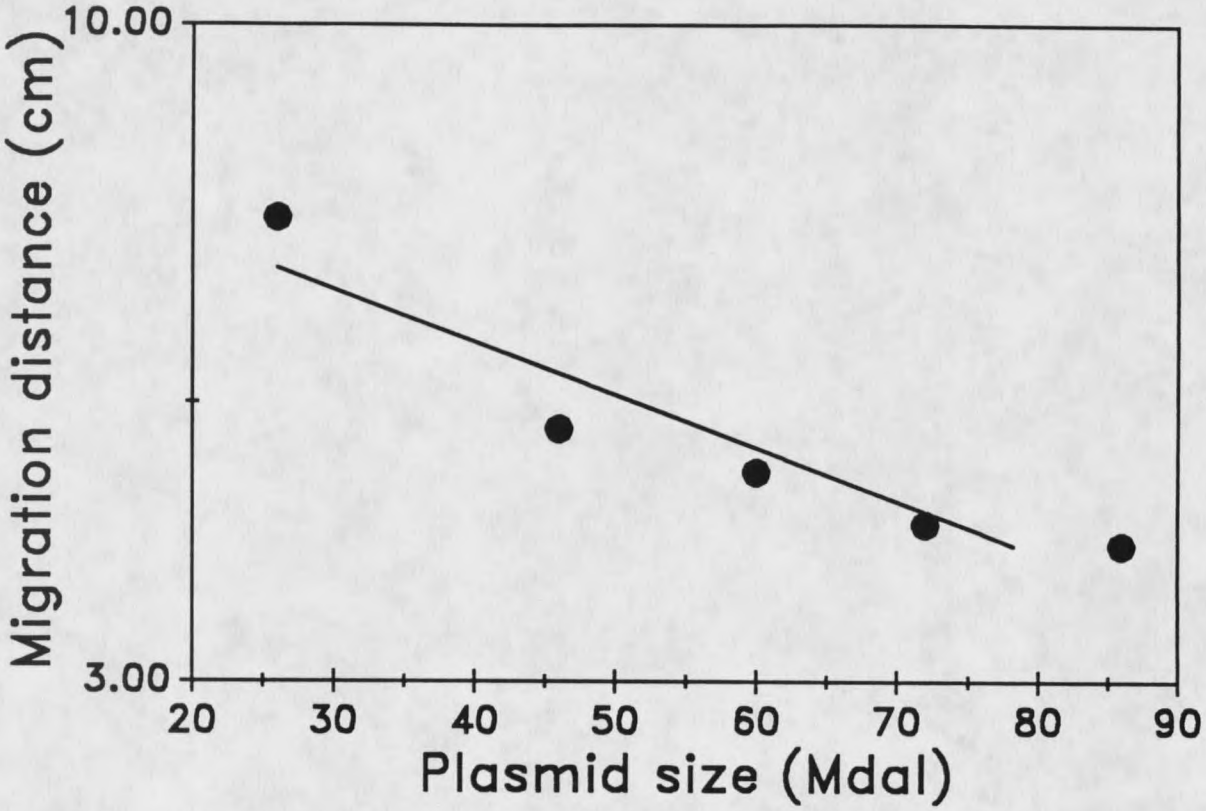

Figure 16. Distance migrated by plasmid standards relative to their size.

APPENDIX F

Supplemental Graphs for Experimental Group SL3235 #1

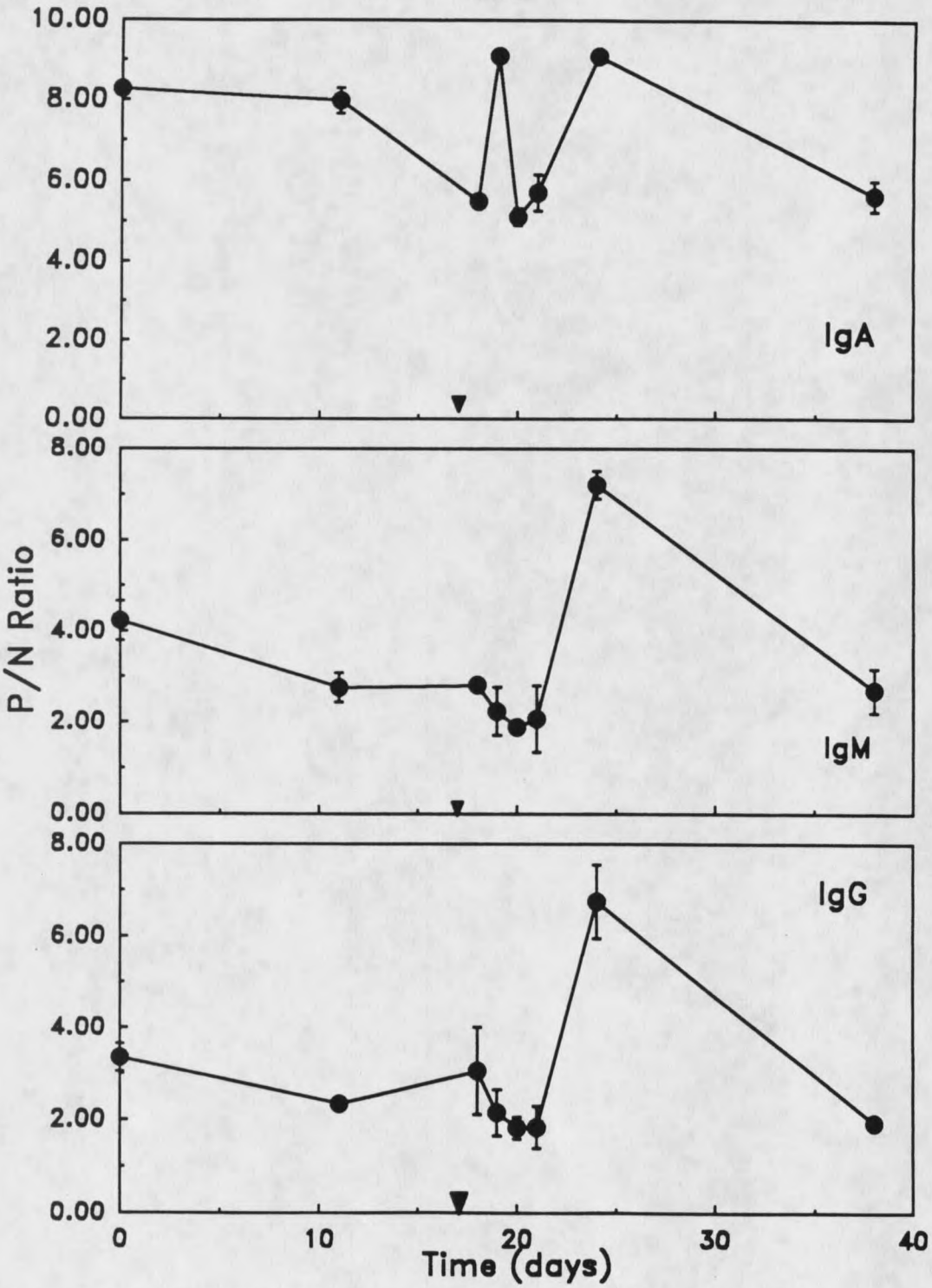

Figure 17. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S2 prior to and following drinking-water immunization with *S. typhimurium* strain SL3235. The time of the drinking-water immunization is indicated by an arrow.

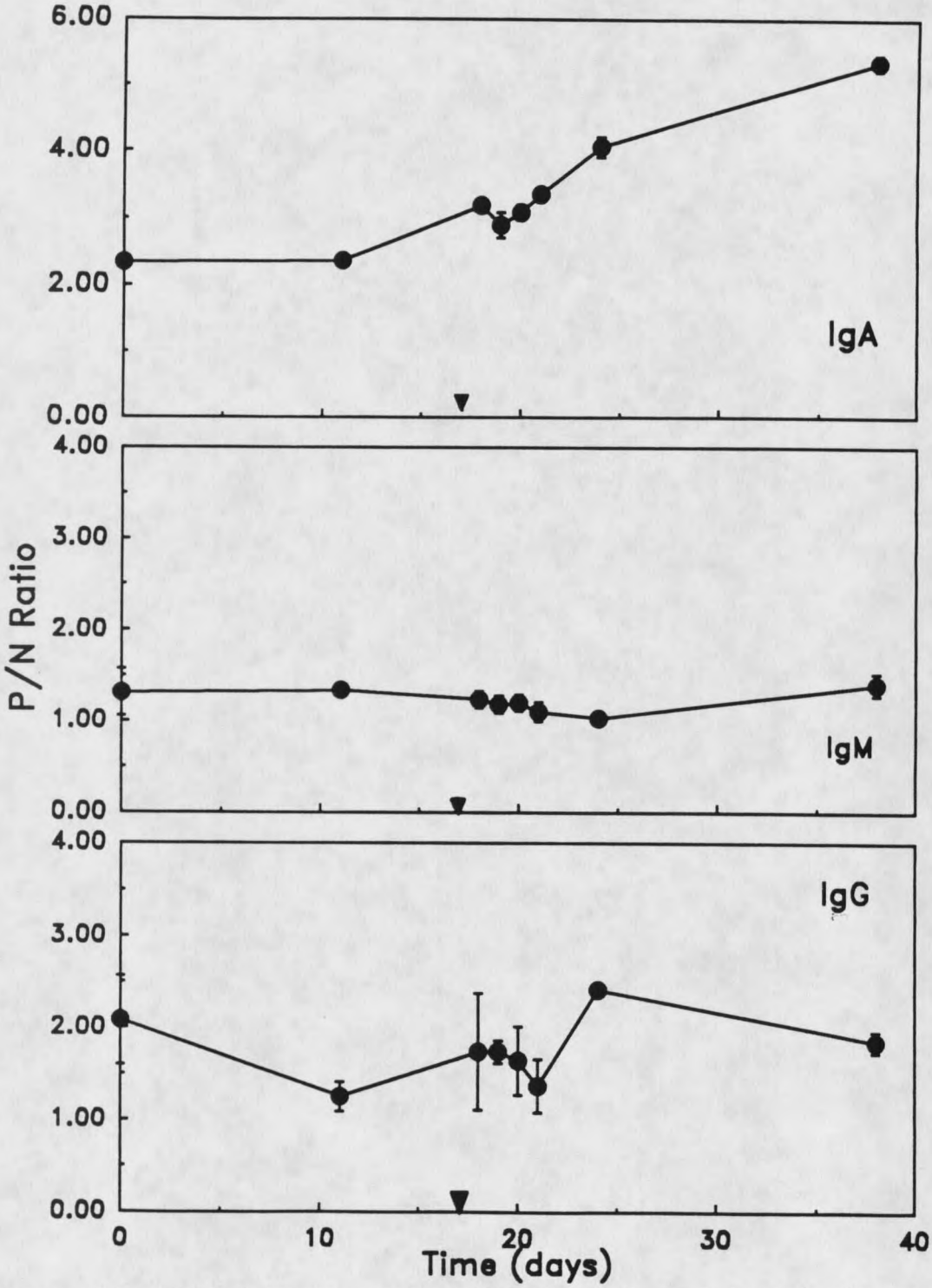

Figure 18. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S3 prior to and following drinking-water immunization with *S. typhimurium* strain SL3235. The time of the drinking-water immunization is indicated by an arrow.

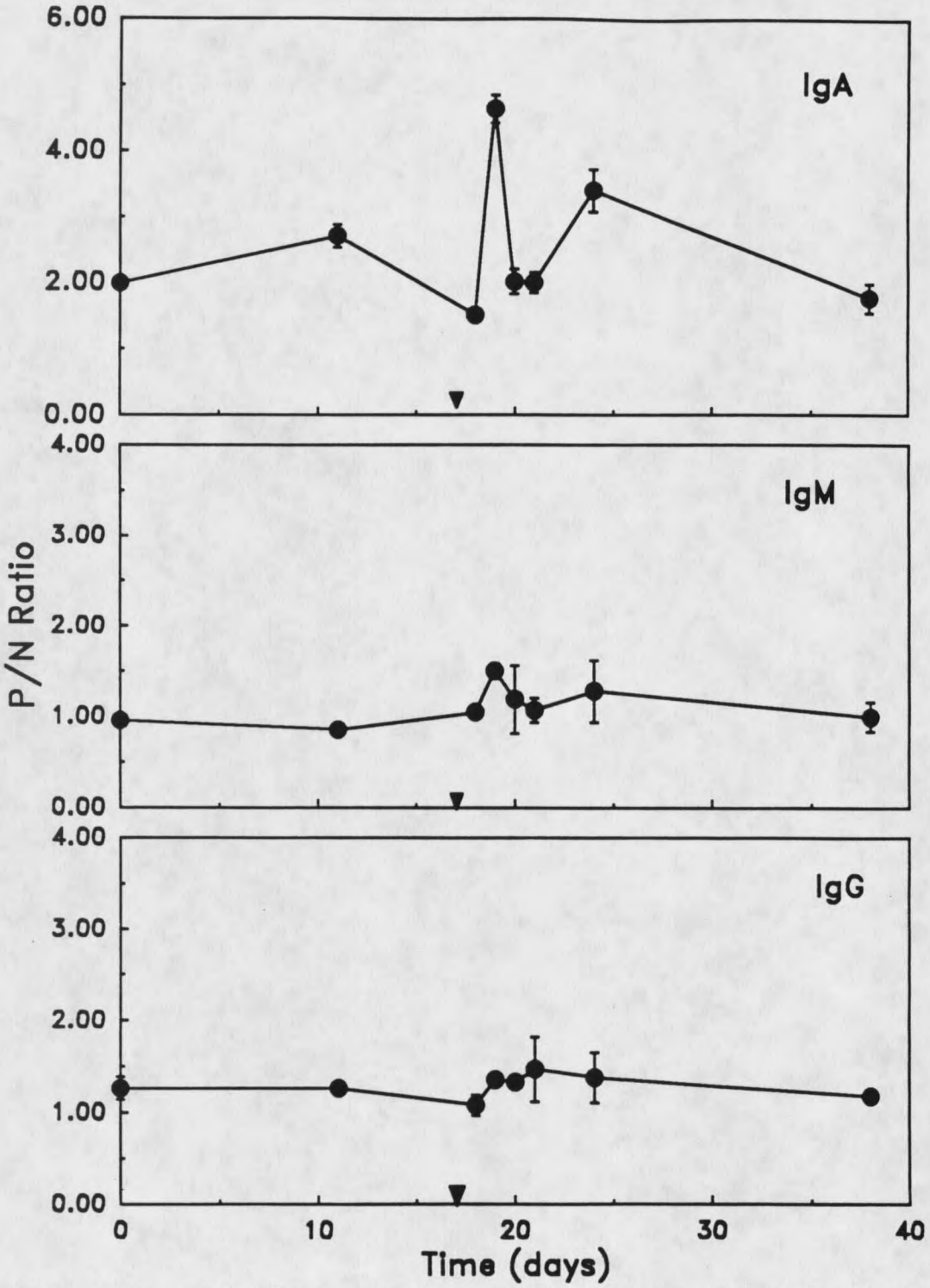

Figure 19. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S4 prior to and following drinking-water immunization with *S. typhimurium* strain SL3235. The time of the drinking-water immunization is indicated by an arrow.

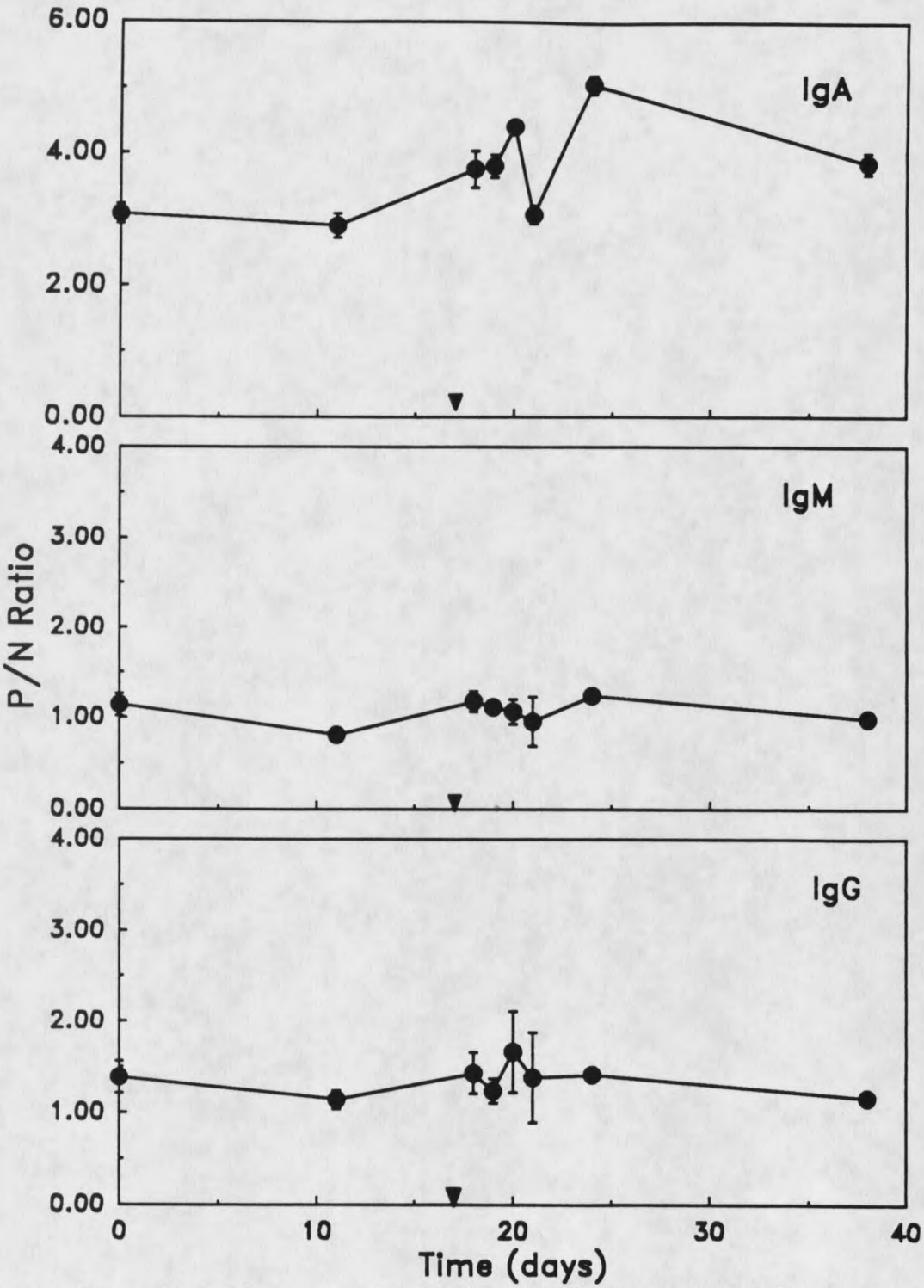

Figure 20. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S5 prior to and following drinking-water immunization with *S. typhimurium* strain SL3235. The time of the drinking-water immunization is indicated by an arrow.

APPENDIX G

Supplemental Graphs for Experimental Group X4064 #1

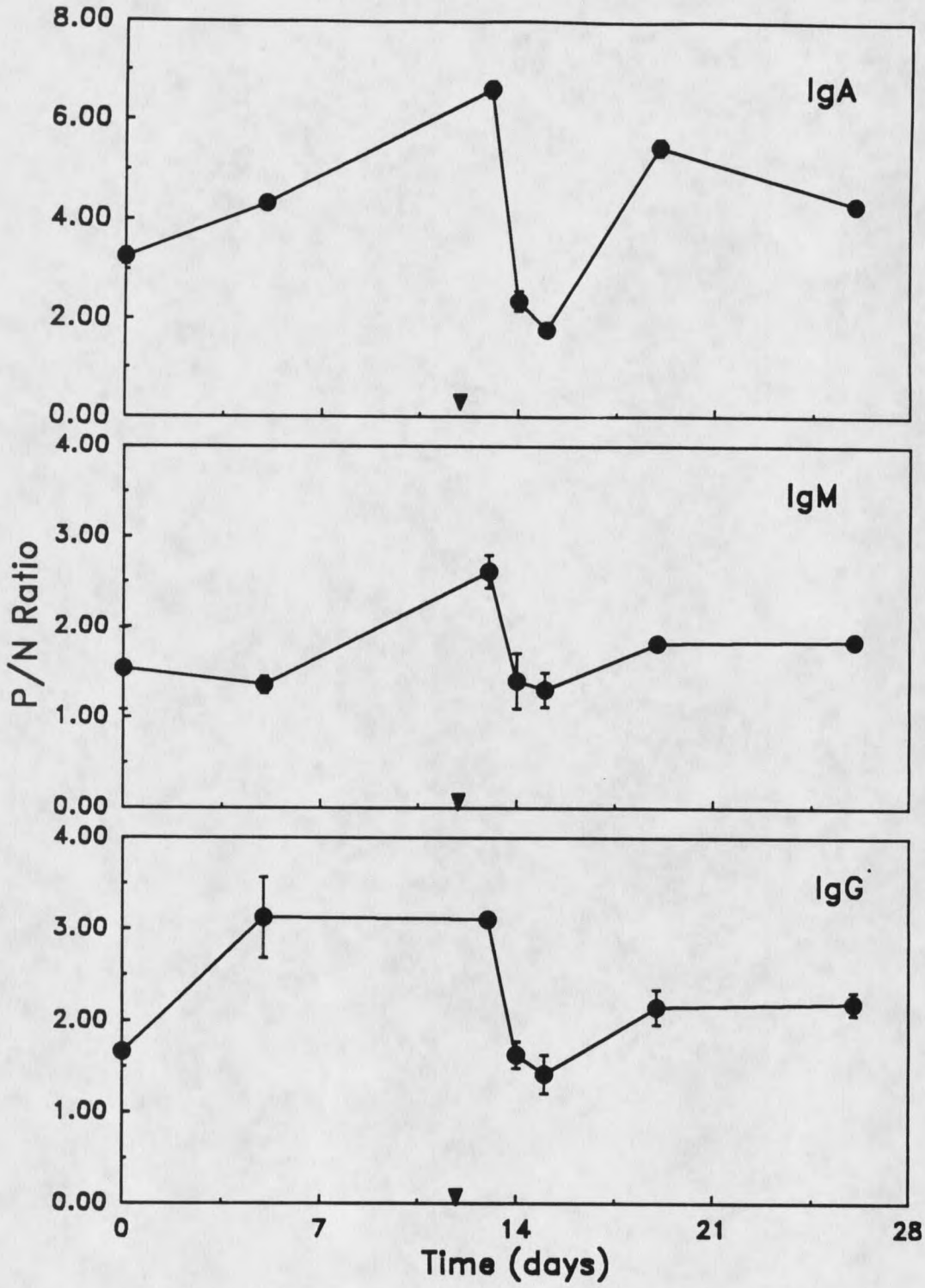

Figure 21. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S7 prior to and following drinking-water immunization with *S. typhimurium* strain X4064. The time of the drinking-water immunization is indicated by an arrow.

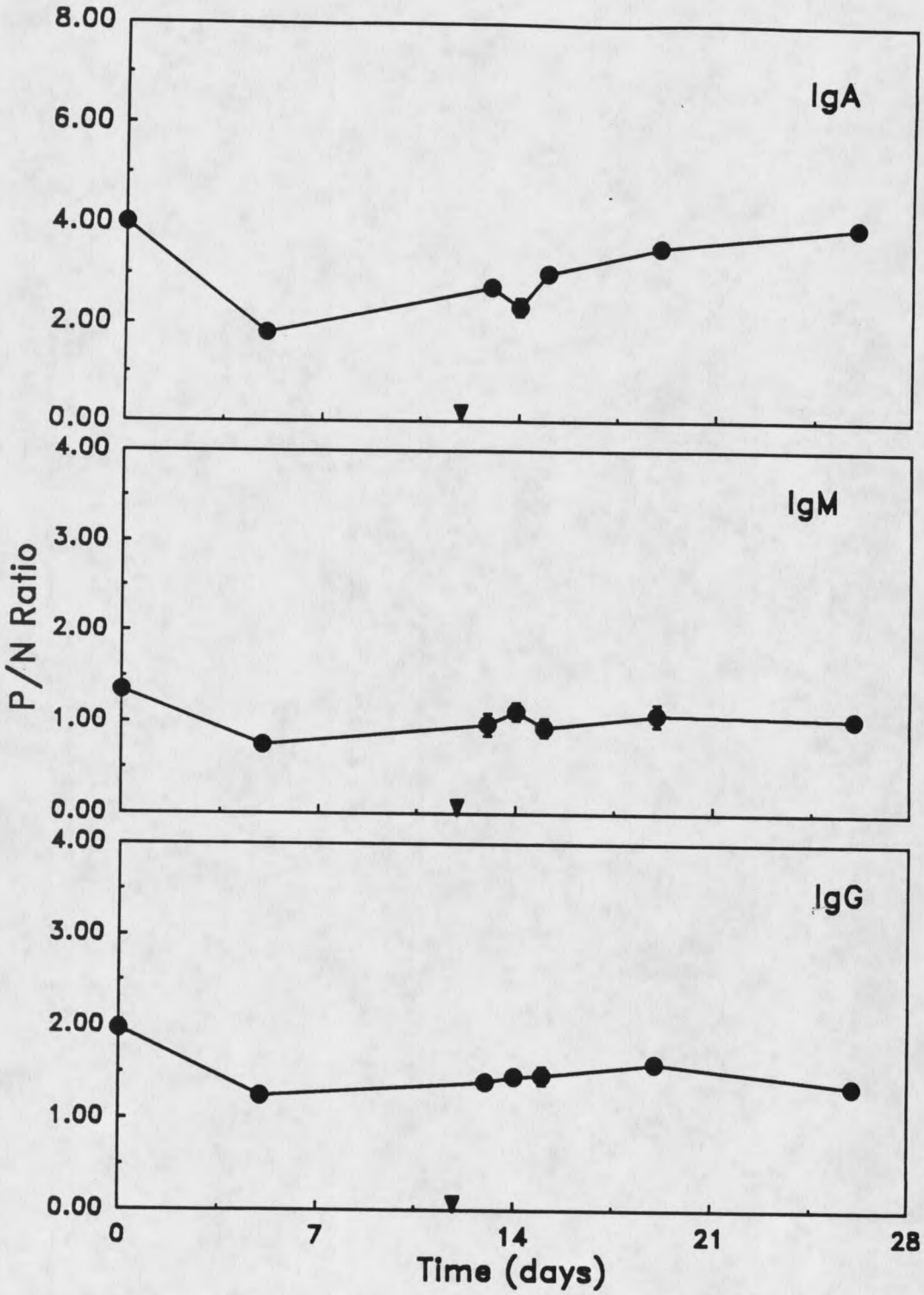

Figure 22. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S8 prior to and following drinking-water immunization with *S. typhimurium* strain X4064. The time of the drinking-water immunization is indicated by an arrow.

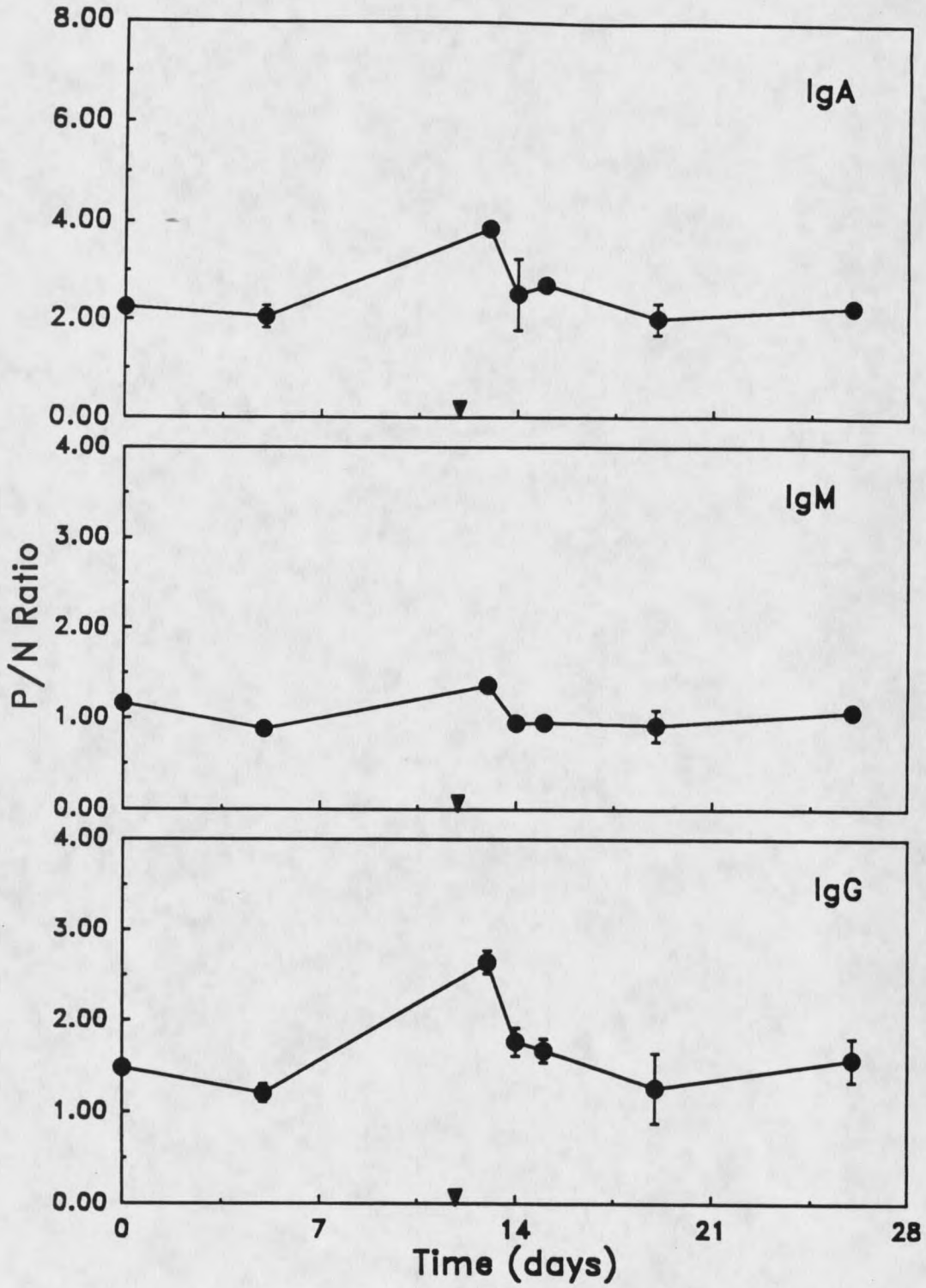

Figure 23. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S9 prior to and following drinking-water immunization with *S. typhimurium* strain X4064. The time of the drinking-water immunization is indicated by an arrow.

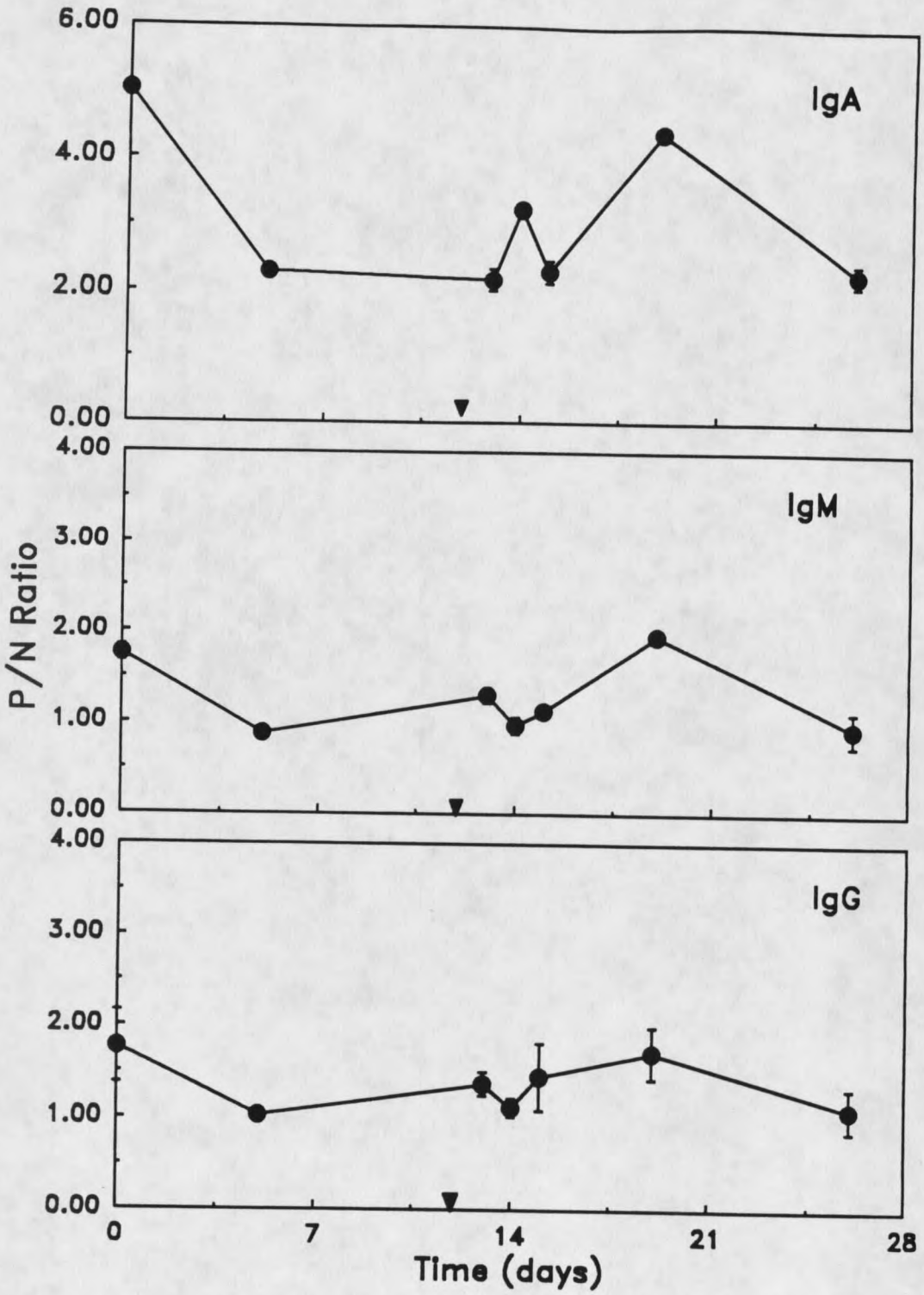

Figure 24. Relative amounts of IgA, IgM, and IgG in the feces of immunized control mouse S10 prior to and following drinking water immunization with *S. typhimurium* strain X4064. The time of the drinking-water immunization is indicated by an arrow.

APPENDIX H

Standard Curve for
Strain SL3201

$$\text{Number of bacteria/ml of culture} = \frac{A540 - 0.0148}{0.0734} \times 10^8$$

This is the linear regression equation of the standard curve developed for *S. typhimurium* strain SL3201. The equation was used throughout the tissue culture assays to adjust the number of bacteria in a suspension to $1x10^7$/ml. In (n=10) determinations the mean number of bacteria/ml in a suspension adjusted by this method was $1.28x10^7$ with a standard deviation of $2.11x10^6$.