A QUANTITATIVE STUDY OF LEUKOCYTE

INFILTRATION IN THE GUT LAMINA PROPRIA OF

GNOTOBIOTIC AND SALMONELLA MONO-CONTAMINATED PIGS

BY

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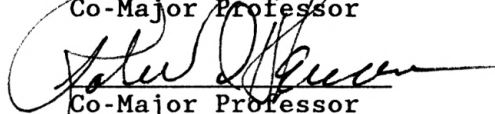Department of Surgery and Medicine

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INTRODUCTION

Swine salmonellosis is an economically important cause of losses to the pig industry worldwide, and is an important zoonosis. The disease, the etiology, and the host response have long been studied; however many questions are still unanswered. Virulent salmonella strains pass all the defense barriers, inducing intestinal and/or septicemic disease. The bacterium is a facultative intracellular parasite and can resist phagocytosis and propagate extracellularly in the host organism. Salmonella choleraesuis var. kunzendorf crosses the intestinal epithelium without induction of cytolysis and elicits an intense inflammatory response in the lamina propria, followed by epithelial destruction and septicemia. The defense against salmonellae involves local, humoral and cellular immunity. Most studies have been conducted on the humoral response, some on the cellular response, but until now, no precise quantitative study on the leukocyte infiltration of the lamina propria has been done.

The intestinal leukocyte composition probably depends on antigenic stimulation, maturity of the tissues, nutrition, stress level, individual variability and other factors. We conducted our study on gnotobiotic piglets in order to achieve a comparable situation between the individuals and a common known starting point in the response to composition in the lamina propria of gnotobiotic piglets and compare newborns to 35 days old piglets. We then studied the rate of appearance of leukocytes in Salmonella choleraesuis-infected pigs at 35 days of age within the first twelve hours of infection. In order to

days of age within the first twelve hours of infection. In order to quantitate the leukocytes infiltrating the lamina propria, we used a recently developed morphometric technique which has not yet been used in veterinary medicine--"the disector." This method allowed an unbiased estimate of number of cells in a unit of volume, which was not possible to obtain utilizing older morphometric methods or other ways of counting tissue cells used in the past. This technique is a revolution in the science of morphometry and together with other new methods, can be applied to many experiments in veterinary medicine, offering possibilities to overcome numerous previously existing obstacles.

Based on earlier studies and available information, we expected to see increasing number of neutrophils as the infection progressed. We also postulated that macrophages and lymphocytes would increase in number, although the response of these cells is usually not evident in early phases of infection, and that the lamina propria of newborn piglets would have a smaller leukocyte population than would 35-day-old piglets.

Since globule leukocytes have been previously observed in pig gut and in intestine of other animals in relation to different pathologic states, we also enumerated these cells in this experiment.

REVIEW OF LITERATURE

IMMUNITY AND LEUKOCYTE INFILTRATION OF THE GUT

The gastrointestinal tract, a large surface area of the body system, is continuously exposed to foreign antigens. The gastrointestinal defense against foreign noxae is specific (humoral and cell mediated immunity) and nonspecific (enzymes, pH, peristalsis, mucins, competitive flora, phagocytosis, and others).

Controlled immunity is important for the selection and the recognition of pathogens and non-pathogens (1). The immune response is regulated in part by genetics and in part by individual response and interaction among several mediators. Genetics plays an important part in controlling the major histocompatibility complex (cell surface molecules involved in cell mediated immunity) and Ig heavy chain molecules (2). After interaction with different antigens, Ig synthesis is regulated resulting in immunization or tolerance. Leukocytes liberate lymphokines (interleukin 1, interleukin 2, interleukin 3) and interferons, regulation mediators of the immune response (2).

Disorders of immunity, cell injury, liberation of oxygen radicals and mediators of inflammation can each lead to pathologic changes.

The gastrointestinal immune apparatus functions independently, but has also an intimate relationship with the extra-intestinal immunity (systemic and mucosal) (1). The Peyer's patches are a known source of cells participating in the immune response. In these sites,

lymphocytes are stimulated to proliferate and emigrate in response to antigens taken up, processed and transported into the patches by dome epithelium and macrophages. Some antigens penetrate junctional complexes and in neonates, some are taken up by villus pinocytosis. B-cells differentiate and migrate to the mucosa, where they secrete Ig as they become plasma cells. Most B-cells produce IgA or IgM. Many T-cells may also derive from Peyer's patches. They are committed to cell-mediated immunity and cooperation with B-cells in humoral responses (3,4).

The mucosal immunity depends on local as well as on systemic antigenic stimulation and on the nature and the dose of the antigen (5). Intrinsic factors and the physiological individual status also influence the defense of the organism.

The lamina propria is composed of loose connective tissue and contains most of the migrating cells. Besides lymphocytes, other leukocytes residing in this site are macrophages and globule leukocytes. Neutrophils, mast cells and eosinophils may be encountered occasionally (3).

CONSIDERATIONS ABOUT MACROPHAGES AND SOME OTHER LEUKOCYTES

Macrophages

Macrophages originate as promonocytes in the bone marrow, migrate in the blood as monocytes and then emigrate to and reside in different

tissues. Their morphology depends on their habitat and on antigenic stimulation. They have an important task in destroying foreign particles, regulating the immune response, and secreting enzymes and immune and inflammatory mediators. These cells are rich in endoplasmic organelles and rarely exhibit cell division. Their life span is 3-4 months or less if they phagocytize (6).

In the inflammatory response, macrophage populations consist of resident as well as emigrating cells (7). Stimulation of macrophages is influenced by microbial status and by other physiological factors. Observations indicate that macrophages in germ free mice are less activated and developed than in normal mice (8). In normal mice, LPS is a potent macrophage activator which is largely lacking in germ-free mice in which Gram-negative bacteria are not present (4). Some facultative intracellular parasites (i.e. Salmonella, Brucella, Listeria, Corynebacterium, Candida, Toxoplasma and Rhodoccocus spp.) are macrophage stimulators. These organisms can resist macrophage activity by preventing release of lysosomal enzymes into phagosomes (6,9).

Activation of macrophages, and the expression of cellular immunity, is mediated by lymphokines secreted from T-lymphocytes. The activation is manifested by certain morphologic and functional changes, such as increased antimicrobial activity, plasma membrane ruffling, enhanced adherence and phagocytosis, increased numbers of phagolysosomes and endocytic vesicles. Certain stimulations also increase macrophage tumoricidal activity (9).

Stress-related hormones suppress certain immunologic responses, i.e. lymphokine production, Ig secretion and macrophage killing ability (10). Epinephrine and norepinephrine block the activation of macrophages by y-interferon and the production of interleukin 1. Tumoricidal activity is also suppressed by catecholamines and ACTH (10).

Mast Cells

Different populations of mast cells are involved in most inflammatory responses, in immediate hypersensitivity, in the late phase of allergic reactions, in delayed hypersensitivity and in regulation of the immune response. They themselves can be directly cytotoxic or can potentiate cytotoxicity of eosinophils and macrophages. Mast cells secrete substances that modulate numerous cells (i.e. histamine, prostaglandin, leukotrienes). Allergic reactions, parasitoses, inflammatory bowel diseases and mastocytosis are some of the diseases where mastocytes play a major role (2).

Globule Leukocytes

Globule leukocytes appear to be large migrating cells of mesenchymal origin characterized by large eosinophilic cytoplasmic granules. They are found in the intestine and in other organs, located mostly within the epithelium, representing an independent cell

population (11). These cells were observed in the gastrointestinal
tract (GIT), biliary system, respiratory apparatus, urinary tract and
uterus of different species, including man, other mammals, and birds
(12).

The origin of globule leukocytes and mast cells is thymus
independent but their proliferation is T-cell dependent. It is not
clear whether they derive from mast cells (13). Some authors have
suggested that globule leukocytes are partially degranulated end-stage
mucosal mast cells (11,14).

Some observations indicated that mast cells and globule leukocytes
are responsible for expulsion of many GIT parasites in various animal
species. (26). In lambs, duodenal globule leukocytes are associated
with resistance to Trichostrongylus colubriformis (16). The number of
globule leukocytes is positively related to the rate of elimination of
Trichinella spiralis from the intestine (15). In parasitized sheep,
Igs were found on the surface and in the granules of globule leukocytes
(14).

Neutrophils

Neutrophils are phagocytic cells representing the first line of
defense against invading microorganisms and particles. They rapidly
migrate to inflammatory sites following chemotactic stimulation. They
exhibit intense phagocytosis and can kill and digest the phagocytized
particles. They can also degranulate, liberating enzymes and other

substances that promote destruction of noxae and enhance inflammatory changes (oxygen radicals, lysosomal enzymes). They are generally the major component of the cellular exudate in acute inflammations.

In the normal intestine, neutrophils are seen occasionally but during some inflammatory processes they can be numerous, infiltrating the lamina propria, the epithelium and the intestinal lumen. The transepithelial migration occurs through the separated apical tight junctions between epithelial cells during inflammation. In the lumen they can phagocytize bacteria and liberate lysosomal enzymes and iron-binding proteins (3).

INFILTRATIVE DISEASES

In many pathologic conditions, the intestinal mucosa is infiltrated with leukocytes, with or without prevalence of one type, depending on the mechanisms involved in the disease. The infiltration may consist of neutrophils, plasma cell, lymphocytes, epithelioid cells, eosinophils, histiocytes and neoplastic leukocytes. In addition to the damage caused by the etiologic agent, the infiltrate itself may damage and alter the function of the intestine. Some of the possible mechanisms include physical impairment of diffusion by occupying space (decreasing perfusion, absorption and elimination of substances), ischemia leading to necrosis, destruction of functional tissue and architecture, liberation of toxins and humoral inflammatory mediators and obstruction of lymph flow (3). Once the inflammation cascade is

initiated, chronic or acute changes may occur, impairing the intestinal function.

PORCINE SALMONELLOSIS

Porcine salmonellosis is a major economic disease. It is manifested mostly as enteric and septicemic disease. In some cases meningitis, pneumonia, encephalitis, rectal stricture and caseous lymphadenitis may be observed (17). Salmonella choleraesuis and S. typhimurium are the most frequently isolated serotypes (18). S. choleraesius was recovered more often from clinically sick as well as healthy pigs and was associated with fatal septicemia (17, 18). It was also isolated in several cases of human food poisoning (19).

Etiology

Salmonella choleraesuis is the etiologic agent of swine paratyphoid (3). It is a host-adapted serotype. The bacteria is a Gram-negative, non-sporing, motile rod, 1-3 x 0.5 -0.7 μm, aerobic and facultative anaerobic (19), considerably resistant in the natural environment.

Epidemiology

Swine paratyphoid is a worldwide disease. The mortality in clinically sick pigs can be high but herd morbidity generally is low

(20) [In one study during 1981-1982, morbidity was 16% and mortality 4% (21)]. The transmission occurs mostly by ingestion of material contaminated with feces of infected animals. Wild animals, rodents, and contaminated feed can also transmit the disease (18). Stress can induce shedding by carriers (20), leading to an outbreak of salmonellosis in groups of animals. Environmental factors such as hygiene, nutrition, population density and stressful conditions play an important epidemiologic role in the prevalence and severity of the disease (19).

Clinical Disease and Pathology

Unlike most other salmonelloses in pigs, S. choleraesuis frequently becomes septicemic and rapidly lethal (21). The septicemia is more characteristic in young pigs, whereas chronic enterocolitis is mostly seen in older ones (22). Many infections are asymptomatic (18) and the mild subclinical disease causes reduced weight gain (21). Acute septicemic salmonellosis near weaning age often occurs as groups of pigs are found dead, while sometimes dull, febrile, weak, and moribund subjects are detected (20).

Necropsied pigs affected by acute salmonellosis have ventral skin discoloration. Petechiae and ecchymoses may also be seen. Internally, splenomegaly, generalized serohemorragic lymphadenitis and icterus are characteristic. Microhemorrages are observed on mucous-membranes, serosae and kidneys. The digestive tract is affected with catarrhal

gastroenteritis. Microscopic lesions consist in disseminated focal necrosis and vascular hyaline thrombosis.

The differential diagnosis includes hog cholera, erysipelas, edema disease, coliform enteritis, mulberry heart disease and streptococcosis (20).

The chronic forms are characterized by prolonged fetid diarrhea with fibrin and/or shreds of mucosa. Pigs are stunted and dehydrated. Hematologic findings consist of slight anemia and leukocytosis with a left shift. On necropsy, ulcerative ileo-colitis and lumenal fluid accumulation are seen. The spleen and mesenteric lymph nodes are hyperplastic. Cranial, middle and anteroventral caudal lung lobes show consolidation and caseous nodules. Other findings consist of lymphadenitis and histiocytic granulomas in renal cortex, meninges and liver.

The differential diagnosis includes swine dysentery, hog cholera and ascariasis (20).

Pathogenesis

S. choleraesuis var. kunzendorf crosses the intestinal epithelium without induction of cytolysis and causes an intense inflammatory response in the lamina propria, resulting in epithelial destruction and later, septicemia. The infection develops in four steps:

1.   Proliferation of salmonellae in the intestinal lumen.

2.   Mucosal invasion.

3.   Stimulation of fluid secretion.

4.   Systemic invasion (17).

The mucosal inflammatory reaction is probably important to the pathogenesis of intestinal fluid secretion. It is possible that prostaglandins are responsible for the secretion, as it is suppressed by indomethacin (22). The local cell damage may be caused by salmonella cytotoxin which inhibits protein synthesis (23). It is also possible that the mucosal tissue is damaged by reactive oxygen radicals released from phagocytes.

Host-Parasite Interaction

The severity of the disease is a function of the host resistance and the virulence of the salmonella. The pathogenicity of virulent salmonella depends largely on its resistance to phagocytosis and its ability to propagate extracellularly (24).

Smooth strains of S. choleraesuis var. kunzendorf are generally refractory to the porcine antibody-complement system (21). It is important to note that the virulence influences the intracellular multiplication and not the rate of ingestion by phagocytes. Rough strains of salmonella lack sugars in the cell wall and are more sensitive to ingestion and to intracellular killing than smooth strains.

The possession of a complete core is important for resisting ingestion and for intracellular survival. The presence of O-specific side chains further contributes to resistance (25,26).

Potentiation of phagocytes and the response of T- and B-lymphocytes are important determinants of host resistance (27). Ig promotes salmonella phagocytosis, thus killed vaccines reduce mortality in otherwise fatal infections. Attenuated live vaccines are more effective as they induce delayed-type hypersensitivity, which inhibits proliferation of challenge inocula of bacteria in the mononuclear phagocyte system (24). Phagocytosis of extracellular bacteria is enhanced by immune serum (opsonization). In addition, cytophilic antibodies on macrophage surface increase clumping and phagocytosis (24).

Lymphocytes from gut-associated lymphoid tissue as well as from peripheral lymphoid organs possess natural and Ig-dependent antibacterial activity (28). In the early phase of the infection, phagocytosis and killing of salmonella by neutrophils play a significant role in the development of the disease. Neutrophils are dominant in the early response and they show more bactericidal activity than macrophages in the non-activated stage (25).

After oral inoculation of S. choleraesuis, neutrophil adherence was significantly increased (in vitro) at 6, 30, and 48 hours post infection. Migration of PMN to the inflammation site involves margination and adherence to vascular endothelium, diapedesis through the capillary wall and migration along increasing chemotactic

gradients. This migration might be related to the effect of endotoxin, which is a potent chemotactic substance (29).

Previous experiments

In a study by Reed et al. (17) four-week old pigs, orally inoculated with 1.2×10^{10} S. choleraesuis, had initial septicemia followed by ulcerative colitis, interstitial pneumonia, and multifocal hepatic necrosis. When inoculation was made into ileal loops (10^9), the bacteria were found within the enterocytes (free in cytoplasm and membrane bound) without detectable cytotoxic effects. Two hours after inoculation, penetrating salmonella were isolated from mesenteric lymph nodes.

Gross lesions of acute enterocolitis were first detected after twenty-four hours consisting of edema, hyperemia, mucus secretion, shallow erosions, fibrin formation, and petechiae.

Microscopic lesions were first seen after 16 hours: the superficial gastric mucosa was congested and infiltrated by neutrophils and macrophages. The lamina propria contained areas of multifocal necrosis, neutrophilic infiltration and congestion of small blood vessels, which were occluded by fibrin and neutrophils. Villi were atrophied and fused; apical enterocytes were eroded.

At twenty-four hours post-infection, more severe lesions were detected, consisting of superficial mucosal erosions and of heavy neutrophilic infiltration of the lamina propria.

Inoculated loops accumulated clear watery fluid and strands of mucus, while mesenteric lymph nodes were moist and congested at 12 hours post-infection.

The infected colonic loops were similar to the control loops. Microscopically, ileal villi were shortened with transmigrating neutrophils between the epithelial cells, detected 4 hours post-infection. No intracellular bacteria were seen after 2, 4 and 6 hours, and only a few bacteria were trapped in mucus over the microvillus border. Eight hours after inoculation, more bacteria were seen over the microvilli and few intracellular bacteria were detected in macrophages of the lamina propria and in the villus absorptive cells. Many neutrophils and eosinophilic cells were present (17).

In other studies, no significant changes were detected 8-16 hours post infection, but mucosal desquamation, erosions and edema were seen at 32 hours post-infection (19).

When <u>S. choleraesius</u> were introduced to the respiratory tract, the bacteria were phagocytized by lung macrophages and neutrophils. Some bacteria survived and multiplied within the phagocytes, and were carried in that way to lymphatics and capillaries, causing the development of bacteremia (30).

STEREOLOGICAL PRINCIPLES AND MORPHOMETRY

Introduction

The three dimensional composition of objects has always been of interest in different branches of science. A solid opaque volume can be studied by obtaining sections of it and applying stereological

principles which <u>explain mathematical relationships</u> between three-dimensional structures and their representation in two-dimensional samples (31).

Morphometry is a powerful instrument which allows measurements of structures using stereological principles. It can furnish estimations of volume (V), length (L), surface area (S), number of items (N), volume density or volume fraction (Vv), numerical density (Nv) and other parameters (31).

Stereology has been a formal branch of science for approximately twenty years (32). The history of stereology began in 1777, when Jean Louis Leclerc, Conte de Buffon, found the probability with which a randomly tossed needle of length "l" intersects a grid of parallel lines of distance "d" (33). Since that time, different problems have been solved, leading to modern stereology. However, there was no interaction between stereology of materials and biological sciences and geometric probability until 1960. Only since then has an effort been made to develop a unified theory and to improve methods (33).

In biological sciences, morphometry is a relatively new area involving quantitative studies of tissue components and is becoming more commonly used in veterinary medical research.

Area and Volume

In 1847, Dellese found that, on average, the fractional area of a component lying in a random transverse section of a solid is directly

proportional to the fractional volume of that component in a solid body: Aa - Vv, so that volume fraction can be estimated from area fraction (31).

In practice, surface area is estimated by counting intersections of profiles with test lines of a known length. Volume fraction (Vv) of particles in a unit of volume can be estimated by counting points (P) within the reference area of tissue sections (31).

Numerical Density

Estimation of particle number has been the most difficult stereological problem (34). Number of structures per unit of volume is very useful for description of tissue and cell structure. Unfortunately, there is not a simple relationship between number of structures per volume unit (Nv) and number of profiles per unit area (Na) (31).

The problem of particle number and size was first treated by S. D. Wicksell in 1925-1926. He derived a relationship between size distribution of spherical and ellipsoidal particles and their profiles on a section. Similar methods were derived by Scheil, Schwarzman and Krumbein in 1931-1935 (33).

Derivations of the basic formula $Nv = n/A\bar{D}$ (where, n = number of particles, A = area, $\bar{D}$ = mean particle diameter) were reported by Henning, Elias, Weibel and Underwood (32). Some methods like those of Dehoff and Rhiness, and Weibel and Gomez (31) were proposed. All these

methods depended on a number of assumptions involving size, shape, distribution and orientation of the particles (31-33).

The New Methods

As mentioned previously, the "old" methods were biased and depended on assumptions such as particle shape and size. It is true that under the stringent assumption that all particles have the same known and simple shape, it is possible to correct the biased sample of particles in sections and to estimate their number and size (35), but in reality this situation is rare. The only possibility is to correct the biased sample after complete three-dimensional reconstruction of the particles or complete mensuration of their individual size without reconstruction. Both procedures require exhaustive serial sectioning of particles (35).

Further biases were due to Holmes effect (the positive thickness of transparent sections) and due to the phenomenon of lost caps (disappearance of small particle fragments in the section) (34).

Recently, new stereological principles and morphometric methods became available, allowing unbiased estimation of structural quantities which could not be estimated at all before, or allowing an easier estimation requiring less assumptions. They are characterized by specific sampling regimes:

1. From vertical sections, one can estimate the surface area of an arbitrarily oriented surface.

2. Mean size of arbitrarily shaped particles in a random section can be estimated by length of point sampled intercepts to the third power (10^3).

3. The disector allows estimation of arbitrary particle number, given the section thickness and the distance between sections.

4. The fractionator allows estimation of particle number without measuring the section thickness or separation and is independent of shrinkage or swelling (36).

5. The selector allows estimation of particle using a disector of unknown thickness (37).

THE DISECTOR (Fig 1)

The disector was the breakthrough. It is a three-dimensional counting rule for obtaining unbiased, direct estimation of arbitrary particle number in a specimen. Together with other stereological methods, it can give estimates of mean particle size, variance of particle volume and individual particle volume distribution (34). On sections, a particle will appear with a chance proportional to its size (35). Test lines hit particles depending on their surface and test points, again, depending on their volume (34). The test frames select particles with a chance proportional to their height (34). The estimation of a particle number is unbiased by size or shape and is independent of section thickness, lost caps and orientation distribution of particles (36).

The disector is composed of an integral test system (a planar of P points) and of a parallel plane, a known distance "h" apart. In practice it consists of two parallel sections (the lower is called the "reference section" and the upper the "look-up section") separated by a known distance "h" (35).

Counting Rule

Q^- particles whose transects are completely or partially inside the counting frame of the two dimensional integral test system are counted, provided that they are not in any way intersected by the two fully drawn edges (or the exclusion lines of the frame) nor by the parallel plane ("look-up" section). The efficiency can be doubled by using the planes ("reference" and "look-up" section) in turn as a "reference section."

The position of the disector must be uniform (systematic random) over all possible positions, where the sampling frame hits the specimen (34). The frame should be in a fixed position on each field. This necessitates the uniform random position of the set of fields, because the estimate is unbiased only if the frames have a uniform random position (36). All disectors containing a visible portion of the reference space (ref.) must be used. It is sufficient to use a subset of "n" disectors taken randomly or systematically. If the particle distribution is inhomogeneous, more positions of the disector are needed (the estimator is not biased by inhomogeneity).

The estimator of the total number N of particles in the reference space of volume V(ref) is:

$$N = \Sigma Q^{-} / \Sigma P \cdot (v(ref) \cdot p / h \cdot a).$$

where:

N = number of particles/unit of volume.

ΣQ^{-} = number of particles selected by the counting rule.

ΣP = number of points hitting the reference space.

V(ref) = volume of specimen measured by the disectors.

p = number of points/sampling frame.

h = disector height.

a = area of sampling frame.

The summation is over all sampled disectors within the reference space. Height "h" should be smaller than the minor diameter of the smallest particles (35). It need not be constant and $\bar{h}$ can be used (36).

Nothing is assumed about the particles except fulfilling the general requirement: i.e., the possibility of identifying the item from the set of profiles produced by one or more sections through it. The orientation of the section planes can be chosen. There is still a problem of shrinkage and swelling but assuming that dimensional changes are uniform and identical, it can be ignored (34).

Holmes effect can be disregarded if section thickness/mean diameter of particles is less than 1.

Statistical errors can be reduced by increasing sample sizes and the distribution of samples over the tissue, using a systematic sampling lattice of points (38).

An interesting note is that three of the principles for estimation of number without shape assumptions were described in 1932 by William R. Thompson, but were overlooked completely. He described the equation of the disector and used the associated point method, which is equivalent to the disector if complete sections are used. He also used the indirect counting method with comparison to the direct one. If those principles had been followed earlier, the development of morphometry in the following half-century could have been different (34).

The Fractionator

This method allows estimation of a total number of items in an object. It is independent of any dimensional measurement and of tissue deformation during processing. Section planes need not be in a random position and orientation; they need not be parallel or of the same thickness (34) (36).

The technique includes systematic random sampling of blocks with known probability 1/p. The blocks are sectioned exhaustively (without intersection of section planes). The counting rule is the same as described in the disector technique and the estimated number of cells is $N = p \cdot \Sigma Q$ (34).

The fractionator is statistically less efficient than the disector when particle distribution is more homogeneous. It requires obtaining many sections and a precise sampling procedure.

For further details, see (34).

The Selector

This method selects particles with identical probability using a disector of unknown thickness and is independent of particle size, shape and orientation. The volume of each particle is estimated by point sampled intercepts, and arithmetic means of particle volume is the estimator of the population mean volume $\overline{VN}$. The volume fraction of the particle aggregate in a reference space Vv = V(par)/V(ref) is estimated by point counting on a random section. The numerical density of particles is estimated (Nv = $Vv/\overline{VN}$) and is multiplied by the reference volume to get an estimation of the total number of the particles in the ref. Volume:

N(par) = V(ref) • $Vv/\overline{VN}$

For further details, see (37).

Indirect Estimation of Particle Number

Cruz-Orive (1986) showed how to transfer size estimators to indirect number estimation. However, the indirect number estimate is less efficient and presents many problems and biases (34).

Vertical Section (Fig. 2)

Vertical sections are plane sections, longitudinal to a fixed but arbitrary axial direction. They can be generated by placing the object on a table and taking sections perpendicularly to the plane of the table.

No assumptions are necessary about the shape or the orientation distribution of the structure, but the requirements are:

1. Directional axis must be identified (intrinsic of the object or generated by the experimenter).

2. All vertical sections must be parallel to the vertical (normal to the horizontal) and the vertical direction must be identified in each section.

3. Relative to the common horizontal plane, the vertical section must have random position and random (isotropic) orientation.

4. On the vertical sections, a test line is given a weight proportional to sine ϕ (where ϕ is the angle between the test line and the vertical direction).

Reasons for using vertical sections can be:

1. Interest in a particular orientation of the section.

2. Need to take particular sections to ensure proper identification of profiles.

3. Practical constraints associated with the physical cutting process.

4. Need to estimate surface area of an arbitrarily oriented surface (39).

Some definitions

<u>Holmes Effect</u>

Inability to distinguish boundaries between two overlapping particles within the section or a small area on one surface from a larger area on the other in a finite thickness section as a consequence of the positive thickness of transparent sections.

<u>Lost Caps</u>

In thin sections, small fractions of particles can be lost and fall off the section surface so that they are not observed and not considered in the analysis.

<u>Isotropic Organ</u>

A homogenous structure for which a random cut through any region will give the same kind of image.

<u>Anisotropic Organ</u>

Structure with a preferred orientation of components (tubule, muscular fibers). The section orientation influences the estimation of volumetric density by lineal or areal measurements but not by point counting.
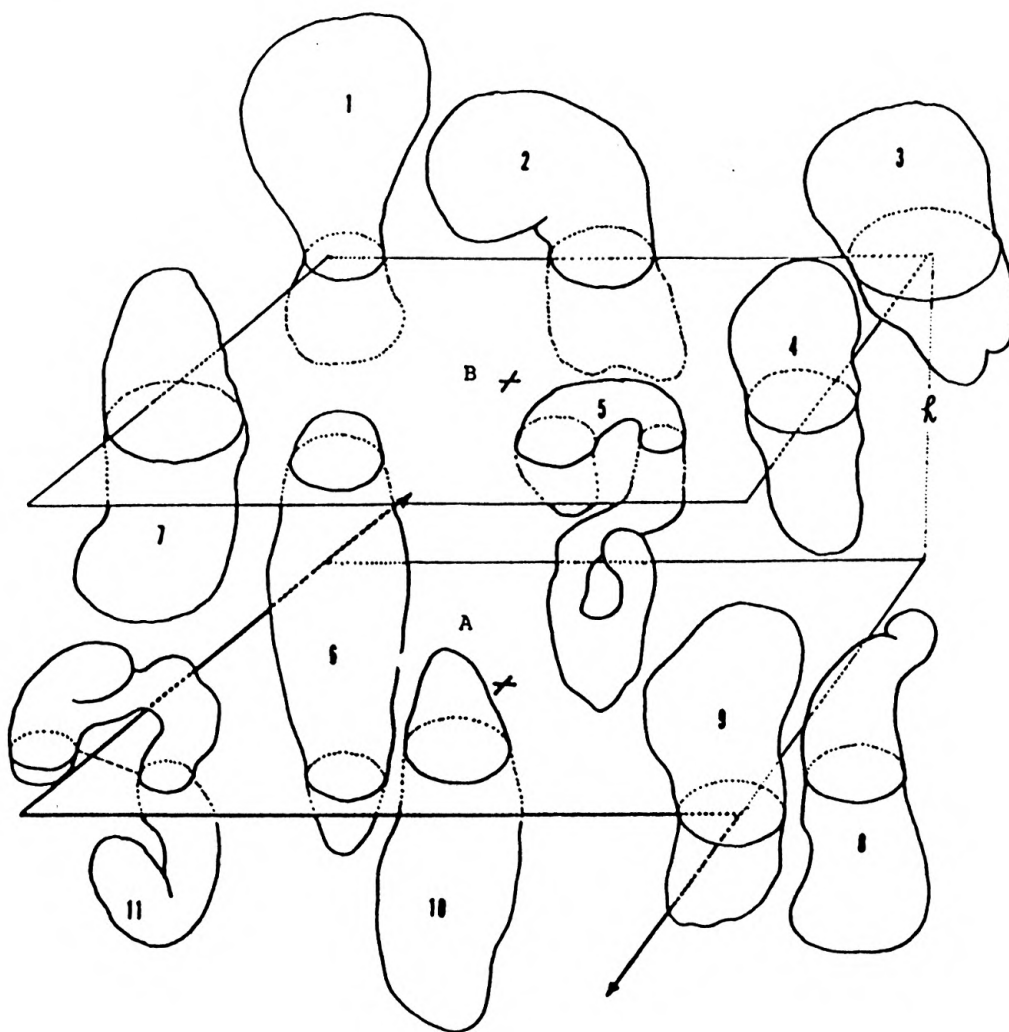
Figure 1. The Disector

a.   Disector composed of two parallel planes (A, B) separated by a
      distance 'h'. The unbiased counting frame of a known area "a" and
      p = 1 point is shown on both sections, since the disector is used
      in both ways.
           One counts all transects which are completely or partially
      inside the frame except for those intersected by the fully drawn
      exclusion lines (heavy lines with arrows) and/or by the parallel
      plane.

Figure 1. (Continued)

b. The two sections are shown as they appear under the microscope,
    each equipped with the selecting counting frame of area "a" and
    p = 1 point.
        Cells are counted if their transects are completely or
    partially inside the frame provided they don't intersect the fully
    drawn exclusion line. All selected cells are looked for in both
    sections and are counted if they appear on one section only:
    1. Cell number 10 is counted in section A since it respects the
        counting route.
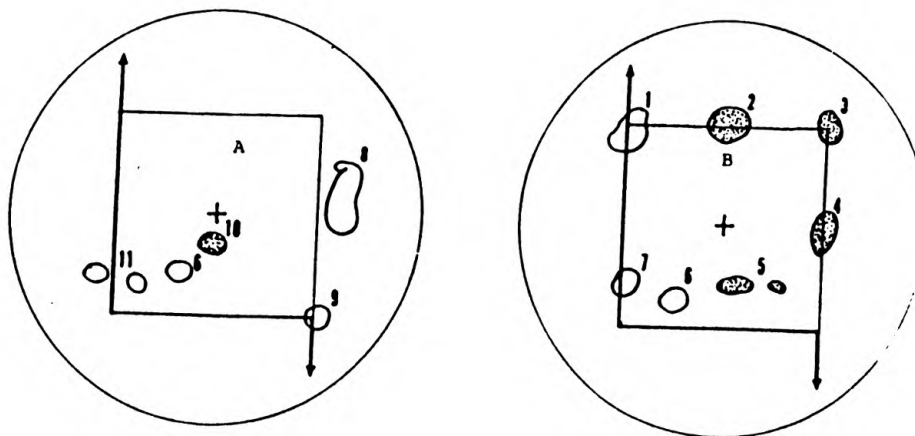   2. On section B, the counted cells respecting the same
        conditions are 2, 3, 4, and 5.
    3. Cells number 6, 8, 9, and 11 are not counted in section A:
        a. Cell number 6 transect is present on both sections.
        b. Cell number 8 transect is outside the counting frame and
            is not counted.
        c. Cell number 11 is not counted because the fully drawn
            exclusion line intersects the dashed line connecting the
            two profiles in its transect.
        d. Cell number 9 is not counted since its transect
            intersects the exclusion line.
    4. Cells number 1, 6, and 7 are not counted in section B:
        a. Cells number 1 and 6 transects intersect the fully drawn
            exclusion line.
        b. Cell number 6 transect appears in both sections.
In conclusion $\Sigma Q^- = 5$ is the total number of counted cells on
both sections.
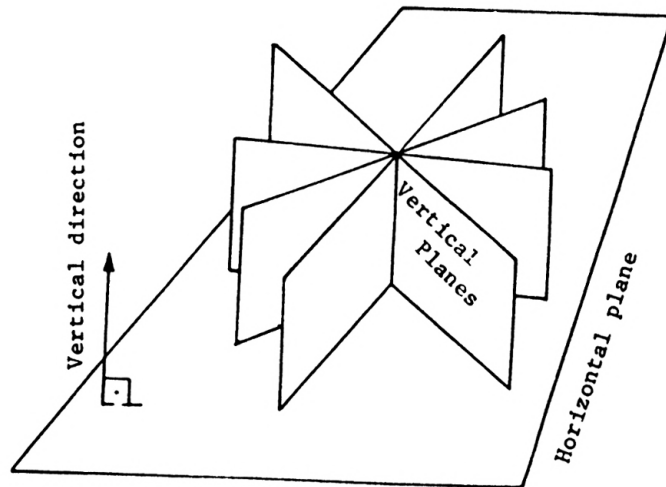
Figure 2. The Vertical Section.

a.   Different plane-sections perpendicular to a given horizontal plane
     and parallel to an arbitrary direction in three-dimensional space
     (the "vertical direction"), generate vertical planes.
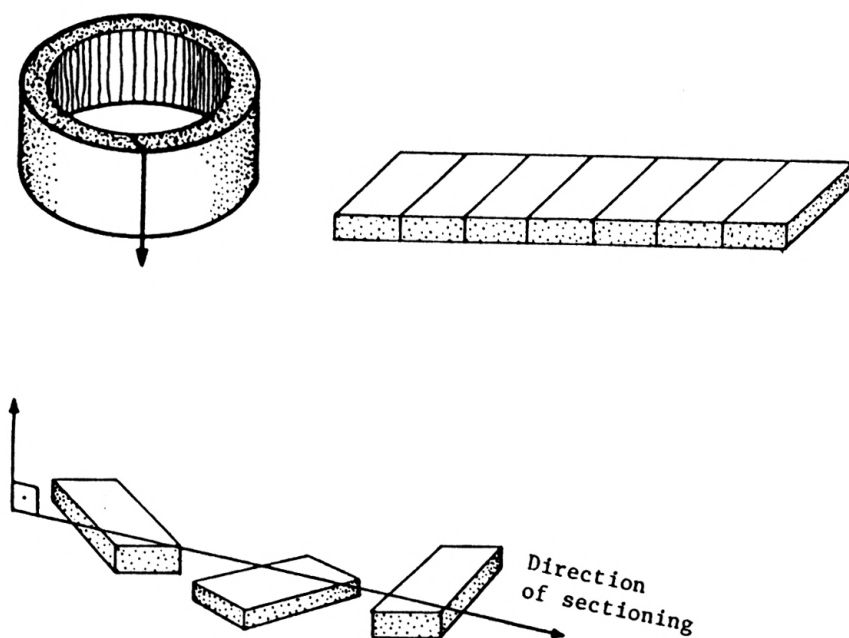
Figure 2. (Continued)

b. Vertical sections of profiles of villi inside a tubular organ
(intestine) can be obtained by a longitudinal cut of the organ,
opening it flat on the table, cutting it into small pieces, of
which "n" are randomly selected and randomly oriented, and
sectioning them perpendicularly to the plane of the external
tubular surface.

MATERIALS AND METHODS (Fig. 3)

ANIMALS

Twenty piglets were derived by cesarotomy at the Department of Veterinary Science, University of Nebraska-Lincoln and maintained in germ free isolators. All members of each experimental group were maintained in germ-free isolators before and during the course of the experiment. The piglets were fed with a sterile balanced liquid ration twice a day and sterile rectal swabs were cultured weekly to ensure accidental bacterial contamination had not occurred. Only those piglets that were successfully maintained in a germfree state were used in the experiments.

EXPERIMENTAL DESIGN

Twenty germ-free piglets were assigned at random to five groups of four animals each as follows:

Group 1--0 day controls

Group 2--35-day old controls

Group 3--35-day old piglets infected for 2 hours with <u>Salmonella</u> <u>choleraesuis</u>

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Group 4--35-day old piglets infected for 6 hours with <u>Salmonella</u> <u>choleraesuis</u>.

Group 5--35-day old piglets infected for 12 hours with <u>Salmonella</u> <u>choleraesuis</u> (Fig. 3a).

TREATMENT

Piglets in Groups 1 and 2 were removed from the isolators, killed by barbiturate overdose at 0 and 35 days of age respectively and tissue samples taken from the jejunum, ileum and spiral colon as described below for the experimental animals. A bacterial culture was done on each intestinal portion prior to fixing the tissue and a complete necropsy performed on each piglet. Piglets in groups 3, 4, and 5 were anesthetized at 35 days of age with xylazine (2.2 mg/kg) and ketamine HCl (2.2 mg/kg), after pre-treatment with atropine sulfate (0.06 mg/Kg). When light anaesthesia was attained, the pigs were placed in dorsal recumbency and a laparotomy performed via midline incision from umbilicus to pubis. Two 5.0 cm loops, each separated by a 5.0 cm intestinal segment, were ligated in the jejunum, the ileum and the spiral colon. One loop in each gut segment was inoculated with 10^9 CFU of <u>Salmonella choleraesuis</u> var. kunzendorf in 1.0 ml sterile PBS. The second loop of each segment was injected with 1.0 ml of sterile PBS to serve as control. After inoculation, the abdomen was closed with #1 Vicryl suture in two layers of simple continuous pattern. The animals

were left in the isolators and monitored closely until the end of the experiment.

TISSUE COLLECTION

Piglets in groups 3, 4 and 5 were removed from the isolators at 2, 6, and 12 hours post-infection, respectively, transported to the necropsy room in sterile boxes, killed with a barbiturate overdose, and examined immediately. The protocol described for the 0 and 35-day controls (Groups 1 and 2, respectively) were followed exactly on the experimental animals. Loops were refilled in situ with 10% buffered formalin and immersion-fixed for 72 hours. Following fixation, the tissue blocks were dehydrated in an ascending series of isopropyl alcohol (70%-absolute), cleared in two changes of toluol and embedded in paraffin following standard protocols. Sections were cut at 6.0 μm, mounted on glass slides, and stained with hematoxylin and eosin and examined by light microscopy to assess acceptability of processing, morphologic integrity of tissues and presence or absence of pathologic changes. In order to provide thin sections for stereological analysis of the tissues, they were cut from the paraffin blocks, placed in a 60° C oven for 45 minutes to melt remaining paraffin, processed for one change of 30 minutes each in warm and cold toluol and then placed in absolute ethanol for an additional 30 minutes. Prior to processing for plastic embedding, each tissue segment was cut into smaller pieces (each approximately 1.5 x 1.5 mm). From these, 2 pieces were randomly

selected from each intestinal segment and re-embedded in glycol

methacrylate (PB-4: Polysciences Co.) using BEEM flat embedding molds.

The recommended protocol for plastic embedding was modified by doubling

the usual quantity of the polymerizing medium (Sol. B) to provide

harder tissue blocks. Tissues were randomly oriented in the plastic

blocks but were always placed such that sections would be taken

according to the vertical section technique (see Review of Literature).

This was done in order to solve problems of villus orientation in the

sections and to obtain consistency in the tested areas of lamina

propria. In addition, sections done in this way can be used for

further stereological investigations such as surface area estimation

(Fig. 3d).

Following the protocol described by Gunderson for the "Disector"

technique of morphometric analysis (see Review of Literature), a series

of consecutive sections each 0.27 μm thick were cut on a Porter-Blum

MT-2 Ultramicrotome, mounted on glass slides and stained with

hematoxylin and eosin. These two randomly selected consecutive

sections served as each disector plane (Fig. 3b, c).

STEREOLOGICAL ANALYSIS

Among the new stereological methods used for estimation of

particle number in a unit of volume, we chose the disector. The choice

was made based on the simplicity of the technique and application to

our needs, cost effectiveness and the possibility to use our existing equipment.

Our disector was composed of a pair of consecutive sections to create the "reference" and the "look up" plane. The distance between the planes of the disector is the actual thickness of the section i.e., 0.27 μm. This ensures that "h" is smaller then the smallest leukocyte nucleus minor diameter and avoids the Holmes effect (see Review of Literature).

Two eye-piece reticles mounted on two Nikon Labophot microscopes, set side by side, were used to obtain the integral test system of the disector. The counting frame consisted of a grid area containing 4 points, systematically positioned until 48 points were counted. Each pair of sections composing a disector was mounted on a pair of microscopes and oriented in the same direction by rotating the stage. The counting frame was positioned at the same point of the lamina propria of each section, covering the same area. Using a 1000 X magnification, the different leukocyte nuclei transects were identified and counted according to the counting rule (see review of literature). Each section was used in turn as a "reference" and a "look-up" plane in order to double the efficiency of the disector. Practically, a leukocyte was counted if its nucleus transect appeared on one but not on the other field within the counting frame and cells common to both fields were not counted. The summation was done for each type of leukocyte (macrophages, lymphocytes, neutrophils and globule leukocytes) counted comparing section "A" to "B" and comparing section

"B" to "A" (Fig. 3e). Since the disector was used in both ways, 96 points were considered for the determination of the reference volume (48 + 48) of each sample.

STATISTICAL ANALYSIS

The sampled data for the statistical analysis included:

1. for the control groups: three intestinal segments, one loop of each segment, two blocks of each loop, and one "two-way disector" of each block.

2. for the infected groups: three intestinal segments, two loops of each segment, two blocks of each loop, and one "two-way disector" of each block.

Figure 3. The experimental design and the technique for quantitative
study of leukocytes in lamina propria of pig gut

a. 5 groups of pigs, 4 pigs in each group: groups 1 and 2 served as
controls and groups 3, 4, and 5 were infected with Salmonella
choleresuis.

Group	1	2	3	4	5

Control	Control	Infected	Infected	Infected
0 day	35 days	2 hours	6 hours	12 hours
		35 days	35 days	35 days

Figure 3. (Continued)

b.   The sampling from each control pig included:
      (1) three intestinal segments, 1 loop of each
      (2) 2 blocks of each loop
      (3) 1 "double disector" of each block

PIG 1

| Intestinal Segment | Jejunum LOOP 1 | Ileum LOOP 1 | Spiral Colon LOOP 1 |

BLK 1   BLK 2   BLK 1   BLK 2   BLK 1   BLK 2

Dis1   Dis2   Dis1   Dis2   Dis1   Dis2   Dis1   Dis2   Dis1   Dis2   Dis1   Dis2

Figure 3 (Continued)

    c. The sampling from each infected pig included:
        (1) 3 intestinal segments, 2 loops of each
        (2) 2 blocks of each loop
        (3) 1 "double disector" of each block

PIG 1

| Jejunum | Ileum | Spiral Colon |

*LOOP1   LOOP2   LOOP1   LOOP2   LOOP1   LOOP2

BLK 1   BLK 2   BLK 1   BLK 2   BLK 1   BLK 2

Dis1   Dis2   Dis1   Dis2   Dis1   Dis2   Dis1   Dis2   Dis1   Dis2   Dis1   Dis2

BLK1   BLK2   BLK1   BLK2   BLK1   BLK2

Dis1   Dis2   Dis1   Dis1   Dis1   Dis2   Dis1   Dis1   Dis1   Dis1   Dis1   Dis2
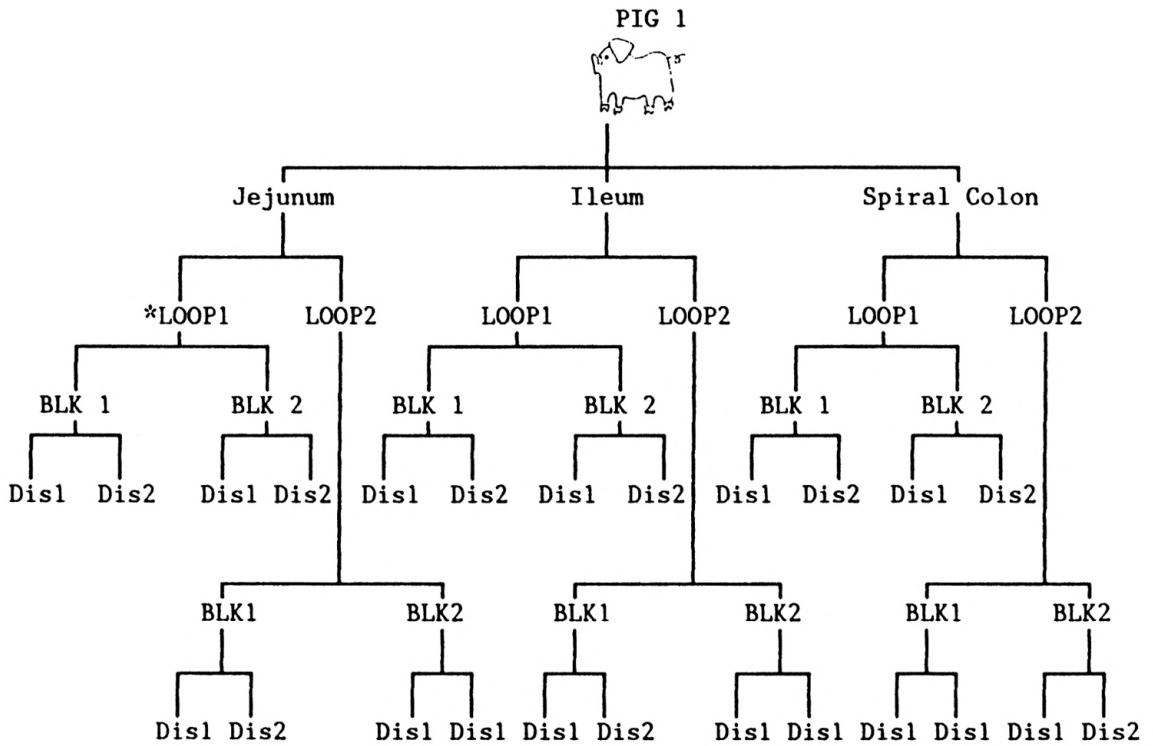
*loop 1 = infected
 loop 2 = control

Figure 3. (Continued)

    d. A selecting frame containing 4 points is imposed on two disector
       sections. In section A, three cells are present but only cell
       number 3 (arrow) is included in the counting frame and does not
       appear in section B. In section B, cells number 1 and 2 appear
       also in section A; two more cells are present (4 and 5) of which
       one (5) intersects the exclusion line and is not counted, and one
       (4) is included in the counting frame area and therefore is
       counted (arrow). A total of 2 cells (3 and 4) are counted using
       the disector in both ways.

A
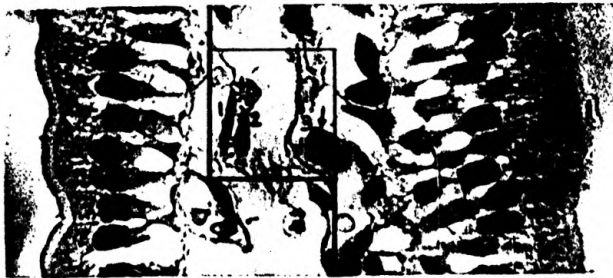
B

Figure 3 (Continued)

e. Profile of a villus obtained by vertical section

RESULTS

QUALITATIVE OBSERVATIONS

In addition to the quantitative analysis, we examined the pathological changes in the intestine of the experimental piglets, in order to obtain a general picture of the effects of early salmonella infection.

Macroscopic Changes

On necropsy, the only evident change was an increased volume of fluid accumulated in the intestinal lumen of infected piglets.

Microscopic Changes

Under low (100 X) and high (1000 X) power magnifications, we observed the changes in the different layers and lumen of the intestine:

GROUP No. 1--newborn piglets

The epithelium showed no alterations and contained very few intraepithelial lymphocytes. More globule leukocytes were present in subvillus than in villus lamina propria. The submucosa was normal and contained few leukocytes.

GROUP No. 2--35-day old piglets

No remarkable changes were seen in the epithelium and in the lamina propria. Some leukocytes were seen in the jejunal and ileal submucosa and the colonic submucosa contained more leukocytes.

GROUP No. 3--35-day old piglets, 2 hrs post-infection

Infected loops contained rod-shaped bacteria and some RBC in the gut lumen. Epithelium of the jejunum contained some migrating lymphocytes and globule leukocytes, but leukocytes were very rare in the ileal and colonic epithelium. A considerable number of globule leukocytes were present in the sub-villus lamina propria but those cells were rare in the colonic segment.

Leukocytes were rare in the submucosa, which was edematous in some places.

The noninfected loops showed no appreciable difference in the leukocyte composition. The intestinal lumen contained some erythrocytes but no bacteria.

GROUP No. 4--35-day old piglets, 6 hrs post-infection

The intestinal lumen of infected loops contained rod-shaped bacteria, erythrocytes, lymphocytes, neutrophils and mononuclear cells. Epithelial disintegration and fragmentation of villi were encountered occasionally in the jejunum, ileum and colon.

In the lamina propria many globule leukocytes occupied the sub-villus zone and neutrophils were seen especially in the colonic part.

The submucosa contained increasing numbers of leukocytes with prevalence of neutrophil increment in some areas. In the colonic segment, the leukocytes were particularly numerous.

Noninfected loops showed basically similar changes but no bacteria were seen in the gut lumen. Submucosal edema was more pronounced in the infected loops, but not so in the noninfected ones.

GROUP No. 5--35-day old piglets--12 hrs post-infection

Infected loops showed bacteria, erythrocytes, and leukocytes in the gut lumen. Lymphocytes, globule leukocytes and neutrophils were observed among the epithelial cells. In some locations, the epithelium was disintegrated and villi were fragmented. A considerable amount of leukocytes infiltrated the lamina propria and neutrophils were numerous in some areas. More globule leukocytes were seen in the subvillus than in the villus lamina propria.

Edema was evident in the submucosa, where leukocytes and numerous neutrophils were observed (especially in the colonic loops).

Uninfected loops did not differ very much from infected loops, except that bacteria were not found.

QUANTITATIVE ANALYSIS

As described previously, according to the disector method, the estimate of the total number of particles in a reference space is:

$$N = \Sigma Q^- / \Sigma P (V(ref) \cdot p/h \cdot a)$$

and the reference space is:

$$V(ref) = \frac{\Sigma P \cdot h \cdot a}{P} \ (35).$$

Since $V(ref) \cdot p/h \cdot a \cdot \Sigma P = 1$ (35), the total number of cells in the reference space is $N = \Sigma Q^-$.

In our experiment:

$\Sigma P = 96$

$a = 400 \ \mu m^2$

$h = 0.27 \ \mu m$

$p = 4$

So: $V(ref) = \frac{06 \cdot 0.27 \cdot 400}{4} = 2,592 \ \mu m^3$ and all cell counts ΣQ^-

are the estimated number of cells N in the same unit of volume of lamina propria V(ref).

STATISTICAL ANALYSIS

Statistical analysis of multiple comparisons between groups and between loops was set at significant level of $\alpha = 0.05$.

The trend in cell number change over infection hours was also tested in order to evaluate the behavior of the infiltrating leukocytes.

Mean estimated leukocyte numbers at the different levels are summarized in table 1.

Multiple comparison F-test results

Multiple comparison F-test results are summarized in Table 2, indicating that there were significant interactions between groups and intestinal segments for macrophages and globule leukocytes. Thus differences in mean number of macrophages and globule leukocytes were not the same for each intestinal segment and the comparisons of groups were done for each intestinal segment separately. However, for neutrophils and lymphocytes there were no interactions between groups and intestinal segments, indicating that differences in mean number of these cells are the same for each intestinal segment and the comparisons of groups were done over all intestinal segments.

TABLE 1--Leukocyte Group-Intestinal Segment Means

Leuko-cyte Type*	Controls					
	0 Days Old			35 Days old		
	Jejunum	Ileum	Spiral Colon	Jejunum	Ileum	Spiral Colon
M	8.375±1.408	7.750±2.659	8.750±4.097	10.625±3.543	10.250±3.370	12.500±5.925
L	6.000±3.207	5.875±3.091	8.625±3.204	14.375±4.534	11.875±3.441	18.667±3.830
N	0.000±0.000	0.125±0.354	0.000±0.000	0.000±0.000	0.250±0.463	0.333±0.516
G	0.500±0.756	0.875±0.835	0.000±0.000	2.625±1.598	1.375±0.916	0.167±0.408

	35 Days Old--2 hrs Post Infection			35 Days Old--6 hrs Post Infection		
	Jejunum	Ileum	Spiral Colon	Jejunum	Ileum	Spiral Colon
M	6.875±3.344	5.625±2.247	5.438±2.128	8.500±2.633	7.875±2.604	4.813±2.073
L	11.250±3.873	10.625±2.473	13.000±2.781	9.438±3.366	10.188±3.507	11.625±1.857
N	1.000±0.966	1.000±1.033	0.438±0.512	1.375±0.957	1.188±0.834	1.125±1.088
G	2.438±1.548	3.250±1.291	0.063±0.250	2.813±1.559	2.313±1.352	0.188±0.750

	35 Days Old--12 hrs Post Infection		
	Jejunum	Ileum	Spiral Colon
M	7.625±2.156	6.625±2.778	5.250±2.463
L	9.438±2.732	10.688±3.321	11.625±2.156
N	2.000±1.673	2.063±2.380	2.250±2.769
G	2.250±1.291	1.938±0.998	0.125±0.342

* M = Macrophages
L = Lymphocytes
N = Neutrophils
G = Globule Leukocytes

TABLE 2--Multiple Comparison F-Test Results

Difference between:	M	L	N	G
Groups	*a	a	a	a
Pigs in Group	b	c	d	-
Loops	-	-	a	-
Blocks in Loop	-	-	-	-
Intestinal Seg.	-	a	-	a
Interaction Between:				
Groups-Loops	-	-	a	-
Pigs-Blocks	-	-	-	-
Groups-Intest Segs	P = 0.0204	-	-	e
Loops-Intest Segs	-	-	-	-
Grps-Lps-Intest Segs	-	-	-	-

* Significant tests are indicated with their letters:

   a: P = 0.0001, b: P = 0.0016, c: P = 0.0470, d: P = 0.0014

Lymphocyte and Neutrophil Group Mean Comparisons

Mean number of both lymphocytes and neutrophils significantly differed in groups (p-value = 0.0001 for lymphocytes and neutrophils); thus, at least two groups were significantly different.

Fisher's LSD multiple comparison procedure was performed to assess where the differences occurred (Table 3).

TABLE 3--LSD Multiple Comparison Procedure for Lymphocyte
and Neutrophil Means in the Different Groups

Lymphocytes			Neutrophils			
Mean		Group	Mean			Group
14.6364 A		2	2.1042 A			5
11.625	B	3	1.2292 A	B		4
10.5833	B	5	0.824	B	C	3
10.5833	B	4	0.1818		C	2
6.833	C	1	0.0417		C	1

(Means with the same letter are not significantly different, α = 0.05)

a. Lymphocytes:

Group 1 mean was significantly different and smaller than group 2, 3, 4, and 5 means. Group 2 mean was significantly different and higher than group 1, 3, 4, and 5 means. Group 3, 4, and 5 means were not significantly different.

b. Neutrophils:

Group 5 mean was significantly different and higher than group 1, 2, and 3 means. Group 4 mean was significantly different and higehr than group 1 and 2 means. Group 1, 2 and 3; 3 and 4; and 4 and 5 were not significantly different.

Macrophage and Globule Leukocyte Group--

Intestinal Segment Mean Comparisons

Since there were significant interactions between groups and intestinal segments for macrophages and globule leukocytes, the analysis was carried out at each intestinal segment and is summarized in tables 4, 5, and 6.

TABLE 4--LSD Multiple Comparison Procedure for Macrophage
and Globule Leukocyte Group Means in the Jejunum

Macrophages			Globule Leukocytes		
Mean		Group	Mean		Group
6.875 A		3	0.5000 A		1
8.500 A		4	2.6250	B	2
7.625 A		5	2.4375	B	3
8.375 A	B	1	2.8125	B	4
10.625	B	2	2.2500	B	5

(Means with the same letter are not significantly different, $\alpha = 0.05$)

a. Macrophages:

Group 2 mean was significantly different and higher than group 3, 4, and 5 means. Group 1, 3, 4, and 5, and 1 and 2 means were not significantly different.

b. Globule Leukocytes:

Group 1 mean was significantly different and smaller than group 2, 3, 4, and 5 means. Group 2, 3, 4, and 5 means were not significantly different.

TABLE 5--LSD Multiple Comparison Procedure for Macrophage
and Globule Leukocyte Group Means in the Ileum

Macrophages					Globule Leukocytes				
Mean		Group			Mean		Group		
4.8125	A			4	3.2500	A			3
5.2500	A	B		5	2.3125	B			4
8.7500	A	B	C	1	1.9375	B	C		5
5.4375	B			3	1.3750	C	D		2
12.5000		C		2	0.8750	D			1

(Means with the same letter are not significantly different, $\alpha = 0.05$)

a.   Macrophages:

Group 4 mean was significantly different and smaller than group 2
and 3 means. Group 3 mean was significantly different and smaller
than group 2 mean. Group 1, 4, and 5; 1, 3, and 5; and 1 and 2
means were not significantly different.

b.   Globule Leukocytes:

Group 3 mean was significantly different and higher than group 1,
2, 4, and 5 means. Group 4 mean was significantly different and
higher than group 1 and 2 means. Group 5 mean was significantly
different and higher than group 1 mean. Group 4 and 5, 2 and 5,
and 1 and 2 means were not significantly different.

TABLE 6--LSD Multiple Comparison Procedure for Macrophage
and Globule Leukocyte Group Means in the Spiral Colon

Macrophages			Globule Leukocytes		
Mean		Group	Mean		Group
5.4375 A		3	0.0000 A		1
4.8125 A		4	0.1667 A		2
5.2500 A		5	0.0625 A		3
8.7500	B	1	0.1875 A		4
12.500	C	2	0.1550 A		5

(Means with the same letter are not significantly different, $\alpha = 0.05$)

a.   Macrophages:

Group 3, 4, and 5 means were significantly different and smaller
than group 1 and 2 means. Group 1 mean was significantly
different and smaller than group 2 mean. Group 3, 4, and 5 means
were not significantly different.

b.   Globule Leukocytes:

Group 1, 2, 3,4, and 5 means were not significantly different.

SUMMARY OF RESULTS:

Analysis of Group Means:

1.   Groups 1 and 2 (0 and 35-day controls)

   a.   There was no difference in numbers of neutrophils.

   b.   There were fewer lymphocytes at day 0 than at day 35.

   c.   Macrophage numbers were the same except in the colon of

        newborns, which had fewer macrophages.

   d.   Globule leukocytes were fewer in number only in the jejunum

        of newborns.

2. Groups 3-5 (35-day infected, 2, 6, and 12 hours respectively)

    a. Neutrophil numbers were significantly higher at 12 hours than
        at 2 hours.

    b. There were no differences in lymphocyte numbers.

    c. Numbers of both macrophages and globule leukocytes were
        significantly higher only in the ileum at 2 hours post
        infection.

3. 35-day control (Group 2) compared with infected (Groups 3-5)

    a. Neutrophil numbers increased in all infected groups.

    b. Lymphocyte and macrophage numbers were higher in the control
        group as compared to all infected groups.

    c. Numbers of globule leukocytes were higher in controls in the
        jejunum. In the ileum numbers were higher only 6 and 12
        hours post infection. There were no differences in colonic
        globule leukocyte numbers.

4. Comparison of loop means

    a. There were fewer neutrophils in non-infected intestinal
        loops.

    b. There were no differences in numbers of lymphocytes and
        globule leukocytes between infected and non-infected
        intestinal loops.

    c. Macrophage numbers were lower in the infected intestinal
        loops.

5. Analysis of regression

    The trend in leukocyte numbers as infection progressed (from 2 to

    12 hours) was as follows:

    a. A quadratic progression for lymphocytes (F - 1.31) and

        neutrophils (F - 2.49);

    b. A linear progression for ileal globule leukocytes

        (F - 1.007).

Plots of these cells are shown in Figure 4.

Figure 4. Plot of Leukocytes vs. Hours of Infection.

a. Plot of lymphocytes vs. hours of <u>Salmonella</u> <u>choleraesuis</u> infection in pig gut lamina propria, showing a quadratic trend ($F = 1.31$).

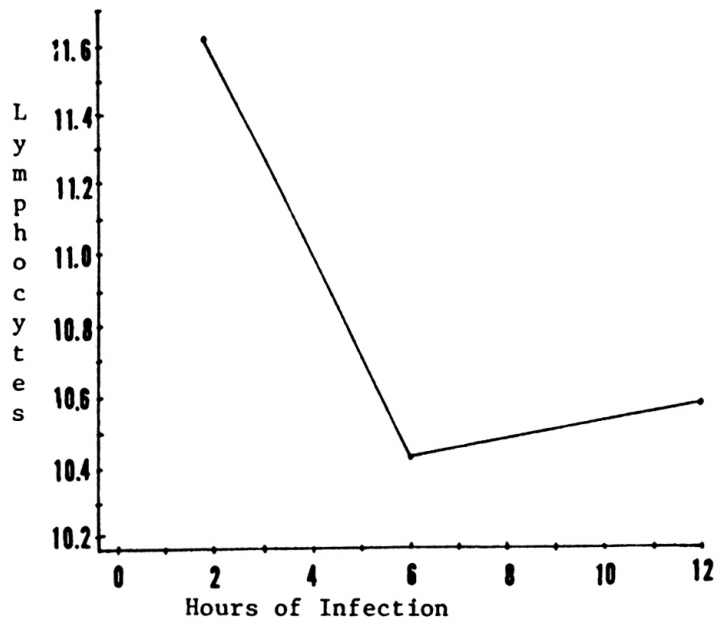Figure 4. (Continued)

b. Plot of neutrophils vs. hours of <u>Salmonella</u> <u>choleraesuis</u> infection
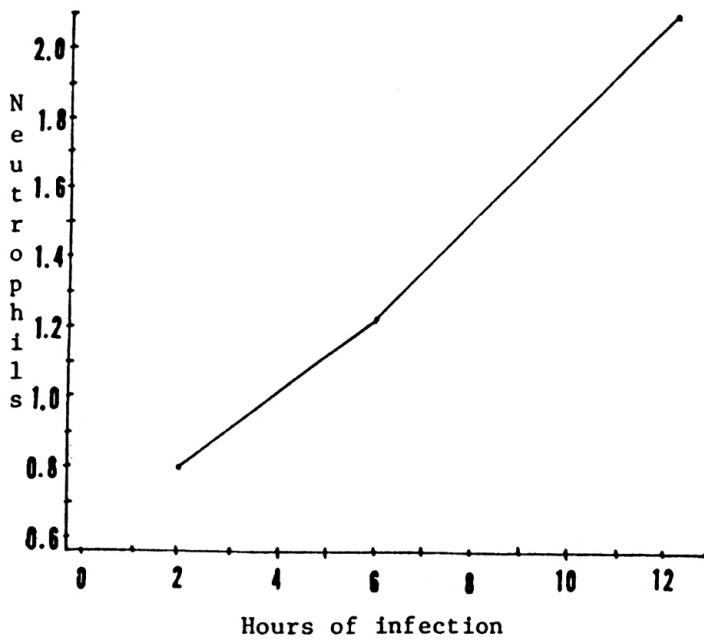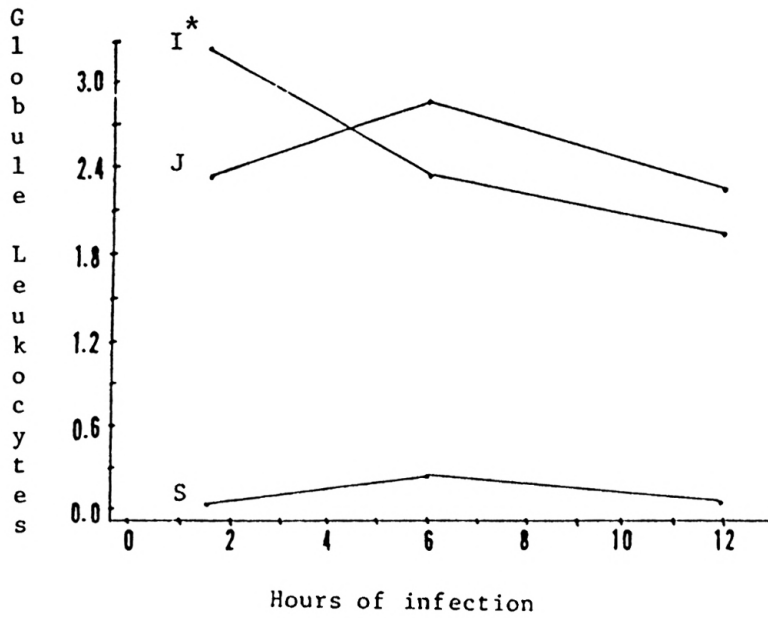   in pig gut lamina propria, showing a quadratic trend (F = 2.49).

Figure 4. (Continued)

  c. Plot of globule leukocytes vs. hours of <u>Salmonella</u> <u>choleraesuis</u>
     infection in pig gut lamina propria, showing a linear trend in the
     ileum (F = 1.007).

Hours of infection

*J = Jejunum, I = Ileum, S = Spiral colon.

DISCUSSION

GENERAL

By morphometrical analysis one can reveal changes in tissue components that are not always easily appreciable by the observing eye. In that way precise measurements are obtained allowing a good general estimation of different parameters of composition and changes of the compartment under study.

In this work, we utilized recently-validated morphometric techniques in order to evaluate the lamina propria cellular composition in salmonella-infected and non-infected swine intestine. The methods described above offer numerous possibilities for quantitative studies of the intestine under different conditions and may be tools for a better understanding of this tissue composition and pathological changes.

THE TECHNIQUE

Quantification of cells in the lamina propria is a problematic task. One cannot simply count cells/villus because villi are not identical in shape and volume and moreover, they are absent in the colonic portion.

Previous quantitative studies of the intestine determined numbers of intraepithelial lymphocytes (40-44) and of leukocytes in the human lamina Propria (45, 46). However, the methods used in these works were

biased and by far not as accurate as the disector. Cells were counted in relation to length of epithelium or to number of epithelial cells (44, 41), but since cells are volume entities and there is no linear relationship between length and volume, these methods are not correct. Cells are particles and should be counted in reference to the volume in which they are present.

The lamina propria is a tissue confined by other tissues (epithelium and muscularis mucosae). Measurement of a sampled volume of it utilizing conventional physical methods (i.e., fluid volume replacement) is quite impossible. A volume unit of lamina propria has to be recovered by other means and the new morphometric methods reviewed in our work give an answer to this problem.

Another obstacle was the extreme variability in villus orientation and surface of villus exposed depending on the direction and the point of its section. By using the vertical section technique this problem was solved.

In order to use the disector method, cell transects should be identified. On "thick sections" different leukocyte types can appear very similar, depending on the portion of the cell present in the section and the distinction of its cytoplasmic membrane. It is obvious that the cell differentiation is subjective and follows as much as possible parameters of shape, size, stain and location in the tissue. Since the cytoplasmic borders are often unclear in light microscopy, one can legitimately count only the nuclei of the cells.

In previous morphometric works, the analysis was done on micrographs. This method may present some difficulties since not all cell profiles on the micrograph appear in focus because of problems of depth of field. Using a pair of microscopes side by side, one can continue to focus while observing the sections. This is a cost-effective and practical way which in addition allows also color distinction and easier identification of cells.

Some authors used a computerized count (46). We also investigated this possibility, but the available equipment was not able to differentiate leukocyte types.

The disector method assumes that tissue processing changes (swelling and shrinkage) are uniform and identical, and therefore can be ignored (34). We observed slight distortion, resulting in small cell location differences between the consecutive sections. These deformities might be generated by stretching or compressing the tissue blocks during the sectioning phase. We solved this problem by using a frame area big enough to include these minor changes.

In the present work we did not consider the volume ratios and changes in lamina propria volume that might result from inflammation. It is possible that the cellular infiltrate can be diluted by serous fluid or vascular artifacts resulting in edema. We also included blood vessels and lymphatics in the considered volume of lamina propria, knowing that changes in these structures are quite possible during inflammation. The calculations of lamina propria volume might be different when these structures are not included.

The results are objective and not biased only for the tissue samples used in the study.

THE RESULTS

Our quantitative analysis demonstrated intestinal composition and changes during salmonella infection similar to those suggested by the quanlitative study.

Some qualitative observations indicated inhomogeneous distribution of leukocytes in some parts of lamina propria. However the systematic random sampling method, used over different locations of lamina propria, took in consideration and reduced the possibility of erroneous quantification of leukocytes.

The assumption that the leukocyte population in the lamina propria of the newborn piglet control group will be smaller than that of the 35-day control group found supporting evidence in some of the results. Lymphocytes were fewer in all intestinal segments of new-born pigs. Macrophages were fewer in the colon, and globule leukocytes were fewer in the jejunum of newborn pigs. It is possible that maturation of tissues and exposure to non-infective antigens (i.e., ingested substances) are responsible for the increased number of macrophages, lymphocytes, and globule leukocytes in some portions of lamina propria of 35-day old piglets.

As mentioned previously, no change was expected in lymphocyte and macrophage numbers in the infected groups as the infection was in its

early phase and these cells generally respond later during inflammation. In fact, quantitative analysis indicated no significant change in macrophage, lymphocyte or globule leukocyte numbers among infected groups, with the exception of higher macrophage and globule leukocyte numbers in the ileum at 2 hours post infection.

As expected, neutrophil number was low in the control groups. Their number increased progressively with the duration of infection as shown in the results of the quatitative analysis and as reported to be typical in salmonellosis. The migration might be a result of endotoxin and other chemotactic stimulants released during salmonella infection. It would be interesting and valuable to determine the increment and behavior of these cells in more prolonged conditions of salmonella infection.

The lower number of macrophages, lymphocytes, and in some instances globule leukocytes in the infected groups compared to the 35-day control group may be only relative. Since we determined only the number of leukocytes/unit of lamina propria volume, it is possible that the volume of the lamina propria increased as a result of early inflammation without any significant change in the number of these cells. The attempt to characterize the behavior of the infiltrating leukocytes in relation to the progression of the infection resulted in quadratic trends for lymphocyte and neutrophil mean numbers and in a linear trend for ileal globule leukocyte mean number. This is an interesting observation which invites more future studies of this kind, considering also the advanced stages of the disease.

SUMMARY

This study provided an objective basis for quantitating cells in the lamina propria of infected and non-infected pig intestine.

The number of different leukocytes in a volume unit of lamina propria was established in the present experiment, using the disector method combined with the vertical section technique.

Further studies can be made to determine other parameters, using these morphometric methods, contributing to a better understanding of salmonella-induced gut inflammation.

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A QUANTITATIVE STUDY OF LEUKOCYTE

INFILTRATION IN THE GUT LAMINA PROPRIA OF

GNOTOBIOTIC AND SALMONELLA MONO-CONTAMINATED PIGS

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ABSTRACT OF A THESIS

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Morphometric analysis of the leukocyte composition in the lamina propria of gnotobiotic and salmonella mono-contaminated pigs was done. The numbers of the different leukocytes were established providing an objective basis for cell quantification.

Twenty gnotobiotic piglets maintained in germ free conditions were assigned at random to five groups of four pigs each, with necropsy done on the day specified:

Group 1--control, 0-day

Group 2--control, 35-day

Group 3-infected with Salmonella choleraesuis (2 hrs) 35-day

Group 4-infected with Salmonella choleraesuis (6 hrs) 35-day

Group 5-infected with Salmonella choleraesuis (12 hrs) 35-day

Pigs from groups 3, 4, and 5 were infected surgically by inoculation of 10^9 CFU of S. choleraesuis var. kunzendorf (1 ml) into ligated loops in the jejunum, ileum and spiral colon. Control loops in all pigs were ligated in the same intestinal segments, but were inoculated with 1 ml sterile PBS. Pigs were removed from isolators at the time indicated above and killed with a barbiturate overdose. Tissues were collected and portions of loops were fixed and processed for light microscopy. Samples were cut into smaller blocks of which two were randomly selected for each loop. The blocks were embedded in glycol methacrylate and consecutive sections, 0.27 μm each, were obtained using the vertical section technique. One pair of sections was randomly selected from each block, mounted on glass slides and stained with hematoxylin and eosin.

The number of each leukocyte type (macrophages, lymphocytes, neutrophils

and globule leukocytes) in a volume unit of lamina propria was determined using the disector method on each pair of consecutive sections.

Macrophages in colon, globule leukocytes in jejunum, and lymphocytes were fewer in the 0-day controls than in 35-day controls, but neutrophils did not differ between the two groups.

Lymphocytes did not differ among infected groups, however neutrophils increased with the time-course of infection. Numbers of macrophages and globule leukocytes were higher only in the ileum at 2 hours post-infection.

Non-infected loops had less neutrophils than infected loops, but no difference in lymphocytes and globule leukocytes was found between infected and non-infected loops. Macrophages were fewer in the infected loops.