IMMUNOFLUORESCENT STUDY OF IgM IN THE CANINE SMALL INTESTINE

by

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INTRODUCTION

The dog, like all mammals studied to date, has a local immune system in the gastrointestinal tract. This immune system in man is thought to function in protecting the body from local invasion of pathogenic or opportunistic organisms and from mucosal penetration of dietary antigens. The length and intensity of antigenic exposure, the presence of local damage concurrent with antigenic exposure, and concomitant exposure to two or more antigens may alter this local immune response just as similar conditions can alter the humoral immune response. The intestinal immune system of the canine has not been adequately characterized even though the canine has some intestinal diseases which appear to be similar to diseases of man having primary or secondary immunologic manifestations. A knowledge of the normal canine intestinal immune system would prove beneficial in studying these diseases in the canine. Knowledge of these may also aid treatment.

Characterization of the number of IgM-containing cells of the canine intestine was the major objective of this study. It was hoped that studies of the IgM-cell population of the canine small intestine would make further studies of pathologic canine intestine more meaningful by establishing normals for some characteristics of the canine intestinal local immune system.

The second objective was to determine the numbers of autofluorescent cells and the total autofluorescence in the same tissues.

THE EXISTENCE OF A LOCAL IMMUNE SYSTEM

Tomasi and Zigelbaum (1963) (131) examined normal human parotid saliva, colostrum, and urine. It was found that normal parotid saliva had large amounts of gamma globulin relative to serum, especially when it is compared to the albumin levels. This increase in the gamma globulin content over the albumin level suggested that some process other than simple diffusion of proteins from blood was needed to explain the presence of the globulin in the local secretions. The principle immunoglobulin found in these secretions was very closely related to if not identical with serum γ_1A (IgA). This statement was based on density gradient ultracentrifugation, double diffusion analysis, and immunoelectrophoretic analysis. There was also a comment that then current studies suggested that γ_1A was also present in high levels [relative to γ_2 (IgG)] in tears, bile, and small intestinal secretions.

Tomasi et al (1965) (130) reported that the γ_1A (IgA) found in parotid saliva had an antigenic determinant which γ_1A found in serum did not have. The γ_1A found in secretions was primarily composed of polymers with an 11S sedemintation coefficient. Local synthesis of the γ_1A was shown to occur in the parotid salivary gland via immunofluorescent techniques. Finally, isohemogglutinins in the saliva were demonstrated to be of the γ_1A type. Thus there "appears to be an [immune system]...characteristic of certain external secretions. Its properties including local production of

a distinctive type of antibody separate from the 'systemic' system...".

Brandtzaeg et al (1968) (23) discovered via fluorescent antibody techniques that oral bacteria, those obtained directly from the human oral cavity, were coated with human IgA. Furthermore, the streptococci obtained from the oral cavity which were coated with IgA tended to grow in long chains much as did bacteria cultured in specific antibody-containing artificial media.

Tomasi and De Coteau (1970) (129) reviewed the concept of mucosal antibodies as they related to gastrointestinal and respiratory disease. It was pointed out that the secretory immunoglobulin, sIgA, was identical to serum IgA except that sIgA occurred as a polymer of two IgA's and one additional "piece" called secretory piece (SP) which carried additional antigenic determinants. Neither IgG or IgM had this secretory piece. sIgA appeared to be synthesized at the mucus membrane by plasma cells and these plasma cells with sIgA were the most populous type of immunoglobulin-containing cells in the mucus membranes. Naturally occurring antibodies to polio, ECHO, coxsackie virus, and parainfluenza were demonstrated in the sIgA class of antibody in nasal secretions. The same was true of E. coli, lactobacillus, and streptococci in other sections. Furthermore, it was demonstrated that isohemoglutimins found in saliva resulted from exposure to certain myxoviruses and E. coli. The antibodies found in the secretions of

mucus membranes were more directly related to the host's ability to resist infection at this location than were the levels of serum antibodies, one example being the effectiveness of live oral cholera vaccine. It was also found that most (but not all) people with selective IgA deficiency were prone to numerous infections of the gastrointestinal and respiratory tracts. IgA-deficiency was also associated with precipitins to foods (i.e. milk) and the question was raised if IgA may protect the mucosal border from being invaded by food antigens. These facts were in accord with sIgA being an integral part of a local immune system.

Brandtzaeg (1972) (14) commented that there appeared to be two lines of defense at the mucus membrane. The first line was locally produced sIgA and IgM. The sIgA had a structure which rendered it resistant to enzymatic degradation and appeared to function by preventing antigenic penetration of mucus membranes, neutralizing virus, and rendering particles osponizable. The fact that IgA was predominant over IgM was noted as potentially important since IgA does not appear to fix complement while IgM does. Excessive complement activation would possibly prove to be harmful to local tissues. The second line of local defense was IgG which appeared to be associated with chronic infections. However, since IgG would activate complement it was wondered if it could prove harmful to the local tissues.

Doe (1972) (53) reviewed the local secretory immune system

of the intestine. The plasma cells found in the intestine were derived from large lymphocytes which seemed to preferentially migrate from the blood to the intestinal lamina propria. These large lymphocytes were formed in intestinal lymphoid tissue and gained access to the blood via the thoracic duct; hence, the local immune system appeared to be separate from the systemic system even in its replenishment. The immunoglobulins found in the intestinal secretion appeared to be the result of local plasma cell-production as opposed to exudation of serum. In vitro culture of intestinal mucosa with ^{14}C-amino acids resulted in immunoglobulins labelled with the ^{14}C. It was thought that the IgA dimers were synthesized in the lamina propria plasma cells and were then passed into the apex of the epithelial cells before going into the intestinal lumen. That these antibodies are protective against local pathogens was shown by comparing locally-inoculated (per os) and parenterally-inoculated guinea pigs. Only the locally-inoculated guinea pigs were protected from Vibria cholera. It was further noted that the local immune response consisted of an IgM response followed by a sIgA response as opposed to the IgM response followed by an IgG response as seen in systemic exposure to antigens.

Brandtzaeg and Baklien (1974) (20) reviewed work concerning the local immune system and diseases of the gastrointestinal tract. They reviewed such facts as the mechanism for selective occurrence of IgA and IgM in local secretions being 1.) preferential local synthesis

and 2.) transmission, which appears to be facilitated through the epithelium, especially the glandular epithelium. This transmission was demonstrated by the presence of IgA and IgM in the epithelial cell as opposed to IgG which though plentiful in the connective tissue ground substance was not found within the epithelial cells. Finally it was noted that most of the IgA cells in the gut produced dimeric IgA as opposed to monomeric IgA, dimeric IgA being the only type which will bind secretory component.

The only functions that were well established at this time were virus neutralization and coating of microorganisms. This coating was thought to be very important since this would prevent the adherence of bacteria to the epithelial cells; a feat necessary before the organism would be able to colonize and invade the mucosa.

It was further noted that some diseases seemed to be associated with marked changes in the local immune system of the intestines. The coeliac syndrome in man was described as one in which the IgM-cell population was increased about three times over normal while the IgA-cells were increased about two times over normal. It was thought that the coeliac syndrome may have a qualitatively-impaired local IgA response. (Currently the authors were characterizing J-chain production and dimer production of IgA as secretory component synthesis and external transport of IgM and IgA appeared normal from previous studies.) It was noted that individuals with selective IgA deficiency were more prone to the coeliac syndrome which would be in line with the possibility of a qualitative defect in

IgA synthesis. Another possibility was that the abundance of IgM present (due to the increase in IgM cells) could be responsible for "excessive" complement activation which though it would be secondary to the inciting cause of increased IgM-cells would cause damage to the tissues.

Selective IgA-cell proliferation was reported. It involved a large increase in immature IgA cells in the intestinal or respiratory tract. In one case the purified protein produced by the proliferated IgA-cells was shown to be polyclonal. Study was currently underway to determine if a specific antigenic stimulus to the IgA cells was responsible.

Ulcerative colitis was reported to commonly have an increase in all classes of immunoglobulin cells, but especially the IgG-cells (up to thirty-fold). The transport of IgA and IgM appeared normal; hence, while a primary defect of the local immune system seemed improbable, quality of the IgA response was considered as worthy of future investigation. It was thought that the increase in IgG-cells could be important in perpetuating the disease via chemotaxis and immune complex formation. Crohn's disease was similar and it was noted that immune complexes were found in many patients with this disease.

Summary of the Review of the Local Immune System

The existence of an immune system localized to body surfaces (mucus membranes in particular) and secretions was demonstrated by:

1.) finding an immunoglobulin which was seemingly preferentially localized by secretion at mucus membranes,[131]

2.) showing that this immunoglobulin had a component generally not found in humoral antibodies even of the same isotype,[129]

3.) demonstrating cells at the mucus membrane which apparently synthesized the immunoglobulins,[130]

4.) showing that this immunoglobulin bound to organisms normally found at mucus membranes[129] as well as to bacteria from the oral cavity affecting their growth,[23] and

5.) finding a tendency toward repeated infections of the gastrointestinal and respiratory tracts in individuals without this local immunoglobulin.[129]

The intestine is one of the organs of the body which has such a system.[53] This system was further shown to not only possibly take part in preventing invasion of the body by bacteria and other antigens, but it may play a part in the pathogenesis of some disease processes.[20] Furthermore, some diseases in man seem to have characteristic immunoglobulin responses as seen by local increases in their respective plasma cells and lymphocytes.[20.]

IgM AS A SECRETORY IMMUNOGLOBULIN

Brandtzaeg et al (1968) (24) demonstrated that patients with a deficiency of IgA had amounts of IgM in local secretions which could not be accounted for by transudation of serum IgM. While secretory component was present in the secretions of such patients, they found evidence that it was not associated with the IgM. The secreted IgM had the same sedimentation rate as serum IgM and double diffusion in agar showed no joining or spurring of lines when anti-secretory component, secretory component, and secreted IgM were allowed to react. It was also noted that while the secreted IgM may be due to enhanced local synthesis (in view of the IgA-deficient state) there was not a consistently increased number of IgM cells in the nasal mucus membranes. "Hence, IgM may not be regarded as a general compensatory secretory immunoglobulin in IgA-deficiency states, although it may function as such in some secretions."

Allen and Porter (1970) (1) found IgM to be distributed in the porcine intestinal glandular epithelium in the same manner as IgA. IgG was not so found in the intestinal glandular epithelium.

Thompson (1970) (127) obtained sera from patients with IgA deficiency. The sera showed the presence of secretory component but IgA could not be detected. It was determined that the fraction

of the serum containing the secretory component would spur with the anti-μ line in double diffusion analysis. Electrophoresis further showed that the secretory component could be associated with IgM. The above findings were not the case in normal individuals with detectable serum levels of IgA.

Chen (1971) (39) described IgM and IgA as both being present (as seen by immunofluorescence) in the columnar epithelium of appendices while IgG was not so seen.

Savilahti (1973) (112) stated that the immunofluorescence of the jejunal mucosa from IgA-deficient patients showed IgM to be present in the apical portion of the intestinal epithelial cells, the basement membrane of the epithelial cells, and the spaces between the cells. He stated that "the deficient IgA is quantitatively replaced by IgM in the intestine of children with IgA deficiency. IgM in the intestine of IgA deficient children has characteristics in common with IgA in normal children".

Brandtzaeg (1975) (18) utilized IgA-deficient parotid secretions to obtain secreted IgM. This IgM would bind in quantitatively small amounts with labelled secretory component while 19S IgM from serum would spontaneously form non-covalently bound complexes with free secretory component in vitro. The same held true for sIgA compared to serum IgA. Even after purification

sIgA and secreted IgM had most of their secretory component binding sites blocked. Ultracentrifugation revealed bound secretory component to have a distribution similar to IgA in normal parotid saliva. In IgA-deficient parotid saliva the distribution of secretory component corresponded to IgM. Double diffusion in agar revealed that this secretory component was indeed associated with the IgM. Furthermore, the "I" determinant of secretory component, which is inaccessible in sIgA, was detectable in secreted IgM which suggested that secretory component was bound less tightly to IgM than to IgA. Finally, IgM was seen by fluorescent antibody techniques to have a distribution in the epithelial cells of the colonic glands identical to sIgA. Thus IgM appeared to be a secretory immunoglobulin similar to sIgA.

Weicker and Underdown (1975) (140) reported that dimeric IgA and pentameric IgM both bound secretory component with approximately one secretory component binding site per molecule while no binding was associated with IgA or IgM monomers.

Allen et al (1976) (4) used electron microscopy and peroxidase-labelled antibody to porcine IgM and demonstrated the presence of IgM in plasma cells and glandular epithelium in the small intestine of three to four week old pigs. The IgM in the epithelial cells was in membrane-bound vacuoles as was IgA in a similar previous study.[3] The major difference between the two studies was that the IgM was not as plentiful as the IgA.

Brown et al (1976) (31) examined IgM, IgA, and IgG in the jejunum and ileum of humans. Light microscopy and electron microscopy with peroxidase-labelled antibodies revealed that IgA and IgM, in contrast to IgG, were seen in the lateral and basal plasma membranes and in cytoplasmic vesicles of intestinal glandular epithelial cells. This evidence supported the concept of IgM being translocated across the intestinal epithelium much as is thought to occur for IgA.

Lindh and Bjork (1976) (90) demonstrated that secretory component does bind to the $(Fc)_5\mu$ fragment of IgM in molar ratio which varies from 2.10 to 0.64 when secretory component is in excess (either 5:1 or 10:1 molar ratio).

Thompson and Reynolds (1977) (128) isolated IgM from canine bronchial secretions and colostrum. Approximately 90% of the "secreted" IgM had covalently bound secretory component.

Summary of the Review of IgM

IgA is commonly thought of as the primary secretory immunoglobulin;[130,131] however, IgM has been shown to bind secretory component[90,127,128,140] and apparently pass into the lumen of the intestine through the intestinal glandular epithelium in the same way as IgA.[1,4,18,24,31,39] Thus even though there is evidence that IgM may not be as important as IgA in the local immune system,

it probably is part of the local immune system. Therefore it is worthwhile determining its "morphology" in the normal small intestine so that changes in the IgM-component of the local immune system of the intestine due to or causing pathology may be characterized.

THE USE OF ETHANOL-FIXED, PARAFFIN-EMBEDDED TISSUE FOR USE IN FLUORESCENT ANTIBODY PROCEDURE

Beginning of Tissue Preparation for Fluorescent Microscopy

Coons et al (1955) (41) reported on the use of unfixed frozen tissue sections in fluorescent antibody procedures. It was noted that "attempts to stain the same field first to reveal antibody and then by conventional histologic methods failed except in a few instances. The cells...were almost invariably unrecognizable when stained afterward with Giemsa."

Comparison of Different Methods of Tissue Preparation

Sainte-Marie (1962) (108) described a technique of cold ethanol-fixation and paraffin-embedding of tissue to be used in immunofluorescent studies. Upon comparing frozen sections and ethanol-fixed, paraffin-embedded sections stained for immunoglobulin or heterologous albumin, he stated that the antibody in the paraffin-embedded slides was "...at least as bright as...the corresponding frozen sections..." and that the heterologous albumin was "...much brighter than in the corresponding frozen sections". He further stated that the "slight shrinkage of tissue resulting from dehydration in alcohol also separates the cells and delineates the potential tissue spaces, which aids in the precise localization of material". He did caution that the paraffin blocks should be cut as soon as possible "as autofluorescence increases with time. However, blocks two or three months old have often been satisfactory".

Eidelman and Berschauer (1969) (56) stated that in their "studies of intestinal mucosal plasma cells...the morphological detail obtained from sections of unfixed, quick-frozen mucosal biopsy specimens have been inadequate for quantitative studies". They used formalin-fixed, paraffin-embedded tissues some of which were stored for up to 1 year at -20°C or -40°C without deterioration of the immunofluorescence.

Friis (1969) (62) used ethanol-fixed, paraffin-embedded samples and unfixed frozen samples of human synovial tissue to demonstrate cells with rheumatoid factor. He stated that "using the cryostat technique we found the cell-limits less sharply defined; the cells were granular, often surrounded by fluorescent particles...using the alcohol technique, the fluorescence was less abundant, the cells well defined, but the thickness of the sections was somewhat greater than that seen when the freezing-method was used, a fact which to some extent has contributed to a veiling of cytological details...If biopsies fixed in alcohol at 4°C were compared with biopsies fixed in alcohol at room temperature (20°C) fluorescent cells were found to be fewer in sections from the latter...This applies...equally well...of IgG and IgM".

Heron (1970) (73) compared ethanol-fixed, paraffin-embedded kidney tissue to frozen kidney tissue for use in fluorescent antibody techniques. He stated that "A major advantage of the cold ethanol-fixation method is that the numerous artifacts present in cryostat

sections are avoided with much better preservation of tissue structures...it is found advantageous that the blocks may be stored at $4^{o}C$ for months and still be suitable for immunofluorescent investigation". He stated that it was his impression "that the glomerular fluorescent staining which is considered specific is less bright following paraffin-embedding" and that there was "only slightly increased autofluorescence in the paraffin sections compared to the frozen sections". It was also noted that interstitial immunoglobulins seemed to be retained more in the ethanol-fixed sections.

Allen et al (1973) (3) stated that intracellular staining for IgA "was more obvious in cryostat sections than in those prepared by the method of Sainte-Marie (1962). Presumably much of the extracellular immunoglobulin was lost during processing by the latter technique".

Husby et al (1973) (77) utilized a cold ethanol-fixation technique with paraffin-embedding in studying liver biopsies. Of this technique he stated, "In our hands the modified Sainte-Marie paraffin-embedding technique...proved...superior to snap-freezing as far as liver biopsies are concerned...it reveals the morphology of cellular elements better than snap-freezing, whereby damage to cells is more likely to occur".

Brown et al (1974) (28) compared unfixed frozen tissues and

ethanol-fixed, paraffin-embedded tissue for immunofluorescent techniques. They noted that primary fluorescence of tissues was more common in paraffin-embedded tissues but that individual fluorescing cells were "clearly seen in wax preparations". However, it was noted that "cryostat sections...showed considerable distortion of tissue architecture, particularly when intestinal tissue was examined...only parts of a section were suitable for use...Tissue structure was better preserved in wax-prepared material...". Furthermore, "A particular problem with labelled IgG antisera was the presence in cryostat sections...of a wide 'background' staining".

Burns et al (1974) (33) compared fresh frozen tissue, cold ethanol-fixed, paraffin-embedded material, and formalin-fixed, routine paraffin-embedded material to determine how the method of fixation affected immunofluorescent demonstration of intracellular immunoglobulins. Using FITC-labelled antibodies to the two separate human light chains and IgM he found that the cold ethanol-fixation, paraffin-embedding technique gave better results than the frozen tissue (for plasma cells; "strong intensity, moderate definition" versus "moderate variable intensity, poor definition" respectively) and equal results to the formalin-fixed tissue. The two disadvantages unique to the ethanol-fixed tissues were 1.) autofluorescent eosinophils, however, the "eosinophils were not prominent" and 2.) the reticulum stained nonspecifically with

a strong intensity. The frozen samples had a general "lack of clear demarcation...not apparent in the...Sainte-Marie [ethanol-fixed] series".

Arnold et al (1975) (6) compared snap-frozen tissue samples cut on a cryostat to cold ethanol and cold formalin-fixed samples later embedded in paraffin and freeze substitution and freeze-dried samples later paraffin-embedded to determine which one(s) were best for immunofluorescent techniques. Mitochondrial antibody and antinuclear antibody staining could not utilize the cold ethanol-fixed samples; however this method of fixation gave good results with skeletal muscle antibodies even after the paraffin blocks had been stored for two years. Immunoglobulins were not studied. "Other than the better preservation of tissue morphology with one of the different tissue preparation procedures (especially freeze substitution, cold ethanol, and freeze drying) the advantage of these methods is that they provide standardized antigenic material that can be preserved for a long time period...".

Use of Cold Ethanol-Fixed Tissue for Fluorescent Microscopy

Brandtzaeg and Baklien (1972) (19) used a cold ethanol-fixation, paraffin-embedding technique to study intestinal biopsy samples. Fixation immediately after obtaining the biopsy caused difficulties in counting IgA cells and made it impossible to

count IgG-cells due to the fact that the 'total' content of interstitial immunoglobulins was contained in the tissues by this method. However "low concentration of IgM were present in the... [connective tissue-ground substance] ...and IgM-cells were easily discerned".

Brandtzaeg (1974) (16) reported that the Sainte-Marie technique of preparing tissues for immunofluorescence was rendered more useful by washing part of the tissue in isotonic saline for 48 hours before fixing it in ethanol. He stated that both washed and unwashed tissues were examined from each biopsy sample. He stated that in "sections of unfixed, frozen specimens there are poor morphologic details and an uncontrolled loss of diffusible protein...". The biopsy samples involved came from the human respiratory and gastrointestinal tracts. It was noted that while extracellular IgG and IgA were largely removed from the sample by washing procedure, "...small granular IgM deposits were often retained, particularly in basement membrane zones of vessel walls and epithelia. A general retention of Ig [immunoglobulin] deposits was noted in traumatized tissue along the periphery of the specimens." He further noted that in unwashed samples IgM showed the least interstitial fluorescence of all isotypes studied and this interstitial fluorescence was primarily located at the basement membrane, around blood vessels, and on the epithelium. Furthermore, the possibility of damage to cells allowing diffusion of immunoglobulin into the cells from the

interstitial areas was shown not to occur. It was noted that "...blocks of tissue exhibit Ig [immunoglobulin] antigenicity for at least 8 years when stored at 4°C. Our observation of preservation of antibody activity is so far limited to 17 months". Finally it should be noted that the modified technique was "gentle" enough to allow the demonstration and semiquantitation of intracellular and membrane antigens (IgA and secretory component).

Feltkamp-Vroom (1975) (60) stated that while cold ethanol-fixation and paraffin-embedding can demonstrate intracellular immunoglobulins, this technique may not be applicable when relatively small amounts of immunoglobulin are present. His reference was to renal immunopathology in particular.

Huang et al (1976) (76) declared that immunofluorescence of intracellular immunoglobulins from paraffin-embedded sections could be improved by partial digestion of the section with trypsin. The immunoglobulins were not significantly harmed by this procedure as regards their demonstration by fluorescent techniques.

Summary of the Review of Techniques for Preparation of Tissues for Immunofluorescence

Snap-freezing of tissue samples in liquid nitrogen has been commonly used for fluorescent antibody techniques.[41] Ethanol-fixation and paraffin-embedding of tissue samples have been

shown to

1.) give better tissue and cellular architecture, [28,33,56,62,77]

2.) perhaps have slightly less brilliant fluorescence than snap-freezing in liquid nitrogen, [73]

3.) be storable for months or possibly years, [6,16] and

4.) be usable for histopathology. [41,62]

There were occasional conflicting reports on some of these statements. [60,73] In general it would appear that whenever tissue and/or cellular morphology was important, the ethanol-fixed tissues provided as good if not better means of preparing tissues than other methods currently available.

THE CLINICAL AND INVESTIGATIONAL USES OF FLUORESCENT ANTIBODY TECHNIQUES TO DISCERN INTRACELLULAR IMMUNOGLOBULINS IN HUMANS

Intracellular Immunoglobulins

Brandtzaeg (1973) (15) showed that two different types of IgA-immunocytes could be distinguished by immunofluorescence. The difference in the two types of IgA-immunocytes was determined by whether or not they bound secretory component. IgA-immunocytes which bound secretory component were thought to be producing dimeric IgA as opposed to monomeric IgA. It was also discovered that some of the cells binding secretory component were IgM-immunocytes.

Brandtzaeg (1974) (17) used immunofluorescent techniques to elucidate the probable mechanism of transfer or secretion of locally produced IgA in the human small intestine and respiratory system. He traced the path from the IgA-containing plasma cells to the intestinal lumen.

Garvin et al (1974) (63) could demonstrate the presence of IgA but not IgM within cells associated with Hodgkin's disease (Reed-Sternberg cells).

Poger and Lamm (1974) (101) studied the local mechanism of IgA formation, transfer, and secretion via immunofluorescent techniques and produced a hypothetical model. No quantitation of the IgA-cells was done.

Weisz-Carrington et al (1976) (141) demonstrated a progressive decrease in the numbers of IgA-cells in neoplasms of the colon as the tumors progressed from benign to malignant, well-differentiated, to malignant, poorly-differentiated. The benign lesions occasionally had an increase in IgA-cells. In all types of tumors examined the IgA-cells were the most common cell type while IgM-cells were the next most common.

The Presence of Intracellular Immunoglobulins in Tissues other than in Alimentary Tissues

Gitlin et al (1952) (68) demonstrated by immunofluorescence the presence of gamma globulin within cells in the spleen and gastrointestinal tract.

Ortega and Mellors (1957) (97) examined human tissues with a fluorescent technique to determine the presence and origin of gamma-globulins (as opposed to alpha-globulins and fast-moving beta-globulins). Three types of cells were seen to produce gamma-globulins: 1.) plasma cells with Russel bodies, 2.) plasma cells without Russel bodies, and 3.) large to medium lymphocytes. The nucleus of these cells was not seen to contain gamma-globulins by this technique. There were large numbers of these cells in the red pulp of the spleen and in the medullary cords of the lymph nodes.

Vasquez (1958) (137) examined three patients with hyper-globulinenemia and high gamma globulin. Bone marrow aspirates

showed findings typical of multiple myeloma. These cells would stain well with anti-human gamma globulin.

Burtin and Buffe (1963) (34) studied several cases of human myeloma bone marrow aspirates. Though not all the cases had cells which stained with anti-gamma globulin, all the cells that did stain were plasma cells and the staining was confined to the cytoplasm of the cells. It was thought that a relatively "insensitive" direct fluorescent technique was responsible for many of the cells apparently not fluorescing.

Mellors and Korngold (1963) (92) examined human lymph node, spleen, thyroid, synovial membrane, fetal thymus gland, and other tissues by immunofluorescence. It was found that with the rare exception of a cell having γ_2 globulin and γ_{1M} globulin simultaneously, all cells had only one type of immunoglobulin. In the above tissues γ_2 cells (individual and focal accumulations) were more numerous than γ_{1M} cells which were more numerous than γ_{1A} cells. Two types of cells were found to have these globulins; "primitive" cells in the germinal centers and plasma cells. However, rare small lymphocytes seemed to contain immunoglobulin within their cytoplasm.

Pernis and Chiappino (1964) (99) examined the human spleen and lymph nodes for the presence of group 1 γ-globulin cells and group 2 γ-globulin cells (these two groups appeared to retain isotypes

or allotypes of light chains). The red pulp of the spleen seemed to be evenly divided into two groups of cells, each producing one type only. There were no groupings of these cells (i.e. nodules or follicles). The white pulp of the spleen and the germinal centers of the lymph nodes, on the other hand, appeared to have very little clear cut differentiation between cell types. Most of the cells stained with both conjugates simultaneously while a few cells stained only with one or the other. The cells staining with both conjugates generally appeared to be "blast" cells.

Bernier and Cebra (1965) (13) examined slide touch prints of human spleen. Quantitation was done to determine the relative percentages of the different types of immunoglobulin-bearing cells. It was found that about 60% of human immunoglobulin-bearing cells had K light chains while 40% had λ light chains. Less than 1% of the cells had mixed staining. It was also noted that these cells had only one light chain and only one heavy chain type per individual cell showing that each cell makes heavy and light chains and only of one isotype for each.

Tomasi et al (1965) (130) used fluorescent antibody techniques to demonstrate the presence of plasma cell-like cells with cytoplasmic 11S IgA in the interstitial tissue of the

human parotid salivary gland. These cells were principally located between the acini of the gland.

Brandtzaeg et al (1967) (22) studied the presence of immunoglobulin-containing cells in the human nasal mucosa, inflamed gingiva, and parotid salivary gland. Most of the fluorescing cells in the salivary gland contained IgA (IgG-cells were present) while most of the fluorescing cells in the chronically inflamed gingiva were IgG-cells. In the nasal mucosa IgA-cells were most populous along the glandular areas and the secretory ducts. There were fewer IgG-cells here but some were definitely present. The ratio of IgA-cells: IgG-cells there was from 8:1 to 3:1. IgG-cells were more numerous at the mucosal surface, being as plentiful as IgA-cells here. IgM-cells were rare, being found less often than IgG-cells in the nasal mucosa but being more common than IgG-cells in the parotid salivary gland.

Crabbé and Heremans (1967) (48) obtained human adenoid tissue and examined it for the type of immunoglobulin-containing cells present. IgG-cells were always the most numerous, IgA-cells being the next most numerous but seemingly always less than the IgG-cells. IgM- and IgD-cells were always the most sparse but neither consistently was greater than the other. There was no obvious predominance of cells with K-type light chains or λ -type light chains. These fluorescing cells seemed to preferentially

occur where histopath techniques showed plasma cells to be most numerous. However there were not areas which favored one type of immunoglobulin-containing cells.

Brandtzaeg et al (1968) (24) examined a patient deficient in IgA. They found an increased number of IgM- and IgG-containing cells in the parotid salivary gland (45 and 35 per field, respectively) compared to samples from patients who were not deficient in IgA (<5 for both). Furthermore, the deficient patient had a greater number of IgM-cells in the gastric mucosa than did normal people.

Martinez-Tello et al (1968) (91) studied bronchial mucosa and bronchial lymph nodes in patients with and without chronic bronchopulmonary disease. Cases without such disease had a ratio of IgA: IgG: IgM cells of 5:5:1 in the bronchial mucosa while in the diseased states the IgA- and IgG-cells were both increased, the IgA-cells significantly more so than the IgG-cells (3 fold versus 2.5 fold). The bronchial lymph nodes were more difficult to count; however, IgG was the principal immunoglobulin present in normal lymph nodes. IgG-cells were also the principal cell which increased during chronic bronchopulmonary disease. However, IgA- and IgM-cells were seen in all lymph nodes. It was further noted that in the bronchial mucosa the fluorescing cells were plasmacytes (mature or immature). IgM-cells also included "small lymphocytic" plasma cells.

Rossen et al (1968) (105) demonstrated that there were very few interstitial cells in the tonsils which contained IgA. The same was true for nasal polyps, salivary glands, bronchi, spleen, and lymph node. There were many cells in the epithelium of the oral and respiratory passages which contained IgA but these appeared to only be epithelial cells; no lymphoid cells appeared present.

Hadziyannis et al (1969) (70) examined immunoglobulin-containing cells in the liver. IgA-cells predominated in hepatic diseases other than primary biliary cirrhosis. In primary biliary cirrhosis, IgM-cells predominated. In non-hepatic diseases which caused a lymphoid infiltrate in the liver IgG-cells were the most common type seen. IgD-cells were rarely seen. The predominant immunoglobulin isotype-cell was not always correlated to an increase in the corresponding serum isotype.

Tourville et al (1969) (132) found few IgG- and IgA-containing cells in the human bronchial mucosa; however, they were about equal in number and each was greater than the number of IgM-containing cells. Salivary glands had few IgA-cells although there were some large foci while there were almost no IgG- or IgM-cells. The gastrointestinal tract (the small intestine and colon in particular) showed a large number of IgA-cells which predominated over the other cell types. Here the IgM-cells were more numerous than the IgG-cells, as opposed to the bronchial mucosa. The gall bladder

had lesser numbers of IgM-cells but large numbers of IgG-cells and IgA-cells. No cellular staining to IgG, IgM, or IgA was seen in the kidney, pancreas, or skin.

Tada and Ishizaka (1970) (125) studied the distribution of IgE-containing cells in humans and monkeys. The human tonsils and adenoids were seen to contain the greatest number of IgE-cells while monkey tonsils had significantly fewer such cells. Human spleen was almost devoid of IgE-cells while monkey spleen had many more IgE-cells. IgE-cells were also found in the lamina propria of the stomach, small intestine, colon, and rectum, but especially around the crypts of Lieburkühn. When compared with IgG-, IgM-, IgA, and IgD-cells, IgE-cells made up 3% of all the immunocytes in bronchial lymph nodes, less than 1% in the subcutaneous lymph nodes and less than 0.1% in the spleen.

Chen (1971) (39) examined the normal and inflamed human tonsil. The normal human tonsil had approximately 10 immunoglobulin-containing cells per high power field, most of which were IgA-cells. IgM-cells were more scarce than IgG-cells. Inflamed tonsils had in some cases more than two times as many immunoglobulin-containing cells as the normal tonsils. These cells were predominantly IgA- and IgG-cells and rarely IgM-cells. There was no predominance of IgA- or IgG-cells in any given area.

Hijams et al (1971) (74) reported that the determination of intracellular immunoglobulins, in this case from bone marrow cells, via immunofluorescence was reproducible and that the percentages of each type of cell in the bone marrow corresponded to the serum level of immunoglobulin.

Green and Fox (1972) (69) presented work that showed that IgA-containing cells increased markedly in the mucosa of the gall bladder proportionately to the degree of inflammation present. In very severe inflammations or in chronic diseases the numbers of IgM- and IgG-cells increased relatively more than did the IgA-cells and this increase occurred in the muscle layers of the gall bladder which appeared to have approximately equal numbers of the different types of immunoglobulin-containing cells.

Ishikawa et al (1972) (78) reported that in human pharyngeal and palatine tonsils, IgA-cells were found primarily near the basement membrane while IgG-cells were scattered throughout. IgM-cells, which occurred in lesser numbers than IgG- or IgA-cells were also scattered in the tissues as were IgD-cells. A few IgE-cells were seen in the tonsils. IgA-cells were generally found in high numbers (i.e. greater than 100 cells per 10 fields at 400 magnification) at the basement membrane.

Franklin et al (1973) (61) examined the human lacrimal gland

by immunohistochemical means and found a significantly higher number of IgA-cells (17 to 47/field) than IgG-cells (1 to 7/field) or IgM-cells (0 to <1/field) or IgE-cells (0 to <1/field.

Husby et al (1973) (77) examined immunoglobulin-containing cells in the livers of patients with various illnesses. Patients with active chronic hepatitis had many plasma cells at sites of inflammation (compared to almost none in the normal liver). About 80% were IgG-cells, 15% were IgA-cells, and 5% were IgM-cells. Rarely were cells seen away from the area of inflammation. Chronic persistent hepatitis had generally fewer cells of all types. Acute hepatitis usually showed there to be as many IgA-cells as IgG-cells. Cases of primary biliary cirrhosis had very few cells, but IgG-cells were the most common type present. Nevertheless, IgM-cells were easiest to find in primary biliary cirrhosis compared to other entities. Lastly, IgG-cells predominated in the periphery of one case of granulomotous hepatitis.

Silverman et al (1973) (114) demonstrated by fluorescent antibody techniques that there were two populations of cells in a patient with a double myeloma, one producing IgM and another IgA. There were no cells seen which contained both isotypes.

Ogra et al (1974) (96) demonstrated IgA-cells in biopsy samples of the human middle ear. No quantitation of cells was done.

Østergaard (1975) (98) found that in tonsils obtained at tonsillectomy 60% were devoid of IgE-cells, 30% were devoid of IgA-cells, 6% were devoid of IgG-cells, and only 4% were devoid of IgM-cells. A combined lack of IgA- and IgE-cells occurred in 25%. The lack of IgE-cells was not distributed according to the age of the patient while a general lack of antibody-containing cells was most common in the younger age group.

The Presence of Intracellular Immunoglobulins in the Stomach

Jeffries et al (1965) (79) observed that the antibody isotype which was responsible for immunofluorescence of parietal cells in pernicious anemia was IgG as demonstrated by specific immunofluorescence.

Brus et al (1968) (32) examined human gastric biopsies for the presence of IgA- and IgG-cells. Normal gastric mucosa had a few IgA-mononuclear cells in the lamina propria and rare IgG-cells. Cases of superficial gastritis usually had increased numbers of IgA-cells, most of which were superficial. Atrophic gastritis usually had increased numbers of IgA-cells, most of which were superficial. Atrophic gastritis without intestinal metaplasia had moderate to large amounts of IgA-cells and very few IgG-cells. Atrophic gastritis with intestinal metaplasia had large numbers of IgA-cells, but generally fewer than were seen in atrophic gastritis without intestinal metaplasia. It

was also noted that the round cells seen in hematoxylin and eosin sections usually exceeded that of similar shaped cells which stained for IgA and IgE.

Odgers and Wangel (1968) (95) found that gastric biopsy samples of three-fourths of the humans with pernicious anemia had a decrease in IgA-containing cells (10% of normal) and an increase in IgG-containing cells (+15% of normal). There was also a 50% decrease in the number of IgM-cells. However, there was no such abnormality seen in one-fourth of the patients examined. Concurrent biopsies of the rectum showed no difference from normal. Furthermore, the ratio of IgA: IgG: IgM cells in gastric mucosa from pernicious anemia patients was 1:2:1 compared to 8:1:1.2 for normal controls.

Baur et al (1970) (12) found a difference in number and distribution of IgG- and IgA-type autoantibody cells in gastric biopsy samples from patients with pernicious anemia. Cells with IgG-type antibody to intrinsic factor were found in less than half the patients while no such IgA-cells were found. Concerning all IgG- and IgA-cells (not just those producing antibody directed against intrinsic factor), the IgA-cells were primarily found among the gastric glands while IgG-cells were found deep to the glands, extending into the muscularis mucosae and submucosa.

The Presence of Intracellular Immunoglobulins in the Colon and Ileum

Crabbé and Heremans (1966) (44) described large numbers of IgD-cells present in the rectal mucosa of a patient with chronic ulcerative colitis who appeared to have diminished numbers of IgA-cells (which were still the predominant cell type).

Kraft et al (1966) (87) tried to show fluorescence of the colonic mucosal cells of ulcerative colitis patients. They showed instead the presence of IgG-cells in the lamina propria and stated that there were increased numbers of IgG-cells compared to both normal colon and colonic diverticulitis.

Eidelman and Davis (1968) (57) described a "reversal of the normal ratio between IgA- and IgM-containing plasma cells in the rectal mucosa" of pediatric patients with ataxis telangiectasia.

Gelzayd et al (1968) (65) examined IgA-, IgM-, and IgG-cells of the lamina propria of the rectal mucosa from patients with ulcerative colitis or abnormal colonic mucosa. The predominant immunoglobulin-containing cell type was the IgA-cell; however, there were relatively fewer IgA- and IgG-cells present than would be expected for a normal patient. The relative numbers of IgM-containing cells appeared equivalent to that of normal patients.

Gelzayd et al (1968) (66) enumerated different classes of antibody-producing cells in the human rectum. It was determined that IgA-cells are the most abundant followed by IgM-cells and IgG-cells. Cells were enumerated in a number of cells per unit area fashion which was found to be "highly reproducible".

Chen (1971) (40) studied the inflamed human appendix. It was seen that IgA-cells were always the most plentiful despite the stage of infection but that IgG-cells increased in absolute and relative numbers as inflammation was prolonged. IgM-cells were rare and increased only slightly and then only in early inflammation.

Brandtzaeg et al (1974) (21) compared normal patients and patients with ulcerative colitis and found that in ulcerative colitis the muscularis mucosa and the submucosa were densely populated with immunocytes, primarily IgG-immunocytes. About 73% of all mucosal immunocytes were found within two hundred μm of the surface, the total number in this area being increased about thirty times over normal. Overall, the diseased biopsy samples had about four times the normal average number of immunocytes, this increase being due to thickening of the mucosa and increased density of the immunocytes. IgA-cells were increased 2.2 times and IgM-cells were increased 5.2 times.

Skinner and Whitehead (1974) (115) examined immunoglobulin-containing cells of the colon in ulcerative colitis and Crohn's disease. IgD- and IgE-cells were present in such small numbers that counting them was abandoned. IgA-, IgG-, and IgM-cells were significantly increased over normal in ulcerative colitis and Crohn's disease. There was a significant increase in IgA-cells in Crohn's disease compared to ulcerative colitis. There was also a significant difference in numbers of IgA-cells in active ulcerative colitis versus inactive ulcerative colitis, the inactive cases having near normal counts.

The Presence of Intracellular Immunoglobulins of the Small Intestine or in the Small Intestine plus other Tissues

Crabbe et al (1965) (43) examined the normal human intestinal mucosa by using fluorescein conjugates of antisera to IgG, IgM, and IgA. It was found that all samples of duodenum and jejunum (ileum was not examined) contained IgA-cells which were primarily seen at the basal part of the lamina propria and at the villus base. Few IgA-cells were seen in between the crypts of Leiberkühn. IgM-cells were distributed much like the IgA-cells. IgG-cells were more difficult to enumerate due to extensive interstitial fluorescence; however, they believed that higher magnification showed the true number of IgG-cells to be small. IgA-cells were quantitated and found to number 181,000 cells/mm^3 of interstitial tissue while IgM- and IgG-cells were found to number 30,000 and 18,000 cells/mm^3 respectively. Lastly it was noted that there was no difference in

the sizes of the different immunoglobulin-containing cells or in their nucleocytoplasmic ratios.

Rubin et al (1965) (106) found that the majority of the cells in the lamina propria of the intestine of human patients with adult celiac disease contained IgG_{1A}.

Crabbé and Heremans (1966) (45) qualitated and quantitated the presence of IgG-, IgM-, IgA-, and IgD-cells in the human gastrointestinal tract. IgD-cells were so sparse that quantitation was not carried out. IgA-cells were always the most numerous (352,000 to 119,000/mm^3) while IgM-cells were always greater than or equal to IgG-cells (52,000 to 7,000/mm^3 for IgM compared to 32,000 to 1,000 cells/mm^3 for IgG).

Crabbé and Heremans (1966) (46) reported the findings that three patients with IgA deficiency and steatorrhea had greatly decreased numbers of IgA-cells in the lamina propria of the small intestine (i.e. 3,423 to 77,594 compared to a normal of 352,290 $\pm$ 122,348 IgA-cells/mm^3). The IgM-cells were increased in number (except for one case) while the IgG-cells were in normal numbers.

Crabbé and Heremans (1967) (47) described a case of steatorrhea associated with a selective IgA-deficiency. Upper jejunal and rectum biopsy samples showed very few IgA-cells and increased

numbers of IgM-cells with a slight increase in IgG-cells. Normal jejunum was earlier seen to have 352,000, 52,000, and 16,000 IgA-, IgM-, and IgG-cells/mm^3 respectively. In this case the observed numbers were 78,000, 432,000, and 17,000 respectively. The same pattern held true for the rectal samples.

Swanson et al (1968) (124) reported a patient with undetectable serum IgA that had "usual numbers" of IgA-producing cells in the gastrointestinal tract.

Douglas et al (1969) (54) reported that "...the small intestinal mucosa in the coeliac disease contains a greatly increased percentage of plasma cells containing IgM". They reported that 10% was the normal percentage of IgM-cells in the human intestine (when compared to other immunoglobulin-cell types).

Hobbs et al (1969) (75) reviewed past work on coeliac disease and stated that "absolute counts of IgA-containing plasma cells... are normal", there is "an excess of IgG-containing plasma cells in the intestinal mucosa of children...but in adults...only minor increases", and that IgM-containing cells are consistently increased in the small intestine only.

Söltoft (1969) (116) studied the jejunal mucosa from normal humans and humans with ulcerative colitis and regional enteritis. It was found that in Crohn's disease there was a significant

difference when IgA- and IgG-cells were paired and when IgM- and IgG-cells were paired but there was not a significant difference when IgA- and IgM-cells were paired. All pairs in the controls were significantly different. Furthermore there was a significant difference in the total number of immunoglobulin-containing plasma cells in the Crohn's disease group and the normal controls, at the 0.05 level; however, the ulcerative colitis disease group was not significantly different from the controls. It was also noted that the ulcerative colitis group had a relative increase in the number of IgM-containing cells compared to IgA-containing cells and in IgG-containing cells compared to IgA-containing cells.

Söltoft and Weeke (1969) (122) examined human cases of non-tropical sprue. The percent IgG-cells (given as percent of interstitial area accounted for by cells) was increased over normal in both treated and untreated cases of sprue. The same was true of IgM-cells but this was not true for IgA-cells. Serum levels of immunoglobulins were determined; however, no correlation existed between the numbers of cells present in the jejunal biopsy samples and the serum levels of the corresponding immunoglobulin in untreated or treated patients.

Douglas et al (1970) (55) stated that IgA-containing cells tended to be slightly decreased in the celiac syndrome while IgM-containing cells were increased in number, in some cases in spite of a lowered serum IgM level.

Søltoft (1970) (117) studied treated and untreated cases of non-tropical sprue in humans. He found that IgM- and IgG-cells were significantly increased in both cases while IgA-cells were comparable to controls. Not only these cell types increased but the total number of immunoglobulin-containing cells was also increased.

Gelzayd et al (1971) (67) reported on a patient with weight loss, steatorrhea, undetectable serum IgA, and diminished levels of serum IgM and IgG. Examination of the small bowel mucosa revealed IgA-cells to be very rare while IgM- and IgG-cells were reduced in number. The same was true of rectal mucosa. Previously calculated normal cell density indices of IgA-, IgM-, and IgG-cells (78.65, 33.65, and 16.65 respectively) in the jejunum were higher than found in this case (2.10, 2.41, and 0.46 respectively). The same held true for the rectal mucosa except for IgG-cells (80.14, 6.04, and 2.45 in the normal compared to 0, 2.76, and 4.87 for IgA, IgM, and IgG respectively).

Johnson et al (1971) (80) examined intestinal tissue from human patients with nodular lymphoid hyperplasia by direct immunofluorescence. They found that there were no cells which stained for IgG or IgA but they did find a few focal accumulations of IgM-cells in the rectal and jejunal mucosa. There was even a lack of interstitial fluorescence to IgA.

Pettingale (1971) (100) reported that in six patients with the coeliac syndrome, three had IgA-cells predominant within the lamina propria of the jejunum, while in the other three there was a decrease in the numbers of IgA-cells compared to IgM- and IgG-containing cells. There was statistically significant difference in the overall means of IgA-containing cells, it being lower in the coeliac patients than in the controls.

Baklien et al (1972) (9) mapped out the distribution of IgA-, IgM-, and IgG-cells in the normal human intestinal mucosa. The mucosa was divided into three zones. The villi comprised one zone, the crypts another zone, and then a measured area between the two comprised the other zone. They determined the ratio of each cell type in the three zones. It was found that the ratio of IgG-immunocytes in the villi: below villi mucosa: crypt area of the mucosa was 8.2:1.9:2.2. For IgM-immunocytes this ratio was 5.3: 18.6:19.6. The entire lower duodenum was compared to the entire upper jejunum. The ratio of IgA-: IgM-: IgG-cells in the entire section (not broken down into zones) was 81.0:16.3:2.8 versus 81.9: 15.7:2.5 respectively.

Brown et al (1972) (30) studied humans with gastrointestinal disorders associated with immune deficiencies. In seven patients they found that immunofluorescent cells to each type of immunoglobulin (IgG, IgM, IgA, IgD, and IgE) were "sparse or absent" except in two cases where IgM-containing cells were normal or

increased and IgE-cells were increased. It was found that this general decrease in total numbers of each cell type was irrespective of the relative severity of the clinical signs. A methyl green pyronine stain histologically supported the lack of plasma cells.

Savilahti (1972) (110) examined the small intestine of clinically normal children. It was seen that IgM-cells were relatively constant in numbers despite age while IgA-cells became more numerous as the child became greater than 2 years old. IgG-cells did not tend to change in numbers.

Savilahti (1972) (111) studied children with coeliac disease. Patients with the disease tended to have increased numbers of IgA-cells (twice as many as normal), IgM-cells (two and one-half times normal), and IgG-cells (slightly increased) in the jejunum. IgD-cells also tended to be slightly increased. There was a subjective increase in lymphocyte-like positive cells compared to plasma cell-like positive cells. No differences were seen between patients above and below two years of age. Treatment resulted in a decrease in IgA-, IgM-, and IgG-cells so that they were indistinguishable from normal controls. In the diseased patients there was no positive correlation between the concentrations of IgA or IgM in intestinal juice and numbers of corresponding cell types in the lamina propria of the intestine.

Søltoft and Søeberg (1972) (120) looked at the numbers of immunoglobulin-containing cells in the small intestine of humans with viral hepatitis. The normal pattern of IgA-cells > IgM-cells > IgG-cells > IgD-cells > IgE-cells was maintained. However the total number of IgA-cells and IgD-cells was significantly increased while the rest were found in normal numbers, IgG-cells not being quantitated. They also found that the majority of these cells appeared to be plasma cells; but there appeared to be a relative increase in the number of lymphocytes which stained for the different immunoglobulins.

Søltoft and Søeberg (1972) (121) showed that the area of IgA-cells and IgM-containing cells in the human small intestine increased significantly during periods of acute enteritis. The area of IgD- and IgG-cells were each unaltered statistically when the acute enteritis cases were compared to the controls.

Söltoft et al (1972) (119) studied two patients with a humoral immune deficiency and an intestinal lesion resembling regional enteritis. Immunofluorescence demonstrated that all classes of immunocyte studied (IgA, IgM, and IgG) were decreased in one patient, while IgA-cells were decreased and IgM- and IgG-cells were increased in the other case studied.

Savilahti (1973) (112) examined several patients with a deficiency of IgA in the serum. IgA-cells were rare in the jejunal

mucosa. The IgM-cells in the deficient patients were almost equal in number to the IgA-cells of controls. The numbers of IgG-cells were slightly elevated. The rectal mucosa was the same except for a lower number of IgG-cells.

Shiner and Ballard (1973) (113) reported on their observations in normal patients versus untreated and treated coeliac patients. An increased number of IgA- and IgM-cells was found in the jejunum of one coeliac patient. An increase in IgG-cells in the jejunal mucosa was found in four out of five untreated coeliacs. A reduction in the number of IgA-plasma cells was seen in one treated patient.

Söltoft et al (1973) (118) examined human patients with ulcerative colitis. The number of IgE-containing cells was generally very small and appeared normal. In active ulcerative colitis, the area of IgA-containing cells in the jejunum was significantly elevated over normal. If the same patients were re-examined when the disease was inactive it was found that there was a significant decrease in the number of IgA-cells. The numbers of IgA-cells in the jejunum "showed no correlation with the duration of the disease, the actual attack, the extension of the disease, or the serum IgA level, either during the active phases or during the inactive phase...". The IgM-, IgG-, and IgD-cells in the jejunum were seemingly unaffected by the disease. Biopsy samples of the rectum showed that the area with

IgA-containing cells in active ulcerative colitis was higher than normal. This increase decreased significantly in states of remission. The same was true of IgG-cells. IgM- and IgD-cells did not appear to be affected by remission.

Gasbarrini et al (1974) (64) studied normal humans and humans with the celiac syndrome. It was found that immunofluorescence "is capable of identifying the three different cell populations individually with a high degree of statistical significance" in normal human mucosa but that the difference between cell types decreased in the pathologic tissue. There was a nonsignificant decrease in the number of IgA-cells while there was a significant increase in IgG-cells and even more so in IgM-cells in the duodenum of celiac patients.

Broom et al (1975) (25) demonstrated immunoglobulin-containing cells in the gastrointestinal tract of individuals without circulatory immunoglobulins. This was true for IgG, IgM, and IgA.

Brown et al (1975) (29) described the localization of IgE-containing cells in the human. IgE-immunocytes were generally found in about the same places as IgA-immunocytes. IgE-immunocytes could be found in all tissues examined (stomach, duodenum-jejunum, and colon-rectum) and were found to comprise about 2% of the total immunofluorescent-positive cell population (i.e. IgA-, IgM-, IgE-,

IgG-, and IgD-immunocytes). Only IgD-cells appeared to be rarer than IgE-cells. IgE-cells tended to be non-uniform within an organ and between individuals. It was also found that the absolute number and the percentage of IgE-cells decreased as the distal end of the alimentary tube was approached. The area of highest density of IgE-cells was at the base of ulcers where they were at least ten times more numerous than normal.

Mul et al (1975) (93) studied a variety of pathologic states in the gastrointestinal tract. In particular the ratio of kappa to lambda IgA-cells was assessed. It was found that there was a ratio of 63:37 (K/λ) with a limited range for all patients, normal or diseased except those with lymphoproliferative processes. Furthermore, it was found that IgA-immunocytes composed 83% of the cells of the lamina propria while IgM- and IgG-cells composed 12% and 5% respectively. There was a very wide range for the individuals.

Baklien and Brandtzaeg (1976) (8) stated that in normal or slightly inflamed ileal mucosa from patients with Crohn's disease, the total number of immunocytes was increased three times, but the ratio of IgA-: IgM-: IgG-cells remained near normal (83.1:11.4: 5.4 respectively). In severely inflamed mucosa with persisting glands, the immunocytes were increased 12.2 times and the IgA-cells decreased while the IgM-cells and especially the IgG-cells increased. The IgG-cells were increased primarily in the deeper areas of the bowel wall.

Baklien et al (1976) (10) reported the finding of a boy with gastrointestinal illness of four years duration. Immunofluorescent examination of the stomach, proximal small intestine, and rectum showed that greater than 95% of the cells were IgA-immunocytes while less than 5% were IgG- or IgM-immunocytes.

Baklien et al (1976) (11) compared the immune morphology of the human proximal jejunum in adult celiac disease and dermatitis herpitiformis. Compared to controls the total number of immunocytes in the lamina propria of the small intestine was raised by a factor of 2.5 and 2.0 respectively. IgA-containing cells were increased 2.2 times and 1.6 times respectively, and IgM-cells were increased 3.0 and 3.1 times respectively. There was no statistically significant difference in the absolute or in the relative cell type distribution between the two diseases.

Summary of the Review of the Uses of Fluorescent Antibody Demonstration of Intracellular Immunoglobulins in Humans

Fluorescent antibody techniques have been used to demonstrate immunoglobulin-containing cells in many normal and pathologic human tissues. These cells have been examined objectively and subjectively by several different methods.[9,22,24,45,67,95,116] Such quantitation, while differing between investigators, has proved consistent for individual investigators. Furthermore, different investigators using different quantitation techniques have arrived

at similar conclusions regarding the normal and pathologic gastrointestinal tract (i.e. sprue was associated with increased numbers of IgM-cells,[11,54,55,64,75,111,113,117,122] while ulcerative colitis had an increase in the numbers of IgG-cells[21,115]) although some disagreement has occurred.[21,65] Fluorescent techniques are now so well established that in some cases the existence of intracellular immunoglobulins in an individual which would have been thought to be devoid of any such cells was proven by this method.[25,124] Therefore, fluorescent antibody demonstration of intracellular immunoglobulins (especially but not exclusively in the gastrointestinal tract) has proved to be a reliable, accurate, and (for at least individual investigators or groups) repeatable technique capable of being used in clinical and basic sciences.

THE CLINICAL AND INVESTIGATIONAL USES OF FLUORESCENT ANTIBODY TECHNIQUES TO DISCERN INTRACELLULAR IMMUNOGLOBULINS IN ANIMALS

Intracellular Immunoglobulins

Vasquez (1961) (138) reviewed prior work on antibody-containing cells and made the conclusions that 1.) plasma cells were the major contributor to antibody formation, 2.) lymphocytes can also contain antibodies, and 3.) antibody-containing cells are identical with gamma globulin-containing cells. This work was done in large part by fluorescent antibody techniques.

Cebra et al (1966) (36) observed that only 2% (at most) of the cells seen had more than one type of heavy chain determinant (α, μ, γ). This held true regardless of the tissue examined. It was also noted that germinal centers were primarily composed of only one type of cell although other types of cells were always seen sparsely scattered throughout. It was also seen that cells could be stained for allotypic markers and, if fluorescein and rhodamine conjugates were used with separate antisera simultaneously (i.e. FITC-anti μ chain and rhodamine conjugated anti-Aa1) one could determine the numbers of cells which had both of these markers versus the number of cells which had only one or the other.

Crabbé et al (1968) (42) used immunofluorescent techniques to demonstrate quantitatively fewer IgA-cells in the germ-free mouse than in the conventional mouse. It was noted in the

conventional mice that IgA-cells were primarily seen at the base of the villi and that not all (although most) of the villi had IgA-cells in them. Germ-free mice rarely had IgA-cells in the villi and when they were found they were usually clustered together.

Crabbé et al (1969) (49) immunized adult germ-free mice with horse spleen ferritin and then determined the classes of antibodies being produced to this antigen by utilyzing fluorescent antibody techniques on cells from various areas. In extra-intestinal areas the cells producing anti-ferritin were of the IgM or IgG class whereas in the intestinal mucosa the cells that resulted from oral exposure to ferritin were primarily of the IgA class.

Crabbé et al (1970) (50) studied adult and growing conventional and germ-free mice from the standpoint of immunocytes. Adult conventional mice were seen to have IgM-cells in large clusters in the red pulp of the spleen. The mesenteric and peripheral lymph nodes had fewer immunoglobulin-bearing cells with IgM being predominant in the peripheral lymph nodes. Adult germ-free mice had fewer IgG_1- and IgA-cells in the spleen.

Kincade and Cooper (1970) (83) reported that IgM-containing cells were the first to appear in the chicken and that IgG-cells appeared later. They could also demonstrate light chains within these cells.

Craig and Cebra (1971) (51) used fluorescent techniques to demonstrate that the Peyer's patches were an enriched source of cells which are IgA-immunocyte precursors in the rabbit. When allogeneic Peyer's patches' cells were inoculated into irradiated hosts, most of the cells in the host's spleen were IgA-cells versus IgG-cells (the latter was the case when a lymph node was the source of inoculation).

Rudzik et al (1975) (107) used immunofluorescence to qualitate and quantitate IgM-, IgG-, and IgA-cells in repopulation experiments and was able to obtain statistically useful results.

The Presence of Intracellular Immunoglobulins in Non-Alimentary Tract Tissues

Coons et al (1955) (41) described the presence of large numbers of plasma cells in the spleen of immunized rabbits by an indirect fluorescent technique designed to demonstrate those cells producing antibody to a specific antigen.

Cebra and Goldstein (1965) (37) studied rabbit spleen imprints and showed that IgG-cells were the predominant immunoglobulin found in the spleen and that these cells usually contained light chains also.

Halliwell (1973) (71) examined IgE bound to mast cells in the canine skin and also checked for the presence of IgA-, IgM-,

and IgG-cells in the dermis. No cells containing any of the three immunoglobulins were detected in the dermis although IgG and IgA were found. IgG was interstitial and IgA was in the sebaceous glands and hair follicles.

Chapman et al (1974) (38) examined the order in which different immunoglobulin-containing cells appeared in the spleen, lymph nodes, thymus, and intestine. In the extra-intestinal tissues IgM-cells were the first to be seen except in the thymus. In the intestine IgM- and IgG-cells appeared at 90 days post conception around the intestinal lymphoid follicles. After birth the IgM- and IgG-cells increased in the gut-associated lymphoid tissue.

Halliwell (1975) (72) studied the IgE-cells in the canine. In skin biopsies it was demonstrated that the IgE-cells (IgE on membrane or in cytoplasm) were mast cells. Staining for IgM, IgG, and IgA did not reveal any cells. IgA- and IgE-cells were rare in the peripheral lymph nodes. Mast cells with IgE in them were distinguishable from IgE-plasma cells due to qualitative staining differences.

Khaleel et al (1975) (82) demonstrated the presence of immunoglobulin-containing cells in the equine. Salivary glands had scattered interstitial mononuclear cells positive for IgG (all subclasses). IgB-cells were not demonstrated but IgM- and

IgA-cells were found in a distribution similar to the IgG-cells. The mesenteric and parotid lymph nodes showed many IgG-cells in the cortex, the cells generally comprising the germinal centers. IgG(T)-cells were located at the periphery of germinal centers. IgM-cells were seen in the germinal centers and medullary cords. IgB-cells were very rare. IgA was confined to occasional cells in the cortex but was never present in the germinal centers.

Pospíšil et al (1975) (103) performed immunofluorescent techniques on swine spleen, mediastinal and mesenteric lymph nodes, duodenum, jejunum, and ileum. The spleen and lymph nodes were seen to primarily have IgG-cells and they were located in the germinal centers of the lymph follicles. IgA- and IgM-cells were present but scarce.

The Presence of Intracellular Immunoglobulins in Alimentary Tract Tissues

Crandall et al (1967) (52) determined the relative proportions of IgA-, IgM-, and IgG-cells in the rabbit small intestine, spleen, popliteal lymph node, and diaphragm during infection with Trichinosis spiralis. "A relative increase in IgM-containing cells in the intestinal mucosa was observed after early infection with Trichinella...".

Vaerman and Heremans (1969) (134) examined the spleen, lymph nodes, and gastrointestinal mucosa of the canine. The spleen and

lymph nodes were predominantly filled with IgG_{2ab}-plasma cells. IgM-, IgG_{2c}-, and IgG_1-cells were inconstant between separate organs from the same or different animals. IgA-cells were generally scarce except in the mesenteric lymph nodes and the gastrointestinal tract. Here IgA-cells were the predominant cells found, IgM being the next most numerous cell type. There appeared to be little difference in the various levels of the gastrointestinal tract sample. IgA-cells were predominantly seen below the villi and the crypts but were also seen in the villi. IgM- and IgG_{2c}-cells were most common in the deeper layers of the lamina propria. Quantitative estimates were made of the different cells in the gastrointestinal tract but since "IgA-type cells were counted in fields where cells were not too closely packed...and other...cells were numbered in fields selected for the abundance of staining cells" these values are of doubtful statistical significance.

Allen and Porter (1970) (1) examined porcine intestinal tissues. IgG was found in the extravascular spaces as well as in plasma cells in lamina propria. IgM-cells were distributed, as were the IgA-cells, in the intercrypt lamina propria.

Crabbé et al (1970) (50) studied adult and growing conventional and germ-free mice from the standpoint of immunocytes. In conventional adult mice IgA-cells predominated in the intestine

and were found primarily between glandular crypts and beneath the villus epithelium. IgA-cells were very abundant in the basal aspect of the villi. IgM- and IgG_1-cells were sparse in the intestine with IgM-cells being more numerous than IgG_1-cells and both types were most commonly found at the periphery of Peyer's patches. The intestines of adult germ-free mice had far fewer IgA-cells, especially at the villi; however, once again more cells were seen next to Peyer's patches. Germ-free mice which were conventionalized showed progressive increases in IgA-cells in the intestine, the villi next to the Peyer's patches being the first to be populated. It was considered that the Peyer's patches and mesenteric lymph nodes generally represented "an immunocyte composition which was intermediate in type between that of the intestinal mucosa and that of the spleen and peripheral lymph nodes".

Porter and Allen (1970) (102) stated that IgA-plasma cells were primarily found in the intercrypt stroma although an occasional IgA-cell was found in the villi.

Atkins et al (1971) (7) examined the intestinal mucus membrane, pyloric glands, and Brunner's glands of the pig for the presence of IgA and IgA-staining cells. All areas of the intestine had intense immunofluorescent staining. The plasma cells which did stain were distributed the same throughout the intestines except

in the ileum where they decreased as the Peyer's patches increased. Very few cells in the pyloric glands or Brunner's glands fluoresced.

Leslie et al (1971) (89) studied chicken tissues to demonstrate the presence of immunoglobulin cells and the types of such cells. Conjugated antisera against light chains demonstrated that most of the antibody cells of the chicken lay in the duodenal mucosa and cecal tonsils despite considerable variation in total numbers of cells between animals. IgY which had earlier been shown to be the predominant secretory immunoglobulin of the chicken was shown to be the predominant cell type present (as compared to IgM-cells) in all tissues except the spleen where there were about equal numbers of IgY- and IgM-cells. IgM- and IgY-cells were determined to constitute the majority of the plasma cells in the tissues examined. The authors considered that there was strong evidence for a local immune system in the chicken.

O'Daly et al (1971) (94) studied rabbits with respect to b-markers, α-chains, and secretory component. They reported the presence of numerous plasma cells surrounding the epithelium of the crypts of Lieberkühn. These plasma cells were stained for an allotypic marker of light chains (b_4) and thus demonstrate the presence of immunoglobulins in these cells.

Allen and Porter (1973) (2) studied the small intestine and determined the relative distribution of IgM- and IgA-cells in

unweaned piglets. IgA-cells were primarily found in the area between the crypts while rarely in the villus itself. IgM-cells were distributed likewise but with a few more being found in the core of the villi. The number of IgM-cells was significantly larger than the number of IgA-cells at three levels of the intestine (duodenum, jejunum, and ileum). While the ratio of IgM-cells to IgA-cells was fairly constant, the absolute numbers were highest in the duodenum and lowest in the ileum being about ten times higher in the duodenum than in the jejunum which was higher than the ileum. Mesenteric lymph nodes showed a similar predominance of IgM-cells over IgA-cells.

Allen et al (1973) (3) reported the presence of many IgA-lymphoid cells in the porcine small intestine. These cells were primarily around the crypts and especially in the duodenum.

Kincade and Cooper (1973) (84) examined IgA-cells in developing chicks. It was found that few circulating lymphocytes carried surface IgA. IgA-containing plasma cells were rare in the spleen but numerous in the lamina propria of the villi in the small intestine.

Brown et al (1974) (28) found many immunoglobulin-containing plasma cells in the porcine small intestine at various levels. He did not quantitate or qualitate these cells.

Halliwell (1975) (72) studied the IgE-cells in the canine. In the gut IgA-cells were the most common followed by IgM- and IgG-cells which were roughly equal and totalled approximately 25% to 50% of the IgA-cells. The numbers of cells were highest in the jejunum. IgE-cells were relatively numerous in the intestine and could be found in the presence or absence of parasites and could also be missing in both cases. IgE-cells were located as far distally as the colon. Locally the IgE-cells tended to be superficial. IgE-cells were particularly common in lymphoid nodules in the duodenum. In mesenteric lymph nodes, the IgG-cells were greatest in number followed by IgM-cells and then IgA-cells. IgE-cells were relatively common here also.

Khaleel et al (1975) (82) demonstrated the presence of immunoglobulin-containing cells in the equine. The duodenum was discovered to have abundant foci of the IgG_a-cells. IgG(T)-cells were present in fewer numbers. IgM-cells were found near the epithelium of the villi and the surrounding lymphoid nodules. IgA-cells were scattered throughout the lamina propria of the duodenum.

Pospíšil et al (1975) (103) performed immunofluorescent techniques on swine spleen, mediastinal and mesenteric lymph nodes, duodenum, jejunum, and ileum. Peyer's patches were similar to spleen and lymph nodes but IgA was the predominant immunoglobulin present. IgA-cells were likewise the main cell type in the

intestines and they were located primarily in the basal portion of the duodenal and cranial jejunal lamina propria. All three cell types were scattered in the vicinity of the intestinal crypts, IgG- and IgM-cells being rare.

Allen et al (1976) (4) reported that IgM-cells appeared in the small intestinal mucosa of 3 to 4 week old piglets. They were most numerous in the duodenum and least frequent in the ileum. Most of the cells present were in the intercryptal tissues although rare IgM-cells were seen in the villi.

Brown and Bourne (1976) (26) studied porcine tissues for the presence and distribution of IgA-, IgM-, and IgG-cells. No such cells were located in the esophogeal lamina propria. Gastric lamina propria had few IgG-cells present, usually associated with lymphocytes, but many IgM- and IgA-plasma cells extending between the glands. The duodenum had fewer IgG-cells than either IgM- or IgA-cells. The IgA- and IgM-cells were both primarily between the crypts of Lieberkühn and occasionally extended up the base of the villi. IgG-cells were primarily in the deeper regions of the lamina propria. The ileal lamina propria with Peyer's patches was much like the duodenum only all the cell types were decreased in number. Lymphoid follicles had few staining cells within them, these being located primarily at the periphery of the follicle. The cecum, rectum, and colon stained much the same as did the duodenum

and ileum without Peyer's patches. The mesenteric lymph nodes had about equal numbers of all three types of cells which were primarily found in the subtrabecular sinuses.

Brown and Bourne (1976) (27) examined the intestine of growing pigs for the presence of IgA-, IgM-, and IgG-cells. At one week of age all cell types were detectable in the duodenum. IgM-cells outnumbered the IgA-cells until 3 weeks of age at which time the IgA-cells became the predominant cell type. IgG-cells were non-uniform and found throughout the intestine in lesser numbers than IgA- or IgM-cells.

Erlandsen et al (1976) (58) showed that IgA can be localized to plasma cells and a subpopulation of Paneth cells in the rat small intestine. The distribution and intensity of the IgA-staining Paneth cells varied from animal to animal but generally appeared to increase in frequency as the anus was approached.

Rodning et al (1976) (104) examined mice and quantitated IgA-cells and Paneth cells in conventional and gnotobiotic mice and conventional mice with iatrogenic self-filling blind loops. There was a decrease in the percentage composition (percent of the area examined composed of a particular cell type) of IgA-cells in gnotobiotic mice compared to conventional mice, but there was only slight reduction in the blind-loop mice. There was an equal decrease in IgA-containing Paneth cells in both

gnotobiotic and blind-loop mice. However, regardless of which class of animal was examined, the IgA-cells were always in the mid and lower villocrypt region.

Summary of the Review of the Use of Fluorescent Localization of Intracellular Immunoglobulins in Animals

The same techniques that have been applied to human tissues have been applied to animal tissues from many different species. Subjectively most of these results are comparable to previously reported results in humans, such as:

1.) one cell generally contains one type of heavy chain and one type of light chain,[3,6]

2.) there is a preponderance of IgA-cells in those areas where they would be expected,[1,2,3,7,26,50,84,102,134] and

3.) there is a preponderance of IgG-cells in the spleen and lymph nodes[37,103]

although some differences have been reported.[82] It has been shown that statistically significant results can be obtained by using the fluorescent antibody technique on animal tissues.[108] In contrast to humans, little quantitation of these cells has been done comparing normal and pathologic states.[52]

MATERIALS AND METHODS

PREPARATION OF MONOSPECIFIC ANTISERA

Antiserum 100 (rabbit anti-human μ obtained from Miles Laboratories, Elkhart, Indiana, see chart 1 for this and all other antisera and sera denoted by numbers) was shown to react with canine serum by double diffusion analysis in 1.25% Noble agar. The line of precipitation between pooled, normal canine serum and antisera 100 formed a "line of identity" with the line of precipitation between pooled, normal canine serum and antisera 101 (goat anti-canine μ obtained from Microbiological Associates, Bethesda, Maryland) thus demonstrating that IgM was the component in canine serum to which antisera 100 reacted.

Antiserum 100 was added to pooled, normal canine serum at a ratio of 1:6 respectively, mixed gently, and placed at 4°C for 12 to 24 hours. This mixture was then spun down at 14,000 rpm for at least 15 minutes in a centrifuge at 4°C. The precipitate obtained was resuspended in 2cc of saline. This mixture was then mixed thoroughly with an equal volume of Freunds Complete Adjuvant (obtained from Difco Laboratories, Detroit, Michigan) by placing the substances in each of two 6cc plastic syringes, connecting the two with a 4 to 6 inch length latex tubing, and repeatedly forcing the contents through the tubing into the other syringe. This was done until the mixture became thick and was difficult to pass back and forth. It was necessary to refrigerate the apparatus from time to time to aid in the thickening process. A New Zealand white rabbit was then

injected with this mixture in 2 or 3 subcutaneous sites. This was done three times at approximately twenty day intervals. Thirty days after the first injection, serum was obtained by ear bleeding the rabbit and it was tested for antibody activity against canine serum by double diffusion in agar. It was seen that this serum (antiserum 200), reacted with several components in the serum; therefore, weekly bleedings of 30 to 40cc per week were begun. Immunoelectrophoresis was performed on an LKB electrophoresis apparatus with 0.85% Noble agar (obtained from Difco Laboratories, Detroit, Michigan) slides in a sodium barbital buffer prepared according to Campbell.[35] This was done at pH 8.4 for 2 hours at 260V in a 4°C refrigerator. Immunoelectrophoresis showed multiple arcs occurring in the anode region and at least one faint arc in the distant cathode region. This serum was stored and frozen.

Four New Zealand white rabbits were injected with 5mg quantities of canine IgG (obtained from Miles Laboratories, Elkhart, Indiana) mixed with equal parts of Freunds Complete Adjuvant. After the first injection, Freunds Incomplete Adjuvant (obtained from Difco Laboratories, Detroit, Michigan) was substituted for the complete adjuvant. The rabbits were inoculated at twenty-five day intervals for a total of four injections. Fifty days after the initial injection serum was collected and found to react strongly with canine serum in double diffusion analysis. Immuno-electrophoresis with normal canine serum electrophoresis and this serum (antiserum 201) in the trough showed at least one to three arcs occurring in the anode region.

Normal canine globulin was obtained by ammonium sulfate precipitation from pooled normal canine serum by the method of Campbell.[35] Approximately 250mg of protein from this procedure was placed on a 80cm x 2.5cm column containing Biorad A 1.5 (obtained from Bio Rad Laboratories, Richmond, California) equilibriated with phosphate buffered saline (0.01 M phosphate in 0.5 M NaCL). Five ml fractions were collected until at least 550cc was passed over the column. A chromotograph was obtained by reading the optical density of the tubes' contents at a wavelength of 280nm on an ultraviolet spectrophometer. Tubes were pooled as shown in figure 1 and concentrated. The fraction represented by AIII (see figure 1 for this fraction and for fractions AI and AII throughout the rest of the paper) was shown by double diffusion analysis against antiserum 102 (rabbit anti-canine γ obtained from Miles Laboratories, Elkhart, Indiana) to contain canine IgG. When the AIII fraction was electrophoresed and antiserum 103 (rabbit anti-whole serum obtained from Miles Laboratories, Elkhart, Indiana) was placed in the trough, a single line of precipitation was seen in the anode region. The protein concentration of the AIII was 1.498mg/ml as determined by using the formula 1.55 (optical density at 280nm) - 0.77 (optical density at 260nm) = mg per ml. The AI fraction was shown by double diffusion analysis with antiserum 101 to contain IgM but was contaminated with a small amount of IgG as seen by double diffusion analysis with antisera 102. The AI fraction was shown to have 1.065mg/ml of protein.

A commercial cold alcohol fraction of normal canine serum (Canalb^{R} obtained from Pitman-Moore, Fort Washington, Pennsylvania, serum 300) was found to have IgM present. There was no line of precipitation visible between antiserum 101 and serum 300 however. Absorption of antiserum 200 with serum 300 rendered antiserum 200 void of any activity against IgM. This was demonstrated by using partially purified canine milk whey, which was previously shown to have IgM present. Serum 300 was then precipitated with ammonium sulfate according to Campbell[35] and the supernatant saved. This supernatant was described as serum 301. Serum 301 was electrophoresed in agar and was tested against antiserum 103. At least four arcs in the early and distal cathode region were seen. None of the arcs appeared in the region where immunoglobulins were thought to be present. When serum 301 was mixed with antiserum 200, the lines of precipitation in the anode region were not abolished while the lines of precipitation formerly seen in the cathode region were abolished. It was then thought that the antisera was reacting with heavy and light chains of IgM and perhaps μ-chain.

In an effort to remove light chain activity from antiserum 200, it was mixed with fraction AIII in addition to serum 301 in a 3:2:3 ratio respectively. This was antiserum 202. This was mixed gently and incubated at 4°C for 12 to 24 hours and then spun at approximately 2,000 rpm for 5 to 10 minutes at room temperature to remove the precipitate. The supernatant was then removed. Double diffusion analysis now showed a single band of precipitation

between antiserum 202 and pooled normal canine serum. Antiserum 202, when reacted against AI formed a "line of identity" with the precipitation line between antiserum 101 and AI as shown in figure 2. Immunoelectrophoresis showed a single arc between pooled normal canine serum (electrophoresed and serum 202 which occurred in the anode region. This arc was nearly identical to the arc formed between antiserum 101 and pooled normal canine serum in an immunoelectrophoresis.

Antiserum 201 was absorbed with AI at 1:4 respectively. This was antiserum 203. Double diffusion analysis in agar now showed lines of precipitation between a.) AIII and antiserum 203, b.) AI and antiserum 203, and c.) antiserum 202 and antiserum 203 which were "lines of identity". This demonstrated that 1.) there was IgG in AI as stated earlier, and 2.) antiserum 202 was active against IgM in the presence of an excess of canine IgG. When pooled normal canine serum was electrophoresed and then reacted against antiserum 203, two partially fused arcs were demonstrated. It was felt that this may have represented a reaction to a subclass of IgG with different electrophoresis mobilities or it may have been due to another component of serum. We did not determine what these components were; however, they were not IgM or IgA. Hence it was felt that antiserum 202 was monospecific for canine IgM while antiserum 203 while not monospecific for IgG, did not react with IgM.

PROVING MONOSPECIFICITY OF FITC ANTISERA OBTAINED COMMERCIALLY

Fluorescein isothiocyanate conjugated goat anti-canine (obtained from Microbiological Associates, Bethesda, Maryland, antiserum 104) was divided into aliquots and stored in a freezer at -70°C. It was found to satisfactorily cause fluorescence at a 1:16 dilution. Specificity of this antiserum was shown by:

1.) Applying antiserum 202 to the tissue and being able to totally block out all green fluorescence. (Compare figures 4 and 5.)

2.) Adding AI to our antiserum 202 which already had excess IgG in it, and using this serum to attempt to block the specific fluorescence. This did not block the fluorescence showing that IgM would block the blocking effect of antiserum 202.

3.) Adding AI to antiserum 104. After this step the conjugated antiserum would not cause fluorescence in the tissue. This showed that addition of IgM with an IgG impurity would prevent binding or tie up the binding sites.

4.) Applying AIII to the tissue (attempting to block fluorescence) and the applying antiserum 104 showed no diminution of fluorescence.

5.) Applying serum 300 and then applying antiserum 104 failed to block specific fluorescence. These last two results showed that the blocking factor(s) found in antiserum 202 were associated with the rabbit serum and not the materials used to absorb the rabbit serum.

6.) Absorbing the tissue first with antiserum 102. This did not abolish fluorescence showing that abolition of IgG binding sites in the tissue (plus whatever else the antisera reacted to) did not affect the reaction of antiserum 104 and the tissue IgM.

7.) Applying antiserum 104 to the tissue first and then absorbing with antiserum 202. This did not abolish the fluorescence which showed that the order of application of the reagents was critical and it was not some nonspecific process in our antisera which abolished fluorescence in number 1 above.

8.) Applying nonspecific goat IgG conjugated with fluorescence (antiserum 105) did not reveal any specific staining which demonstrated that the specific staining was not a property of goat IgG conjugated with fluorescein.

9.) Finally, sections which were not treated with antiserum 104 showed autofluorescence in the villi and at the submucosa. This autofluorescence was not in the same place where the specific staining with antiserum 104 occurred and was also different in quality of staining. This autofluorescence varied from intense to absent in different sections. While black and white film does not distinguish between the two, the autofluorescence was yellow to orange as opposed to green. (See figure 6) No green autofluorescence was seen in the sections not treated with antiserum 104 except occasionally in the submucosa of the ileum.

COMPARISON OF DIFFERENT METHODS OF TISSUE PREPARATION

Intestinal tissue from a dog was obtained and was treated in the following ways:

1.) frozen in liquid nitrogen, cut at 6 μ on a cryostat, air-dried, fixed in 75% ethanol for thirty minutes, air-dried, and then stained,

2.) as in 1 only it was not fixed in ethanol,

3.) fixed in cold 10% neutral buffered formalin for four hours, left in 30% sucrose overnight at 4°C, frozen in liquid nitrogen, sectioned on a cryostat, air-dried, and stained,

4.) as in 3 except that Modified Millonig's Fixative was used in place of formalin,

5.) as in 1 except a thirty minute wash in phosphate buffered saline at pH 7.3 was interposed before the ethanol-fixation,

6.) a cold ethanol-fixed, paraffin-embedded technique.

The ethanol-fixed, paraffin-embedded technique was chosen. It is described under Preparation of Tissues for FA.

DOGS USED IN THE EXPERIMENT

Seven intact, mature, male and female, mongrel dogs having weights ranging between 20 pounds to 65 pounds were used. All of the dogs were fed commercial dry dog food (Waynes Dog Food, Chicago, Illinois) once per day in the morning for at least three days prior to use. The dogs were allowed to exercise outside in wire enclosed runs at least once per day. Within 24 hours before the biopsy procedure a blood sample was drawn and a fecal sample collected. (see chart 2)

At the time of tissue sampling, the dogs were anesthetized with either sodium pentobarbital or 1:1 mixture of sodium pentobarbital and sodium thiamylal. While under general anesthesia, the abdomen was opened and the intestine exposed. Biopsy samples were taken by isolating a segment of gut, clamping the intestine and associated vessels on either side of the area with Kelly or Halstead hemostats to prevent exsanguination and to avoid getting excess blood on the mucosa which would contain IgM, and quickly cutting out that entire section of gut with a scapel. Three tissue sites were

sampled. The duodenum (defined here as the point at which the right lobe of the pancreas diverges from the intestinal wall), the jejunum (about 15 to 20 centimeters past the Ligament of Tritz), and the ileum (approximately 4 to 6 centimeters cranial to the cecum) were the sites chosen. After the tissues were collected the dog, still under general anesthesia, was euthanatized by an overdose of intravenous barbituate. A complete necropsy was not performed; the intestines were checked and a cursory inspection of other abdominal organs was made.

PREPARATION OF TISSUES FOR FA

The tissue sample was immediately cut longitudinally with scissors to allow the entire mucosa to face upward. This section of intestine was then divided into two parts and each was placed on a separate wooden tongue depressor and fastened to it with the mucosa side up by at least four needles. The purpose of the needles was to keep the tissue from curling before or during fixation. One sample was immediately placed in an ice-chilled normal saline solution to try to remove as much intestinal debris and blood as possible. This sample was allowed to remain in the saline for no more than thirty minutes and then was placed, still on the wooden tongue depressor, in 95% ethanol at $4^{\circ}C$ for 18 to 24 hours, then in 100% ethanol at $4^{\circ}C$ for 4 hours, and then in xylene at $4^{\circ}C$ for 36 to 48 hours. The other sample of the same tissue was meanwhile placed in neutral buffered formalin at room

temperature while still on the tongue depressor and allowed to fix for at least 48 hours.

When the fixation process was finished the tissues were examined for the most level area and from here at least one block (at least 3mm x 5mm) was cut and placed in a metal "cricket". The blocks from the formalin-fixed tissue were placed in an automatic processor, paraffin-embedded, cut at six microns (6μ), and stained with hematoxylin and eosin. The tissues from the cold xylene were likewise cut into blocks and placed in "crickets". These "crickets" were then put in paraffin at 56°C for 90 minutes and then placed in a vacuum of at least 20mm Hg for 30 minutes after which they were embedded in paraffin blocks and stored in a closed dessicator in a refrigerator at 4°C. These blocks were later placed in a microtome and sections were cut at 6μ. Whenever the blocks were cut on the microtome, the tissue was oriented so that the villi were pointed away from the blade of the knife and were perpendicular to the knife. This seemed to help prevent folding of the villi onto other parts of the tissue. Replicate paraffin sections were conventionally mounted and stained with hematoxylin and eosin.

The sections used for fluorescent microscopy were processed as follows: the excess paraffin was trimmed off with scissors and the tissue was placed on a 45°C acetone-cleaned microscope slide with a drop of demineralized water already on the slide to facilitate placing the section. The tissue was blotted onto the slide and

allowed to stay on the 45°C slide for 30 minutes after which it was placed in a refrigerator at 4°C. The slides remained at 4°C for at least 24 to 48 hours before being used. If they were used prior to this the tissue would often come off the slide and float away when placed in the different solutions. It was found that leaving the slides in the refrigerator was superior to using gelatin, albumin, or a commercial tissue adhesive to make the tissue sections stick to the slides. Excess heat (56°C for 30 minutes) proved too harsh for the immunoglobulins, the fluorescence in sections treated in this way being almost nonexistent.

When the slides were to be used they were taken out of the refrigerator and dipped repeatedly but gently into two separate sequential baths of xylene at 22°C for 10 to 30 seconds per bath. The excess xylene was then blotted off and the slides were placed in three sequential baths of 95% ethanol at 4°C for 5 to 30 seconds per bath and then three sequential baths of pH 7.3 phosphate buffered saline for at least one minute per bath.

All excess phosphate buffered saline was blotted off the slides with tissue paper. There was left a small drop of phosphate buffered saline in which the tissue section lay undisturbed.

Antiserum 104 was drawn up in a 0.8 to 1.1mm x 75mm capillary tube and one-half to one-third of the contents of this tube were placed on the tissue sample. The slide was then placed in a petri plate with moistened paper and covered and put in a darkened area for 15 minutes. The petri plate and slide were gently rocked from

two to five times for several seconds per time during this fifteen minute interval. At the end of this 15 minutes the conjugate was removed from the slide by gently washing the tissue section with phosphate buffered saline administered via a Pasteur pipette while the slide was at an incline. The slide was again blotted and the above staining procedure was repeated two more times. (This was due to an initial error in the dilutions in which the conjugate was diluted too much.) After the last staining procedure the slides were placed in at least two baths of pH 7.3 phosphate buffered saline at $4^{o}C$ for at least five minutes per bath. At the end of this time, the slides were partially blotted and a drop of a mixture of neutral glycerin and phosphate-buffered saline (9:1) at pH 8.5 was placed on the tissue and a coverslip applied with an effort to avoid air bubbles. The slides were now ready to be observed under the fluorescent microscope (as described below).

For autofluorescent studies the slides were prepared as before except after they were taken out of the third phosphate-buffered saline bath in the deparaffinizing series. The slides were then coverslipped without any further treatment and photographed. The coverslips were then removed gently and the glycerin was washed off in phosphate-buffered saline at pH 7.3 for 10 to 30 minutes. At this time they were stained with hematoxylin and eosin and coverslipped and examined under the light microscope.

FLUORESCENT MICROSCOPY

The slides were examined with a Leitz Orthoplan microscope with Ploem illumination. The light source was a HBO-200 bulb with a BG-38 heat sink, a K460 suppression filter, and a KP490 interference blue filter. For the most part the slides were examined at 100x to minimize the oxidation of the fluorochrome.

PHOTOGRAPHY

The largest area of each section with the fewest artifacts was selected for photography. The area was photographed with the edges of sequential pictures overlapping so that a composite could be made later by taping the pictures together. The area photographed included the lamina propria from its submucosa junction to at least the base of the villi.

A Leitz Orthomat camera was used to take black and white and color photographs of the slides. Kodak Tri-Pan 400 ASA black and white film was used with the camera setting at 200 or 100 ASA and between 10 to 25 optic field ratio. All pictures for counting were taken at 100x magnification (using a 10x objective plus a 10x eyepiece) plus 3.2:1 magnification in the camera. The film was then hand developed at 23.8^{o}C with Kodak Midrodol developer in a 1:3 dilution for 13 to 15 minutes depending on changes in the temperature. Development generally was performed at 23.8^{o}C. The negatives were hand printed on Kodak F-3 Kodabromide paper. To allow fluorescing cells and the outline of the tissue to both

be plainly seen, the prints were slightly underexposed. For color photography Kodak EH-135 reversal film was used at 19 to 20 DIN and at a 10 to 40 optic field ratio on the above equipment. This film was developed commercially.

A print of a slide micrometer measured in hundreths of a millimeter was used to provide a scale for measurement. The negative for this print was taken under the same set of conditions as were the negatives of the IgM-cells in the lamina propria. The negative enlarger was then set at a specific location and left there after this negative was printed. In this way a direct measure of the area of the intestine concerned was possible.

After the photos were printed the margins were cut off and they were taped together on the back in accordance with the outline of the tissue. Then the largest area which had minimal artifacts was visually determined and marked off with a pen and a straight edge. In the ileum an area was chosen where there were no lymph follicles or nodes. In all cases a quadrilateral was constructed with the base at the mucosal-submucosal junction and the top line running at the base of the villi. Then another line was drawn at both ends forming the quadrilateral. These latter two lines were then marked at their midpoint and a line was drawn through these two points. (see figure 7) The area denoted as "I" in the figure was the area counted.

DETERMINING THE AREA COUNTED

The area of the quadrilaterals counted was determined by drawing

the two diagonals of the quadrilateral and measuring their length in centimeters and the acute angle formed by their intersection being measured in degrees. The values were then used in the equation $[A = \frac{1}{2}k(a)(b)(\sin\phi)]$ where A= actual area counted in mm^2, a= length of one diagonal in centimeters, b= length of other diagonal in centimeters, $\sin\phi$= sin of the acute angle formed by the diagonals when the angle was expressed in degrees, and k= a constant which will convert centimeters on the photograph to millimeters of intestine. This value was obtained by using the micrometer negative previously mentioned. It was determined how many centimeters on a ruler equalled a certain number of millimeters on the micrometer (i.e. $\frac{0.14mm}{1.19cm}$). This value was then squared (to account for the two diagonals measured in centimeters). The area was calculated to seven significant decimals. The area was determined one time for each photograph and served as the area for all counts from that photograph.

COUNTING THE CELLS

The cells were counted three times in each section. The author counted them two separate times (using techniques 1 and 2) and an assistant counted them once using technique 2. For each technique an IgM-cell was defined as that which:

1.) had a nucleus that did not fluoresce,

2.) had discernible fluorescent cytoplasm which was homogenous as opposed to granular,

3.) was located in the tissue as opposed to the lumen of an intestinal gland, and

4.) was of the size of the other fluorescing structures believed to be cells.

The first technique consisted of moving an opaque material (i.e. sheet of paper) across the photo and counting the IgM-cells before they were covered with the paper. The second technique consisted of placing a sheet of transparent plastic over the photo and marking over each IgM-cell with a black wax pencil. Then the black marks were counted. The number of cells per unit area ($1mm^2$) was obtained by dividing the total number of cells on a slide obtained from one of the three counts by the area.

The above was also done on the untreated slides; however, two different counts were made. These were 1.) any autofluorescence which resembled a cell, and 2.) anything of any shape or form which fluoresced. When counting the former, it was recognized that many, or at least some, of the "cells" counted would be so faint on stained sections that they normally would not be counted there and hence would not affect the absolute counts. They were counted despite this fact in order to be more consistent in counting the autofluorescent cells. If one would have to make a judgement as to whether or not a cell was bright enough to count, this would certainly make the counts less reproducible. A least two slides were used for each block and each slide was counted two times, once by counter M_2 and once by counter N.

STATISTICAL ANALYSIS

The data on the number of cells (IgM, autofluorescent, or any fluorescence) was analyzed by an unequal subclass analysis

of variance. The data was fitted to the two-way factorial, with interaction, model y= U + D + T + D*T, where y= the number of cells found per unit area, U= average number of cells per unit area for the entire experiment, D= the effect on y due to dog type, T= the effect on y due to tissue type, and D*T= the effect on y due to the interacting of dog type and tissue type.

QUANTITATION OF PLASMA CELLS AND EOSINOPHILS FROM HEMATOXYLIN AND EOSIN SECTIONS

The hematoxylin- and eosin- (H and E) stained sections from the formalin-fixed tissue and the ethanol-fixed tissue were examined under a Leitz Ortholux scope with a 3.2:1 magnification graticule in the eyepieces. The slides were examined at 400x. Two fields were chosen for quantitation as follows: the fourth villi from each end was determined and the eyepiece graticule was aligned with its long side on the base line of the villi (where the villi stopped and the mucosa began). The center of the rectangle was aligned with the center of the villi. If artifacts (i.e. folds or tears) were in the field or if engorged blood vessels were in the fields, the slide was moved progressively toward the middle of the section until the first area without such artifacts was located. Once such an area was found the plasma cells seen within the 3.2:1 graticule were counted. The following criteria determined if a cell was a plasma cell

1.) a round, dark-staining nuclei (in some sections the "cartwheel" appearance was uniformly absent from the plasma cells, if the cartwheel

appearance was present it was used; however if it was absent hyperchromic, round nuclei were sought instead),

2.) not being associated with intestinal glands (either as part of the gland or within the gland), and

3.) not of the appearance of fibroblasts or smooth muscle nuclei.

The eosinophils were not easily or accurately countable in the ethanol-fixed tissue therefore they were counted only on the formalin-fixed tissues. Eosinophils were defined as cells which had multiple, distinct red granules in the cytoplasma. Size was not useful in distinguishing eosinophils due to the variety of angles of cuts possible, which resulted in a multitude of shapes and sizes of cytoplasm seen with eosinophilic granules.

The photographic graticule was used so that the area counted could be easily determined by photographing a stage micrometer, measuring the size of the photograph, and then using a comparison factor to see how many mm^2 the photograph represented.

RESULTS

Data which lends Itself to Objective Evaluation with Numbers

CONSISTENCY OF OBSERVATIONS OF FLUORESCENT CELLS

Comparison of average numbers of IgM-cells per dog per mm^2 of a 6 μ section of a specfic tissue shows (see chart 3) that with the exception of the three counts noted, all counts are within two standard deviations of one another which is approximately a 95% confidence interval. The three counts which were outside this interval were from the same counter. This shows that there is a consistency between trained individuals: the results are repeatable. Dog 8 is excluded here since only M_1 counted it due to technical considerations.

UNEQUAL SUBCLASS ANALYSIS OF VARIANCE OF COUNTS OF IgM-CELLS (CHARTS 4 & 5)

1.) The null hypothesis Ho: "There is no significant dog by tissue interaction" is rejected at p= 0.00000.

2.) The null hypothesis Ho: "There is no significant difference between the three tissues" is rejected at p= 0.00041.

3.) The null hypothesis Ho: "There is no significant difference between the dogs" is rejected at p= 0.02857.

4.) The order of the tissue means going from the highest cell per mm^2 density to the least based on the averages of all dogs is J > D > I. However, means separations show that statistically there

is no difference between the duodenum and jejunum but the ileum is significantly lower than both.

UNEQUAL SUBCLASS ANALYSIS OF VARIANCE OF AUTOFLUORESCING CELLS (CHARTS 6 & 7)

1.) The null hypothesis Ho: "There is no dog by tissue interaction" is rejected at p= 0.027870.

2.) The null hypothesis Ho: "There is no difference among tissues" is not rejected at p= 0.24697.

3.) The null hypothesis Ho: "There is no difference among dogs" is not rejected at p= 0.13948.

UNEQUAL SUBCLASS ANALYSIS OF VARIANCE OF ALL AUTOFLUORESCENCE (CHARTS 8 & 9)

1.) The null hypothesis Ho: "There is no dog by tissue interaction" is rejected at p= 0.01019.

2.) The null hypothesis Ho: "There is no difference among the tissues" is rejected at p= 0.06222.

3.) The null hypothesis Ho: "There is no difference among the dogs" is not rejected at p= 0.10535.

4.) The order of the means from greatest to least in fluorescing structures per mm^2 of a 6 μ section is ileum, jejunum, duodenum; however, means separations shows that the ileum is significantly greater than duodenum or jejunum (p= 0.03050 and p= 0.04967 respectively).

COMPARISON OF AUTOFLUORESCENT CELLS TO TISSUE EOSINOPHILS (CHART 10)

Visual comparison of autofluorescent cell means for each dog-tissue block and the numbers of eosinophils from the same tissue (but not from the same paraffin block) shows little or no correlation between eosinophils and autofluorescent cells.

COMPARISON OF PLASMA CELL DENSITIES TO IgM-CELL DENSITIES (CHART 11)

Comparison of the total number of plasma cells per mm^2 of a 6 μ section to the total number of IgM-cells per mm^2 of a 6 μ section revealed that in every case the total number of plasma cells was greater than the total number of IgM-cells. This was true for formalin-fixed tissue and the ethanol-fixed tissue used in the fluorescent antibody procedure.

Data which did not lend Itself to Objective Evaluation with Numbers

1.) Interstitial IgM appeared most plentiful near the mucosa-submucosa junction. This interstitial IgM made it difficult to accurately count cells in the deeper mucosa while there was not this impediment to counting cells in the outer or superficial layers.

2.) The sections treated with antisera 104 included specific fluorescing material within the intestinal glands. This was found predominantly in the duodenum and jejunum where there were more IgM-cells present and it also was seen clearest in the deeper aspects

of the glands. However, this finding was not constant and there were some glands in which no staining was detected. (see figure 8) Although not shown by black and white photography there was minimal staining of the apical portions of the glandular epithelium.

3.) There was a definite lack of detectable IgM-cells in the villi of all dogs except dog 10. Dog 10 was the only one found to have intestinal parasites. (see figure 9) Treating the sections with antisera 106 (which like antisera 102 was shown to probably not be *monospecific* for IgG but also probably did not react to IgM) showed numerous cells extending up into the villi of all sections observed. (see figrue 10) These cells had a morphology consistent with plasma cells.

4.) The cells which fluoresced appear to be either plasma cells or lymphocytes. There was characteristically a nonstaining nucleus eccentrically located in a fluorescing cytoplasm. Some cells had narrow rims of cytoplasmic fluorescence which may be due to a.) their being lymphocytes or b.) the plane through which the plasma cells were cut. (see figures 11a and 11b)

5.) Isolated lymphoid follicles were *common* in the ileum. These follicles were observed both on hematoxylin and eosin and under fluorescent microscopy to originate in the submucosa and form a dome lined by epithelium which projected into the intestinal lumen. These were not typical Peyer's patches. The latter are usually much larger and have several follicles adjacent to one another. The solitary follicles observed were interspersed among the villi, there generally being from zero to four seen

per section. These follicles had two areas which characteristically fluoresced when stained with antiserum 104. The base of these follicles had substantial fluorescence in the interior, but this fluorescence generally was difficult to demonstrate due to intracellular IgM and appeared to be extracellular. (see figure 12) Fluorescing cells were primarily seen near or in the apex of the follicle where it protruded into the lumen. It was further noted from examining serial sections of tissue that these cells were most numerous in the center of the apex (i.e. the first and last cuts into a follicle would tend to be negative for cells in this region).

6.) Examination of Peyer's patches on two of the dogs revealed very few fluorescing cells. There was substantial intercellular fluorescence; however, rare positive cells could be demonstrated within the Peyer's patch but this was primarily at the apex.

7.) Three sections of intestine from two separate dogs included an unusual response. Fluorescent cells were only seen at the very periphery of either end of a longitudinal section of gut. The rest of the tissue was distinctly yellowish to orangish. This effect was unaltered by prolonged deparaffinization or prolonged and/or repeated antibody exposure. (see figure 13)

8.) Examination of formalin-fixed tissues stained with H and E revealed no gross changes. The only unusual findings included a.) a very large lymph follicle in the duodenal submucosa extending

to the intestinal lumen in dog 11, and b.) many eosinophils seen in some animals, especially at the mucosa-submucosa border. Occasionally a sample would have blood vessels engorged with red blood cells which was thought to be due to the clamping of the vessel immediately prior to resection of the biopsy sample. In no case were neutrophils or destruction of tissue seen.

9.) Hematoxylin- and eosin-stained tissue sections from all the paraffin blocks used in the fluorescent study (ethanol-fixed tissue) revealed two main facts. a.) There is usually definite shrinkage of interstitial elements between the intestinal crypts. (see figure 14) No evidence for a gross loss of cells was seen. b.) Plasma cells were generally discernible but the morphology of eosinophils was rather poor when compared to formalin-fixed tissue.

10.) Autofluorescent studies revealed three types of autofluorescing cells. The first type was yellowish or orange and very granular. (see figure 6) The granules were of different sizes and intensities. These cells were primarily seen in the apical portions of the villi or at the mucosa-submucosa border. They were not present in all tissues observed. These cells in the villi appeared on H and E stained sections to be very pale with an indistinct nucleus. (see figures 15a and 15b) The autofluorescing cells at the mucosal-submucosal junction were not identified. The second type of autofluorescing cell was orange, fainter, and more homogenous. The section in which these showed up the best was also a section which would not stain with antiserum 104 as stated

earlier. In the section in which they fluoresced the best they were shown to be tissue eosinophils. (see figures 16a and 16b) These were found throughout the mucosa. The third type of autofluorescing cell may be identical to the first. It was much fainter and was not very granular. It was seen primarily thoughout the villi. The cell type was not identified. (see figure 9a)

11.) Autofluorescent cells also appeared in the base of the lymphoid follicles in the ileum. This autofluorescence was of the bright yellow to orange granular type seen in the tops of the villi. (see figure 17)

12.) Peyer's patches showed bright, yellow, granular fluorescence throughout most of the base; however, there appeared to be a noticeable increase in the fluorescence around the periphery of the base.

13.) Autofluorescence of the submucosa, particularly in the ileum, also occurred. This autofluorescence appeared to be primarily due to collagen or fibrous connective tissue as evidenced by the morphology of the material. This did not occur in all sections of ileum. (see figure 18)

Chart 1

Serum Components - Code versus Reagent

Code	Reagent
100	rabbit anti-human μ (Miles Laboratories)
101	goat anti-canine μ (Cappel Laboratories)
102	rabbit anti-canine δ (Miles Laboratories)
103	rabbit anti-whole canine serum (Miles Laboratories)
104	FITC-goat anti-canine μ (Cappel Laboratories)
105	FITC-normal goat IgG (Microbiological Associates)
106	FITC-rabbit anti-canine δ (Miles Laboratories)
200	rabbit anti-canine IgM (unabsorbed)
201	rabbit anti-canine IgG (unabsorbed)
202	antiserum 200 (2 parts) + serum 300 (2 parts) + AIII (3 parts)
203	antiserum 201 (1 part) + AI (4 parts)
300	Canalb^{R} (Pitman-Moore)
301	supernatant from ammonium sulfate precipitation of serum 300

Chart 2

Clinical and Laboratory Parameters
of the Dogs Used in the Project

Factor Observed	Dog 5	6	8	9	10	11	12
PCV %	45	42	45	48.5	32	48	42
total WBC count/mm^3	11,100	15,800	10,800	11,800	6,700	8,600	6,600
Metamyelocytes/mm^3	0	0	0	0	0	0	0
Bands/mm^3	111	632	324	944	67	172	396
Neutrophils/mm^3	6,438	11,376	6,372	9,086	3,149	3,870	5,148
Lymphocytes/mm^3	2,997	2,212	3,456	944	1,742	2,150	792
Monocytes/mm^3	1,332	0	108	708	0	1,548	66
Eosinophils/mm^3	222	0	540	118	1,474	860	198
Basophils/mm^3	0	0	0	0	268	0	0
Amylase (dye units)	994	752	470	1,503	982	721	695
Sodium (mEq/L)	146	149	152	153	153	155	146
Potassium (mEq/L)	4.3	4.0	5.05	4.3	5.0	5.0	3.9
BUN (mg/dl)	18	12	12	21	24	18	22

Chart 2 (continued)

Factor Observed	Dog 5	6	8	9	10	11	12
Alkaline Phosphatase (mU/ml)	38	25	15	15	20	50	15
SGPT (mU/ml)	40	50	40	35	30	30	30
Total Protein (gm/dl)	6.75	6.5	6.6	6.25	6.5	6.25	6.3
Albumin (gm/dl)	3.25	2.95	3.45	3.5	2.8	3.85	2.75
Calcium (mg/dl)	10.4	9.9	10.2	10.5	9.8	11.4	9.95
Inorganic Phosphorus (mg/dl)	4.6	3.7	5.2	3.8	4.5	5.5	not determined
Creatinine (mg/dl)	0.75	0.65	0.6	0.8	0.7	0.9	0.9
Fecal flotation for parasites	-	not determined	-	-	hooks	-	-
approximate age (years)	≈3	>10	>5	>1	>1	≈1	≈5
clinical signs of gastrointestinal disease (diarrhea or vomiting)	-	-	-	-	-	-	-

Chart 3

Comparison of Different Counters; showing homogeneity between the three Counters*
$\bar{x} \pm 1$ standard deviation where $\bar{x}$ is in cells per mm^2 of a 6 μ section

	Tissue-Counter								
	Duodenum			Jejunum			Ileum		
Dog	M_1	M_2	N	M_1	M_2	N	M_1	M_2	N
5	267 ± 53	284 ± 50	266 ± 68	270 ± 47	304 ± 45	291 ± 61	75 ± 53	73 ± 50	59 ± 68
6	353 ± 47	373 ± 45	366 ± 61	495 ± 61	485 ± 58	395 ± 79	27 ± 53	27 ± 50	20 ± 68
9	341 ± 53	336 ± 50	219 ± 68**	404 ± 53	391 ± 50	442 ± 68	92 ± 53	98 ± 50	89 ± 68
10	971 ± 47	930 ± 45	770 ± 61**	991 ± 61	1038 ± 58	760 ± 79**	134 ± 53	124 ± 50	90 ± 68
11	711 ± 47	690 ± 45	710 ± 61	714 ± 75	685 ± 71	764 ± 96	122 ± 75	122 ± 71	103 ± 96
12	269 ± 61	257 ± 58	271 ± 79	636 ± 53	666 ± 50	581 ± 68	106 ± 47	114 ± 45	65 ± 61

M_1 = counter "M", technique 1

M_2 = counter "M", technique 2

N = counter "N", technique 2

* Dog 8 excluded here

** indicates values which are more than two standard deviations from the other two values

Chart 4

Analysis of Variance for IgM-cells for Counter M_1

(M_1 was the only counter who was able to count the duodenum of dog 8)

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	Probability
Dog	6	1966411.34	327735.19	3.5815	.02857
Tissue	2	2922646.22	1461323.00	15.9696	.00041
Dog x Tissue	12	1098019.49	91506.56	8.3649	.00000
Residual	61	667300.31	10939.35		
Total	81	6991431.00			

Chart 5

IgM-cells per mm^2 of a 6 μ section for counter M_1

Tissue	Means ± 1 standard error
Duodenum	452.2399 ± 60.0871
Jejunum	572.6804 ± 62.1245
Ileum	107.4716 ± 59.5669

Assuming the overall test of equality of means for tissues (α = .00041) is significant, we have the following means separations:

duodenum vs. jejunum $t_{(26)} = \frac{-120.4402}{86.4287} = -1.3935; \alpha = .17528$

duodenum vs. ileum $t_{(26)} = \frac{344.76855}{84.6090} = 4.0748; \alpha = .00038$

jejunum vs. ileum $t_{(26)} = \frac{465.2088}{86.0678} = 5.4051; \alpha = .00001$

Chart 6

Analysis of Variance for Autofluorescent Cells
for Combination of Results of Counters M_2 & N

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	Probability
Dog	6	38955.1817	6492.5273	2.0314	.13948
Tissue	2	10066.6629	5033.3281	1.5749	.24697
Dog x Tissue	12	38352.7167	3196.0596	2.4961	.02870
Residual	23	29449.5156	1280.4136		
Total	43	114740.2500			

Chart 7

Autofluorescence which Resembles Cells;
Average of all Dogs in Cells per mm^2
of a 6 μ section for Counters M_2 & N

Tissue	Means ± 1 standard error
Duodenum	25.3326 ± 15.4648
Jejunum	26.1239 ± 15.1092
Ileum	58.1757 ± 14.7451

Since α = .24697 for overall test of equality of means, there is no point in looking at individual comparisons because we fail to reject overall equality.

Chart 8

Analysis of Variance of all Autofluorescent Structures for Combination of Results of Counters M_2 & N

Source	Degrees of Freedom	Sum of Squares	Mean Square	F-Value	Probability
Dog	6	2321020.750	386836.750	2.2833	.10535
Tissue	2	1197146.270	598573.125	3.5315	.06222
Dog x Tissue	12	2033959.365	169496.563	3.0633	.01019
Residual	23	1272608.000	55330.781		
Total	43	6660287.000			

Chart 9

All Autofluorescence, Averages of all Dogs in Structures per mm^2 of a 6 μ section for Counters M_2 & N

Tissue	Means ± 1 standard error
Duodenum	286.6467 ± 112.6201
Jejunum	330.7986 ± 110.0307
Ileum	661.0801 ± 107.3790

Assuming the overall test of equality for tissues (α = .06222) is significant, we have the following means separations:

duodenum vs. jejunum $t_{(14)} = \frac{-44.1519}{157.4486} = -.2804$; α = .78327

duodenum vs. ileum $t_{(14)} = \frac{-374.4335}{155.6070} = -2.4063$; α = .03050

jejunum vs. ileum $t_{(14)} = \frac{-330.2815}{153.7434} = -2.1483$; α = .04967

Chart 10

Autofluorescence which resembles cells-individual dogs' average number of cells per mm^2 of a 6 μ section $\pm$ 1 standard deviation versus numbers of eosinophils per mm^2 of a 6 μ section from 2 separate counts (standard deviation not determined due to reasons stated in Discussion).

	Tissue - cell type counted					
Dogs	D-A	D-E	J-A	J-E	I-A	I-E
5	3.23 $\pm$ 12.78	23/0	0.00 $\pm$ 15.65	71/71	0.00 $\pm$ 12.78	286/310
6	-0.00 $\pm$ 15.65	71/23	7.74 $\pm$ 15.65	0/0	-0.00 $\pm$ 15.65	95/71
8	42.61 $\pm$ 15.65	95/95	-0.00 $\pm$ 15.65	75/597	4.79 $\pm$ 15.65	621/621
9	8.36 $\pm$ 22.13	47/47	10.35 $\pm$ 15.65	47/71	24.83 $\pm$ 15.65	47/47
10	14.46 $\pm$ 12.78	167/71	6.74 $\pm$ 15.65	47/71	91.84 $\pm$ 15.65	884/836
11	-0.00 $\pm$ 15.65	430/281	12.81 $\pm$ 15.65	310/191	-0.00 $\pm$ 15.65	860/1099
12	26.38 $\pm$ 15.65	310/310	32.48 $\pm$ 15.65	71/95	113.20 $\pm$ 15.65	549/549

D = duodenum
J = jejunum
I = ileum

A = autofluorescent cells
E = eosinophils

Chart 11

Number of Plasma Cells per mm^2 of a 6 μ section
Compared to the Number of IgM-Cells per mm^2 of a 6 μ section

Dog - Tissue	Plasma Cells from Ethanol-Fixed Tissue		Plasma Cells from Neutral Buffered Formalin Tissue		IgM-Cells from Ethanol-Fixed Tissue
	count 1	count 2	count 1	count 2	count M_1
5 - D	4112	3132	3299	2056	267
5 - J	3539	3730	2534	3012	270
5 - I	860	239	1721	1386	76
6 - D	3395	3993	5428	4902	354
6 - J	1960	2821	4471	3299	496
6 - I	382	908	1123	1482	28
8 - D	3299	3108	2702	4184	251
8 - J	1899	1769	2534	3084	497
8 - I	1339	549	980	813	194
9 - D	2462	2415	4495	3634	342
9 - J	2056	2343	2176	2582	404
9 - I	239	430	1649	597	92

D = duodenum
J = jejunum
I = ileum
M_1 = counter "M", using technique 1

Chart 11 (continued)

Dog - Tissue	Plasma Cells from Ethanol-Fixed Tissue		Plasma Cells from Neutral Buffered Formalin Tissue		IgM-Cells from Ethanol-Fixed Tissue
	count 1	count 2	count 1	count 2	count M_1
10 - D	2391	2941	1912	4686	972
10 - J	2821	3586	2104	2104	991
10 - I	932	1076	741	932	134
11 - D	1363	980	884	1123	711
11 - J	2080	1171	1458	1386	714
11 - I	286	143	1482	717	122
12 - D	4089	2966	1673	3108	270
12 - J	2486	1626	2630	2941	636
12 - I	980	454	502	1578	106

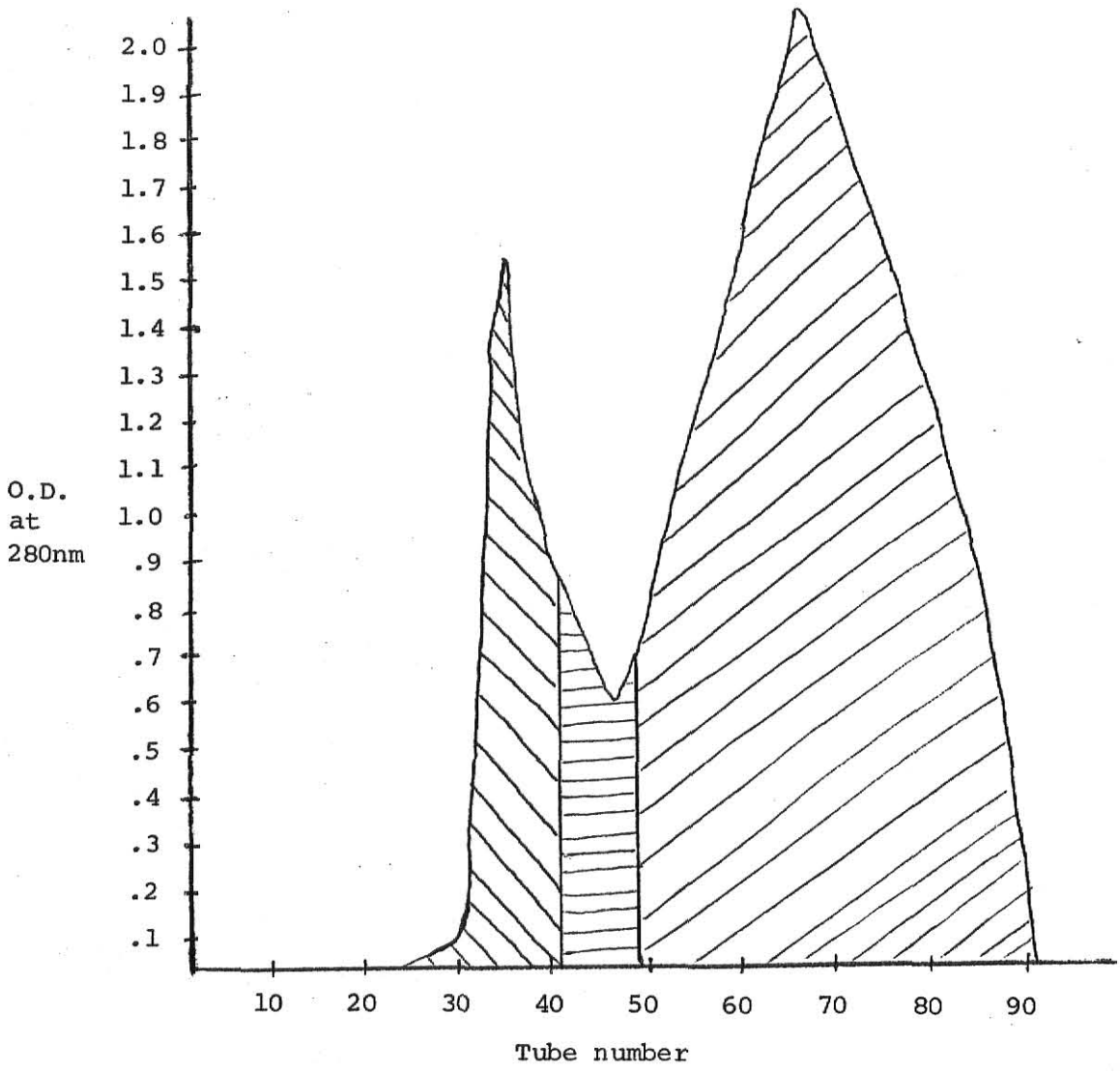
D = duodenum
J = jejunum
I = ileum
M_1 = counter "M", using technique 1

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Figure 1

Chromatogram of Ammonium Sulfate Precipitated Dog Globulins



$(NH_4)_2SO_4$ precipitated normal canine serum eluted from a Bio-Rad A1.5 column with 0.01 M PO_4 and 0.5 M NaCl at pH 7.4 using a 5ml fraction/tube

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= AII

= AIII

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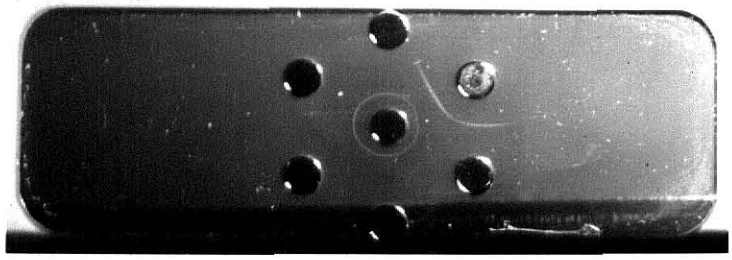
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Figure 2

Comparison of Antisera 101 & 202 by Double Diffusion Analysis



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IgM-rich whey

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antiserum 202 (see chart 1)

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antiserum 101 (see chart 1)

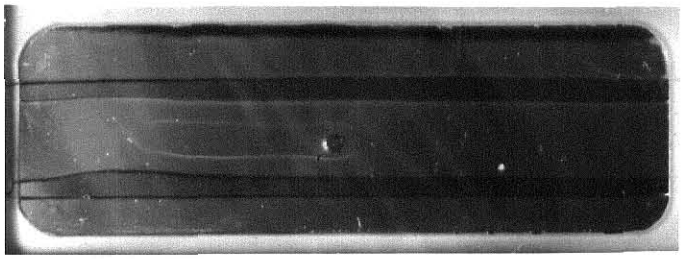
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Figure 3

Immunoelectrophoretic Comparison of Antisera 101 & 202

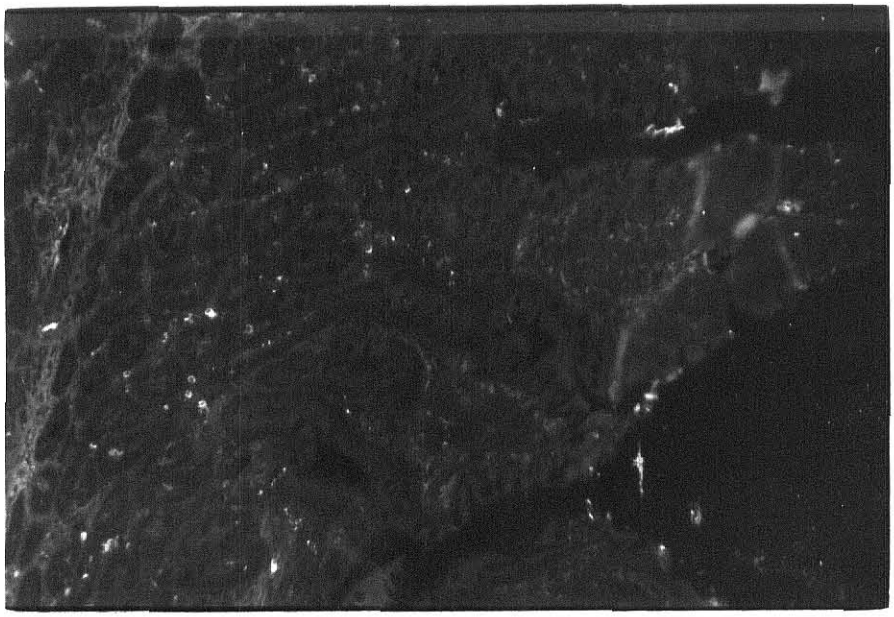


———— antiserum 101 (see chart 1)

o pooled normal canine serum

———— antiserum 202 (see chart 1)

Figure 4



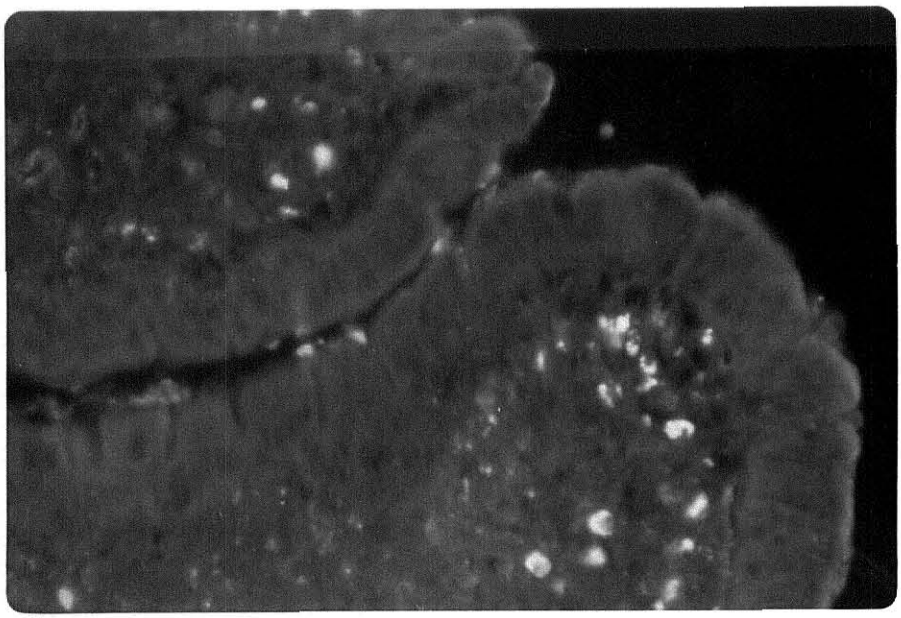
Canine jejunal tissue stained with antiserum 104 (see chart 1) showing IgM-cells and interstitial IgM.

Figure 5



Jejunal tissue from same dog as figure 4. Absorbed with antiserum 202 (see chart 1) six times at ten minutes per time. Then stained with antiserum 104 (see chart 1) as in figure 4.

Figure 6



Comparison of yellow autofluorescent cells
and specific green fluorescent IgM-cells.

Figure 7

Diagramatic representation of area of small intestine mucosa selected for enumeration of IgM-cells

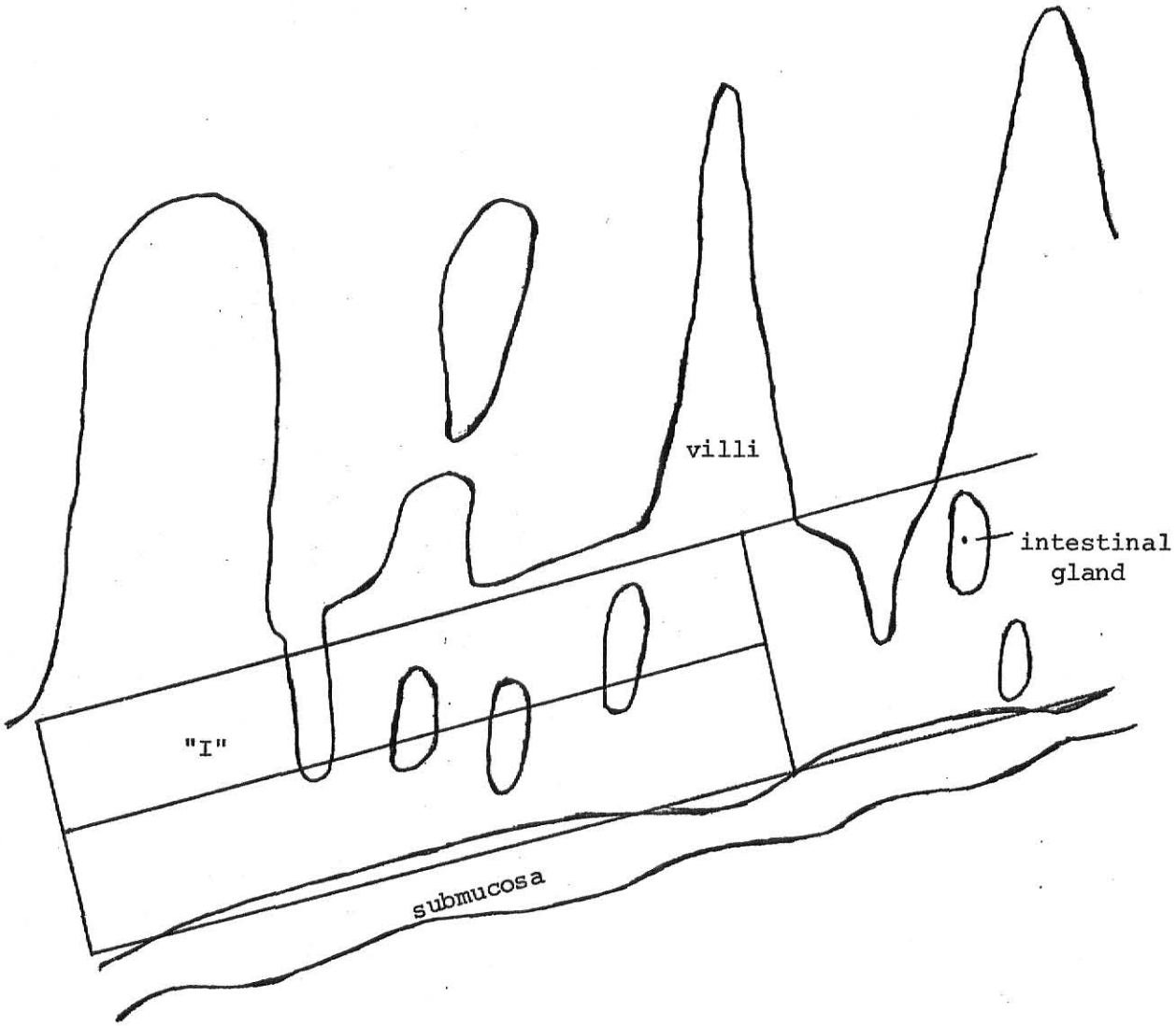
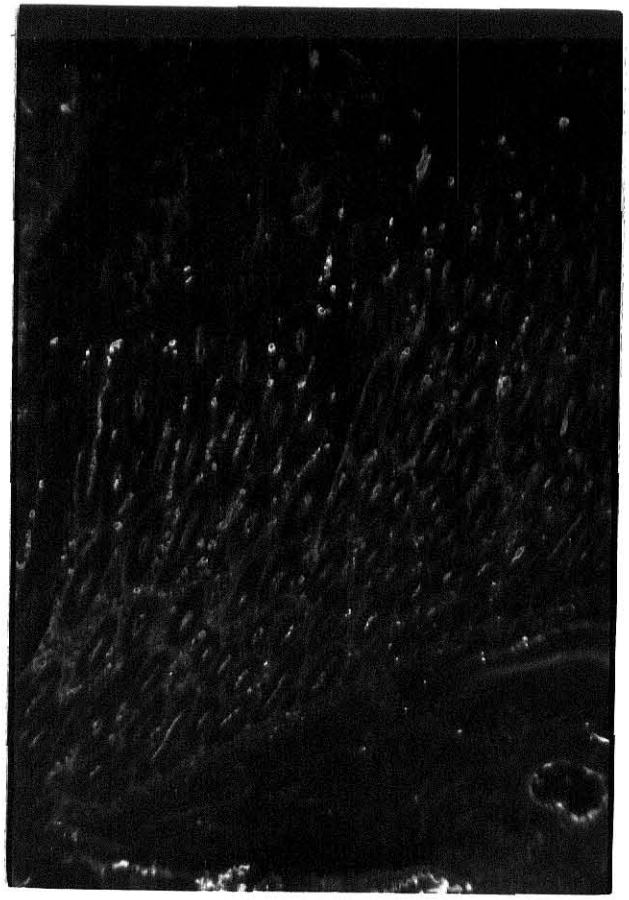


Figure 8

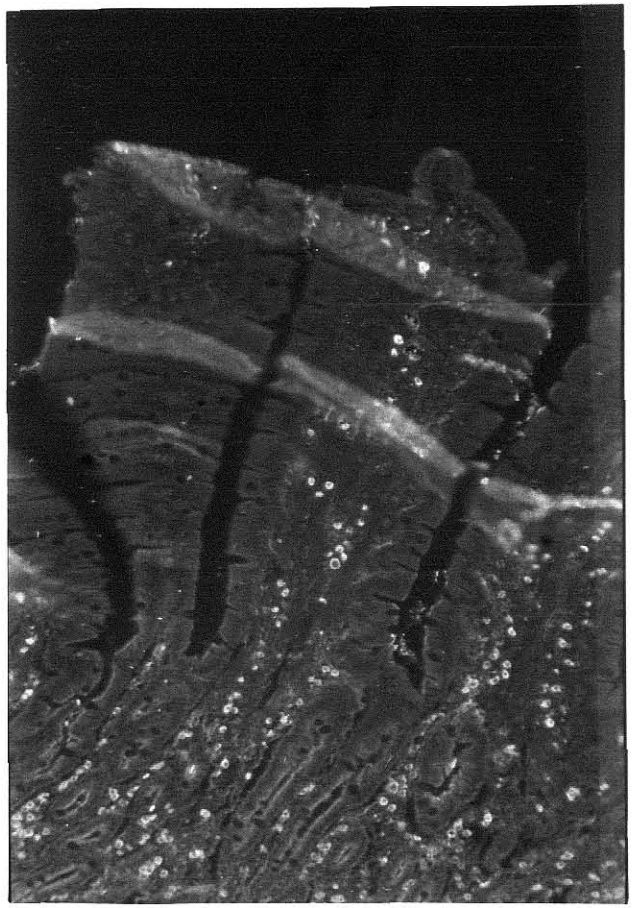


Duodenal tissue demonstrating fluorescence within the glands and interstitial fluorescence which is brightest near the submucosa.

Figure 9

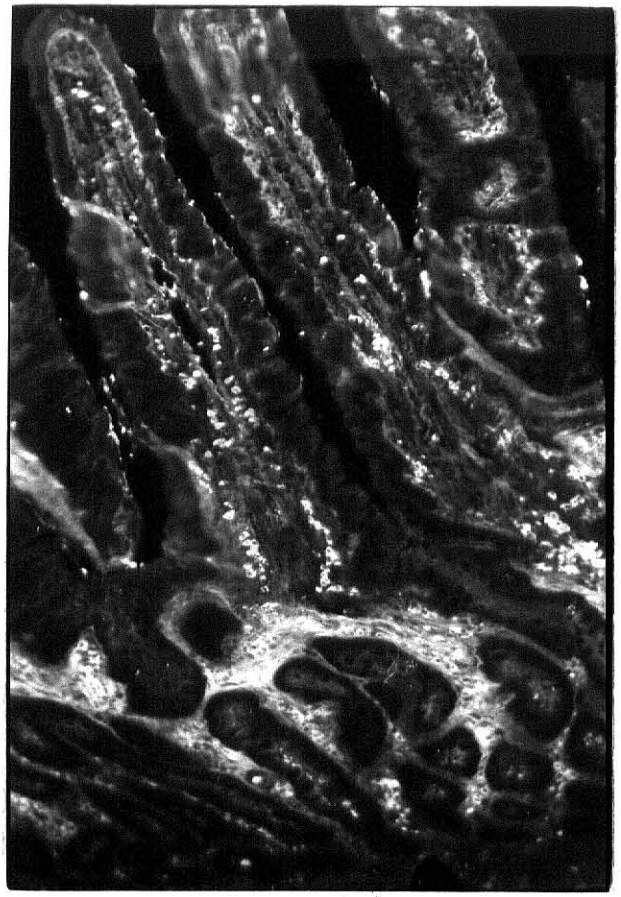


a.) Intestinal villi showing no fluorescing cells within the villi although fluorescing cells can be seen below the villi. Some autofluorescence is noted throughout the villi.



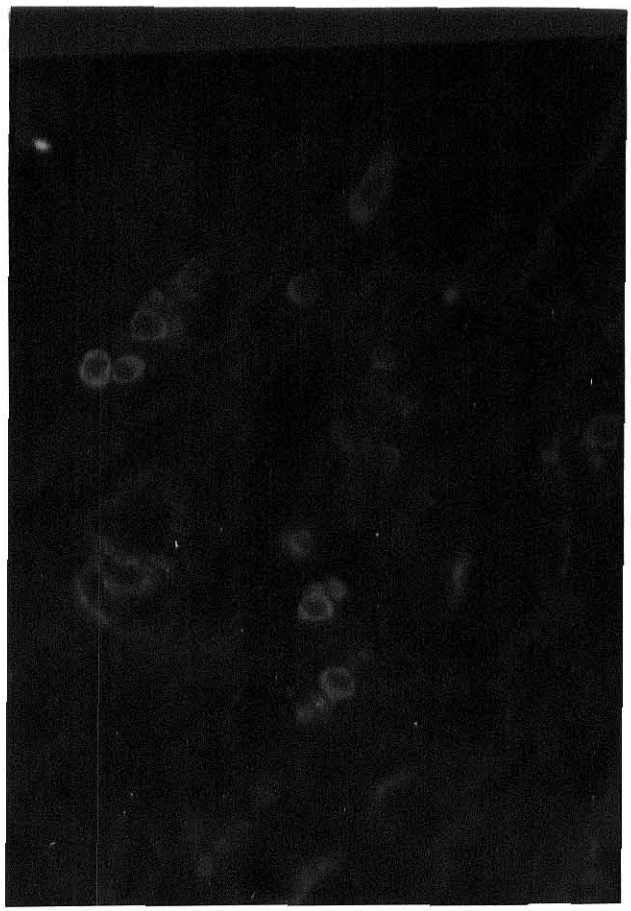
b.) Intestinal villi from dog 10 (which had hookworms) showing IgM-cells extending into the duodenal villi.

Figure 10



Antiserum 106 staining cells in the villi. Also note the marked interstitial staining compared to figures 4 and 8.

Figure 11



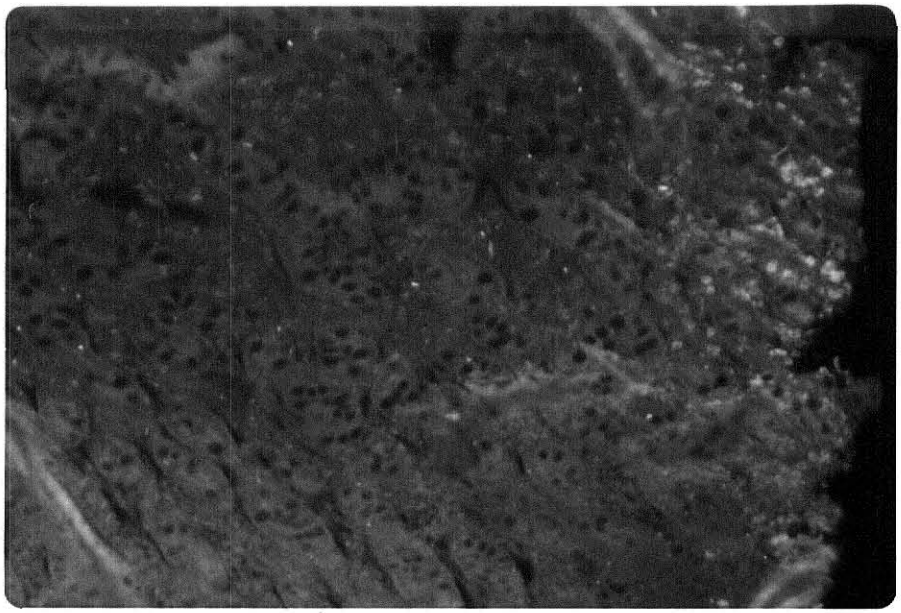
540x magnification of cells showing a.) cells with eccentric nuclei compatible with plasma cells and b.) cells with a thin rim of cytoplasm comparable to lymphocytes.

Figure 12



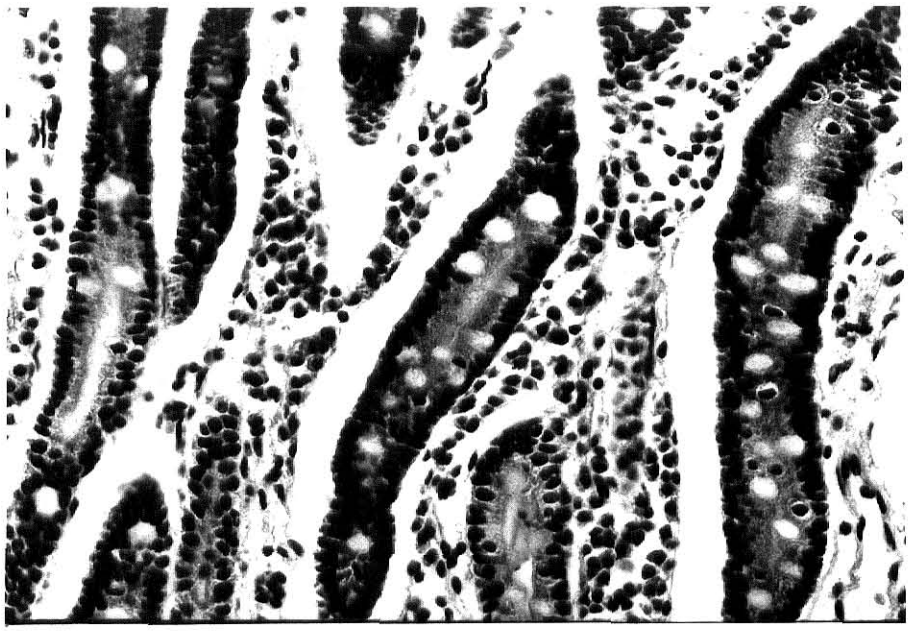
Ileum stained with serum 104 (see chart 1) showing an isolated lymphoid follicle with a few IgM-cells near the apex and prominent interstitial fluorescence at the base.

Figure 13



Duodenum from dog 8 showing "odd" staining reaction with specific fluorescence only occurring at the cut edge of the tissue.

Figure 14



An H and E section of tissue prepared for fluorescent antibody examination by cold ethanol-fixation.

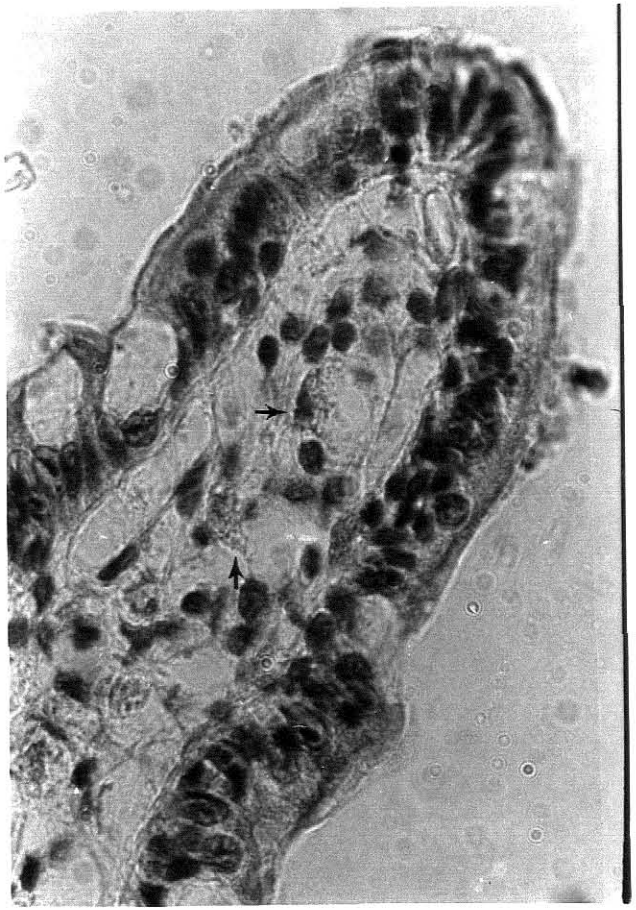
Figure 15

a



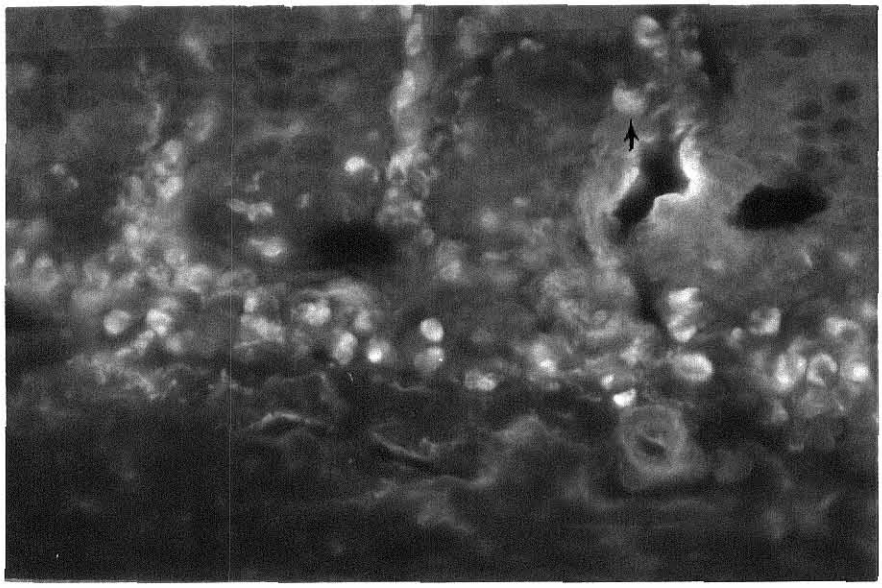
Autofluorescent cells in a villi.

b



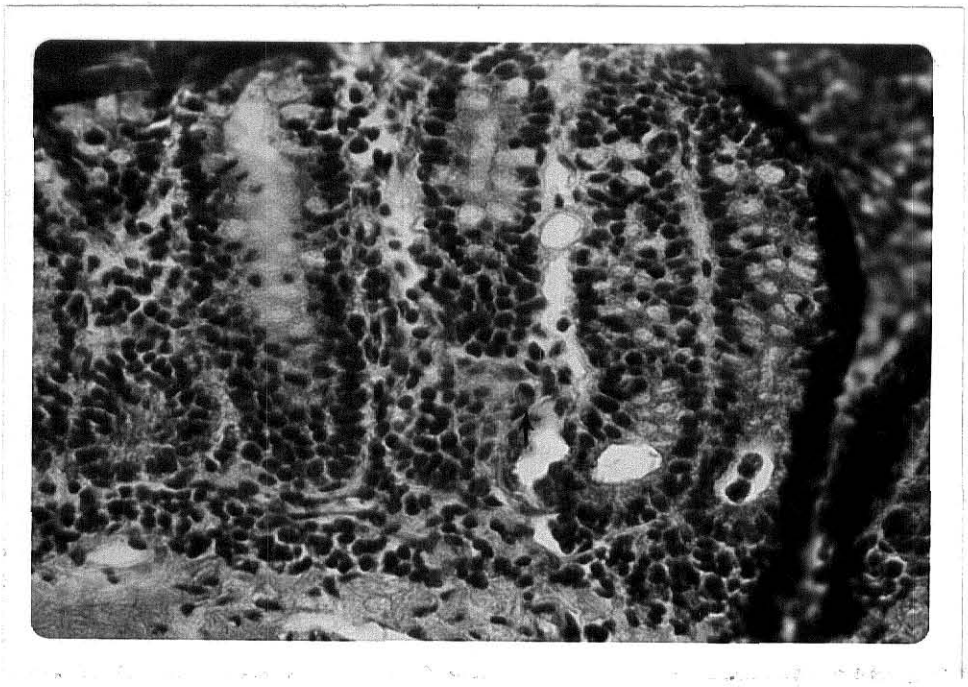
The same villi in a, now stained with H and E.

Figure 16a



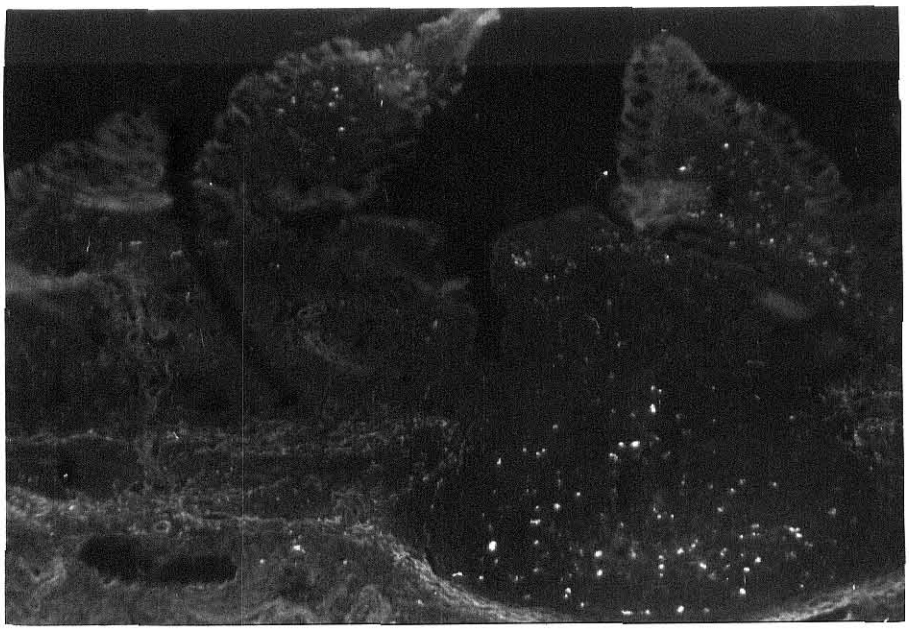
Ileum of dog 7. Autofluorescence and lack of staining with antiserum 104 (see chart 1) characterized this and two other tissues.

Figure 16b



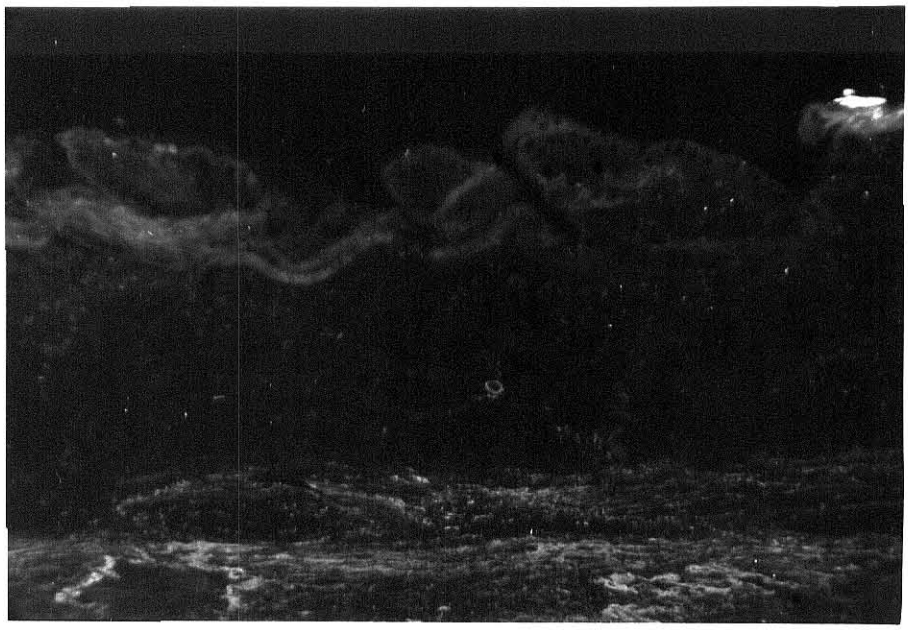
The same slide in a, now stained with H and E showing the autofluorescent cells in a to be eosinophils.

Figure 17



Autofluorescence in a lymphoid follicle in the ileum.
This is the same type of follicle as seen in figure 12.

Figure 18



Autofluorescence of submucosa of ileum. No FITC antiserum was applied. The autofluorescence as viewed was nearly as green as specific IgM fluorescence.

DISCUSSION

The cold ethanol-fixed tissue appeared equal to or superior to other tissue preparation methods. The ethanol-fixation procedure yielded results with minimal effort compared to the other techniques tried. This reduced effort was in large part due to the longer time periods for which the tissue could be stored in the various baths, allowing one to do this and other things simultaneously instead of committing a full hour or two to the tissue preparation only. The cold ethanol procedure was mild enough to be used for fixing tissues to be used for examining immunoglobulins. Another reason for using the ethanol-fixation was that ethanol and xylene were inexpensive, easily stored, and to some degree reusable. If fluorescent antibody techniques could be simplified and made more readily available to more practitioners through this method of tissue preparation, more of this type work might be done and hence more information on the subject would be available.

Antiserum 104 was demonstrated to be specific for IgM by many criteria. Antiserum 202 was made by a group (i.e. the author) separate from the group which produced antiserum 104. This fact is important in that it adds credence to the controls (as opposed to the situation where one group makes both the control antiserum and the fluorescein-conjugated antiserum). More evidence of the specificity of antiserum 104 for μ chain was given by consistency of our results with prior reported data in other species. The cell type which fluoresced:

1.) generally had an eccentric nucleus comparable to a plasma cell or had a thin band of fluorescence comparable to a lymphocyte,

2.) was primarily located between the intestinal glands (which is where IgM-plasma cells are primarily found in man [1,7,26,50,102]), and

3.) was not located in the submucosa, within intestinal glands, or in the muscle sheaths (although on one occasion a few lymphocyte-like cells were seen in the blood vessels of the muscle sheath).

These resluts suggested that the cells which fluoresced were plasma cells or lymphocytes and not fibroblasts, glandular epithelial cells, smooth muscle cells, or endothelium (plasma cells and lymphocytes being the cells which were reported to contain immunoglobulins by other authors [41,92,138]).

With one exception (dog 10) there were no IgM-cells seen in the villi. The fact that plasma cells were present in the villi was seen in that 1.) the apparently impure antiserum 106 stained numerous cells with plasma cell-like qualities and 2.) sections stained with H and E showed plasma cells to be present in the villi. In other work IgM-cells were generally considered to be most numerous in the area of the intestinal glands and were rare in the villi.[9,43]

Recent evidence has also suggested that IgM is a secretory immunoglobulin.[4,18,24,31,39,112,128] In the present study the epithelium itself showed at most minor fluorescence in the apical portion of the cell similar to what some authors have demonstrated.[31,101,112] The thin line of fluorescence within the gland

near the apices of the glandular epithelium was by far the brightest. The fluorescence generally appeared brightest in the duodenum or jejunum where the numbers of IgM-cells were the greatest and was radically lacking in the ileum. This fluorescence was not seen in all intestinal glands but was seen in the majority of the glands, especially in the deeper parts of the glands. Since IgG has been shown to not cross through the epithelium,[31,39] staining for δ-chain would have been the best way to see whether or not this staining pattern did or did not represent local secretions of immunoglobulins. However, as stated earlier, the FITC-anti-canine δ (antiserum 106) was polyspecific, it therefore came as no surprise that glandular fluorescence was seen in sections stained by it. There are at least two possibilities. One is that the anti-δ contained activity against antigens naturally found in the intestinal glands. (Remember that this antiserum, while polyspecific, did not react with IgA or IgM.) The other possibility was that the fluorescing material within the glands was IgG and that it was present in exuded serum which had all the serum immunoglobulins present in it.

It is conceivable that the biopsy technique could have permitted exudation of serum into the glands; however, in such a case one would probably have expected to see this fluorescence occurring in all the glands in all the tissues which was not the case. The ileum, which had the fewest IgM-cells of all the tissues, had very little of this type of fluorescence. If a monospecific anti-gamma and anti-alpha had been available, comparison of the

obtained results with the results from these antisera may have allowed conclusions to be drawn. However, it was felt that no conclusions could be drawn about IgM being or not being secreted into intestinal glands based on our work.

The results reported here are comparable with previous reports of immunoglobulin-containing cells in Peyer's patches. Generally very few if any IgM-cells were seen in Peyer's patches although there was substantial interstitial fluorescence.[107] There was an absence of large numbers of IgM-cells around the patches as previously reported.[26] The same held true for the sparse lymphoid follicles in the ileum. The deeper areas of the follicle (particularly the part of the follicle which extended into the submucosa) had very few IgM-cells. The part of the follicle which projected into the lumen had a few cells; however, no report of fluorescent antibody examination of this structure for immunoglobulins or cells was found in the literature, hence, no conclusion can be drawn.

The fact that interstitial IgM was detected in relatively small amounts was consistent with previous results. IgG and IgA were reported to be present in large and moderate quantities (respectively) in the intestinal interstitium while IgM was reported to be present in the least amounts interstitially.[16,17,19,21] It was seen that the FITC-anti-canine γ (antiserum 106) did stain the interstitium to a much greater extent than did the FITC-anti-canine μ (antiserum 104). There was so little IgM present

interstitially by this technique, that it was believed that the IgM-cells could be counted successfully without modifying the technique by washing the tissue prior to ethanol-fixation.[16]

The fact that there were always more plasma cells than IgM-cells (see chart 14) indicates that the results are not inconsistent with what would be expected in that the IgM-cells are plasma cells or lymphocytes.[28,41,138] In man IgM-cells generally accounted for about 15% to 30% of the intestinal plasma cells.[43,45,54] While that figure ranged from below 3% to above 80% in this work, the percentages were not critically evaluated here because the primary purpose of the enumeration of the plasma cells was simply to confirm the fact that there were never more IgM-cells than plasma cells. The accuracy of numeration of plasma cells was considered only fair because these cells, being counted on the microscope through a photographic graticule, represented cells from a much smaller area than the area used to count the IgM-cells. The area used here was so much smaller, any focal variation in the number of cells or the chance positioning of the slide at a particularly big or small gland would render the obtained count significantly different than the true count. Possibly of greatest significance was that the total plasma cell count was always greater than the IgM-cell count. In view of the above findings, the canine may have IgM-production as a part of the local immune system.

In combined use of the ethanol-fixed, paraffin-embedded tissue with antiserum 104 provided a reproducible method for determining numbers of IgM-cells, even when coupled with black and white

photography which does not allow distinction between colors (i.e. specific green fluorescence from yellow autofluorescence). Problems encountered in counting cells included finding areas of the stained section which were suitable for photography (i.e. no tears, no folding, all of the field in focus) and accurately counting the fluorescing cells seen. The former problem was partially alleviated by observing many sections from the same block of paraffin-embedded tissue. The latter problem was alleviated by 1.) making a definition of what constituted a fluorescing cell and 2.) counting only a part of the mucosa. Interstitial IgM, though sparse, appeared to be prominent enough in the deepest parts of the mucosa to cause confusion between IgM-cells and negative cells surrounded by interstitial IgM. When a portion of a negative cell was surrounded by interstitial IgM the negative cytoplasm and/or nucleus would appear to be the nonstaining nucleus of a fluorescing plasma cell. Since the interstitial IgM was evenly dispersed throughout the interstitium, no demarcation of a cell outline was visible and hence it was difficult to determine whether a nonstaining circumscribed area was an IgM-cell nucleus (which did not stain but was surrounded by fluorescing cytoplasm) or a negative cell's cytoplasm surrounded by interstitial IgM. Both situations were almost certainly present and probably many gradations in size of the negative cells' cytoplasms. Depending on the angle of the cut, an IgM-cell could have changed slightly in size and shape; determination of which were IgM-cells was difficult when interstitial fluorescence in the deeper parts of the mucosa was present.

When three different individuals (well-trained, trained, and untrained) counted cells from the same marked off area of a black and white photographic print there was little consistency between counters. When the lower one-half of the mucosa was omitted the count became consistent between and within the well-trained and the trained individuals which was attributed to decreasing the interstitial IgM content of the tissue counted thus rendering the IgM-cells more distinct and discrete.

The reason why the interstitial IgM was predominantly found in the lower portion of the mucosa is unclear. The submucosa was always seen to have substantial specific green fluorescence when stained with antiserum 104. This requires careful interpretation since on occasion substantial green autofluorescence apparently due to collagen was seen in the submucosa.[33] (This occurred primarily in the ileum and was focal.) Assuming that the submucosa did have substantial interstitial IgM present, it might have been present due to at least two reasons. The submucosa, as seen in histopath sections, was very vascular and perhaps there was substantial transudation of serum here. Staining with antiserum 106 also showed fluorescence in the submucosa hence there may indeed be substantial transudation normally occurring here. Another possibility was that the biopsy procedure may in part account for this finding. Clamping off the intestine may have caused a sudden increase in the hydrostatic pressure in the intestines which in turn may have caused excessive abnormal transudation of serum into the tissue.

Indeed, the submucosal blood vessels were often engorged with erythrocytes. However, the mucosal blood vessels were occasionally also engorged with erythrocytes and this occurred in areas of the intestinal mucosa which had minimal interstitial fluorescence. Furthermore, when the intestine was clamped it was clamped with the express purpose of occluding both artery and vein. Except in the ileum, which has a dual blood supply bleeding was very rarely seen.

Perhaps during the time that the clamps were applied there was more of an occlusion of the veins than the arteries and increased hydrostatic pressure resulted in transudation of serum.

The results of the counts of IgM-cells were expressed in numbers of cells per mm^2 of a 6 μ section. It was decided to not take into account the area of the tissue accounted for by glands, (expressing the result in percent of the interstitial area accounted for by positive cells). Since an effort was made to use the largest possible area when counting cells, it was felt that the possibility of having a particularly large or small intestinal gland in any area of the tissue became less important and that the result as expressed in cells per mm^2 of a 6 μ section was reproducible and would not be affected by focal changes in the size of glands or numbers of cells. Also it was felt that such a technique would give the desired information with minimal effort.

The counts of the IgM-cells indicated a significant dog by tissue interaction. This means that the results for the numbers of

cells per unit area for a given tissue type depends on the specific dog sampled. This study was a mixed analysis (dogs being random and tissues being fixed); therefore, it comes as no surprise that the dogs are judged to be different ($p= 0.02857$). It is of significance that in none of the seven dogs did the number of IgM-cells in the ileum equal or exceed the number of IgM-cells in the duodenum or jejunum. This means that while the degree of difference between the tissues is different, the order of the tissues (largest number of IgM-cells to lowest number of IgM-cells) is constant. If another dog was examined, we could expect the ileum to have significantly fewer IgM-cells then the jejunum or duodenum; however, we could not predict how much smaller the ileum would be compared to the other tissues.

Autofluorescent cells probably caused little or no alteration in the counts of the IgM-cells. This was thought to be so because

1.) the brightest autofluorescence was seen in the tips of the villi and at the mucosa-submucosa junction, both being areas which were not included in our counts,

2.) the autofluorescence present in the area being counted did not have the characteristics assigned to IgM-cells (i.e. nonstaining nucleus and homogenous staining cytoplasm) and was so much dimmer that the positive fluorescence that it was very unlikely that it would have been counted even if it did have the above characteristics, and

3.) despite significant dog by tissue interaction ($p= 0.02870$) there was no statistical difference between tissues or between dogs; therefore, any alteration in the IgM-cell counts caused by this

autofluorescence should be evenly distributed among the tissues.

Even if there was an influence on the IgM-cell counts by the autofluorescence, chart 7 shows that the ileum would be the most likely to be altered by the autofluorescent cells (assuming that this autofluorescence is the same in each tissue which it usually appeared to be) and that this alteration would tend to increase the counts. It seems significant that in spite of this the ileum was very significantly lower than the other two tissues.

Though the autofluorescent cells did not appear to affect the IgM-cell counts when the cells were counted as described in Materials and Methods, they may or may not be important in their own right. The literature generally reports that tissue eosinophils and mast cells were the major cause of autofluorescent cells.[43,111,117] Eosinophils did produce some autofluorescence; however, as mentioned earlier this was usually so much fainter than the IgM-cells that it was unlikely that they were important in this project. The numbers of eosinophils seen by histopath section did not seem to be correlated to the numbers of autofluorescent cells seen in a tissue. (see chart 10) The numbers of eosinophils could not be evaluated critically since they were counted from the same fields as the plasma cells were and hence, as mentioned earlier, counting from such a small area may have allowed focal variation in numbers of eosinophils present to distort the calculated number of eosinophils. In addition, it has been reported that eosinophils are extremely variable in the intestines in regard to numbers present in sections

only millimeters apart.[85,88] Therefore, no conclusion can be drawn from the lack of correlation of autofluorescent cells and tissue eosinophils.

It was not established that only the eosinophils autofluoresce. As mentioned earlier the sections on which the eosinophils autofluoresced most brightly were sections which did not stain well [Subjective Evaluation (7)]; it was on one of these sections where the correlation between the autofluorescent cells and the eosinophils was made. The questions were 1.) was some chance mistake responsible for fixing the tissue in such a way as to render it more likely to demonstrate eosinophils? and 2.) could the results of this "odd" section be extrapolated to other tissue sections which did not react this way? The "odd" reaction of the three tissues was not understood. Of the three tissues which reacted in this manner, only one was used in the study, the other two tissues (which were from the same dog) were omitted as was the unaffected tissue from this dog.

It is tempting to state that this "odd" reaction was a result of some property of the tissue and was not an accident. In the case of the dog which was discarded, as in the case of dog 8 which was not discarded, all the tissues from each individual dog were placed on wooden tongue depressors (in one case a tissue which reacted "odd" and another which did not so react were placed on the same tongue depressor) and then placed in the same solution (all the tissues from each individual dog were in the same solutions simultaneously) for almost identically the same time (i.e. the

tissues would be in the solution within three to four minutes of the same time of each other. The main difference came when they were cut in and one would be in xylene 47 hours and 30 minutes as opposed to 47 hours and 35 minutes for another.) Duplicate blocks of these tissues were not made which was regrettable. Such duplicates may have proved informative. On the other hand, the fact that this phenomenon appeared to be all-or-nothing (i.e. there were no tissues which appeared to be affected and were between the unaffected ones and the affected ones) and that there were distinctly positive fluorescing cells only on the periphery of the section suggested that there was some "accident" in the processing. What it may have been was unknown. The fact that there was fluorescence at the periphery of the tissue allowed the duodenum from dog 8 to be counted because at higher magnification the green fluorescence was brighter and could be visually discerned from the yellow-orange non-specific fluorescence. Black and white film did not differentiate the colors well and hence it was evaluated directly on the microscope.

The other type of autofluorescent cell (the bright granular cell in the villi and near the submucosa) may be similar to what Sainte-Marie called lipid-laden cells.[109] He investigated these cells in relationship to the thymus of rats. While lipid stains were not used in this work, the description of these cells fits well with what Sainte-Marie described. They were not present in all villi or even all tissues and being primarily in the villi were not enumerated in this study; therefore no comment on their significance can be made at this time.

Overall autofluorescence (which differed from autofluorescent cells probably due to autofluorescence of collagen and other supportive structures) showed that, despite significant dog by tissue interaction, there appeared to be a statistically significant increase in such autofluorescence in the ileum. If the autofluorescent cells seemed to not affect the IgM-cell counts, then, for similar reasons plus the fact that here morphology was not considered (i.e. the autofluorescent structure did not have to resemble anything, it just had to autofluoresce), it would seem very probable that the IgM-cell counts were not affected by these structures.

The finding that the ileum had fewer IgM-cells than the jejunum or duodenum may be useful in describing some of the intestinal diseases of the canine. A sprue-like disease, which in man has been consistently characterized by an increase in the number of IgM-cells,[11,55,111,123] has been reported in the canine (though based only on histopathology and history[81,136,139]). Ulcerative colitis which in man has been described as having an increased number of IgG-cells with normal or increased numbers of IgM-cells[21,109] has also been reported in canine.[59] In dogs there are possibly three forms of the disease. Dogs have also been reported to have regional enteritis,[123,135] gastrointestinal tumors,[86] and acute enteritis[5] all of which have been examined immunologically in man. Diseases which man does not have or which have not been examined immunologically include eosinophilic gastroenteritis[85,88,136] and

lymphocytic-plasmacytic enteritis.[136] The benifits in examining these diseases immunologically may include:

1.) having an understanding of the etiology, (is it an autoimmune phenomenon or are autoantibodies merely the result of alteration of tissues being nonpathogenic in themselves),

2.) understanding the cause of perpetuation or exacerbation of the disease, (does the immune response prolong the disease as it is thought to possibly do in human ulcerative colitis[20]),

3.) seeing if diseases of animals are intrinsically different or similar to apparently similar human diseases, (i.e. eosinophilic gastroenteritis), and

4.) possibly finding animal models of human diseases.

CONCLUSIONS

1.) The ileum had significantly fewer IgM-cells than either the duodenum or jejunum. There is no statistically significant difference between the duodenal and jejunal counts.

2.) Autofluorescent cells were equivalent in all three tissues examined and do not appear to cause much variation in the determination of IgM-cell counts.

3.) There was more total autofluorescence in the ileum than in the duodenum or jejunum.

4.) The method of counting IgM-cells was reproducible within and between counters.

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IMMUNOFLUORESCENT STUDY OF IgM IN THE CANINE SMALL INTESTINE

by

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ABSTRACT

Canines have several intestinal diseases which appear similar to human diseases and which are possibly immunologically-mediated. These diseases can only be studied after a thorough knowledge of the normal immune system is available. The first objective of this study was to quantitate IgM-cells in the duodenum, jejunum, and ileum. The second objective was to quantitate the number of autofluorescent cells and the total autofluorescence in the same tissues.

Seven mongrel dogs of various breeds, ages, and unknown backgrounds were used in the study. Samples of intestine were obtained under surgical anesthesia and were studied by fluorescent antibody techniques and by light microscopy. Cells were enumerated from black and white films taken with the fluorescence microscope. Each photographed area was counted by two people for a total of three times (twice by one counter).

The results were interpreted as follows:

1.) the order of the tissues in regards to IgM-cells per mm^2 of a 6μ section was, from least to greatest, ileum, duodenum, and jejunum; however, statistically the duodenum and jejunum were equivalent,

2.) autofluorescent cells were equivalent in the three tissues observed. Furthermore, they probably caused no variation in the IgM-containing cell counts,

3.) there is more total autofluorescence in the ileum than in the duodenum or jejunum, and

4.) the method of counting immunofluorescent cells was reproducible for IgM-cells.