STUDIES OF THE CELLULAR BASIS OF IMMUNITY AT MUCOSAL SURFACES

By

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ABSTRACT

The origins of the immunoglobulin-containing cells in the intestinal, respiratory, mammary, and genital tissues were studied in mice, rats, and guinea pigs by using an adoptive lymphocyte transfer method. Proliferating lymphocytes (lymphoblasts) were obtained from various donor lymphoid sources and radiolabelled *in vitro* with either ^{3}H-thymidine or ^{125}I-deoxyuridine. Radiolabelled lymphoblasts were then injected into the circulation of recipient animals. Twenty-two - 24 h later, various tissues taken from recipient animals were examined for the presence of radiolabelled donor cells by either radiocounting or autoradiographic techniques. Furthermore, radiolabelled B-cells containing different immunoglobulin isotypes were identified in mouse tissues by a combination of immunofluorescent staining with autoradiographic procedures. It was found possible to greatly accelerate this procedure by the use of liquid scintillation fluid so that ^{3}H-thymidine-labelled cells could be identified in less than 24 h as opposed to 4-6 wks in the absence of scintillation fluid.

In mice and rats, mesenteric lymph node (MLN) lymphoblasts showed a propensity to selectively localize in the gut mucosa. Careful examination of other mouse tissues revealed that MLN lymphoblasts were present, not only in the intestinal lamina propria, but also beneath the mucosal epithelia of the respiratory and

genital tracts, the mammary glands and in the MLN. In these mucosal tissues approximately 60% of these cells contained IgA and 25% contained IgG. In peripheral lymph nodes (PLN), a few labelled MLN cells were observed and 40% of these contained IgG, whereas only 8 % were of the IgA isotype. The preference of MLN to populate mucosal sites was clear from the results.

In marked contrast, when labelled PLN cells were adoptively transferred, the majority returned to their sites of origin and contained IgG. Of the small number of labelled PLN cells found in mucosal tissues, approximately equal percentages (30%) of IgA- and IgG-containing cells were seen.

Dividing cells prepared from bronchial (mediastinal) lymph nodes (BLN) showed a propensity to localize in the lungs rather than in the intestine or lymph nodes. However, the predominant immunoglobulin content of these donor cells in the gut, lungs, and MLN was IgA. Thus, the BLN made a quantitatively minor, but presumably significant, contribution to the IgA plasmacytes found in the gut lamina propria; in the lungs, BLN made a quantitatively major contribution to population of IgA-containing cells residing in the lung mucosa.

It was concluded that, in rodents, the MLN was a major contributor of the immunoglobulin-containing cells beneath the mucosal epithelia of the gut, lungs, cervix, and vagina and gestational mammary glands. Thus, lymph nodes draining two mucosal surfaces, though differing in IgA plasmacyte precursor content, both possessed

cells destined to localize in mucosal tissues. Furthermore, there was organ specificity for the distribution of these cells; those immediately derived from the lymph nodes draining the bowel tended to return to the bowel, whereas those from the lungs tended to return to the lungs.

To further explore the properties of MLN lymphoblasts, the influence of the mouse estrous cycle on MLN lymphoblast localization in the cervix and vagina was investigated. Compared to proestrus and estrus, a 2-fold reduction in the number of MLN lymphoblasts localizing in the diestral cervix and vagina was observed. Detection of the immunoglobulin isotype of these cells suggested that this reduction was restricted to the IgA plasmacyte progenitor population, i.e., the major B-lymphocyte subpopulation entering these sites. No significant changes in B-lymphoblast subpopulation lodging in the small intestine were observed over the course of the estrous cycle. Moreover, although the small intestine more than doubled in wet weight by late gestation, this increased quantity of gut tissue did not appear to compete for a finite number of donor MLN lymphoblasts. It was concluded that changes in the sex hormone status of mice may influence selective localization of MLN lymphoblasts in sex hormone target tissues.

Since subpopulations of cells can often be separated from each other on the basis of cell size, some preliminary studies were done to purify, on the basis of size, subpopulations of mouse MLN lymphoblasts. These results indicated that the labelled cells being transferred were all large in size, likely lymphoblasts, and that it

was possible to use this technique to obtain donor cell populations highly enriched in the proportion of labelled cells. Some evidence was obtained to suggest that the MLN contained a minor population of lymphoblasts with a propensity to localize in the spleen.

To examine the possibility that some lymphocytes in the gut mucosa may themselves have a predisposition to localize in other mucosal tissues (assuming that they were able to migrate), lymphocytes were mechanically prepared from the lamina propria of the guinea pig small intestine. A subpopulation of these cells incorporated ^{125}I-deoxyuridine and localized (within 24 h after adoptive transfer) in the gut mucosa in a manner similar to transferred MLN lymphoblasts and different from either PLN or Peyer's patch lymphoblasts. It was concluded that a portion of the lymphocytes seen in the gut mucosa were similar in migration characteristics to cells found in the MLN.

The results of these studies support the concept of a common mucosal immunologic system in which different mucosal surfaces are linked by migrating IgA plasmacyte precursors originating primarily in the lymphoid tissue associated with the intestinal and respiratory tracts.

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ACC	Anti-cholera toxin containing cells (IgA isotype)
α-chain	Heavy (high molecular weight) polypeptide chain of IgA
BALT	Bronchus-associated lymphoid tissue
B-cells	Bone marrow-derived lymphocytes
BLN	Bronchial (mediastinal) lymph nodes
BSA	Bovine serum albumin
Cpm	Counts per minute
Dpm	Disintegrations per minute
ESR	External standard ratio
FCS	Fetal bovine (calf) serum
FITC	Fluorescein isothiocyanate
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IP	Intraperitoneal
I.T.	Intratracheal
^{125}I-Udr	^{125}I-deoxyuridine
HBSS	Hanks' Balanced Salts solution
HEPES	N-2-hydroxyethylpiperazine-N-2'-ethane sulfonic acid
HPF	High power microscopic fields (0.105 mm^2 at magnification of 400 diameters)
^{3}H-Tdr	^{3}H-thymidine

LIST OF ABBREVIATIONS (cont'd)

κ-chain	κ-type light (low molecular weight) polypeptide chain of immunoglobulins
λ-chain	λ-type light (low molecular weight) polypeptide chain of immunoglobulins
MALT	Mucosa-associated lymphoid tissue
MLN	Mesenteric lymph nodes
MPC-11	IgG2b-secreting plasmacytoma
MOPC-315	IgA-secreting plasmacytoma
MOPC-774	IgM-secreting plasmacytoma
OVA	Ovalbumin
PBS	Phosphate buffered saline, pH 7.2
PFC	Plaque-forming cells
PLN	Peripheral lymph nodes
POPOP	1,4-bis(2-(5-phenyloxazolyl))benzene
PP	Peyer's patches
PPO	2,5-diphenyloxazole
S	Sedimentation velocity
SC	Secretory component
sIgA	Secretory form of immunoglobulin A
T-cells	Thymus-derived lymphocytes
TD	Thoracic duct

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 Introduction

A fundamental evolutionary achievement of vertebrates is a refinement of the ability to recognize certain chemical configurations as being non-self; to respond effectively to foreign invaders, a specialized population of cells termed lymphocytes arose with the potential to recognize non-self structures (antigens).

The portals of the body provide alien agents with easy access to mucosal sites interfacing the external environment. Here, local architectures and environments can promote proliferation of, and invasion by, such aliens. The vast area which mucosae present to the outside world ensures frequent encounters with potentially harmful intruders at different sites. Thus, an individual who is to survive must develop the ability to counteract harmful invasions at mucosal surfaces. An example of the early evolutionary development of defenses to protect mucosal surfaces is found in the gills and gut of species more advanced than cyclostomes as diffuse aggregates of lymphoid tissue (Good & Papermaster, 1964; Marchalonis, 1974). In mammalia and aves, the mechanism of host resistance to antigens contacting mucosal surfaces appears to be quite specialized. This chapter will be concerned with the understanding of humoral immunity at mucosal surfaces at the time that the work contained in

this thesis began. More recent information will be dealt with in Chapter 5.

Following a parenteral encounter with antigen, antibodies are produced by cells of the immune system and appear in serum. Serum antibody titres have commonly been considered representative of the effectiveness of the systemic immune response and, in a number of situations, there is good correlation between serum antibody titres and resistance to infections, although the mechanism by which serum is protective against potentially harmful agents is not always clear. Moreover, the benefits of passive transfer of serum antibodies in some cases are well recognized.

There is evidence, however, that immunity which protects mucosal surfaces is not reflected by the level of circulating serum antibodies. Besredka (1927) reported that rabbits orally immunized with heat-killed *Shigella* developed resistance to experimentally-induced bacillary dysentary. Since this protection was seemingly unrelated to serum antibody titre, it was postulated that a local type of humoral immunity in the gut could occur which was independent of systemic antibody responses. In support of this idea was the finding that anti-*Shigella* antibodies in the feces of humans with bacillary dysentary preceded the appearance of specific antibodies in serum (Davies, 1922). Similarly, when guinea pigs were orally immunized with *Vibrio cholera*, a high correlation between local fecal antibodies (coproantibodies) and protection against experimental cholera infection was found (Burrows *et al.*, 1947). Coproantibody titres were transient; both the presence of coproantibodies and

excretion of vibrios disappeared by 3 weeks post-infection. In contrast, parenteral immunization provided long-lasting systemic immunity but resistance to enteric *V. cholera* infection was poor unless coproantibodies were also present. Moreover, fecal and serum antibody titres appeared unrelated which, therefore, suggested that coproantibodies were not derived by transudation of serum into the intestinal lumen (Burrows *et al.*, 1950b). The observation that the intestinal antibody response was abolished by X-irradiation while the systemic response remained unaffected, further emphasized the independence of systemic and intestinal antibodies (Burrows *et al.*, 1950a). These studies clearly indicated that the intestinal type of antibody plays a major role in gut mucosal immunity.

Results have been obtained to suggest that a local immune response plays an important role in defense of the respiratory tract. Amoss and Taylor (1917) demonstrated the capacity of nasal secretions to neutralize poliovirus which was later shown to be due to specific antibodies (Francis *et al.*, 1943). Bull & McKee (1929) reported that intranasal immunization with killed pneumococcus could render rabbits resistant to pneumococcal respiratory infections and, in some resistant animals, anti-pneumococcal antibodies were undetectable in serum. These findings were confirmed by Walsh & Cannon (1936, 1938) who also showed that animals lacking detectable serum antibodies had, nevertheless, acquired significant immunity to pneumonia. Intranasal instillation of influenza virus resulted in the appearance of antibody levels in nasal secretions which were over ten-fold those produced by subcutaneous inoculation

(Fazekas de St. Groth, 1951; Fazekas de St. Groth *et al.*, 1951). Thus, the lungs seemed very similar to the gut in terms of local immune responses.

Evidence for local mucosal immunity has also been obtained for the female genital tract. Intravenous injection of viable *Trichomonas foetus* protozoa resulted in very high serum antibody titres although these animals exhibited the same susceptibility to intravaginal infection by trichomonads as normal, unimmunized animals (Byrne & Nelson, 1939). Furthermore, uterine antibodies specific for *Trichomonas* occurred in the absence of circulating antibodies whereas parenteral administration of trichomonads resulted only in serum antibodies (Kerr & Robertson, 1953). Intrauterine instillation of *Brucella abortus* led to vaginal and uterine antibody titres 28-fold higher than serum titres. *Brucella abortus* antibodies were absent in vaginal and uterine secretions following parenteral administration of antigen although serum titres were extremely high (Kerr, 1955). In addition, Batty and Warrack (1955) showed that local production of antibodies specific for diphtheria and tetanus toxoids can occur in the uterus and vagina. Taken together, the preceding examples indicate that the generation of local immunity is best achieved by local immunization while non-mucosal immunity is most successfully realized by parenteral administration of antigen.

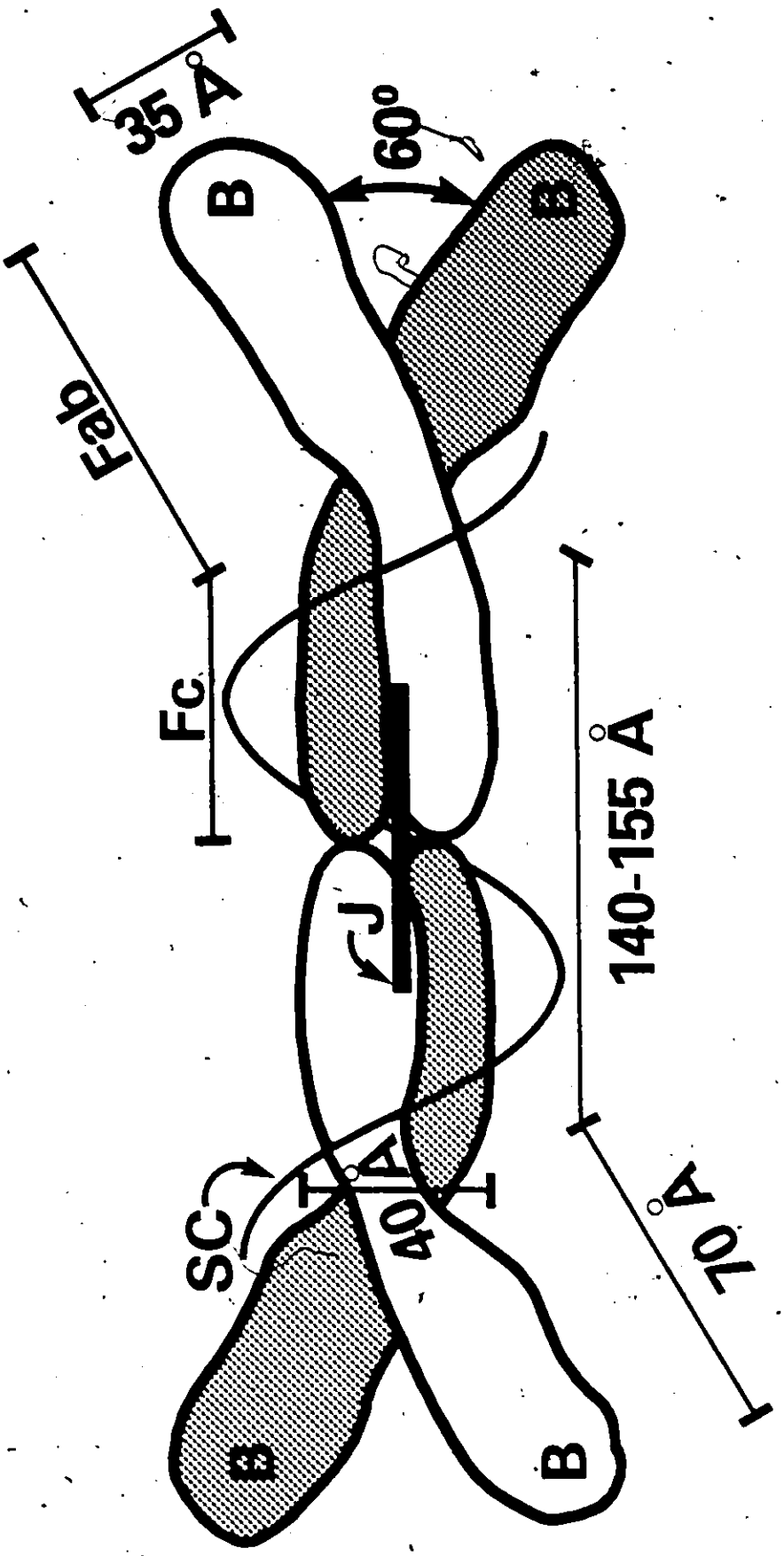
1.2 Humoral mechanism of local mucosal immunity

The discovery of immunoglobulin A (IgA) (Heremans *et al.*, 1959), its predominance over other immunoglobulins (Ig's) in most external secretions (Chodirker & Tomasi, 1963), and the preponderance of IgA-containing cells beneath secretory epithelia (Tomasi *et al.*, 1965) formed the basis for the suggestion that a distinct secretory immune system(s) might exist, whose properties were dissimilar, at least in part, from those responsible for systemic humoral immunity (Tomasi *et al.*, 1965).

Secretory IgA (sIgA) differs from serum IgA both in size and structure. Secretory IgA appears to be an 11S molecule (Tomasi *et al.*, 1965) having a molecular weight of 3.85×10^5 daltons (Newcomb *et al.*, 1968; Tomasi & Bienenstock, 1968). As illustrated by Figure 1.1, sIgA is composed of two 7S serum-type IgA molecules (each 1.7×10^5 daltons) covalently linked to each other by a glycoprotein (MW 1.5×10^4 daltons), termed the J-chain, (Halpern & Koshland, 1970; reviewed by Koshland, 1975) which is also present in polymeric IgM (Mestecky *et al.*, 1971). Additional antigenic determinants of sIgA are primarily due to a second covalently bound glycoprotein now called secretory component (SC) (South *et al.*, 1966) which has a molecular weight of 7.0×10^4 daltons (Newcombe *et al.*, 1968). However, additional conformational determinants have been described (Brandtzaeg *et al.*, 1970). *In vitro*, SC selectively binds to polymeric IgA, and to a lesser extent polymeric IgM, of a variety of species (Mach, 1970) which, therefore, suggests that IgM and IgA are phylogenetically related. Analysis of the structure of sIgA by electron

Figure 1.1

Schematic representation of the secretory form of an immunoglobulin A molecule. B, antigen-binding site; Fab, antigen-binding fragment; Fc, crystallizable fragment; J, J-chain; SC, secretory component. The positions of SC and J-chain remain speculative. This figure is a composite prepared from Bloth & Svehag (1971), Heremans (1975), and Koshland (1975).


35 Å
60°
B
B
Fab
Fc
J
140-155 Å
SC
40 Å
70 Å
B
B

microscopy has indicated that the two 7S IgA components are oriented in a "double Y" fashion (Bloth & Svehag, 1971). The positions of both J-chain and SC in the molecule remain speculative (Tomasi & Grey, 1972; Heremans, 1974).

The biological properties of sIgA further distinguish it from those Ig's predominating in the circulation. Perhaps the most significant feature of sIgA is its resistance to proteolytic degradation compared to serum (monomeric) IgA or IgG (Shuster, 1971; Underdown & Dorrington, 1974). Although the presence of SC in the molecule has been implicated in resistance to proteolysis (Brown *et al.*, 1970; Tax & Korngold, 1971; Underdown & Dorrington, 1974), the structure of the α-chain is certainly involved since serum IgA was more resistant than IgG to proteolysis (Underdown & Dorrington, 1974). By resisting proteolysis, sIgA could, in principle, remain functional in the enzymatically hostile environments found at mucosal surfaces such as in the gut lumen.

Some evidence indicates that IgA plays a role in defense against infection at mucosal surfaces by neutralizing toxins (Holmgren *et al.*, 1975) and viruses such as poliovirus (Ogra *et al.*, 1968; Ogra & Karzon, 1969a, 1971). It has also been shown that sIgA could block the adherence of bacteria to mucosal epithelia and thus could prevent colonization (Williams & Gibbons, 1972; McClelland *et al.*, 1972; Freter, 1969, 1970, 1974; Hill & Porter, 1974). Furthermore, sIgA may play a role in regulating the uptake and transport of certain antigens. Complexes formed by soluble antigens and secretory antibodies might prevent uptake

by intestinal epitheliocytes. Walker and colleagues (Walker *et al.*, 1972, 1973) found that oral immunization led to a specific decrease in intestinal uptake of antigen administered subsequently to both germ-free and conventional rats. Similar findings were obtained by André (André *et al.*, 1974). The high incidence of both antibodies specific for dietary antigens and autoimmune phenomena in IgA-deficient individuals (Buckley & Dees, 1969; Tomasi & Katz, 1971) is consistent with a model of sIgA-mediated antigen handling at mucosae. If prevention of antigen contact with the elements of the immune system is often the primary function of sIgA, it is not surprising that IgA does not activate complement by either the classical or alternate pathway (Ishazaka *et al.*, 1965; Adinolfi *et al.*, 1966; Gotze & Muller-Eberhard, 1971; Colten & Bienenstock, 1974). Moreover, sIgA lacked opsonization properties and was incapable of promoting phagocytosis of antigen by neutrophils and monocytes in either the presence or absence of complement (Lipursky *et al.*, 1973). Thus, these mechanisms for antigen elimination did not appear to be required for effective resistance to mucosal invasion and, if present, could lead to mucosal inflammation with concommitant exposure of antigens to immune elements.

Several models can be entertained to explain the predominance of IgA in external secretions. Simple filtration from serum into mucosal fluids and reabsorption of serum proteins with the exception of IgA seems unlikely. Transudation of serum proteins into secretions seems possible especially since Fazekas de St. Groth (1951) showed that astringents could promote transudation of serum antibodies,

specific for influenza virus, into the respiratory passages. This phenomenon was described by Fazekas de St. Groth (1951) as "pathotopic potentiation". Since sIgA is resistant to proteolytic digestion (Brown *et al.*, 1970; Tax & Korngold, 1971; Underdown & Dorrington, 1974), it might appear predominant to other serum proteins which are less resistant to proteases. Against such a possibility are the observations that parotid fluid lacked proteolytic activity and would not degrade IgG (Chauncey, 1961), but nevertheless contained predominantly sIgA (Tomasi *et al.*, 1965). Furthermore, 7S serum IgA was much less resistant to proteolytic degradation than 11S sIgA (Underdown & Dorrington, 1974). Alternatively, IgA may have a selective transport advantage into secretions. Intravenous administration of either radiolabelled 7S serum IgA or 11S sIgA did not indicate a major selective transport from serum to secretions (Tomasi *et al.*, 1965; South *et al.*, 1966; Stiehm *et al.*, 1966; Haworth & Dilling, 1966; Strober *et al.*, 1970). Moreover, serum agglutinating antibody could not be correlated with antibody in secretions (reviewed by Tomasi & Bienenstock, 1968). Taken together, these data indicate that the serum is not a major source of IgA present in secretions at least for those mucosal surfaces which had been examined (see Chapter 5 for some exceptions).

Most of the IgA found at mucosal surfaces originates from local synthesis. Compared to the other Ig isotypes, plasma cells containing IgA were predominant beneath secretory epithelia (Tomasi *et al.*, 1965; Crabbé *et al.*, 1965; Tourville *et al.*, 1969, 1970). *In vitro* synthesis of all of the components of sIgA has been

demonstrated in the gut (Bull *et al.*, 1971; Kagnoff *et al.*, 1973), salivary glands (Hochwald *et al.*, 1964; Hurlimann & Darling, 1971) and mammary glands (Asofsky & Small, 1967; Lawton & Mage, 1969; Lawton *et al.*, 1970). Antibody synthesis of unknown isotype was shown to occur *in vitro* by rabbit vaginal tissue immunized with diphtheria toxoid prior to extirpation (Bell & Wolf, 1967).

The synthesis of dimeric IgA appears to occur within a single plasma cell. Lawton & Mage (1969) and Bienenstock & Straus (1970) demonstrated that 11S IgA contained either κ- or λ- chains but not both. These results suggested that sIgA molecules were synthesized as 11S dimers. If 11S molecules were either the collaborative product of two cells making 7S monomers or the association of serum-derived IgA monomers then the 11S dimers would be expected to show light-chain heterogeneity. One report (Costea *et al.*, 1968) suggested that 11S dimeric IgA may be heterologous for light chains but this finding is open to criticism in regard to technical artifacts. Thus, a mucosal plasma cell synthesizing identical 7S IgA monomers, as well as J-chain, constructs a 9S dimer (O'Daly & Cebra, 1971; Della Corte & Parkhouse, 1973) which is transported toward external secretions. In fact, IgA-containing plasma cells in the intestinal mucosa appeared to be a major source of the serum IgA pool (Vaerman & Hereman, 1970).

Secretory component, an epithelial cell product (Tourville *et al.*, 1969; Brandtzaeg, 1974; Poger & Lamm, 1974) is coupled to dimeric IgA either in or near to the mucosal epitheliocytes and granular cells. The completed sIgA molecule is then secreted externally.

1.3 Humoral immune response in gastrointestinal tract

A large body of literature indicates that oral immunization may lead to the appearance of specific IgA antibodies in gastrointestinal secretions (reviewed by Tomasi & Bienenstock, 1968; Heremans, 1974). In mice, oral immunization with either soluble or particulate antigen can result in an antigen-specific, predominantly IgA plasmacyte response in the lamina propria of the gastrointestinal tract and mesenteric lymph nodes (Crabbé *et al.*, 1969; Bazin *et al.*, 1970). However, the site(s) of initiation of mucosal antibody responses following oral immunization have yet to be identified. The most likely candidates for initiation of the intestinal immune responses are the mesenteric lymph nodes (MLN) and the gut-associated lymphoid tissue (GALT) which includes lamina propria lymphocytes, unorganized lymphoid aggregates, the Peyer's patches (PP) and appendix.

Many studies have directly revealed that the mature intestinal epithelium is indeed permeable to antigenically significant quantities of intact macromolecules. Such antigens included equine ferritin (4.66×10^5 daltons) (Bockman & Winborn, 1966; Casley-Smith, 1967), horseradish peroxidase (4.4×10^4 daltons) (Cornell *et al.*, 1971; Warshaw *et al.*, 1971, 1972), cholera toxin (8.4×10^4 daltons) (Kao *et al.*, 1972), *Clostridium botulinum* type A toxin (1×10^6 daltons) (May & Whaler, 1958), and bovine serum albumin (6.8×10^4 daltons) (Warshaw *et al.*, 1974; Rothberg *et al.*, 1969). Thus, contact between some kinds of antigens and immunocompetent lymphocytes could occur in the intestinal lamina propria.

Unfortunately, little is known about the identities of gut lamina propria lymphocytes. Approximately 11% of these cells were killed by antiserum specific for rabbit thymic lymphocyte antigen and complement (Rudzik *et al.*, 1975a). Although cells containing IgA predominate in the gut lamina propria compared to other Ig isotypes (Tomasi *et al.*, 1965), only 16% of the rabbit gut lamina propria lymphocytes had surface Ig. Nevertheless, 50% of cells bearing Ig had surface IgA. These results suggest that antigen reactive cells might reside in the intestinal lamina propria, at least in the rabbit.

Much attention has been directed toward the PP and appendix with regard to initiation of immune responses to enteric antigens. PP are discrete islands of organized lymphoid tissue arising within the lamina propria of the gut. Friedenstein & Goncharenko (1965) identified bacteria, presumably of enteric origin, within macrophages in the rabbit appendicial lymphoid follicles. Similarly, viable *Salmonella typhimurium* was recoverable from mouse PP shortly after infection (Carter & Collins, 1974). Administration of either carbon particles or radiolabelled, aggregated bovine γ-globulin via the rabbit appendicial artery or lumen resulted in the appearance of these antigens within reticular cells in appendicial lymphoid tissues (Hanaoka *et al.*, 1971). Electron microscopic examination of normal, unobstructed small intestine obtained from humans and rodents has revealed a unique epithelial cell type located in areas of non-columnar epithelium overlying the lymphoid follicles in PP (Bockman & Cooper, 1973; Owen, 1974). This cell type was characterized

by luminal surface microfolds and has been termed the M-cell. It was not identified as an epithelial component of villus areas. Since M-cells were pinocytotic and allowed lymphocytes to closely approach the gut lumen, it was suggested that they might prepare and present antigen to lymphocytes, similar to a function postulated for macrophages (Bona *et al.*, 1972). Indeed, exposure to antigens via the intestinal lumen appears to be essential for the normal development of the GALT. PP were poorly developed in germ-free mice but matured following oral but not parenteral immunization (Pollard & Sharon, 1970). Moredver, when fetal gut was transplanted into presumably antigen-free environments, the GALT remained immature (Ferguson & Parrot, 1972; Ferguson, 1974; Milne *et al.*, 1975). These studies clearly indicate that the PP and appendix were equipped to present enteric antigens to the immune system.

Some information has been obtained about the identity of cells found in the PP. PP contain both thymus-derived and bone marrow-derived lymphocytes (T-cells and B-cells, respectively). In the mouse, approximately 10% to 25% of PP lymphocytes were identified as T-cells either by immunofluorescence (using a heterologous anti-T-cell serum) or by cytotoxicity with homologous anti-theta antigen serum and complement (Raff & Owen, 1971; Guy-Grand *et al.*, 1974). Approximately 70% of PP cells stained positive using a fluorescent heterologous antiserum specific for mouse B-cells and about 60% of cells had κ-light-chain determinants on their surfaces (Guy-Grand *et al.*, 1974). Of the PP cells bearing surface Ig, about 10% to 25% bore surface IgA (McWilliams *et al.*, 1974;

Guy-Grand *et al.*, 1974). However, some evidence from work in rabbits indicated that much of the IgA on the surface of PP cells may not be of endogenous origin but acquired by cytophillic absorption (Jones & Cebra, 1974). Approximately 3.2% PP cells were B-lymphoblasts and the majority of these made surface α-heavy-chains. Extremely few PP lymphocytes contained intracellular Ig (McWilliams *et al.*, 1974; Guy-Grand *et al.*, 1974), and Dolezel & Bienenstock (1971) did not detect antigen-specific Ig within PP cells following oral or parenteral immunization. Collectively, these results indicate that although PP contain many B-lymphocytes, very few of these cells are completely differentiated. This conclusion is consistent with the hypothesis that the PP occupy a very early position in the afferent immune pathway.

PP contain both T- and B-cell-dependent regions similar to lymph nodes and spleen. De Sousa *et al.* (1969) observed that congenitally athymic (nude) mice possessed few cells in PP inter-follicular regions. Similarly, Sprent (1973) found that chronic thoracic duct lymph drainage (in which 75% of cells are T-lymphocytes (Guy-Grand *et al.*, 1974)) resulted in the selective depletion of interfollicular regions in PP. Depletion of the B-dependent regions of PP took nearly three times longer. Thus, it seems that only a small portion of both T- and B-cells found in PP arise from stem cells located therein.

Following the passage of antigens into the intestinal lamina propria and PP, the next lymphoid structures to which antigens might penetrate are the MLN (Carter & Collins, 1974). Here,

cells emigrating from the PP (Sprent, 1973) (and perhaps the gut lamina propria) could interact with antigens and eventually pass into the thoracic duct lymph (Yoffey & Courtice, 1970).

There are significant differences in the cells found in the MLN compared to those in the PP. The proportion of B-cells in the MLN was found to be considerably lower (35%) and although only 0-8% of these cells were synthesizing DNA, the majority of the B-lymphoblasts had surface and/or intracellular IgA (Guy-Grand *et al.*, 1974; McWilliams *et al.*, 1974) and lacked receptors for complement components (Bianco *et al.*, 1970). Indeed, the great majority of thoracic duct (TD) lymphocytes (0.25% were B-lymphoblasts) had both surface and intracellular IgA (Guy-Grand *et al.*, 1974). The fact that 80% of TD lymphocytes came from the MLN is in keeping with these findings (Yoffey & Courtice, 1970). Thus, it seems that the MLN probably contains a population of B-lymphocytes at a later stage of differentiation than those located within the PP.

In contrast to cells in the PP, MLN and thoracic duct, lymphocytes obtained from either peripheral (axillary, brachial, inguinal, and popliteal) lymph nodes (PLN) or spleen bore and contained predominantly IgG or IgM (Guy-Grand *et al.*, 1974; McWilliams *et al.*, 1974; Rudzik *et al.*, 1975a). Thus, it could be suggested that PP might be an important source of the immediate precursors of IgA plasmacytes, whereas plasmacyte precursors in peripheral lymphoid tissue are destined to synthesize either IgG or IgM.

Perhaps the most compelling evidence to support the contention that PP are involved in the IgA immune response by providing an enriched source of the immediate precursors of IgA plasma cells comes from the work of Craig & Cebra (1971). Six days after adoptive transfer into lethally irradiated, allogeneic host rabbits, donor lymphocytes prepared from the PP appeared almost exclusively as IgA plasmacytes in the gut and spleen. In contrast, transferred lymphocytes obtained from popliteal lymph nodes or peripheral blood did not repopulate the intestine but appeared primarily as IgG-containing cells in the spleen. Usually, IgA plasma cells are uncommon in the rabbit spleen (Crandall *et al.*, 1967) and would not be expected in this site after transfer of IgA plasma cell precursors. Rudzik *et al.*, (1975b,c) corroborated the results of Craig & Cebra (1971) and showed that repopulation of the irradiated recipient spleen after transfer of PP cells was most likely due to an unphysiologic allogeneic effect.

Subsequently, it was shown that a sub-population of PP cells was responsible for the generation of IgA-containing cells after adoptive transfer. This precursor population, which comprised about 80% of the total cells, had no detectable endogenous heavy-chain determinants, but did have light-chain determinants (Jones *et al.*, 1974). When cultured *in vitro*, these cells could be stimulated by pokeweed mitogen to synthesize IgA, whereas this was not the case when PLN were cultured and stimulated. Circumstantial evidence suggested that these cells might have been identical to a subpopulation of PP cells which had surface IgA

allotypic determinants (Jones & Cebra, 1974). It could be that IgA antigenic determinants on the cell membrane are arranged in such a way as to be undetectable by heterologous anti-rabbit α-chain serum. Taken together, these studies are consistent with the idea that the PP are a very fertile source of the immediate precursors of IgA plasmacytes which require several days to mature and ultimately take up residence in the gut lamina propria appearing as IgA plasma cells. Since adoptive transfer of cells is essentially an artificial release of lymphocytes from lymphoid sources, it seems likely that the IgA plasma cell progenitors migrated at some time from the PP to the MLN and then via the circulation to the gut.

Some evidence indicates that the MLN harbours a population of rapidly-dividing B-lymphocytes which are committed to the synthesis of IgA and are destined to become IgA-plasmacytes in the intestinal lamina propria in much less than 6 days. Within 24 h following adoptive transfer of radiolabelled lymphoblasts into syngeneic recipient rodents, MLN lymphoblasts (and to a lesser degree TD lymphoblasts) showed a remarkable propensity to localize in the gut mucosa and these cells had little affinity for PLN. Approximately 60% of radiolabelled MLN donor lymphoblasts in recipient intestine contained intracytoplasmic IgA (Gowans & Knight, 1964; Griscelli et al., 1969; Hall & Smith, 1970; Hall et al., 1972; Guy-Grand et al., 1974; Parrot & Ferguson, 1974). By comparison, labelled PLN donor lymphoblasts returned primarily to recipient PLN and spleen and became IgG plasmacytes. Moreover, in contrast to lymphoblasts from either source, ^{3}H-uridine-labelled small

lymphocytes obtained from the same anatomical sources showed little or no tendency to selectively localize in any site. These results indicated that the MLN contained a population of blastogenic, IgA plasmacyte progenitors which were much more differentiated than those present in the PP. The observations that either lymphoblasts or small lymphocytes obtained from PP did not preferentially localize within 24 h in either recipient PP or anywhere else (Guy-Grand *et al.*, 1974; Zatz & Lance, 1970), is consistent with this conclusion. B-lymphocytes in the PP which were destined to become IgA plasma cells might have been at a differentiation stage that did not provide them with the means to recognize the gut mucosa as their localization site.

Since oral immunization could lead to an IgA antibody response in the gut mucosa and the MLN (Crabbé, 1969; Bazin, 1970), it could be suggested that certain B-cells in the GALT became sensitized to enteric antigens and began differentiating into IgA plasmacytes; this process required several days. However, once these cells migrated to the MLN, they were totally committed to develop into IgA plasma cells within a short period of time and had also acquired the capacity to quickly localize primarily in the intestinal lamina propria. Thus, it might have been this localization which accounted for the predominance of IgA antibodies in mucosal secretions and little specific antibody in serum. In contrast, cells sensitized to antigens in PLN would be expected to eventually return to PLN and synthesize specific IgG class antibodies which are predominant in the serum and not in external secretions.

The nature of the developmental alterations that cause IgA plasma cell progenitors in the MLN to localize in the gut mucosa is unknown. One model suggests that localization of MLN lymphoblasts in the gut mucosa might be antigen-directed and dependent upon antigen-specific cell surface receptors. Although antigen may play a part in lymphoblast localization, it is unlikely to be the only cause. Rat thoracic duct lymphoblasts localized in the intestinal lamina propria upon injection into neonatal, colostrum-deprived rats and mice (Halstead & Hall, 1972; Moore & Hall, 1972). GALT was present in transplanted fetal gut which was presumably antigen free (Ferguson & Parrot, 1974), and thoracic duct and MLN lymphoblasts localized in transplanted fetal gut (Moore & Hall, 1972; Guy-Grand *et al.*, 1974; Parrot & Ferguson, 1974). An alternative model suggests that other types of cell surface receptors play key roles in lymphocyte localization. For example, procedures calculated to either enzymatically destroy or alter cell surface molecules interfered with cell lodging (Gesner & Ginsburg, 1964; Woodruff & Gesner, 1968; Gesner *et al.*, 1969; Woodruff, 1974). If the enzymatically treated cells were allowed to repair their cell membranes *in vitro* and then injected, lymphocyte localization was unaffected. Finally, the fact that IgA-containing cells are found close to mucosal epithelia suggests that SC, an epithelial cell product (Tomasi & Bienenstock, 1968) might serve as a receptor for localization of cells bearing surface IgA (Brandtzaeg, 1973, Uhr & Vittetta, 1974; McWilliams *et al.*, 1974). At present, the roles of antigen, Ig, and receptors such as SC in explaining selective lymphoblast localization are unknown, but all may contribute.

If the PP, in fact, contain primarily IgA plasmacyte progenitors destined to localize beneath the gut epithelium it might be expected that they would lack mature immunocompetent B-cells. The evidence to support this expectation is conflicting. Antigen-specific antibody-containing cells were not found in hamster PP following either oral or systemic immunization although such cells appeared in other tissues (Bienenstock and Dolezel, 1971). Following either feeding, parenteral administration, or injection of antigen directly in PP, no antibody-forming cells were found in rabbit PP (Henry *et al.*, 1970). Neither intraperitoneal nor intraduodenal primary immunization with *Vibrio cholera* resulted in the appearance of IgM plasma cells in rodent PP (Veldkemp *et al.*, 1973). However, secondary immunization by either of these routes led to an IgM response in PP. In opposition to these data, direct injection of antigen into PP in rats, hamsters and mice elicited antibody-producing cells in the injected patch (Cooper & Turner, 1967; Dolezel & Bienenstock, 1971; Veldkemp *et al.*, 1973). However, these findings might be explained by antigen-mediated recruitment of cells into the injection site. It should be noted that the number of antibody-forming cells detectable in the immunized patch of the rat (Cooper & Turner, 1967) was only 10% of the number appearing in MLN. Moreover, *in vitro* investigations of PP immuno-competancy are also confusing. For example, primary cultures of PP cells obtained from normal rabbits responded to the presence of antigen by the generation of IgM-secreting cells (Henry *et al.*, 1970). Both IgM- and IgG-secreting cells appeared in antigen stimulated cultures of PP cells prepared from systemically immunized rabbits.

Perhaps the presence of IgG-secreting cells in these cultures reflected the presence of antigen-stimulated, circulating cells from the PLN being present in the PP at the time of culture preparation. Thus, the available data do not allow a firm conclusion as to presence of immunocompetent cells within the PP. Nevertheless, it seems that the PP contain a population of cells which ultimately become IgA plasma cells in the gut mucosa.

1.4 Humoral immune response in the respiratory tract

In health, the bronchial and nasal mucosae contain substantially fewer immunoglobulin cells than does the lamina propria of the gastrointestinal tract (Crabbé & Heremans, 1966; Brandtzaeg *et al.*, 1967; Martinez-Tello *et al.*, 1968). Nonetheless, the immune response in the upper respiratory tract is characterized by a predominance of sIgA over other immunoglobulins in secretions. Infections with rhinovirus (Cate *et al.*, 1965; Perkins *et al.*, 1969), influenza virus (Bellanti *et al.*, 1965; Alford *et al.*, 1967; Mann *et al.*, 1969; Rosen *et al.*, 1970), parainfluenza virus (Smith *et al.*, 1966; Blandford & Heath, 1974), and adenovirus (Smith *et al.*, 1970), led to humoral immune responses in bronchial and nasal secretions in which IgA was found in the greatest amount compared to other Ig isotypes. Since there was a preponderance of IgA-containing cells beneath the bronchial and nasal epithelia (Tourville *et al.*, 1969; Brandtzaeg *et al.*, 1967), IgA in respiratory tract secretions was most likely derived from local synthesis. Moreover, Cassal and coworkers (Cassal *et al.*, 1974) observed that antibody-containing cells of the IgA

isotype appeared to be the major population infiltrating the lungs of pathogen-free mice infected with *Mycoplasma pulmonis*. In humans, locally produced IgA antibodies may be related to resistance to *M. pneumoniae* infection (Brunner & Chanock, 1973). Indeed, the protective effects of pulmonary IgA antibodies are emphasized by the observations that IgA-deficient humans suffered from an increased prevalence of either respiratory tract infections or associated disorders (Ammann & Hong, 1971). However, species and physiological differences in the humoral immune response to respiratory infections have been reported. Fernald and colleagues (Fernald *et al.*, 1972) found that *M. pneumoniae* infection of hamsters resulted in a predominantly IgM antibody containing cell response when compared to IgA. Asymtomatic, IgA-deficient individuals appear to replace IgA in their secretions with IgM, much of which is seemingly locally produced (Stobo & Tomasi, 1967; Eidelman & Davis, 1968). Perhaps the species and physiologic state of the host and the type of antigen contacted by the pulmonary mucosa play a significant role in both the quantity and quality of the humoral immune response in the upper airways. Collectively, the above results indicate that the immune response in the upper respiratory tract is, in many ways, similar to that found in the gut.

Compared to the upper airways, some significant differences can be found in the humoral immune response in the lower respiratory passages. Infection of rabbits with either *Diplococcus pneumoniae* or *Listeria monocytogenes* led to the appearance of antigen-specific IgA antibodies in the lower respiratory tract accompanied by a

marked increase in local synthesis of IgG (Hand & Cantey, 1974). Similarly, intrabronchial immunization of dogs with sheep erythrocytes produced an antibody response which was confined to the IgM and IgG isotypes (Kaltreider *et al.*, 1974). The results of these studies imply that functional differences exist between the immunological apparatus associated with the upper and lower respiratory tracts.

Although little is known about the mechanisms by which inhaled antigens can cross the pulmonary epithelium, some evidence indicates that this phenomena does occur. Drinker & Hardenbergh (1947) reported that after intratracheal administration of either bovine serum albumin (BSA) or ovalbumin (OVA) to dogs, foreign albumin could be detected in the right lymphatic duct. More recently, it has been shown that lung tissue in dogs and guinea pigs was capable of absorbing up to 33 mg of radioiodinated human albumin within 48 h after intratracheal instillation, i.e., mean absorption rate of 0.69 mg/h (Dominguez *et al.*, 1967). It is interesting that intact radioiodinated albumin was absorbed from the rat intestinal lumen at a rate at least two orders of magnitude less than that absorbed from the lungs (Warshaw *et al.*, 1971, 1974). Thus, the lungs appear to be as capable as the gut in regard to absorption of intact antigens, but the site(s) of antigen absorption and interaction with the pulmonary immune elements are unknown.

One possible location for interaction between antigen and the pulmonary lymphoid elements might be the lymphoid follicles in the bronchial walls described by Klein (1875). Bienenstock and colleagues (Bienenstock *et al.*, 1973a,b) have studied these organized

lymphoid aggregates in detail and termed them the bronchus-associated lymphoid tissue (BALT). In lagomorphs, rodents, swine, canines, birds and humans, BALT bore a striking morphological resemblance to the PP. BALT possessed a lymphoepithelium and contained both T- and B-cells. Approximately 18% of BALT lymphocytes in the rabbit were killed by antiserum specific for rabbit T-cells in the presence of complement (Rudzik *et al.*, 1975a). Of the BALT B-lymphocytes (50% of the total cells) bearing surface Ig, the majority were found to have membrane α-heavy-chain determinants. Moreover, BALT lacked antibody-containing cells within the follicles. Although BALT was randomly distributed along the bronchial tract, it was concentrated at bronchial bifurcations, sites known to impact inhaled antigens. Thus, BALT was very much similar to GALT and dissimilar to peripheral lymphoid tissues in all parameters tested.

In view of the fact that PP and MLN were a fertile source of IgA plasma cells found beneath intestinal epithelia, and that BALT possessed some properties similar to GALT, it was proposed that BALT might be part of a more universal mucosal lymphoid system in which GALT and BALT might be the sources of antigen sensitized cells which are disseminated and ultimately localize and differentiate into IgA-secreting plasma cells beneath mucosal epithelia (Bienenstock *et al.*, 1973a,b). On the other hand, cells present in peripheral lymphoid tissues would likely be relatively deficient in this capacity. Accordingly, this hypothesis was tested using the experimental animal model originally described by Craig & Cebra (1971). It was demonstrated (Rudzik *et al.*, 1975c) that cells derived from the BALT

had virtually the same capacity as PP cells to repopulate the spleen of allogeneic recipients with IgA-containing cells. Of greater significance, cells derived from the BALT were able to equally repopulate the lungs and gut with IgA plasmacytes. Similarly, GALT lymphocytes repopulated the gut and to nearly the same extent the bronchus, with IgA-containing cells. In marked contrast, cells prepared from the popliteal lymph nodes were much less capable of repopulating the bronchus, bowel or spleen with IgA-containing cells. From these studies it was concluded that cells from one mucosal site may localize at another mucosal site. Thus, it seemed that cells sensitized to antigens at one mucosal site might journey to distant mucosae and provide antigen-specific immunity in secretions.

A second location at which inhaled antigens might interact with the immune system is the bronchial (mediastinal) lymph nodes (BLN) draining the lungs. Either intratracheal or intraperitoneal immunization will lead to the presence of antigen in the BLN (Yoffey & Courtice, 1970) accompanied by a substantial increase in BLN size.

Unfortunately, the functional properties of cells within the BLN have not been adequately studied. Investigations in rabbits (Holub & Hauser, 1969; Ford & Kuhn, 1973), mice (Nash, 1973) and dogs (Kaltreider *et al.*, 1974) showed that plaque-forming cells (PFC) appeared in the BLN following intrapulmonary immunization with sheep erythrocytes, but were absent following parenteral administration of antigen. The antibody-containing cell response was primarily of the IgM isotype and controversy exists concerning the presence of IgA class PFC in the BLN. Thus, the nature of BLN lymphocytes

remains a fertile area for further exploration.

The origin(s) of the antibody-containing cells which appear to populate the lungs in response to infection are unknown (Cassel *et al.*, 1974; Blandford & Heath, 1974). Similarly, the sources of lymphocytes found in pulmonary washings have not been identified. Since both the GALT and BALT appear to be productive sources of the immediate precursors of IgA plasmacytes (Craig & Cebra, 1971; Rudzik *et al.*, 1975a,b,c), lymphocytes in the respiratory tract might have originated in these sites (Clancy & Bienenstock, 1974). Thus, like the MLN, the BLN might be expected to contain a population of cells more differentiated than those contained in either the GALT or BALT. It could be predicted that both MLN and BLN cells are committed to IgA synthesis and would selectively localize beneath mucosal epithelia, particularly in the lungs and gut. If this were so, antigen interacting with the lymphoid tissues of the intestinal and respiratory tracts (the major portals of entry by intruders) would provide an organism with mucosal immune defenses capable of functioning interchangeably and therefore, in principle, more effectively.

1.5 Humoral immune response in the female reproductive organs

1.5.1 Mammary glands

Unlike the intestinal and respiratory tracts, the mammary glands do not contain any organized lymphoid aggregates. However, the mammary secretions (colostrum and milk) contain significant quantities of several Ig isotypes (Tomasi & Bienenstock, 1968).

In ungulates, the origin of these antibodies is diverse. The mammary glands of swine and ruminants have been shown to be capable of selectively concentrating and secreting serum Ig's across the ductal mucosa (Lascelles & McDowell, 1970; Porter, 1972; Bourne, 1973). In these animals 7S IgG was the predominant immunoglobulin in colostrum and milk. Neonatal ungulates selectively absorb intact Ig's from milk in the gut lumen directly into the circulation. Clearly, this phenomena is necessary for survival since epitheliochorial placentation in ungulates does not allow the passive transfer of humoral immunity from mother to offspring *in utero* (Brambell, 1970). Nevertheless, antigen infusion into ungulate udders led to a local immune response which was manifested as sIgA in milk (Lee & Lascelles, 1970; Wilson *et al.*, 1972; Bohl *et al.*, 1972). Most evidence indicated that milk sIgA was synthesized locally (Lee & Lascelles, 1970; Bourne & Curtis, 1973). Thus, it appears that humoral immunity in ungulate mammary tissue is derived from both serum transudation and local synthesis of Ig's.

In primates, rodents, and lagomorphs, sIgA is the predominant Ig in milk and colostrum (Tomasi & Bienenstock, 1968), and appears to be locally synthesized (Hochwald *et al.*, 1964; Bienenstock & Straus, 1970). In these animals, breast milk sIgA did not appear to be absorbed from the neonatal gut lumen (Brambell, 1970; Hemmings *et al.*, 1973), but seemingly acted locally in the intestinal tract to protect against enteric infection. [Similar functions have been proposed for sIgA in the swine alimentary tract although much sIgA is absorbed (Porter *et al.*, 1970; Porter, 1973).]

In order to perform a protective role in the gut, milk antibodies must have specificity for the antigens of intestinal microorganisms. Bohl and co-workers (Bohl *et al.*, 1972) showed that porcine milk could contain sIgA antibodies specific for a gastro-intestinal virus. Similarly, infection of pregnant women with *Salmonella typhimurium* was observed to result in *S. typhimurium*-specific IgA antibodies in colostrum (Allardyce *et al.*, 1974). Furthermore, when pregnant rabbits were orally immunized with a haptenated antigen, sIgA antibodies specific for the hapten appeared in colostrum and milk (Montgomery *et al.*, 1974). These findings suggested that the IgA plasma cells lying beneath the mucosal epithelium of the mammary ducts had previously encountered enteric antigen although the mechanism(s) by which this occurred was unknown.

1.5.2 Vagina, cervix and uterus

Little is known about the humoral immunity of the female reproductive organs. The vagina, cervix, and uterus are lined with secretory epithelia but organized lymphoid aggregates are absent. Vaginal and cervical mucus contains significant quantities of Ig with sIgA being the predominant isotype (Chodirker & Tomasi, 1963; Tomasi *et al.*, 1965). Following exposure of the cervico-vaginal canal to microbial antigen, an antigen-specific antibody response may be detected in cervical-vaginal secretions (Kerr, 1953; Kerr & Robertson, 1953; Parish *et al.*, 1967; Waldman *et al.*, 1972; Chipperfield *et al.*, 1972; Wilkie *et al.*, 1973; Ogra & Ogra, 1973). Usually, sIgA antibodies predominate over other Ig isotypes with these types of immunizations in the cervix and vagina. For example,

infection with either *Candida albicans*, *Neisseria gonorrhoeae*, *Vibrio fetus* or poliovirus caused the appearance of specific antibodies in which sIgA had the highest titre (Waldman *et al.*, 1972; Chipperfield *et al.*, 1972; Wilkie *et al.*, 1973; Ogra & Ogra, 1973). However, one report (Chipperfield *et al.*, 1972) indicated that the type of infection might be important in eliciting the sIgA antibody response; *Trichomonas* caused a predominantly IgM antibody response. It should be noted here that although cervico-vaginal secretions are collected from the vagina and cervix, this does not necessarily mean that they originated at these sites. Although such secretions could have been a product of the uterus or oviducts, this does not seem to be the case. Ogra & Ogra (1973) observed that intravaginal immunization with poliovirus resulted in a predominantly sIgA response in the cervix and vagina. Intrauterine immunization led to an IgG response in the uterus which was not mirrored in the cervico-vaginal secretions. Moreover, IgA appears to be produced locally since there is a preponderance of IgA plasma cells beneath the mucosal epithelium of the cervix and vagina (Tomasi *et al.*, 1965; Tourville *et al.*, 1969, 1970) and rabbit cervix synthesized IgA antibodies *in vitro* (Behrman, 1970). Thus, sIgA-mediated immunity in the vagina and cervix seemingly protects the uterus from infection. The observation that the uterus is aseptic and might lack the capabilities for local sIgA synthesis (Ogra & Ogra, 1973) is consistent with this idea.

In health, there is a paucity of Ig-containing cells in the cervico-vaginal mucosae (Tourville *et al.*, 1969, 1970). However, intravaginal infection often leads to a marked increase in the

frequency of IgA plasma cells in these sites (Waldman *et al.*, 1972; Chipperfield *et al.*, 1972; Ogra & Ogra, 1973). The sites of origin of the IgA plasmacytes in the genital tract are unknown. Such sites are likely important if control of genital infections is to be accomplished.

1.6 A common mucosal immunologic system

Parenteral immunization can lead to humoral immunity at parenteral locations quite distant from the immunizing site. Therefore, it might be expected that immunization at one mucosal site could lead to humoral immunity (predominantly sIgA) at other mucosal surfaces. However, the degree of dissemination of the IgA immune response to distant mucosae seems to be quite variable. When poliovirus vaccine was introduced into the distal colon of patients with double-barrelled colostomies, a primary IgA response was elicited which diminished in magnitude with increasing distance from the site of vaccination (Ogra & Karzon, 1969b). Similarly, Mann and colleagues (Mann *et al.*, 1969) reported that aerosol immunization of humans with influenza virus was associated with an increase in specific anti-viral IgA antibodies in the sputum and nasal washings but not in saliva. These data demonstrated that the secretory response is confined to the site of immunization, i.e., a local immune response.

In contrast, several pieces of more recent evidence have suggested that the secretory immune response might be dispersed to distant sites under some circumstances. For example, oral immunization of pregnant rabbits with dinitrophenylated (DNP-) pneumococcal

vaccine led to the appearance of a high titre, anti-DNP IgA antibody response in colostrum which was not accompanied by a detectable serum anti-hapten antibody response (Montgomery *et al.*, 1974). Moreover, Bohl and co-workers (Bohl *et al.*, 1972) showed that either natural or experimental infection of swine with transmissible gastroenteritis virus (which remains confined within the gut) elicited significant IgA antibody response to virus in colostrum and milk. In contrast, direct immunization of the mammary tissue with virus led to specific IgG antibodies in the milk. However, it should be noted that anti-DNP IgA antibody appeared in colostrum after intramammary immunization of pregnant rabbits with DNP-bovine γ-globulin (DNP-BGG). Nevertheless, parenteral immunization with DNP-BGG did not elicit anti-DNP antibodies in colostrum. In humans, oral administration of encapsulated adenovirus vaccine was able to provide significant resistance to pulmonary infection by the virus (Edmonson *et al.*, 1966; Smith *et al.*, 1970). Similarly, Allardyce and colleagues (Allardyce *et al.*, 1974) observed colostral sIgA antibodies specific for *Salmonella typhimurium* in women suffering from enteric infection by this organism. These results suggest that the nature and quantity of antigen, the route of its administration and the species in question might be extremely important in restriction or dissemination of the secretory immune response. Nevertheless, it is clear that given the appropriate set of circumstances, dissemination of the secretory immune response can occur.

Several hypotheses may be considered to explain dispersion of the sIgA antibody response from one mucosal surface to another.

Firstly, antigen might cross the mucosal epithelia, enter the circulation and ultimately reach distant mucosal surfaces whereupon it could induce completion of differentiation of antigen-specific, IgA precursor cells. This possibility seems, for the most part, to be unlikely because parenteral administration of antigen should have a similar effect and this is usually not the case.

Secondly, antigen might in some way be specially "processed" by the gut such that upon reaching distant mucosae, only IgA plasmacyte precursors are activated. Of course, parenterally administered antigen would be expected to remain "unprocessed" and, therefore, incapable of activating IgA plasma cell precursors. Some evidence indicates that intestinal macrophages can ingest orally administered antigen (Kao, 1972) and macrophages have been shown to accumulate in developing mammary glands (Lee & Lascelles, 1970). It is possible that such cells might aid in the processing and transport of mucosally absorbed antigen.

Thirdly, it seems possible that specific dimeric IgA made at a mucosal surface in response to local immunization at the same mucosal surface, might enter the circulation and be transported across distant mucosal epithelia. If such transport were selective, very low serum concentrations of dimeric IgA could be effectively concentrated at distant mucosal epithelia. Considerable experimental evidence is inconsistent with this hypothesis (Tomasi *et al.*, 1965; South *et al.*, 1966; Stiehm *et al.*, 1966; Haworth & Dilling, 1966; Strober *et al.*, 1970) but some exceptions have been recently reported (see Chapter 5).

Lastly, IgA plasma cell precursors situated in mucosa-associated tissues might receive antigen-mediated stimulation, emigrate via the lymphatics and circulation, and ultimately localize not only at the mucosa from which they were generated, but also at distant mucosae. The great majority of experimental findings are most compatible with this hypothesis and suggest that the sites at which antigen stimulates IgA plasmacyte precursors are the GALT and BALT. As previously described, both the PP and the BALT were fertile sources of the IgA-containing cells observed beneath both the gut and bronchial epithelia (Craig & Cebra, 1971; Rudzik *et al.*, 1975c,d). Similarly, mesenteric lymph node cells selectively localized in the gut mucosa and the majority of these localized cells contained IgA (Griscelli *et al.*, 1969; Hall & Smith, 1970; Guy-Grand *et al.*, 1974; Parrot & Ferguson, 1974). On the basis of these findings, Bienenstock and colleagues (Rudzik *et al.*, 1975c) postulated the existence of a common mucosal immunologic system involving several mucosal surfaces linked together by migrating IgA plasma cell progenitors. Although the various pieces of evidence clearly showed that migration and selective localization of cells could occur between mucosal tissues containing organized lymphoid aggregates, they did not indicate that IgA plasma cells at other mucosae originated from precursors harboured in either the PP, mesenteric lymph nodes or BALT. If precursors destined to become IgA-secreting cells were specifically disseminated amongst the various mucosae, an organism might have a major survival advantage. For example, secretory lymphoid aggregates stationed at strategic locations along

the gut and bronchus would continuously sample the antigenic external environment and then pass humoral immunity to distant mucosal sites where environmental antigens might also be present. Such a common mucosal immunologic system would, in principle, provide a means by which mucosal immunity might be harnessed and brought to bear as a first line of defense, not only at the primary site of exposure to potentially harmful agents, but at secondary sites as well.

CHAPTER 2

PURPOSE OF THE STUDY

2.1 Summary of the literature and conclusions

Much of the literature concerning humoral immunity has focused on the systemic immune responses. The cells responsible for the production of serum IgM and serum IgG antibodies have been examined with respect to their location at the time of response to antigen and subsequent travels. For example, the presentation of antigen to both T- and B-cells stationed in a particular lymph node can result in the emigration of cells from the lymph node which will ultimately lead to the presence of IgM and IgG antibody producing cells in distant lymphoid tissue such as that found in lymph nodes, spleen and bone marrow. Most of the antibody in serum originates in such cells.

In contrast, relatively little is known about the mechanisms which lead to a humoral immune response at mucosal surfaces. Immunization at a mucosal surface often results in production of antibodies which are confined to the vicinity of immunization. Thus, the mucosal immune response may be almost entirely localized.

Unlike systemic immune responses, local immune responses are characterized by a predominance of dimeric IgA in external secretions relative to the other immunoglobulin isotypes. Dimeric IgA appears to be synthesized locally since there is a preponderance of IgA-containing cells beneath mucosal epithelia and because

monomeric IgA appears to be the form in which IgA is made in peripheral lymphoid tissue. Although the precise site(s) at which mucosally presented antigen and immunoreactive cells interact is unknown, the vast majority of evidence is consistent with the idea that this interaction occurs at the mucosal surface and/or in mucosa-associated lymphoid tissue (MALT).

Presentation of immunogens to the luminal surface of the small intestine can lead to the appearance of antigen-specific IgA antibodies in gut secretions. Remarkably, following oral immunization, specific IgA antibodies may also appear in mucosal secretions distant from the gut (e.g. colostrum and milk). One explanation for these observations could be the dimeric IgA synthesized by cells in the intestinal lamina propria was not only transported into the gut but also into the circulation whereupon it ultimately appeared in distant mucosal secretions. At the outset of this study, most evidence did not support this possibility.

Alternatively, it was suggested that IgA plasmacyte progenitors sensitized to enteric antigens in the gut lamina propria, PP or MLN might have the capacity to emigrate from these sites and selectively localize in the gut and other mucosal sites, complete differentiation and secrete dimeric IgA across the epithelia. Several observations suggested that the latter hypothesis might be the major mechanism. After oral immunization, resistance to enteric infection by antigenically related organisms was not correlated with circulating antibodies. In rabbits, PP were found to be a rich source of the precursors of IgA plasmacytes that appeared in not only the gut,

but in the bronchus as well several days after adoptive transfer into allogeneic or autologous hosts. Furthermore, a population of B-lymphoblasts obtained from rodent MLN became IgA plasmacytes in the intestinal lamina propria 24 h after adoptive transfer into normal recipients. Finally, a population of rapidly-dividing lymphoblasts in thoracic duct lymph failed to recirculate but instead localized in the lamina of the gut. The contention that cells derived from one mucosal surface could localize at another mucosal surface was further supported by the demonstration that the BALT, like the PP of the gut, was a fertile source of IgA plasmacytes eventually seen beneath the mucosal epithelium of both the gut and bronchus. In these types of studies, cells derived from PLN showed radically different properties in that they were nearly incapable of supplying IgA-plasmacyte precursors to any mucosal site.

Collectively, these findings constituted the basis for the hypothesis that there might exist a mucosal immunologic system in which different mucosal surfaces are linked by migrating IgA plasmacyte precursors. If lymphoid tissue associated with the gut and lungs was capable of providing mucosa-associated tissues with IgA plasma cells, it may be hypothesized that both the MLN and BLN (as opposed to PLN) would be rich sources of blastogenic cells destined to become IgA plasmacytes beneath the secretory epithelia of the gut and other mucosa-associated tissues. Moreover, it might be that the intestinal lamina propria contained cells which behave like those cells harboured in the MLN. The purpose of the study was to test these hypotheses.

2.2 Specific objectives of the study

In order to examine these hypotheses, answers were sought to the following questions.

1) Do dividing cells in MLN, as compared to those from PLN, preferentially localize beneath the mucosal epithelia of the intestinal, respiratory and genital tracts and mammary glands?

2) Do dividing cells in BLN behave like those from MLN in terms of their localization characteristics?

3) Is there a tendency for the precursors of IgA plasma cells, compared with those of other Ig isotypes, to preferentially localize beneath the mucosal epithelia of the gut, lungs, genital tract and breast?

4) If the precursors of Ig-containing cells localized beneath the mucosal epithelia of the genital tract and mammary glands, was their localization influenced by sex hormones?

5) Does the intestinal lamina propria contain dividing cells, and if so, do these cells behave like those in the MLN, BLN, PP or PLN?

2.3 Outline of the animal model

Rodents were chosen as experimental animal models. Lymphoblasts, taken from various lymphoid sources in groups of donor animals, were labelled by *in vitro* incubation with a radioactive nucleotide and were then injected intravenously into recipients. It was assumed that this adoptive transfer of cells was analogous

(at least in part) to the normal physiologic release of lymphocytes from the respective lymphoid sources *in vivo*. Approximately 24 h later, various organs in recipients were examined for the presence of donor lymphoblasts and the intracytoplasmic immunoglobulin isotype (if any) of the labelled donor cells.

Two major obstacles in detecting ^{3}H-labelled cells in recipient organs were: 1) solubilization of the tissues in scintillation fluid to produce a homogeneous, translucent, single-phase preparation that could be radioassayed, and 2) correction for quenching. To surmount these problems, the development of a method capable of solubilizing recipient tissues and detecting ^{3}H was undertaken.

In order to visualize radiolabelled lymphocytes in recipient organs, autoradiography of thin tissue sections has been the method of choice. Although this technique had been successfully combined with immunofluorescent staining of intracytoplasmic immunoglobulin in labelled cells, it required 1-2 months for performance. Therefore, the development of a method capable of drastically reducing the time requirement for combined immunofluorescence and autoradiography was also undertaken.

CHAPTER 3

MATERIALS AND METHODS

3.1 Animals

CBA/J male and female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. In the adoptive lymphocyte transfer system described in sections 3.3-3.5, virgin female mice, ages 8-16 wk, were used as lymphocyte donors regardless of their stage in the estrous cycle. Either age matched virgin mice or 10-20 wk old animals undergoing their first syngeneic pregnancy served as recipients of donor lymphocytes. Significant changes in the size and structure of the cervix, vagina and uterus occur during the course of the estrous cycle and such physiological alterations in recipients might have influenced the movement of transferred cells. Thus, in some experiments, the stage of estrus in virgin recipients was determined by examination of vaginal cell smears as described by Bronson et al. (1974). Pregnant mice were produced in a controlled breeding situation. The morning that a vaginal plug was sighted was designated day 0.5 of gestation (Theiler, 1972).

Some adoptive transfer studies were done using either rats or guinea pigs in order to corroborate and extend the observations made in the mouse studies. Outbred Sprague/Dawley male rats were purchased from Biobreeding Laboratories Ltd., Ottawa, Ontario, and

used at ages 7-8 wks. Outbred Hartley strain male guinea pigs, obtained from Dutchland Lab Animals, Inc., Denver, Pa., were used at 3 mo of age. Inbred Strain 13 male guinea pigs, kindly supplied by Dr. David Nelson, National Institutes of Health, Bethesda, Md., were used at 8-9 mo of age. When either rats or guinea pigs were studied, both lymphocyte donors and recipients were age and strain matched. All animals were housed in a 12 h light/12 h darkness regime and fed and watered *ad libitum*. Occasional mite infestations were controlled by judicious use of Shell Vapona 20 insecticide (Ketchum Manufacturing Co., Ltd., Ottawa, Ontario).

3.2 Immunizations

In mice and rats, the BLN are extremely small and frequently cannot be seen. Therefore, donor animals were immunized in order to increase the size of the BLN. Groups of mice were primarily and secondarily immunized intraperitoneally (I.P.) with tetanus toxoid and *Bordetella pertussis* vaccines (Connaught Laboratories Ltd., Toronto, Ontario) according to the methods of Gerbrandy & Bienenstock (1976). Briefly, 2 LF tetanus toxoid together with 1×10^8 *B. pertussis* cells in 0.5 ml saline were injected. For secondary immunizations, the same tetanus toxoid doses as for primary immunizations, but without *B. pertussis*, were given 7 days after priming. Mice were used 7 days after the secondary immunization.

Rats immunized with 4×10^3 viable third stage *Nippostrongylus brasiliensis* larvae were kindly provided by Dr. Dean Befus, Department of Pathology, McMaster University. Rats were used 14 d post-infection.

Since both MLN and PLN were easily observed in donor mice, no immunization of either MLN or PLN donors was required.

3.3 Preparation of lymphocyte suspensions

Animals were sacrificed by cervical dislocation and either their MLN, PLN, BLN or PP removed and placed into ice cold Hank's Balanced Salts solution (HBSS; Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% v/v fetal bovine serum (FCS; Grand Island Biological Co.) and 20 mM N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid (HEPES; Sigma Chemical Co., St. Louis, Mo.) and adjusted to pH 7.2 ± 0.05 and 310, 280 or 300 mOsM/Kg for use with either mouse, rat or guinea pig tissues, respectively. In guinea pig studies the small intestine was also removed and placed into ice cold 0.87% w/v NaCl solution.

Either pooled MLN, PLN, or BLN taken from mice were disrupted by pressing through 60-mesh stainless steel wire sieves into ice cold HBSS. Because of their size and connective tissue content, lymph nodes and Peyer's patches obtained from either rats or guinea pigs could not be effectively disrupted with wire sieves. Accordingly, either pooled MLN, PLN, BLN or PP taken from these species were disrupted in siliconized ground glass tissue homogenizers containing HBSS.

Cell suspensions produced by either of the above methods were layered over and centrifuged through FCS at 4°C to remove cell aggregates and fine debris as described by Shortman et al. (1972). Cell pellets were resuspended in ice cold HBSS and washed once by

centrifugation at 200 x g for 10 min. Viable nucleated cells were enumerated in a hemocytometer using 0.4% (w/v) trypan blue dye in saline as diluent (Pappenheimer, 1917).

The isolation of lymphocytes from the guinea pig small intestine was conducted, in part, by Mr. Michael O'Neill according to previously described methods (Rudzik & Bienenstock, 1974). Briefly, the small bowel was freed from its mesenteric connections, resected and flushed free of luminal contents with 0.87% w/v NaCl solution. Macroscopically visible PP were excised and the gut was cut into segments of approximately 20 cm. The ends of each segment were clamped and segments were filled with HBSS in which the FCS had been replaced by 3% w/v BSA. Segments were gently rubbed by hand until the gut wall became transparent. Luminal contents (which included lamina propria lymphocytes) were collected and large cell aggregates and debris allowed to settle at room temperature under unit gravity for 15 min. Supernatants were removed and centrifuged at 200 x g for 10 min at 4°C. Cell pellets were resuspended and washed once with HBSS. Cell suspensions were then passaged over siliconized glass bead columns at room temperature to remove epithelial cells. Column eluants were collected, pooled and centrifuged at 200 x g for 10 min at 4°C. Cell pellets were resuspended in ice cold HBSS and viable nucleated cells were enumerated in 0.4% trypan blue.

3.4 Radiolabelling of dividing cells

All cell suspensions were adjusted to 1 x 10^7 viable nucleated cells per ml. Dividing cells were radiolabelled by addition of either

[^{3}H]-thymidine (^{3}H-Tdr, 6.7 Ci/mM; New England Nuclear Corp., Boston, Mass.) or its synthetic analogue [^{125}I]-deoxyuridine (^{125}I-Udr, 35 Ci/mM; Amersham/Searle, Ltd., Oakville, Ontario) to the cell suspensions. The final concentration of ^{3}H-Tdr was 5 µCi/ml whereas that of ^{125}I-Udr was only 2 µCi/ml because ^{125}I-Udr has been shown to be more toxic than ^{3}H-Tdr (reviewed by Rooijen, 1977). Cell suspensions were allowed to warm to room temperature and incubated at 37°C for 90 min. Cell suspensions were then centrifuged at 200 x g for 10 min at 4°C and the radioactive supernatants discarded. Cell pellets were resuspended and the cells were washed thrice with ice cold HBSS to remove exogenous radiolabel. Viable nucleated cells were enumerated in 0.4% trypan blue.

Occasionally, large cell aggregates (composed primarily of dead cells) formed in cell suspensions after the radiolabelling procedure. To remove these, cell suspensions were passed through stainless steel wire sieves having mesh sizes ranging from 60 to 400. The choice of mesh was dictated by the size of the aggregates. Usually multiple passes through sieves of progressively increasing mesh-size were required.

3.5 Adoptive transfer of radiolabelled cells

For transfer into recipient mice, cell suspensions containing ^{3}H-Tdr-labelled cells were adjusted to 5 x 10^8 cells/ml. Mice under light ether anaesthesia were injected with 1 x 10^8 cells in 0.2 ml HBSS via a lateral tail vein. An aliquot of each ^{3}H-Tdr-labelled cell suspension was retained for determination of injected radioactivity (section 3.7) and stored at -70°C until used.

Preliminary experiments indicated that in order to detect ^{125}I-Udr-associated radioactivity in various tissues, the recipient mice, rats or guinea pigs must have received a quantity of radioactivity not less than 6×10^4, 1×10^5 or 2×10^5 counts per min (cpm), respectively. The cpm of each ^{125}I-Udr-labelled cell suspension was, therefore, determined in a Packard Auto Gamma model 5220 scintillation spectrophotometer prior to transfer. Recipient mice or rats were anaesthetized with ether while guinea pigs received nitrous oxide. The appropriate quantity of cell-associated radioactivity was injected into mice via the lateral tail veins, into rats via the lateral penile veins, and into guinea pigs via cardiac puncture.

A small aliquot of each cell suspension was retained and used to prepare cell smears (section 3.6).

Twenty-two to 24 h after receiving radiolabelled cells, recipient animals were exsanguinated by cardiac puncture under ether anaesthesia. Various organs were removed and assayed for the presence of either radioactivity or radiolabelled cells.

3.6 Preparation of gelatin-coated slides and cell smears

Gelatin-coated slides were used throughout in order to insure adhesion of cells to the glass. Frosted end microscope slides (22 x 75 mm) were immersed in a 1% w/v gelatin solution, drained and air dried. Approximately 2×10^5 cells in 15 µl of HBSS were placed on each slide and spread with a round bottom test tube. Cell smears were air dried, fixed in a 95% v/v ethanol solution, dried and stored at room temperature until used.

3.7 Detection of ^{3}H - Tdr-associated radioactivity in recipient organs

Tissues removed from recipient mice were freed of extraneous material such as fat, mesentery, blood clots, etc., and rinsed with normal saline. Large and small intestines were flushed of fecal contents using saline. After blotting dry on filter paper, organs were weighed (section 3.17) and stored in scintillation vials at -70°C until assayed for the presence of radioactivity.

Either frozen tissues or aliquots of ^{3}H-Tdr-labelled cells were thawed and digested with NCS solubilizer (Amersham/Searle, Ltd.) according to the manufacturer's instructions. Briefly, a predetermined optimal volume of NCS per gram organ wet weight was added to each vial and vials were heated at 50°C until digestion was complete. Each sample was adjusted to pH 6.5 - 7.0 by addition of a previously determined volume of glacial acetic acid and divided into 1.0 ml portions. Ten ml of the recommended scintillation fluid was added to each vial. Vials were assayed for radioactivity in a Beckman LS-233 liquid scintillation spectrophotometer.

3.8 Quench correction

The contributions of NCS and tissue pigments to radioquenching were determined by the use of the external standard ratios (ESR). The radiodetection efficiency of solubilized tissues was determined by the following method. Various tissues were removed from 15 normal mice which had not received radiolabelled cells. Tissues were digested with NCS as described in section 3.7 and 1.0 ml aliquots of each tissue

digest removed. To each aliquot a known quantity of radioactivity as ^{3}H-Tdr was added followed by 10 ml of scintillation fluid. Vials were assayed for the presence of radioactivity in a Beckman LS-233 liquid scintillation spectrophotometer adjusted to operate at maximum sensitivity by use of a ^{3}H-toluene standard. By assaying samples prepared from the same organs taken from individual mice, the relationships between ESR and counting efficiency for a particular organ were determined. Counting efficiency was computed by the function,

$$\text{efficiency} = \frac{\text{detectable radioactivity per vial}}{\text{actual radioactivity per vial}} \times 100\%$$

and used to convert tissue radioactivity (measured as cpm) into disintegrations per minute (dpm). This method, in principle, normalized the radioassays by correcting for quenching.

3.9 Detection of ^{125}I-Udr-associated radioactivity

Tissues removed from mice, rats or guinea pigs were freed of extraneous material such as fat, mesentery and blood clots and placed into scintillation vials. Vials were assayed for radioactivity in a Packard Auto Gamma model 5220 scintillation spectrophotometer.

3.10 Preparation of antisera

Rabbit antisera specific for mouse Ig was kindly prepared by Dr. Ross Milne, Brussels, Belgium, as described in detail elsewhere (Milne *et al.*, 1975; Milne, 1976). In brief, Ig was isolated from the sera of BALB/c mice bearing the IgA plasmacytoma MOPC-315, the

IgG2b plasmacytoma MPC-11, or the IgM plasmacytoma MOPC-774. New Zealand white rabbits were immunized with purified mouse IgA, IgG2b or IgM. Rabbits were bled and their sera tested for specific antibody against mouse Ig by double radial immunodiffusion and immunoelectrophoresis using normal mouse serum.

3.11 Fluorescein conjugation of antisera

Antisera were conjugated by a modification of the method of Holborrow & Johnson (1967). An Ig rich precipitate of each rabbit antiserum was prepared by addition of an equal volume of saturated ammonium sulfate. After 30 minutes at room temperature, precipitates were collected by centrifugation at $2.5 \times 10^4 \times g$ for 15 min at 4°C. Each precipitate pellet was dissolved in the original serum volume of saline and exhaustively dialyzed against saline for 24 h at 4°C. Protein concentrations of the Ig rich fractions were estimated by the method of Lowry et al. (1951) using human Cohn fraction II as a standard. Fractions were diluted with two volumes of 0.01 M sodium bicarbonate buffer, pH 8.9, and powdered fluorescein isothiocyanate (FITC; Sigma Chemical Co.) added to a final concentration of 20 µg/mg protein. The reaction mixtures were magnetically stirred for 18 h at 4°C. Unbound FITC was removed from fractions by passage over columns containing CGA-541 ion exchange resin (J.T. Baker Chemical Co., Phillipsburg, N.J.) which had been previously chlorinated with 1.0 m HCl followed by exhaustive washing with distilled H_2O. Column eluants were concentrated to their original serum volumes with Aquacide® (Calbiochem Corp., La Jolla, California) and exhaustively

dialyzed against normal saline at 4°C. After conjugation, rabbit anti-mouse IgG was absorbed with purified IgA generously provided by Dr. Brian Underdown, University of Toronto. Both rabbit anti-mouse IgM and anti-mouse IgA were absorbed with neonatal mouse serum (IgG). The heavy-chain specificity of each reagent was tested by fluorescent staining of mouse tissues. All antisera were stored in 300 µl aliquots at -20°C until used.

3.12 Immunofluorescent staining of mouse tissues

Various tissues were removed from mice and fixed at 4°C in 10% neutral formalin (BDH Chemicals Ltd., Toronto, Ontario) for 4 h and placed into 30% w/v sucrose solution for 24 h at 4°C (Eidelman & Berschauer, 1969).

Tissues, appropriately positioned for sectioning, were rapidly frozen in O.C.T. Compound (Lab-Tek Products, Inc., Naperville, Ill.) with isopentane cooled by liquid nitrogen and stored under isopentane at -70°C until used. Lymph nodes and small pieces of mammary gland were randomly sectioned while uterus, cervix/vagina and coiled jejunum were sliced longitudinally. The lobes of the lungs were oriented such that the bronchi were cut longitudinally. Frozen tissues were serially sectioned (4 µ) and five consecutive sections mounted per slide. Successive slides were alternately stained with fluoresceinated rabbit antisera specific for either mouse IgA, IgG or IgM according to described methods (Dolezel & Bienenstock, 1970; Bienenstock & Dolezel, 1970). Briefly, mounted sections received a 5 min secondary fixation in 10% neutral formalin.

Sections were washed for 30 min in phosphate buffered saline (PBS; pH 7.2) and incubated for 30 min in Lendrum's chromotrope to abolish non-specific eosinophil fluorescence (Johnston & Bienenstock, 1974). After washing with PBS for 30 min, sections were overlayed with fluoresceinated rabbit antiserum specific for mouse IgA, IgG or IgM and incubated at room temperature for 45 min. Sections were washed for 30 min, post-fixed in 95% ethanol for 15 min and air-dried. Slides were stored at -20°C until used.

3.13 Combined immunofluorescence and high speed scintillation autoradiography

Autoradiography has proved to be a very powerful tool for biological investigations. Unfortunately, this technique suffers from the disadvantage that the time required for its performance is usually measured in weeks. To overcome this inexpedience, methods have been developed to enhance the exposure of the photographic emulsion by the secondary photo-emission from fluors in a liquid scintillator interfaced with the emulsion (Panayi & Neill, 1972; Dube & Salmon, 1975). In order to expedite the detection of the immunoglobulin isotype (if any) of radiolabelled, transferred cells, immunofluorescence was combined with high speed scintillation autoradiography.

All autoradiographic procedures were performed in complete darkness. Mounted tissue sections were stained with fluoresceinated antisera, and then overlaid with NTB-2 photographic emulsion (Kodak Canada, Ltd., Toronto, Ont.) diluted 1:1 with distilled water. After thorough drying at room temperature, emulsion-coated slides

were placed in light-proof slide boxes containing a packet of Drierite® (Fisher Scientific Co. Ltd., Toronto, Ont.) and stored for 24 h at room temperature to facilitate complete desiccation. Slides were then transferred to glass staining racks and immersed in a toluene-based scintillation fluid (6 g 2,5-diphenyloxazole (PPO) and 75 mg 1,4-bis (2-(5-phenyloxazolyl))-benzene (POPOP) per litre of toluene) for a period of 9-24 h at -20°C. Following sequential washings with toluene, 100% ethanol and distilled water, the slides were developed at room temperature for 3 min in Dektol® (Kodak Canada, Ltd.) diluted 1:3 with distilled water, rinsed with distilled water and fixed for 5 min. After a thorough rinsing with distilled water and air drying, slides were stored at -20°C until microscopic examination. Autoradiographs of donor cell smears were similarly prepared and used to determine optimal exposure periods.

This autoradiographic technique has resulted in considerable reduction in exposure time (from 4-6 wks to less than 24 h) while preserving both immunofluorescent and microscopic resolution. Although its use was feasible with ^{125}I-Udr, this DNA precursor caused the exposure of many silver grains not directly over the labelled cell. Accordingly, ^{3}H-Tdr was chosen to label cells which would be detected autoradiographically as this nucleotide caused only those silver grains directly over the labelled cell to be exposed.

3.14 Microscopy

Coverslips were applied with 80% v/v glycerol in PBS to developed autoradiographic-immunofluorescent slides.

Tissue sections were examined using a Leitz Orthoplan microscope equipped with a Ploempak II vertical fluorescence illuminator and HBO 50 mercury lamp. The number of labelled cells and their immunoglobulin isotype content (if any) per 10^3 high power microscopic fields (HPF) was recorded for each tissue. Each HPF encompassed 0.105 mm^2 at a magnification of 400 diameters. An HPF was included if the total luminal space was judged to be less than 0.25 HPF. Autoradiographs of cell smears prepared from donor cell suspensions were stained with hematoxylin and eosin and the percentage of cells which were radiolabelled was determined.

3.15 Separation of cells by velocity sedimentation

Separations of radiolabelled mouse MLN or PLN cells on the basis of size were achieved by velocity sedimentation at 4°C in the STAPUT system (O.H. Johns Scientific Co. Ltd., Toronto, Ont.) originally described by Miller & Philips (1969). The theoretical basis of this biophysical separation technique is described briefly in Appendix I (for a more elaborate discussion, see Miller (1973)). Briefly, radiolabelled cells were prepared (sections 3.3-3.5) and elevated as a thin band beneath a layer of PBS in a STAPUT sedimentation chamber (diameter, 24.5 cm). Beneath the cell layer a discontinuous stabilizing gradient ranging from 0.35% (w/v) to 2.0% (w/v) BSA in PBS was formed. Cells were allowed to sediment through the gradient for 3.5-4.5 h. The average density of the gradient in the region through which the cells sedimented was 1.010 g/cm^3. Fifty ml fractions were collected and centrifuged at 200 x g for 10 min at 4°C.

Cell pellets were resuspended in ice cold HBSS and cells were enumerated in 0.4% trypan blue. The mean sedimentation velocity(s) of cells appearing in each fraction was computed by the method described in Appendix II. The cells were kept at 4°C until used.

3.16 Detection of cell-associated radioactivity in STAPUT fractions

A known number of cells from each STAPUT fraction was collected on nitrocellulose fibre membranes and osmotically lysed with H_2O. Membranes were dried at 37°C and placed into scintillation vials together with 10 ml of scintillation fluid. Incorporated radioactivity was assayed in a Beckman LS-233 liquid scintillation spectrophotometer.

Cell smears were prepared (section 3.6) from aliquots of each fraction and the percentage of radiolabelled cells in each fraction was determined by autoradiography without previous immunofluorescent staining (sections 3.13 and 3.14).

3.17 Organ weights

Twenty-six cm of jejunum and the cervix and vagina (5 mm) were removed from proestral, estral and diestral mice. Guts were rinsed free of luminal contents with PBS. Other tissues were removed and freed of extraneous material. Organs were blotted on filter paper and immediately weighed on a Mettler H20T analytical balance (Fisher Scientific Co.).

3.18 Statistical analysis

Tests for significant differences between groups were conducted by use of Student's unpaired t-test.

CHAPTER 4

EXPERIMENTAL RESULTS

4.1 Isolation and radiolabelling of cells

Cell suspensions were prepared from rodent MLN, PLN, BLN, PP or LP and cells were radiolabelled with either ^{3}H-Tdr or ^{125}I-Udr. Representative results of these procedures are presented in Table 4.1. MLN were routinely the richest source of nucleated cells in all species examined with the exception of both PP and LP in guinea pigs. By comparison, the number of PLN cells obtained from donor animals were relatively few. Although immunization of donor mice and rats caused a dramatic increase in size of the BLN, the actual yield of BLN cells per animal was considerably less than that obtained from each MLN cell donor. Notably, Hartley strain guinea pig lymphoid sources yielded a great many more labelled cells than those taken from Strain 13 animals. Possibly these differences reflected the fact that the Strain 13 guinea pigs were approximately 3-fold older than the Hartley strain animals.

After radiolabelling of lymph node cells and passage through wire sieves (section 3.4) greater than 99% of nucleated cells were not stainable with trypan blue. Although ^{3}H-Tdr- and ^{125}I-Udr-labelled lymph node cells are usually considered to be large, DNA-synthesizing lymphocytes (Yoffey & Courtice, 1970), it might have been that a portion of these lymph node cells were either small

Table 4.1

Preparation of [^{3}H]-thymidine- or [^{125}I]-deoxyuridine-labelled lymphocytes from rodents

Species	Cell source[a]	Number of animals examined	Number of nucleated cells obtained per animal ($\times 10^{-6}$)[b]	Fraction of cells containing radiolabel (%)[c]
CBA/J mouse	MLN	777	33.8 ± 2.1	1.51 ± 0.07
	PLN	628	10.4 ± 0.9	0.80 ± 0.40
	BLN	398	4.5 ± 0.4	4.01 ± 1.69
Sprague/Dawley rat	MLN	14	782.0 ± 45.1	ND[d]
	PLN	16	23.5 ± 1.9	ND
	BLN	16	73.2 ± 2.4	ND
Hartley strain guinea pig	MLN	32	117.6 ± 33.8	ND
	PLN	32	74.8 ± 16.7	ND
	PP	35	129.3 ± 4.7	ND
	LP	14	103.3 ± 13.3	ND
Strain 13 guinea pig	MLN	4	9.0	0.99
	PLN	4	45.0	0.38
	PP	4	37.0	1.26
	LP	4	195.0	1.16

Table 4.1 (Footnotes)

[a] MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial and inguinal) lymph nodes; BLN, bronchial (mediastinal) lymph nodes; PP, Peyer's patches; LP, small intestinal lamina propria. MLN, PLN, PP or LP cells were obtained from donor animals which were not specifically immunized. BLN were obtained from either mice that had been immunized with *Bordetella pertussis* and tetanus toxoid vaccines, or rats infected with third stage *Nippostrongylus brasiliensis* larvae.

[b] Figures represent the arithmetic mean ± standard error.

[c] Figures represent the arithmetic mean ± standard error as determined by autoradiography on cell smears.

[d] ND, not determined.

lymphocytes (with intracellular DNA-precursor pools) or other cell types.

Some evidence was obtained to show that the radiolabelled nucleotides were incorporated only by large, blastogenic cells. The sedimentation and radiolabelling profiles of mouse cells obtained from either MLN or PLN, which were pulse labelled with ^{3}H-Tdr and separated from each other by velocity sedimentation at unit gravity, are displayed in Figures 4.1 and 4.2. When mouse cells were labelled with ^{125}I-Udr, the sedimentation and radiolabelling profiles conformed very closely to those generated by separation of ^{3}H-Tdr-labelled cells. Routinely, greater than 90% of either MLN or PLN nucleated cells subjected to velocity sedimentation separation were recovered after the sedimentation period. Separation revealed that greater than 85% of loaded cells were small in size (sedimenting at 2.0-3.4 mm/h) and these had incorporated less than 5% of recoverable radioactivity. In contrast, the vast majority of recoverable radioactivity was associated with large cells sedimenting at more than 4.0 mm/h. The small radioactivity peak between 1.0-2.0 mm/h represented radiolabel associated with dead nucleated cells and debris. Radioactivity in fractions with s values greater than 7.7 mm/h was primarily due to doublets and triplets of nucleated cells. When all fractions above 4.0 mm/h were pooled, the percentage radiolabelled cells in this pool routinely exceeded that of the unseparated cell suspension by 25-to 30-fold (cf. Table 4.1). Morphologically, the large, radiolabelled cells sedimenting at more than 4.0 mm/h were mononuclear in character. These results indicated that lymph node cells incorporating radiolabelled

Figure 4.1

Sedimentation and radiolabelling profiles of mouse mesenteric lymph node cells after pulse-labelling with ^{3}H-thymidine. Cells were incubated for 90 min with ^{3}H-thymidine and allowed to sediment at unit gravity for 4 h. ● , total nucleated cells/ml; ▲ , incorporated radiolabel collected on nitrocellulose filters, cpm/10^{6} nucleated cells; ○ , percentage of cells containing radiolabel as identified by autoradiography. Sedimentation velocities (mm/h) are related to a gradient in which the average density in the region through which the cells sediment is 1.010 g/cm^{3}.

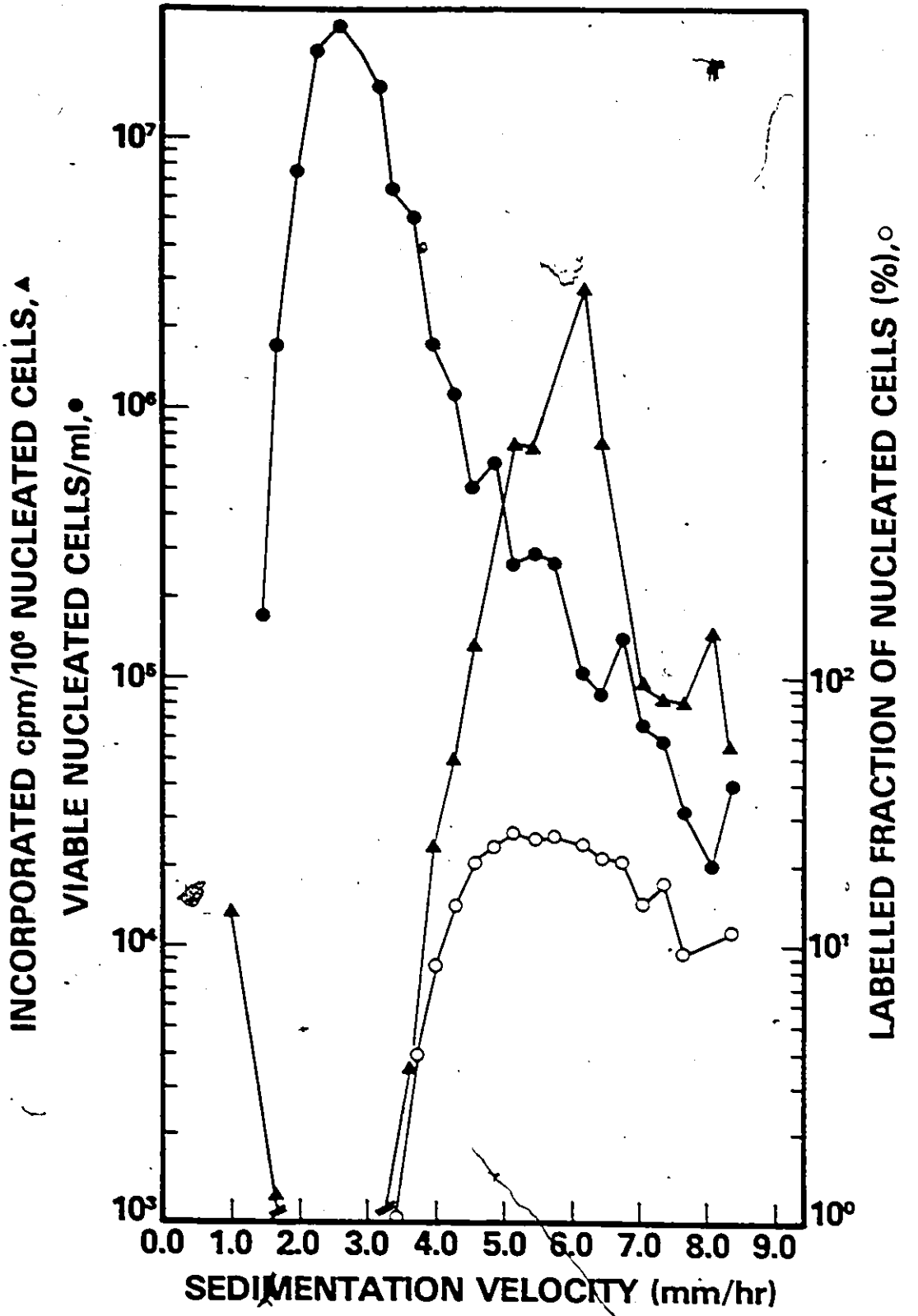
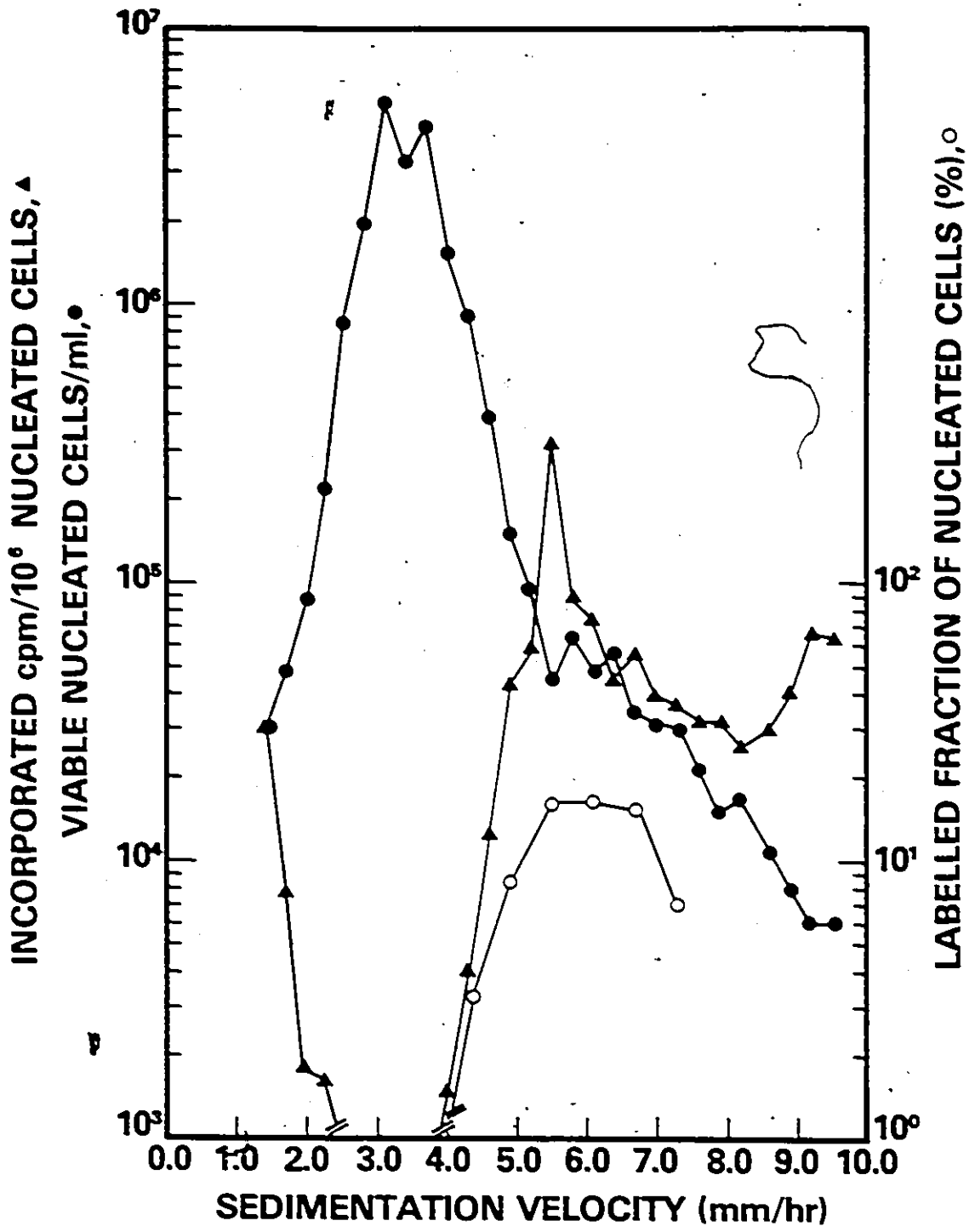

INCORPORATED cpm/10⁶ NUCLEATED CELLS, ▲
VIABLE NUCLEATED CELLS/ml, ●
LABELLED FRACTION OF NUCLEATED CELLS (%), ○
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0
SEDIMENTATION VELOCITY (mm/hr)

Figure 4.2

Sedimentation and radiolabelling profile of mouse peripheral lymph node cells after pulse labelling with ^{3}H-thymidine. Cells prepared from axillary, brachial, and inguinal lymph nodes were incubated for 90 min with ^{3}H-thymidine and allowed to sediment at unit gravity for 4 h. ● , total nucleated cells/ml; ▲ , incorporated radiolabel collected on nitrocellulose filters, cpm/10^{6} nucleated cells; ○ , percentage of cells containing radiolabel as identified by autoradiography. Sedimentation velocities (mm/h) are related to a gradient in which the average density in the region through which the cells sediment is 1.010 g/cm^{3} at 4°C.


INCORPORATED cpm/10⁶ NUCLEATED CELLS, ▲
VIABLE NUCLEATED CELLS/ml, ●
LABELLED FRACTION OF NUCLEATED CELLS (%), ○
10⁷
10⁶
10⁵
10⁴
10³
10²
10¹
10⁰
0.0 1.0 2.0 3.0 4.0 5.0 6.0 7.0 8.0 9.0 10.0
SEDIMENTATION VELOCITY (mm/hr)

nucleotides were most likely large lymphocytes (lymphoblasts) and not small lymphocytes or other cell types.

4.2 Quench correction for ^{3}H-radiodecay detection

It was assumed that 22-24 h after adoptive transfer of radiolabelled cells the quantity of radioactivity recovered from various recipient tissues was directly related to the numbers of radiolabelled cells localizing in these sites. In order to estimate the actual quantity of ^{3}H-Tdr in recipient organs, it was necessary to correct each sample for varying radioquenching effects caused by NCS Solubilizer, endogenous water and tissue pigments. To accomplish this, solubilized tissue samples containing known quantities of ^{3}H-Tdr were prepared (section 3.8) and the relationships between radiodetection efficiencies and ESR determined. A representative example of these relationships is shown in Figure 4.3. Within the limits investigated, radiodetection efficiency and ESR were linearly related for solubilized samples of small intestine. Similar linear relationships were found when either solubilized large intestine, MLN, PLN, BLN, cervix and vagina, uterus or cell suspension were examined. The algebraic functions used to compute relative counting efficiencies of samples are listed in Table 4.2. From these functions, the quantity of radioactivity (as dpm) in individual tissues could be determined. All organs did not lend themselves to this method of analysis (for example, liver, spleen, lungs and blood) because of excessive quenching by endogenous pigments.

Figure 4.3

Relationship between [^{3}H]-radiodecay detection efficiency and external standard ratio in samples of solubilized mouse small intestine. The small intestine was removed from 15 mice and individually solubilized with NCS Solubilizer. A known quantity of ^{3}H-thymidine was added to each sample followed by scintillation fluid. Samples were assayed for detectable radioactivity in a Beckman LS-233 liquid scintillation spectrophotometer. The radiodetection efficiency was calculated as,

$$\text{efficiency} = \frac{\text{detectable radioactivity (cpm) in sample}}{\text{actual radioactivity (dpm) in sample}} \times 100\%.$$

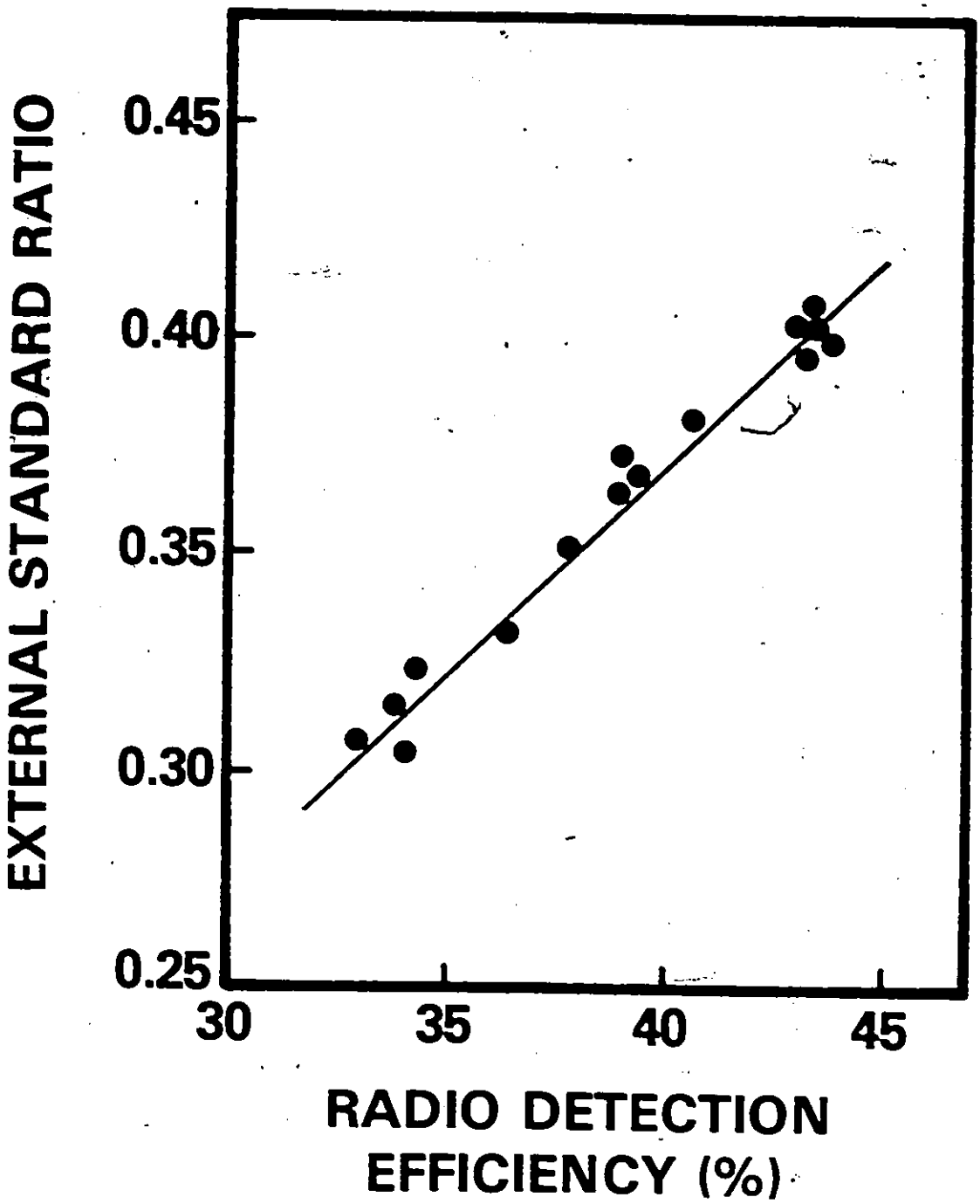

EXTERNAL STANDARD RATIO
0.45
0.40
0.35
0.30
0.25
30
35
40
45
RADIO DETECTION EFFICIENCY (%)

Table 4.2

Detection of [^{3}H]-radiodecay in solubilized tissue samples prepared from virgin CBA/J female mice

Tissues examined[a]	ml NCS/g tissue[b]	ml solubilized tissue assayed	Radiodetection efficiency[c] (x 100%)	Correlation coefficient (r)[d]
Small intestine	9.0	1.0	$\frac{ESR + 0.109}{0.012}$	0.99
Large intestine	9.0	1.0	$\frac{ESR + 0.034}{0.011}$	0.99
MLN	7.0	1.0	$\frac{ESR + 0.074}{0.007}$	0.99
PLN	7.0	1.0	$\frac{ESR - 0.062}{0.009}$	0.99
BLN	7.0	1.0	$\frac{ESR - 0.043}{0.009}$	0.99
Cervix and vagina	7.0	1.0	$\frac{ESR + 0.254}{0.014}$	0.98
Uterus	7.0	1.0	$\frac{ESR - 0.103}{0.007}$	0.99
Mammary glands	8.0	1.0	$\frac{ESR - 0.004}{0.010}$	0.96
Cell suspension (0.10 ml)	6.0	1.0	$\frac{ESR - 0.248}{0.004}$	0.99

Table 4.2 (Footnotes)

a MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial and inguinal) lymph nodes; BLN, bronchial (mediastinal) lymph nodes.

b Figures represent the volume of NCS Solubilizer required per gram tissue wet weight to effect complete solubilization.

c To each solubilized tissue sample a known quantity of ^{3}H-thymidine was added together with scintillation fluid. The detectable radioactivity was measured in a liquid scintillation spectrophotometer. The relationships between the external standard ratios and the radio-detection efficiencies as a percentage were determined for samples of each tissue.

d Figures represent the linear correlation values associated with the function described in footnote c.

4.3 Distribution of radioactivity in mice receiving ^{3}H-Tdr-labelled lymphoblasts

Table 4.3 shows the percentage of injected radioactivity recovered from various solubilized mouse tissues 22-24 h after transfer of ^{3}H-Tdr-labelled MLN, PLN or BLN cells. Total recoveries of injected radioactivity in these experiments were approximately 18.9% , 11.9%, and 8.97% from recipients of labelled MLN, PLN, and BLN cells, respectively. When compared to lymphoblasts prepared from PLN, those obtained from MLN showed a preferential migration into the small intestine. MLN-derived cells showed little tendancy to localize in peripheral lymph nodes draining either mucosal or non-mucosal regions. In marked contrast, PLN-derived labelled cells returned to the PLN and were seemingly more common in axillary, brachial and inguinal sites. Investigation of other recipient organs did not reveal any dramatic differences when comparing the distributions of labelled MLN and PLN donor cells.

Since the BLN drain the lungs, a major mucosa-associated organ, it seemed likely that BLN lymphoblasts would show a localization pattern similar to that of labelled MLN cells. Surprisingly, the tissue distribution of transferred BLN lymphoblasts was not very different from that of transferred PLN lymphoblasts except that BLN cells seemingly avoided recipient MLN and PLN. Radioactivity associated with donor MLN, PLN or BLN lymphoblasts was not detectable in recipient BLN, cervix and vagina, uterus or mammary glands.

These results suggested that cells prepared from the MLN, PLN, and BLN possessed different migration properties. This inference

Table 4.3

Distribution of radioactivity in various tissues 22-24 h after adoptive transfer of [^{3}H]-thymidine-labelled lymphoblasts into virgin syngeneic CBA/J female mice[a]

Recipient tissues examined[b]	Source of donor cells injected	% injected radioactivity recovered[e]
Small intestine (including PP)	MLN	13.42 ± 0.35
	PLN[c]	5.75 ± 1.44
	BLN[d]	5.90 ± 0.78
Large intestine	MLN	3.06 ± 0.37
	PLN	2.30 ± 0.43
	BLN	1.13 ± 0.20
MLN	MLN	1.20 ± 0.46
	PLN	1.40 ± 0.84
	BLN	0.61 ± 0.22
Non-mucosal PLN	MLN	0.66 ± 0.26
	PLN	1.38 ± 0.19
	BLN	0.73 ± 0.20
Mucosal PLN	MLN	0.59 ± 0.03
	PLN	1.07 ± 0.07
	BLN	0.60 ± 0.05
BLN, cervix and vagina, or uterus	MLN	NDt[f]
	PLN	NDt
	BLN	NDt

Table 4.3 (cont'd)

Recipient tissues examined[b]	Source of donor cells injected	% injected radioactivity recovered
Mammary glands	MLN	NDt
	PLN	NDt
	BLN	NDt

[a] Results shown are pooled from 3,4 or 5 experiments involving transfer of BLN, PLN, and MLN cells, respectively. Each recipient received at least 6 x 10^4 cpm. A total of 19 MLN cell recipients, 16 PLN cell recipients and 6 BLN cell recipients were examined.

[b] PP, Peyer's patches, MLN, mesenteric lymph nodes; non-mucosal PLN, axillary, brachial and inguinal lymph nodes; mucosal PLN, lumbar and salivary lymph nodes; BLN, bronchial (mediastinal) lymph nodes.

[c] PLN, peripheral (axillary, brachial and inguinal) lymph nodes.

[d] BLN were obtained from donor mice previously immunized intraperitoneally with tetanus toxoid and *Bordetella pertussis* vaccines.

[e] Tissues were solubilized with NCS Solubilizer and assayed by liquid scintillation spectrophotometry. Figures represent the arithmetic mean ± standard error of recovered radioactivity as a percentage of injected radioactivity.

[f] NDt, not detectable.

assumed that the number of dpm detected in an individual tissue is directly related to the number of radiolabelled cells in the tissue. Unfortunately, the assay method for detection of ^{3}H-Tdr in recipient tissues lacked the versatility and sensitivity deemed necessary to best locate transferred labelled cells in recipient tissues. For example, small numbers of cells in a particular organ might have contained a quantity of radioactivity that was not significantly above background. Moreover, certain organs such as the lungs, liver and spleen could not be examined because of excessive tissue quenching. Finally, the technique was cumbersome and expensive. To overcome these problems, an alternative method of labelling lymphoblasts by use of the thymidine analogue ^{125}I-Udr was chosen. Because ^{125}I-Udr is a γ-emitter, radiodecay detection was for the most part, unaffected by chemical and pigment quenching, thereby allowing convenient radioassay of recipient tissues. In addition, ^{125}I-Udr is not reutilizable while ^{3}H-Tdr can be reincorporated (reviewed by Van Rooijen, 1977). This method was also less costly.

4.4 Distribution of radioactivity in virgin mice receiving ^{125}I-Udr-labelled lymphoblasts

The distribution of radioactivity in tissues taken from mice 22-24 h after receiving ^{125}I-Udr-labelled lymphoblasts is presented in Table 4.4. The percentage of injected ^{125}I-label recovered from recipients was always less than recoverable ^{3}H-label. Nevertheless, the distribution of ^{125}I-Udr label in recipients conformed closely to the apportionment of radioactivity in animals receiving ^{3}H-Tdr-labelled

Table 4.4

Distribution of radioactivity in various tissues 22-24 h after adoptive transfer of [^{125}I]-deoxyuridine-labelled lymphoblasts into virgin syngeneic CBA/J female mice[a]

Recipient tissues examined[b]	Source of donor cells injected	% injected radioactivity recovered[c]
Small intestine	MLN	5.50 ± 0.30
	PLN	2.30 ± 0.15
Large intestine	MLN	1.40 ± 0.17
	PLN	1.00 ± 0.11
Spleen	MLN	1.80 ± 3.45
	PLN	3.45 ± 0.27
Liver	MLN	1.10 ± 0.10
	PLN	1.70 ± 0.24
Lungs	MLN	0.47 ± 0.07
	PLN	0.61 ± 0.16
MLN	MLN	0.48 ± 0.04
	PLN	0.60 ± 0.07
PLN	MLN	0.14 ± 0.01
	PLN	0.97 ± 0.17
Cervix and vagina	MLN	NDt[d]
	PLN	NDt
Uterus	MLN	NDt
	PLN	NDt

Table 4.4 (cont'd)

Recipient tissues examined[b]	Source of donor cells injected	% injected radioactivity recovered
Mammary glands	MLN	0.30 ± 0.14[a]
	PLN	NDt

[a] Results shown are pooled from 4 or 5 experiments involving transfer of PLN and MLN cells, respectively. A total of 17 MLN cell recipients and 13 PLN cell recipients were examined.

[b] MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial, and inguinal) lymph nodes.

[c] Figures represent the arithmetic mean ± standard error of recovered radioactivity as a percentage of injected radioactivity.

[d] NDt, not detectable.

[e] Four recipient animals of 17 examined had detectable radioactivity in the mammary glands.

cells. MLN lymphoblasts showed a predilection to migrate into the small intestine whereas labelled PLN cells localized primarily in their sites of origin and the spleen. The consistency of these results produced by either ^{125}I-Udr or ^{3}H-Tdr labelling of cells is illustrated by the following example. The ratio of MLN-associated radioactivity to PLN-associated radioactivity (computed from Tables 4.3 and 4.4) in the small intestine was 2.39 and 2.33 after transfer of ^{125}I-Udr and ^{3}H-Tdr labelled cells, respectively. In contrast, these ratios when calculated for recipient MLN, were 0.14 (^{125}I-Udr) and 0.34 (^{3}H-Tdr).

Transfer of ^{125}I-Udr-labelled MLN or PLN cells did not reveal any impressive differences in either MLN or PLN lymphoblast migration into either large intestine, liver, lungs or MLN. Furthermore, ^{125}I-Udr-associated radioactivity was not detectable in the cervix, vagina or uterus. However, in 4 of 17 recipient mice examined, a significant quantity of radioactivity was detectable in virgin mammary glands.

4.5 Distribution of radioactivity in male rats receiving ^{125}I-Udr-labelled lymphoblasts

Since mouse BLN yielded a small number of labelled lymphoblasts when compared to rat BLN (Table 4.1), it was more convenient to explore the migration of labelled BLN cells in rats. The objectives of these experiments in rats were to corroborate the observations obtained from mouse studies (Tables 4.3 and 4.4). The distribution of radioactivity in tissues taken from rats 22-24 h after receiving ^{125}I-Udr-labelled cells is shown in Table 4.5.

Table 4.5

Distribution of radioactivity in various tissues 22-24 h after adoptive transfer of [^{125}I]-deoxyuridine-labelled lymphoblasts into outbred male rats[a]

Recipient tissues examined[b]	Source of donor cells injected	% injected radioactivity recovered[d]
Small intestine	MLN	3.90 ± 0.34
	PLN	2.51 ± 0.54
	BLN[c]	1.22 ± 0.11
Large intestine	MLN	0.78 ± 0.05
	PLN	0.71 ± 0.16
	BLN	0.47 ± 0.05
Spleen	MLN	2.40 ± 0.19
	PLN	2.10 ± 0.66
	BLN	2.70 ± 0.15
Liver	MLN	11.40 ± 1.10
	PLN	3.22 ± 0.63
	BLN	5.70 ± 0.57
Lungs	MLN	3.90 ± 0.81
	PLN	2.20 ± 0.11
	BLN	3.70 ± 0.59
MLN	MLN	0.77 ± 0.06
	PLN	1.03 ± 0.21
	BLN	0.42 ± 0.01

Table 4.5 (cont'd)

Recipient tissues examined[b]	Source of donor cells injected	% injected radioactivity recovered[d]
PLN	MLN	0.32 ± 0.04
	PLN	1.05 ± 0.40
	BLN	0.97 ± 0.12

[a] Results shown are pooled from 2 experiments. A total of 9 MLN cell recipients, 2 PLN recipients and 8 BLN recipients were examined.

[b] MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial, and inguinal) lymph nodes.

[c] BLN were obtained from donor rats previously immunized 14 d previously with 4×10^3 viable third stage *Nippostrongylus brasiliensis* larvae.

[d] Figures represent the arithmetic mean ± standard error of recovered radioactivity as a percentage of injected radioactivity.

Not surprisingly, labelled PLN cells returned to PLN, and MLN lymphoblasts preferentially migrated into the small intestine. Remarkably, pulmonary MLN-associated radioactivity was approximately twice that associated with transferred PLN lymphoblasts. By comparison, labelled BLN cells avoided the gut and lymph nodes but showed a remarkable propensity to localize in the lungs. Presumably, the differences in recoverable hepatic radioactivity after transfer of either MLN, PLN or BLN cells reflected the relative proportion of damaged cells in the inocula.

The results obtained from both mice and rats strongly suggested that lymphoblasts obtained from MLN were distinctly different, in terms of localization patterns, compared to those prepared from PLN. In addition, BLN lymphoblasts seemingly possessed localization capabilities different from those cells derived from either MLN or PLN.

4.6 Interpretation of radiocounting data

Two possible explanations were entertained to account for the lack of detectable radioactivity in some of the tissues examined. Either labelled lymphoblasts of donor origin might not have localized in these sites or the methods employed might have lacked the sensitivity necessary to detect a small number of labelled cells. Moreover, a large organ such as the recipient gut might have contained only a few labelled MLN cells per unit of tissue volume but assayed as a great deal of radioactivity per whole organ. Thus, the observed preferential localization of cells in a particularly large organ might be due to organ size whereas such cells might actually have greater

preference for an organ of smaller size.

One solution to these problems was to directly enumerate the number of radiolabelled cells localizing per unit volume of tissue by the use of autoradiographical methods (sections 3.13 and 3.14). This technique greatly increased the sensitivity of detection and allowed one to determine the location of labelled cells in recipient tissues. Moreover, by combining this technique with immunofluorescence, the behavior of distinct subpopulations of cells, such as dividing plasma cell precursors, could be examined.

4.7 Frequency of radiolabelled lymphocytes in recipient tissues

Table 4.6 shows the frequencies of radiolabelled cells in tissue sections prepared from various organs of recipient mice 22-24 h after adoptive transfer of ^{3}H-Tdr-labelled MLN, PLN or BLN cells. The preferential seeking of the various tissues by either MLN or BLN as compared to PLN lymphoblasts is conveniently expressed by the homing index (HI), defined as,

$$HI = \frac{\text{mean number of labelled cells per } 10^{-3} \text{ HPF in MLN or BLN recipients}}{\text{mean number of labelled cells per } 10^{-3} \text{ HPF in PLN recipients}}$$

where HPF represents high power microscopic fields each having a total area of 0.105 mm^2 at a magnification of 400 diameters. An HI larger than 1.0 indicates a greater tendency of either MLN or BLN cells than PLN cells to selectively localize in a particular tissue, i.e., homing. An index near 1.0 indicates little difference in the homing behavior of labelled donor cells from each of the lymphoid sources.

Table 4.6

Distribution of [^{3}H]-thymidine-labelled lymphoblasts in various tissues 22-24 h after adoptive transfer into syngeneic CBA/J female mice[a].

Recipient tissues examined	Source of donor cells injected[b]	Number of radiolabelled cells per 10^3 HPF[c]	Homing index[d]
Small intestines	MLN	93.4 ± 6.0	10.9
	PLN	8.6 ± 1.8	
	BLN	5.1 ± 0.8	0.6
MLN	MLN	123.8 ± 17.4	6.3
	PLN	19.6 ± 3.4	
	BLN	44.6 ± 3.7	2.3
Lungs	MLN	38.6 ± 3.8	3.0
	PLN	12.7 ± 1.3	
	BLN	127.1 ± 24.1	10.0
PLN	MLN	42.4 ± 3.4	1.0
	PLN	42.6 ± 4.8	
	BLN	44.8 ± 5.4	1.1

Table 4.6 (Footnotes)

[a] Results shown are pooled from 2 experiments. Three to four proestral animals were studied in each experiment.

[b] MLN, mesenteric lymph nodes; PLN, peripheral lymph nodes (axillary, brachial, and inguinal); BLN, mediastinal (bronchial) lymph nodes.

[c] HPF, high power microscopic fields (0.105 mm^2, magnification of 400 diameters). 2700-3300 HPF per tissue per animal were examined. Figures represent the arithmetic mean ± standard error.

[d] Homing index =

$$\frac{\text{mean number of labelled cells per } 10^3 \text{ HPF in MLN or BLN recipients}}{\text{mean number of labelled cells per } 10^3 \text{ HPF in PLN recipients}}$$

The larger the homing index, the greater the preference of MLN cells or BLN cells compared to PLN cells for a particular organ.

Indices from 3.0 to 10.9 were observed in recipient small intestines, MLN and lungs after transfer of labelled MLN cells. In the gut, substantial numbers of donor MLN lymphoblasts were found close to the crypts and glands and occasionally within the lamina propria of the villi. Labelled MLN cells did not preferentially localize either in or around the Peyer's patches or in the epithelium as inspection of more than 3.5×10^5 HPF revealed only six labelled cells in these locations. Labelled MLN cells selectively localized in the lungs and seemed to lodge adjacent to, or very near the BALT but rarely within the BALT. Donor MLN blasts were most commonly observed within the T- and B-dependent regions of recipient MLN. Although labelled MLN cells were seen in the T- and B-dependent regions of recipient PLN, homing *per se* was not evident (HI = 1.0).

BLN-derived lymphoblasts showed a propensity to localize in the lungs (HI = 10.0) and were seen less frequently than either labelled MLN or PLN cells in the gut (HI = 0.6). Examination of recipient lymph nodes revealed that BLN cells did have some affinity for recipient MLN (HI = 2.3) and were also observed as frequently in PLN as labelled cells obtained from MLN. The intraorgan distributions of labelled BLN were similar to those of transferred MLN cells except that BLN cells seemed to be more dispersed throughout the pulmonary parenchyma than MLN lymphoblasts.

In marked contrast to the localization of both MLN and BLN cells, PLN lymphoblasts returned primarily to their sites of origin. It should be noted, however, that the PLN did contain a few cells capable of localizing in the gut, lungs and MLN and these showed an

intra-organ distribution similar to that of transferred MLN cells.

4.8. Specificity of immunofluorescent staining reagents

In order to determine the intracytoplasmic immunoglobulin content (if any) of radiolabelled donor cells, it was necessary to obtain immunofluorescent staining reagents which were monospecific for mouse Ig's. After fluorescein conjugation and absorption (section 3.11), rabbit anti-mouse immunoglobulin sera were tested for specificity against either mouse IgA, IgG or IgM by immunofluorescent staining of mouse tissues (section 3.12).

4.8.1 Rabbit anti mouse IgA:

Fluorescein-conjugated rabbit anti-mouse IgA stained the cytoplasm of many cells present in the lamina propria of the normal mouse small intestine. Moreover, intracytoplasmic staining of cells located in the medullary cords and within follicles was observed. Staining of cells in the PLN and spleen was rare. Intracellular fluorescence in the gut lamina propria could be blocked by prior incubation with unconjugated rabbit anti-mouse IgA but not with unconjugated rabbit antisera specific for either mouse IgG2b or IgM.

4.8.2 Rabbit anti mouse IgG2b:

Fluorescein-conjugated rabbit anti-mouse IgG stained the cytoplasm of few cells in the intestinal lamina propria. However, groups of cells in MLN, PLN and spleen were stained with this reagent. Intracellular fluorescence in these tissues was abolished by prior incubation with unconjugated rabbit antisera specific for mouse IgG2b but not with rabbit anti-mouse IgA or IgM.

4.8.3 Rabbit anti mouse IgM:

Fluorescein-conjugated rabbit anti-mouse IgM stained the cytoplasm of cells in the MLN, PLN and spleen, but only a few cells in the gut lamina propria. Intracellular fluorescence was abolished by prior incubation with unconjugated rabbit anti-mouse IgM but not with rabbit antisera specific for either mouse IgA or IgG2b.

The reagents used in this study were originally prepared by Dr. Ross Milne (Milne, 1976) who showed that when either rabbit anti-mouse IgA, IgG or IgM were conjugated and used to stain frozen tissue sections prepared from Ig-secreting plasmacytomas, the reagents were specific for the appropriate Ig heavy-chain. However, in the present work, each of these fluorescent reagents (as well as fluoresceinated normal rabbit serum) stained a population of granulated cells present in all tissues except the small intestine and lymph nodes. Staining was deemed to be non-specific since prior incubation with either unconjugated antiserum or normal rabbit serum failed to abolish granulated intracellular fluorescence. The granulated cells were amine-containing as indicated by a positive argentaffin staining reaction (Pearse, 1972) and were subsequently identified as mast cells after staining with alcian blue at pH 0.30 (Enerback, 1966). Accordingly, care was taken so as not to confuse fluorescing mast cells with fluorescing plasmacytes.

These results indicated that the immunofluorescent reagents were monospecific for either IgA, IgG or IgM and were suitable for the purposes intended.

4.9 Immunoglobulin isotype of radiolabelled cells in recipient tissues

The large majority (50-61%) of donor MLN and BLN lymphoblasts found in recipient small intestines, lungs and MLN tissues contained intracytoplasmic IgA (Table 4.7). A lower percentage of labelled MLN cells observed in mucosal tissues and MLN contained IgG (20-28%) and still fewer (1-7%) IgM. The majority of MLN blasts which lodged in the PLN contained IgG.

Although BLN lymphoblasts demonstrated specific localization in the lungs and not in the gut or lymph nodes, these cells were also detected as IgA- and IgG-containing cells in proportions comparable to those observed following MLN lymphocyte transfer. Moreover, it was interesting to note that the BLN contained a substantial portion of lymphoblasts which appeared in the PLN as IgA plasmacytes.

A few donor PLN blasts seen in the intestinal lamina propria of recipients contained primarily IgA or IgG. In other mucosal sites and the MLN, 25-50% of donor PLN blasts contained IgG. In the lungs and MLN, 16-20% of labelled PLN cells were identified as IgM plasmacytes.

In no tissue examined did the percentage of either labelled MLN or PLN cells containing IgA, IgG and IgM total 100% (Table 4.7). These results suggested that the unstained labelled cells seen in various tissues might have been either immature B-cells, plasmacytes containing other immunoglobulin isotopes, or T-cells.

Collectively, the results presented in Tables 4.6 and 4.7 indicated that the murine MLN contained primarily IgA and to a lesser extent IgG and IgM plasma cell precursors destined to leave the MLN

Table 4.7

Detection of the immunoglobulin isotype of [^{3}H]-thymidine-labelled lymphoblasts in various tissues 22-24 h after adoptive transfer into syngeneic CBA/J female mice[a]

Recipient tissues examined	Source of donor cells injected[b]	Percentage of radiolabelled cells containing immunoglobulin[c]			
		IgA	IgG	IgM	Total
Small intestines	MLN	60.8 ± 3.3 (57)[d]	28.4 ± 1.8 (26)	1.8 ± 0.6 (2)	91.0
	PLN	38.2 ± 10.6 (3)	26.7 ± 18.1 (2)	0 (0)	64.9
	BLN	68.2 ± 4.2 (3)	36.4 ± 5.8 (2)	NDt[e]	–
MLN	MLN	50.0 ± 4.0 (6[illegible])	20.9 ± 3.6 (26)	2.8 ± 0.8 (3)	73.7
	PLN	28.7 ± 6.6 (6)	33.6 ± 9.5 (7)	20.3 ± 5.9 (4)	82.6
	BLN	50.2 ± 4.1 (22)	38.2 ± 4.1 (17)	NDt	–
Lungs	MLN	58.1 ± 3.1 (22)	23[illegible] ± 8.5 (9)	5.2 ± 1.7 (2)	87.1
	PLN	32.0 ± 8.1 (4)	34.8 ± 3.1 (4)	16.2 ± 7.1 (2)	83.0
	BLN	52.4 ± 2.5 (67)	37.6 ± 4.2 (48)	NDt	–
PLN	MLN	7.9 ± 3.6 (3)	48.1 ± 10.9 (20)	6.7 ± 4.0 (3)	62.7
	PLN	6.1 ± 2.1 (3)	32.4 ± 7.4 (14)	11.3 ± 5.7 (5)	49.8
	BLN	17.6 ± 4.0 (9)	34.9 ± 4.7 (16)	NDt	–

Table 4.7 (Footnotes)

[a] Results shown are pooled from 2 experiments. Three to four proestral animals were studied in each group in each experiment.

[b] MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial, and inguinal) lymph nodes; BLN, bronchial (mediastinal) lymph nodes.

[c] Figures represent the arithmetic mean ± standard error.

[d] Figures in parentheses were computed using Table 4.6 and represent the approximate number of radiolabelled cells containing immunoglobulin per 10^3 high power microscope fields (0.105 mm^2, magnification of 400 diameters).

[e] NDt, not determined.

and selectively localize beneath the mucosal epithelium of the gut and lungs, and within the MLN itself. The BLN were also a rich source of IgA (and IgG) plasmacyte precursors, but unlike those harboured within the MLN, BLN-derived precursors showed a remarkable propensity to localize in the lungs. In marked contrast, PLN were a fertile source of IgG plasma cell precursors which returned primarily to PLN. It should be noted that lymphoblast localization was not absolutely restricted to certain tissues. For example, although MLN lymphoblasts localized primarily in mucosa-associated tissues, they were also found in PLN. These observations suggested that the lymph nodes may have contained major and minor populations of lymphocytes with regard to their homing properties.

4.10 Selective localization in the reproductive organs

The ability of transferred lymphoblasts obtained from MLN and PLN to localize in the female reproductive organs are compared in Table 4.8. Proestral animals were chosen as recipients in order to keep constant any potential effects of sex hormones on cell migration. As indicated by an HI of 7.9 (described in section 4.7), MLN lymphoblasts preferentially localized in the cervix and vagina when compared to labelled PLN cells. Here, both labelled MLN and PLN cells did not lodge in any particular site but were distributed throughout the lamina propria and were occasionally seen in luminal material. Both MLN and PLN lymphoblasts were found infrequently in the uterine lamina propria of recipients (HI = 1.5).

Table 4.8

Distribution of [^{3}H]-thymidine-labelled mesenteric and peripheral lymphoblasts in the reproductive organs 22-24 h after adoptive transfer into syngeneic CBA/J female mice[a]

Recipient tissues	Source of donor cells injected[b]	Number of radiolabelled cells per 10^3 HPF[c]	Homing index[d]
Proestral cervix and vagina	MLN	15.7 ± 1.6	7.9
	PLN	2.0 ± 0.2	
Proestral uterus	MLN	6.9 ± 2.3	1.5
	PLN	4.7 ± 1.7	

a Results shown are pooled from 2 experiments. Three proestral animals in each group were studied in each experiment.

b MLN, mesenteric lymph nodes; PLN, peripheral lymph nodes (axillary, brachial, and inguinal).

c HPF, high power microscopic fields (0.105 mm^2, magnification of 400 diameters). 2700-3300 HPF per tissue per animal were examined. Figures represent the arithmetic mean ± standard error.

d Homing index =

$$\frac{\text{mean number of labelled cells per } 10^3 \text{ HPF in MLN recipients}}{\text{mean number of labelled cells per } 10^3 \text{ HPF in PLN recipients}}$$

The larger the homing index, the greater the preference of MLN cells compared to PLN cells for a particular organ.

The immunoglobulin isotypes of labelled MLN or PLN cells seen in the female reproductive organs are displayed in Table 4.9. As in the gut, lungs, and MLN, labelled MLN cells in the cervix, vagina, and uterus appeared chiefly as IgA-containing cells and secondarily as cells containing IgG. No MLN lymphoblasts containing IgM were identified in these sites. In the genital tract, the sparsely localized PLN lymphoblasts contained primarily IgG and secondarily IgA. No PLN lymphoblasts containing IgM were seen here.

These results indicated that the MLN was a rich source of the precursors of IgA (and to a lesser extent IgG) plasma cells seen beneath the mucosal epithelia of the murine genital tract. PLN were very much deficient in this capacity but, nevertheless, were capable of providing these locations with a small number of both IgG and IgA plasmacyte precursors.

4.11 Influence of pregnancy and the stages of the estrous cycle on the localization of lymphoblasts

Significant changes in the size and structure of the reproductive organs occur during the course of either pregnancy or the estrous cycle. Therefore, it was decided to investigate whether or not either pregnancy or the stages of the estrous cycle might influence the tissue localization of both MLN- and PLN-derived lymphoblasts, particularly in sex hormone target organs.

4.11.1 Pregnancy

In gestational mammary tissue, MLN lymphoblasts were seen nearly 3-fold more often than those of PLN origin (Table 4.10). Analogous

Table 4.9

Detection of the immunoglobulin isotype of [^{3}H]-thymidine-labelled lymphoblasts in the reproductive organs 22-24 h after adoptive transfer into syngeneic CBA/J female mice[a]

Recipient tissues examined	Source of donor cells injected[b]	Percentage of radiolabelled cells containing immunoglobulin[c] IgA	IgG	IgM	Total
Proestral cervix and vagina	MLN	66.0 ± 1.8 (10)[d]	20.2 ± 8.9 (3)	0 (0)	86.2
	PLN	10.0 ± 1.0 (1)	50.0 ± 16.7 (3)	0 (0)	60.0
Proestral uterus	MLN	58.8 ± 10.3 (4)	0.0 ± 0.0 (0)	0 (0)	58.8
	PLN	0.0 ± 0.0 (0)	24.6 ± 10.4 (1)	0 (0)	24.6

a Results shown are pooled from 2 experiments. Three proestral animals in each group were studied in each experiment.

b MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial, and inguinal) lymph nodes.

c Figures represent the arithmetic mean ± standard error.

d Figures in parentheses were computed using Table 4.8 and represent the approximate number of radiolabelled cells containing immunoglobulin per 10^3 high power microscopic fields (0.105 mm^2, magnification of 400 diameters).

Table 4.10

Distribution of [^{3}H]-thymidine-labelled mesenteric and peripheral lymphoblasts in various tissues 22-24 h after adoptive transfer into syngeneic CBA/J pregnant mice[a]

Recipient tissues examined[b]	Source of donor cells injected	Number of radiolabelled cells per 10^3 HPP[c]	Homing index[d]	Change in tissue wet weight (%)[e]
Small intestine	MLN	110.8	7.0	130
	PLN	15.8		
MLN	MLN	200.0	2.7	52
	PLN	74.8		
PLN	MLN	46.7	0.66	8
	PLN	70.4		
Mammary glands	MLN	26.8	2.8	-
	PLN	9.6		

Table 4.10 (Footnotes)

[a] Results shown are those obtained from one recipient animal in each group examined on the 19*th* day of a syngeneic pregnancy.

[b] MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial, and inguinal) lymph nodes.

[c] HPF, high power microscopic fields (0.105 mm^3, magnification of 400 diameters). 2700-3300 HPF per tissue per animal were examined. Figures represent the arithmetic mean ± standard error.

[d] $$\text{Homing index} = \frac{\text{mean number of labelled cells per } 10^3 \text{ HPF in MLN recipients}}{\text{mean number of labelled donor cells per } 10^3 \text{ HPF in PLN recipients}}$$

The larger the homing index, the greater the preference of MLN cells, compared to PLN cells for a particular tissue.

[e] $$\% \text{ change in tissue wet weight} = \frac{\text{tissue wet weight in pregnant mice} - \text{tissue wet weight in virgin mice}}{\text{tissue wet weight in virgin mice}} \times 100\%$$

Tissue wet weights were determined by examination of 26 pregnant and 26 virgin animals.

to the gut, lungs, MLN, cervix, vagina, and uterus, a few PLN lymphoblasts localized in the mammary tissue on the 19th day of gestation. Of the labelled donor MLN cells seen in the mammary glands, 53.6%, 19.5%, and 17.2% contained IgA, IgG, and IgM, respectively. In contrast, the few transferred PLN lymphoblasts localizing in recipient mammary tissue were identified primarily as IgG plasma cells (20%) and secondarily as either IgA (10%) or IgM (10%) containing cells. These investigations of the mammary glands indicated that the MLN is a fertile source of the IgA plasma cells seen beneath ductal epithelium on the 19th day of gestation.

Just after the initiation of this study an abstract appeared (and was subsequently published (Roux *et al.*, 1977)) which demonstrated selective localization of MLN lymphoblasts in the mammary glands. Although investigations of selective localization during pregnancy were abandoned at this time, the results shown in Table 4.10 (based on a single animal in each group) provided some insight into the regulation of lymphoblast localization.

Some preliminary experiments indicated that the small intestine increased substantially in mass over the course of pregnancy in mice. An investigation was initiated to determine if the frequency of MLN cells preferentially localizing in the gut lamina propria would be reduced due to more intestinal tissue competing for a finite number of labelled cells. The distributions of transferred ^{3}H-Tdr-labelled MLN or PLN cells in various organs taken from pregnant mice are presented in Table 4.10. Although the small intestine had more than doubled in weight, the frequency of either labelled MLN or

PLN cells seen here was not very different when compared to the gut of virgin recipients (cf. Table 4.10 and Table 4.6). However, a 50% increase in the size of MLN during pregnancy seemingly caused a substantial increase in both the migration of MLN- and PLN-derived lymphoblasts into the MLN. The frequency of MLN-derived lymphoblasts found in the PLN was unchanged although the frequency of labelled PLN cells was nearly doubled. The weights of the PLN were not influenced by the pregnant state.

Collectively, these results suggested that for a given quantity of intestinal tissue a relatively constant number of either MLN or PLN lymphoblasts will localize in the lamina propria, i.e., an increased quantity of tissue does not compete for a finite number of cells. However, the increased size of lymph nodes during pregnancy may have influenced lymphoblast localization at least for those cells derived from MLN.

4.11.2 Stages of the estrous cycle

The frequencies of labelled MLN cells in the cervix and vagina (and small intestine) of proestral, estral and diestral female mice are presented in Table 4.11. Due to differences in the percentage of labelled cells in the inocula (determined after transfer), the proestral animals received approximately two-fold more labelled cells than the estral and diestral recipient (3.6% into proestral mice versus 1.4% into estral and diestral mice). The disparity in frequencies of labelled cells in the proestral compared to estral and diestral small intestines presumably reflected this difference. However, in the absence of evidence describing the

Table 4.11

Influence of the estrous cycle on the migration of [^{3}H]-thymidine-labelled mesenteric lymph node cells into the small intestine, cervix and vagina 22-24 h after adoptive transfer into syngeneic CBA/J female mice[a]

Recipient tissues examined	Stage of estrus	Number of radiolabelled cells per 10^3 HPF[b]	Tissue wet weight (mg)[c]
Small intestines	Proestrus[d]	93.4 ± 6.2	805 ± 39[*e]
	Estrus	53.9 ± 5.8	784 ± 74[*]
	Diestrus	56.3 ± 5.1	781 ± 70[*]
Cervix and vagina	Proestrus	15.7 ± 1.6	95 ± 11[†]
	Estrus	10.4 ± 1.2	109 ± 9
	Diestrus	4.0 ± 0.6	93 ± 11[†]

Table 4.11 (Footnotes)

[a] Results shown are pooled from 2 experiments. Three virgin animals in each group were studied in each experiment.

[b] HPF, high power microscopic fields (0.105 mm^2, magnification of 400 diameters). 1800-2200 HPF per tissue per animal were examined. Figures represent the arithmetic mean ± standard error.

[c] Twenty-six cm of jejunum and 5 mm of cervix and vagina taken from 5 animals in either proestrus, estrus or diestrus. Tissues were blotted on filter paper and weighed immediately.

[d] Data for proestral recipients are reproduced from Tables 4.6 and 4.8 for comparison. The percentage of labelled cells in the inocula was 2.6% for the proestral group and 1.4% for estral and the diestral group.

[e] Groups were tested for significant differences by using Student's unpaired t-test. Figures marked with either * or † were not significantly different ($p \geq 0.95$).

relationships between the number of labelled cells transferred and the frequency of labelled cells in a particular tissue, a comparison of the proestral recipients to the estral and diestral recipients was considered to be tentative. Nevertheless, the data showed that although the transition from the estral to diestral state had no effect on MLN lymphoblast localization in the gut this transition was seemingly correlated with a dramatic alteration in MLN cell migration into the cervix and vagina. Only one half as many MLN lymphoblasts entered the diestral cervix and vagina compared to these sites in estral recipients. The wet weights of the small intestine did not show any significant differences ($p \leq 0.001$) when compared at different stages of the estrous cycle. The cervix and vagina showed a significant ($p \leq 0.001$) increase in weight during estrus. Changes in the mass of the cervix and vagina alone did not appear to be able to account for the alteration in the frequency of labelled cells localizing in the cervix and vagina during various stages of the estrous cycle.

The possibility existed that the decrease in the frequency of MLN blasts in cervix and vagina during diestrus (Table 4.11) might have been due to a selective alteration in the localization of IgA plasma cell progenitors (Table 4.9). Table 4.12 shows the immunoglobulin isotype of labelled donor MLN lymphoblasts in the gut, cervix, and vagina taken from recipients during various stages of the estrous cycle. Because the data are expressed as percentage, the proestral groups can be compared to the estral and diestral groups. No change in the portion of labelled MLN cells containing the IgA isotype was detectable in the small intestine taken from either

Table 4.12

Detection of the immunoglobulin isotype of [^{3}H]-thymidine-labelled mesenteric lymph node cells in the small intestine, cervix, and vagina 22-24 h after adoptive transfer into syngeneic CBA/J female mice[a]

Recipient tissues examined	Stage of estrus	Percentage of radiolabelled cells containing immunoglobulin[b] IgA	IgG
Small intestine	Proestrus[c]	60.8 ± 3.3 (56)[d]	28.4 ± 1.8 (26)
	Estrus	63.0 ± 3.3 (33)	40.3 ± 2.1 (21)
	Diestrus	60.1 ± 4.0 (33)	36.9 ± 4.0 (20)
Cervix and vagina	Proestrus	66.0 ± 1.8 (11)	20.2 ± 8.9 (3)
	Estrus	57.3 ± 4.0 (6)	24.8 ± 11.2 (2)
	Diestrus	41.0 ± 4.5 (2)	25.9 ± 14.8 (1)

[a] Results shown are pooled from 2 experiments. Three virgin animals in each group were studied in each experiment.

[b] Figures represent the arithmetic mean ± standard error.

[c] Data for proestral recipients are reproduced from Tables 4.7 and 4.9.

[d] Figures in parentheses for estral and diestral recipients were computed using Table 4.11 and represent the approximate number of radiolabelled cells containing immunoglobulin per 10^3 high power microscopic fields (0.105 mm^2/magnification of 400 diameters).

proestral, estral or diestral recipients. A slight increase in the percentage of labelled MLN cells containing IgG was noted in the gut from estral and diestral mice compared to those in proestrus. The portion of donor IgA-containing MLN lymphoblasts found in the estral cervix and vagina was two-fold greater than the portion seen in these organs when taken from diestral animals, although no change in the percentage of IgG donor lymphoblasts occurred. Collectively, the results shown in Tables 4.11 and 4.12 indicated a reduction in the cervico-vaginal localization of IgA plasmacyte progenitors occurring during diestrus.

4.12 Localization of lymphoblast subpopulations purified by velocity sedimentation

Cell function is often correlated with physical parameters such as cell size. Some studies were done to investigate whether or not cell populations separated from each other on the basis of size would show different tissue localization patterns. Furthermore, it seemed likely that this technique could also be used to increase the sensitivity of detection of ^{125}I-Udr-labelled cells in various tissues; presumably, a large number of labelled cells could be transferred with minimal concern for effects on localization caused by the creation of extremely high, artificially induced lymphocyte numbers in recipients.

The sedimentation and radiolabelling profiles of cells obtained from either MLN or PLN, which were pulse labelled with ^{3}H-Tdr and separated from each other by velocity sedimentation at unit

Table 4.13

Distribution of radioactivity in various CBA/J mouse tissues 22-24 h after adoptive transfer of [^{125}I]-deoxyuridine-labelled syngeneic mesenteric lymph node cells separated by velocity sedimentation

Sedimentation velocity(s) of donor cells injected[a]	Recipient tissues examined[b]								
	Small intestine	Large intestine	Spleen	Liver	Lungs	MLN[c]	PLN[d]	Proestral cervix, vagina and uterus	Carcase
≥ 4.0 mm/h	40.0 ± 0.56	5.1 ± 0.16	7.3 ± 0.61	7.0 ± 0.29	2.1 ± 0.11	1.8 ± 0.18	0.73 ± 0.10	1.66 ± 0.04	31.7 ± 1.12
< 4.0 mm/h	23.8 ± 0.15	4.9 ± 0.70	16.5 ± 1.27	7.8 ± 0.40	3.2 ± 0.90	5.6 ± 0.15	3.4 ± 0.75	3.24 ± 1.19	33.7 ± 2.00

Table 4.13 (Footnotes)

[a] Cells were separated from each other on the basis of size by sedimentation through a gradient in which the average density in the region through which the cells sedimented was 1.010 g/cm^3 at 4^{0}C. Results shown are pooled from 2 experiments. Three proestral animals in each experiment received cells with s values ≥ 4.0 mm/h and one proestral animal in each experiment received cells sedimenting at less than 4.0 mm/h.

[b] Figures represent arithmetic mean ± standard error of the radioactivity in an organ as a percentage of the total recoverable radioactivity.

[c, d] MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial, and inguinal) lymph nodes.

gravity have already been presented in Figures 4.1 and 4.2. As described in section 4.1, the sedimentation and ^{125}I-Udr-radiolabelling profiles of either MLN or PLN lymphoblasts were very similar to those obtained with ^{3}H-Tdr-labelled cells. Table 4.13 shows the distribution of radioactivity in recipient tissues after transfer of ^{125}I-Udr-labelled MLN cells separated by velocity sedimentation at unit gravity. These results indicate that the MLN contains at least two populations of lymphoblasts in terms of tissue localization. Lymphoblasts with s values greater than or equal to 4.0 mm/h localized predominantly in the small intestine. In contrast, the small population of labelled cells sedimenting at less than 4.0 mm/h (3.0-3.7 mm/h, Figure 4.1) was composed not only of cells primarily localizing in the small intestine, but also of some lymphoblasts localizing in the spleen, lungs, lymph nodes, and genital tract. The difference in splenic radioactivity was probably not due to transfer of radiolabelled, dead nucleated cells and debris ($s \leq 2.0$ mm/h) since hepatic radioactivity was similar in both groups. It should also be noted that since large cells ($s \geq 4.0$ mm/h) localized preferentially in the gut, the presence of small cells in the inocula was not necessary for this localization to occur.

4.13 Localization of lymphoblasts from MLN, PLN, Peyer's patches and intestinal lamina propria of guinea pigs

The foregoing results indicated that the MLN were a very fertile source of the precursors of IgA plasmacytes seen beneath the various mucosal epithelia. However, it was also possible that a

Table 4.14

Distribution of radioactivity in various tissues 22-24 h after adoptive transfer of [^{125}I]-deoxyuridine-labelled lymphoblasts into guinea pigs[a]

Recipient tissues examined	Source of donor cells injected[b]	% of total recoverable radioactivity in recipient tissues[c]	
		Hartley strain recipients	Strain 13 recipients
Small intestine	MLN	24.4 ± 3.7	9.8 ± 2.3
	LP	34.4 ± 4.2	9.8 ± 1.1
	PLN	8.2 ± 0.6	1.4 ± 0.4
	PP	5.8 ± 0.1	15.0 ± 0.9
Large intestine	MLN	13.6 ± 0.9	9.0 ± 2.5
	LP	8.5 ± 1.2	4.4 ± 0.3
	PLN	6.5 ± 0.7	NDt[d]
	PP	3.2 ± 0.9	22.9 ± 3.7
Spleen	MLN	9.8 ± 1.1	7.7 ± 0.8
	LP	6.8 ± 1.1	11.3 ± 1.5
	PLN	6.9 ± 1.3	6.4 ± 0.4
	PP	18.6 ± 0.3	8.8 ± 0.2
Liver	MLN	18.5 ± 1.4	63.0 ± 0.3
	LP	35.6 ± 2.4	65.4 ± 0.8
	PLN	11.7 ± 1.8	58.8 ± 7.9
	PP	51.7 ± 2.9	49.9 ± 1.7

Table 4.14 (cont'd)

Recipient tissues examined	Source of donor cells injected[b]	% of total recoverable radioactivity in recipient tissues[c]	
		Hartley strain recipients	Strain 13 recipients
Lungs	MLN	18.5 ± 1.4	63.0 ± 0.3
	LP	5.8 ± 1.6	6.4 ± 0.8
	PLN	3.7 ± 2.9	2.1 ± 0.7
	PP	20.3 ± 4.0	14.3 ± 2.3
MLN	MLN	10.0 ± 1.4	1.9 ± 0.1
	LP	1.4 ± 0.4	0.6 ± 0.0
	PLN	7.6 ± 1.7	0.5 ± 0.2
	PP	0.2 ± 0.1	0.5 ± 0.1
PLN	MLN	6.5 ± 1.8	2.6 ± 0.2
	LP	0.7 ± 0.0	2.0 ± 0.4
	PLN	50.4 ± 4.7	0.9 ± 0.5
	PP	0.1 ± 0.0	1.9 ± 0.7

Table 4:14 (Footnotes)

[a] Results shown are pooled from 3 experiments in Hartley strain guinea pigs (6 animals per group per experiment) and 2 experiments in Strain 13 guinea pigs (3 animals per group per experiment).

[b] MLN, mesenteric lymph nodes; PLN, peripheral (axillary, brachial, and inguinal) lymph nodes; LP, lymphocytes obtained from the lamina propria of the small intestine; PP, Peyer's patches.

[c] Figures represent the arithmetic mean ± standard error of the radioactivity in an organ as a percentage of the total recoverable radioactivity.

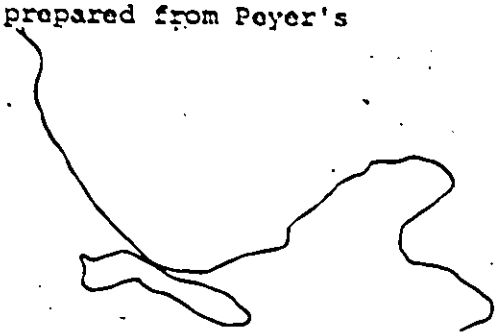
[d] NDt, not determined.

portion of these cells in these sites arose by clonal expansion of precursors situated beneath mucosal surfaces. Some evidence was obtained to show that a portion of cells derived from the intestinal lamina propria were capable of DNA synthesis and possessed a specific predilection to selectively localize in the gut. Because methods were not available to mechanically isolate lymphocytes from the mouse intestinal lamina propria, either 3 month-old Hartley strain or 8-9 month-old Strain 13 guinea pigs were used.

The distributions of cell-associated radioactivity in various guinea pig tissues 22-24 h after adoptive transfer of syngeneic or allogeneic lymphoblasts are presented in Table 4.14. As expected, MLN lymphoblasts preferentially localized in the gut, lungs and MLN of either allogeneic (Hartley strain) or syngeneic (Strain 13) recipients. The smaller percentage of organ-associated radioactivity after syngeneic adoptive transfer compared to allogeneic transfer presumably reflected a large fraction of damaged donor cells being trapped by the liver. Syngeneic and allogeneic lymphoblasts derived from gut lamina propria showed a seemingly preferential localization in the small intestine and avoided other tissues when compared to labelled MLN cells. In contrast, allogeneic PLN lymphoblasts returned primarily to their sites of origin. However, labelled PLN cells used in the syngeneic transfers did not show a predilection to localize in any site including recipient PLN. A similar type of contrast between allogeneic and syngeneic recipients was observed after transfer of labelled Peyer's patch cells. In the allogeneic system, Peyer's patch lymphoblasts did not show a preferential

localization in any tissue except perhaps the lungs. Pulmonary radioactivity might have been due to a large number of either dead or damaged cells in the inocula since considerable hepatic radioactivity was also detectable. However, when 8-9 month-old Strain 13 animals were investigated, a great deal of radioactivity was found in both the large and small intestines.

These experiments indicated that DNA-synthesizing cells exist in the gut lamina propria and showed a rather specific localization in the gut upon either syngeneic or allogeneic adoptive transfer. Furthermore, these lamina propria cells showed a distribution similar to MLN-derived cells and dissimilar to those prepared from Peyer's patches or PLN.



CHAPTER 5

DISCUSSION OF THE EXPERIMENTAL RESULTS

5.1 Preamble

The contents of this chapter will be concerned primarily with the movements of lymphoblasts. Considerable information about this subject has appeared in the literature during the course of the work presented in Chapter 4 and the experimental results will be discussed in view of these new findings as well as those surveyed in Chapter 1.

5.2 Technical considerations

Some important technical considerations have emerged from this study. The use of radiocounting methodologies for the detection of radiolabelled cells in recipient tissues was suitable only for those tissues containing a substantial portion of the labelled cells transferred. Comparisons of Tables 4.3 - 4.5 with Table 4.6 clearly show that if the numbers of labelled cells in certain tissues was small (e.g., genital tract and mammary glands) radiocounting procedures were unable to detect these cells. Of equal significance, high speed autoradiography revealed that donor lymphoblasts were more abundant, on a tissue volume basis, than was evident by radiocounting (cf., Tables 4.3 - 4.5 and Table 4.6). Clearly, high-speed autoradiography provided the greatest amount of information.

When combined with immunofluorescence, autoradiography has proved to be a powerful tool for detecting B-lymphocyte subpopulations

in recipient tissues (Dolezel & Bienenstock, 1971; Guy-Grand *et al.*, 1974; McWilliams *et al.*, 1977; Roux *et al.*, 1977). However, autoradiography usually requires several weeks for performance. In the present work, high-speed autoradiography using a toluene-based scintillation fluid (Panayi & Neill, 1972; Durie & Salmon, 1975) was successfully combined with immunofluorescence without loss of either autoradiographic or immunofluorescent resolution. The drastic reduction in autoradiographic exposure time (from 4-6 wk to < 24 h) has made this technique quite useful. Furthermore, by combining double immunofluorescence (using both rhodamine- and fluorescein-conjugated reagents) with high-speed autoradiography, both the Ig isotype and antigen specificity of transferred lymphoblasts might be detected. Therefore, it was concluded that high-speed autoradiography combined with immunofluorescence might, in many cases, be the method of choice for detecting subpopulations of radiolabelled cells in tissues.

5.3 Origins of lymphoblasts in mucosa-associated tissues

Humoral immunity at mucosal surfaces is effected by sIgA antibodies in external secretions (Chodirker & Tomasi, 1963; Tomasi *et al.*, 1965). The sIgA in exocrine secretions is believed to arise from IgA-containing cells which are remarkably abundant beneath mucosal epithelia (Tourville *et al.*, 1969, 1970). In the rabbit, 85% of all plasma cells in mucosa-associated tissues contained IgA whereas 2.5% of all plasma cells in peripheral lymph nodes and spleen contained IgA (Crandall *et al.*, 1967). The mechanism of both establishment and maintenance of this differential IgA

plasmacyte distribution has only begun to be unraveled, and the MALT appears to play a central role.

A very persuasive body of evidence indicates that the GALT is a productive source of the IgA-containing cells found beneath mucosal epithelia. In rabbits, PP were found to be a rich source of the precursors of IgA plasma cells appearing in the mucosa of the gut (and bronchus) several days after adoptive transfer into allogeneic or autologous irradiated hosts (Craig & Cebra, 1971; Rudzik *et al.*, 1975b, c). In this regard, similar studies revealed that the rabbit appendix functionally resembled the PP (Craig & Cebra, 1975) and, recently, Cebra and colleagues (Cebra *et al.*, 1977) have demonstrated that PP were the sole source of IgA plasma cells several days after adoptive transfer into syngeneic recipient mice. In these sorts of studies, peripheral lymphoid sources were very deficient in IgA plasmacyte progenitors. Thus, it seems that PP and appendix are a major source of the precursors of IgA plasmacytes found beneath the gut (and bronchial) mucosa. However, these precursors require a long period of time to differentiate. The observations by Zatz & Lance (1970) that within 24 h after adoptive transfer PP lymphocytes showed little tendency to localize in recipient tissues including the gut and PP is consistent with the notion that PP occupy an early position in the differentiation steps generating IgA plasma cells that mediate mucosal immunity.

If the PP and appendix harbour a subpopulation of cells which are in the process of differentiating, where are these cells located just before differentiation is completed? Although one might predict

that these cells would be either in the PP or in mucosae of the intestinal and respiratory tracts, ample evidence indicates that this is not the case. In rodents, transferred lymphoblasts derived from MLN had the property of selectively localizing within 24 h in both the intestinal lamina propria and MLN of recipients (Griscelli *et al.*, 1969; Guy-Grand *et al.*, 1974; Parrot & Ferguson, 1974; McWilliams *et al.*, 1975, 1977; Rose *et al.*, 1976a, b; Roux *et al.*, 1977). The majority of MLN lymphoblasts belonged to a B-lymphocyte subpopulation which was committed to the synthesis of IgA (McWilliams *et al.*, 1977), and, appeared in the gut as IgA-containing cells (Guy-Grand *et al.*, 1974; McWilliams *et al.*, 1975, 1977; Roux *et al.*, 1977). In contrast, when PLN lymphoblasts were transferred, most were found in recipient PLN and only a few were detected in the gut mucosa. Other Ig isotypes were not studied and PLN lymphoblasts were not examined for any cytoplasmic isotype.

The present work has confirmed and extended these findings (Tables 4.3 - 4.7). Following adoptive transfer, labelled MLN cells were always found more frequently than those of PLN origin in the gut lamina propria and MLN. Fifty to 60% of the transferred MLN lymphoblasts that appeared in the MLN and gut of mice contained IgA (Table 4.7) whereas only 28-38% of PLN lymphoblasts appearing in these organs contained IgA. These findings are consistent with the view that the MLN harbours IgA plasmacyte progenitors more differentiated than those in the PP.

It is interesting that the MLN also contained an additional lymphoblast subpopulation which appeared as IgG plasmacytes in the gut

and MLN (Table 4.7). The early progenitors of IgG plasma cells have been identified in the PP of rabbits and mice (Craig & Cebra, 1971, 1977; Rudzik *et al.*, 1975b, c), and a small percentage of MLN lymphoblasts were shown to have surface and/or intracellular IgG (Guy-Grand *et al.*, 1974). Moreover, a significant number of IgG-containing cells were observed in both the mouse and human intestinal mucosae (Milne *et al.*, 1975; Milne, 1976; Crabbé *et al.*, 1965; Crabbé & Heremans, 1966; also see section 4.8.2). Thus, the presence of the immediate precursors of intestinal IgG plasmacytes in the MLN is not improbable.

The BALT has been shown to bear a remarkable morphologic and functional resemblance to the PP (Klein, 1875; Bienenstock *et al.*, 1973a, b; Rudzik *et al.*, 1975a; Bienenstock & Johnston, 1976). Like the PP, the BALT has been identified as a major precinct of the early precursors of IgA plasma cells, and, to a smaller extent, IgG progenitors. Six days after adoptive transfer into irradiated allogeneic rabbits, cells derived from BALT were able to repopulate the gut (and bronchus) mucosa primarily with IgA plasma cells; a few IgG-containing cells were also observed (Rudzik *et al.*, 1975c). Indeed, it seems that the BALT also contains IgA progenitors at a very early stage of differentiation. Accordingly, one might predict that the BLN would contain BALT-derived cells which had achieved a more advanced state of differentiation and that these cells (as lymphoblasts) would selectively localize in the gut mucosa and rapidly develop into IgA plasma cells. IgA plasmacytes did develop from BLN lymphoblasts within 24 h after transfer (Table 4.7). However, results show that BLN lymphoblasts did not localize in the intestine

any greater extent than did PLN lymphoblasts (Table 4.3 - 4.6). Unlike PLN lymphoblasts localizing in the gut, the percentages of BLN lymphoblasts seen here containing IgA (68%) and IgG (36%) were similar to those observed after MLN lymphoblast transfer (Table 4.7). Moreover, the frequency of BLN lymphoblasts localizing in recipient MLN was intermediate between those observed after MLN and PLN lymphoblast transfer and the proportions of BLN lymphoblasts containing IgA and IgG were also intermediate (50% IgA, 38% IgG). It seemed, therefore, that BLN lymphoblasts differed functionally from those prepared from either MLN or PLN. Although the BLN does seem to contain many BALT B-lymphocyte subpopulations which can rapidly differentiate, there is a significant difference in the BLN lymphoblast localization pattern. The reasons for this difference are not clear. Perhaps the BLN does not contain many BALT B-lymphocyte subpopulations at a later stage of differentiation. Alternatively, B-lymphocytes emigrating from the BALT might have undergone a unique developmental alteration in the BLN which did not provide them with the ability to selectively localize in the gut mucosa. This developmental event might have been circumvented by adoptive transfer of BALT cells (Rudzik *et al.*, 1975c) which avoided their passage through the BLN. Unfortunately, the available information does not allow a firm choice to be made between these hypotheses. It should be noted that in rodents the BALT is not nearly as organized as in the rabbit (Bienenstock *et al.*, 1973a). Thus, comparison of rodent BLN to rabbit BALT must be viewed with caution.

Evidence indicates that although adoptive transfer is an artificial release of cells from lymphoid sources, this experimental technique is closely representative of the *in vivo* situation. In rodents, a population of thoracic duct (TD) B-lymphoblasts (collected by cannulation) did not recirculate but very quickly localized selectively in the intestinal lamina propria (Gowans & Knight, 1964; Griscelli *et al.*, 1969; Hall & Smith, 1970; Hall *et al.*, 1972; Guy-Grand *et al.*, 1974). When compared to MLN cells, the proportion of TD cells which were dividing was substantially reduced yet the numbers of TD lymphoblasts bearing and/or containing IgA was more than doubled. Thus, these findings suggest that the TD contains populations of MLN B-lymphoblasts which have differentiated even further toward IgA-containing cells. Furthermore, the observations that lymphoblasts derived from the efferent lymph of MLN in sheep behaved like MLN and TD lymphoblasts after adoptive transfer (Hopkins & Hall, 1976) indicates that release of lymphocytes from lymph nodes by mechanical means, as used in the present study, has little effect on their natural migratory properties.

As already mentioned, both the PP and BALT were found to be rich sources of the early precursors of IgA plasma cells seen in the mucosae of both the intestinal and respiratory tracts (Craig & Cebra, 1971,1975; Rudzik *et al.*, 1975b,c). Since the MLN seemingly contains more differentiated precursors of IgA plasmacytes, one could predict that MLN lymphoblasts would selectively populate the bronchial mucosa within 24 h after adoptive transfer. The results indicated that this was indeed the case. When compared to PLN lymphoblasts,

cells derived from MLN selectively localized in the lungs and these contained IgA and IgG in proportions similar to labelled MLN cells localizing in the gut (Tables 4.6 and 4.7). Quite surprisingly, BLN lymphoblasts had an even greater propensity than labelled MLN cells to localize in the lungs and these contained IgA and IgG in proportions similar to the few BLN cells localizing in the gut. These observations further emphasized the dissimilarities between MLN, PLN, and BLN lymphoblast subpopulations.

An explanation for the high frequency of BLN lymphoblasts in the lungs and not in the small intestine and MLN might be that these cells were either dead or damaged when injected or shortly thereafter, and were non-specifically trapped in the pulmonary vasculature. Several observations argue against this possibility. Greater than 99% of BLN cells injected were not stainable with trypan blue. Furthermore, hepatic radioactivity after transfer of ^{125}I-Udr-labelled BLN cells amounted to only 50% of that detected after transfer of labelled MLN cells; one would have predicted much more radioactivity in the liver if the transferred BLN cells were either dead or damaged (Table 4.5). In addition, no labelled BLN cells were seen in the pulmonary vasculature; a labelled MLN cell was observed in pulmonary capillaries on two occasions. Finally, the frequency of labelled BLN cells in recipient PLN did not decrease as one would have predicted had trapping in the lungs reduced the number of labelled cells entering the arterial bloodstream. Thus, non-specific trapping of BLN lymphoblasts in the lungs is unlikely to account for the failure of BLN lymphoblasts to localize in the gut and MLN.

The current findings suggest that the Ig-containing cells in the lungs are from both the MLN and BLN. Moreover, the BLN is seemingly restricted to a specific pulmonary defense role. Both intratracheal (I.T.) and I.P. immunization have been shown to lead to a humoral response in the mouse BLN (Nash, 1973; Kaltreider *et al.*, Gerbrandy & Bienenstock, 1976; McLeod *et al.*, 1978), whereas no response was evident after oral immunization (McLeod *et al.*, 1978). The observation that BLN lymphoblasts were more distributed through the pulmonary parenchyma than were labelled MLN cells (section 4.7) suggests that perhaps BLN lymphocytes find their way into distal pulmonary sites. Thus, it is interesting to speculate that the PFC recovered from mouse alveolar washings after either I.T. or I.P. immunization (Nash, 1973; Kaltreider *et al.*, 1974; McLeod *et al.*, 1978) might have been sensitized in, and emigrated from, the BLN, and that the BLN are the source of IgA plasmacytes infiltrating the lungs after *Mycoplasma pulmonis* infection (Cassel *et al.*, 1974). It is possible that the GALT might provide lung defenses mainly in the bronchus and not in the alveoli. This view is consistent with the recent observations by Montgomery and co-workers (Montgomery *et al.*, 1978a) that oral immunization led to the appearance of specific sIgA antibodies in bronchial washings. On the other hand, the BLN might confer a more generalized defense which is restricted to the lungs. The cellular and molecular determinants of this restriction are unknown.

In rodents, as in humans and rabbits, IgA is the predominant Ig in milk (Ammann & Stiehm, 1966; Guyer *et al.*, 1976). The most likely source of IgA in mammary secretions is the plasmacytes which lie beneath the ductal epithelium in the breast (Tourville *et al.*,

1969, 1970). In mouse mammary glands, IgA plasma cells increase dramatically in number in late gestation and during lactation (Weisz-Carrington *et al.*, 1977).

The present work has demonstrated that IgA-containing cells in the gestational mammary gland most likely originated in the MLN (Table 4.10 and section 4.11.1) thus confirming the work of Lamm & colleagues (Roux *et al.*, 1977). These results provide an explanation for the findings that after oral immunization, sIgA antibodies specific for the antigens of the gastrointestinal tract can be found in milk (Bohl *et al.*, 1972; Montgomery *et al.*, 1974, 1978a; Allardyce *et al.*, 1974; Ahlstelt *et al.*, 1975, 1977). Indeed, cells containing IgA antibodies specific for the antigens of the gut microflora could be found in the mammary secretions of lactating humans (Ahlstelt *et al.*, 1975; Goldblum *et al.*, 1975). Thus, intestinal humoral immunity in the mother could be passively transferred to the neonatal intestine which has sIgA receptors on enterocytes (Guyer *et al.*, 1976; Nagura *et al.*, 1978). The function of cells from mammary secretions in the newborn gut is unknown.

Until now, no information has existed as to the origin of plasma cells in the reproductive tract. The results presented in Tables 4.8 and 4.9 indicate that the MLN are a fertile source of the IgA and IgG plasmacytes seen in the proestral cervix and vagina. The PLN were quite deficient in this capacity. The proportions of MLN lymphoblasts containing IgA and IgG in the cervix and vagina conformed closely to these proportions of labelled MLN cells in the intestinal tract (cf., Tables 4.7 and 4.9). Although a similar conclusion may

be drawn from studies of the proestral uterus, an insufficient number of labelled cells was seen to substantiate this.

In health, there is a relative paucity of plasma cells in the female genital tract compared to some other mucosal sites and IgA levels in cervical and vaginal secretions are quite low (Tourville *et al.*, 1970; Waldman *et al.*, 1972; Chipperfield *et al.*, 1972; Chipperfield & Evans, 1975; Hurlimann *et al.*, 1978). The current findings are in keeping with these reports; only a few labelled MLN cells were seen in the cervix and vagina. Moreover, the strikingly low frequency of labelled MLN cells in the uterus is consistent with the observation that intrauterine immunization produced primarily an IgG response (Ogra & Ogra, 1973). These results indicate that the MLN can supply the immediate precursors of IgA plasma cells to a mucosa-associated organ which does not possess organized lymphoid aggregates similar to PP and BALT.

Finally, it should be mentioned that although IgM-containing lymphoblasts were identified in the various mucosa-associated tissues, no conclusion as to their origin was secured because insufficient numbers of labelled IgM-containing cells were observed.

The notion that <u>all</u> Ig-containing cells beneath mucosal epithelia migrated from external sources has no foundations. For example, it is possible that a portion of the plasmacytes seen in mucosa-associated tissues arose by clonal expansion of precursors in this site. This hypothesis has been examined to some extent in the gut. The results presented in Table 4.14 support this latter idea. Clearly lymphocytes which were mechanically isolated from

the guinea pig intestinal lamina propria were capable of incorporating DNA precursors (Tables 4.1 and 4.14; also see Rudzik & Bienenstock, 1974). Although these lymphoblasts behaved very much like MLN-derived lymphoblasts in that they selectively localized in the gut mucosa, the fact that they did not localize in the lungs suggests that gut lamina propria lymphoblasts are dissimilar to labelled MLN cells. Certainly, gut lamina propria lymphoblasts were functionally unlike either PP or PLN lymphoblasts.

Adoptive transfer of Strain 13 guinea pig PP or PLN lymphoblasts showed a remarkable disparity with those results obtained using Hartley strain guinea pigs (Table 4.14). The reason for this difference is unknown but the result may reflect the fact that the Strain 13 animals were three times older than those of the Hartley strain (section 3.1). Certainly, the yield of lymphoid tissue taken from these animals was unequal (Table 4.1). No information is available in the literature as to the influences of age on lymphocyte localization.

It is generally assumed that mature plasma cells do not proliferate. However, Clafton & Smithies (1967) reported that cells beginning to secrete Ig were still capable of division. More recently, Husband & Gowans (1978) showed that a portion of MLN-derived, IgA-containing cells seen in the gut mucosa incorporated ^{3}H-Tdr. It is, therefore, conceivable that the proliferating gut lamina propria cells which selectively localized in the intestinal wall of guinea pigs were beginning to secrete IgA. However, without appropriate immunofluorescent investigation, this point cannot be

decided. If these cells are dividing plasmacytes, do they return to the lymph *in vivo*? A recent report has indicated that they do not migrate from the gut but probably die in the lamina propria (Husband & Gowans, 1978).

On the other hand, some evidence has demonstrated that the intestinal lamina propria, like PP and BALT, contains precursors of IgA plasmacytes appearing in the gut mucosa. Approximately 40% of the B-lymphocytes bearing surface Ig bore surface IgA; only 0.4% of these lymphoid cells were IgA plasmacytes (Rudzik & Bienenstock, 1974; Rudzik *et al.*, 1975a). When transferred into allogeneic irradiated rabbits, gut lamina propria B-lymphocytes repopulated recipient guts with IgA-containing cells (Befus *et al.*, 1978). These B-cells did not appear to be IgA progenitors from PP that were more differentiated since gut repopulation was not observed prior to 6 days post transfer.

Taken together, these results suggest that the B-cells located beneath mucosal epithelia, at least in the intestine, are a mixture of several subpopulations. One of these subpopulations may clonally expand and give rise to Ig-secreting cells. Whether or not these cells can seed distant mucosae remains to be explored.

Not all radiolabelled donor cells appeared to be the immediate progenitors of either IgG, IgA or IgM plasma cells since a portion of these cells in recipient tissues did not stain with fluorescent antisera specific for these Ig's (Tables 4.7 and 4.9). These unstained lymphoblasts might represent non-lymphoid cells such as accessory cells, other B-cell isotypes, T-cells or a

mixture of these. Studies employing velocity sedimentation at unit gravity have revealed that all labelled MLN and PLN cells were morphologically large cells ($s \geq 3.8$ mm/h) (section 4.1). Only investigation of cell surface markers can decide what proportion of these cells represent non-lymphocytic cell types.

It seems likely that the precursors of IgE plasmacytes can be found in greatest numbers in lymph nodes draining mucosal sites such as the lungs (Gerbrandy & Bienenstock, 1976) and gut (Waksman & Ozer, 1976). Therefore, some of the labelled non-fluorescent cells in mucosal tissues might be of the IgE class.

A more attractive alternative might be that the unstained lymphoblasts in recipient tissues were T-cells. In the MLN, 0.8% of all lymphoblasts were T-cells; in the PLN this figure was 0.9% (Guy-Grand et al., 1974). MLN T-lymphoblasts have been shown to selectively accumulate in the gut during health and with parasitic infestation (Parrot & Ferguson, 1974; Rose et al., 1976a,b, 1978). One report indicates that T-lymphoblasts localize in the gut to a lesser extent than B-lymphoblasts (McWilliams et al., 1975). Furthermore, Guy-Grand and colleagues (Guy-Grand et al., 1974) observed that T-lymphoblasts derived from MLN tended to localize in the gut epithelium and Sprent (1976) has shown that some TD T-lymphoblasts (H-2 activated) localized in the epithelium of the gut. Indeed, both athymic (nude) and neonatally thymectomized mice had far fewer intraepithelial cell lymphocytes than normal mice (Ferguson & Parrot, 1972; Parrot & de Sousa, 1974). Out of all the gut tissue sections examined in this study, a total

of only 2 MLN lymphoblasts were found in the epithelium. In this respect, the present findings are similar to those of Parrot and Ferguson (1974); MLN lymphoblasts were not observed between epithelial cells of the gut. The available data allow no firm conclusion as to the identity of the labelled, non-fluorescent cells which were observed in the various mucosal tissue.

In summary, evidence has been presented which indicates that the MLN and BLN are the sources of the immediate precursors of mucosal plasmacytes, principally those synthesizing IgA. The MLN appears to be a versatile source capable of providing IgA plasma cell progenitors to a variety of mucosal surfaces. On the other hand, the BLN is a more restricted source and supplies chiefly the lungs with IgA plasma cells. In addition, such precursors might arise in intestinal mucosa itself. These results are consistent with IgA being the predominant Ig isotype in mucosal secretions. Some factors which might influence selective lymphoblast localization are discussed in section 5.4.

5.4 Factors affecting selective localization of lymphoblasts

5.4.1 Numbers of lymphoblasts injected

An explanation for the high frequency of localization of MLN and BLN lymphoblasts relative to PLN lymphoblasts in the various mucosae-associated tissues could be the differences in numbers of labelled cells present in the respective donor cell sources; in mice, approximately 1.5% of MLN cells, 4.0% of BLN cells and 0.8% of PLN cells which were transferred incorporated ^{3}H-Tdr (Table 4.1).

One might predict that the relationship between the number of labelled cells in the donor inoculum and the frequency of labelled cells appearing in a particular recipient tissue would be linear with a slope of unity. Accordingly, the results (e.g., Table 4.6) could be normalized. Recent evidence (S. Link, M. McDermott & J. Bienenstock, manuscript in preparation) has indicated that for MLN-derived lymphoblasts entering the mouse gut this relationship is indeed linear and it has a slope of 1.0. Whether or not this relationship holds true for lymphoblasts derived from the various lymphoid sources entering the gut and other mucosal sites has yet to be investigated. In the present study labelled cells from both the MLN and BLN were observed up to 11 times more often than PLN blasts in mucosal sites. It was concluded that the differences in the numbers of labelled cells in the inocula were unlikely to account for the selective localization of MLN- and BLN-derived lymphoblasts compared to PLN cells. Nevertheless, knowledge of the relationship described above for each cell source and each recipient tissue is required for a more precise interpretation of the results. Furthermore, the possibility exists that various subpopulations of lymphoblasts are represented in varying proportions in the different donor lymphoid sources. For all of the reasons listed above, it is not possible to normalize the data obtained in the present study but it is possible to draw general conclusions as to the tendency of cells to localize in different tissues.

5.4.2 Antigen

A large body of evidence has indicated that antigen can cause localization of lymphocytes in peripheral lymphoid tissue (reviewed by Ford, 1975). However, the role of antigen in selective localization of lymphoblasts in mucosa-associated tissues is unclear. Some studies suggest that MLN lymphoblast localization is antigen independent. Both BALT and GALT were present in transplanted fetal gut and lungs presumed to be antigen-free (Ferguson & Parrot, 1972; Ferguson, 1974; Milne *et al.*, 1975), and thoracic duct and MLN lymphoblasts localized in transplanted fetal gut (Moore & Hall, 1972; Parrot & Ferguson, 1974; Guy-Grand *et al.*, 1974). Indeed, lymphoblasts in sheep intestinal lymph selectively localized in the gut of fetuses *in utero* (Hall *et al.*, 1977). Similar findings were obtained when either suckled or unsuckled neonatal rats were injected with TD lymphoblasts (Halstead & Hall, 1972). In fact, even when sheep intestinal lymphoblasts were transferred xenogeneically into rats, enhanced localization of lymphoblasts occurred in the gut of recipients (Moore & Hall, 1972). Recently, Husband & Gowans (1978) demonstrated that cells (likely from the PP) which made anti-cholera toxoid IgA antibodies could selectively localize in the gut mucosa of recipients independent of the presence of cholera toxoid.

The present work is consistent with these findings. MLN cells localized in the breast tissue and uterus, the former organ being apparently free of enterically absorbed antigens (Montgomery *et al.*, 1974; Goldblum *et al.*, 1975), while the latter is considered

to be aseptic (Ogra & Ogra, 1973). Moreover, BLN lymphoblasts, the majority of which were presumably generated in response to immunization with *Bordetella pertussis* and tetanus toxoid, localized in the lungs of unimmunized recipients. Thus MLN and BLN lymphoblast selective localization might be, at least in part, independent of the presence of antigen.

On the other hand, antigen may have a profound effect on MLN lymphoblast localization, both in magnitude and persistence. In immunized rats, the frequency of cells in the intestinal lamina propria synthesizing IgA antibodies against cholera toxoid was always greatest in that region of the gut which had been challenged with antigen (Pierce & Gowans, 1975). Similar observations have been made in dogs (Pierce *et al.*, 1978). When Thiry-Vella intestinal loops prepared in rats immunized with cholera toxoid were challenged, the number of anti-toxin-containing cells (ACC) in the loop lamina propria always exceeded that seen in unchallenged loops (Husband & Gowans, 1978). A time course study revealed that ACC continued to accumulate and persist in the challenged loop but only appeared transiently in unchallenged loops. The persistence of ACC in challenged loops might have been due to proliferation of ACC and/or their precursors in the lamina propria; both the present work (section 4.13) and Husband & Gowans (1978) demonstrated that some cells in the gut lamina propria could incorporate radiolabelled nucleotides. These results suggest that selective localization and proliferation of MLN lymphoblasts chiefly requires the presence of antigen.

Several models can be proposed to explain both antigen-independent and antigen-dependent localization of MLN lymphoblasts and three points, at which regulation of this phenomenon might occur, can be considered: 1) entry into mucosa-associated tissues, 2) retention in these sites, and 3) emigration from such locations.

5.4.2.1 Entry

Taken together, the results described above suggest that a MLN B-lymphoblasts might possess two types of receptors on their surface; one type specific for the sorts of antigens encountering mucosal sites, i.e., antigen-dependent receptors; the other type specific for a non-antigen structure present on the surface of cells in mucosal sites, i.e., antigen-independent receptors. In this model, antigen-independent receptors would be crucial for entry into the mucosa. The absence of antigen-independent receptors on PLN lymphoblasts could be postulated to explain their lack of affinity for mucosae. It is not known exactly where or how lymphoblasts enter mucosa-associated tissues. MLN lymphoblasts might have to pass through the PP to gain access to the intestinal lamina propria. Husband & Gowans (1978) have shown that the presence of a PP in a Thiry-Vella intestinal loop was an absolute requirement for ACC localization in the loop mucosa. After adoptive transfer, donor PP cells tended to repopulate recipient gut in areas closer to the PP (Rudzik *et al.*, 1975b) and transferred MLN lymphoblasts were frequently seen adjacent to PP (Guy-Grand *et al.*, 1974). Moreover, IgA-containing cells are most common in regions close to PP (Crabbé *et al.*, 1965). These results suggest that the PP

might be one avenue for MLN lymphoblasts to enter the gut. A similar function could be postulated for the BALT. Since structures analogous to the PP and BALT are absent in the genital tract and mammary glands, these organized lymphoid structures would appear not to be essential for MLN lymphoblast localization here. However, unorganized, discrete lymphoid aggregates in mucosa-associated tissues might serve as entry pathways to the mammary and cervico-vaginal mucosae. Lastly, MLN lymphoblasts might enter mucosal sites in the absence of any lymphoid structures, either organized or otherwise. Appropriate experiments have not been done to identify the sites at which lymphoblasts enter mucosal sites although post capillary venules, the sites of entry of lymphocytes into lymph nodes, are known to be present in BALT and PP.

5.4.2.2 Retention

Once in the mucosa, an appropriate number of antigen-dependent and antigen-independent receptors might be necessary for MLN lymphoblasts to be retained in this site. Gowans (see transcribed discussion following Husband *et al.*, 1977) has suggested that some MLN-derived cells might proliferate once in the mucosa and be retained through this mechanism. Thus, the purported MLN localization in presumably antigen-free environments might be mediated by antigen-independent receptors. Here, the lack of antigen would account for the paucity of donor MLN lymphoblasts (Ferguson & Parrot, 1972; Guy-Grand *et al.*, 1974) and IgA-containing cells (Milne *et al.*, 1975) compared to conventional

intestines. Since most MLN lymphoblasts would likely possess receptors specific for gastrointestinal antigens, MLN cells would be expected to localize in the gut in the largest numbers (Table 4.6). The lungs and genital tract, having a lesser intestinal type antigenic load, could be predicted to have proportionately fewer cells localizing. This hypothesis is consistent with the present results (Table 4.6). The relative restriction of BLN lymphoblasts to the lungs might be due to a unique antigen-independent receptor conferring upon these cells the ability to enter only the respiratory tract. Such unique receptors might exist in individual tissues. The results of this study have clearly shown that MLN lymphoblasts tended to return primarily to the gut, BLN returned primarily to the lungs, and PLN returned to PLN. One could predict, therefore, that BLN would not selectively localize in the genital tract and mammary glands but that lymphoblasts prepared from the paraaortic and renal lymph nodes (which drain the genital tract) would exhibit selective localization beneath the epithelia of the cervix, vagina, uterus and perhaps the mammary glands. Experiments to support or refute this speculation have not been conducted.

5.4.2.3 Emigration

It seems possible that antigen might retain lymphoblasts in mucosal sites by causing the closure of departure pathways. Lymphoblasts entering the gut by virtue of their antigen-independent receptors might be unable to leave and thus could interact with antigen and complete differentiation in the mucosa. Such a

mechanism of lymphocyte trapping seemingly exists in lymph nodes (Ford, 1975). This possibility remains to be explored in mucosa-associated tissues.

Whether or not lymphoblast surface receptors play a role in selective lymphoblast localization is unknown. Some evidence suggests that such receptors may in fact exist.

5.4.3 Cell surface receptors

One approach to determine whether or not "localization" receptors exist on lymphocytes has been to enzymatically alter certain lymphocyte surface molecules. Either trypsin or neuraminidase treatment of small TD lymphocytes dramatically reduced the extravascularization of these cells into lymph nodes (Gesner & Ginsberg, 1964; Woodruff & Gesner, 1968; Gesner et al., 1969; Woodruff, 1974; reviewed by Schlesinger, 1976). Similar findings have been obtained using an *in vitro* model of lymphocyte localization (Stamper & Woodruff, 1976, 1977; Woodruff et al., 1977). Although these results illustrated the importance of intact surface membrane structures, no B-lymphocyte subpopulation was studied. Nevertheless, it is clear from these investigations that receptors on lymphocytes can interact with a complementary structure on high-walled endotheliocytes of the post-capillary venules in lymph nodes. This sort of biochemical approach might be suitable for detection of antigen-independent receptors on lymphoblasts entering mucosal tissues. Since high-walled endotheliocytes are found nowhere outside of lymphoid tissue, it seems likely that

such receptors on MLN lymphoblasts are not specific for this type of endothelium.

Antibody molecules attached to the surface of lymphoblasts are the most likely candidates for antigen-dependent receptors. IgA, of endogenous origin, was certainly present on the surface of MLN lymphoblasts (Guy-Grand *et al.*, 1974; McWilliams *et al.*, 1977). Thus, IgA might serve as an antigen-dependent localization receptor. It is interesting that recently Strober and colleagues (Strober *et al.*, 1978) have identified mouse lymphocytes with IgA receptors. Their tissue distribution is not known.

SC, an epithelial cell product (Poger & Lamm, 1974) possibly associated with the transport of dimeric IgA into secretions (South *et al.*, 1966; Tourville *et al.*, 1969; Brandtzaeg, 1973; Crago *et al.*, 1978) might serve as an antigen-independent receptor for MLN and BLN lymphoblasts displaying surface IgA. In support of this hypothesis, a patient with absolute SC deficiency had no IgA plasmacytes in the intestinal lamina propria but possessed circulating IgA (Strober *et al.*, 1976). Some evidence, however, argues against this idea. First, J-chain is necessary for dimeric IgA binding to SC (Eskeland & Brandtzaeg, 1974) and J-chain has not been identified on circulating lymphocytes (unfortunately MLN cells were not examined) (Brandtzaeg, 1976). Secondly, both monomeric IgA and IgM had no measurable affinity for SC (Weicker & Underdown, 1975) although the polymeric forms of both of these Ig isotypes had similar affinities for SC. Since IgA (and IgM) antibodies are expressed as monomers on the cell surface and not

as polymers (Sigal & Klinman, 1978), it is unlikely that cells bearing either monomeric IgA or IgM would recognize SC as a receptor. Finally, procedures calculated to interfere with SC as a receptor had no effect on selective localization of MLN lymphoblasts in the gut (McWilliams et al., 1975). These findings offer no support for SC as a "localization" receptor.

At present, the roles of antigen, Ig and receptors in explaining the selective lymphoblast localization of lymphoblasts are unknown, but all might contribute. Antigen is seemingly very important in development and maintenance of the IgA distribution. However, MLN lymphoblast localization in antigen-deficient gestational mammary glands is inconsistent with this idea. Lymphoblast localization in sex hormone target organs appears to be under unique regulation and this topic is discussed in section 5.4.5.

5.4.4 Lymphocyte chemotaxis and blood flow

The selective localization of lymphoblasts in mucosal tissues might be, at least in part, due to a chemotactic stimulus. However, investigations attempting to look at this question have not met with much success (Parrot et al., 1976; Wilkinson et al., 1976). Thus, chemotactic attraction of lymphoblasts does not appear to be an immunologically specific mechanism for inducing cells to enter mucosal sites, although it must be considered in any discussion of this question.

One factor which might influence selective lymphoblast localization in mucosal tissues could be the number of lymphoblasts

arriving at entry sites via the circulation. Hay & Hobbs (1977) have clearly shown that hyperemic lymph nodes have an enhanced lymphocyte output. Prostaglandins (PG) of the E type were shown to be potent inducers of hyperemia (Johnston *et al.*, 1976). It has been suggested that PGE may increase blood flow to the gut (Nordstrom, 1972) and mammary glands (Dhondt *et al.*, 1977). Thus, consideration of blood-flow on lymphoblast localization in mucosae deserves further study, particularly in light of the fact that the proestrous and estrous genital tract is hyperemic.

5.4.5 Influences of sex hormones

In the present work, IgA precursor localization in mouse cervix and vagina was greatest during proestrus and estrus and least during diestrus. Several explanations for these observations have been entertained. Possibly, the increased wet weight of the cervix and vagina during estrus reflects more tissue capable of trapping MLN lymphoblasts. However, Table 4.10 shows that although the small intestine doubled in wet weight by late gestation, the recovery of radioactivity 24 h following transfer of labelled MLN lymphoblasts was equal, per mg of gut, to that in virgin recipients. This suggests that for a given quantity of tissue a constant number of MLN-lymphoblast will localize. Lamm and co-workers reached a similar conclusion after studying MLN localization in the developing mammary glands (Roux *et al.*, 1977). It seems unlikely that cervical and vaginal size is the sole factor controlling lymphocyte localization during the estrous cycle;

between estrus and diestrus these organs decreased 15% in wet weight while the frequency of labelled cells was reduced by more than 50%. Alternatively, the hyperemia and endothelial permeability may have been altered since vascular permeability changes during pregnancy. One of the factors responsible for this phenomenon might be estrogen, which reaches a maximal serum concentration in proestrus (Austin & Short, 1972) and could cause degranulation of local mast cells (Spozini & Szego, 1958) with a concomitant localized hyperemia and an increase in vessel permeability. Although hyperemia is certainly a major factor in the lymphocyte traffic through lymph nodes (Hay & Hobbs, 1977), Rose & Parrot (1977) have demonstrated a lack of simple association between vascular permeability and lymphoblast immigration into tissue sites. Preliminary evidence obtained in the present study (data not shown) corroborated these findings.

Alterations in mucosal immune functions by sex hormones in target tissues warrants careful investigation. Wira & Sandoe (1977) have reported sex steroid regulation of IgA and IgG in rat uterine secretions. It is known that women are more susceptible to bacterial genital infections such as gonorrhea during menstruation (Alqvist, 1976), a time which corresponds to the reduction in cervico-vaginal IgA plasma cells in mice. If receptors for B-lymphoblasts destined to make IgA are found to be under the influence of sex hormones, these might be open to manipulation to maximize host resistance to potential pathogens.

In mice, the number of IgA plasma cells in mammary glands increases dramatically in late gestation and during lactation, and

declines after weaning (Weisz-Carrington *et al.*, 1977). In contrast, equivalent changes were not observed in the numbers of IgM- and IgG-containing cells in the breast. Evidently, the immediate precursors of IgA plasma cells arriving from the MLN are responsible for the predominance of IgA plasmacytes in the breast (Roux *et al.*, 1977; Table 4.10 and section 4.11.1). Moreover, treatment of virgin female mice with estrogen, progesterone and prolactin caused the development of mammary tissue with concomitant increase only in MLN-derived IgA plasma cells here (Weisz-Carrington *et al.*, 1978). These authors suggested that tissue cells in developing mammary glands possess a receptor for MLN lymphoblasts destined to produce IgA.

The presence of an antigen-independent receptor for IgA precursor lymphoblasts in the cervix and vagina could be postulated. If present, the display of this receptor might be influenced by the estrous cycle and thus affect lymphoblast localization in sex hormone target organs. Because there is a dramatic change in cervico-vaginal epithelium during the estrous cycle, perhaps such a receptor could be an epithelial cell product such as secretory component. Moreover, a small subpopulation of MLN lymphoblasts might be especially sensitive to the presence of such a receptor. Inspection of Table 4.13 suggests that a subpopulation of some lymphoblasts may have affinity for the genital tract.

It seems unlikely that hormone induced receptors are the main factor determining the localization of lymphoblasts in the small intestine; the number of radiolabelled cells localizing in the gut remained unchanged over the course of the estrous cycle

(Table 4.11) and the gut is not known as a sex hormone target tissue. Whether or not an analogous situation exists in the male intestinal and genital tract remains to be investigated.

It is possible that sex hormones influence cell surface characteristics. Whether or not the putative receptor in the mammary glands is the same as that in the genital tract is not known. Whether or not the factors responsible for localization of IgA plasmacyte precursors in both the mammary gland and reproductive tract are the same, or even totally different from those responsible for localization in the bronchus and gut cannot be determined at this point. As mentioned previously, the lymph nodes draining the genital tract may contain B-lymphocytes, destined to make IgA, which localize specifically in the reproductive organs.

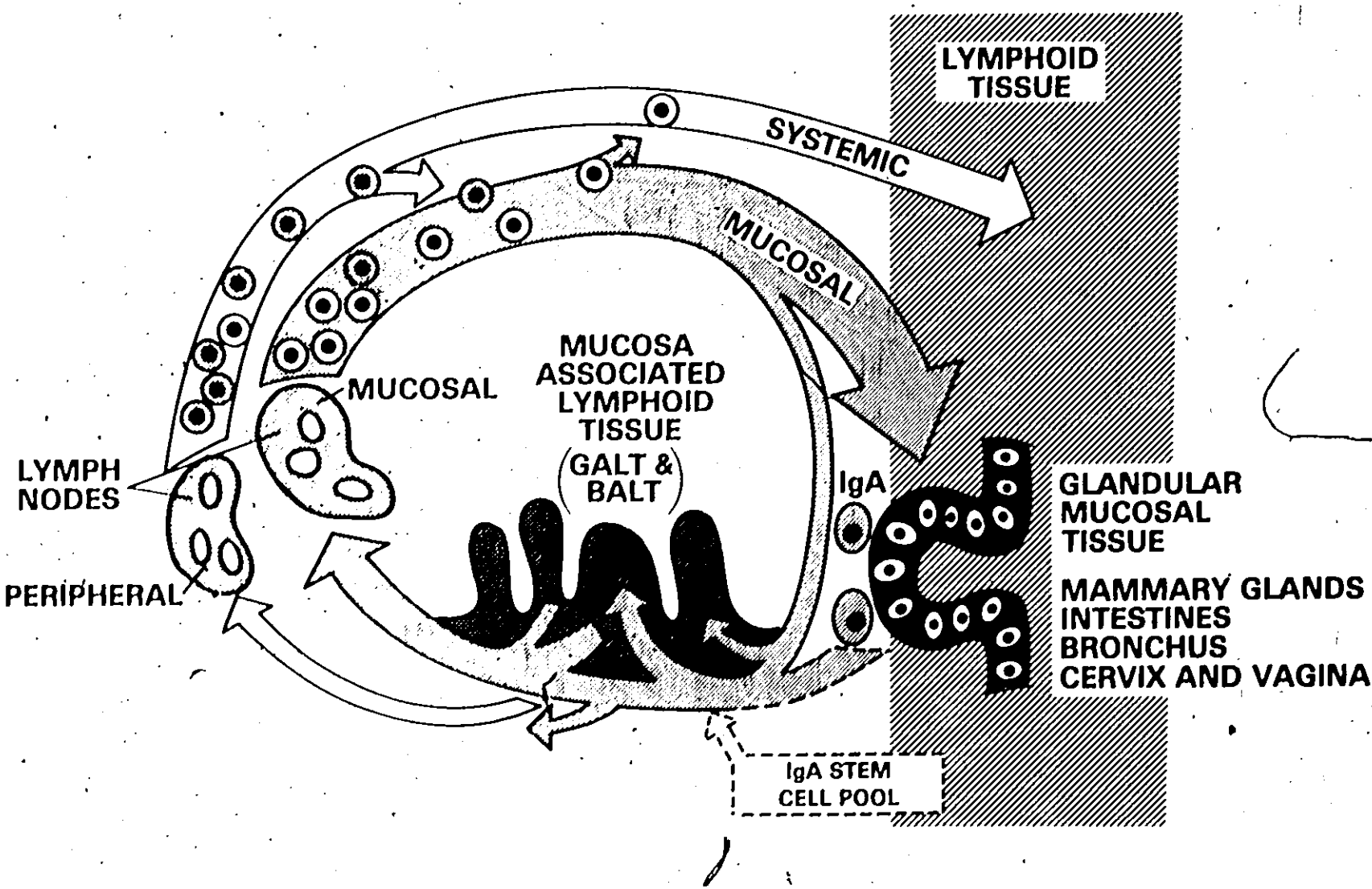
5.5 Model of mucosal immunity

The results of this study strongly support the concept of a common mucosal immunologic system (Bienenstock et al., 1973a) in which the precursors of IgA plasma cells sensitized at one mucosal site may migrate to a variety of mucosal sites. A model of this system is presented in Figure 5.1 and will be described below.

The GALT and BALT appear to be two of the primary sources of the early precursors of IgA plasmacytes. How these precursors are generated is unknown but they might arise from an external stem cell pool, presumably bone-marrow derived. Regardless, IgA precursors begin to differentiate and at some point are found in

Figure 5.1

Model of a common mucosal immunologic system. GALT, gut-associated lymphoid tissue; BALT, bronchus-associated lymphoid tissue. The mucosa-associated lymphoid tissue, mainly the GALT and BALT are two of the major source precursors of IgA plasma cells. These precursors migrate primarily through mucosal lymph nodes although a few may pass through peripheral lymph nodes. Precursors leaving the mucosal lymph nodes ultimately take up residence in glandular mucosal tissues. A portion of these cells may return to their sites of origin. In contrast, peripheral lymph nodes contain few IgA plasmacyte precursors and provide mainly systemic immunity. However, some IgA plasma cell progenitors in peripheral lymph nodes may take up residence in mucosal sites. The systemic and mucosal lymphoid systems are integrated to maximize host resistance to intruders.


LYMPHOID TISSUE
SYSTEMIC
MUCOSAL
MUCOSA ASSOCIATED LYMPHOID TISSUE (GALT & BALT)
MUCOSAL
LYMPH NODES
PERIPHERAL
IgA
GLANDULAR MUCOSAL TISSUE
MAMMARY GLANDS
INTESTINES
BRONCHUS
CERVIX AND VAGINA
IgA STEM CELL POOL

1384(a)

mucosal lymph nodes. Here, IgA precursors are nearly completely differentiated and quickly enter the TD and circulation. Ultimately, most of these cells localize beneath a variety of mucosal epithelia.

Although this study has emphasized the role of MLN in mucosal defenses, they may not be an absolute requirement. Husband & Gowans (1978) reported that the presence of PP was an absolute requirement not only for the generation of ACC but for their localization as well; removal of the MLN had little effect on the appearance of ACC in the gut as long as PP were present. Perhaps, cells programmed to selectively localize in the gut, briefly enter the PP so they can examine the antigenic environment through receptors and then localize outside of the PP. These findings suggest that both the MLN and BLN may be merely a staging ground for cells committed to IgA synthesis.

A salient feature of the lymphocyte migration patterns observed in this study is that selective localization in mucosal sites is not the sole property of MLN or BLN cells nor is homing restricted to PLN cells. Although the MLN contained IgA and IgG plasma cell progenitors destined to lodge in mucosae-associated organs, and BLN blasts demonstrated an impressive migration into the lungs, a few labelled cells from each of these lymphoid sources, nevertheless, were found in recipient PLN. Similarly, the PLN contained a small number of lymphoblasts appearing in mucosal sites as IgA plasmacytes. These results suggest, not surprisingly, that mucosal and peripheral immunity are not totally separate systems; each contains a portion of the cellular elements found

in the other. Thus, as shown in Figure 5.1, some exchange of cells occurs between mucosal and systemic IgA precursor streams. The degree that one system overlaps with the other may be influenced by the physiologic state of the mucosae. Integration of peripheral and mucosal immunity would be consistent with the observations of Blandford & Heath (1974) that although IgA-containing cells predominated in the lungs shortly after a primary Sendai virus infection, their number was soon equalled by the appearance of local IgG plasmacytes, as well as by a dramatic increase in the number of IgM-containing cells. Similar observations have been made in humans with genital and respiratory tract infections (Hurlimann *et al.*, 1978; Callerame *et al.*, 1971). The present results suggest that, in health, some pulmonary IgG and IgM plasmacytes may originate in the PLN (Table 4.7). In chronic lung disease in humans (cystic fibrosis) the numbers of pulmonary IgA plasma cells are dramatically increased (Martinez-Tello *et al.*, 1968). Perhaps either pulmonary immunization or infection causes expansion and recruitment of this population into the lungs. Cells already in the bronchial lamina propria may proliferate *in situ* as discussed earlier in regard to the intestinal tract.

In human inflammatory bowel disease, a substantial increase in the number of mucosal IgG and IgM plasmacytes occurs and it has been proposed that this represents the inability of the IgA system, as a first line of defense, to halt the ingress of antigens (Baklein & Brandtzaeg, 1976). However, an alternative explanation can be offered to explain the results in Table 4.7.

PLN lymphoblasts appearing as IgA plasma cells in the gut lamina propria might actually represent circulating MLN lymphoblasts (committed to IgA synthesis) coincidentally located in the PLN at the time of animal sacrifice. The apparent migration of some MLN lymphoblasts to PLN could be explained in a similar way. The data allow no firm choice between these two possibilities and, therefore, both ideas have been included in Figure 5.1. It does seem that a model of integrated peripheral and mucosal immunity is most consistent with the available literature.

Some cells localizing in mucosal tissues might not yet be differentiated. The proliferation and localization of intestinal lamina propria cells (Table 4.14) exemplifies this possibility. These cells might leave the mucosa but it is likely that this departure is a minor component of their traffic. Moreover, Husband & Gowans (1978) obtained some evidence which suggested that once in the mucosa, even in the absence of antigen, TD-derived IgA-containing lymphoblasts did not depart. This observation discredits the possibility that antigen causes closure of exist passages (see section 5.4.2.3).

This model of mucosal immunity lacks one critical component. Although early studies indicated that little dimeric IgA in mucosal secretions originates from serum (Tomasi et al., 1965; South et al., 1966; Stiehm et al., 1966; Haworth & Dibling, 1966; Strober et al., 1970), recent investigations indicate that dimeric IgA can be selectively transported into saliva (Montgomery et al., 1977; Mestecky et al., 1978), and bile (Lemaitre-Coelho

et al., 1978; Orlans *et al.*, 1978). Thus, it seems that selective transport of antibodies derived from one mucosal site may play a role in providing passive immunity to distant mucosal sites. Since both the salivary glands and liver are devoid of organized lymphoid structures analogous to GALT and BALT, selective transport of IgA makes a significant contribution to immune defenses in these sites.

Possibly, the number of IgA plasma cell progenitors in such mucosal sites might be proportional to the extent of antigenic stimulation the site receives. This might explain the paucity of IgA plasmacytes in the healthy genital tract and bronchus. It would be interesting to investigate whether or not selective IgA transport into either cervico-vaginal or breast secretions occurs. The possibility that this sort of transport mechanism might be common to all mucosal glandular tissues has only begun to be explored.

The existence of a common mucosal immunologic system integrated with the peripheral system has profound implications in terms of harnessing these systems to best achieve effective mucosal immunity. The protocols necessary to accomplish this are yet to be delineated but might be approached using a combination of mucosal and peripheral immunization routes. Indeed, Pierce & Gowans (1975) reported that an I.P. primary immunization followed by oral challenge resulted in the greatest ACC response in the gut; oral priming was no more effective than parenteral priming. Immunization of the lungs and female genital tract might require analogous combinations. Moreover, such an example would allow a rational strategy for immunization of the intestinal and respiratory tracts and, in particular, the

male and female genital tracts against infections such as gonorrhea, candidiasis, trichomoniasis and *Herpes Simplex* viruses.

APPENDIX I

The Separation of Cells on the Basis of Size

When a spherical body such as a cell is suspended in a fluid of lesser density, it will sink (sediment) under the influence of gravity. Assuming that the environmental conditions are such that the density of a particle remains constant, then the downward force on the particle is given by

$$F_g = mg \tag{1}$$

where F_g, m and g represent the gravitational force, the particle mass and the acceleration due to gravity, respectively.

Since the mass of a particle is related to its density, ρ, and volume, V, then

$$F_g = \rho V g \tag{2}$$

However, the effective density of a particle of fluid is the difference between its actual density, ρ, and ρ', the buoyant density of the medium in which the particle is positioned. Therefore, the net downward force is given by

$$F_g = (\rho - \rho')Vg \tag{3}$$

As the cell accelerates, it is subject to a second retarding force due to friction which is in the opposite direction. When the frictional force, F, equals F_g, acceleration will cease and the cell will reach a terminal velocity. Hence, when

$$F = fs \tag{4}$$

where f represents the coefficient of friction, then s, the terminal velocity, is given by,

$$s = \frac{(\rho - \rho')Vg}{f} \tag{5}$$

If the cell is taken as a sphere of radius r, f is defined by,

$$f = 3\eta(\pi r^2) \tag{6}$$

where η represents the viscosity of the medium and, $2\pi r$, the cross-sectional area of the sphere. Since the volume of a sphere is given by,

$$V_s = \frac{4}{3}\pi r^3 \tag{7}$$

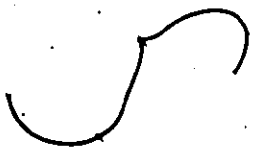
then,

$$s = \frac{2(\rho - \rho')gr}{9\eta} \tag{8}$$

defines the limiting sedimentation velocity of a spherical particle (e.g., a cell) falling through a fluid under the influence of gravity. In theory, it is possible, therefore, to use differences in terminal sedimentation velocities to separate cells on the basis of size (volume).

APPENDIX II

Determination of Mean Sedimentation Velocity of Cells in Fractions Separated by the STAPUT System



The following program, entitled SVAL 3, was prepared by Dr. David A. Clark, Department of Medicine, McMaster University and used to compute the mean sedimentation velocity, s, of cells in fractions collected after separation by the STAPUT system. SVAL 3 was designed for use in a Texas Instruments SR-52 programmable calculator (Texas Instruments, Inc., Dallas, Texas).

In calculating s values of cells, the distance a cell sediments is related to the volume through which it falls. However, this calculation requires several corrections because a number of variables are involved. For example, the equations presented in Appendix I assumed that all cells begin sedimenting from the same location at the same time and individually recovered after a known period of time. Obviously, it was impractical to achieve these sets of circumstances. Cells were loaded in a thin layer and began to sediment while the gradient was being formed. Moreover, fractions containing groups of cells were recovered, not individual cells. A complete discussion of these problems has been provided by Miller (1973). SVAL 3 corrected for these deficiencies and computed s values under a constant set of circumstances.

SVAL 3

Input	Display
lrn	00
2nd	00
LBL	1
A	2
STO	3
O	4
1	5
HLT	6
2nd	6
LBL	7
B	8
STO	9
O	10
2	11
HLT	12
2nd	12
LBL	13
C	14
STO	15
O	16
3	17
HLT	18
2nd	18
LBL	19
D	20
STO	21
O	22
4	23
HLT	24
2nd	24
LBL	25
E	26
STO	27
O	28
5	29
HLT	30
2nd	30
LBL	31
2nd	31

Input (cont'd)	Display (cont'd)
A	32
STO	33
O	34
6	35
HLT	36
2nd	36
LBL	37
2nd	37
B	38
STO	39
O	40
7	41
HLT	42
2nd	42
LBL	43
2nd	43
C	44
STO	45
O	46
8	47
HLT	48
2nd	48
LBL	49
2nd	49
D	50
STO	51
O	52
9	53
HLT	54
RCL	55
O	56
8	57
-	58
.	59
4	60
X	61
(	62
RCL	63
O	64
6	65

Input (cont'd)	Display (cont'd)
+	66
RCL	67
0	68
8	69
-	70
RCL	71
0	72
7	73
)	74
=	75
STO	76
1	77
0	78
(	79
RCL	80
0	81
9	82
-	83
RCL	84
0	85
8	86
)	87
÷	88
RCL	89
0	90
3	91
=	92
STO	93
1	94
1	95
RCL	96
0	97
3	98
-	99
.	100
5	101
+	102
(	103
RCL	104
0	105
4	106
-	107

Input (cont'd)	Display (cont'd)
RCL	108
0	109
5	110
)	111
÷	112
RCL	113
0	114
1	115
=	116
STO	117
1	118
2	119
HLT	120
CE	121
1	122
SUM	123
0	124
0	125
RCL	126
0	127
0	128
HLT	129
(	130
RCL	131
0	132
1	133
X	134
(	135
RCL	136
1	137
2	138
-	139
RCL	140
0	141
0	142
)	143
÷	144
RCL	145
0	146
2	147
)	148
÷	149

Input (cont'd)	Display (cont'd)
(	150
(	151
RCL	152
1	153
0	154
+	155
(	156
RCL	157
1	158
1	159
X	160
RCL	161
0	162
0	163
)	164
)	165
+	166
6	167
0	168
)	169
=	170
HLT	171
GTO	172
1	173
2	174
0	175
LRN	175

At this juncture the measured parameters from the separation in question were added to memories A through D'.

Fraction volume (ml)	177
A	177
Chamber constant (ml/mm)	178
B	178
Number of last fraction	179
C	179
Volume of last fraction(ml)	180
D	180

Input (cont'd)	Display (cont'd)
Volume (ml) of PBS top layer plus one half the volume (ml) of cell susp. loaded	181
E	181
Elapsed time (min) to chamber cone rim	182
2nd	182
A	182
Elapsed time (min) to beginning of draining chamber cone	183
2nd	183
B	183
Elapsed time (min) to beginning of collecting fraction 1	184
2nd	184
C	184
Elapsed time (min) to end of collecting last fraction	185
2nd	185
D	185

At this point, the calculator will automatically compute the fraction number containing the centre of the unseparated cell input layer. This number will be displayed. The mean sedimentation velocities, s (mm/h), of the individual fractions can now be computed.

Input (cont'd)	Display (cont'd)
Run	Figures displayed represent the fraction containing the centre of the unseparated cell layer
Run	number of first fraction
Run	s value (mm/h) of first fraction
Run	number of second fraction
Run	s value (mm/h) of second fraction

Continued pressing of run key alternately causes display of successive fraction numbers and s values (mm/h).

To reset and check all s values, press

0
STO
0
0

The calculator will display the fraction number of the unseparated cell input layer. The mean sedimentation velocities of the individual fractions can be computed again.

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