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ANALYSIS OF THE GALACTOSE-BINDING LECTIN DURING THE DEVELOPENT OF THE CHICK YOLK SAC.

by

Christopher Kenneth Guay

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

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Christopher K. Guay

Nay, the same Solomon the king, although he excelled in the glory of treasure and magnificent buildings, of shipping and navigation, of service and attendance, of fame and renown, and the like, yet he maketh no claim to any of those glories, but only to the glory of inquisition of truth; for so he saith expressely, "The glory of God is to conceal a thing, but the glory of the king is to find it out"; as if, according to the innocent play of children, the Divine Majesty took delight to hide his works, to the end to have them found out; and as if kings could not obtain a greater honour than to be God's play-fellows in that game.

-FRANCIS BACON, *The Advancement of Learning* (1605)

Would to God your horizon may broaden every day! The people who bind themselves to systems are those who are unable to encompass the whole truth and try to catch it by the tail; a system is like a lizard; it leaves its tail in your fingers and runs away knowing full well that it will grow a new one in a twinkling.

-IVAN TURGENEV TO LEO TOLSTOY (1856)

Where the telescope ends, the microscope begins. Which of the two has the grander view?

-VICTOR HUGO, *Les Misérables* (1862)

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled: Analysis of the Galactose-binding lectins During the Development of the Chick Yolk Sac, submitted by Christopher K. Guay in partial fulfillment of the requirements for the degree of Master of Science.

Professor Sara E. Zalik
Supervisor

Professor Esmond J. Sanders
External Examiner

Professor Warren J. Gallin
Committee Member

Professor Mike Belosevic
Committee Member

Professor S. K. Malhotra
Chairman

Date: Oct 6/93

Abstract

Lectins are proteins that recognize specific carbohydrates, are non-enzymatic, and are of non-immune origin. In animals most of the soluble lectins (S-type) bind carbohydrates bearing terminal β-galactosides. It has been shown that in the chicken, the specific activity of the S-type lectins is high in the area opaca of the gastrulating primitive streak embryo, the extraembryonic tissue that gives rise to the yolk sac membranes. In the cells of the extraembryonic endoderm of this tissue lectin in the medium affects cell adhesion. We therefore decided to study the galactose-binding lectins during the development of the yolk sac. Lectin activity is associated with two proteins of 14 and 16 kD and increases rapidly in the yolk sac until 4 days post-incubation, after which activity remains at plateau levels. Because these results suggested a functional role for lectins in cell-matrix interactions, and since the lack of specific probes for lectins has been the major problem in investigating their role(s) in development, we have prepared monoclonal antibodies (mAbs) specifically reactive with the 16 kD yolk sac lectin. Immunolocalization studies indicate that the 16 kD lectin is abundant in the area vasculosa endoderm where it is present around the intracellular yolk platelets and nuclei of the endoderm. The 16 kD lectin is also found in discrete organelles associated with the yolk platelets in 5 day yolk sacs. The mAbs inhibit haemagglutinating activity in extracts of yolk sac, embryonic pectoral muscle, and adult liver, but have no effect on the haemagglutinating activity of the 14 kD adult intestinal lectin. Taken together, these findings suggest that the 16 kD lectin is involved in expansion of the yolk sac membrane. The yolk sac lectins may also be involved in the processing of yolk for use in the embryo, and in haematopoiesis.

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I add to this long list tribute to the animals used in this study. This would be a barren document if not for the mice who were sacrificed to raise the probes, and the chicks, on whom the probes were ultimately used.

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Abbreviations

aAR	anti-aggregation receptor
AF	aggregation factor
APL	*p*-amino-phenyl-β-D-lactoside
anti-E16	anti-16 kD lectin antiserum to whole chick embryos
anti-I14	anti-14 kD lectin antiserum to adult chick intestine
anti-L16	anti-16 kD lectin antiserum to adult chick liver
anti-M16	anti-16 kD lectin antiserum to embryonic chick muscle
anti-S14	anti-14 kD lectin antiserum to embryonic chick skin
AR	aggregation receptor
BSA	bovine serum albumin
CAM	cell adhesion molecule
CBP35	35 kD carbohydrate binding protein
CHO	Chinese hamster ovary
Con A	concanavolin A
CRD	carbohydrate recognition domain
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagles medium
DSPG	dermatan sulphate proteoglycan
DTT	1,4-dithio-L-threitol
ECM	extracellular matrix
EGF	epidermal growth factor
EHS cells	Englebreth-Holm-Swarm cells
E/L	elastin/laminin
E-selectin	endothelial leucocyte adhesion molecule
FITC	fluorescein isothiocyanate
FN	fibronectin
GAG	glycosaminoglycan
GalNAc	N-acetylgalactosamine
GAR-HRP	goat-anti-rabbit horseradish peroxidase
GlcNAc	N-acetylglucosamine

HA	hyaluronic acid
HAT medium	containing hypoxanthine, aminopterin, thymidine
HT medium	containing hypoxanthine, thymidine
HU	haemagglutinating unit
Hz	hydrazide
IgE	immunoglobulin E
IgG	immunoglobulin G
kD	kilodalton
L16	16 kD lectin from adult chick liver
LAMP	lysosome-associated membrane glycoprotein
L-CAM	liver cell adhesion molecule
LEC-CAM	lectin cell adhesion molecule
L-selectin	lymph node homing receptor
mAb	monoclonal antibody
mGBP	murine galactoside-binding protein
Mw	molecular weight
N-cadherin	neural-cadherin
N-CAM	neural-cell adhesion molecule
PAP	peroxidase-anti-peroxidase
PBS	phosphate-buffered saline
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
P-selectin	platelet granule membrane protein
RAM-HRP	rabbit-anti-mouse conjugated to horseradish peroxidase
RGD	arginine-glycine-asparagine
S14	14 kD lectin from embryonic chick skin
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSEA	stage specific embryonic antigen
TBS	Tris-buffered saline
TTBS	Tris-buffered saline containing Tween
WGA	wheat germ agglutinin

Introduction

The development of organisms from early embryos involves both spatial and temporal regulation of five primary cellular processes: mitosis, recognition and adhesion, movement, differentiation, and death. Glycoconjugates at the cell surface and in the extracellular matrix play a role in all of these processes, but the study of carbohydrate-binding proteins, the lectins, and their carbohydrate receptors in animal development is a relatively recent phenomenon (Drickamer 1987). The role of lectins in development is gradually being determined by combining information from experiments in the rapidly advancing field of molecular biology with immunohistochemical localization techniques. In the following essay I will describe lectins, discuss why the soluble β-galactoside-binding lectins have been implicated in development, and outline the biological system used for our experiments. This discussion will be prefaced by a brief review of the expression and involvement of extracellular glycoconjugates in development.

Extracellular Carbohydrate Expression During Development:

The Cell Surface:

Most investigations of the lectins in development have focused on their possible involvement in mediating interactions between apposing cells, and between cells and the extracellular matrix (ECM). The motivation for such studies centers on the belief that these interactions are primarily responsible for the communication of morphogenetic information during development. The extracellular regions contain many varied proteins carrying complex glycans. Glycosylation is not limited to proteins; specific oligosaccharide moieties are also found attached to membrane glycosphingolipids, and as complex polysaccharides (Fenderson

et al. 1990). Carbohydrates are better suited than proteins to carry structurally encoded information because the variation in monosaccharide sequences, the anomeric configuration of each monosaccharide within the sequence, and the nature and extent of oligosaccharide branching allows for far greater diversity than is possible with proteins (Cook and Stoddart 1973; Sharon and Lis 1989). Furthermore, carbohydrate diversity is often enhanced by the covalent attachment of sulphate, phosphate, and acetyl groups (Sharon and Lis 1989). The enormous potential for carbohydrates to encode biological information, and the extreme diversity of complex oligosaccharides at the cell surface, suggests that they may function to decode extracellular information gathered from interactions with other cells and ECM molecules (Rademacher *et al.* 1988; Hart 1992).

Cell surface glycosylation has been implicated in developmental events such as migration, adhesion and deadhesion, and differentiation. In the primitive streak stage of chick development, mesenchymal cells migrating into the streak temporally lose carbohydrate moieties recognized by the plant lectins concanavalin A (Con A) and wheat germ agglutinin (WGA), and then reacquire these moieties following passage through the streak (Hook and Sanders 1977; Sanders and Anderson 1979; Sanders 1986). Recently, Augustin-Voss and Pauli (1992) demonstrated that when endothelial cells differentiated from a non-migrating to a migrating cell type they became hyperglycosylated. By probing the cell surface with a series of plant lectins they further determined that specific surface glycans were expressed during cell-type changeover. Additional evidence of a putative role for carbohydrates in development came from immunolocalization studies using monoclonal antibodies (mAbs) raised against stage-specific embryonic glycans (SSEAs). The SSEAs have been used to demonstrate that the spatial and temporal expression of certain cell surface glycans changes

dramatically during avian and mammalian development (Feizi 1985; Muramatsu 1988a; Muramatsu 1988b; Rademacher *et al.* 1988; Thorpe *et al.* 1988; Didier *et al.* 1990; Fenderson *et al.* 1990; Loveless *et al.* 1990). Cell surface-associated glycosyltransferases also act to modify extracellular carbohydrate ligands during development by catalyzing the transfer and addition of monosaccharides from donor sugar nucleotides to specific carbohydrate acceptors (Shur 1977a; Shur 1977b). The modification of specific extracellular glycans by cell surface-associated glycosyltransferases may allow for cell migration over the carbohydrate domain of the ECM and on apposing cells (Shur 1982; Shur 1989).

Cell adhesion molecules (CAMs) consist of an extremely large family of integral membrane glycoproteins that were first detected in homophilic cell-cell adhesion assays and have subsequently been isolated from all vertebrate tissues (reviewed in Cunningham and Edelman 1990). They are comprised of two subfamilies, the Ca^{2+}-dependent CAMs (cadherins), and the Ca^{2+}-independent CAMs. The liver and neural cell adhesion molecules (L-CAM and N-CAM), and Ca^{2+}-dependent neural CAM (N-cadherin), are expressed in the epiblast of the early chick embryo very early in development, but their expression is downregulated during cell-type transformation into migratory mesenchymal cells (reviewed in Thiery *et al.* 1990). With the cessation of migration, these CAMs become re-expressed at the cell surface and regulate the establishment of secondary epithelia. All the CAMs contain carbohydrates, but the function of carbohydrate moieties in CAM-mediated cell adhesion is not clearly understood. The N-CAM molecule is a major carrier of polysialic acid residues, and the chain length of these negatively charged carbohydrates gradually decreases in brain, muscle, and kidney tissues of various vertebrates concomitantly with development (reviewed in Finne 1990). The polysialic acid chains are believed to negatively modulate N-CAM-mediated homophilic binding between cells since removal of the sialic

acid residues in the presence of neuraminidase or endosialidase enhances cell binding (Rutishauser *et al.* 1988; Finne *et al.* 1990).

More recently, a large family of heterodimeric membrane-spanning receptors, collectively called the integrins, have been implicated in adhesion and transmembrane communication between cells and the ECM (Turner and Burridge 1991; Hynes 1992; Juliano and Haskill 1993). Integrins are glycoproteins that act as receptors for various ECM glycoproteins including vitronectin, fibrinogen, collagens, laminin, fibronectin (FN), and thrombospondin (Hynes 1985; Ruoslahti and Pierschbacher 1987; Hynes *et al.* 1990; Springer 1990; Hynes 1992). The generic integrin molecule consists of an α- and a β-subunit, each containing a large extracellular domain, a single membrane-spanning domain, and a short cytoplasmic domain (Hynes 1992; Juliano and Haskill 1993). Evidence suggests integrins are important in development. Antibodies to β_1 integrins inhibit amphibian gastrulation (Darribere *et al.* 1988), and in the chick embryo the α_1 integrin, a laminin-collagen receptor, is transiently expressed during development and disappears with tissue differentiation (Duband *et al.* 1992).

The Extracellular Matrix:

The ECM is an insoluble meshwork of glycoproteins and carbohydrates produced and externalized by cells. It contains three distinct components: 1) collagens; 2) proteoglycans and 3) the non-collagenous glycoproteins. Of the latter, the most extensively studied are FN and laminin, however, other developmentally significant glycoproteins within this class include elastin, fibrinogen, tenascin, thrombospondin, and vitronectin (Adams and Watt 1993). The ECM of vertebrates can be divided into two distinct matrix types: interstitial matrices, that provide the structural foundation in bones, cartilage, tendons, and skin; and basement membranes, the thin matrices underlying epithelial

and endothelial cell layers, and sometimes associated with nerve, muscle, and fat cells. Structural changes that take place in the ECM during development have been shown to affect cell morphology, gene expression, and ultimately tissue differentiation (Bissell and Barcellos-Hoff 1987; reviewed in Adams and Watt 1993).

The basement membrane is the first definitive ECM structure to appear in early development (Martin and Timpl 1987; Adams and Watt 1993). The basement membrane is primarily composed of homomolecular and heteromolecular glycoprotein aggregates of laminin, collagen IV, nidogen/entactin, and heparan sulphate proteoglycan (Martin and Timpl 1987; McDonald 1988; Timpl 1989). Basement membranes separate epithelial and endothelial cell layers from the interstitial matrix, and during development may segregate embryonic cells allowing for cell-type specific differentiation (Timpl and Dzaidek 1986; Martin and Timpl 1987). Basement membranes may also be responsible for maintaining the structural integrity of the embryo prior to gastrulation (Sanders 1986).

Assigning developmental functions to the ECM remains elusive, primarily because the evidence for involvement of the ECM in cell differentiation is mostly circumstantial (Sanders 1986). In the following sections I will briefly describe some of the ECM proteins believed to play critical roles during the development of the vertebrate embryo. It should be stressed that the function of the ECM in development is dependent on its three-dimensional construction and the regulated incorporation of the components within that structure. The separation of the ECM into individual components in the following discussion has been made for convenience.

Collagen:

The collagens are the major components of the ECM and are distinguished by their triple-stranded helical arrangement of three similar, but not necessarily identical, polypeptides. Collagens are defined by Martin *et al.* (1985) as any protein containing repeats of the tripeptide Glycine(Gly)-X-Y, where -X-Y denotes unspecified amino acids, and containing hydroxyproline and hydroxylysine residues. The relative rigidity of different collagen types is controlled by the frequency of interruption between Gly-X-Y repeats, with less rigid collagens containing many more interruptions (Vuorio and de Crombrugghe 1990). There are at least 14 types of collagen (Shaw and Olsen 1991). Interstitial collagens (types I - III) are extremely rigid and are the major structural components of tendons, bone, skin, cartilage, and other fibrous tissues (Martin *et al.* 1985).

Collagen type IV is specifically localized in the basement membrane and contains binding domains for laminin, heparan sulphate proteoglycan, and the 67 kD galactoside-binding lectin (Glanville 1987; Senior *et al.* 1989). This collagen does not form fibres and is more extensively glycosylated than other collagens. The amino acid sequence of this collagen contains many Gly-X-Y interruptions, allowing for the increased flexibility apparently required in the basement membrane. The function of collagen IV in development is not known, but Glanville (1987) has suggested that collagen IV acts as a substrate for laminin attachment and orientation. It is likely that the primary function of collagens during development is to stabilize cells to allow for differentiation. This is evidenced by the finding that morphogenesis of mammary and colonic epithelial cell layers *in vitro* is dependent on a collagen substrate (Hall *et al.*

1982; Lee *et al.* 1985).

Proteoglycans:

Proteoglycans are a large ubiquitous family of proteins that have covalently-attached sulphated carbohydrate groups, or glycosaminoglycans (GAGs) (Ruoslahti 1988a; Ruoslahti and Yamaguchi 1991). The GAGs are composed of long polymers of repeating anionic disaccharide moieties, and have been classified into four main types that include: 1) heparin/ heparan sulphate; 2) chondroitin sulphate/dermatan sulphate; 3) keratan sulphate; 4) and hyaluronic acid. Hyaluronic acid exists as a free GAG and is not sulphated. The disaccharide pairs in heparin, heparan sulphate, and hyaluronic acid consist of N-acetylglucosamine (GlcNAc) and uronic acid, in chondroitin and dermatan sulphate of N-acetylgalactosamine (GalNAc) and uronic acid, and in keratan sulphate of GalNAc and galactose (Ruoslahti 1988a; Kjellen and Lindahl 1991). Proteoglycans can have remarkably complex carbohydrate structures; Kjellen and Lindahl (1991) point out that many proteoglycans carry a diverse range of GAG chains, and within chains there is often a high degree of polysaccharide heterogeneity. Proteoglycans are also likely to carry both N- and O-linked oligosaccharides. The number of GAGs attached to the protein cores can vary from 1 to >100 (Kjellen and Lindahl 1991).

Proteoglycans are primarily localized in the ECM, however, some are membrane-associated proteins such as the mixed heparan sulphate/chondroitin sulphate proteoglycan of mouse mammary epithelial cells (Rapraeger *et al.* 1986; Saunders *et al.* 1989), and the chondroitin sulphate proteoglycan NG2 found in both the nerve tissues and mesenchyme of the developing rat embryo (Levine and Stallcup 1987; Nishiyama *et al.* 1991). Proteoglycans have also been localized in intracellular granules, and evidence suggests that they are released from the cell upon stimulation to

become incorporated into the ECM (reviewed in Ruoslahti 1988a). Various proteoglycans will precipitate out of solution specifically with the basement membrane proteins collagen types I and IV, FN, and laminin (Kleinman *et al.* 1986; Ruoslahti 1988a). These precipitates appear ECM-like, suggesting that proteoglycans could be important in mediating the three dimensional conformation of the matrix. Sulphation of the carbohydrate GAG chains is important in the assembly of basement membranes since inhibitors of sulphate synthesis reversibly block the matrix-organizing activity of heparan sulphate proteoglycan (Brauer *et al.* 1990).

Proteoglycans have been implicated in the developmental regulation of cell adhesion/deadhesion events and in cell differentiation. Heparan sulphate proteoglycans promote cell adhesion to most ECM components (Cole *et al.* 1985; Saunders and Bernfield 1987). However, chondroitin sulphate proteoglycan, serglycin, and various small dermatan sulphate proteoglycans (DSPGs) interfere with cell attachment on FN substrates by binding to GAG recognition domains present on the FN molecule (Brennan *et al.* 1983). The DSPGs bind to the heparin-binding domain of the FN molecule and may compete for attachment with heparan sulphate proteoglycans (Schmidt *et al.* 1987). Proteoglycan-mediated cell deadhesion has been implicated in regulating chondrocyte differentiation (Knox and Wells 1979; West *et al.* 1979; Goetinck 1982; reviewed in Ruoslahti 1988a). Other proteoglycans, such as syndecan, are associated with the plasma membrane and bind to numerous components of the basement membrane (Gallagher 1989).

Hyaluronic acid (HA) is the most common GAG expressed during early development in the chick, comprising as much as 80%-90% of the total GAG content in the ECM (reviewed in Sanders 1986). The large size of this molecule (200-20,000 sugar residues) and its hydrophilic nature provide for structural resiliency in the ECM (Fisher and Solursh 1977). In the chick embryo, this compound is localized between the

epiblast cells, in the basement membrane, and surrounding the mesenchymal cells. Hyaluronic acid may provide pathways for cell migration, sterically inhibit cell-cell contact and binding, and perhaps direct cell migration along concentration gradients (Fisher and Solursh 1977; Sanders 1986). The compaction and adhesion of cells during development is often associated with reduced expression of HA (Kosher *et al.* 1981), increased hyaluronidase activity (Toole and Trelstad 1971), or both (Toole 1973).

Laminin:

The major component of all basement membranes is laminin, a complex glycoprotein consisting of three polypeptide chains (A, B1, B2) linked together by disulphide bonds to form a molecule of Mw 850 kD (Mercurio 1990; Mecham 1991). First isolated from Engelbreth-Holm-Swarm (EHS) cells, laminin is heavily glycosylated (17-27% Asn-linked oligosaccharide), containing primarily polylactosamine-type carbohydrates (Mercurio 1990). Laminin is a multidomain macromolecule having attachment sites for type IV collagen, $\alpha_{(1\text{-}3,6,V)}\beta_1$, $\alpha_V\beta_3$, and $\alpha_6\beta_4$ integrins, $\beta_{1,4}$ galactosyltransferase, heparin, heparan sulphate proteoglycan, and the 14 kD, 35 kD, and 67 kD galactoside-binding lectins (Glanville 1987; Shur 1989; Mercurio 1990; Mecham *et al.* 1989; 1991).

The biological activities mediated by laminin during development include cell growth, attachment, migration, differentiation, and neurite outgrowth (Martin and Timpl 1987; Kleinman and Weeks 1989). There is tissue-specific differential expression of the three laminin chains, and during development expression of the A chain is lost from many tissues (Klein *et al.* 1990). All three laminin chains contain epidermal growth factor (EGF)-like repeats. The EGF-like domains induce mitogenic activity, suggesting laminin may be important in regulating early embryonic cell division

(Kleinman and Weeks 1989). This role for laminin is supported by the immunohistochemical localization of laminin as early as the two-cell stage in mouse embryos (Kleinman and Weeks 1989).

Several lines of evidence suggest laminin carbohydrates are important in development. Following the discovery that the plant lectin Con A specifically inhibited laminin-induced cell-spreading and neurite outgrowth (Dean *et al.* 1988), Dean *et al.* (1990) demonstrated that unglycosylated laminin produced by EHS cells in the presence of tunicamycin, a glycosylation pathway inhibitor, also blocked cell-spreading and neurite outgrowth, but had no effect on cell binding. The length of the oligosaccharide side-chains appears to regulate the extent of cell-spreading and neurite outgrowth since laminin with smaller chain oligosaccharide moieties has no biological activity, while laminin having longer chain oligosaccharide sequences is partially active (Chandrasekaran *et al.* 1991). The above laminin carbohydrates restore laminin-dependent cell-spreading and neurite outgrowth on unglycosylated laminin (Chandrasekaran *et al.* 1991). Further investigations have found that cell surface galactosyltransferases are also required for neurite outgrowth on laminin (Thomas *et al.* 1990), and may be necessary for cell migration along laminin matrices (Shur 1989). It is possible that soluble lectins may regulate the function of laminin carbohydrates during development, but this has not been investigated.

Fibronectin:

Fibronectins are complex ECM glycoproteins containing 4-10% carbohydrate, and are major components of the basement membrane. They have also been found in vertebrate plasma, and in the connective tissue of certain embryonic and wound healing tissues (Hynes 1985; McDonald 1988; Ruoslahti 1988b). Fibronectin is a unique ECM protein, existing in both soluble

form and as large insoluble crosslinked complexes (McDonald 1988). Structurally, FN is a dimer (subunit Mw = 250 kD) connected via a pair of disulfide bonds at the C-termini. The FN molecule contains three series of homologous repeats (Types I, II, and III) encoded by individual exons. Variations between subtypes of FN are often categorized based on the relative differences in the number of repeats of each type found within the peptide (Hynes 1985; Ruoslahti 1988b). Fibronectin binds to collagen, heparin, fibrin, and to cells via both integrin and non-integrin binding domains. The defined cell-binding region of FN is composed of the tripeptide Arg-Gly-Asp (RGD) and is recognized specifically by the $\alpha_5\beta_1$, $\alpha_V\beta_5$, $\alpha_V\beta_3$, $\alpha_{IIb}\beta_3$, and $\alpha_V\beta_6$ integrins (Buck and Horwitz 1987; Ruoslahti 1988b; Hynes 1992). *In vitro*, synthetic RGD peptides effectively block cell attachment and migration on FN substrates, while immobilized RGD substrates allow cell attachment similar to that seen with FN (Ruoslahti and Pierschbacher 1987; McDonald 1988).

Like laminin, FN is believed to mediate several biological activities during development. Fibronectin-rich matrices have been localized along the paths of major cell migrations seen during gastrulation and neurulation, and purified FN promotes the adhesion and migration of embryonic mesenchymal cells *in vitro* (Mayer *et al.* 1981; Boucaut and Darribere 1983; Sternberg and Kimber 1986; Duband *et al.* 1986). In the chick embryo, neural crest cells preferentially migrate along FN-rich matrices (Rovasio *et al.* 1983; Dufour *et al.* 1988; Duband and Delannet 1992). This migration can be blocked by anti-FN cell-binding fragment antibodies. In the chick embryo, FN is involved in the migration of chick primordial germ cells (Critchley *et al.* 1979; ffrench-Constant *et al.* 1992), and expansion of the blastoderm by mediating the interactions between the edge cells of the epiblast and the vitelline membrane (Lash *et al.* 1990). Additional evidence of a putative role for FN in cell migration during development came from observations that mAbs

raised against FN, as well as RGD-containing peptides, inhibited amphibian gastrulation and neural crest cell migration in avian and mammalian embryos (Boucaut *et al.* 1984a; Boucaut *et al.* 1984b; Bronner-Fraser 1986; McDonald 1988). The carbohydrate moieties of FN may be important in cell migration and adhesion since *in vitro* studies comparing unglycosylated and glycosylated FN synthesized by fibroblasts have demonstrated that removal of carbohydrates from FN promotes cell adhesion (Jones *et al.* 1986).

Lectins:

Definition and History:

Lectins have been defined as proteins that recognize specific carbohydrate moieties, are non-enzymatic, and are not antibodies. Originally isolated from plants, they were considered to have divalent or multivalent carbohydrate binding sites because they could agglutinate vertebrate erythrocytes (reviewed in Sharon and Lis 1987). However, increasing structural and functional knowledge of the lectins has led to a revision in the definition of lectins to include non-agglutinating proteins having only one carbohydrate-binding site (Barondes 1988). Barondes (1988) also emphasizes the importance of lectin-protein interactions, but the activity of lectins is still defined by their carbohydrate-binding characteristics.

The carbohydrate specificity of lectins is defined by the monosaccharide or disaccharide that most effectively interferes with cell agglutination, or lectin precipitation of glycoconjugates (Goldstein *et al.* 1980). However, the carbohydrate specificity of any single lectin is generally given as a hierarchy of binding affinities it has for a series of related oligosaccharide sequences. As a result, assays of sugar-binding specificity do not necessarily implicate these simple sugars as the natural ligands of the

various lectins. Different plant lectins are capable of distinguishing very subtle differences in the carbohydrate structures present in animal tissues and are commonly used to probe the structure of cell surface glycans (Cook and Stoddart 1973; Nicolson 1974; Sharon and Lis 1987). It has been demonstrated that many of the endogenous animal lectins also exhibit subtle carbohydrate-recognition capabilities, a necessary characteristic if endogenous lectins are involved in the transduction of glycan-encoded biological information (Sharon and Lis 1987; 1989).

Classification of Animal Lectins:

Drickamer (1988) divided the animal lectins into two groups on the basis of amino acid sequence homologies within the carbohydrate recognition domains (CRDs): 1) the C-type lectins, and 2) the S-type lectins. The C-type lectins require calcium and the formation of intramolecular disulfide bonds between the cysteine residues to maintain their activity (Drickamer 1988; Hughes 1992). These lectins are found integrated into cell membranes with the CRD exposed to the extracellular domain, or sometimes as soluble proteins circulating in the haemolymph (Ashwell and Harford 1982; Taylor and Summerfield 1986; Drickamer 1987). The carbohydrate specificity of C-type lectins is diverse. The S-type lectins have no requirement for calcium to maintain their activity, but do require the presence of reducing agents (Barondes 1986). The S-type lectins are easily solubilized and have been localized in the nucleus, the cytoplasm, and at the surface of cells and the extracellular matrix. Most of the S-type lectins are specific for β-galactosides (reviewed in Barondes 1986; Lotan 1990).

The C-type Lectins:

The first evidence for the C-type lectins came from experiments examining the uptake and removal of desialyated serum glycoproteins by the rabbit liver (Morell *et al.* 1968). Removal of the terminal sialic acid groups of these circulating glycoproteins exposed terminal galactose residues that made the proteins highly susceptible to removal by hepatocytes (Ashwell and Morell 1974). A β-galactoside-specific lectin recognizing desialyated serum glycoproteins was subsequently isolated in detergent-solubilized membrane fractions from liver (Hudgin *et al.* 1974). Similar receptors specific for terminal β-galactosyl glycoproteins were found in the rat and the human (Achord *et al.* 1978; Maynard and Baenziger 1982; Drickamer 1987). The mammalian asialoglycoprotein receptors are now called galactose/N-acetylgalactosamine (GalNAc) lectins because it was determined that they bound N-acetylgalactosamines with higher affinity (Maynard and Baenziger 1982; Steer and Ashwell 1980; Taylor and Summerfield 1986).

Another group of membrane-bound C-type lectins mediating the endocytotic clearance of serum glycoproteins, the mannose/N-acetylglucosamine (GlcNAc) receptors, have been isolated from the rat (Stockert *et al.* 1976; Stahl *et al.* 1976), human (Achord *et al.* 1978), and chicken liver (Kawasaki and Ashwell 1977). The mammalian mannose receptors were first localized on the hepatic sinusoidal cells (Schlesinger *et al.* 1978), but have subsequently been found on hepatocytes (Maynard and Baenziger 1982), alveolar and peritoneal macrophages (Stahl *et al.* 1978; Imber *et al.* 1982), and cultured monocytes (Kataoka and Tavassoli 1985). The C-type lectins of the liver function primarily in recycling glycoproteins intracellularly for lysosomal degradation. The purpose for clearing glycoproteins from the serum remains controversial, although it has been alternatively suggested that the lectins may function in the

retrieval of toxic lysosomal enzymes (Shepherd *et al.* 1985), the elimination of mannose-bearing bacteria (Perry and Ofek 1984), and the intracellular trafficking of metabolites (Mori *et al.* 1984; Taylor and Summerfield 1986).

Within the last five years a novel class of carbohydrate-binding adhesion molecules has been found having an amino-terminus lectin domain homologous to the C-type lectins (Drickamer 1988; 1989). These membrane-associated proteins, alternatively termed lectin-cell adhesion molecules (LEC-CAMs) or selectins, have similar primary structures and have been implicated in carbohydrate-mediated attachment of leucocytes to the vascular endothelium (Siegelman 1991; Hughes 1992). Amino-acid sequence analysis has revealed that these proteins also have domains homologous to epidermal growth factor (EGF) and a varying number of repeats homologous to complement regulatory proteins (Siegelman 1991). The selectins have been localized on the surface of the high endothelial venules (alternatively called endothelial leucocyte adhesion molecule-1, ELAM-1 or E-selectin), on lymphocytes and neutrophils (alternatively called lymph node homing receptor, LHR or L-selectin), and on platelets (alternatively called granule membrane protein 140, GMP 140 or P-selectin) (Bevilacqua *et al.* 1989; Springer and Lasky 1991; Siegelman 1991; Lasky 1992; Moore *et al.* 1992; Mills 1993). An interesting feature of these receptors is that they are all share a carbohydrate-binding specificity for the fucosylated and sialylated tetrasaccharide sialyl Lewis X, named after the antigenic determinant of the Lewis X blood group (Imai *et al.* 1993). Although the selectins have been implicated in thrombosis, leucocyte recirculation, and inflammation responses, a role for these adhesion molecules in development has not been described.

The S-type Lectins in Development:

Invertebrate Lectins:

Sponges:

Examination of cell aggregation in the sponge *Geodia cydonium* provides perhaps the best evidence of a putative developmental role for soluble lectins in cell recognition events. A Ca^{2+}-dependent soluble cell aggregation factor (AF) has been purified and characterized from *G. cydonium* (Müller and Zahn 1973; Gramnzow *et al.* 1986). The AF mediates cell binding via attachment to a membrane-associated aggregation receptor (AR) that is necessary for cell reaggregation (Müller *et al.* 1976; Müller *et al.* 1979a). Cell aggregation involving the AR-AF-AR complex can be inhibited non-enzymatically by an endogenous membrane-associated glycoprotein, or anti-aggregation receptor (aAR) (Müller *et al.* 1979b). A terminal galactose group on the aAR has been shown to be responsible for dissociation of the AR-AF-AR complex (Müller *et al.* 1988). The dissociative activity of the aAR can be inhibited by the reversible binding of an endogenous 36.5 kD galactoside-binding lectin (Müller *et al.* 1983). Addition of the lectin exogenously to non-aggregating cells, or preincubation of non-aggregating cells with β-galactosidase, promotes cell reaggregation (Müller *et al.* 1983). The ECM of *G. cydonium* contains large aggregates of this lectin, and the lectin may act temporally as a substrate for cell attachment (Müller *et al.* 1988). It has been suggested that lectin-mediated interactions may be regulated by an endogenous β-galactosidase present at the cell surface (Müller *et al.* 1979c).

Sarcophaga peregrina:

The humoral lectin from the larvae of the flesh fly *S. peregrina* is a trimer of 32 kD α- and 30 kD β-subunits in a molar ratio of 2:1 respectively (Komano *et al.* 1980). The lectin is essential in the clearance of foreign antigens, and is first observed in the pupal stage, at the time when larval tissues become recognized by the organism as nonself and are ingested by haemocytes, thus suggesting a possible regulatory role for this lectin in development (Komano *et al.* 1980; Komano *et al.* 1983; Komano and Natori 1985). The lectin may be important in programmed cell death during metamorphosis via the activation of haemocytes (Itou *et al.* 1984; Takahashi *et al.* 1985). The *Sarcophaga* lectin also regulates the hormone-induced differentiation of pupal-stage imaginal discs into adult appendages *in vitro*. The lectin is synthesized by imaginal disc cells in the presence of 20-hydroxyecdysone and is secreted to the cell surface where it has been shown to stimulate neighboring imaginal disc cells to differentiate (Kawaguchi *et al.* 1991). The imaginal discs do not differentiate in the presence of either 10 mM galactose or anti-lectin antibodies (2 µg/ml), but adult appendages develop when the discs are incubated in the presence of 10 mM glucose, a nonspecific sugar hapten, and preimmune serum (Kawaguchi *et al.* 1991).

Vertebrate Lectins:

Galactoside-specific lectins have been isolated from a large number of vertebrate tissues ranging from teleosts to mammals. They can be catagorized into three distinct groups based on their relative subunit molecular weights as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE): 1) the 14-16 kD lectins; 2) the 29-35 kD lectins; and 3) the 67-72 kD lectins. The widespread and ubiquitous distribution of S-type lectins in

developing tissues suggests that they are important in morphogenetic processes involving protein-carbohydrate interactions (Paroutaud *et al.* 1987; Lotan 1990; Zalik 1991). Studies utilizing anti-lectin antibodies and fluorescent-labelled glycoproteins have shown that the S-type lectins are primarily localized in the cytosol of the cell, but they are also found in the nucleus, at the cell surface and in the ECM (Barondes 1986; Lotan 1990; Zalik 1991). These proteins have been isolated from normal tissues including: heart, lung, spleen, intestine, skin, muscle, kidney, thymus, bone marrow, brain, and body fluids (Lotan 1990). Several S-type lectins are also highly expressed in various cancer tissues and may mediate tumour cell adhesion (reviewed by Lotan and Raz 1988). The S-type lectins share extensive amino-acid sequence homology, are not glycosylated, and contain no hydrophobic leader sequences (Paroutaud *et al.* 1987; Hirabayashi and Kasai 1988; Jia and Wang 1988; Hirabayashi *et al.* 1989; Drickamer 1988; 1989; Laing *et al.* 1989; Lotan 1990; Sakakura *et al.* 1990; Harrison 1991). It has been proposed that the 14-16 kD lectins, and perhaps the 29-35 kD lectins, are derived from a common ancestral gene (Sakakura *et al.* 1990; Lotan 1990). Paroutaud *et al.* (1987) have stated that the conservation of amino-acid sequence between the vertebrate S-type lectins is further evidence of their physiological significance. Although the S-type lectins have overlapping saccharide-recognition-specificities, and can generally be isolated by β-lactoside affinity chromatography, these lectins have higher affinities for the dimeric *N*-acetyllactosamine residue, and for the polysaccharide poly-*N*-acetyllactosamine (Harrison 1991).

Early in the examination of the S-type lectins at the molecular level a conceptual problem arose in the evaluation of these proteins as extracellular mediators of development. How were they externalized? The S-type lectins have been localized outside cells, but they lack a hydrophobic

secretory signal sequence. At least some of these lectins are translated on free cytosolic ribosomes, as demonstrated for the 14 kD lectin from rat lung (Wilson *et al.* 1989). It is now known that several other proteins synthesized within the cytosol and lacking hydrophobic signal peptides, such as acidic and basic fibroblast growth factors, and interleukin-1 (α and β), are translocated out of the cell (Kuchler and Thorner 1990; Muesch *et al.* 1990). The 14 kD lectin is found in the secretory vesicles of intestinal goblet cells and may be secreted with mucin to the intestinal epithelial surface (Beyer and Barondes 1982). In immunolocalization studies Cooper and Barondes (1990) showed that cultured mouse myoblasts released the 14 kD lectin via blebbing-off of lectin-rich cytoplasm. Release of the 14 kD lectin from the ectoplasmic vesicles was not observed. However, since most of the lectin synthesized by mouse myoblasts was ultimately found in the culture medium, it was presumed that the lectin was released by vesicle disintegration.

Lectins as growth factors:

The examination of possible growth-regulatory activities of endogenous animal lectins followed the discovery that many plant lectins stimulated mitosis in T-cells. Addition of 14 kD and 16 kD adult chick lectins to cultured mouse spleen cells dramatically increased the incorporation of radiolabelled thymidine into cellular DNA (Lipsick *et al.* 1980). Mitogenic activity was found to be dependent on the carbohydrate-binding activity of the lectins. Similar mitogenic activity was seen in mouse lymphocytes when cultured in the presence of the embryonic 15 kD lectin from chick kidney (Pitts and Yang 1980). Since these lectins were found in high quantities in various embryonic tissues, Lipsick *et al.* (1980) proposed that the S-type lectins might regulate mitotic activity in the developing chick embryo. The 16 kD lectin has been reported to stimulate

chondrogenesis in chick limb bud cells (Matsutani and Yamagata 1982). Recently, Wells and Malluci (1991; 1992) isolated and characterized an endogenous 15 kD β-galactoside-binding protein (mGBP) from mouse embryonic fibroblasts having cytostatic activity. The lectin was an autocrine negative growth factor that reversibly inhibited passage of cells from the G_0 and G_2 stages of the cell cycle. Since control over cell growth was independent of the carbohydrate-binding activity of the lectin, the role of the carbohydrate-binding domain in this process is not known.

Lectins in cell-cell and cell-matrix interactions:

During development the S-type lectins are differentially expressed in various embryonic chick tissues including liver, kidney, pancreas, brain, skin, and heart (Kobiler and Barondes 1977; Kobiler *et al.* 1978; Beyer *et al.* 1979; 1980; Oda and Kasai 1983). Two S-type lectins, having relative subunit molecular weights of 14 kD and 16 kD, were found to be responsible for this activity. The two lectins have different isoelectric points and their relative abundance varies within the tissues at different developmental stages. The 16 kD lectin is found in large quantities in embryonic pectoral muscle, while the 14 kD lectin is prevalent in the adult intestine (Beyer and Barondes 1982). The 14 kD and 16 kD lectins are present in the gastrulating chick embryo (Zalik *et al.* 1990a; 1990b), and these lectins are believed to be present at the earliest stages of development because galactose-inhibitable haemagglutinating activity was isolated from unincubated blastoderms (Cook *et al.* 1979; Zalik *et al.* 1983). In the 13-somite stage embryo, following gastrulation, the 14 kD lectin has been immunolocalized in the basement membranes of the notochord, the endoderm and mesoderm of the foregut, the internal and external limiting membranes of the rhombencephalon, and lining the coelomic surface of the myocardium (Zalik 1991; Zalik *et al.* 1992).

Staining with anti-16 kD adult liver lectin antibodies has shown that the 16 kD lectin has a similar extracellular distribution as the 14 kD lectin, but is also present at the periphery of the somites, and is abundant in a group of migratory neural crest cells and in the primordial germ cells (Zalik 1991). The 16 kD lectin is present in fibrous material in the ECM between organ primordia, the matrix material overlying the epiblast (Zalik 1991), and on the cell surface of the developing mesonephros and metanephros (Didier *et al.* 1988). Expression of lectins at the cell surface, and their colocalization with components of the basement membrane, suggests that they are secreted from cells and remain bound to the acetyllactosaminoglycan chains of ECM glycoconjugates.

The S-type lectins may be physiologically significant mediators of extracellular interactions between cell and matrix glycoconjugates. Endogenous β-galactoside-binding lectins decrease adhesion of disocciated endodermal cells of the chick embryo area opaca, the cells that later form the yolk sac membrane (Zalik 1991). Lectin-induced inhibition of adhesion can be reversibly blocked with saccharide inhibitors to the lectins (Milos and Zalik 1982). Endogenous lectin also reduces the binding affinity of endodermal opaca cells to the substrate (Milos and Zalik 1981). Zalik (1991) has speculated that secreted lectin binds to surface galactosyl residues and modulates carbohydrate-mediated cell adhesion during development. The modulatory activity of lectins in cell adhesion is supported by the localization of galactose-bearing ligands for soluble lectins at the cell surface in the developing primitive streak embryo of the chick (Zalik and Cook 1976; Phillips and Zalik 1982; Sanders 1986). A regulatory role for lectins in cell adhesion has also been demonstrated for the 14 kD lectin from mouse embryos, which has been shown to enhance the homophilic binding of embryonal mouse brain cells *in vitro* (Joubert *et al.* 1987; 1988). The

14 kD lectin is differentially expressed during mouse embryogenesis (Poirier *et al.* 1989; 1991), and has been implicated in attachment of the blastocyst to the uterine wall (Poirier *et al.* 1992). Differences in the relative adhesive activities of chick and mouse lectins may involve differences in either specific cell surface carbohydrate ligands, or the developmentally regulated expression of similar carbohydrate ligands, since the 14 kD lectin from mammals has also been reported to mediate cell deadhesion (Cooper and Barondes 1990; Cooper *et al.* 1991).

The regulation of lectin expression during development is correlated with the differential expression of a multitude of glycoproteins in the ECM that could act as potential ligands for extracellular lectins (see above). The major basement membrane glycoproteins laminin, FN, and collagens I and IV all carry acetyllactosamine residues, and several S-type lectins have been reported to bind to laminin-bound carbohydrates (Mecham *et al.* 1989; Cherayil *et al.* 1990; Mercurio 1990; Woo *et al.* 1990; Zhou and Cummings 1990; Cooper *et al.* 1991; Sato and Hughes 1992; Massa *et al.* 1993). Sato and Hughes (1992) recently presented evidence that basement membrane FN, but not plasma FN, binds the β-galactoside specific 30 kD lectin from embryonic hamster kidney cells in a lactose-inhibitable manner. The cell-surface associated 67 kD lectin from mammals has been described as the elastin/laminin (E/L) receptor (Mecham *et al.* 1989). Attachment of this lectin to the actin cytoskeleton increases the binding affinity for its ligands (Mecham *et al.* 1991). The affinity of the lectin for extracellular ligands is reduced in the presence of 30 mM lactose (Mecham *et al.* 1991), and the lectin is removed from the cell surface in the presence of 5 mM lactose (Hinek *et al.* 1988). Low concentrations of lactose block the ability of cultured chondrocytes to form elastic fibers; this suggests a potential regulatory role for carbohydrates on E/L

receptor-ECM mediated matrix construction (Hinek *et al.* 1988). Cooper *et al.* (1991) presented evidence that the 14 kD lectin secreted by mouse myoblasts during differentiation promotes cell-matrix deadhesion by binding to laminin present in the nascent basement membrane. Lectin-induced myoblast detachment from the laminin substrate and subsequent differentiation was blocked by competitive saccharide-ligands for the lectin, and by enzymatic cleavage of the laminin polylactosamine side-chains. The authors suggest that this lectin is involved in the detachment of cells from the substrate during myoblast fusion and myotube formation. Although, previous binding-affinity studies have provided little information on the biological significance of S-type lectins, considering the importance of laminin and FN in development, and that stage-specific glycosylation of ECM glycoconjugates occurs during tissue differentiation (see above), endogenous lectins may play a significant role in mediating cell-matrix interactions in development.

Development of the Yolk Sac Membrane:

Gastrulation of the Chick Embryo:

The early chick embryo is a small flat disc of cells, the germinal disc, that lies over a large yolk mass. Eleven hours after fertilization the germinal disc becomes separated from the underlying yolk by a large fluid filled cavity and forming the blastoderm (Eyal-Giladi and Kochav 1976). Soon after, cells at the lower surface of the blastoderm begin to polyinvaginate into the sub-blastodermic cavity. The tissue overlying the cavity is called the area pellucida because of its clear appearance (Eyal-Giladi and Kochav 1976; Eyal-Giladi 1991). At the periphery of the area pellucida the blastoderm cells remain in contact with the yolk to form the area opaca, which will later form the yolk sac and contribute to the formation of other extraembryonic

membranes.

Separation of the blastoderm dorso-ventrally forms two distinct cell layers: an upper layer that will later form the embryo, called the epiblast, and a lower layer that later forms the extraembronic endoderm, called the hypoblast. The primitive streak develops as a central thickening of cells in the epiblast and later forms the primitive groove (Azar and Eyal-Giladi 1979; Eyal-Giladi 1991). Gastrulation involves the directed migration of epiblast cells through the primitive groove into the blastocoele. These cell movements subsequently allow for the formation of three distinct germ layers: endoderm, mesoderm, and ectoderm. The extraembryonic mesoderm of the yolk sac originates from epiblast cells that migrate through the primitive groove and subsequently become localized in the in the area opaca.

The Avian Yolk Sac:

In early avian development the yolk sac appears as a highly vascularized extraembryonic membrane overlying the yolk. It is primarily involved in the uptake, synthesis, and transport of nutrients from the yolk mass to the embryo, but it also mediates secretory, respiratory (New 1956), excretory (Clark and Fisher 1957), and haemopoietic functions (Romanoff 1960) until later stages of development. Yolk sac organogenesis begins when cell aggregates from the area opaca start to expand over the yolk. Mesodermal cells subsequently invade the newly formed tissue and give rise to the yolk sac vasculature.

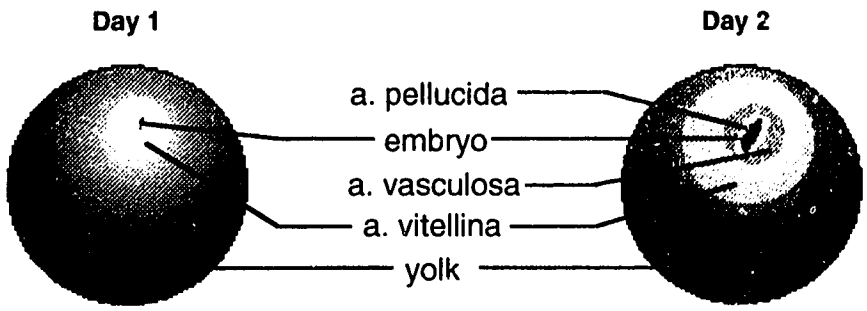
Formation of the yolk sac membrane:

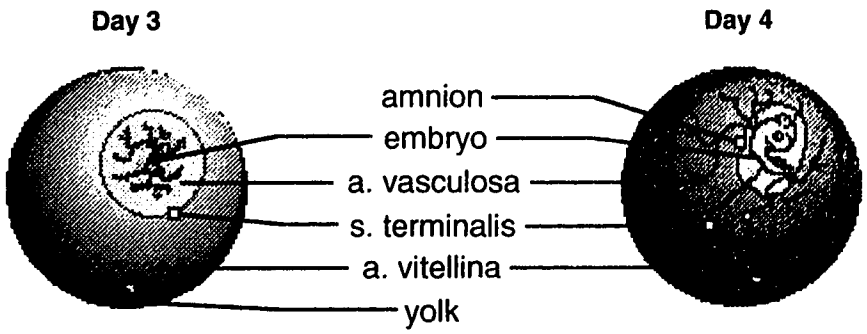
The chick embryo can be demarcated into three distinct regions: 1) the area vitellina, 2) the area vasculosa, and 3) the area pellucida (Fig. 1). The area vitellina lies distal to the embryo and is composed of endoderm and ectoderm

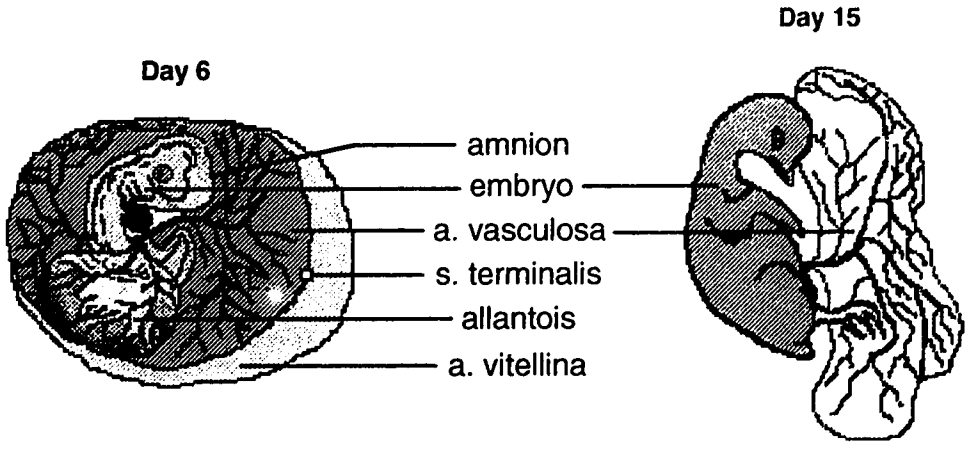
overlying the yolk mass. The outer margin of the area vitellina marks the leading edge of the expanding yolk sac. The area vasculosa is radially contiguous with the area vitellina and is positioned between the area vitellina and the area pellucida. The area vasculosa is derived from endodermal and splanchnic mesodermal cell layers and forms at approximately 30 hours with the appearance of the blood islands (Lillie 1952). The mesoderm of the yolk sac is initially derived from the primitive streak mesoblast (Patterson 1909). The mesoblast cells in this region condense into numerous cell clusters to form the blood islands and appear at approximately the same time the first somite is seen. The blood islands fuse to form vascular tissue in the area opaca and the area vasculosa, readily visible by 40 hours, becomes the primary site of haematopoiesis in the developing embryo. A large marginal vein, the sinus terminalis, marks the transitional boundary between the area vitellina and the invading area vasculosa. The development of the yolk sac membrane is illustrated in Figure 1.

Formation of the yolk sac occurs following the epibolic migration of two distinct cell populations over the yolk mass. The first is the ectoderm of the area vitellina and the associated endodermal cells. Expansion of the area vitellina is rapid, reaching the equator of the yolk by 3 days, and surrounding most of the yolk after 6 days (Romanoff 1960). The second cell migration involves the splanchnic mesoderm of the area vasculosa. At the end of 2 days the area vasculosa surrounds the perimeter of the embryo, and then expands quickly into the area vitellina (Romanoff 1960). At 5 days the area vasculosa has extended centrifugally past the equator, following the route previously marked by the area vitellina (Juurlink and Gibson 1973). After this, the area vasculosa encroaches on the area vitellina at a slower rate, finally encompassing the latter by 15 days incubation

Figure 1. Expansion of the yolk sac membrane during chick development. This figure is modified from illustrations by Romanoff (1960). The choriallantoic membrane is not shown in the 15 day embryo to better visualize the yolk sac membrane. Days refer to post-incubation. Abbreviations: area (a.); sinus (s.).


Day 1
Day 2
a. pellucida
embryo
a. vasculosa
a. vitellina
yolk


Day 3
Day 4
amnion
embryo
a. vasculosa
s. terminalis
a. vitellina
yolk


Day 15
Day 6
amnion
embryo
a. vasculosa
s. terminalis
allantois
a. vitellina

(Romanoff 1960). At 4 days simple folds into the yolk appear in the area vasculosa. With development the folds become more complex, concomitant with the increase in the absorptive function of the yolk sac (Romanoff 1960).

The sinus terminalis, the largest blood vessel in the early embryo, appears at approximately 45 hours of incubation and surrounds the embryo circumferentially by 56 hours (Fig. 1). This vein remains intact until the appearance of the definitive system of veins linking the embryonic circulation and the extraembryonic circulation during the third day. Between 6 days and 10 days the sinus terminalis atrophies and is replaced by an expanding ring of arteries and veins (Romanoff 1960).

Tissue differentiation during formation of the yolk sac:

The endodermal epithelium of the yolk sac lines the surface of the yolk mass. Maturation of this epithelium occurs in a proximo-distal direction to the embryo. This is reflected in the cell-type morphology of the endoderm observed in the different regions of the yolk sac. The most mitotically active cells, and the least mature, are at the periphery of the leading edge of the area vitellina (Romanoff 1960). These irregular-shaped cells are organized loosely into pseudostratified layers and are responsible for expansion of the yolk sac. When observed using light microscopy the cell boundaries are indistinguishable and the nuclei appear scattered indiscriminately throughout the tissue layer (Bellairs 1963). In the proximal margin of the area vasculosa, constituting the most differentiated region, the endoderm exists in a single layer of cylindrically shaped cells containing large, basally-located nuclei. Approaching the embryo from the leading edge of the yolk sac, the number of endodermal cell layers in the peripheral stratified zone becomes reduced, eventually giving way to a single layered epithelium of cuboidal cells that first appear at 3 days in

the area vasculosa (Romanoff 1960; Mobbs and McMillan 1979). The maturation of the latter into cylindrically shaped columnar cells soon after, coincides with the rapid advancement of the area vasculosa into the area vitellina (Mobbs and McMillan 1979). The area vasculosa surrounds the equator of the yolk by 5-6 days. Although the endoderm in the region of the sinus terminalis remains partially stratified, the endoderm associated with the vascular tissue of the yolk sac rapidly organizes into a single-layered epithelium. By 12 days the stratified zone disappears and the endoderm becomes entirely epithelial (Romanoff 1960).

Some authors have suggested that interaction of the mesodermal endothelium with the endodermal cells is responsible for maturation of the latter into a columnar epithelium (Bellairs 1963). However, Milos *et al.* (1979; 1984) showed that cultured extraembryonic endoderm cells isolated from the area opaca cavitate in the absence of mesoderm. They suggested that the ability to form an epithelium was intrinsic to the endodermal cells and that the mesoderm played only a permissive role in development of the endodermal epithelium.

Formation of the yolk sac endothelium and blood cells is dependent on the interactions between the splanchnic mesoderm and the endoderm (Wilt 1965; Augustine 1978, 1981). Expansion of the area vasculosa is mediated by a group of mesodermal edge cells localized slightly peripheral to the sinus terminalis (Augustine 1970; Mayer and Packard 1978; Flamme 1987). These actively motile cells migrate along the overlying ectoderm, invasively separating the ectoderm and endoderm of the area vitellina, and allowing for the formation of the extraembryonic somatopleura and splanchnopleura (Mayer and Packard 1978; Flamme 1987).

Function of the yolk sac membrane:

The yolk sac is responsible for the absorption and transfer of nutrients from the yolk to the embryo, and the early production of blood and blood proteins (Romanoff 1960). During expansion of the yolk sac, the endodermal cells within the epithelium acquire the ability to selectively transport different yolk constituents. This ability is apparently dependent upon the maturational stage of individual cells within the vascular epithelium (Kram and Klein 1976; Young and Klein 1983). The nutrients are actively passed to the blood vessels where they are transported to the embryo proper. The proposed functions of the yolk sac are the processing and transfer of metabolites to the embryo (Hassel and Klein 1971), transfer from yolk of maternal antibodies (Brierley and Hemmings 1956) including immunoglobulin G (Kowalczyk *et al.* 1985), and in the calbindin-mediated uptake and transfer of calcium (Ono and Tuan 1991). The yolk sac is possibly the major excretory organ before development of a functional mesonephros, and has the ability to concentrate urea and ammonia, and to synthesize uric acid (Clark and Fisher 1957). The yolk sac endoderm is the exclusive site of serum protein synthesis in the early avian embryo (Young *et al.* 1980; Young and Klein 1983), and the relative synthesis of individual serum proteins changes with development (Kram and Klein 1976). Changes in the levels of circulating serum proteins may provide stage-specific cues to the embryo during development (Klein *et al.* 1978; Hsu 1980; Young and Klein 1983).

Purpose of Study:

Maturation of the chick yolk sac epithelium involves the developmentally ordered transformation of the peripheral, irregularly-shaped and mitotically active, area vitellina cells into a monolayer of tightly-packed cuboidal cells

underlying the vascular tissue of the yolk sac. The change in cell-type morphology is concomitant with differentiation from a migratory cell-type responsible for expansion of the yolk sac endoderm into non-migratory epithelial cells responsible for delivering and processing metabolites from the yolk for ultimate use in the embryo. The possibility that lectins are important mediators of migration and adhesion in the expanding yolk sac was indicated by evidence that cells from the area opaca, the progenitor of the yolk sac, contain endogenous lectins that are released extracellularly and have regulatory activity in cell adhesion (see above). Lectin activity is present in early stage yolk sac tissues Mbamalu and Zalik (1987), and is approximately four times greater in area vasculosa extracts than in extracts from the area vitellina, suggesting lectin activity is associated with differentiation of the vascular yolk sac (Mbamalu 1986).

As a continuation of studies performed previously in this laboratory by Geraldine Mbamalu, I examined the developmental regulation of lectin activity in yolk sac tissues from 2 to 18 days post-incubation. The results indicate that the specific activity of lectin extracts dramatically increases until 4 days, and then remains stationary. Lectin activity is primarily asociated with protein bands of 14 kD, 16 kD and 67-72 kD. This was observed in lactoside-affinity purified preparations of yolk sac tissues at all developmental stages.

To understand the regulatory role of extracellular lectins in development it is necessary determine their localization, synthesis, and physiological activity using specific probes. I report here on the isolation and characterization of mAbs to the yolk sac 16 kD haemagglutinating lectin. In chick tissues, this lectin is abundant in the endoderm of the area vasculosa, adult liver, and embryonic pectoral muscle, but is not found in adult intestine. This lectin is responsible for most of the

haemagglutinating activity present in the 15 day yolk sac; and the specific lectin activity from yolk sac extracts is developmentally regulated. The findings indicate that the 16 kD lectin may be important in the differentiation of the yolk sac membrane, and possibly in haematopoiesis and in the processing of yolk.

Experimental Procedure

Preparation of Yolk Sac Lectin Extracts:

Yolk sac membranes were removed from White Leghorn embryos (Univ. of Alberta Farm) following incubation at 39°C (relative humidity 55%) for 2 days or for 4, 5, 6, 7, 8, 10, 12, 14, 16, and 18 days. At stages later than 5 days the chorion was separated from the yolk sac with forceps and discarded. Lectin was extracted according to previously reported procedures (Cook *et al.* 1979; Zalik *et al.* 1983). The membranes were dissected out in phosphate buffered saline (PBS) containing 0.15 M NaCl in 0.005 M $NaKHPO_4$ buffer, pH 7.2, and centrifuged at 900 × *g* for 10 minutes to determine packed tissue mass. The membranes were then homogenized in a 10 × volume of lectin extraction solution (LES) consisting of PBS, 300 mM β-lactose, 4 mM β-mercaptoethanol, and 0.25 mM phenylmethylsulphonyl fluoride (PMSF, Sigma) using a ground glass homogenizer. In subsequent experiments a Polytron homogenizer (Brinkmann) was used to homogenize the tissues. Particulate matter was removed after centrifugation at 88,000 × *g* for 1 h and the lectin-containing supernatants were transferred to dialysis tubing (Mw cutoff 6000-8000, Spectrapor). Lactose was removed from the supernatants by exhaustive dialysis in PBS containing 4 mM β-mercaptoethanol (MEPBS, pH 7.2). Supernatants were concentrated to approximately their original volume in the same dialysis tubing on a bed of polyethylene glycol (PEG, Mw 20,000). The concentrates were combined and centrifuged at 88,000 × *g* for 1 h before affinity purification of the lectins. Protein concentration in both the crude homogenates following the final centrifugation and the affinity-purified lectin solutions was determined by the method of Bradford (1976) using the Biorad kit and bovine γ-globulin as the standard.

Affinity Chromatography:

Lactoside-affinity chromatography was performed as described by Mbamalu and Zalik (1987). Following concentration, crude tissue homogenates were loaded onto a column containing Sepharose 6B (Pharmacia) to which para-aminophenyl-β-D-galactoside (APL, Sigma) had been linked according to Nowak *et al.* (1977). Lectin extract from 2 day yolk sacs was purified on a column having an approximate volume of 11 ml (diameter = 1.2 cm, height = 10 cm); yolk sac lectin from all other developmental stages was purified on columns having volumes ranging between 80 ml and 100 ml (diameter = 2.6 cm, height = 18 cm). Affinity chromatography was performed using a LKB 2111 Multirac fraction collector, and protein peaks observed with a Uvicord S 2138 monitor (OD_{280}). After elution of the void volume the column was washed sequentially with MEPBS, 0.3 M sucrose in MEPBS, and MEPBS. Galactose-binding lectins were eluted in 6 ml fractions from the column by addition of 0.1 M or 0.3 M lactose in MEPBS. The first 3-4 fractions eluted with lactose, previously found in our laboratory to have the highest agglutinating activity, were pooled and subsequently dialyzed extensively in MEPBS. Purified lectin was concentrated on PEG (Mw 20,000) in dialysis tubing as described previously (Procedure A).

To increase the purity of lectins isolated from yolk sac tissues, I purified lectins from 15 day yolk sacs using a modification of the above procedure (Procedure B). The tissues were homogenized, and the nonsoluble fraction removed by centrifugation as described above. Following concentration of the crude extract on a bed of PEG, the extract was centrifuged at 81,000 × *g* for 1 h. To remove the lactose, the crude extract supernatant was passed through a Sephadex G-25 column (diameter = 2.6 cm; height = 20 cm) previously equilibrated in MEPBS, and the extract loaded onto the above mentioned APL-Sepharose column. After the column

was washed, the lectin-rich fractions were eluted according to procedure A. The eluant was concentrated on a bed of PEG and then reapplied to a clean Sephadex G-25 column to remove the lactose as described for the crude extract.

Determination of Lectin Activity:

Haemagglutination assays were used to determine lectin activity in both crude extracts and lactoside-affinity purified lectin (Nowak *et al.* 1977). The protein concentration in lectin extracts was determined, and the specific activity of each extraction calculated by the procedure of Cook *et al.* (1979). Briefly, 25 µl of lectin-containing fractions were serially diluted two-fold in microtitre 'V' plates (96 well, Dynatech Laboratories) against MEPBS. To block non-specific agglutination, 50 µl of 0.05% bovine serum albumin (BSA)-MEPBS was added prior to addition of the erythrocytes. To each well 25 µl of 4% trypsinized rabbit erythrocytes was added to give a final volume of 100 µl according to Zalik *et al.* (1983). The titre of visible haemagglutinating activity was determined after 1 h using two replicate dilutions, and the specific activity of each fraction given as the reciprocal of the titre per mg of protein. Specific activity of the crude extracts at different developmental stages was determined in 4 separate experiments.

Sodium Dodecylsulphate Polyacrylamide Gel Electrophoresis:

Crude lectin extracts and purified lectin fractions were analyzed by gradient SDS-polyacrylamide gel electrophoresis (4.5%-22%, or 10%-18%) under reducing conditions (Weber and Osborn 1969) using the Biorad Mini-Protean II gel apparatus; the gels were 1 mm thick. Proteins were reduced in O'Farrel's buffer containing 50 mM dithiothreitol (DTT), 0.07 M Tris, 5% SDS, 22% glycerol, 30% urea, and 0.01% bromo-

phenol blue (pH 6.8) by heating at 95°C for 5 min., or at 60°C for 1 h. Gels were run at 200 V for approximately 1 h following electrophoresis through the stacking gel at 100 V for 10 min. Proteins were then visualized with either Coomassie Blue R250 stain, or in some experiments with silver stain (Wray *et al.* 1981). The molecular weight of prominent protein bands was determined by comparing their relative migration in the gels with known molecular weight standards. Standards were from BRL, Amersham, and Sigma.

Immunoblot Analysis:

Crude lectin extracts and affinity-purified lectins were separated by gradient SDS-PAGE (4.5%-22%; 10%-18%) and transferred to nitrocellulose (pore size 0.2 µm, Biorad) according to Towbin *et al.* (1979). Gels were equilibrated in transfer buffer containing 25 mM Tris, 0.2 M glycine, and 20% methanol for 30 min. Proteins were then transferred to nitrocellulose at 0.20 A for 2.5 h in the same buffer using a Biorad Mini Transblot transfer cell packed in ice.

Nitrocellulose strips were incubated against a number of polyclonal antisera raised against either the 14 kD or the 16 kD lectins from several chicken tissue sources. All antisera were obtained from rabbits. Antiserum to the lactoside affinity-purified 16 kD lectin (anti-L16), isolated from the liver tissue of laying hens, was obtained in our laboratory as described previously (Sanders *et al.* 1990). Antisera to the 16 kD lectin from whole chick embryos (anti-E16) and the 14 kD lectin from embryonic chick skin (anti-S14) were kindly provided by Drs. Y. Oda and K.I. Kasai (Teikyo University, Japan). Dr. E. Didier (Universite de Clermont Blaise-Pascal, France) provided us with the antiserum to the 16 kD lectin from 15 day chick embryonic muscle (anti-M16). The antiserum to the 14 kD lectin from adult chicken intestine (anti-I14) was a gift from Dr. S. H. Barondes (University of California, San Francisco, USA). Preimmune rabbit serum corresponding to

the anti-L16 antiserum was used as the control in all immunoblots.

Immunoblot analysis was performed at room temperature as described by Zalik *et al.* (1990b). Strips of nitrocellulose were blocked in 5% bovine serum albumin (BSA, Sigma) in Tris-buffered saline (TBS) having 50 mM Tris-HCl in 0.15 M NaCl, pH 7.5. After 2 h in blocking solution the strips were incubated overnight in primary antisera diluted in TBS containing 1% BSA. All antisera were diluted 1:1000 with the exception of the anti-I14 antiserum, which was diluted 1:500. Strips were removed and washed in 2 changes of TBS supplemented with 0.05% Tween 20 (Biorad, TTBS) for 10 min. each. This was followed by 3 × 10 min. changes in TBS. The blots were then incubated for 2 h in goat-anti-rabbit immunoglobulin G (IgG) coupled to horse-radish peroxidase (GAR-HRP, Biorad) diluted 1:3000 in TBS-1% BSA. Blots were washed as before in 2 times changes in TTBS, followed by 3 changes in TBS. Immunoreactive protein bands were visualized on the strips by detection of peroxidase using 4-chloro-1-naphthol (Biorad), as outlined by Hawkes *et al.* (1982).

Preparation and Isolation of Monoclonal Antibodies:

Six-week-old female BALB/c mice were injected intraperitoneally with 100 µl (~1 µg/µl in complete Freund's adjuvant, Sigma) of lactoside-affinity purified lectin(s) isolated from 15 day yolk sacs. After 3 weeks the mice were boosted with an additional 100 µg of pure lectin in incomplete Freund's adjuvant (Sigma). All subsequent procedures were performed using sterile culture media, supplements, and antibiotics purchased from Gibco. Three days later 1 mouse was sacrificed, the spleen aseptically removed, and perfused by multiple injections with 5 ml pre-warmed Dulbecco's modified Eagle's medium (DMEM;37°C). The spleen was cut into several small pieces and the splenocytes collected in a petri dish containing 10 ml warm DMEM by

scraping the spleen pieces over a stainless steel screen mesh (80 holes/inch), previously sterilized by flaming in alcohol. The cell suspension was washed through the screen by gently pipetting with the DMEM. The screen was then rinsed with 2 ml fresh DMEM to remove cells still attached to the mesh. The suspension was transferred into a 15 ml centrifuge tube and left to stand at room temperature for 2 min., allowing the non-dissociated cell clumps to settle to the bottom of the tube. The upper supernatant was transferred to a clean centrifuge tube, the cells pelleted by centrifugation at 400 × *g* for 3 min., and the supernatant removed by aspiration. The pellet was then resuspended vigorously in 5 ml cold NH_4Cl (0.75% in millipore filtered H_2O) and left standing on ice for 5 min. to lyse the erythrocytes. The lymphocyte-containing suspension was added to 45 ml warm DMEM in a 50 ml clinical centrifuge tube and the cells pelleted.

Myeloma cells (Sp2/0; American Type Culture Collection #CRL 1581), previously stored in liquid nitrogen, were thawed and cultured in DMEM to log phase growth at 37°C in a CO_2 incubator at 10%-in-air CO_2 with 98% relative humidity. The myeloma cells were maintained in DMEM for 1 week prior to the fusion to ensure that the cells were rapidly dividing, and to culture enough cells for fusing. Immediately before fusion, the myeloma cells were centrifuged at 400 × *g* for 3 min. and the cell pellets were resuspended in 50 ml warm DMEM. The lymphocytes and the myeloma cells were pelleted separately, resuspended in 10 ml DMEM, and the cells counted using a cytometer. Spleen cells (2.1×10^8) were mixed with 4.5×10^7 myeloma cells (5:1 ratio), and the cells pelleted tightly at 600 × *g* for 3 min. The pellet was flicked loose in the tube and left at 37°C for 2 min. The cells were fused by spinning in 50% PEG (Mw 4,000, stored in Ca^{2+}/Mg^{2+}-free PBS) according to the following schedule: a) PEG added to the cell pellet at 37°C and mixed gently for 1 min., b) cell mixture centrifuged at 160 × *g* for 110 sec., c) PEG removed by aspiration, d)

warm DMEM (4.5 ml) was added to the fused cells for 3 min., e) additional 5 ml DMEM added to cell suspension for 2 min. (Pontecorvo 1975). Following fusion, the tube was filled with DMEM, the cells centrifuged at 160 × *g* for 5 min., and the supernatant removed by aspiration. According to the procedure of Gallin and Sanders (1992), cells were resuspended in a nonselective medium, consisting of DMEM containing 10% fetal calf serum, 10% NCTC 109 medium, 2 mM L-glutamine, 0.1 mM sodium hypoxanthine, 16 µM thymidine, 600 µl gentamycin, and 2 µl β-mercaptoethanol (BDH) (HT medium), and then incubated at 37°C in a 10% CO_2 overnight. Following incubation, cells were pelleted and then resuspended for selection of viable hybrids in 100 ml HT medium containing 0.4 µM aminopterin (HAT medium)(Kohler and Milstein 1975; 1976). Immediately after, the cells were plated dropwise in 96 well culture plates and incubated at 37°C. When cell colonies appeared (~5 days later) the hybridomas were fed dropwise with HT medium and again at 10 days.

Antibodies from growing hybridoma colonies were screened for specific reactivity against purified 15 day yolk sac lectins by enzyme-linked immunosorbent assays (ELISA) as described by Engvall and Perlman (1971). Briefly, 100 µl of purified lectin in PBS (4 µg/ml) was adsorbed to each well of 4 × 96 well Immulon microtitre plates (Dynatech Laboratories Inc.) by incubation overnight at 4°C. Following removal of the unadsorbed material, the bound protein was incubated in the presence of a PBS blocking buffer containing 0.05% Tween 20 and 1% rabbit normal serum at room temperature for 1 h. The supernatant from growing colonies containing the antibodies (50 µl) was added to separate wells and incubated overnight at 4°C. Subsequently, excess media was discarded, and the wells washed vigorously with 5 × changes PBS-0.05% Tween 20. The wells were incubated for 1 h with 100 µl of peroxidase-conjugated rabbit-anti-mouse IgG (RAM-HRP), diluted 1/1000 in the above mentioned blocking buffer. The

presence of specific anti-lectin antibodies was indicated by development of colour in the presence of a peroxidase substrate solution containing 1 mg/ml ø-phenylenediamine in 0.1 M citrate buffer.

Screening of Hybridoma Clones for Removal of Haemagglutinating Activity:

a) *Preparation of immobilized antibody:*

Clones producing antibodies to the yolk sac lectins were selected by ELISA following limiting dilution of the hybrid cells, and the culture media supernatants from positive clones collected for immunoblot analysis (Towbin *et al.* 1979). Antibodies were precipitated from the hybridoma supernatants in an equal volume of 100% saturated ammonium sulfate, centrifuged and the pellets collected. The pellets were then resuspended to approximately 20% their original volume in PBS (~9 ml) and dialyzed extensively against PBS. The mAbs were bound to polyacrylamide beads covalently coupled to rabbit-anti-mouse (RAM) IgGs (Biorad) (1:10 v/v ratio, beads to culture supernatant) according to Harlow and Lane (1988).

b) *Removal of haemagglutinating activity:*

Crude lectin extract from yolk sacs with a titre of 8 was incubated with the mAb-conjugated immunobeads overnight at 4°C with rocking. The beads were pelleted and haemagglutination assays performed on the extract supernatants. Controls consisted of RAM immunobeads linked to mAbs raised against chicken liver cell adhesion molecule (L-CAM; kindly provided by Dr. W. Gallin), RAM immunobeads incubated with Sp2/0 myeloma culture supernatant, and RAM immunobeads incubated in the presence of culture medium alone. Three clones of anti-lectin antibodies, 5E5, 5F9, and 10E10, were selected for their ability to remove

haemagglutinating activity and were subjected to further study.

Determination of Antibody Sub-isotypes for Positive Clones:

The isotypes of the antibodies for each agglutinating-inhibitory clone were determined using a Biorad Mouse Typer Sub-Isotyping kit according to the manufacturer's instructions. Briefly, antibodies in ascites fluid were diluted 1:1000 in PBS-1% bovine serum albumin (BSA) and incubated against purified 15 day yolk sac lectin that had previously been adsorbed to a 96 well Immulon microtitre plate (Dynatech Laboratories Inc.). The lectin-bound antibodies were exposed to a panel of RAM antibodies specific for different antibody sub-isotypes. The rabbit antibodies bound to the well were screened with GAR-HRP antibodies, and the colour reaction developed in a peroxidase substrate solution containing 2,2'-Azino-di (3-ethyl-benzthiazoline sulfonate). Ascites fluid to NS-1 (Sigma) used at the same dilution was the negative control; the positive control consisted of serum from a non-immunized mouse adsorbed directly to the plate (1:100 dilution in PBS-1% BSA). One column was reserved for the colour substrate blank where no antibodies had been incubated against the adsorbed lectin.

Inhibition of Haemagglutination Assays:

The inhibition of haemagglutinating activity mAbs in crude lectin extracts was determined. Crude lectin extracts of 15 day yolk sac tissue, adult liver, and adult intestine were used. Ammonium sulfate precipitates of 5E5, 5F9, and 10E10, stored at -70°C, were thawed and resuspended in cold PBS. The antibody suspensions were desalted on a P6-DG column, and the concentration of antibody in solution was determined by UV absorption (OD_{280}). The mAbs were aliquoted at concentrations of 2 mg/ml and 10 mg/ml in PBS and refrozen at -70°C until used for the inhibition assays. The control

antibody for these experiments consisted of technical grade mouse IgG in PBS (Sigma). Prior to initiating the haemagglutinating inhibition assays, crude extract were diluted in MEPBS to a titre of HU = 16/25 µl in order to increase the sensitivity of the assay.

Inhibition assays were performed by adding 30 µl of lectin extract to an equal volume of antibody (final antibody concentrations ranging from 0.01-5.0 mg/ml) and incubated at 37°C for 30 min. in microcentrifuge vials. The ability of the antibody to inhibit lectin activity in extracts was assessed by determining the decrease in the haemagglutination titre after incubation.

Preparation of Immunoaffinity Columns:

Ascites fluid was prepared from clones 5E5, 5F9, and 10E10, and the ascites antibodies were precipitated with an equal volume of saturated ammonium sulfate (1:1, v:v), resuspended, and dialyzed against PBS. Immunoaffinity columns were prepared using antibodies from the three clones by covalently linking them to Affi-prep Hz beads (Biorad), a support matrix consisting of hydrazide groups covalently linked to polyacrylamide beads via a 10-atom spacer arm. Prior to linking, the antibodies were incubated in the presence of 10 mM sodium periodate in order to oxidize the carbohydrates in the Fc region of the mAbs. The aldehyde groups formed by this oxidation react with the hydrazide groups to form stable covalent hydrazone bonds.

The oxidized antibody was buffer exchanged into coupling buffer containing 0.1 M sodium acetate in 1.0 M NaCl, pH 4.5. The volume of antibody solution was kept to ~3× the volume of beads to be incubated. Approximately 5 mg of antibody was incubated with 1 ml of beads; the volume of all columns was 2 ml with the exception of that used for 10E10 (1 ml). Unbound antibody was eluted from the columns in PBS, and subsequently non-specifically bound antibody was removed by elution with

PBS containing 0.5 M NaCl. The protein concentration was recorded in the eluants containing the unbound antibody, and the amount of bead-coupled antibody determined by subtracting the amount of unbound antibody from the total amount of antibody used for coupling. The columns with the bound antibody were incubated with crude lectin extract for periods of 1 to 2 hours. After incubation the void volume was collected and the columns were eluted sequentially with PBS, PBS with 0.5 M NaCl (high salt), PBS, PBS with 10 mM β-lactose, and PBS. Proteins bound specifically to the antibodies were removed in 1 ml fractions from the columns by eluting in 0.2 M Glycine-HCl, pH 2.5, and delivered into tubes containing 200 µl of 1.0 M sodium phosphate buffer, pH 8.0, to neutralize the acid eluant. Haemagglutination assays were performed on the void volume, the 0.5 M salt wash, and all glycine eluted fractions. The glycine and lactose eluants from each column were dialyzed extensively against millipore filtered distilled H_2O, frozen at -70°C, and lyophilized. The proteins from these eluants were resuspended in gel sample buffer, analyzed by gradient (10%-18%) gel electrophoresis under reducing conditions (Weber and Osborn 1969), and stained with silver. The molecular weight of prominent protein bands was determined by comparing their relative migration in the gels to known molecular weight standards (Biorad Low, BRL prestained High). Proteins eluted in PBS-0.5 M NaCl were also examined by gel electrophoresis. Control columns consisted of beads coupled with non-immune mouse IgG (Sigma), Affi-prep Hz support alone, and Macro-prep Q beads. The latter are base beads used in the preparation of Affi-prep Hz beads; Macro-prep Q beads behave as an ion exchange matrix. The underivatized Affi-prep Hz beads and Macro-prep Q beads were used to characterize proteins that bound non-specifically to the polyacrylamide matrix.

Immunoprecipitation Experiments:

The procedure of immunoprecipitation used to determine the lectins recognized by the anti-lectin mAbs was modified from protocols described by Harlow and Lane (1988). A flow chart of the method used is shown in Figure 2.

a) *Preparation of tissue extracts:*

Crude chicken lectin extracts were prepared from 15 day yolk sac, 15 day embryonic pectoral muscle, adult liver, and adult intestinal mucosa using the method described for the extraction of lectins from yolk sac membranes with minor modification. Following homogenization of the tissue in LES and the initial centrifugation, the lectin-containing supernatants were immediately concentrated in dialysis tubing on a bed of PEG at 4°C. The extracts were then recentrifuged at 81,000 × *g* for 1 h, and the lactose was removed from the concentrated extracts by passage through P6-DG columns (diameter = 1.3 cm, height = 17 cm) and elution with MEPBS. These lectin fractions were stored at either 4°C or at -70°C until used for the immunoprecipitation experiments. The supernatants from crude extracts were filtered prior to antibody precipitations to remove remaining particulates (0.2 µm pore size, Nalgene). Haemagglutinating activity in the extracts was standardized immediately before performing the immunprecipitations by diluting the lectin-containing solutions with MEPBS (HU = 1024-2048/25 µl).

b) *Removal of proteins in crude lectin extract that bind non-specifically to the beads:*

Crude lectin extracts contain proteins that bind non-specifically to both agarose- and polyacrylamide-based gel matrices. The majority of these proteins were removed from extracts by exhaustive preincubation with underivatized

Figure 2. Procedure for the immunoprecipitation of lectins using anti-lectin mAbs. All extract incubations were performed at room temperature with rocking unless otherwise stated. Before the immunoprecipitation stage of the procedure, the crude extracts were incubated several times with underivatized beads to remove proteins binding to the beads nonspecifically. Prior to immunoprecipitation, the extracts were also precleared with rabbit normal serum, and proteins that bound to the antibodies nonspecifically were removed with Protein A beads. A mAb against peroxidase was used as the control in all immunoprecipitation experiments. Abbreviations: lectin extraction solution (LES); polyethylene glycol (PEG); haemagglutinating units (HUs); phosphate buffered saline (PBS); PBS containing 4 mM β-mercaptoethanol (MEPBS); rabbit-anti-mouse (RAM); sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

Preparation of Tissue Extract

- Homogenize 15 day yolk sacs in 10 × volume of LES
- Centrifuge at 81,000 × g for 1 h and collect supernatant
- Concentrate extract on a bed of PEG (Mw 20,000)
- Centrifuge at 81,000 × g for 1 h and collect supernatant
- Remove lactose from extract over P6-DG column
- Dilute haemagglutinating titre of extract to 1024-2048 HU with MEPBS

Preclearing

- Incubate 2.2 ml of extract against 4 × changes of 220 µl Affi-prep Hz beads; remove beads
- Incubate extract overnight at 4°C with 220 µl Affi-prep Hz beads; remove beads
- Incubate extract with 110 µl of rabbit normal serum (30 min.)
- Incubate extract with 2 × changes of 170 µl Protein A agarose beads (30 min. each); remove beads

- Adjust agglutinating titre of extract to 512 HU with MEPBS; divide extract into 2 × 1 ml aliquots (one used for the control antibody)

Immunoprecipitation

- Add 5 µl of primary mAb in ascites fluid to 1 ml of extract and incubate for 30 min.
- Add 2 µl of RAM IgG and incubate for 30 min.
- Add 100 µl of Protein A agarose beads, previously equilibrated in PBS, and incubate for 1 h
- Pellet beads by centrifugation at 16,000 × g for 30 s

- Determine haemagglutinating activity of extract

- Resuspend beads and wash in 2 × changes of PBS-0.1% Tween, followed by 3 × washes in PBS (beads pelleted after each wash at 16,000 × g for 30 s)

- Add 65 µl of reducing sample buffer and heat beads at 95°C for 5 min.

SDS-PAGE

Immunoblot Analysis

agarose beads. Affi-gel Hz beads (Biorad) were chosen because in previous immunoaffinity experiments these beads bound large quantities of protein without affecting lectin activity.

Affi-gel Hz beads were washed, and then equilibrated in MEPBS (pH 7.2). The beads were allowed to settle on ice, and the saline was then removed by aspiration. The bead slurry was added to the tissue extract at a 1:10 v/v ratio in 1.5 ml microcentifuge tubes. The mixture was incubated with rocking at room temperature for 1 h and the beads pelleted by centrifugation at 16,000 × *g* for 5 min on an Eppendorf Centrifuge 5415. The supernatant was removed with a fine-tipped glass pipette, transferred to a new microcentrifuge vial, and incubated as before with Affi-gel beads. This procedure was repeated 3 more times, with the final incubation overnight at 4°C.

c) *Preclearing of tissue extracts:*

Extract supernatant was collected the next day and precleared using rabbit serum bound with Protein-A agarose beads (Biorad) to remove proteins present in the tissue extracts that bound nonspecifically to the beads and to rabbit antibodies. The Protein-A agarose beads were washed and equilibrated in PBS, pH 7.1, using the same procedure used for the Affi-gel. Fifty µl of rabbit normal serum was added to the extract and incubated at room temperature with rocking. After 30 min., Protein-A beads were added to the extract (8% v/v extract), and the beads incubated with the extract for another 30 min. The supernatant was removed following centrifugation for 2 min., and incubated again with Protein-A as before to ensure complete removal of the rabbit immunoglobulins. The beads were pelleted, and the haemagglutinating activity of the supernatant was determined. The lectin activity in crude tissue extracts from yolk sac, liver, and muscle was adjusted prior to beginning the anti-

lectin immunoprecipitation to a titre of 512 HU/25 µl. Lectin activity of the crude intestine extract was 256 HU/25 µl.

d) *Immunoprecipitation:*

All the immunoprecipitation procedures were performed at room temperature. Five µl of ascites fluid was added to 1.0 ml of the crude lectin extract and the mixture was incubated for 30 min. with rocking. Subsequently, 2 µl of RAM-IgG (Sigma) was added and the extract incubated for another 30 min. Antibody-bound lectins recognized were removed from the solution by incubation for 1 h with 100 µl of the Protein-A beads. The supernatants were collected following a 30 s centrifugation and used for haemagglutination assays that were performed immediately. Supernatants were frozen at -20°C for future analysis. The beads were resuspended and washed in 2 × 1 ml changes of PBS containing 0.1% Tween 20, followed by 3 × 1 ml changes in PBS. Following each change, the beads were pelleted by centrifugation and the wash solutions removed by aspiration. The material bound to the beads was released by heating at 95°C for 5 min. in SDS-PAGE sample buffer. Following a 5 min. centrifugation, the sample buffer containing the released material was carefully removed, and proteins present in the immunoprecipitates characterized by gradient (10%-18%) gel electrophoresis, and immunoblot analysis. Immunoblot analyses were also conducted on aliquots of yolk sac tissue extract taken before and after the immunoprecipitation experiments. A mouse mAb to horseradish peroxidase in ascites fluid (Sigma), having the same sub-isotype as the anti-lectin mAbs, was used as the control for all the immunoprecipitation experiments. Control immunoprecipitations were performed in concert with the anti-lectin mAb precipitations.

Immunoprecipitation of lactoside-affinity purified lectin from 15 day yolk sacs was performed according to the

above protocols, with some exceptions. The purified lectin was not incubated with underivatized beads, nor was it precleared using rabbit normal serum. The activity of the pure lectin was adjusted to 1024 HU/25 µl prior to addition of the antisera. A summary of the approaches used in characterizing the yolk sac lectins with antibodies is shown in Figure 3.

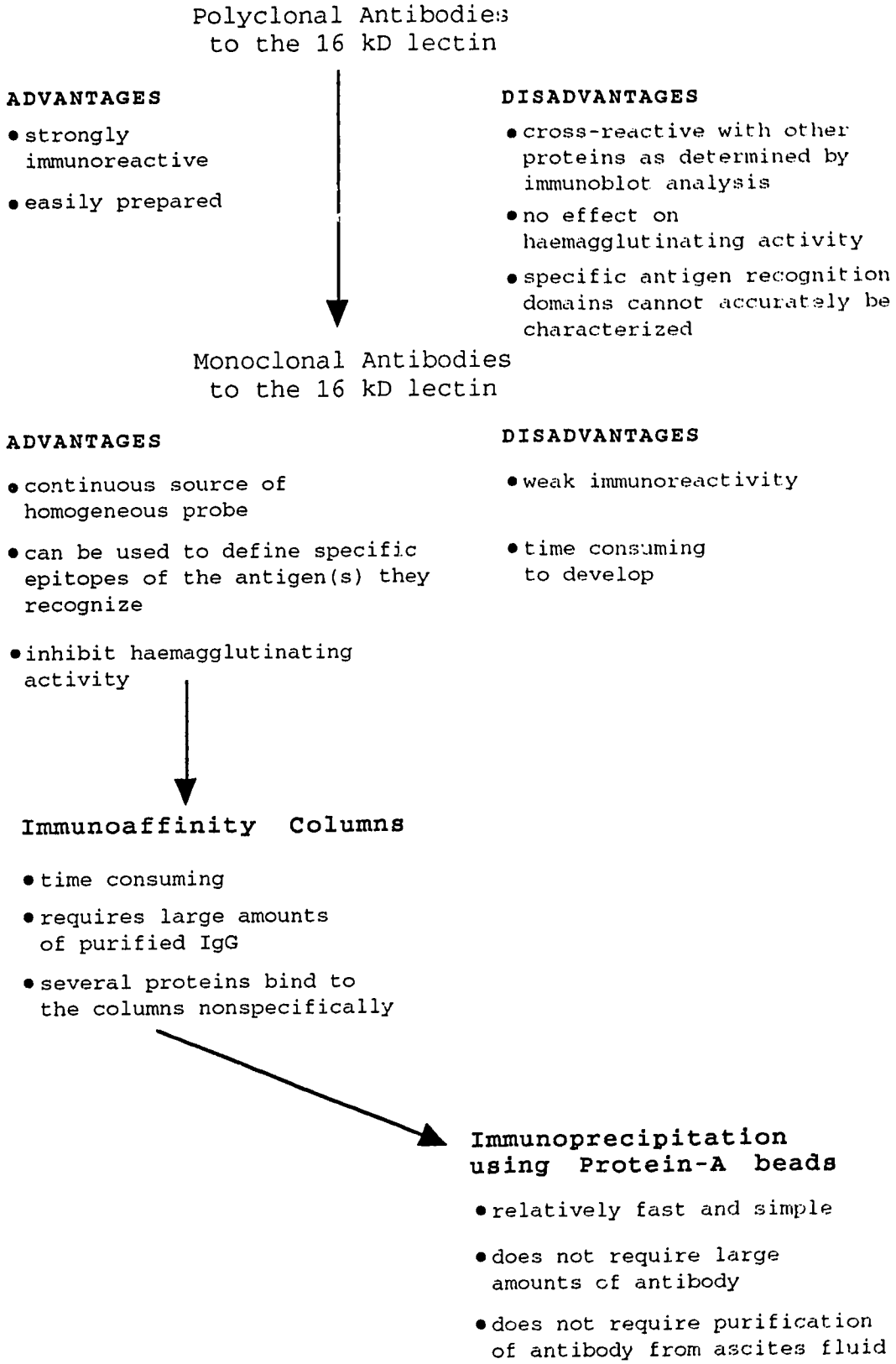
Immunohistochemistry:

a) *Immunoperoxidase staining:*

Yolk sac tissue from embryos incubated for 5 days was removed and adherent yolk washed off by gentle pipetting in cold PBS. The tissue was then immediately fixed at room temperature for 1 h in freshly prepared 3.7% paraformaldehyde in Pannett and Compton's saline (PCS), pH 7.5. Dehydration and embedding in PEG was done according to the procedure of Zalik *et al.* (1987) by a modification of the method used by Drews (1975). Yolk sacs were rinsed briefly in PCS and dehydrated in the following series of polyethylene glycol (PEG, Sigma) solutions mixed in H_2O: 25%, 50%, 75%, and 100% PEG (Mw 400) respectively for 30 min. at room temperature. Tissues were then left in a mixture of PEG 400/PEG 1000 (1:1, v/v) at 45°C for 60 min., followed by incubations in PEG 1000 for 1 h and PEG 1500 for 2 h. at 45°C. The yolk sacs were then embedded in PEG 1500 and sectioned. Sections 5 µm thick were mounted on slides previously coated with rubber cement (Drews 1975). Sections were left in the dark at 4°C until immunostaining could be performed.

All immunostaining procedures were carried out at room temperature. Sections were rinsed twice in acetone for 3 min., followed by a wash in TBS, pH 7.6, for 5 min. They were blocked in TBS containing 3% goat serum for 30 min. prior to overnight incubation with either anti-L16 or anti-S14 antisera diluted 1:1000 in TBS-1% goat serum. Sections

Figure 3: Flow ch[illegible]rating the reasoning behind the procedures used in [illegible]cterization of the 16 kD chick yolk sac lectin.


Polyclonal Antibodies to the 16 kD lectin
ADVANTAGES
• strongly immunoreactive
• easily prepared
DISADVANTAGES
• cross-reactive with other proteins as determined by immunoblot analysis
• no effect on haemagglutinating activity
• specific antigen recognition domains cannot accurately be characterized
Monoclonal Antibodies to the 16 kD lectin
ADVANTAGES
• continuous source of homogeneous probe
• can be used to define specific epitopes of the antigen(s) they recognize
• inhibit haemagglutinating activity
DISADVANTAGES
• weak immunoreactivity
• time consuming to develop
Immunoaffinity Columns
• time consuming
• requires large amounts of purified IgG
• several proteins bind to the columns nonspecifically
Immunoprecipitation using Protein-A beads
• relatively fast and simple
• does not require large amounts of antibody
• does not require purification of antibody from ascites fluid

were rinsed in three changes of TBS for 10 min. each and then incubated with GAR-HRP (Biorad) diluted 1:20 in TBS-1% goat serum for 30 min. Sections were rinsed in three changes of TBS as before and then incubated with peroxidase-conjugated anti-peroxidase (PAP) IgG, diluted 1:400 with TBS-1% goat serum. Tissue sections were washed again in three changes of TBS. Bound peroxidase in the tissues was detected by a brown colour reaction in the presence of 0.05% diaminobenzidine (DAB) in 10 ml TBS to which 3 µl of hydrogen peroxide had been freshly added. The colour reaction was allowed to proceed for 10 min., the sections were washed in three changes of TBS, and then mounted in Mowiol 4-88 medium (Calbiochem) (Osborn and Weber 1982) containing 2,4-diazabicyclo (2,2,2) octane (DABCO, Sigma) as outlined in an earlier report (Zalik *et al.* 1990b). In some cases, sections were exposed to the nuclear stain DAPI (4,6-diamidino-2-phenylindole, Sigma) dissolved in PBS (0.675 µg/ml) for 2 min. prior to mounting. In such instances the sections were washed again in two changes of TBS, fo. lowed by a quick rinse in H_2O. Sections were observed on a Zeiss Photomicroscope III using the appropriate filters and photographs taken using Kodak Ektachrome 200 film.

b) *Immunofluorescence staining:*

The fixation, dehydration, and embedding were performed as for the immunoperoxidase staining. All immunostaining procedures were carried out at room temperature. Sections from 5-, 7-, and 15 day yolk sac membranes were incubated in PBS for 5 min (pH 7.1) prior to blocking in 1% BSA-PBS for 30 min. Sections were then incubated for 3 h in mouse ascites fluid from clones 5E5, 5F9, and 10E10 (dil. 1:50 in 1% BSA-PBS). The control consisted of a mAb to human muscle actin at the same dilution (Dako), concentrated from hybridoma culture supernatant. The control for 7 day yolk sac sections consisted of non-immune serum from a Balb/c female mouse.

Sections of yolk sac tissue at 15 days development were also incubated in a mixture of polyclonal anti-L16 antiserum (final dil. 1:100 in 1% BSA-PBS) and mAb 10E10 at the previously specified final dilution for 3 h. The control for this experiment consisted of a mixture of preimmune rabbit serum (final dil. 1:100 in 1% BSA-PBS) with anti-human muscle actin antiserum (final dil. 1:50 in 1% BSA-PBS). All the sections were then rinsed in three changes of PBS prior to incubation with FITC-conjugated and rhodamine-conjugated secondary antibodies in the dark. Tissues incubated initially with the mAbs were exposed to RAM-IgG diluted 1:32 (ICN) in 1% BSA-PBS for 1 h. Fifteen day yolk sac tissue first exposed to mixed polyclonal and monoclonal antisera were reacted against a mixture of rhodamine-conjugated GAR-IgG (dil. 1:30 in 1% BSA-PBS; Sigma) and FITC-conjugated RAM-IgG (dil. 1:64 in 1% BSA-PBS; Sigma). Diluted secondary antibodies were centrifuged at 80,000 × *g* prior to incubation of sections. Sections were again washed in 3 × 5 min. changes of PBS, and then incubated with DAPI as described earlier. Sections were observed and photographed as described for the immunoperoxidase staining. Fifteen day yolk sac tissue exposed to mixed polyclonal and monoclonal primary antibody solution was observed on a Zeiss Axiophot Photomicroscope and photographs taken using Kodak T-Max 100 black and white film.

Results

Changes in lectin activity during yolk sac development:

The log specific haemagglutinating activity, as determined by haemagglutination assay of crude yolk sac lectin extracts, is relatively low at 2 days (1.9 ± 0.03 HU/mg protein) and increases rapidly until 4 days (3.8 ± 0.14 HU/mg protein). Thereafter, lectin activity remains at approximately the same levels until 18 days, the latest stage examined in this study (Fig. 4).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):

Analysis of crude yolk sac lectin extracts by SDS-PAGE revealed a doublet. From its rate of migration in gels this doublet was tentatively identified as the 14 kD lectin following Coomassie staining (Fig. 5, plate A). This doublet was present in all developmental stages, however, two day extracts showed only one band (Mw = 14 kD), while in extracts from 4 to 18 days the 14 kD doublet was clearly evident. A 16 kD band could not be positively identified in crude extracts separated by SDS-PAGE.

Affinity purified lectin preparations from 2 to 16 days of incubation, when examined by SDS-PAGE showed two major bands at Mw 14 kD and 16 kD (Fig. 5, plate B). In some gels a third band could be visualized between the 14 kD and 16 kD bands (not shown). In some gels a distinct doublet having a Mw 66-70 kD was also present (plate B; see also Fig. 8, Plate A).

Figure 4. Specific lectin activity in the yolk sac during development. Yolk sac membranes were isolated at stages of development from 2 to 18 days post-incubation and homogenized in PBS containing lactose and β-mercaptoethanol. Following removal of lactose, haemagglutination assays were performed on the extracts and the protein concentration for each determined. Specific activity is given as the log of the titer per mg protein. Each point is expressed as the geometric mean of 4 experiments ± SEM. Lectin activity increases rapidly from 2 to 4 days, but does not change significantly from 4 to 18 days ($p \leq 0.01$).

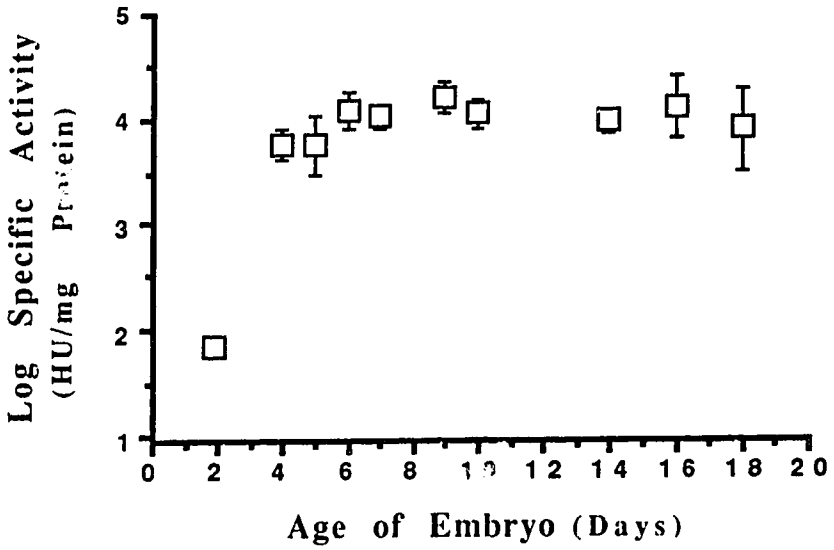
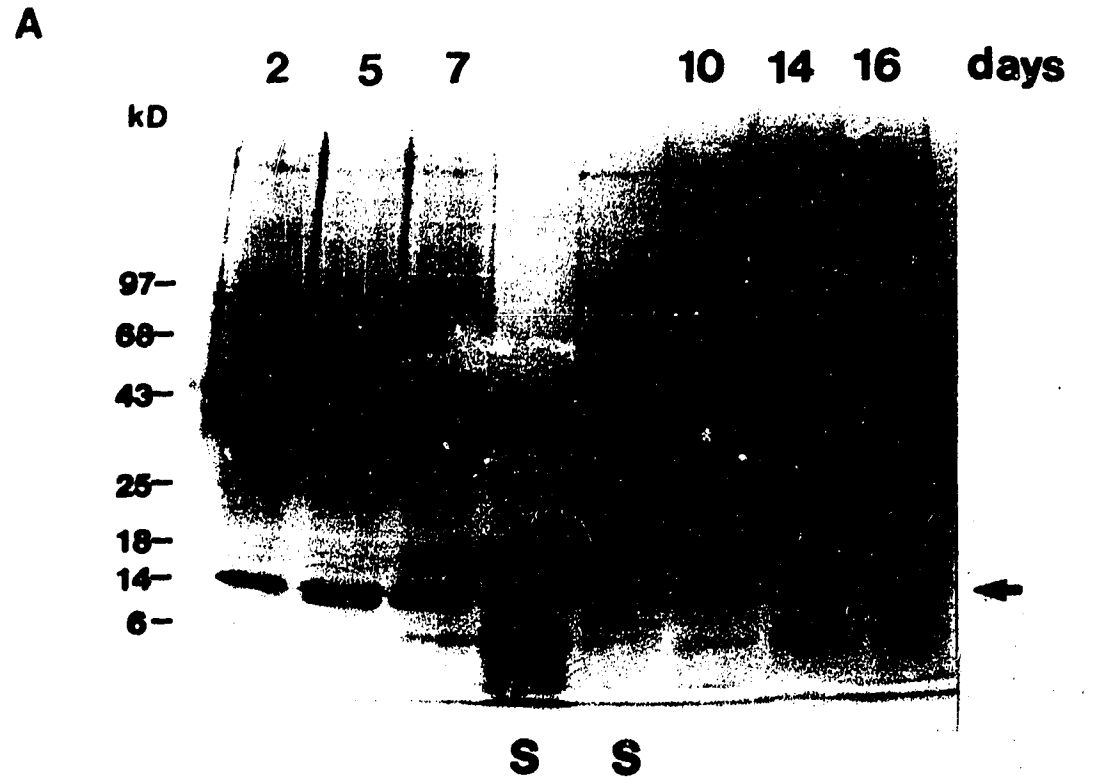

Log Specific Activity
(HU/mg Protein)
5
4
3
2
1
0
2
4
6
8
12
14
16
18
20
Age of Embryo (Days)

Figure 5. SDS-polyacrylamide gel electrophoresis of crude lectin extracts and lactoside-purified lectins from the yolk sac at different stages of development. Protein samples were prepared as described in the experimental procedures and examined on 4.5%-22% gradient gels (1 mm thick) under reducing conditions. Gels were stained with Coomassie brilliant blue. Numbers over the lanes designate the days of development. Standards are expressed in kilodaltons (kD) and are as follows: phosphoryase b (97 kD); bovine serum albumin (BSA, 68 kD); ovalbumin (43 kD); α-chymotrypsinogen (25.7 kD); β-lactoglobulin (18.4 kD); lysozyme (14.3 kD); bovine trypsin inhibitor (6.2 kD).

Plate A. Crude lectin extracts (40 µg) isolated from yolk sacs at 2-, 5-, 7-, 10-, 14-, and 16 days were loaded onto the gel (well width = 5 mm) following incubation in a reducing sample buffer at 60°C for 1 h. The arrow indicates a protein doublet having a relative Mw of 14 kD and corresponding to the 14 kD chicken lectin. The doublet was seen at all stages except at 2 days, where only the higher Mw component of the doublet was observed. S indicates lanes containing standards.

Plate B. Purified lectin preparations. Twenty µg of yolk sac lectin, from extracts of different developmental stages, were purified on an APL-Sepharose column and loaded onto gels (well width = 13 mm) at 5-, 6-, 8-, 14-, and 16 days development. Staining revealed two proteins having relative Mw's of 14 kD and 16 kD in all stages examined.


A
2
5
7
10
14
16
days
kD
97–
68–
43–
25–
18–
14–
6–
S
S
B
5
6
8
14
16
kD
97–
68–
43–
25–
18–
14–
6–
43–
25–
18–
14–
6–
43–
25–
18–
14–
6–
43–
25–
13–
14–
6–
43–
25–
18–
14–
6–

Immunoblot analysis of yolk sac lectins:

Crude lectin extracts:

Crude lectin extracts from yolk sacs at 2 to 18 days of development were examined by immunoblot analysis using the antisera to the 14 kD and the 16 kD lectins. Reactivity of the blots with all antisera tested was similar at all stages of development (Fig. 6). Anti-L16 antiserum detected both the 14 kD and 16 kD bands (strip B), however, often it was necessary to load high amounts of protein on the gels (>200 µg/13 mm wide well in a mini-gel) to detect the 16 kD band on the blots. The 16 kD band stained moderately when incubated with anti-E16 (strip C), and was never seen after incubation with anti-S14 (strip D). However, at all stages anti-S14 detected a Mw doublet of 68-70 kD. In these strips a band was also observed having a relative Mw of 25 kD. The anti-S14 antisera generally did not detect the 14 kD yolk sac lectin in blots of crude yolk sac extract.

Purified lectin preparations:

Purified lectin extracts from 2 to 16 day yolk sacs were examined by immunoblotting with the same antisera used to analyze the crude extracts. Most immunoblot analyses were conducted on fractions containing the highest haemagglutinating activities. The anti-L16, anti-E16 (Fig. 7, strips B and C), and anti-M16 antisera (Fig. 8, plate B, strip C) generally gave strong reactions against both the 14 kD and 16 kD lectin bands of the yolk sac, however, in purified preparations of 2 day yolk sacs the 14 kD band gave a weak signal in the presence of all the antisera tested (Fig. 7). In some extracts a third band of Mw 15 kD was recognized by anti-L16 between the above bands (not shown). The anti-S14 antiserum either stained weakly or failed to stain the 16 kD band throughout development (Fig. 7, strip D;

Figure 6. Immunoreactivity of crude yolk sac extracts against rabbit antisera prepared against the 14 kD lectin from chick embryonic skin (S-14), the 16 kD lectin from whole chick embryos (E-16), and the 16 kD adult chick liver lectin (L-16). Yolk sac extracts from 2-, 5-, and 10 days (400 µg protein) were reduced in sample buffer at 95°C for 5 min. The samples were loaded into 13 mm wide wells, subjected to gradient (4.5%-22%) SDS-PAGE, and transferred to nitrocellulose. Nitrocellulose strips were incubated with preimmune serum corresponding to anti-L16 (strip A), anti-L16 antiserum (strip B), anti-E16 antiserum (strip C), and anti-S14 antiserum (strip D). Standards (not shown) are: phosphorylase b (97 kD); BSA (68 kD); ovalbumin (43 kD); α-chymotrypsinogen (25.7 kD); β-lactoglobulin (18.3 kD); lysozyme (14.3 kD).

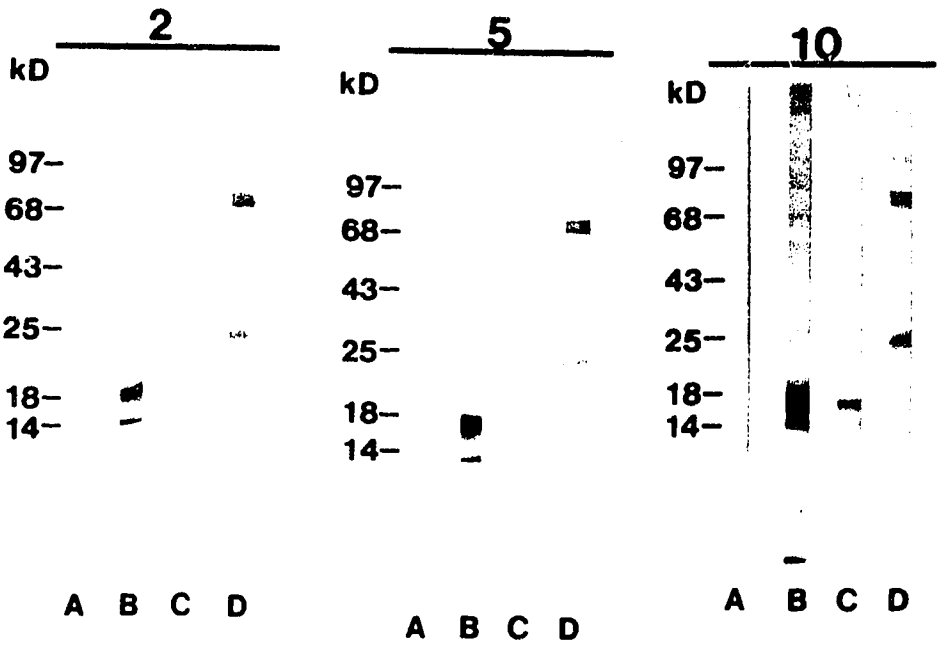

2
kD
97–
68–
43–
25–
18–
14–
A B C D
5
kD
97–
68–
43–
25–
18–
14–
A B C D
10
kD
97–
68–
43–
25–
18–
14–
A B C D

Figure 7. Immunoblot analysis of lactoside-affinity purified yolk sac lectins at different stages of development. Yolk sac tissue was removed at 2-, 5-, 10-, 14-, and 16 days and the lectins lactoside-affinity purified as described in the Experimental Procedures. Purified preparations were reduced in sample buffer at 95°C for 5 min. and 20 μg of each loaded into wells (13 mm wide). The lectin was transferred to nitrocellulose following gradient (4.5%-22%) SDS-PAGE, and nitrocellulose strips incubated with the following rabbit antisera: preimmune serum corresponding to anti-L16 (strip A); anti-L16 antiserum (strip B); anti-E16 antiserum (strip C); anti-S14 antiserum (strip D). Standards (not shown) are phosphorylase b (97 kD); BSA (68 kD); ovalbumin (43 kD); α-chymotrypsinogen (25.7 kD); β-lactoglobulin (18.4 kD); lysozyme (14.3 kD); bovine trypsin inhibitor (6.2 kD).

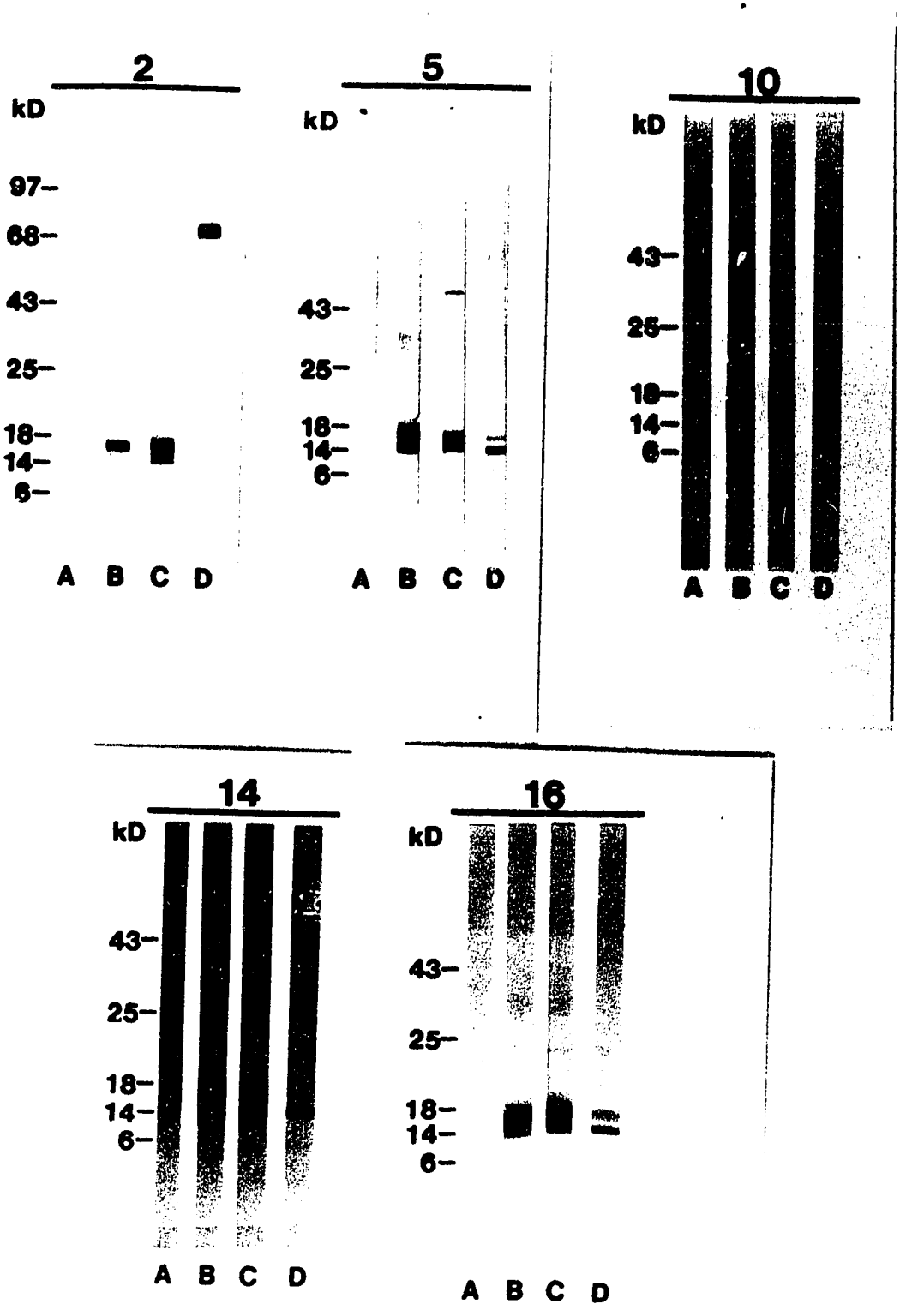
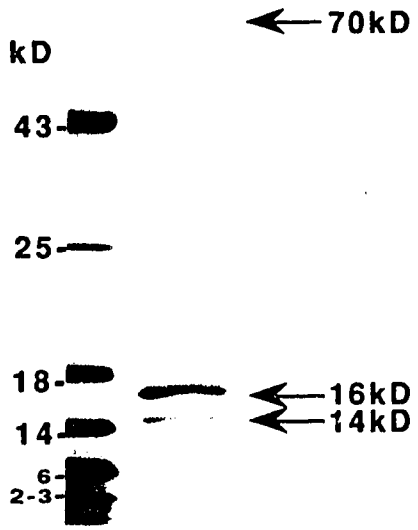

2
kD
97–
68–
43–
25–
18–
14–
6–
A B C D
5
kD
43–
25–
18–
14–
6–
A B C D
10
kD
43–
25–
18–
14–
6–
A B C D
14
kD
43–
25–
18–
14–
6–
A B C D
16
kD
43–
25–
18–
14–
6–
A B C D

Figure 8. Characterization of the 15 day yolk sac lectins by SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Yolk sac tissue was extracted from 15 day embryos in LES. Following dialysis out of lactose, the extract was concentrated and lectin purified on a lactoside-derivatized Sepharose column. Lectin was eluted from the column in MEPBS-lactose, and lactose was removed by passage through a column of P6-DG. Subsequently, the lectin was further purified by repeating the lactoside-affinity procedure on the same Sepharose column.

Plate A. Pure yolk sac lectin was precipitated out of solution by addition of 10 volumes of acetone, then resuspended and reduced in sample buffer at 95°C for 5 min. The lectin (10 µg; well width = 5 mm) was loaded onto a gradient gel (10%-18%) and analyzed by SDS-PAGE. Bands were visualized following overnight incubation of the gel in Coomassie Brilliant blue stain. Molecular weight standards include ovalbumin (43 kD); α-chymotrypsinogen (25.7 kD); β-lactoglobulin (18.4 kD); lysozyme (14.3 kD); bovine trypsin inhibitor (6.2 kD); insulin a and b (3.4 and 2.3 kD respectively).

Plate B. Immunoblot analysis of the purified lectin. SDS-PAGE was performed on pure 15 day yolk sac lectin (20 µg; well width = 13 mm) as described above and the lectins transferred to nitrocellulose. Nitrocellulose strips were incubated with the following antisera: anti-S14 (lane A); anti-I14 (lane B); anti-M16 (lane C); anti-L16 (lane D); preimmune serum corresponding to anti-L16 (lane E). Protein standards were visualized following Ponceau red staining and are at the right-hand side of the blot. Standards are phosphorylase b (97 kD); BSA (66.2 kD); ovalbumin (45 kD); bovine carbonic anhydrase (31 kD); soybean trypsin inhibitor (21.5 kD); lysozyme (14.4 kD). Observe that all antisera react with both the 14 kD and 16 kD lectins with the exception of anti-S14, which reacts specifically with the 14 kD band.

A


70kD
kD
43-
25-
18-
14-
6-
2-3-
16kD
14kD

B

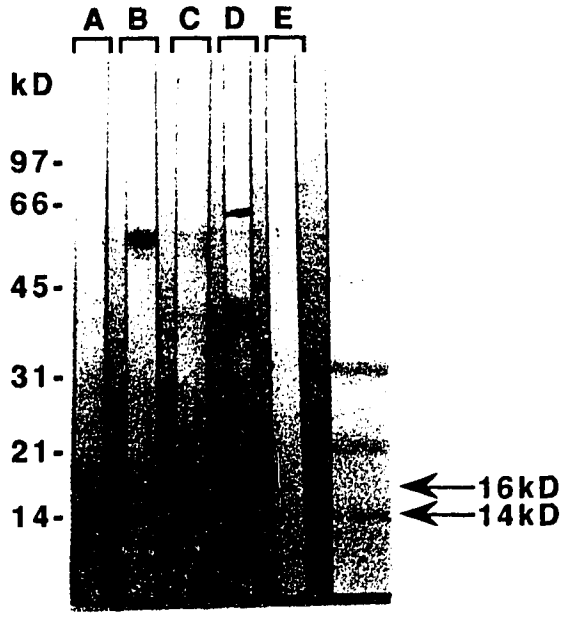

A B C D E
kD
97-
66-
45-
31-
21-
14-
16kD
14kD

Fig. 8, plate B, strip A). Some reactivity against the 16 kD lectin was observed in blots reacted with the anti-I14 antiserum (Fig. 8, plate B, strip B). In immunoblots of pure yolk sac lectins from developmental stages older than 4 days, antisera to the 14 kD lectins stained the 14 kD lectin band (Fig. 7, strip D; Fig. 8, plate B, strips A and B). Previous reports have dealt with the immunological cross-reactivity of the 14 kD and 16 kD lectins (Hirabayashi *et al.* 1987, Zalik *et al.* 1990b; 1991). In our studies we have determined that both the 14 kD and 16 kD lectins of the yolk sac are recognized by all antisera tested. The anti-16 kD antisera frequently recognized a 35 kD band at all stages of development, but the staining in most blots was weak (Fig. 7, strips B and C; Fig. 8, plate B, strip D). Protein constituents having Mw of 70 ± 2 kD were recognized by anti-S14 in 2 day yolk sacs (Fig. 7, strip D). Antiserum to I14 and M16 also recognized a 70 kD band at later stages of development (not shown). However, when the yolk sac lectins were purified by double-affinity chromatography, and precipitated with acetone, the staining of the 35 kD and 70 kD bands was less evident (Fig. 8, plate B). Following further analysis of 15 day yolk sacs it became clear that in both crude and purified lectin preparations the anti-14 kD antisera reacted strongly with a protein having a Mw of approximately 10 ± 2 kD. This protein was observed more frequently in extracts prepared for immunoaffinity and immunoprecipitation procedures and will described later.

Screening for inhibition of haemagglutinating activity by mAbs:

Crude extracts from 15 day yolk sacs were incubated with mAbs from clones 5E5, 5F9 and 10E10 coupled to beads via a rabbit-anti-mouse antibody. Results from these experiments showed that these mAbs removed all haemagglutinating activity from diluted (titre = 6-8 HUs/25 µls) yolk sac extracts

(Table 1). Four other mAbs, also previously found to react in ELISA with purified yolk sac lectins, had no effect on the agglutinating activity of these extracts relative to the controls. The 3 mAbs were found to have identical IgG_1, λ chain sub-isotypes (data not shown). This isotype was confirmed in subsequent experiments requiring the use of IgG-specific secondary antibodies.

Inhibition of haemagglutinating activity by mAbs:

Haemagglutinating activity present in solution was inhibited by the anti-lectin mAbs. In Fig. 9 it can be seen that haemagglutinating activity of yolk sac extracts (16 HUs/ 25 µls) was inhibited at mAb concentrations of between 0.1-0.2 mg/ml (Fig. 9). The inhibitory activity of all three mAbs was approximately the same. The anti-lectin mAb 10E10 also inhibited the haemagglutinating activity of crude lectin extracts from adult liver at concentrations similar to those effective for the yolk sac lectins (not shown).

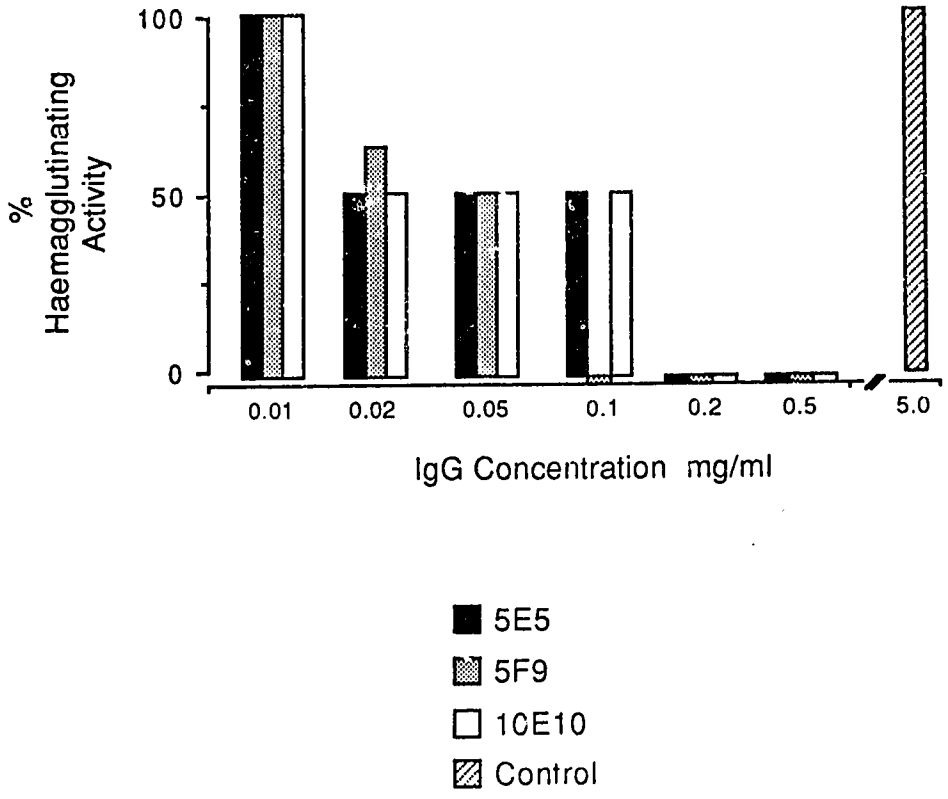
Immunoaffinity experiments:

Anti-lectin mAbs (mAb 10E10) were immobilized on beads, packed into columns, and the lectin components retained by the column examined after elution with lactose and acid-glycine. The material from lectin extracts that was retained specifically by the immunoaffinity columns was examined by immunoblot analysis using polyclonal antibodies. The eluted material was separated by SDS-PAGE, transferred to nitrocellulose, and incubated against the polyclonal antisera described earlier. We examined the effect of washing the column with 10 mM lactose prior to elution of the mAb-bound lectin(s) to determine if some of the lactose-specific lectins were bound by the beads, and not by the mAbs, or whether antibody-lectin binding was affected by the presence of lactose. A major band with a Mw of 10 ± 2 kD reactive

Table 1. Ability of bound mAbs raised against pure yolk sac lectins to remove haemagglutinating activity from crude 15 day yolk sac homogenates extracted in lactose. Antibodies from the hybridoma supernatants of ELISA positive clones to purified yolk sac lectins were concentrated from media in saturated ammonium sulfate and then linked covalently to RAM immunobeads. The bound mAbs were incubated against crude yolk sac extract previously diluted to an agglutinating titer of 8 or 6. Controls consisted of RAM immunobeads linked to mAbs raised against chicken L-CAM, and beads incubated in the presence of supernatant to the myeloma cell line Sp2/0. The titer haemagglutinating activity remaining in the extract following a 1 h exposure to the bead-bound antibodies is given as the average of 2 separate experiments. The mAbs from clones 5E5, 5F9, and 10E10 removed all agglutinating activity from the extracts; all other clones and the controls removed no agglutinating activity.

ELISA Positive Clones	Starting Titre	Void Titre
a) 3C5	7	4
b) 3G4	"	4
c) 4G11	"	3
d) 5C3	"	4
e) 5E5	"	0
f) 5F9	"	0
g) 10E10	"	0
h) anti-LCAM	7	5
i) Sp2/0	"	4

Figure 9. Inhibition of haemagglutinating activity by mAbs raised to lactoside-affinity purified lectins from 15 day yolk sac tissue. Antibodies from clones 5E9, 5F9, and 10E10 were incubated with crude extract from 15 day yolk sacs at various IgG concentrations ranging from 0.01 to 0.5 mg/ml at 37°C for 30 min. The titre of the extract was adjusted to 16 haemagglutinating units (HUs) before addition of the mAbs. Control antibodies were added at the same concentrations as the anti-lectin mAbs and consisted of technical grade mouse IgG. Control IgG antibodies at concentrations of 5 mg/ml were never observed to remove haemagglutinating activity from lectin extracts.


100
50
0
% Haemagglutinating Activity
0.01
0.02
0.05
0.1
0.2
0.5
5.0
IgG Concentration mg/ml
5E5
5F9
1CE10
Control

with anti-S14 and anti-I14 was found in the lactose eluant (Fig. 10, strips C and E). A 16 kD band was seen in the lactose eluant in strips incubated with the anti-L16 and anti-M16 antisera (strips D and F). A 14 kD band developed in the presence of anti-L16 (strip D), similar to results obtained incubating anti-L16 with crude yolk sac lectin extracts.

The 16 kD lectin was the only immunoreactive protein specifically bound by the 10E10 immunoaffinity column and recognized by the polyclonal antisera (Fig. 10). The protein eluted in acid-glycine following incubation of the immunoaffinity column in lactose was stained by anti-L16 and anti-M16 (strips D and F). Anti-14 kD lectin antisera did not react with any protein bands (strips C and E), but a weakly immunoreactive 14 kD band was observed in the blot incubated with anti-L16. These results suggest that some of the lectin bound by the antibody was released by lactose. The 16 kD lectin was never observed in immunoblots of proteins eluted from control immunoaffinity columns in either the lactose or the acid-glycine eluates (not shown).

Immunoprecipitation experiments were done to isolate lectins specifically recognized by the mAbs. This was performed using crude lectin extracts from 15 day yolk sacs. In Figure 11, SDS-PAGE shows that mAbs from clone 10E10 specifically precipitated the 16 kD lectin. Subsequent immunoprecipitation experiments (Fig. 12) revealed that mAbs from the clones 5E5, 5F9, and 10E10 all recognize the 16 kD lectin. The 16 kD protein band was never immunoprecipitated by the control antibody. In the course of the partial purification steps outlined in the procedures text, a protein doublet migrating to the same relative position in gels as the 14.4 kD Mw standard was observed in all of the immunoprecipitates of the above mentioned antibodies. These protein bands were also bound by the control beads. Further

Figure 10. Immunoblot analysis of lectins eluted from an anti-lectin immunoaffinity column in the presence of lactose, and in glycine-HCl. Anti-lectin mAbs (10E10), previously screened for their ability to remove haemagglutinating activity from crude lectin extracts, were covalently linked to polyacrylamide beads (see Experimental Procedures). The bead-bound mAbs (450 µg bound to 1 ml beads) were incubated with 10 ml crude lectin extract for 1 h at 4°C in the column. The void volume was removed and the column washed sequentially with PBS, 0.5 M NaCl in PBS, PBS, 10 mM lactose-PBS, and PBS; specifically bound protein was eluted in 0.2 M glycine-HCl (pH 2.5). The lactose and glycine-HCl eluates were dialyzed thoroughly against H_2O, lyophilized, and the proteins solubilized and reduced in sample buffer at 95°C for 5 min. Gradient (10%-18%) SDS-PAGE and transfer to nitrocellulose of the eluates was performed as described earlier. Strips were incubated with antisera previously raised to S-14 (strip C); L-16 (strip D); I-14 (strip E); M-16 (strip F). The control blot was incubated with preimmune serum corresponding to anti-L16 (strip B). Strip A shows protein transferred to nitrocellulose and stained with Ponceau red. Standards (not shown) are represented and were: phosphorylase b (97 kD); BSA (66.2 kD); ovalbumin (45 kD); bovine carbonic anhydrase (31 kD); soybean trypsin inhibitor (21.5 kD); lysozyme (14.4 kD).

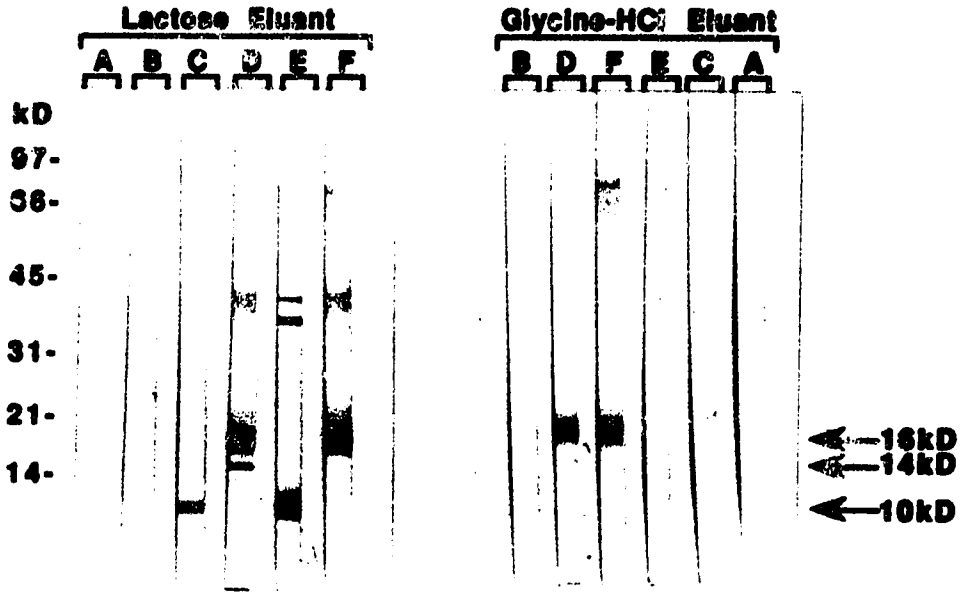
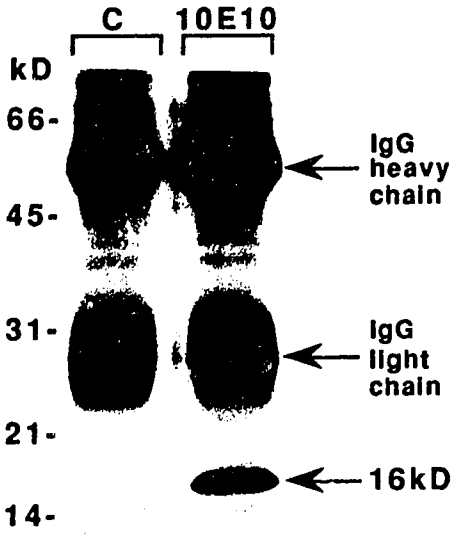

Lactose Eluant
A B C D E F
Glycine-HCl Eluant
B D F E C A
kD
97-
66-
45-
31-
21-
14-
16kD
14kD
10kD

Figure 11. Immunoprecipitation of the 16 kD lectin from crude 15 day yolk sac extracts with the mAb 10E10. Protein bound by the anti-lectin mAb 10E10 was removed from crude lectin extract by incubation with Protein A beads as reviewed in the Experimental Procedures section. The beads were washed thoroughly and the lectin-antibody complexes released from the beads in sample buffer by heating at 95°C for 5 min. The sample was loaded into a 5 mm well of an acrylamide gel, analyzed by gradient (10%-18%) SDS-PAGE, and then stained in silver. A mAb recognizing peroxidase was used as the control (C). Standards are BSA (66.2 kD); ovalbumin (45 kD); bovine carbonic anhydrase (31 kD); soybean trypsin inhibitor (21.5 kD); lysozyme (14.4 kD).


C
10E10
kD
66-
45-
31-
21-
14-
IgG heavy chain
IgG light chain
16kD

examination showed that these bands bound nonspecifically to underivatized beads and were not recognized by the mAbs (not shown). No protein of 14 kD, reactive with the anti-14 kD antibodies was observed in these immunoprecipitates. The broad protein bands having approximate Mw of 55 kD and 25 kD in these gels correspond respectively to the heavy chain and light chain regions of the IgG molecules. All subsequent immunoprecipitation procedures were performed using only the mAbs produced by clone 10E10, since it was apparent that all 3 mAbs recognized the same protein in these experiments.

Immunoblots of crude yolk sac lectin extract were compared before and after immunoprecipitation with mAb 10E10 using a battery of polyclonal antisera described earlier. The blots showed that the 16 kD lectin was absent following immunoprecipitation with the 10E10 mAb (Fig. 13, strips B and C). Subsequent immunoblot analysis of mAb-bound proteins revealed that only 16 kD lectin was found by the 10E10 mAb (not shown). The immunoreactive 14 kD band seen in the presence of anti-L16 in the extract before incubation was absent in the extract supernatant following immunoprecipitation (strip B). It is possible that this lectin precipitates with the Protein A-mAb complex with low affinity, but becomes dissociated during the wash procedure (refer to Fig. 3). This band was detected by anti-M16 (strip C), anti-I14 (strip D), and anti-S14 (strip E') before and after the immunoprecipitation procedure, but staining was weak and is considered to be nonspecific. Aside from some nonspecific staining of the IgG light and heavy chain molecules in blots of the extract after incubation with the mAbs, no staining was found in the controls (strip A).

Figure 12. Immunoprecipitation of the 16 kD lectin from 15 day yolk sac extracts using mAbs against lactoside-affinity purified lectin(s) from 15 day yolk sacs. Antibodies secreted by hybridoma cells that inhibited haemagglutinating activity were used. Monoclonal antibodies from 3 clones: 5E5, 5F9, and 10E10, were incubated with crude lectin extracts from 15 day yolk sacs for 1 h, and the antibody-antigen complexes isolated on Protein A beads according to experimental protocols. The bead-bound complexes were reduced in sample buffer, the proteins separated by gradient (10%-18%) SDS-PAGE, and the gels stained with silver as described earlier. All 3 clones specifically recognize the 16 kD lectin from yolk sac tissues. The control mAb (C) was against horseradish peroxidase. Standards loaded on gels include: BSA (66.2 kD); ovalbumin (45 kD); bovine carbonic anhydrase (31 kD); soybean trypsin inhibitor (21.5 kD); lysozyme (14.4 kD).

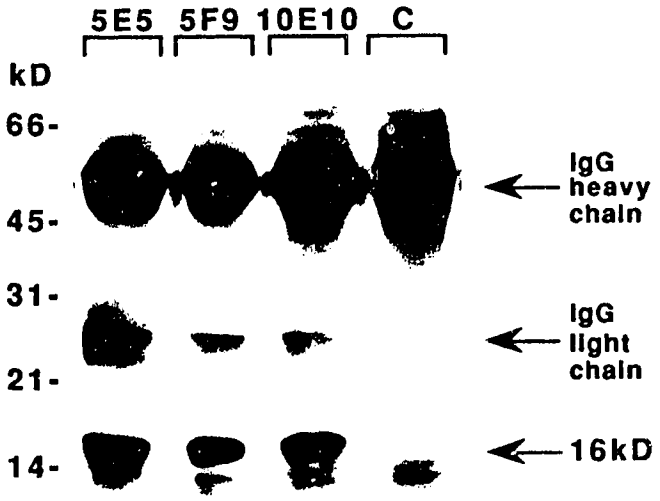
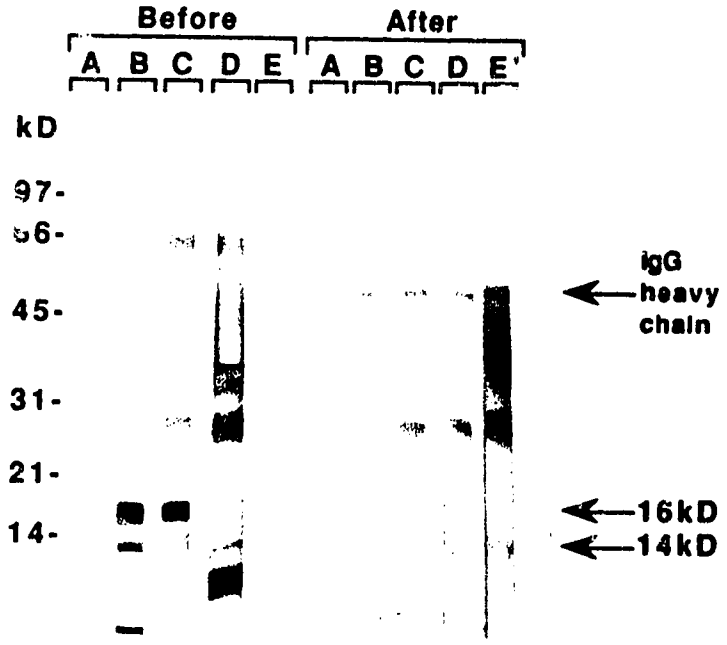

5E5
5F9
10E10
C
kD
66-
45-
31-
21-
14-
IgG heavy chain
IgG light chain
16kD

Figure 13. Immunoblot analysis of crude 15 day yolk sac lectins before and after immunoprecipitation with the anti-lectin mAb 10E10. Twenty µl aliquots were taken from lectin extract immediately prior to, and immediately following incubation of the extract with the anti-lectin mAb. The extract fractions were reduced in sample buffer, run on a gradient (10%-18%) SDS-polyacrylamide gel (well width = 13 mm), and transferred to nitrocellulose. Strips of nitrocellulose were incubated with rabbit antisera to L-16 (strip B); M-16 (strip C); I-14 (strip D); and S-14 (strip E'). Strip E shows Ponceau red staining of blotted proteins (not visible). Controls strips (A) were incubated with preimmune serum corresponding to anti-L16. Standards (not shown) are: phosporylase b (97 kD); BSA (66.2 kD); ovalbumin (45 kD); bovine carbonic anhydrase (31 kD); soybean trypsin inhibitor (21.5 kD); lysozyme (14.4 kD). Observe the absence of the 16 kD lectin from the extract following immunoprecipitation.

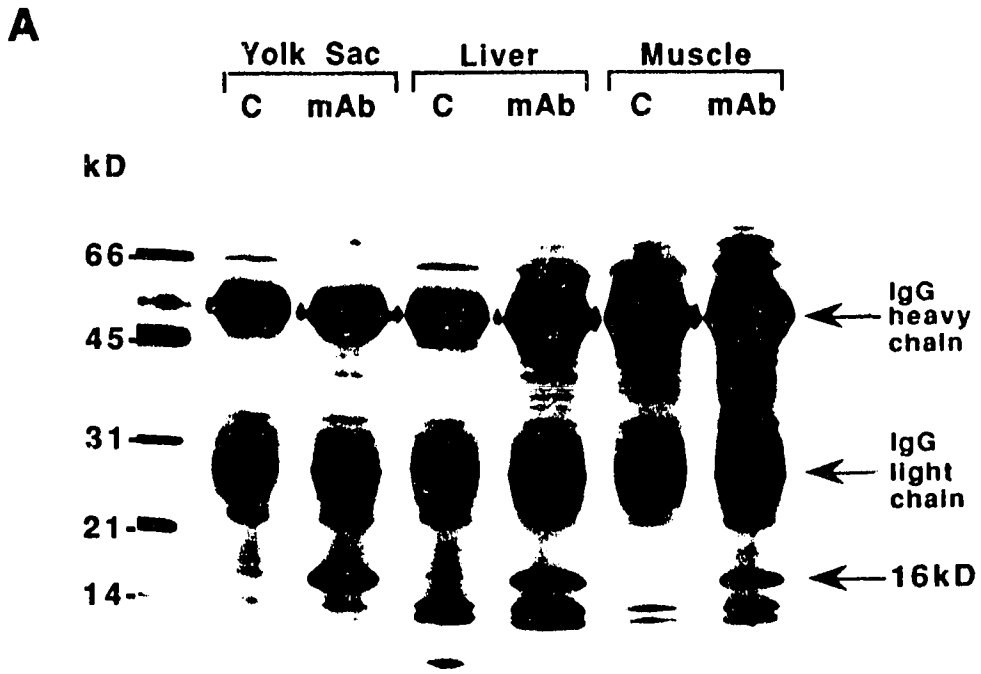

Before
After
A B C D E
A B C D E'
kD
97-
66-
45-
31-
21-
14-
IgG
heavy
chain
16kD
14kD

Immunoprecipitation of the 16 kD lectin from various chick tissues using the anti-lectin mAb 10E10:

Monoclonal antibodies that recognize the 16kD yolk sac lectin also recognize the haemagglutinin present in the adult chicken liver and the embryonic chick muscle. The 16 kD lectin was immunoprecipitated specifically from lectin extracts of liver and muscle by mAb 10E10 (Fig. 14, plate A). The 16 kD lectin was also immunoprecipitated from lactoside-purified 15 day yolk sac preparation (refer to Fig. 16, plate A) with the anti-lectin mAb 10E10 (Fig. 14, plate B). The mAb did not recognize a 16 kD lectin in extract derived from the adult intestinal mucosa (Fig. 14, plate B), and did not recognize the 14 kD haemagglutinating lectin in this tissue (refer to Fig. 15). The 16 kD lectin was never observed in control lanes containing anti-peroxidase mAb immunoprecipitates.

A 14 kD Mw doublet was found in both the control lanes and the anti-lectin mAb lanes following immunoprecipitation of all the crude tissue extracts. Repeated incubations of the crude extracts with underivatized agarose beads prior to the immunoprecipitation procedure failed to remove all the proteins that bound nonspecifically to the agarose beads. We observed the 14 kD doublet in all the crude extract immunoprecipitates (Fig. 14). These bands were also present in immunoprecipitates from control beads containing anti-immunoperoxidase mAbs. This doublet corresponds to the 14 kD doublet seen in reducing gels of crude lectin extracts from the yolk sac (refer to Fig. 5, plate A). In lanes containing liver and muscle lectin immunoprecipitates the 14 kD bands appear in higher quantities relative to the controls (Fig. 14, plate A), suggesting that some of these lower Mw proteins may also be recognized by the anti-lectin mAbs. The 14 kD lectin was not seen in immunoprecipitates of pure yolk sac lectins (Fig. 14, plate B).

Figure 14. Immunoprecipitation of the 16 kD lectin from lectin extracts of various chicken tissues by the anti-lectin mAb 10E10, followed by SDS-PAGE. The anti-lectin mAb 10E10 was incubated for 1 hour with lectin extracts from the following tissues: the 15 day yolk sac; adult liver of the hen; 15 day pectoral muscle **(plate A)**; the mucosal lining of the adult intestine; lactoside-affinity purified lectin from 15 day yolk sacs **(plate B)**. The antibody-antigen complexes were removed with Protein A; immunoprecipitates were electrophoresed on a gradient (10%-18%) SDS polyacrylamide gel, and the protein bands visualized with silver stain. The control was a mAb raised against horseradish peroxidase (C). Standards are shown and consist of: BSA (66.2); ovalbumin (45 kD); bovine carbonic anhydrase (31 kD); soybean trypsin inhibitor (21.5 kD); lysozyme (14.4 kD). The 16 kD lectin was specifically immunoprecipitated from all extracts with the exception of the intestinal extract.


A
Yolk Sac
Liver
Muscle
C
mAb
C
mAb
C
mAb
kD
66
45
31
21
14
IgG heavy chain
IgG light chain
16kD

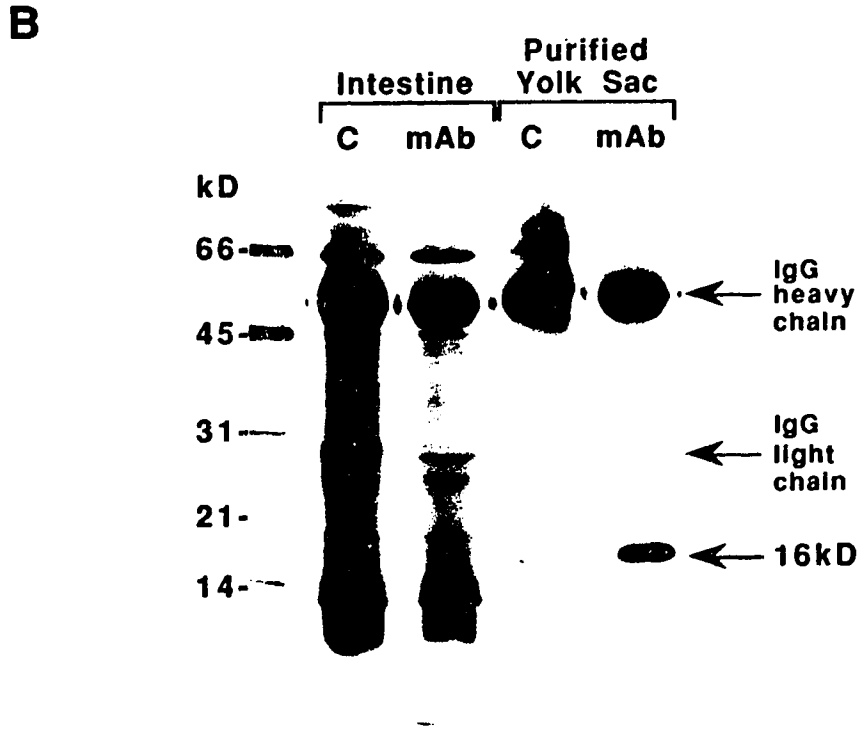
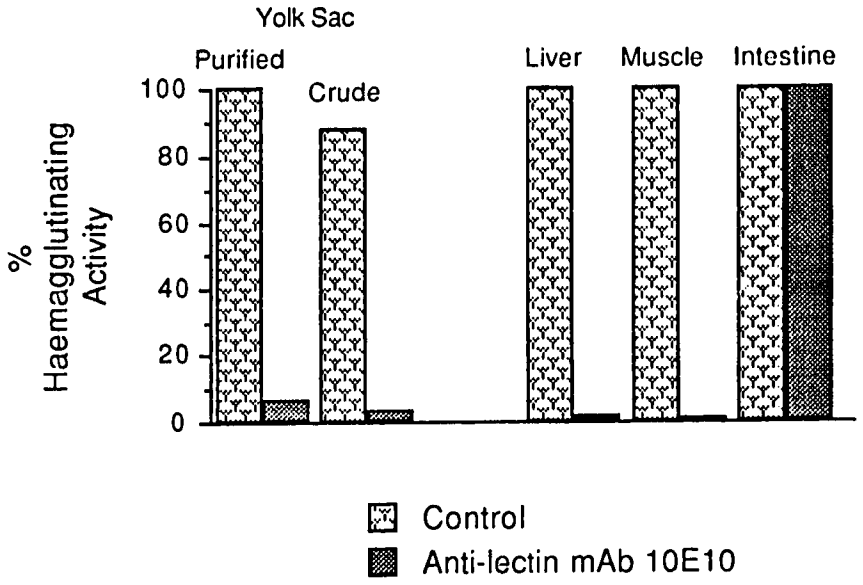

B
Intestine
Purified Yolk Sac
C
mAb
C
mAb
kD
66
45
31
21
14
IgG heavy chain
IgG light chain
16kD

Figure 15. Analysis of lectin activity in crude tissue extracts following immunoprecipitation in the presence of mAb 10E10. The haemagglutinating activities of crude chick lectin extracts from 15 day yolk sac, adult hen liver, embryonic pectoral muscle, adult intestine, and from lactoside-affinity purified yolk sac lectin were determined following the immunoprecipitation procedure of Fig. 22. The agglutinating titre of the extracts before immunoprecipitation was 512, except for the intestinal extract (256 HUs) and the purified yolk sac lectin (1024 HUs). Observe that the mAb 10E10 removed most of the activity from all the tissues with the exception of the intestine.


Yolk Sac
Purified
Crude
Liver
Muscle
Intestine
100
80
60
40
20
0
% Haemagglutinating Activity
Control
Anti-lectin mAb 10E10

Haemagglutination assays were used to determine the amount of lectin activity removed from the above mentioned crude chicken tissue extracts and from purified yolk sac preparations by the anti-lectin mAbs (Fig. 15). Following immunoprecipitation, most of the haemagglutinating activity was removed from crude yolk sac, adult liver, and embryonic muscle extracts by the mAbs. The mAb also bound the protein responsible for agglutination in pure yolk sac preparations. The mAb did not recognize the 14 kD agglutinin in crude lectin extracts from the intestine.

Immunolocalization of the 14 kD and 16 kD lectins in the developing yolk sac membranes:

Localization using polyclonal antibodies to the 14 kD lectin from embryonic chick skin and to the 16 kD adult liver lectin:

Immunoperoxidase staining was performed on yolk sacs from 5 day embryos using anti-L16 and anti-S14 antisera. With each antiserum staining was observed around the intracellular yolk platelets of the endoderm. However, although concentrated staining was seen around the periphery of the endodermal yolk platelets following incubation with anti-S14 (Fig. 16, plates A and B), anti-L16 reacted with discrete organelles associated with the yolk platelets (Fig. 17, plates B and D). No noticable difference was observed between the staining of endoderm from the area vasculosa and the area vitellina in these sections. Staining was not detected in 5 day (Fig 17, plate A), or in 15 day yolk sac sections following incubation with control rabbit serum (not shown).

In 15 day yolk sac sections staining was present around several large intracellular vacuoles following incubation with anti-L16 and visualization with rhodamine-conjugated secondary antibodies (not shown). In these sections punctate staining was dispersed intracellularly throughout the

Figure 16. Immunoperoxidase staining of 5 day yolk sac sections incubated with anti-S14 antiserum. Staining is concentrated intracellularly around the endodermal yolk platelets in the area vasculosa **(plate A)** and in the area vitellina **(plate B)**. Staining was absent in sections incubated with control rabbit serum and can be seen in Figure 17, plate A. Abbreviations: Ec (ectoderm); M (mesoderm); En (endoderm); V (blood vessel). Bars are 50 μ.

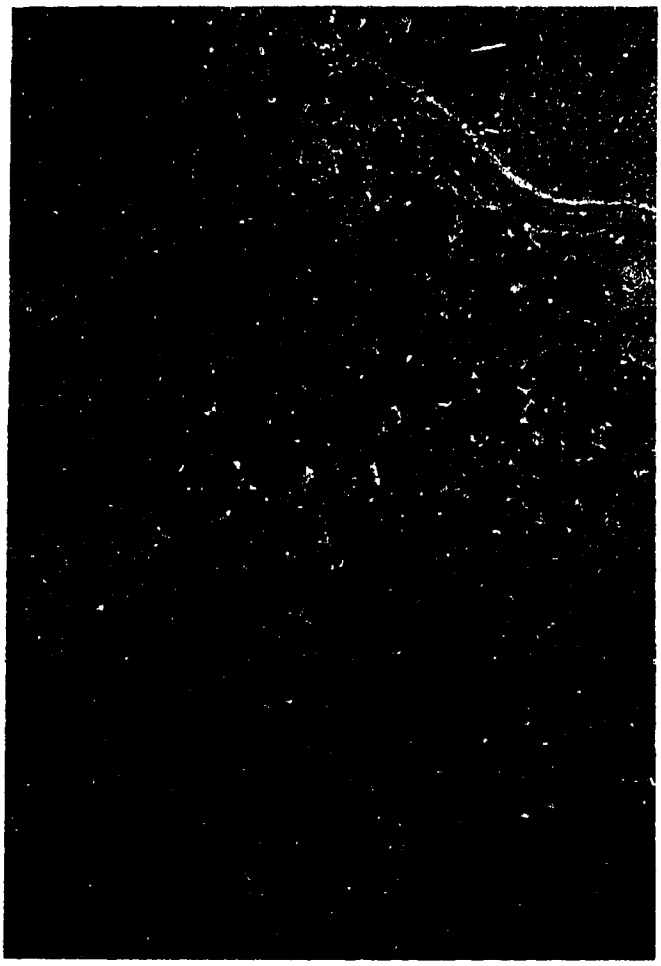
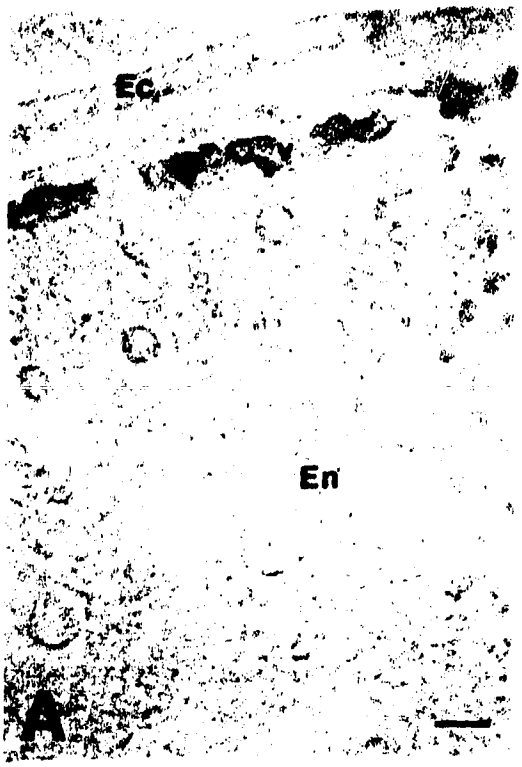
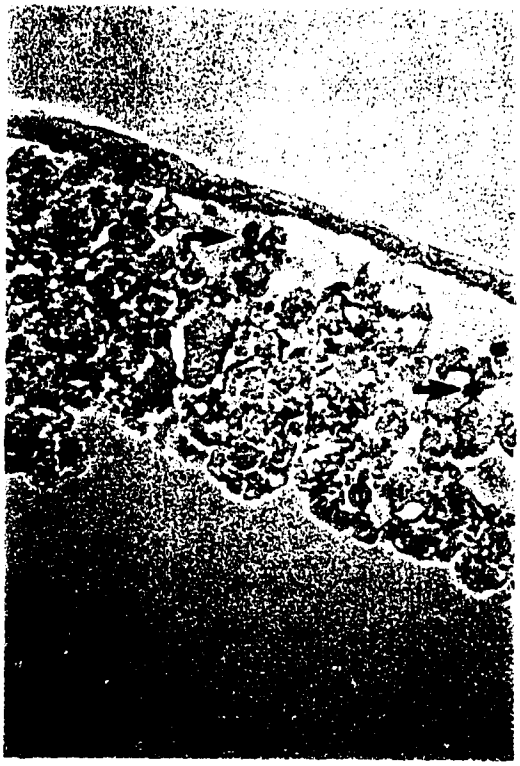


Figure 17. Immunoperoxidase staining of 5 day yolk sac membrane with anti-L16 antiserum. Observe staining in the discrete organelles (small arrows) associated with the yolk platelets of the endoderm in both the area vasculosa **(plates B and C)** and the area vitellina **(plate D).** Plate C shows a section stained for nuclei with DAPI, which shows nuclei of the blood cells and of the endodermal epithelium (large arrow). Staining was not present in tissues reacted with preimmune control serum **(plate A).** Abbreviations: Ec (ectoderm); M (mesoderm); En (endoderm); V (blood vessel). Bars are 25 μ.


Ec
En
A


B


En
V

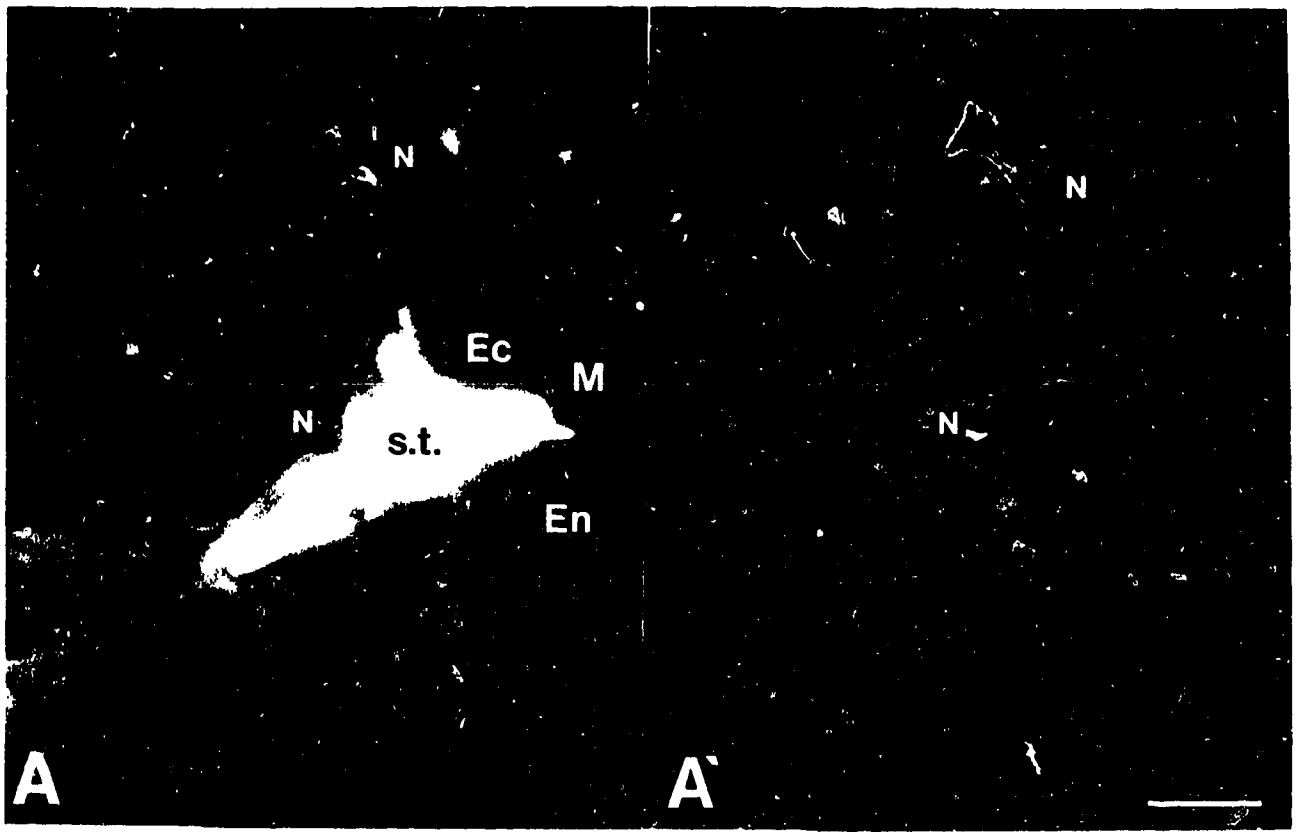


endoderm, similar to staining observed in 5 day yolk sac sections following incubation with the anti-L16 antiserum (refer to Fig. 17, plates B, C and D).

Localization using monoclonal antibodies to the 16 kD yolk sac lectin:

The antibodies raised against the 16 kD lectin from 15 day yolk sac membranes reacted specifically with endodermal constituents at all stages of development. Staining in the area vasculosa of 5 day (Fig. 18, plate A) and 7 day yolk sacs (Fig. 19, plate A) was localized throughout the endoderm around the periphery of the yolk platelets and nuclei, and was also present in the cytoplasmic material associated with the large intracellular vacuoles. Staining in the area vitellina of these membranes was primarily observed around the yolk platelets (Fig. 18, plates B; Fig. 19, plate B). In 5 day sections of the area vitellina staining of discrete organelles associated with the yolk platelets was observed (Fig. 18, plate B), this staining is similar to that seen in sections of 5 day yolk sac tissue in the presence of the anti-L16 antiserum (refer to Fig. 17, plates B, C, and D). In the endoderm of 15 day yolk sac tissue the anti-16 kD mAbs reacted with amorphous cytoplasmic material surrounding the large intracellular vacuoles (Fig. 21; Fig. 22). In some sections the staining was closely associated with the cell nuclei (Fig. 22, plate B). Control staining is shown for 5 day (Fig. 18, plate C), 7 day (Fig. 20), and 15 day yolk sac tissues (Fig. 23). Staining of yolk sac endoderm was never observed in the presence of control antibodies.

Figure 18. Immunofluorescence staining of 5 day yolk sac tissue with mAbs to the 16 kD yolk sac lectin. Tissue sections (5 μm) of the area vasculosa **(plate A)** and the area vitellina **(plate B)** were stained with mAb 10E10. Respective DAPI staining of cell nuclei in the area vasculosa is shown **(plate A')**. Endodermal cell nuclei in this section are indicated (N). Plate B shows double staining with both DAPI and FITC. Staining in the area vasculosa is present surrounding the periphery of the yolk platelets and nuclei, and is also seen in the region of the basement membrane overlying the endoderm. In the area vitellina reactivity is primarily localized in discrete organelles associated with the yolk platelets (arrows). Overall, reactivity in the area vitellina is much weaker than in the area vasculosa. Staining can be seen intracellularly in the ectoderm of tissues incubated with a control antibody to muscle actin, but is absent in the endoderm **(plate C)**. Abbreviations: Ec (ectoderm); M (mesoderm); En (endoderm); s.t. (sinus terminalis). Bars are 25 μ.


N
Ec
M
N
s.t.
En
A
N
N
A'

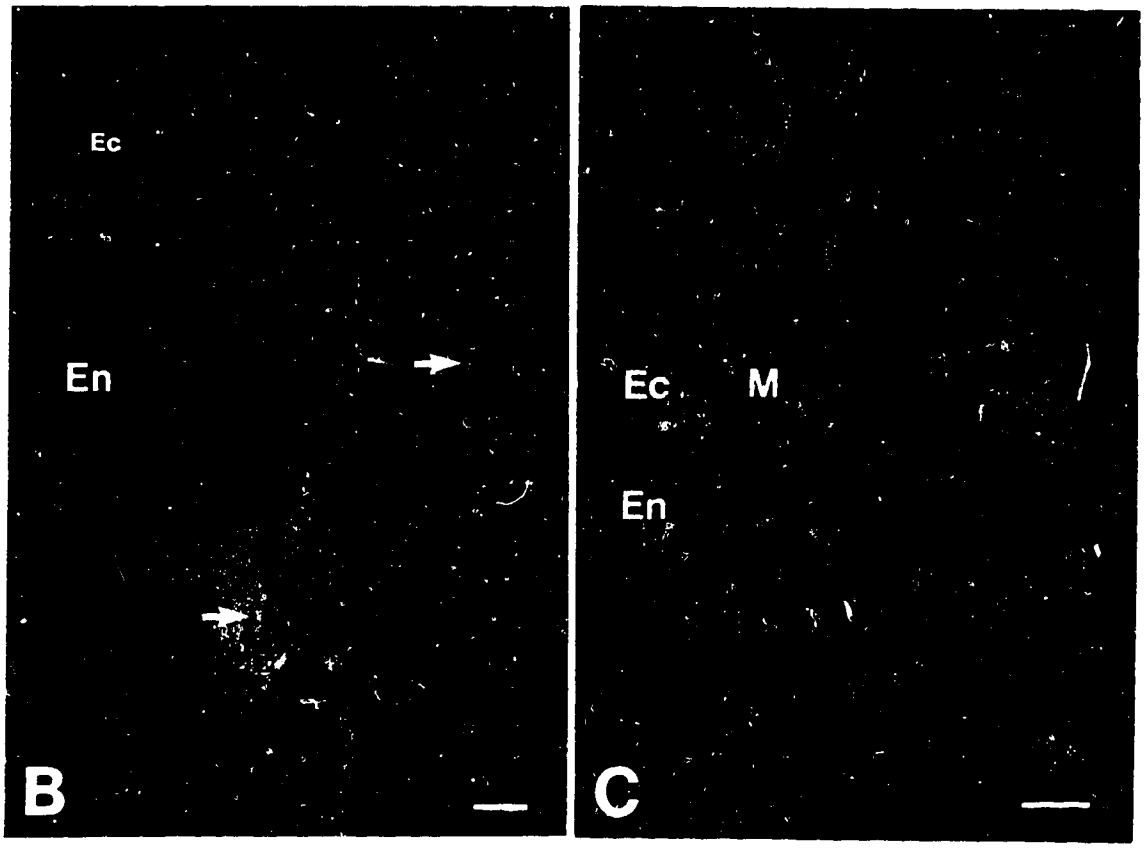

Ec
En
B
Ec
M
En
C

Figure 19. Immunofluorescence staining of 7 day yolk sac tissue with mAbs to the 16 kD yolk sac lectin. Sections of the area vasculosa **(plate A)** and area vitellina **(plate B)** were reacted with mAb 10E10. Respective DAPI staining of cell nuclei for each section can be seen in plates A′ and B′. Staining is present throughout the intracellular endoderm surrounding the yolk platelets and cell nuclei. In the area vasculosa, observe fluorescence in the peripheral material of the large intracellular vacuoles. Blood cells can be seen within the vascular mesoderm and are indicated by the small asterisk (*). Abbreviations: Ec (ectoderm); M (mesoderm); En (endoderm). Magnifications are identical between all plates; bar in plate B′ is 50 μ.

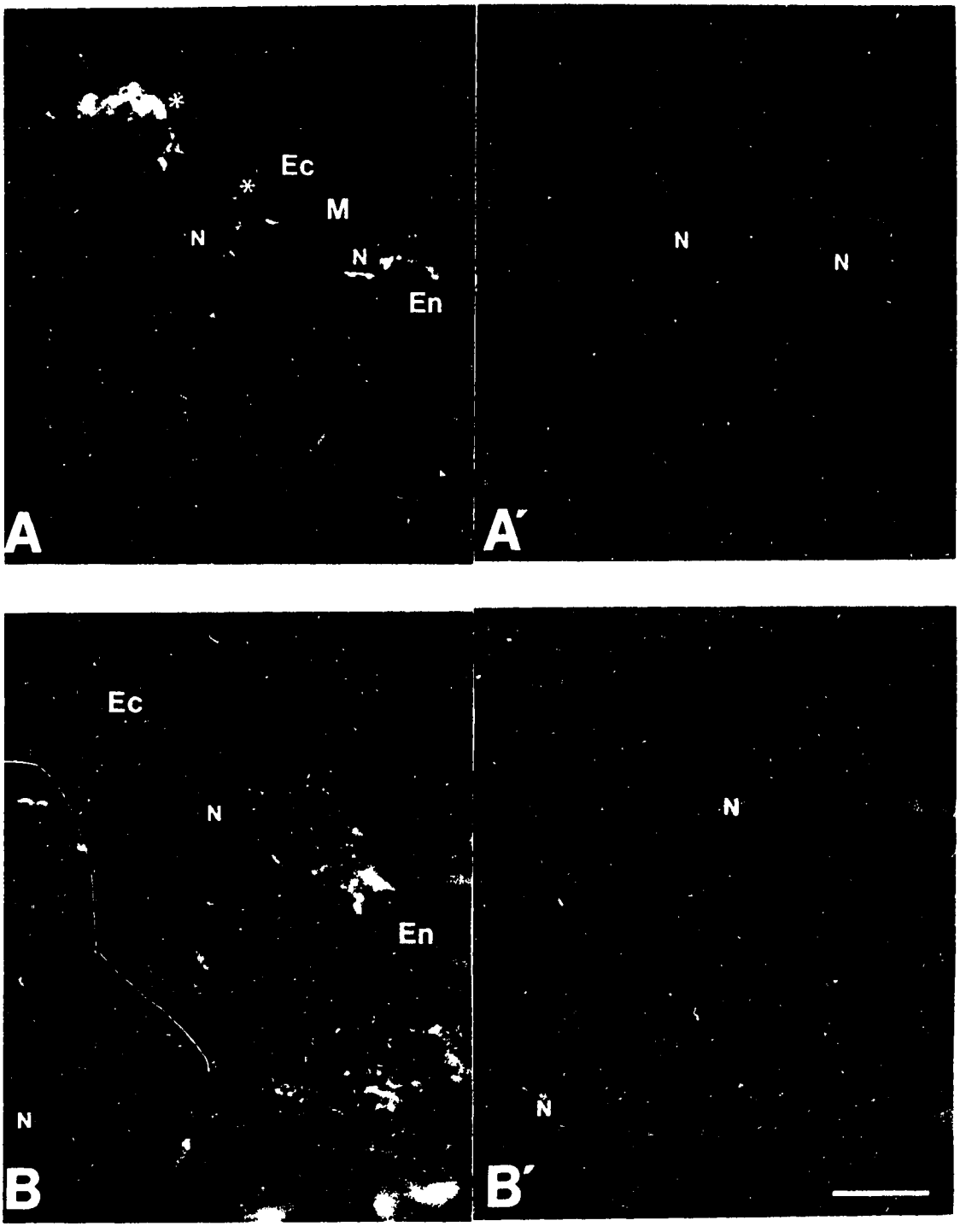

Ec
M
N
N
En
A
N
N
A'
Ec
N
En
N
B
N
B'

Figure 20. Immunofluorescence staining of 7 day yolk sac tissue with non-immune mouse serum. Note, no staining is present. Ec (ectoderm); En (endoderm). Bar is 25 μ.

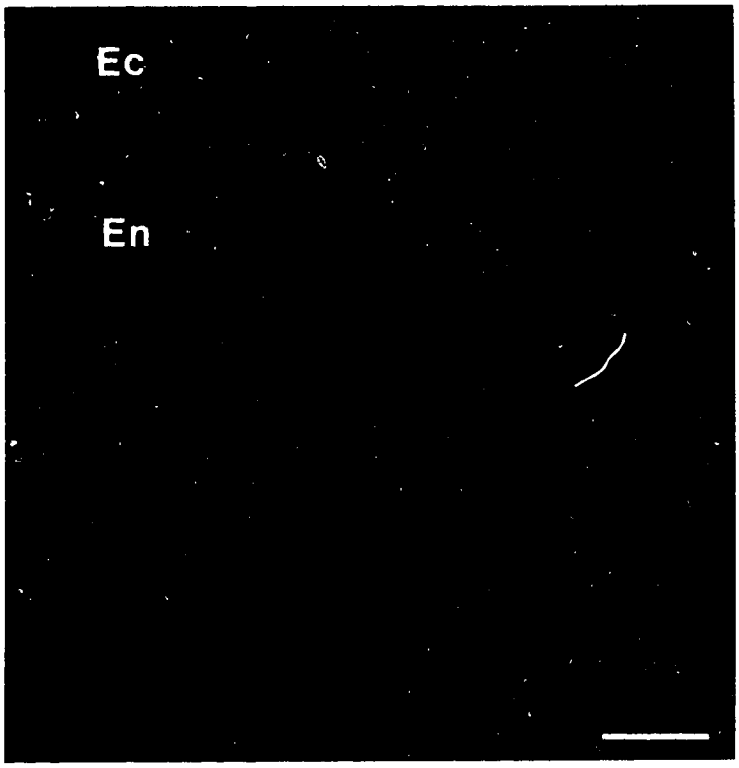
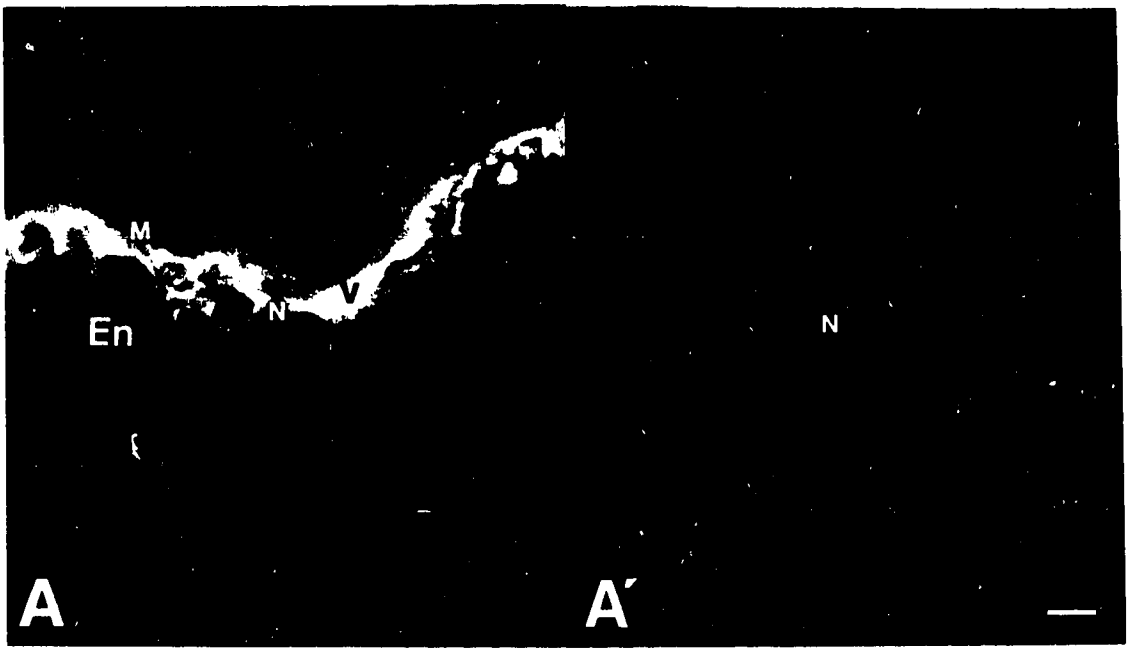

Ec
En

Figure 21. Immunofluorescence staining of the 16 kD yolk sac lectin in 15 day yolk sac membranes using the anti-16 kD lectin mAb 10E10. Staining can be seen in the basal areas of the endodermal cells in regions of the yolk sac membrane located medially to the embryo and the peripheral edge of membrane **(plate A)**. At higher magnifications the staining appears in cytoplasmic material between large intracellular vacuoles of the endodermal cells **(plate B)**. Note the autofluorescence of the blood cells located within the blood vessels (V), seen stained as yellow. Plates A' and B' show DAPI staining of the nuclei for the corresponding sections. Abbreviations: M (mesoderm); En (endoderm); N (nuclei). Bars for plates A and B are 25 μ.


M
N
V
En
N
A
A´

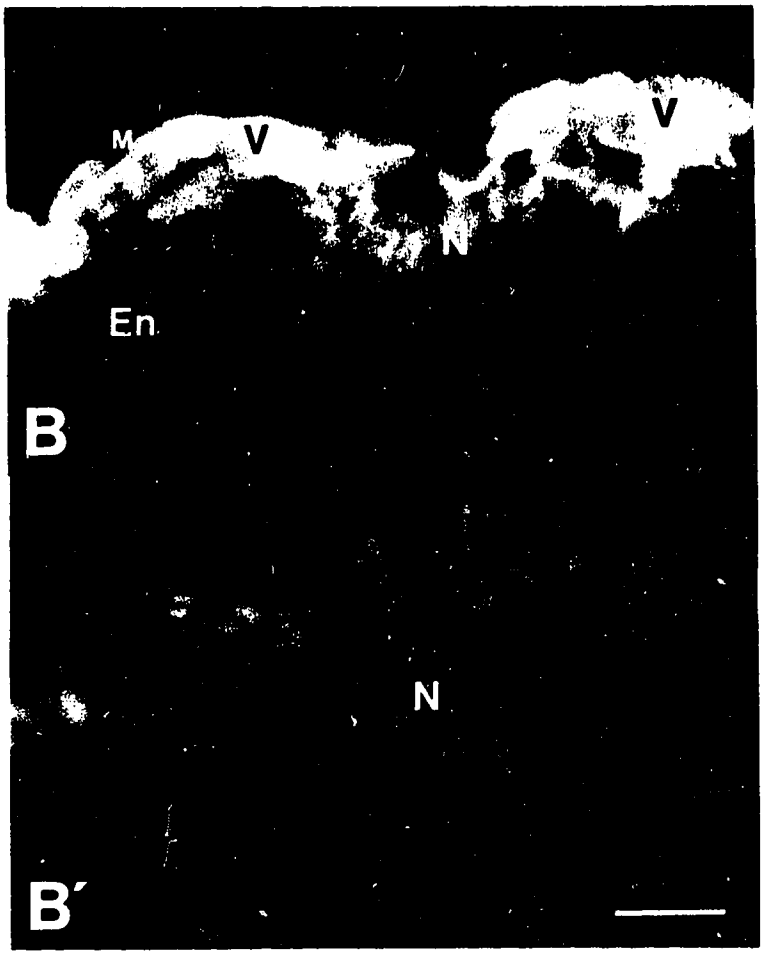

M
V
V
N
En
B
N
B´

Figure 22. Localization of the 16 kD lectin in 15 day yolk sac endoderm cells associated with the blood vessels of the yolk sac plica. Observe fluorescence staining of cytoplasmic material throughout the endoderm cells **(plate A).** Staining of nuclei within this section using DAPI is shown in plate A', and the large, basally located endodermal nuclei are clearly visible (N). Abbreviations: V (blood vessel). Bar is 25 μ.

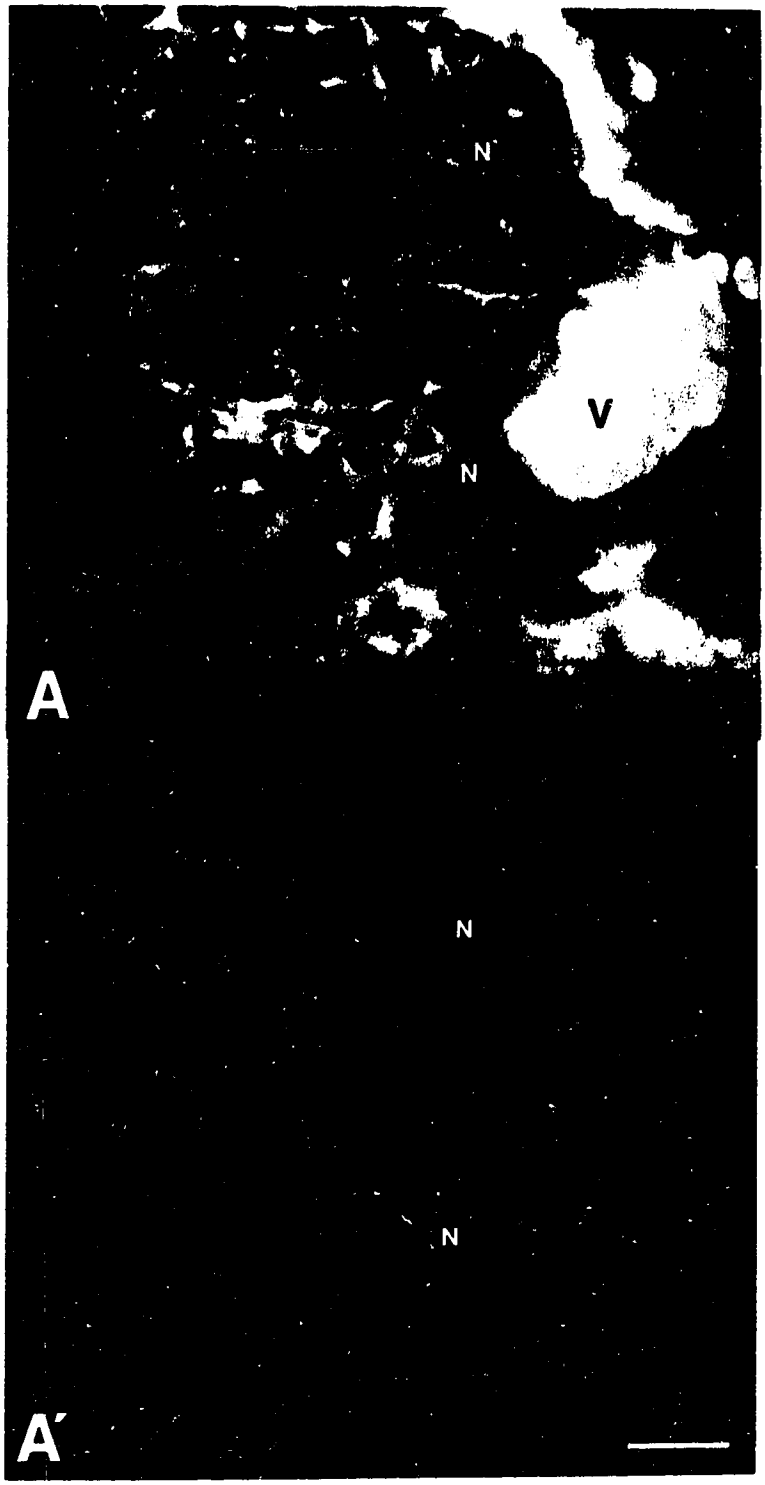
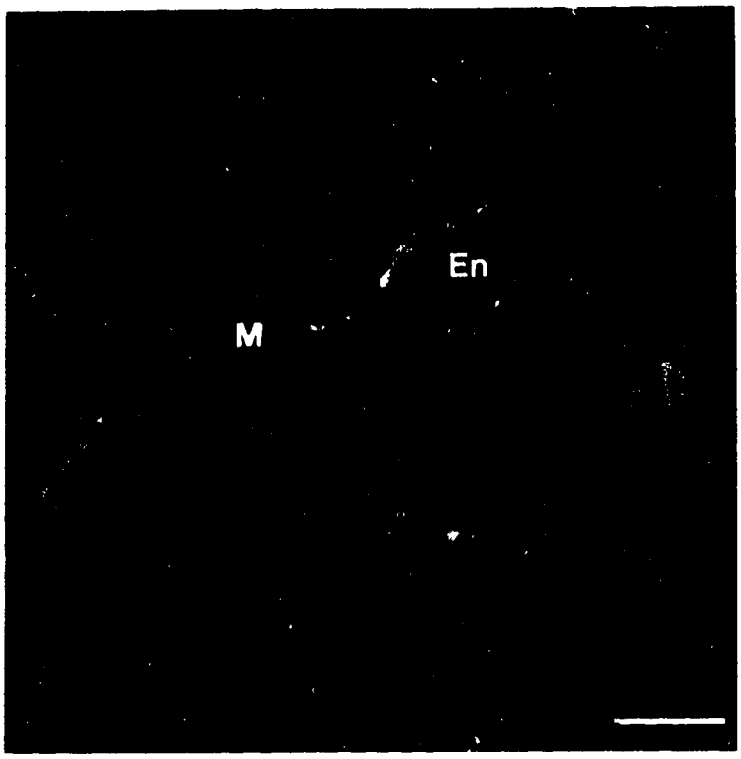

N
V
N
A
N
N
A´

Figure 23. Control staining of 15 day yolk sac membrane using a mAb to human muscle actin. Observe staining is present intracellularly in the mesoderm cells (M), but not in the endoderm (En). Bar is 50 μ.


En
M

Discussion

The experiments described in this thesis indicate that lectin activity is relatively low in the yolk sac at 2 days post-incubation and increases rapidly until 4 days, whereupon it remains stable. Previous studies have shown that lectin activity is highest in the area vasculosa, as compared to the area vitellina (Mbamalu 1986). The increase in lectin activity between 2 and 4 days occurs at the time when the area vasculosa is growing most rapidly and the definitive circulatory system of the yolk sac becomes established (Romanoff 1960). The association between specific lectin activity and expansion of the area vasculosa suggests that the lectins could be involved in the morphogenesis and maintenance of the yolk sac vascular epithelium. These lectins may further function in haematopoiesis and in the processing of yolk for use in the embryo. Harrison and Chesterton (1980) found high levels of a 13 kD S-type lectin on the surface of differentiating rabbit erythroblasts. Since the lectin agglutinated these cells *in vitro*, they suggested that the lectin acted to bridge erythroblasts transiently until they left the bone marrow.

Characterization of the yolk sac lectins:

In our experiments, examination of crude yolk sac lectin extracts by SDS-PAGE revealed the presence of a 14 kD doublet at all stages of development. A 16 kD band could not be adequately visualized in these gels, suggesting that in the yolk sac tissues this lectin is present in lower concentrations than are the 14 kD lectins. Analysis of lactoside-affinity purified yolk sac lectins by SDS-PAGE shows that lectin activity is associated with proteins having relative subunit molecular weights of 14 kD, 16 kD, 28-35 kD, and 66-70 kD. The 14 kD and 16 kD lectins were the most prominent bands seen in these gels and were found at all

stages of development. The weak 28-35 kD band which was found in lactoside-affinity purified fractions from yolk sac at some stages of development could represent homodimers of either the 14 kD or the 16 kD lectins that were not fully reduced. The 16 kD chick lectin has been described previously as a homodimer (Barondes 1986), and the embryonic 14 kD chick lectin from skin has also been found to exist, under non-dissociating conditions, mainly as a dimer (Oda and Kasai 1983). When the lactoside-affinity purified yolk sac lectins were analyzed by SDS-PAGE under nonreducing conditions a weak protein band was seen that migrated to an approximate molecular weight of 32 kD (not shown). This band was not observed when the same pure lectin extract protein fraction was run on gels in the presence of reducing agents. Immunoblot analyses of lactoside-affinity purified yolk sac lectins indicate that anti-L16 and anti-M16 recognized a 35 kD band. Whether this band represents remnant 16 kD dimer or is a lectin homologous with the mammalian 35 kD carbohydrate-binding protein (CBP35) remains to be determined. The latter lectin, also known as IgE-binding protein and Mac-2 antigen, has been localized at the cell surface and is the major laminin-binding protein synthesized by macrophages (Raz *et al.* 1989; Woo *et al.* 1990; Truong *et al.* 1993).

In the yolk sac the polyclonal anti-14 and anti-16 kD antisera exhibit cross-reactivity with the 14 kD and 16 kD yolk sac lectins. Overlapping reactivities between the chick lectins using the same antibodies has been reported in the lectins of the gastrulating chick embryo (Zalik *et al.* 1990a; 1990b; Zalik 1991), and mAbs to the 14 kD lectin from embryonic skin have been shown to cross-react with a 16 kD band (Hirabayashi *et al.* 1987). These findings are not unexpected since the vertebrate lectins share highly similar amino acid sequences, and probably contain a number of related immunoreactive epitopes. Immunoblot analyses of crude extracts showed similar lectin staining profiles

throughout development. In crude lectin extracts, anti-L16 stained bands at 14 kD and 16 kD. On the other hand, in immunoblots of crude lectin preparations anti-S14 stained bands at 25 kD and 70 kD at all stages examined and did not recognize a 14 kD band. The latter antibodies recognized the 14 kD lectin in lactoside-affinity purified preparations. The observation that a 14 kD band consistently stained in the presence of anti-S14 following purification suggests the presence of two isolectins of 14 kD in the yolk sac: 1) a 'liver-like' 14 kD lectin immunologically similar to the 16 kD liver lectin, and 2) a 'skin-like' 14 kD lectin immunologically similar to the 14 kD lectin from skin. Further investigations are required to support this hypothesis.

Following SDS-PAGE a doublet migrating at a relative Mw of 14 kD was sometimes seen in lactoside-affinity purified preparations of yolk sac membranes. A similar 14 kD doublet has been observed in lectin extracts of adult chick liver following affinity purification on asialofetuin columns (Beyer *et al.* 1980). The existence of more than one galactose-binding 14 kD lectin in the chicken has not been confirmed, but cannot be discounted. Kakita *et al.* (1991) reported that the chick 14 kD lectin from skin, which appeared as a homogeneous band following SDS-PAGE, could be separated into at least two peaks by immunoaffinity chromatography. The presence of multiple lectins having the same molecular weight from a single tissue was demonstrated by Sparrow *et al.* (1987), who were able to isolate at least five 14 kD lectin isoforms from human lung tissue by isoelectric focusing.

In the present studies mAbs were immobilized by attachment to derivatized polyacrylamide and agarose beads. In immunoprecipitation experiments it was shown that, in crude extracts, the 14 kD protein bound to derivatized antibody-free beads. In immunoaffinity experiments the 14 kD doublet was removed from the beads with low concentrations of

lactose suggesting these proteins were lectins.

Immunoblot analyses of the lactose eluates from columns containing immobilized control mouse antibodies or immobilized anti-lectin mAbs showed that the anti-S14 and anti-I14 recognized a band of 10 kD. This band was the only band present in these preparations, and no 14 kD band was present. The molecular weight of the 10 kD band does not correspond to that of any of the previously described chick lectins, but its immunoreactivity with the anti-14 kD antisera may be due to post-translational modifications of the 14 kD yolk sac lectin. Modifications in protein structure and conformation may have occurred during the eluting process or other experimental conditions. Changes in the migration of proteins in gels could be the result of events involving phosphorylation/dephosphorylation, acetylation, deglycosylation, and proteolytic degradation. Other lectins such as carbohydrate-binding protein 35 (CBP35) undergo phosphorylation, resulting in a significant change in the isoelectric point of the protein (Cowles *et al.* 1990), and P-selectin becomes selectively phosphorylated in response to platelet activation (Crovello *et al.* 1993). Although not considered to be glycosylated, little else is known of the post-translational modifications of the chick lectins (Lotan 1990; Sakakura 1990). Amino acid analysis of the chick 14 kD and 16 kD lectins has shown that the two have almost identical molecular weights of 14.9 kD (Hirabayashi *et al.* 1987; Sakakura *et al.* 1990). The anomalous mobility of the 16 kD lectin on gels is thought to involve a specific feature of its primary structure (Sakakura *et al.* 1990). It remains to be determined if the 16 kD lectin is phosphorylated or is susceptible to other modifications.

Two bands removed in the lactose eluants and having approximate molecular weights of 34 kD and 38 kD also stained following incubation with anti-I14. These protein bands were present in the eluates from both the control and the anti-lectin antibody columns. The nature of these proteins has

not been investigated, but they may be aggregates of the 14 kD lectin. Another possibility is that these protein bands are homologous with the mammalian 35 kD carbohydrate-binding protein (see above). At present we do not know the nature of these proteins. Cloning and amino acid sequence analysis of the various 'lectins' observed during these investigations is necessary to determine what their structural relationship is with the known chick lectins.

The higher molecular weight component of 68-70 kD eluted from lactoside-affinity columns corresponds to a protein previously isolated from the gastrulating chick blastoderm and the chick yolk sac having molecular weights of 70-72 kD and 68 kD respectively (Mbamalu and Zalik 1987; Zalik 1991). It is possible that this protein is a storage form of the 14 kD lectin since immunoblot analysis shows that it reacts with antibodies to S14. Another possibility is that this band represents a different lectin sharing sequence homology with the 14 kD lectin. Further work is needed to characterize this protein, and to determine if it has lectin activity. An interesting possibility exists that this protein is homologous to the 67 kD elastin/laminin (E/L) receptor isolated from mammals (Lotan 1990; Mecham *et al.* 1991; Mecham 1991). The presence of the E/L receptor in chick tissues has not been investigated.

Properties of the anti-16 kD lectin mAbs:

The function of the 14 kD and 16 kD lectins in development are not yet known. In this study, we raised mAbs to one haemagglutinating yolk sac lectin in an attempt to raise specific probes that could be used to determine the localization, synthesis, and possible physiological roles of these proteins in the developing chick embryo. Not all lectins are necessarily agglutinins; however, since haemagglutinating activity of the yolk sac lectins is developmentally regulated we initially chose to screen anti-

lectin mAbs by their ability to inhibit haemagglutination. It was also hoped that mAbs that could inhibit lectin activity could be used to perturb their physiological function *in vivo*. The mAbs were found to specifically recognize the 16 kD lectin in extracts from 15 day yolk sac, embryonic pectoral muscle, and adult liver. Since the mAbs almost completely inhibit haemagglutinating activity in crude extracts of these tissues, it is probable that the 16 kD lectin is primarily responsible for the haemagglutinating activity present in these tissues. The 16 kD lectins of various embryonic and adult tissues have identical relative electrophoretic mobilities in SDS-PAGE. The results presented here also indicate that binding of the anti-16 kD lectin mAbs to the 16 kD lectin is inhibited by lactose, raising the possibility that the mAbs may recognize an epitope within the carbohydrate-recognition domain of the 16 kD lectin. It is important to point out that the anti-16 kD lectin mAbs did not react with the 16 kD lectin band in immunoblots of SDS-PAGE. This suggests that the mAb recognizes only the non-denatured form of the lectin. Similar findings have been reported for mAbs raised to the 14 kD embryonic lectin (Oda and Kasai 1984; Oda *et al.* 1986)

In crude extracts from adult chicken intestine the anti-16 kD lectin mAbs do not inhibit the 14 kD haemagglutinin; by extension, the mAbs may not recognize the carbohydrate recognition domain of the 14 kD lectin. This finding is particularly interesting because it suggests that the β-galactoside-binding 14 kD and 16 kD chick lectins have distinct carbohydrate-recognition domains and could recognize different galactose-bearing oligosaccharides. It should be noted that the 14 kD lectin from the intestine has not been sequenced, and it is not known if it is the same as the 14 kD lectin from embryos. These proteins share several epitopes in common, since anti-I14 antiserum reacts strongly with the 14 kD yolk sac lectin. In this regard, it would be useful to determine if the anti-16 kD lectin mAbs inhibit the activity

of the 14 kD lectin present in embryonic chick skin. Antibodies that could be used to interfere with the ability of the 16 kD lectin to bind carbohydrates, but not affect 14 kD lectin binding, could potentially be used analytically to examine the respective functions of these lectins in development. Embryonic chick 14 kD and 16 kD lectins may recognize subtle configurations in oligosaccharides bearing terminal galactose residues. Knibbs *et al.* (1993) recently showed that CBP35 more actively binds tetrasaccharides than either di- or trisaccharides carrying terminal β-galactosyl residues. These authors suggest that the carbohydrate recognition domain of CBP35 contains at least three binding sites for different carbohydrate monomers, and conformation of the first three monomers within the tetrasaccharide is important in lectin-carbohydrate binding.

Localization of the lectins in the yolk sac and possible functions during development:

The localization of the lectins in the tissues of the yolk sac was examined at 5-, 7- and 15 days of development using polyclonal anti-S14 and anti-L16 antisera, and the anti-16 kD lectin mAbs to yolk sac. Lectin immunoreactivity to polyclonal antibodies is not well preserved in tissues embedded in paraffin, and preliminary experiments indicated that this was also the case when the anti-16 kD lectin mAbs were used. For this reason we decided to perform immunofluorescence staining on yolk sac tissue embedded in PEG, as this procedure had been used successfully with the polyclonal antisera to the 16 kD liver lectin and to the 14 kD skin lectin (Zalik *et al.* 1987). Results from these experiments show that the 14 kD and 16 kD lectins are distributed intracellularly throughout the extraembryonic endoderm of the yolk sac, where they are associated with the yolk platelets. In sections of yolk sac tissue the 14 kD lectin was present in the area vitellina and area vasculosa

surrounding the periphery of these platelets. In contrast, staining with anti-L16 antiserum in 5 day yolk sac resulted in localization of the 16 kD lectin in discrete organelles associated with the yolk platelets as well as at the periphery of the yolk platelets (refer to Fig. 17, plates B and D). For the purpose of this discussion I will refer to these discrete organelles as lectinosomes. The lectinosomes also stained in the presence of the anti-16 kD lectin mAbs. The nature of the lectinosomes has not been determined, but the localization of the 16 kD lectin in these structures suggests they might serve to store lectin.

At present, there is no clear understanding of how the yolk produced during oogenesis is processed in the yolk sac endoderm. However, since the yolk sac membrane is involved in the processing of yolk for utilization in the embryo, it is possible that some organelles, such as the lectinosomes and the yolk platelets, are 'lysosome-like' in their function. Ultrastructural immunocytochemistry of early embryos indicate that lectins are found at the periphery of the yolk platelets. Since yolk platelets are considered to be modified endosomes, and since lysosomes traffic and fuse with endosomes, it is possible that the lectinosomes and yolk platelets contain some lysosomal components. Lysosomal associated membrane glycoproteins, or LAMPs, are extensively glycosylated and have been identified as the major carriers of poly-N-acetyllactosamines in many cell types. In these proteins glycosylation is restricted to the lumenal portion of the glycoprotein (Lippincott-Schwartz and Fambrough 1986; Fukuda 1991). Early work on the LAMPs focused on their selective targeting to lysosomes, and their possible role in intracellular trafficking. A physiological function has not been assigned to these proteins, although evidence gathered suggests that the carbohydrate side-chains may serve to protect the lysosomal membrane from degradation by sterically inhibiting its own hydrolases (Kornfeld and Mellman 1989). The LAMPs have been localized at the cell surface, and in

chick fibroblasts they have been found to circulate continuously between lysosomes, endosomes, and the plasma membrane (Lippincott-Schwartz and Fambrough 1987; Fambrough *et al.* 1988). Fukuda (1991) has postulated that the LAMPs may be the major source of polylactosaminoglycans at the cell surface, and may act as ligands for various galactose-binding selectins. Do *et al.* (1990) have shown that the 14 kD calf lectin is a potent agglutinin of Chinese hamster ovary (CHO) cells. Experimental evidence also indicated that LAMPs at the cell surface were the primary lectin ligands. It is possible that lectin-LAMP complexes at the cell surface mediate communication and adhesion events with other cells and the ECM. Additionally, the recycling of LAMPs at the cell surface could potentially remove extracellular lectins and transport them into the cell for degradation, thus regulating the concentration of lectins outside the cell. In this scenario, down-regulation of lectins at the cell surface might control lectin-mediated cell-cell and cell-matrix interactions.

The recycling of LAMP-lectin complexes at the apical cell surface of the yolk sac epithelium might allow for the uptake of yolk constituents. Lectin activity was highest in differentiated yolk sac endoderm, and remained high throughout the stages of development examined in this study, indicating lectins may play a role in the uptake of yolk metabolites. Lectins might be secreted to the apical cell surface of the yolk sac epithelium where they could bind to appropriately glycosylated yolk constituents. One such protein, ovalbumin, is a major glycoprotein component of the yolk and carries numerous galactoside-bearing glycans (Yamashita *et al.* 1975). The area opaca in early chick embryos is the specific site for ovalbumin absorption and conversion to serum proteins (Hassel and Klein 1971). This protein is thought to enter the yolk as the vitelline membrane is displaced (Burley and Vadehra 1989). The ability of the yolk sac lectins to bind carbohydrate ligands suggests

they could bind to galactosylated yolk components and cell surface LAMPs at the same time. The LAMPs could then be cycled into the cell, and the metabolites sorted for further processing. It is not known if LAMPs are present in the cells of the yolk sac endoderm, or if the yolk sac lectins are externalized. These possibilities will require further investigation.

The rearrangement of cells within the area vitellina from a loose collective of stratified cells to a single-layered columnar epithelium in the area vasculosa is associated with differential expression of the 16 kD lectin within each region. In the area vasculosa of the 5 day yolk sac, the 16 kD lectin was localized around the periphery of the yolk platelets, and not in lectinosomes. Staining in these tissues was stronger in the area vasculosa than in the area vitellina. At 7 days, however, there was no difference in the intensity of staining between the two regions of the yolk sac, and staining of lectinosomes was not observed. By 7 days, the yolk sac has almost encompassed the yolk and consists mainly of area vasculosa and a very small margin of area vitellina. At this time the endodermal cells within the remaining area vitellina are rapidly becoming incorporated into the area vasculosa. Increased expression of the 16 kD lectin in the yolk sac endoderm appears to accompany differentiation of the area vasculosa. Staining of the 16 kD lectin remains strong in the yolk sac epithelium until at least 15 days, the latest stage studied by immunofluorescence microscopy. The cells of the endoderm at these late stages are highly vacuolated and the contents of these vacuoles are poorly preserved with the immunohistochemical procedures used in these experiments. In late-stage yolk sac tissue, during histochemical preparation, the loss of the vacuolar contents makes it impossible to determine if lectin is present in these structures. Longer fixation times might preserve more vacuolar structure prior to sectioning. Immunocytochemical analysis of non-sectioned endoderm cells using confocal

microscopy might also allow for the preservation of intracellular structure. The continued expression of high amounts of 16 kD lectin within the cytosol of differentiated yolk sac endoderm suggests that this lectin might regulate various activities inside the cell. A 15 kD lectin from mouse embryonic fibroblasts has been found to inhibit mitosis by blocking passage of these cells from the G_0 and G_2 stages of the cell cycle (Wells and Malluci 1991; 1992), and the 16 kD chick lectin has been found to promote *in vitro* the differentiation of chondrocytes from mesenchymal cells (Matsutani and Yamagata 1982). It is possible that the 16 kD lectin is involved in the differentiation of yolk sac endoderm into an epithelial cell type, and could further function in maintaining mitotic quiescence within the differentiated epithelium of the area vasculosa.

The possibility exists that the yolk sac lectins may also be involved in cellular interactions within the embryo. The 16 kD lectin has been localized at the extracellular surface of the developing mesonephros and metanephros, but is not expressed by cells of these tissues *in vitro* (Didier *et al.* 1988). Didier *et al.* (1988) suggested that lectins produced in other regions of the embryo might be transported to the developing kidney tissues where they could bind to receptors present at the cell surface. A potential source of these lectins could be the yolk sac membranes since they produce relatively large quantities of lectins (Mbamalu and Zalik 1987; Guay and Zalik 1990).

I was not able to determine if any of the lectins were present in the basement membrane of the developing yolk sac. Since the basement membrane of the yolk sac is an extremely thin structure, immunolocalization of the lectins will require the use of immunoelectron microscopy to ascertain if the lectins are present in this structure. Evidence has accumulated to support a role for galactose-binding lectins in adhesion-deadhesion processes during development. Almost all the soluble lectins from mammals have been found to bind

laminin including: the 14 kD mouse lectin (Cooper *et al.* 1991; Zhou and Cummings 1990; 1993); CBP35 (Woo *et al.* 1990); the 30 kD baby hamster kidney lectin (Sato and Hughes 1992; Sato *et al.* 1993); the 31 kD human galactoside-binding protein, hL31 (Ochieng *et al.* 1992); and the 67 kD E/L receptor (Mecham *et al.* 1989; 1991). Recent studies showed that the chick 14 kD and 16 kD lectins are localized extracellularly in embryonic skin during development (Akimoto *et al.* 1992; 1993). These authors suggested that the lectins might regulate epidermal-dermal interactions during skin differentiation by binding to the polylactosaminoglycan moieties of laminin and fibronectin. *In vitro* studies with extraembryonic endoderm cells from the area opaca have shown that blastoderm lectins inhibit the ability of these cells to adhere to each other (Milos and Zalik 1982). Cooper *et al.* (1991) have described a mouse muscle 14 kD lectin that has the ability to inhibit the binding of differentiating myoblast cells to laminin. These authors suggest that the inhibition of adhesion to the extracellular carbohydrate moieties of laminin would result in the promotion of myoblast-myoblast adhesion and myotube differentiation. In contrast, the 14 kD lectin from the pig heart enhances cell binding of CHO cells and F9 mouse teratocarcinoma cells to laminin *in vitro* (Zhou and Cummings 1993). Recently in our laboratory, experiments performed by Jeeva (unpublished) indicate that anti-L16 antiserum as well as thiodigalactoside and lactosylated BSA block expansion of early chick blastoderms in culture. Normal development was observed when blastoderms were incubated in the presence of preimmune serum, maltose, and maltosylated BSA. These experiments suggest that the 16 kD lectin may play an important role in cell adhesion during early chick development. It is hoped that her study will soon be repeated using the anti-16 kD lectin mAbs and Fab fragments from the mAbs.

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