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LIVER CELL ADHESION MOLECULES

IN EMBRYONIC DEVELOPMENT

A thesis submitted to the Faculty of The Rockefeller University

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

by

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ABSTRACT

Cell-cell adhesion is one of the essential processes for normal embryogenesis. The isolation and characterization of the neural cell adhesion molecule, N-CAM, provided the first substantial opportunity to study this process at a molecular level. Moreover, studies of N-CAM expression in embryos suggested that cell adhesion molecules may play a critical role in induction.

This thesis describes the isolation of a glycoprotein, L-CAM, that mediates adhesion between liver cells, using an assay similar to the one used to isolate N-CAM.

L-CAM was released from liver cell membranes by proteolysis with trypsin, and purified by ion-exchange chromatography, gel filtration chromatography and isoelectric focusing. The purified L-CAM was used to produce monospecific antibodies in rabbits and monoclonal antibodies from murine hybridomas. The identity of the L-CAM activity with the isolated molecule was confirmed using these immunological reagents, by demonstrating their capacity for disrupting cell adhesion and by showing that these antibodies were neutralized by the purified L-CAM molecule. L-CAM is an acidic intrinsic membrane glycoprotein of $M_r=124,000$ daltons. It is approximately 12% neutral sugar, with a small but demonstrable amount of sialic acid. The carbohydrate is distributed as four approximately equal sized asparagine-linked oligosaccharide chains. Only one of the oligosaccharide chains is susceptible to cleavage by endoglycosidase H, suggesting that it is of the high-mannose type; the other three are probably of the complex type.

Antibodies to L-CAM inhibit calcium-dependent cell-cell adhesion between liver cells in a rotary suspension culture. The same antibodies inhibit the formation of histotypic liver cell colonies that form when dissociated liver cells are cultured on a solid substrate. Colonies that have already formed can be disrupted by the antibodies to L-CAM. These histotypic colonies have some physiological properties comparable to intact livers. These results suggest that it is possible to investigate the role of cell-cell contact in some of the physiological functions of the liver using anti-L-CAM as a perturbing agent.

N-CAM and L-CAM are two different molecules, mediating different processes of cell adhesion. L-CAM, however, does bear striking structural and functional homologies to a molecule (uvomorulin) that mediates adhesion between cells in very early embryos. This similarity, considered with the known appearance of N-CAM in very early embryos, suggests that N-CAM and L-CAM may play complementary roles in the processes of early embryogenesis. In accord with this hypothesis, L-CAM was found to be widely distributed in organs of embryos and adult animals. L-CAM was found on organs that arise from all three germ layers; in the organs where it was detected, the molecule was restricted to epithelial cells, and in some cases to only part of the cell surface. This result provides support for Edelman's hypothesis that a few cell-adhesion molecules can play major roles in a variety of developing organs and tissues. L-CAM, along with N-CAM, appears to be among these cell adhesion molecules.

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PREFACE

In the twenty-one days after a chicken lays a fertile egg, the simple matter of the yolk and white is metabolized to form the cellular structures of a functioning chick. The processes that occur during this development are complex and interrelated, as are the organs that finally arise from them. All during development, cells of different lineages from diverse areas of the embryo move together and form progressively more complex tissues and organs. The combination of these cellular mixings with the differentiation of various cell lineages into functionally discrete and complex populations gives rise to the organs and tissues of the adult animal.

During development, the units of differentiation appear to be groups of contiguous cells. The premise of this thesis is that the processes that hold cells together in functional units during embryogenesis are important in development, and that characterization of these processes at a molecular level can aid in understanding how development occurs. Specifically, this thesis describes the isolation and characterization of a molecule, L-CAM, that plays a role in adhesion among liver cells.

N-CAM, a molecule that mediates the binding of neuronal cells to each other, has been isolated and characterized (Hoffman et. al., 1982). It appears to play an important role in neural development (Buskirk et. al., 1980; Edelman and Chuong, 1982). N-CAM also is present in very early embryos, and appears on and disappears from a number of cell groups during early inductive processes (Thiery et. al.,

1982). As the information on N-CAM accumulated, it became apparent that the isolation of another cell adhesion molecule would be of interest, both for purposes of comparison to N-CAM, and as an independent study of a cell adhesion molecule from another tissue.

The liver was chosen as the source of cells for this study for a number of reasons: it is a large, discrete organ containing only a few cell types; the large majority of the cells are of one type, the parenchymal cells; the liver of the chick embryo is accessible at an early stage of development; and there is a large body of information on liver histology, function, structure, and development.

As background to describing this work, the introductory section of this thesis contains a description of the functions of the liver as they are related to the histological structure of the organ, an outline of the general development of the embryo and the detailed development of the liver, and a summary of the field of cell adhesion that begins with a discussion of the potential role of cell-cell adhesion molecules in development. The next two sections describe experiments, first on the isolation and characterization of L-CAM, a molecule mediating the adhesion of liver cells to each other, and then on the localization of L-CAM in tissues of embryonic and adult chickens using immunochemical techniques. The final section is a discussion of the results in terms of the structural similarities and differences of cell adhesion molecules and their potential role in developmental processes.

HISTORY AND BACKGROUND

LIVER FUNCTION

In a fully developed and functioning animal, the liver is one of the most important organs for maintaining metabolic homeostasis. All circulation from the digestive tract passes through the liver before going to the rest of the body. The liver also produces the bile salts, which are secreted into the digestive tract to help dissolve dietary fats, and resorbs and recycles them from the blood and lymph (Hendrix, 1980). It is the main organ for detoxifying compounds either generated by the animal or introduced from the outside; drugs, for example, are oxidized, conjugated and the products secreted by the liver. A common test for liver function consists of injecting a dye into the bloodstream and monitoring the clearance of the dye into the bile. Plasma glycoproteins that have become desialated are cleared from the blood stream by the parenchymal cells (Ashwell and Harford, 1982) and particulate blood contaminants, ranging from cell fragments to artificially introduced colloids, are phagocytosed by the Kupffer cells (Benacerraff, 1958). Blood sugar is maintained by glycogenesis, glycogenolysis, and gluconeogenesis, all processes occurring in the liver. The liver is thus the primary organ buffering blood glucose levels under the influence of insulin and glucagon (Goodman, 1980).

Aside from the immunoglobulins, most plasma proteins are synthesized by the liver. Perfusion of the liver with radioactively labelled amino acids leads to the appearance of radioactively labelled

albumin, fibrinogen, alpha and beta globulins, but not gamma globulins in the perfusate. Perfusion of the hepatectomized carcass gives only trace incorporation into alpha and beta globulins in the perfusate and a high incorporation into gamma globulins (Miller and Bale, 1954).

The liver is like a large spongy filter surmounting the portal circulation, cleansing the blood and replenishing it with appropriate nutrients and serum proteins as it passes through. Topologically the liver is three continuous compartments: 1) the lacunae, continuous with the circulatory system, 2) the bile ducts and canaliculi, continuous with the interior of the digestive tract, and 3) the parenchymal cells, which form a continuous layer a single cell thick between the other two compartments, and which perform most of the metabolic functions of the liver. A necessary consequence of this topology is that the parenchymal cell itself has three kinds of surface; one facing the lacuna, another the bile canaliculus, and the third the adjoining parenchymal cell surfaces. Within the lacunae are the blood sinusoids, lined with endothelial cells and Kupffer cells. The Kupffer cells appear to be macrophages, because they phagocytose particulate material from the blood stream.

The three compartments of the liver are folded into a highly convoluted network, so that the surface area of contact between them is huge. In mammals the organization of the liver is lobular, with perforated sheets one cell thick leading from the incoming blood vessels to the outgoing blood vessels. In the chicken, the liver is a network of anastomosing tubes, two cells thick, with no real plates of

cells; the net effect is the same, however. The blood flows through the sinusoids in layers a few cells thick, allowing highly efficient filtering of the plasma and maintenance of plasma nutrient and protein concentrations.

OVERVIEW OF EMBRYONIC DEVELOPMENT

By the time a chicken's egg is laid, it has been developing approximately 20 hours (Eyal-Giladi & Kochav, 1976). The ovum proper consists of the yolk and the membrane surrounding it. The egg white and the shell are deposited around the ovum as it moves through the oviduct and the uterus. When the egg is finally laid, the embryo is a disc of cells on the surface of the yolk, the blastula; it splits into two layers, the epiblast, which gives rise to most of the embryo, and the hypoblast, which gives rise to some of the extraembryonic endoderm and the germ cells. The epiblast and the hypoblast are separated by a fluid-filled pocket, the blastocoel. Unlike the amphibian, the early cell division in the avian embryo occurs with only partial cleavage. Due to the huge mass of inert yolk, the whole ovum cannot divide, and the partial cleavages that do occur result in a thin layer of cells on part of the surface of the yolk, with marginal cells opening directly onto the yolk (Bellairs et al., 1978). Thus, the whole process of gastrulation, the migration of cells to form the three germ layers of the embryo, is different from that in the holoblastic amphibian egg.

Shortly after laying (3-4 hours) the primitive streak forms (Hamburger and Hamilton, 1951). This feature is a concave area with

ridges along each side, which marks the location of cells moving to the interior of the embryo. The primitive streak stage is equivalent to gastrulation in amphibian embryos and leads to the formation of the primordial cell layers; the ectoderm (cells remaining on the upper surface), the endoderm (cells in direct contact with the yolk), and the mesoderm (cells migrating between the other two layers).

Cells of the epiblast migrate into the primitive streak, where they undergo a change of shape and loss of tight junctions (Granhölm & Baker, 1970, Revel et. al., 1973), and then migrate downwards and outwards, under the epiblast. These cells push aside the hypoblast and form first the endoderm (8-22 hours) and then the mesoderm (after 15 hours) (Vakaet, 1970). There is not a sharp delineation between the timing of the formation of the two cell layers; a large proportion of the cells that first pass through the primitive streak, and mainly through the most cephalad part of the primitive streak (Hensen's node), become endoderm, and later the proportion of cells becoming mesodermal increases as the proportion of cells becoming endodermal decreases (Nicolet, 1970). At this stage, the cells are moving laterally from the primitive streak, forming somitic and lateral plate mesoderm, and cephalad from Hensen's node, forming the early notochord, or head process.

During the latter part of gastrulation the primitive streak is retracting caudally, leaving in its wake the three primordial cell layers. While this is occurring, two major foldings of the flat embryo, which will give rise to the nervous system and the gut of the embryo,

are starting. These processes convert the embryo from a flat sheet several cells thick into a three dimensional structure that can develop into the adult organism. After 22 hours of incubation the anterior part of the embryo has folded up and over the anterior, or proamnion, region of the blastoderm. The neural groove starts forming at the cephalic end, and the formation of this groove progresses caudally with time. The neural ectoderm folds up into twin ridges, forming the neural groove. The ridges grow up, then the dorsal edges move together medially, until the neural groove fuses at its dorsal edges, forming the neural tube, which will give rise to the brain, spine, nervous system and neural crest derivatives. The neural tube is a closed tube of ectoderm, surrounded by mesoderm.

At the same time, the lower surface of the embryo starts the tube closing that will eventually separate the body entirely from the substance of the yolk. The lateral mesoderm splits into two layers to form the coelom, and the lower layer of mesoderm and the endoderm (collectively called the splanchnopleure) on each side of the midline fold towards each other, starting at the subcephalic fold and proceeding caudally, forming the foregut. Later a similar process starts at the tail and proceeds cephalically, forming the hindgut. The net effect is that of pulling two folds of flat fabric together and zipping them up along the edges of the folds; the result is the formation of a closed tube lined with endoderm and surrounded by mesoderm. This simple tube will eventually grow and curl together to form the complete digestive tract; evaginations of this tube will bud off, interact with mesodermal tissue, and form the liver, gall bladder

and bile ducts, pancreas, and respiratory structures (Carlson, 1981).

LIVER DEVELOPMENT

The liver starts forming as a morphologically detectable rudiment in the middle of the second day of incubation (Kingsbury et. al., 1956). At this time the anterior intestinal portal, the leading edge of the closing foregut, is just caudal to the heart. The first sign of liver formation is the appearance of two evaginations of the endodermal layer at the ventral lip of the anterior intestinal portal. These evaginations continue to grow during the next two or three days, in turn sprouting off lumenated buds which invade mesenteric mesenchyme and mesocardium. By the end of the fourth day, the endodermal cells of the liver have invaded the ductus venosus, a large embryonic blood vessel that collects blood from the vitelline veins. These endodermally derived cells will become the functional parenchymal cells of the liver, and the mesodermal cells that are infiltrated by the endoderm will form the endothelial cells lining the blood sinusoids and capillaries of the liver. The developing liver tissue forms a network of anastomosing tubules, with walls two cells thick, totally occupying the ductus venosus. Thus, all blood flowing through the vitelline veins to the heart must pass through the liver. By the seventh day of incubation, the liver is a discrete organ within the coelom and its gross form is set. The major developmental changes in the liver after day seven are differentiation of the cells and growth of the organ to fill the available space (Kingsbury et. al., 1956).

At this stage the ductus venosus and other circulatory vessels in the septum transversum have been invaded by the budding hepatic endoderm and its associated mesoderm, resulting in a finely divided blood filter. During this development into a highly ramified filter structure, the topology of the liver does not change. There are two extracellular surfaces separated by a layer of tightly connected cells; one surface is exposed to the portal circulation, the other surface is still continuous with the lining of the gut. The first surface becomes lined with a fenestrated layer of endothelial cells and some connective tissue. The second surface consists of the bile fronts of the cells. Secretion of metabolized and detoxified breakdown products and bile salts from the liver parenchymal cells occurs on this surface, into the bile canaliculi, which feed into bile ducts, and finally lead into the gall bladder and the common bile duct.

Along with this morphological description of the development of the embryo leading to the formation of the liver, it is important to consider the tissue interactions that lead to the induction of liver formation. This problem has been approached by Le Douarin (see review by Croisille and Le Douarin, 1965) using techniques of ablation and transplantation of tissues in different areas of the early embryo at different times of development, and by using implanted barriers in specific locations within the embryo at different times. By using these techniques, she has described a series of interactions between mesoderm and endoderm that, although not correlated with detectable morphological changes at the time they occur, are essential for the normal development of the liver. The origins of the cells have been

traced by transplanting tissues from quail to chick embryos. The constructed chimeras develop normally, but the quail cells have a highly heterochromatic nucleus, which makes them easily distinguishable from chick cells.

Two induction steps have been defined by these approaches. The first is an interaction between the prehepatic endoderm and the hepatocardiac mesoderm prior to the five somite stage (approximately 29 hours after laying). Presumptive hepatic endoderm removed up to the four somite stage (approximately 25 hours) and implanted into the area of presumptive hepatic mesenchyme will fuse with the local endoderm and integrate into the normal gut structures. This finding indicates that up to this point in development the endoderm has not been induced to differentiate through a hepatocyte pathway. The same endodermal tissue taken from 5-somite and later stage embryos, implanted onto presumptive hepatic mesoderm will differentiate into hepatoblasts. This induction is specific; only hepatocardiac mesoderm will induce, and only the prehepatic endoderm will respond. The primary induction is necessary but not sufficient for the prehepatic endoderm to form hepatocytes. If the prehepatic endoderm after primary induction is isolated from the overlying mesoderm, it will not develop into hepatocytes; in fact, it will dedifferentiate if not associated with some mesenchyme. A secondary induction, by hepatic mesenchyme, is required.

If the prehepatic endoderm is grafted onto a host embryo's hepatic mesenchyme after primary induction, with a barrier inserted to prevent the host hepatic endoderm from invading, it forms normal liver

structures. Even hepatic mesenchyme from a five-day embryo cultured in vitro will support the development of a lobe of liver tissue from prehepatic endoderm. This secondary induction is not as specific as the primary induction. Any mesenchyme of mesenteric or peritoneal origin will mediate the secondary induction, but other non-hepatic mesenchymes (somitic, cephalic or mesonephric) will only permit survival without inducing differentiation.

To elucidate the contributions of mesoderm and endoderm to the final liver structure, a barrier of shell membrane can be inserted in an embryo caudal to the primary hepatic primordium, preventing the endodermal invasion of the posterior hepatic mesoderm. By the fifth day of incubation, the posterior mesoderm has formed a network of cellular strands arrayed around the central vein, with the interior of the meshwork communicating with the vein. Grafts of presumptive hepatic endoderm into this area allow the progress of the endoderm to be traced. Such grafting studies show that, at the fourth day of incubation, endoderm invades the mesenchyme, pushing the mesodermally derived cells aside, until, by the sixth or seventh day, the strands are exclusively endodermally derived, and the only cells of mesodermal origin are those that make up the endothelium lining the blood sinusoids. The interpretation of this result is that the mesoderm forms a loose mesenchymal network into which endodermal cells migrate; these endodermal cells will develop into the functional hepatocytes. The mesenchyme seems to act as a framework, defining the architecture of the organ, onto which the cells that will form the liver parenchyma grow; later it differentiates to form the lining of the sinusoids.

Thus there is a very intricate relationship between the inducing tissue (mesoderm) and induced tissue (endoderm). Not only does contact with the mesoderm cause the endoderm to differentiate in a defined pathway, it also participates in the morphogenesis of the organ.

CELL ADHESION

The origin of the notion that cell adhesion plays a role in embryogenesis and organogenesis is usually traced to the work of Wilson (1907), who dissociated sponges by forcing them through a fine mesh. The cells reassembled into multicellular clusters, and over the period of a few days reformed into small sponges. Galtsoff (1925) later found that cells from different sponge species would preferentially reaggregate with cells from the same species in a mixture of cells from two species of sponge. It was observed that the cells, after aggregation, underwent motion in the clumps to form the morphologically recognizable tissues of a functioning sponge. On the surface, these processes appear similar to those observed in the normal developmental sequence of sponges.

The aggregation of cells from dissociated tissues from higher organisms has also been found to be a common phenomenon. Sea urchin gastrulas and blastulas dissociated by removal of calcium ions from their sea-water will dissociate into single cells. When calcium is replenished, the cells reaggregate and can form embryos that develop normally (Giudice, 1962). Because the different kinds of blastomeres of sea urchins can be readily separated once dissociated (Hynes & Gross,

1970), it is possible to make aggregates lacking one or two of the three types of cells. If aggregates lacking any of the blastomeric cells are allowed to develop, they produce structures lacking the tissues that are the lineal descendents of the missing blastomeres. From these experiments it is clear that reaggregated cells do not dedifferentiate to stem cells and then redifferentiate to form a complete embryo, but rather maintain their level of differentiation, and upon reaggregation reassemble themselves into such a configuration that normal development can continue. This finding indicates that adhesion and subsequent cell movement can be taken to be minimum requirements for organogenesis and histogenesis, assuming that this reassembly process is analogous to normal developmental processes.

Similarly, tissues from gastrulas of amphibia can be dissociated by raising the pH and will reaggregate when the pH is dropped to normal. Townes and Holtfreter (1955) showed that dissociated cells of different embryonic germ layers reassociated and then underwent sorting out and differentiation producing aggregates with very specifically arranged cell layers; recognizeably differentiated tissues arose from the appropriate germ cell layers, and the differentiated tissues often appeared to move as cell groups, rather than individual cells.

All of these phenomena argue that cell-cell adhesion plays a role in the sorting out of tissues during development, as well as some role in induction of differentiation, if only in mediating contact between tissues that then interact in an inductive process. It also suggests that the adhesion must be plastic to allow movement of cells within an

aggregated structure, i.e. a set of moving seals rather than a rigid skeletal framework is required.

To use an in vitro cell aggregation system to study the role of cell adhesion in development requires a single-cell suspension of the desired tissue. Whereas simple invertebrates and early embryos of the higher animals can be dissociated by simple mechanical means or by alterations in the ionic environment, the compact cellular tissues of later stage embryos require dissociating conditions such as adding proteases to disrupt the connective tissue (collagenase, elastase, hyaluronidase, or ill-defined protease mixtures), proteases to disrupt cell-cell interactions (usually trypsin), and some manipulation of calcium ions, which appear to be involved in stabilizing many cell-cell interactions and the structure of some proteins.

The key to studying the molecular basis of cell-cell adhesion is the design of meaningful assays. Once cells are dispersed, there are a number of ways to study their interactions (Frazier & Glaser, 1979). Cells can be grown on tissue culture plates and their behaviour observed (Rubin et. al., 1977, Damsky et. al. 1981). A monolayer can also be used as a target surface for the binding of cells in suspension (Thiery et. al, 1977a).

The most commonly used systems for studying cell adhesion are those using cell suspensions in rotary cultures. Moscona (1961) pioneered the use of rotating cultures of single cells in studying aggregation, and Roth and Weston (1967) developed a modification of this technique by studying the binding of single cells to preformed

aggregates. Rotation cultures of single cells yield, after 24-48 hours, aggregates of cells with a distinctive and reproducible morphology. These aggregates typically show a recovery of some of the histological structures present in the tissue from which the cells originated, and are thus called histotypic aggregates. It appears that some normal developmental steps may be occurring in these aggregates, but this is not a simple phenomenon. As Holtfreter (1944) realized, adhesion is occurring, followed by histotypic sorting. The assay in which simple binding of cells to an aggregate over a period of several hours is measured (Roth and Weston, 1967) isolates the first step, adhesion, from the latter sorting steps, but even this assay requires 3-6 hours, during which time considerable cell movement can occur. To study the very early events of cell adhesion, aggregation detectable on a much shorter time scale is necessary. Many of the current assays used for studying cell adhesion use rotating culture assays, but under conditions in which aggregation is detectable in less than thirty minutes (Orr and Roseman, 1969; Brackenbury et al., 1977; Bertolotti et al., 1980; Ocklind and Obrink, 1982).

Analysis of cell adhesion at a molecular level also requires assays that can detect adhesion mediating molecules on cells and in solubilized extracts during fractionation procedures. Such assays have taken two main forms. One approach is to study the effects of extracts of cells, cell membranes, or material from conditioned medium directly on adhesion, either showing inhibition of aggregation or enhancement of aggregation. The underlying assumption in this kind of assay is that the molecules involved in the adhesion events will show some direct

effect in spite of the fact that they are no longer present on a cell. This assumption might be valid for an extracellular molecule that serves a bridging function; such a molecule would be expected to enhance cell-cell adhesion, as has been shown in sponge cell-cell adhesion systems (Humphreys, 1963). Such an assumption would also be appropriate for a molecule that binds adhesion sites, competes with cell-surfaces for the sites and thus inhibits aggregation, or even (through some more complex mechanism) enhances aggregation. Assays of this type have been used by Henkart and his colleagues (1973) to isolate an aggregation molecule from sponge, by Jakoi and Marchase (1979) to isolate a protein from retina, called ligatin, and by Hausman and Moscona (1975) to isolate a protein from retina, called cognin.

An alternative approach was developed by Huesgen and Gerisch (1975) for studying adhesion sites in slime mold, and was adapted in our laboratory (Brackenbury et al., 1977) for studying mechanisms of adhesion in cells of the embryonic chicken. This approach is based on the idea that an antiserum containing an antibody to an adhesion molecule, when incubated with cells, will bind to adhesion sites on the cell surface and block adhesive interactions between cells. This inhibition was achieved with antisera raised against whole cells of the chicken embryonic neural retina and liver. The assay is more useful when the antibody is incubated with material (e.g. membrane extracts) containing the adhesion molecule prior to incubating the antibody with the cells. The ability of the antibody to block the adhesion is neutralized, and the assay is then equivalent to a radioimmunoassay, with the measured response being cell aggregation instead of bound

radioactive antigen. Neutralization of this type was accomplished with whole cells, cell membranes, and soluble extracts of the membranes for the antibodies to chicken embryonic neural retina cells and liver cells. These findings formed the basis of an assay for a cell adhesion molecule that was successfully identified (Rutishauser et. al., 1976; Brackenbury et. al., 1977; Thiery et. al., 1977) and isolated (Hoffman et. al., 1982).

Although both ligatin and retinal cognin are purified proteins that affect cell adhesion, experiments on their activity have been restricted to the in vitro sytems in which their activities were first defined. N-CAM is the first cell adhesion molecule that has been structurally characterized and shown to have a role in in vivo developmental processes. N-CAM is a cell-surface glycoprotein, containing 30% sialic acid, probably as polysialic acid (Hoffman et. al., 1982). The purified molecule is subject to degradation by some intrinsic activity to a protein fragment of $M_r=65,000$ daltons, free of detectable sialic acid, that appears to include the domain responsible for cell adhesion. N-CAM in adult neural tissue has the same polypeptide structure as N-CAM in embryonic tissue, but contains only 10% sialic acid (Rothbard et. al., 1982). N-CAM has been shown to function in the adhesion of neurites to each other in the outgrowth of neurite bundles from explanted ganglia (Rutishauser et. al., 1978). Anti-N-CAM F_{ab}' fragments disrupt the development of normal cell layers in cultured whole retinas (Buskirk et. al., 1980). Anomalies in the normal conversion of N-CAM from embryonic to adult form have been implicated in the abnormal development of the brain of the staggerer

mouse mutant (Edelman and Chuong, 1982). N-CAM was also shown to undergo temporal and spatial alterations in amount in tissues of the early embryo that are undergoing induction (Thiery et.al., 1982).

These findings suggest that N-CAM is of special importance in development. Another cell adhesion molecule would be of value, both for comparison with N-CAM, and for furthering our knowledge of the roles that cell adhesion molecules play in developmental processes. Because N-CAM, isolated from neural cells, had a general distribution in the early embryo, it also seemed possible that another cell adhesion molecule would reveal information about embryogenesis that was not obvious from morphological studies alone. The reasons for choosing the liver cell adhesion molecule were enumerated above.

The approach used for the isolation of N-CAM was adapted for the isolation of the cell-cell adhesion molecule from liver (L-CAM). Prior to the studies described here F_{ab}' fragments of IgG from rabbit antisera raised against chick embryo hepatocytes were shown to inhibit cell aggregation in a concentration-dependent manner and plasma membranes of embryonic chicken liver and extracts of the membranes were shown to neutralize the effect of the F_{ab}' fragments in a concentration-dependent manner (Bertolotti et. al., 1980). We extended this approach and isolated a tryptic fragment of a membrane protein that would neutralize the inhibitory activity of anti-hepatocyte F_{ab}' fragments. Antibodies to this single molecule, L-CAM, would in turn inhibit aggregation of liver cells (Gallin et. al., 1983). These studies comprise a major portion of this thesis. In addition, we have

prepared monoclonal mouse antibodies and specific polyclonal antibodies to L-CAM. I will describe how these antibodies have been used to disrupt liver cell aggregates in culture, a model system for liver cell function. The antibodies have also been used to localize L-CAM in the tissues of the developing chick embryo and the adult animal. From these studies it appears that L-CAM, although originally isolated from the developing liver is widely distributed throughout the developing embryo, and is present in many organs of the adult chicken besides the liver.

PURIFICATION AND CHARACTERIZATION OF L-CAM

INTRODUCTION

Cell-cell adhesion of embryonic liver cells is tissue specific, when compared to neural cells (McGuire and Burdick, 1976) and species specific, when compared with rat, mouse, and guinea pig (Grady and McGuire, 1976; Obrink et. al., 1977). Earlier findings suggesting lack of tissue and species specificity (Moscona, 1957) were probably due to the fact that static culture conditions were used, which forced cells into contact for extended periods of time. Such conditions could well allow formation of aggregates via non-specific cell interactions.

A series of in vitro assays have demonstrated three independent adhesion mechanisms in juvenile rat hepatocytes: cell-cell, cell-collagen, and cell-fibronectin (Rubin et. al., 1977; Rubin et. al., 1978; Rubin et. al., 1981). The three systems were shown to be structurally independent by the use of antisera of limited specificity to inhibit the three processes independently (Ocklind et. al., 1980; Rubin et. al., 1979).

The fact that antibodies can inhibit an adhesion phenomenon has been used to develop an assay for the adhesion molecule with which the antibodies interact (Huesgen and Gerisch, 1975; Brackenbury et. al., 1977). This approach was used to define a liver cell adhesion molecule (L-CAM) (Bertolotti et. al., 1980; Nielsen et. al., 1981; Ocklind & Obrink, 1982) as the cell surface molecule(s) having the ability to neutralize the inhibition of cell-cell adhesion by a

polyspecific antiserum. A number of studies achieved the partial purification of small amounts of cell surface molecules enriched in such neutralizing activity. An activity in the neutralization assay described above was extracted from embryonic chicken liver membranes with either EDTA or sodium deoxycholate and the molecule responsible for the activity was tentatively identified as a protein of $M_r=68,000$ daltons (Bertolotti et. al.,1980; Nielsen et.al.,1981) whereas the molecule associated with the activity from juvenile rat liver was identified as a protein of $M_r=105,000$ daltons (Ocklind and Obrink, 1982). This discrepancy suggested that either there was a significant difference between liver cell adhesion molecules between species, or that the intact L-CAM had not yet been identified.

To gain insight into the mechanisms of cell adhesion it was crucial that the L-CAM molecule be unequivocally identified, and sufficient L-CAM be prepared to characterize it in chemical terms. It was also important to have specific, high titer antibodies for immunochemical and immunohistological experiments designed to elucidate the function of L-CAM.

This section of the thesis describes the identification of L-CAM, the purification of L-CAM in milligram quantities, the production of mouse monoclonal antibodies to L-CAM, and the preparation of highly specific rabbit antibodies to L-CAM. In the course of these studies, it became clear that the earlier descriptions of the L-CAM molecule were incomplete, most probably because L-CAM is readily degraded to small fragments, many of which can be detected by most adhesion-

blocking antisera. L-CAM is an acidic intrinsic membrane protein of $M_r=124,000$ daltons. The remainder of this section describes the identification of some of the proteolytic fragments and preliminary studies on the composition and structure of the molecule.

MATERIALS AND METHODS

Polyacrylamide gel electrophoresis in SDS (SDS-PAGE) for analytical and preparative purposes was performed by published methods (Laemmli, 1970). Protein was assayed by Coomassie blue binding (Bradford, 1976) in complex protein mixtures. Purified L-CAM protein was measured by the method of Lowry et. al. (1951) using bovine serum albumin as a standard.

Chick embryos were obtained from fertile White Leghorn eggs that were stored at 4-10°C for up to a week, until development was started by incubation in a commercial egg incubator. Embryos from eggs incubated 10 or 11 days were used for preparing liver cells for immunization, assay and culture, because they gave good yields of cells that demonstrated robust aggregation behaviour. Embryos after 14 days of incubation were used for preparing membranes for the purification of L-CAM.

Preparation of cells for immunization, assay, and culture- 30 to 90 embryos of 10-11 days of age were removed from the eggs and the livers were immediately dissected and placed in ice-cold Eagles minimum essential medium with salts modified for suspension culture (SMEM,

Gibco). When all of the livers were collected, the cold medium was decanted and the cells were washed twice, for 5 minutes each time, at 37°C, in 40 ml SMEM, on a rotary shaker shaking at 150 rpm. Following this procedure the cells were incubated, at the same temperature and shaking conditions, in 15-20 ml an enzyme solution called CTB [0.1% collagenase (Worthington CLS), 0.1% trypsin (Difco, 1:250), 10% heat inactivated calf serum, in 0.8% NaCl, 0.03% KCl, 0.005% NaH_2PO_4 , 0.0025% KH_2PO_4 , 0.1% NaHCO_3 , 0.2% glucose]. The first CTB supernatant was discarded, and the livers were shaken with 15-20 ml. of fresh CTB for 5 minutes. The livers were dissociated in the second CTB supernatant by trituration with a wide-mouthed Pasteur pipette. The cell suspension was then transferred to two iced centrifuge tubes with 1 ml of heat inactivated calf serum in each, cold SMEM-DNAase (10 ug/ml) was added to bring the final volume to 15 ml per tube and the cells were pelleted at speed 3 in a clinical centrifuge (International Equipment, Model CL) for 4 min. The supernatant was decanted and the cells were resuspended to 5-8 ml in cold SMEM-DNAase. Clumps of cells were removed by passing the cell suspension through a coarse Nitex filter (80 mesh), the volume was brought to 15 ml with cold SMEM-DNAase and the cells were pelleted again. The pellet was resuspended in 5-8 ml of SMEM-DNAase and the suspension was passed through a fine Nitex filter (225 mesh). The suspension was brought up to 15 ml in cold SMEM-DNAase, the cells pelleted, and the pellet was resuspended to 5-8 ml in SMEM-DNAase. The cells were counted and the suspension used as described in the following sections.

Rabbit antibodies- For anti-hepatocyte antibodies, $1-2 \times 10^7$ hepatocytes were washed twice with Hanks balanced salt solution (Gibco) to remove DNAase and restore calcium. A final volume of 1.5-2 ml of cell suspension was emulsified with an equal volume of Freund's adjuvant (complete for first injection, incomplete for subsequent injections) and the emulsion injected subcutaneously into a rabbit. Injections were at six week intervals, and blood was collected on four occasions, one week apart, starting 10 days after each injection.

Antibodies to samples in solution were prepared by emulsifying the solution with Freund's adjuvant and injecting subcutaneously. The first injection was followed by a boost three weeks later, with four weekly bleedings started 10 days after the second injection. Subsequent injections were at six week intervals with similar bleeding schedules.

Antigens in polyacrylamide from preparative SDS-PAGE were prepared by homogenizing the polyacrylamide in PBS (8 g/l NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4 , 0.15 g/l Na_2HPO_4 , pH 7.4) with a loose-fitting Dounce homogenizer, and then emulsifying the suspension with Freund's adjuvant. The injection and bleeding schedules were the same as for the soluble antigens.

IgG was prepared from the sera by precipitating with 37% ammonium sulfate followed by chromatography on DE-52 (Williams and Chase, 1967). The IgG was stored lyophilized at -20°C .

F_{ab} fragments were prepared from IgG by pepsin digestion, followed by reduction and alkylation (Brackenbury et. al., 1977).

Stock solutions that were 10 mg/ml IgG before digestion were stored frozen in PBS until ready for use.

Hepatocyte primary cultures- $2-3 \times 10^6$ cells were plated in 2 ml of DMEM (Dulbecco's minimum essential medium)-10% calf serum in 35 mm tissue culture dishes (Costar), or $5-10 \times 10^6$ cells in 5 ml of medium in 60 mm dishes, and grown at 37°C in 10% CO_2 . Cells stuck down and formed colonies within 5 hours of plating.

Aggregation assay- In this assay aggregation is measured as the disappearance of single cells during an incubation which allows cells to bind to each other, forming a few large aggregates from many small cells. F_{ab} ' fragments of antibodies that bind to molecules mediating adhesion block adhesion by preventing the normal interactions of such molecules. If, however, the F_{ab} ' fragments are preincubated with the appropriate antigen, the antibodies bind to the antigen, not to the cell surface, and the effect of the antibody on aggregation is neutralized.

5 ml plastic Falcon tubes were set up at room temperature, appropriate volumes of the F_{ab} ' solution to be used (usually 50 to 200 μl) were aliquotted, and the sample to be tested for L-CAM activity was added, in a total of 400 μl of assay buffer (8 g/l NaCl, 0.35 g/l KCl, 0.16 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.18 g/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2.4 g/l HEPES, pH 7.4). The neutralization was allowed to proceed for 15' at room temperature, then the samples were cooled on a water-ice slurry. $2-3 \times 10^6$ cells from a freshly prepared suspension were added to each tube, and the reaction mixtures were incubated 15' on ice.

To start the aggregation, Eagles minimum essential medium (EMEM), which contains calcium, was added to a final volume of 2 ml. A 200 μ l aliquot of the cell suspension was taken as the $t=0$ timepoint. The remainder of the suspension was transferred to a clean 20 ml scintillation vial and shaken at 37°C, at 90 rpm in a shaker-incubator (New Brunswick Scientific). 200 μ l aliquots were taken from the vials at 25' and 40' after the incubation was started.

All aliquots were immediately diluted into 1 ml of PBS-1% glutaraldehyde if counting was to be in a Cytograf counter, or into 12 ml of PBS-1% glutaraldehyde if the counting was to be done using a Coulter counter. The PBS-1% glutaraldehyde totally halts aggregation, maintaining a stable cell count for up to 24 hours in the case of these hepatocytes.

The fixed cells were counted in electronic cell counters. Calculations of aggregation, inhibition of aggregation, and neutralization of that inhibition are shown in Table 1.

Preparation of liver cell membranes- Livers from approximately 1800 14-day embryos were collected into ice-cold SMEM. The livers were then homogenized in 15-20 ml batches in 1.4 l 1 mM NaHCO_3 , 0.5 mM CaCl_2 , 2 mM iodoacetamide (Sigma), 1 mM PMSF (phenylmethylsulfonyl fluoride, Eastman), with 6-8 strokes of a loose-fitting Dounce homogenizer, and diluted to a final volume of 18 l in the same buffer. Crude membranes were collected in a Sorvall SZ-14 rotor with continuous-flow attachments, at 7500 rpm with a flow rate of 235 ml/min. The crude membrane pellet was resuspended using a loose-fitting Dounce

Table 1 - Calculations for the assay for L-CAM activity

1) Calculation of percent aggregation at time=t

$N_{i,t}$ = number of particles counted in tube i at time t

$$\%aggregation = \frac{N_{i,0} - N_{i,t}}{N_{i,0}}$$

2) Calculation of percent inhibition of aggregation by antibody

$$\%inhibition = \frac{(\%aggregation, control - \%aggregation, antibody)}{(\%aggregation, control)}$$

3) Calculation of percent neutralization of F_{ab}' by a sample

$$\%neutralization = \frac{(\%agg. \text{ with } F_{ab}' + \text{sample}) - (\%agg. \text{ with } F_{ab}')}{(\%agg. \text{ with no } F_{ab}') - (\%agg. \text{ with } F_{ab}')$$

homogenizer, the volume measured, and the membrane suspension was made to 48% in sucrose by adding 24/11 volumes of a 70% stock solution. A 1350 ml discontinuous sucrose gradient was set up in the SZ-14 rotor, with zonal run attachments, with the sample overlaid with equal volumes of 37%, 41%, and 45% sucrose. The gradient was centrifuged 10 hours at 17000 rpm and the material above the 45%-48% interface was collected, diluted 1:1 with cold distilled water, and centrifuged 40' at 12,000 rpm in a Sorvall GSA rotor to collect the membranes. The membrane pellet was resuspended to a final volume of 40 ml in L-CAM assay buffer, and stored at -70°C.

Crude membrane preparation- For monoclonal antibody (see below) affinity purification of L-CAM, a membrane fraction of lower purity but giving a much higher yield of L-CAM was prepared. The 1800 livers were handled in three batches. 600 livers were homogenized in a total of 500 ml of homogenizing buffer. Step gradients consisting of 12 ml of 48% sucrose overlaid with 50 ml of sample, in polycarbonate tubes, were centrifuged at 35,000 rpm for 30' in Beckman Ti45 rotors. The supernatant was carefully decanted and the membranes floating as a loose layer on top of the sucrose were collected. All material adhering to the walls of the tube was discarded. The combined pellets were resuspended by using a loose-fitting Dounce homogenizer and stored at -70°C.

Extraction of L-CAM from plasma membranes- Membranes were thawed and washed twice with L-CAM assay buffer by resuspending and then centrifuging at 19,000 rpm in a Sorvall SS-34 rotor. The final washed

pellets were resuspended, the protein concentration measured using the Bradford (1976) assay, and the concentration adjusted to 0.7 mg/ml protein.

The membrane suspension was then prewarmed 5' at 37°C in a water bath, and trypsin (Worthington, 2X crystallized) was added to 50 ug/ml. This suspension was incubated 30' at 37°C, with occasional swirling. The digestion was stopped by adding 1/100 volume of 35 mg/ml PMSF in ethanol (final concentration, 2mM) in four equal aliquots with mixing between additions. The mixture was then centrifuged at 19,000 rpm for 20' (Sorvall SS-34 rotor) and the clarified supernatant was collected and dialysed against two 2 liter changes of HC buffer (10 mM HEPES, 1 mM CaCl_2 , pH 7.4) overnight.

Fractionation of activity- The dialysed extract was centrifuged (10,000rpm for 20') and the clear solution (approximately 150 ml) was loaded onto a column (2.5 X 1.2 cm) of DEAE-cellulose (Whatman, DE-52) equilibrated in HC buffer. The column was eluted with a linear gradient of 25 ml each of HC buffer and HC buffer that was 0.3 M in NaCl; the column was then washed with 0.5 M NaCl in HC buffer. Fractions (2 ml) were collected, and those containing activity were pooled and dialysed against 5 mM HEPES, 0.5 mM CaCl_2 , pH 7.4, and lyophilized. The dried material was redissolved in 3 ml of assay buffer and loaded onto a column (75 X 1.7 cm) of Sephadex G-150 (Pharmacia) equilibrated in the same buffer. Fractions (2 ml) containing activity were dialysed against 5 mM HEPES, 0.5 mM CaCl_2 , pH 7.4, and lyophilized.

The lyophilized material was dissolved in 2 ml of a 1.5% solution of LKB Ampholines (pH3.5-10) and dialysed against 100 ml of the same Ampholine solution for four hours. The sample was then fractionated by isoelectric focussing in a sucrose gradient (10-40%) in a column (15 X 1 cm) (Holtfund and Kristensen, 1978). Fractions (0.5 ml) were collected by aspiration from the meniscus. Fractions containing active material were pooled, dialysed into L-CAM assay buffer, and stored frozen at -20°C.

Preparation of monoclonal antibodies- Mice were injected intraperitoneally at two week intervals with approximately 2 ug of L-CAM in 200 ul of L-CAM assay buffer, emulsified with Freund's adjuvant (complete for the first injection, incomplete for subsequent injections). Three days after the last injection spleens were taken from the mice, and cells prepared by forcing the spleen through an 80 mesh stainless-steel screen. The cells were washed in DMEM and the red cells lysed by incubation with NH_4Cl . The isolated spleen cells were counted and mixed 1:5 with P3U myeloma cells harvested in mid-log phase of growth. The cell mixture was pelleted together and fused using polyethyleneglycol (Marshak-Rothstein et. al., 1979). The fused cells were cultured in growth medium overnight to allow recovery, and the fusion mixture was then plated in 96-well plates in HAT selective medium (Marshak-Rothstein et. al., 1979) at a dilution of 10^6 original spleen cells per ml.

Ten days later the supernatants from all wells were assayed for immunoglobulin which bound to liver cell membranes (prepared as

described above and bound to 96-well plastic plates) and to brain cell membranes (prepared and assayed as described by Hoffman et. al., 1982). All cultures that showed binding to liver membranes but not to brain membranes were grown to higher cell number and reassayed. Those clones that were still positive for liver binding and negative for brain binding were cloned by the method of limiting dilution. Positive clones from this step were grown in culture, several aliquots were frozen, and mice previously primed with pristane were injected intraperitoneally with hybridoma cells. Ascites fluid was collected, usually starting 10 to 14 days after injection. Clarified ascites fluids were made to 45% saturation with ammonium sulfate and the precipitated protein was collected by centrifugation (15,000 rpm, 10', Sorvall SS-34 rotor). The protein was redissolved in a volume of PBS equal to the original volume of the ascites fluid, dialysed against several changes of PBS, clarified by centrifugation, and used for further analysis.

Coupling of antibodies to Sepharose CL-2B- Approximately 60 g of Sepharose CL-2B was washed thoroughly with water and resuspended in 150 ml of water. This slurry was stirred and the pH adjusted to 11.0-11.5 with 6 N NaOH. 10 g CNBr dissolved in 250 ml of water were added to this stirring slurry and the pH maintained between 11.0 and 11.5 with 6 N NaOH. The activation reaction usually took 5-10 minutes, and was considered complete when the pH remained unchanged without addition of further NaOH.

The reaction was then cooled by adding crushed ice. This cooled mixture was washed on a 350 ml coarse sintered glass funnel by sucking

the pellet to a moist cake, then washing with 2 l of ice-cold water, followed by 500 ml of ice-cold PBS. Equal volumes of the final washed moist cake and 2 mg/ml solutions of partially purified ascites Ig in PBS were mixed and gently shaken at room temperature for 3 hours, then at 4°C overnight. Coupling was usually 75-90%, and never less than 50%. The slurry of derivatized beads was washed with whatever buffers were appropriate for a given experiment.

Amino acid composition- Duplicate samples of protein were hydrolysed in vacuo, in 6 N HCl, at 110°C, for 24, 48, and 72 hours. Amino acid analyses were performed on a Beckman M-121-MB amino acid analyser. Amino acid composition was determined by extrapolating the values obtained from the analyses back to the zero time for easily hydrolysed amino acids (serine and threonine) and to maximum yield for slowly released amino acids (valine and isoleucine). Tyrosine and phenylalanine were maximum at the 48 hour timepoint, so that value was used for these two amino acids.

Sugar analyses- Neutral sugars were determined by the phenol-sulfuric acid assay (Ashwell, 1966) using glucose as a standard. Analyses were performed on several aliquots of the same sample that was used for amino acid analysis.

Lectin binding experiments-Crude trypsin extracts of liver membranes were shaken with lentil lectin bound to Sepharose (Pharmacia) at a ratio of 1 ml of extract to 0.1 ml of packed beads, for one hour at 4°C. The supernatant was reabsorbed the same way and aliquots of each supernatant were saved for analysis. The lentil lectin bead

pellets were washed twice with assay buffer, then eluted with 10 volumes of 0.1 M alpha-methyl-D-glucoside in L-CAM assay buffer for two hours at 4°C. The eluates were saved for analysis.

200 ul aliquots of the original extract and each of the supernatants and eluates were dried down under vacuum, dissolved in SDS-PAGE sample buffer, and run on 7.5% SDS-polyacrylamide gels. The separated proteins were electrophoretically transferred to nitrocellulose (Towbin et. al., 1979), and the resulting immunoblots were visualized with a rabbit anti-L-CAM antibody or a rabbit anti-L-CAM antibody, followed by ^{125}I -labelled protein A.

Glycosidase digestions- Three enzymes were used for digesting carbohydrate portions of the L-CAM molecule: endoglycosidase H, specific for high mannose type oligosaccharide chains (Tarentino and Maley, 1974); endoglycosidase F, which cleaves all asparagine linked oligosaccharides (Elder and Alexander, 1982); and neuraminidase, which removes sialic acid from oligosaccharides.

For endoglycosidase H digestion, samples were prepared by adding SDS to a final concentration of 1%, Tris-HCl, pH 7.4, to 1 mM, and dithiothreitol to 50 mM. The sample was then incubated in a boiling water bath for three minutes and cooled to room temperature. The sample was diluted 1:1 with 0.3 M sodium citrate, pH 5.5. Ten milliunits of enzyme (Miles, Lot 13) was added to the sample, and the reaction mixture was incubated at 37°C.

For Endoglycosidase F digestion, the sample was adjusted to 50 mM dithiothreitol, 2% NP-40, 2 mM PMSF, and 1 unit of trasytol was added, in a final volume of 28 μ l. The enzyme was prepared by mixing 5 μ l of a stock solution in 50% glycerol (a gift from Dr. John Elder) with 4 μ l of 1 M NaPO_4 , pH 6.1, 4 μ l 500 mM EDTA pH 7.0, and 11 μ l water. The enzyme mixture was then incubated at room temperature for 5 minutes, added to the substrate solution and the mixture was incubated at 37°.

Samples for neuraminidase digestion were adjusted to 100 mM sodium acetate, pH 5.0, 0.2 mM EDTA, 2 mM CaCl_2 . 5 milli-units of neuraminidase (Vibrio cholerae, Calbiochem) were added in 5 μ l, and the reaction mixture was incubated at 37°C.

Preparation of intrinsically labelled L-CAM- Samples of L-CAM intrinsically labelled with radioisotopes were prepared from cultured embryonic hepatocytes. After the cells had been allowed to attach to the culture dish for 2-5 hours the medium was replaced with medium lacking the amino acid being used for the labelling. The culture was incubated a further 2-6 hours to allow depletion of any pools of the amino acid that remained, and fresh medium containing the labelled amino acid was added. The culture was then grown overnight (10-16 hours), the medium removed, the cells washed with PBS and the cells remaining on the culture dish extracted with 2.5 ml of boiling 1% SDS. The extract was clarified, adjusted to 1% NP-40 and immune precipitated, half with a rabbit antibody raised against SDS-denatured L-CAM, and half with antibody from an unimmunized rabbit. 50 μ l of 5 mg/ml IgG solution was used for each precipitation. After incubation

with Protein A-Sepharose (Pharmacia), each mixture was centrifuged and the beads were washed four times with wash buffer (.15 M NaCl, 25 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 100 units/ml trasylol, 20 mM Na_2HPO_4 , pH7.4), twice with wash buffer made to 1 M NaCl, and twice more with wash buffer.

Tunicamycin treated cells were prepared by adding tunicamycin (1 ug/ml) to all medium changes after the cells had attached to the substrate.

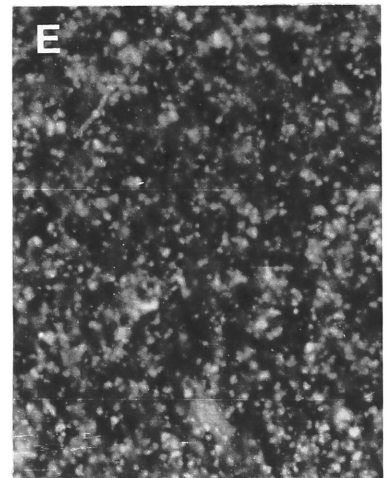
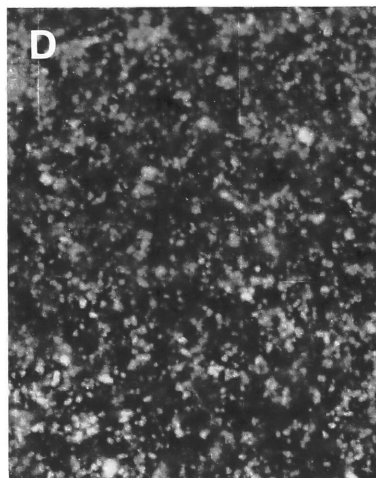
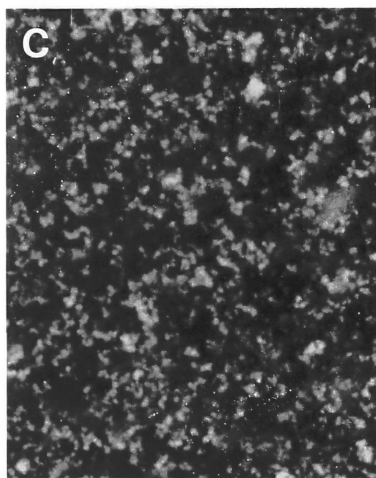
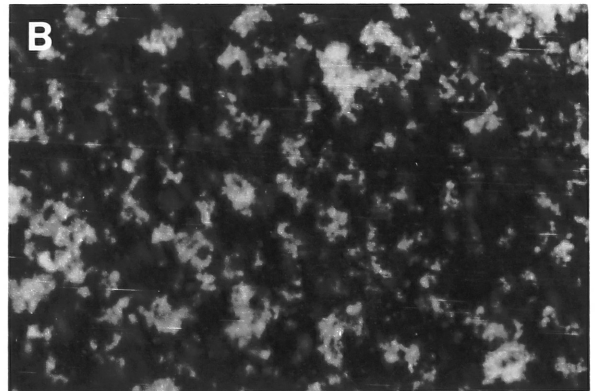
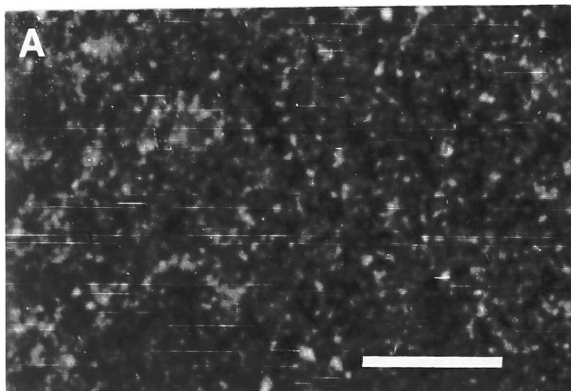
RESULTS

Liver cells dissociated with collagenase and crude trypsin with serum present yield a suspension of single cells and clumps of a few cells (Fig 1a). When the suspension is warmed in the presence of calcium ions, and incubated on a rotary shaker, the cells adhere to each other, forming large aggregates and decreasing the total number of particles in the suspension (Fig 1b). If the cells are preincubated with F_{ab} ' fragments of rabbit antibodies raised against whole cells, the aggregation is inhibited, the aggregates are smaller, and there are more cells and aggregates than in parallel incubations with F_{ab} ' fragments from non-immune rabbit serum present (Fig 1 c-e).

When aliquots of the cell suspensions are counted at different times after the beginning of the aggregation assay the time course shows a rapid aggregation to a plateau (Fig. 2). In the absence of calcium the decrease in cell number is much less, and is totally

Figure 1 - Inhibition of liver cell aggregation by anti-L-CAM

(A) unaggregated cell suspension. (B) the same cells as in A, after incubation at 37°C for 25' while shaking at 90 r.p.m. on a gyrorotary shaker to allow aggregation. (C) suspension treated as in B, except the cells were preincubated for 15' with 10 ul of anti-L-CAM F_{ab}' on ice before the aggregation was initiated. (D) same conditions as C, but 50 ul of anti-L-CAM F_{ab}' added. (E) same conditions as C, with 125 ul of anti-L-CAM F_{ab}' added. The F_{ab}' was prepared from IgG from a rabbit (623) immunized with a purified 81,000 dalton fragment of L-CAM. Cells were photographed under darkfield illumination using a Wild dissecting microscope. All panels are the same magnification. Scale bar=1 mm.



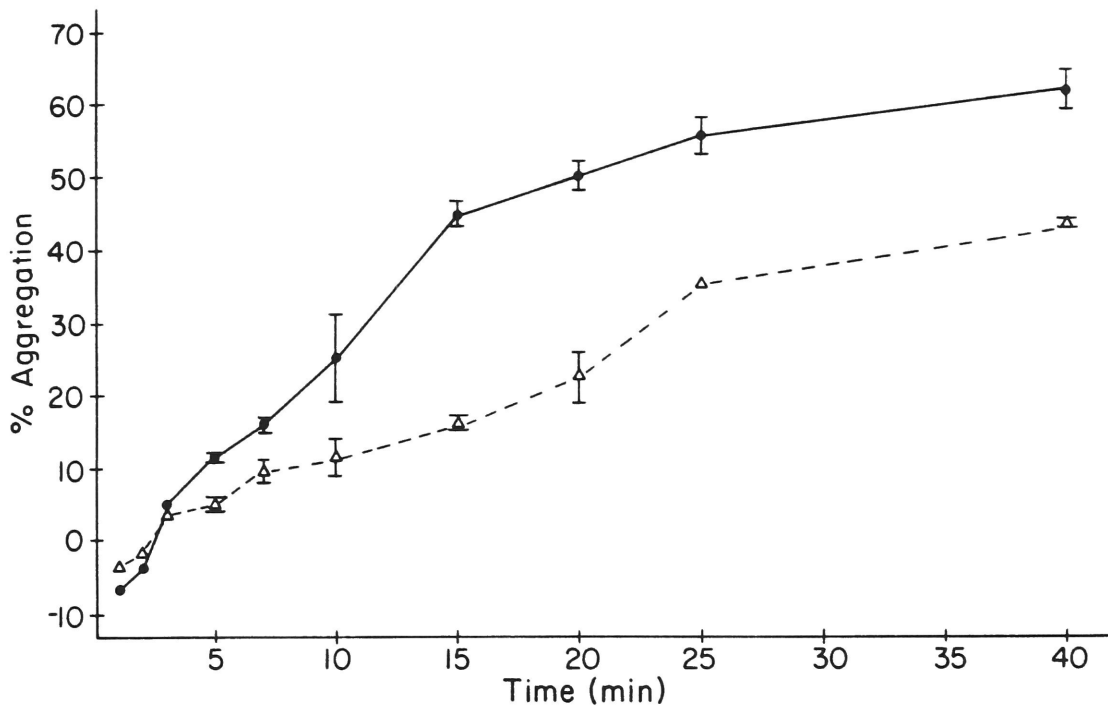


Figure 2 - Kinetics of liver cell aggregation. 3×10^6 cells in 2 ml of EMEM-DNAase were incubated at 37°C , shaking at 90 r.p.m. in scintillation vials. Aliquots of cells were taken at the indicated times, fixed in 1% glutaraldehyde in PBS, and counted using a Cytograf electronic cell counter. Percent aggregation was calculated as shown in Table 1. All points are averages of duplicates: the range is indicated by error bars. —●— 125 ul non-immune rabbit F_{ab}' in each incubation. —△— 125 ul anti-hepatocyte F_{ab}' in each incubation. These are the amounts of F_{ab}' solutions used in the standard neutralizing assay. Abscissa, time of incubation, ordinate, percent change in cell number.

insensitive to the presence of F_{ab}' fragments (not shown). Thus, the aggregation phenomenon depends upon the presence of calcium and is inhibited by blocking with antibody.

Figure 3 shows that the inhibition of aggregation by the F_{ab}' can be neutralized by a plasma membrane fraction made from embryonic livers, and that the neutralization increases with the amount of this material. The neutralization gives a roughly linear response when percent neutralization is plotted against the logarithm of the amount of the neutralizing sample added, so the assay can be used for quantitation of the amount of neutralizing material present. Thus, this is a quantitative assay for the molecules that mediate the adhesion.

Using this assay to trace the neutralizing activity, several treatments to extract the activity from membranes were tested. Treatment of membranes with high concentrations of EDTA (Bertolotti et. al., 1980) gave variable and low yields. Detergent extractions, using NP-40, sodium deoxycholate, and octyl-glucoside, all gave yields that varied extensively from preparation to preparation, often giving yields so low as to be useless for further purification. These approaches had the additional disadvantage that detergent had to be rigorously removed before the assay, which involves live cells, could be performed.

Subsequent tests indicated that treatment of membranes with trypsin, in the presence of calcium, consistently released 75-100% of the activity from the membranes, in a form that was soluble in the absence of detergents. Optimization of this approach showed that a 30' incubation of membranes at 0.7 mg/ml protein concentration with

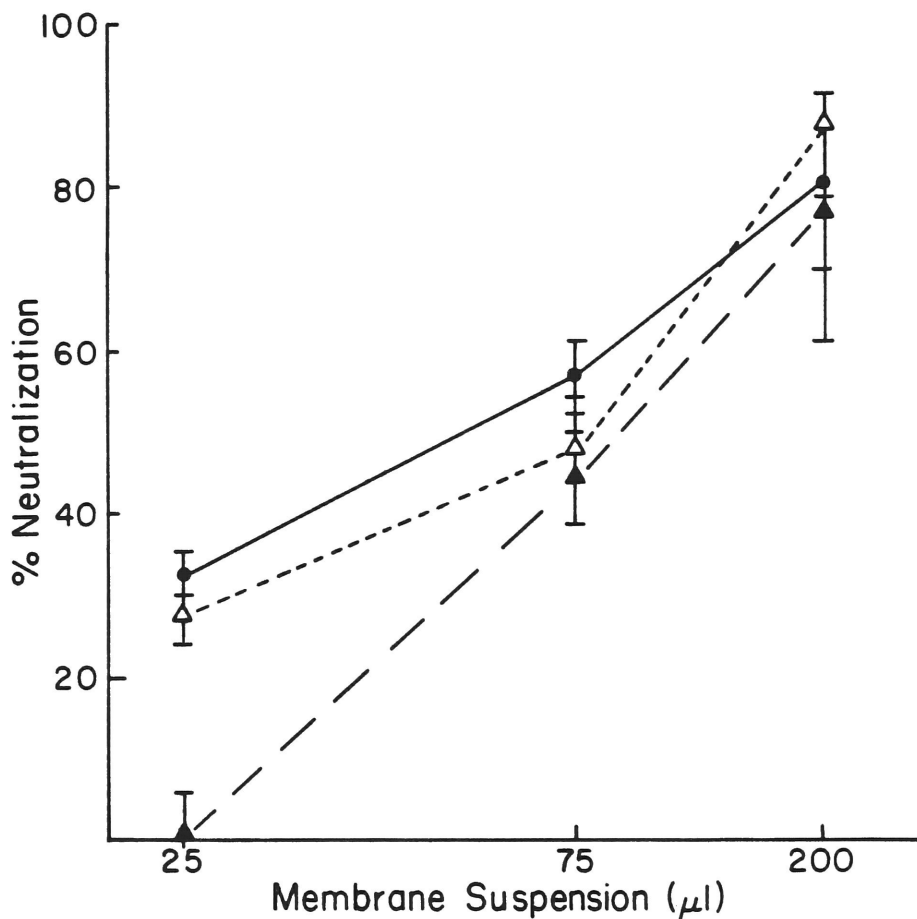


Figure 3 - Neutralization of anti-hepatocyte F_{ab}' by chicken liver membranes. The three lines summarize data from three different preparations of membranes, assayed on three different days using the same conditions. Membrane suspensions were adjusted to 0.7 mg/ml protein before assay. Abscissa, volume of membrane suspension used for neutralization; ordinate, percent neutralization.

50 ug/ml trypsin gave minimum release of protein with the maximum yield of activity (Fig 4).

Using this soluble extract as a starting point, L-CAM activity was purified in three steps (Fig 5). Ion-exchange chromatography using elution with a linear salt gradient removed nucleic acids and some protein (Fig 5a). Subsequent gel filtration gave a large purification because the trypsin used for the extraction and large protein aggregates were removed while the L-CAM activity ran in an area of the column with little other protein present (Fig 5b). The pooled activity from gel filtration, when fractionated by isoelectric focusing under non-denaturing conditions, gave a single large peak of activity with an isoelectric point of 4.0-4.5 (Fig. 5c). When aliquots of the active pools from each of the purification steps were separated by SDS-PAGE on 8.5% acrylamide gels the purification of a single component of $M_r=81,000$ daltons was seen (Fig 6).

An aliquot of the pooled active material from gel filtration was used to immunize mice for the production of monoclonal antibodies. A single fusion yielded 42 colonies that produced antibodies that bound to liver membranes but not brain membranes, and that survived cloning. Twenty-five of these clones were grown in ascites form in mice and the immunoglobulin from the ascites fluid was coupled to Sepharose. Aliquots (2 ml) of trypsin-released material from liver membranes were incubated 1 hr at 4°C with 0.1 ml of the antibody-bead conjugate and the supernatants were tested for neutralizing ability. As shown in figure 7, five of the 25 monoclonal antibodies depleted essentially all

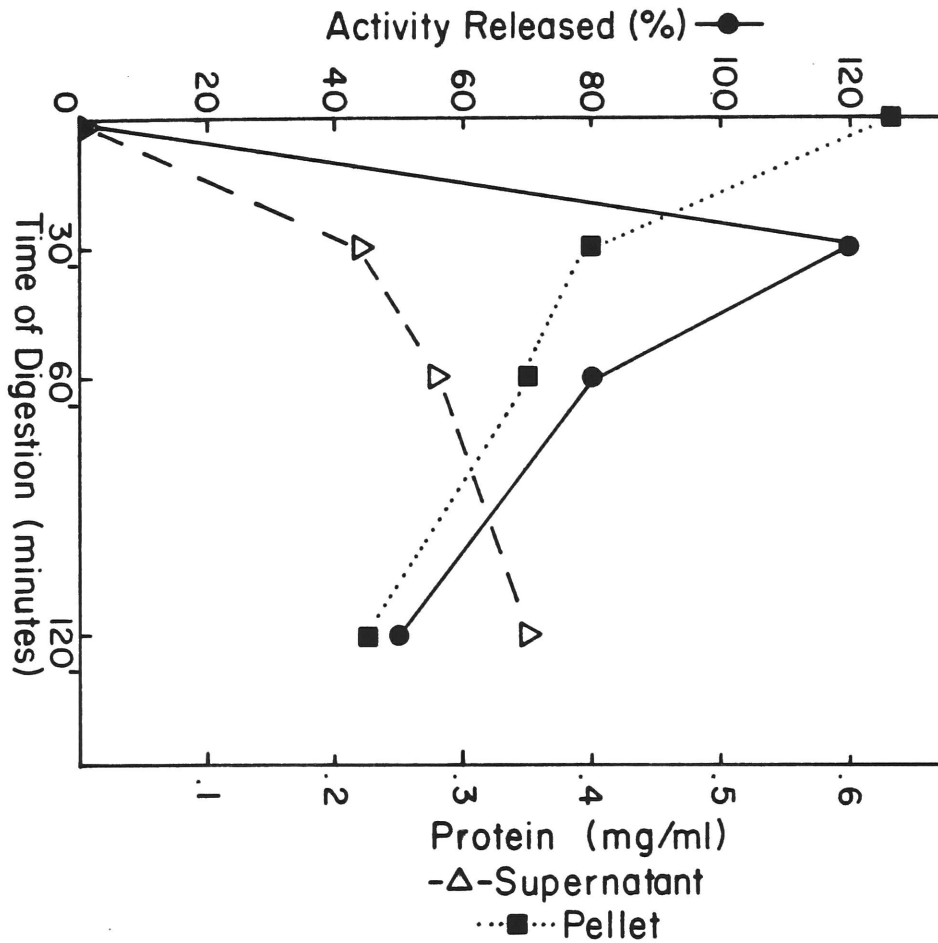


Figure 4 - Rate of L-CAM release from membranes by trypsin. Membranes were adjusted to 0.7 mg/ml protein in assay buffer and digested at 37°C with 50 ug/ml trypsin. Aliquots of the digestion mixture were taken at the indicated times and the reaction was quenched with 2 mM PMSF. The samples were clarified by centrifugation and the activity in the supernatants was assayed. Protein in the pellet and the supernatant was measured using the Coomassie blue binding assay (Bradford, 1976). Abscissa, time of digestion; left ordinate, activity as percent neutralization; right ordinate, protein in pellet and supernatant.

Figure 5 - Purification of trypsin-released L-CAM.

- (A) Chromatography of trypsin-released material on DEAE-cellulose. Proteins were eluted with a linear gradient of 0-0.3 M NaCl (vertical arrows) and then with 0.5 M NaCl. (—) Absorbance at 280 nm; (—●—) neutralizing activity.
- (B) Gel filtration of fractions pooled from column shown in A on Sephadex G-150 in assay buffer. (—) Absorbance at 230 nm. (—●—) neutralizing activity. Proteins used for calibration were: IG, IgG ($M_r=205,000$); OA, ovalbumin ($M_r=43,000$); and CC, cytochrome C ($M_r=12,400$).
- (C) Isoelectric focusing of fractions pooled from the column shown in B in Ampholines (pH 3.5-10) in a sucrose gradient (10-40%). (—) pH; (—●—) neutralizing activity.

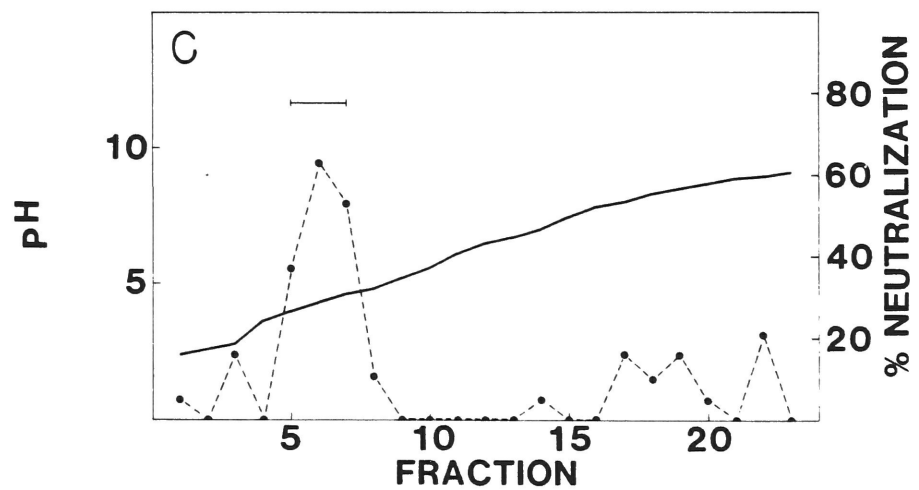
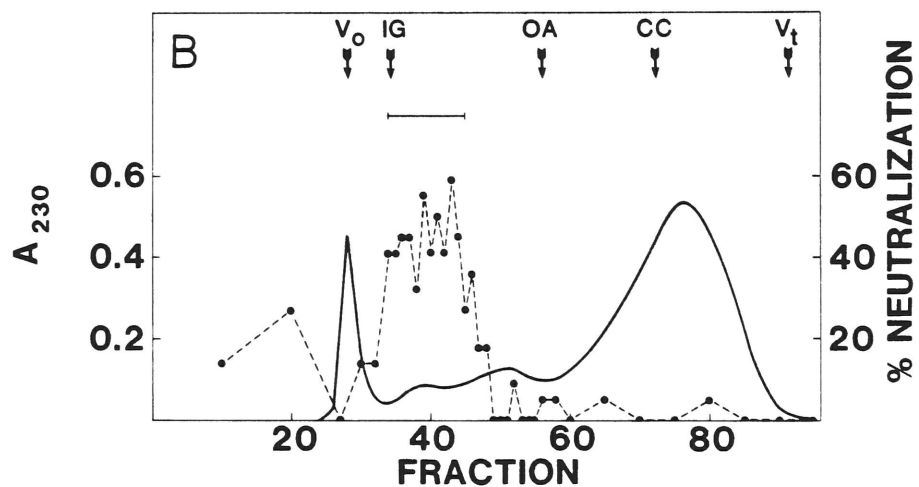
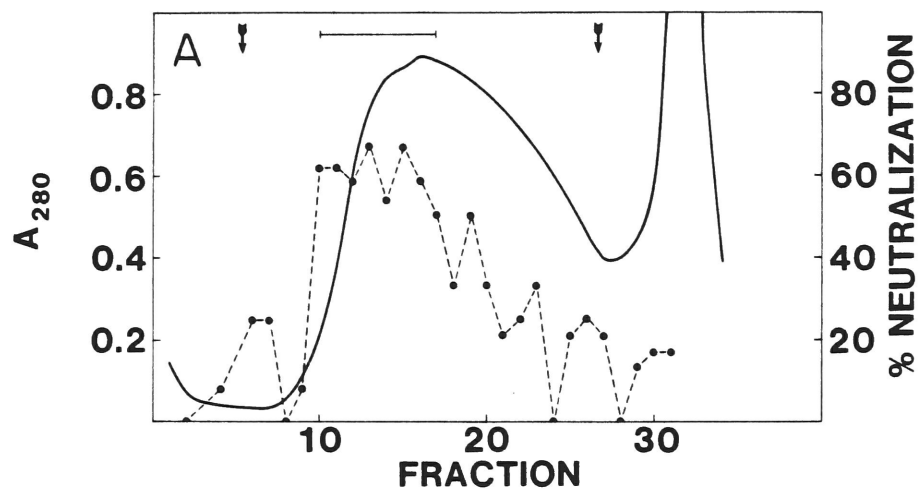


Figure 6 - SDS-PAGE analysis of partially purified L-CAM fractions. Samples from fractions having L-CAM activity at different stages of purification were analysed by SDS-PAGE on 7.5% acrylamide gels. Protein was detected by staining with Coomassie blue. a, liver membranes; b, trypsin-released material; c, pooled fraction from the DEAE-cellulose column (Figure 5A); d, pooled fractions from the gel filtration column (Figure 5B); e, pooled fractions from the isoelectric focusing column (Figure 5C)

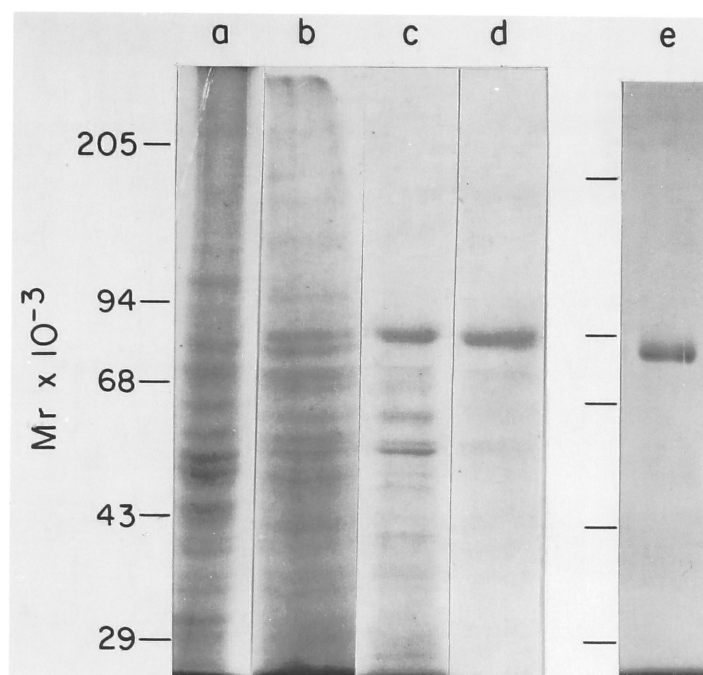


Figure 7 - Screening of monoclonal antibodies. Ig from hybridoma ascites fluid was coupled to Sepharose. 100 ul of the antibody derivatized beads were then incubated with 2 ml of a trypsin extract of liver membranes prepared as described in Materials and Methods. 50 and 300 ul aliquots of the absorbed extracts were assayed for neutralizing activity. The bars show the average of duplicate neutralization measurements for the two amounts of absorbed extract; the first bar in each pair represents 50 ul of extract and the second bar represents 300 ul. C is the unabsorbed extract; numbers were arbitrarily assigned to the clones for identification. Arrows indicate the five clones (6, 9, 18, 20, 25) scored as positive by virtue of their ability to deplete neutralizing activity from the extract.

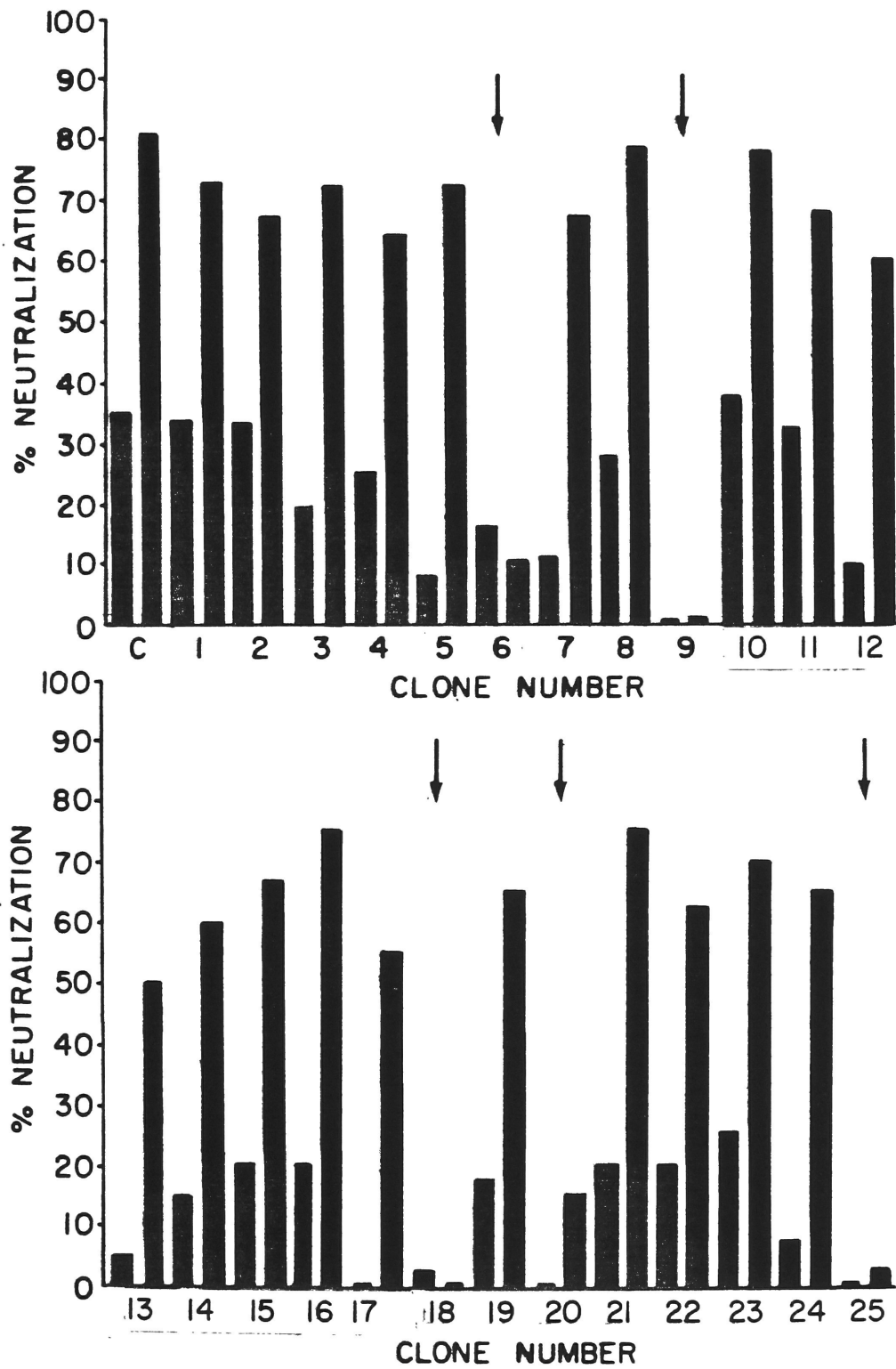


Figure 7

activity from the extracts. These five antibodies were further screened for use in immunoblotting and affinity purification. Clone 7C5 (Figure 7, clone 6) was selected for immunoblotting because it was the only one of the five that would produce bands on the protein transfers using just ^{125}I -labelled protein A for visualization. Clone 12G4 (Figure 7, clone 25) was chosen for use in affinity purification because it totally depleted activity from crude extracts and yielded at least 75% of the depleted activity on elution with 50 mM diethylamine/1 mM CaCl_2 , pH 11.5.

Rabbit antibodies were raised against the L-CAM material collected from the isoelectric focusing step. This material was also further purified on a preparative SDS polyacrylamide gel. The slice of the gel containing the component of $M_r=81,000$ was cut out and used to immunize rabbits.

To confirm that the material of $M_r=81,000$ was indeed L-CAM, the F_{ab}' fragments of the antibodies from the rabbits injected with the material purified by isoelectric focussing, and with material further purified by preparative SDS-PAGE, were tested for their ability to inhibit the aggregation of liver cells. Both gave good inhibition of aggregation (Fig. 8), at concentrations of F_{ab}' much lower than those required for good inhibition by F_{ab}' fragments from anti-whole cell sera.

Both a crude trypsin extract and liver membranes dissolved in SDS-PAGE sample buffer were separated by SDS-PAGE on 7.5% acrylamide gels and transferred to nitrocellulose (Towbin et. al., 1979); these

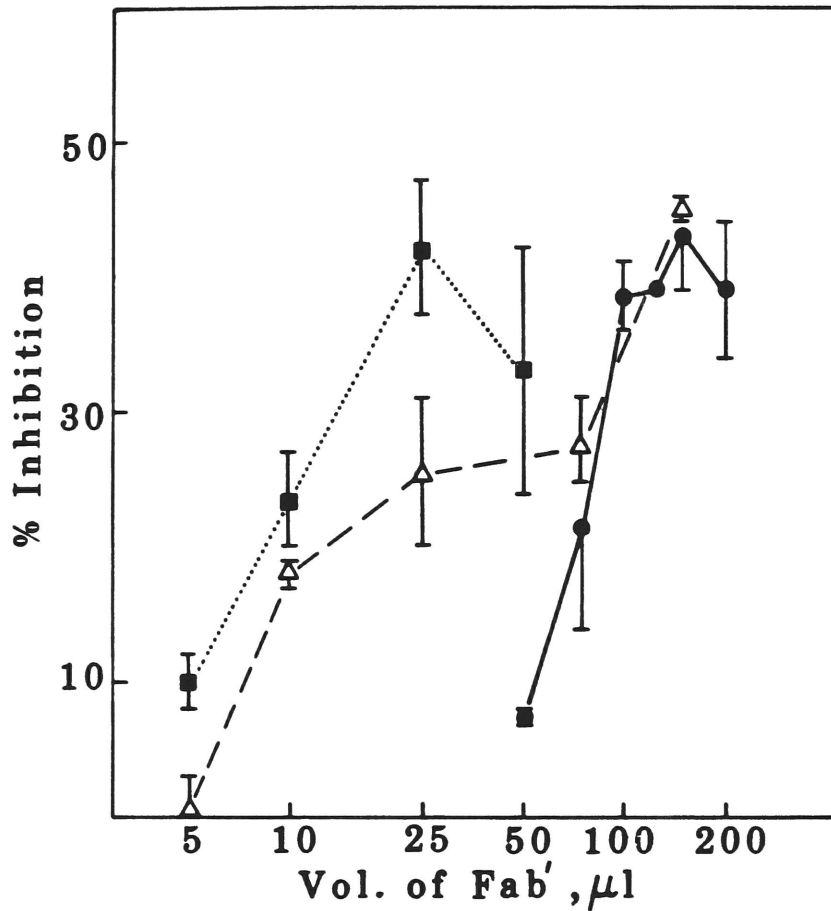


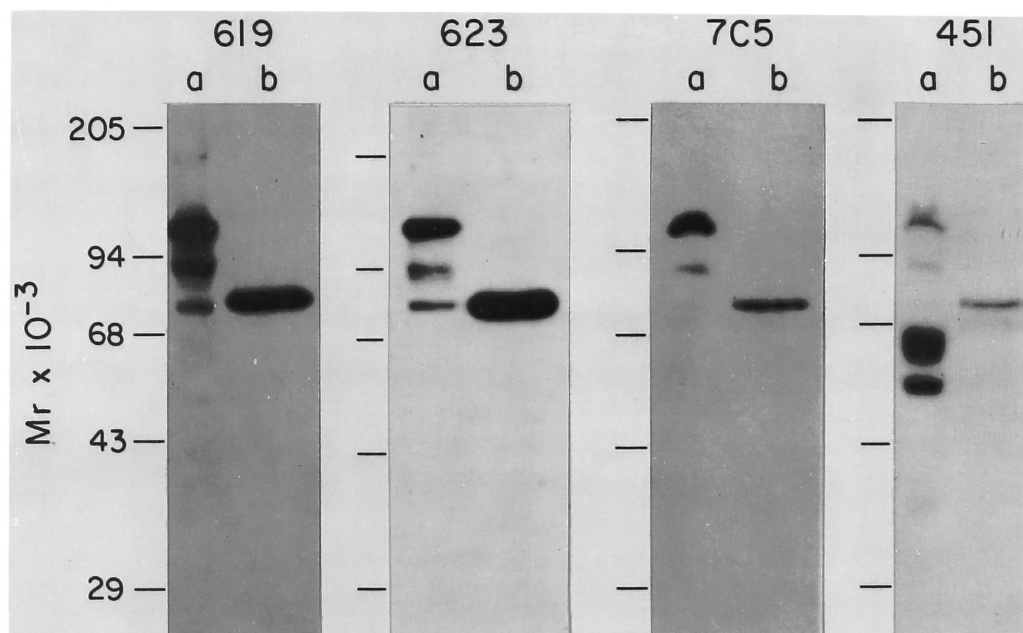
Figure 8 - Inhibition of aggregation by anti-L-CAM F_{ab}' .
 Abscissa, μ l of F_{ab}' fragments (prepared from a solution of IgG originally at a concentration of 10 mg/ml); ordinate, percent inhibition of aggregation. (—●—) Antibodies to trypsin-dissociated embryonic (10-11 day) chick hepatocytes; (···■···) Antibodies to trypsin-released L-CAM after isoelectric focussing (Figure 5C and Figure 6e)(Rabbit 6190); (—△—) Antibodies to the 81,000 dalton component in trypsin-released L-CAM after SDS-PAGE purification (Rabbit 623).

immunoblots were visualized with the various anti-L-CAM antibodies (Fig. 9). As can be seen, the monoclonal antibody (7C5), the rabbit antibody to material purified through the isoelectric focusing step (619), and the rabbit antibody to the material further purified by preparative SDS-PAGE (623) all showed only one component out of the whole crude trypsin extract, material with $M_r=81,000$. This result is a strong indication that the material of $M_r=81,000$ is indeed an L-CAM fragment. The immunoblot on the whole membranes shows three bands with each of these antibodies; a predominant component of $M_r=124,000$ and two minor components of $M_r=94,000$ and $81,000$. This result suggests that L-CAM is present on the membrane as an intrinsic membrane protein of $M_r=124,000$; it is probable that the $94,000$ dalton and particularly the $81,000$ dalton species are fragments of the parent molecule. This question is further addressed below.

The monoclonal antibodies made it possible to develop a single step purification of the $81,000$ dalton fragment by affinity chromatography. Membranes prepared by centrifuging a homogenate of embryonic livers onto a 48% sucrose cushion contained all detectable L-CAM. This crude membrane fraction could be digested with trypsin (2.5 mg/ml membrane protein, 150 ug/ml trypsin) to give a crude extract containing the $81,000$ dalton fragment of L-CAM.

The trypsin extract was shaken for 1 hr at 4°C with approximately 20 ml of affinity adsorbent, containing 30 mg of monoclonal antibody, per liter of extract. This slurry was then poured into a 2.5 cm diameter column, the flowthrough discarded, and the packed beads washed

Figure 9 - Analysis of anti-L-CAM antibodies using immunoblots. Material was separated on 8.5% acrylamide gels by SDS-PAGE. The separated material was electrophoretically transferred to nitrocellulose (Towbin et. al., 1979) and visualized with the appropriate antibody. Lane a, whole liver membranes; lane b, trypsin-released material. 619, rabbit antibody to trypsin-released L-CAM after isoelectric focusing; 623, rabbit antibody to the 81,000 dalton component purified by SDS-PAGE; 7C5, mouse monoclonal antibody 7C5; 451, rabbit anti-L-CAM prepared previously (Bertolotti et. al., 1980)

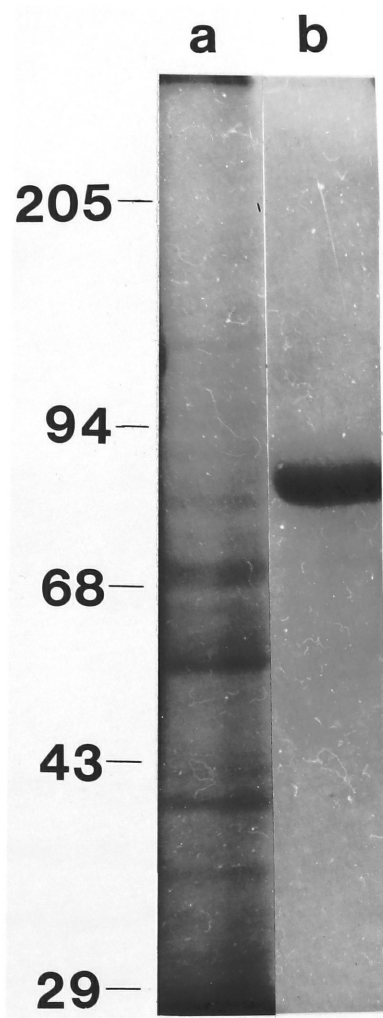


with 15 volumes of L-CAM assay buffer. The column was eluted with 3 volumes of 50 mM diethylamine/1 mM CaCl_2 , pH 11.5, and the eluate collected into 1/10 of its volume of 1 M HEPES, pH 7.0. The eluate was dialysed against several changes of distilled water and lyophilized. The lyophilized material was redissolved in 1/15 of its original volume of distilled water, centrifuged 5 min in a Beckman microfuge to remove any insoluble material, and stored frozen for later use. The resulting clear solution contained the 81,000 dalton L-CAM fragment; SDS-PAGE showed only trace amounts of contaminants when the material was heavily overloaded (Fig. 10). The yield of L-CAM by this method was approximately 2.5 mg protein from 1800 embryonic livers.

Only small amounts of the affinity purified material were needed to neutralize the anti-hepatocyte F_{ab} ' fragments: 50% neutralization of F_{ab} ' that causes a 50-60% inhibition of aggregation is accomplished by 1.5-5 ug of protein (Fig 11).

The affinity purified material, although containing only a single protein species, had a U.V. absorbance spectrum that indicated a chromophore absorbing maximally at 260-265 nm. (Fig 12). This spectrum is suggestive of a mixture of protein and nucleic acids. Because the affinity purification uses only one step from a crude (albeit a proteolytic) extract of membranes to the purified material, with no separations under extreme ionic conditions, it seemed possible that there was a nucleic acid contaminant non-specifically co-purifying with the L-CAM.

Figure 10 - SDS-PAGE of unfractionated trypsin extract of crude membranes and affinity purified 81,000 dalton tryptic fragment of L-CAM. Lane a, 200 μ l of trypsin extract of crude membranes prepared by digesting a suspension of crude embryonic chicken liver membranes (2.5 mg/ml protein) in assay buffer with 150 μ g/ml trypsin for 30' at 37°C, lane b, 60 μ g of affinity purified 81,000 dalton fragment of L-CAM.



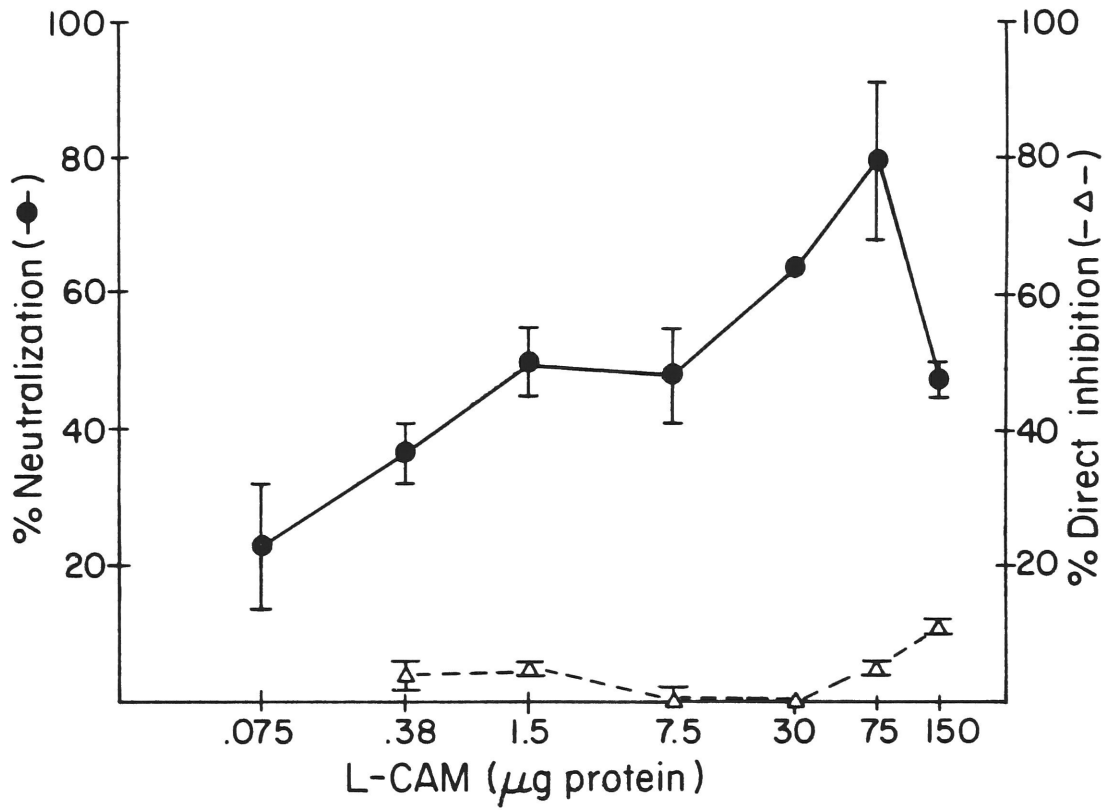


Figure 11 - Assays of affinity purified 81,000 dalton fragment of L-CAM, testing its ability to neutralize anti-hepatocyte F_{ab}' (left ordinate, —●—) and its direct effect on aggregation (right ordinate, —Δ—).

Figure 12 - a) UV absorbance spectrum of affinity-purified 81,000 dalton tryptic fragment of L-CAM. b) spectrum of same material as in (a) after absorption with DEAE-cellulose in 0.3 M NaCl, 20 mM Tris-HCl, pH 7.4. c) spectrum of material absorbed from the L-CAM by DEAE-cellulose and released by 1 M NaCl.

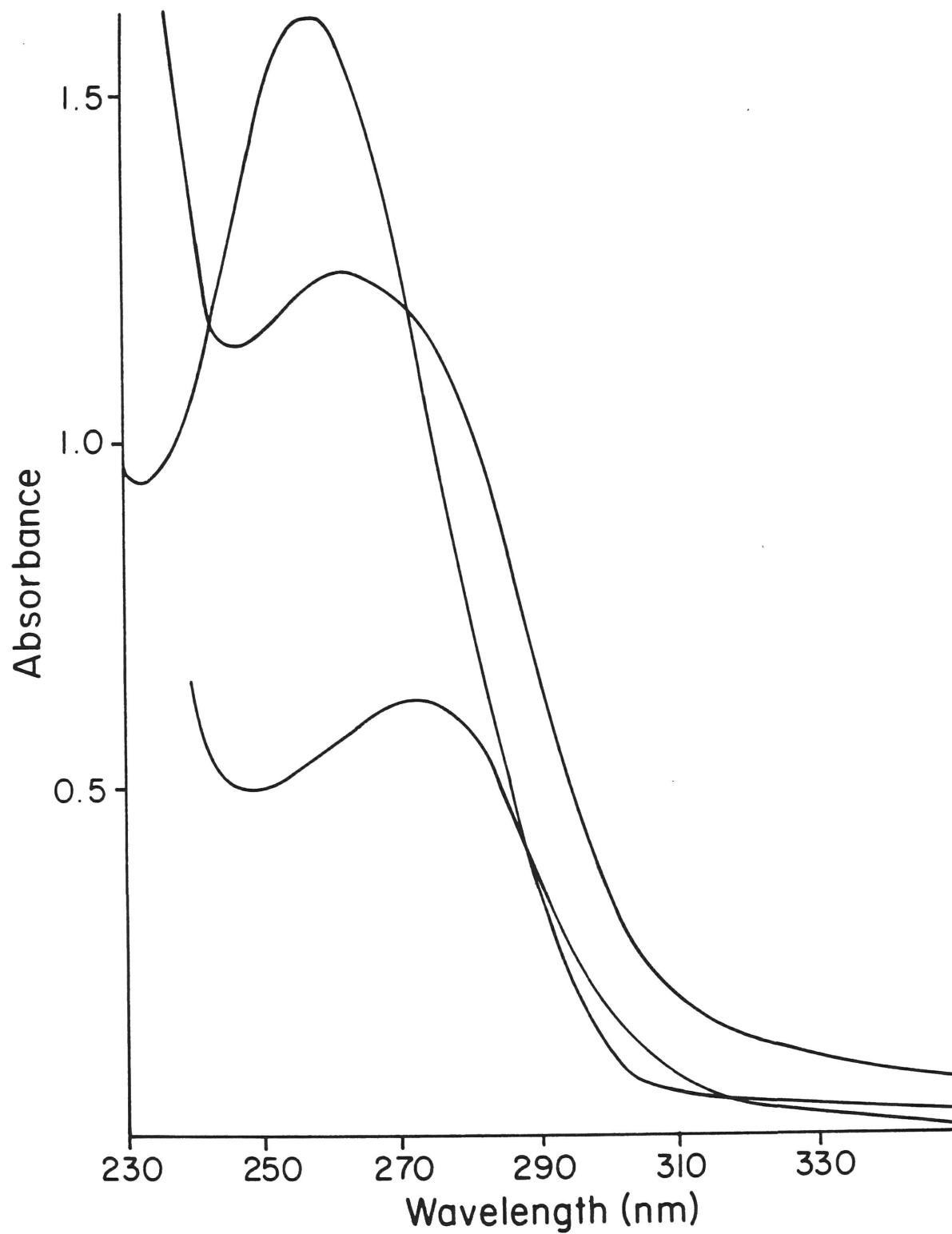


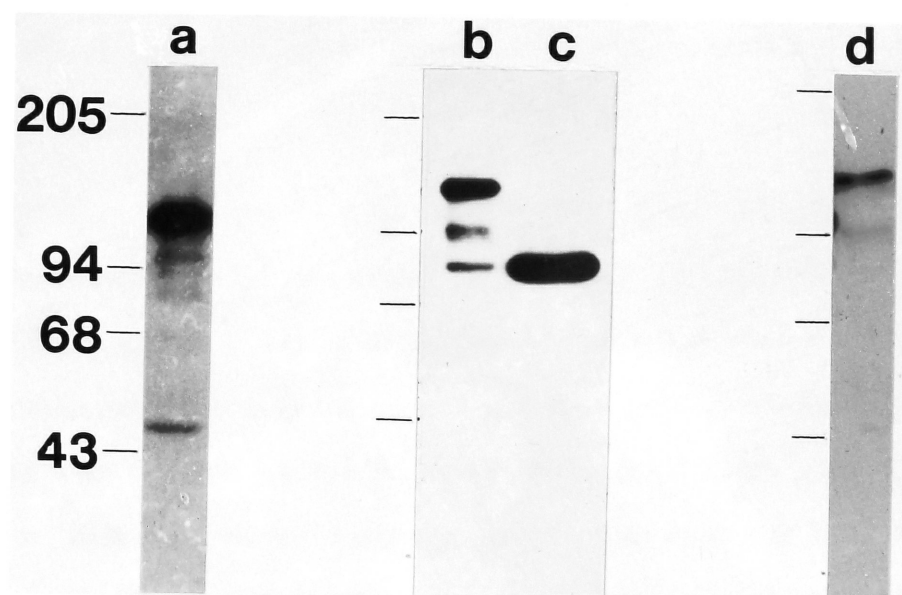
Figure 12

To test this possibility, an affinity purified sample of the trypsin released L-CAM was adjusted to 0.3 M NaCl, 20 mM Tris-HCl, pH 8.0, and passed over a 1 ml DEAE-cellulose column (Whatman, DE-52) equilibrated in the same buffer. The unbound fraction contained all of the L-CAM material, and the spectrum was typical for a protein, with an absorbance maximum at around 278 nm (Fig 12). When the column was eluted with 1 M NaCl, a fraction was obtained which had a spectrum typical for nucleic acids. When the two fractions were tested for activity in the neutralization assay, all the activity was in the flowthrough (L-CAM) fraction.

Finally, to address the question of the nature of the L-CAM molecule in vivo, livers were extracted with a boiling 1% SDS solution, conditions which have been shown to minimize degradation of other proteins during extraction from cultured cells and whole tissues. When this extract was separated on SDS-PAGE and electrophoretically transferred to nitrocellulose, the resulting immunoblot showed predominantly the 124,000 dalton component (Fig 13) with small amounts of material of lower molecular weight. Thus, it appears that the 94,000 dalton and 81,000 dalton L-CAM components are enzymatic degradation products of a 124,000 dalton membrane protein that mediates cell adhesion in the liver.

The characterization of L-CAM activity from embryonic chicken liver by Nielsen et. al. (1981) suggested that the activity was bound to lentil lectin supports and could be released from the affinity resin with an appropriate glycoside. Material released from membranes by

Figure 13 - Comparison of L-CAM extracted with SDS from whole liver, with NP-40 extract of isolated membranes, trypsin extract of isolated membranes and SDS extract of cells used for the aggregation assay. Lane a, 14 day embryonic livers were extracted with boiling 1% SDS (200 mg wet weight organ per ml. of extraction solution), 15 μ l of the resulting clarified extract was separated by SDS-PAGE on a 7.5% acrylamide gel, and the separated proteins were transferred to nitrocellulose (Towbin et. al., 1979). The resulting immunoblot was visualized with rabbit anti-L-CAM (623) and 125 I-labelled Protein A. Lane b, an NP-40 extract of purified membranes. Lane c, a trypsin extract of purified membranes. Lane d, a boiling SDS extract of single cells, as shown in figure 1, that were capable of aggregation in the DSC assay.



trypsinization was therefore tested for its ability to bind to lentil lectin. As shown in figure 14, the L-CAM 81,000 dalton fragment was separated into two fractions, one which bound lentil lectin and was eluted with alpha-methyl-D-glucoside, and another fraction, which did not bind to lentil lectin even when re-incubated with fresh lentil Sepharose. This suggests that there is a structural heterogeneity in the carbohydrate of the L-CAM molecule: it is not clear whether this is an artifact of the preparation, an in vivo synthetic variation that does not affect adhesion, or a structural variation that has a functional role.

The results from the amino acid analysis of the affinity purified trypsin fragment of L-CAM are shown in Table 2. Hydrophobic residues comprise 30.7% of the amino acid residues, basic residues comprise 10.3%, acidic residues comprise 23.7% and hydrophilic residues comprise 18.3%. The high content of acidic residues is consistent with the low isoelectric point of the molecule (4.0-4.5). The apparent lack of cysteine residues means that there are no disulfide bonds to stabilize the tertiary structure of the molecule.

Analysis of aliquots of the same sample of 81,000 dalton L-CAM fragment by the phenol-sulfuric acid sugar assay showed approximately 90 ug of neutral sugar (using glucose as a standard) associated with 355 ug of amino acids, indicating that this fragment is approximately 20% carbohydrate. This result means that intact L-CAM (124,000 daltons) is approximately 12% carbohydrate, assuming that the trypsin fragment contains all of the carbohydrate of L-CAM (see below).

Figure 14 - Binding of L-CAM to lentil lectin. A crude trypsin extract of purified membranes was absorbed with lentil lectin-Sepharose, the unbound material was reabsorbed with fresh lentil lectin-Sepharose a second time, and the unfractionated, bound, and unbound fractions were all analysed by SDS-PAGE followed by immunoblotting. Lane a, unfractionated crude trypsin extract of membranes. Lane b, material from (a) that bound to lentil lectin. Lane c, material from (a) that did not bind to lentil lectin. Lane d, material from (c) that bound to lentil lectin during a second absorption. Lane e, material from (c) that did not bind to lentil lectin during a second absorption. Rabbit 623 antibody, to the 81,000 dalton trypsin-released fragment of L-CAM, was used to visualize the blots.

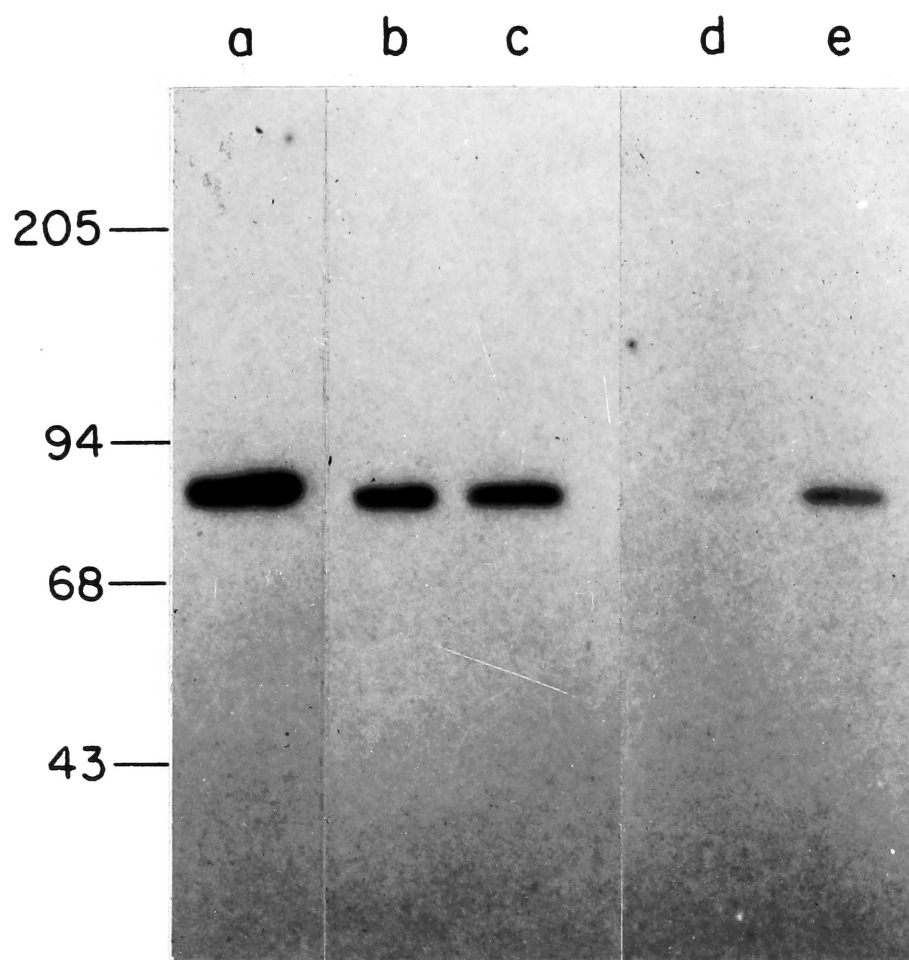


Table 2 - Amino acid composition of 81,000 dalton fragment of L-CAM

Residue	moles %	moles/ 81,000 D
Lys	5.2	38.3
His	1.6	11.8
Arg	3.5	25.8
Cys	-	-
Asp	11.0	81.0
Thr	7.6	55.9
Ser	7.8	57.4
Glu	12.7	93.5
Pro	7.4	54.5
Gly	8.2	60.4
Ala	6.2	45.6
Val	8.9	65.5
Met	1.5	11.0
Ile	6.5	47.8
Leu	7.0	51.5
Tyr	2.9	21.3
Phe	2.1	15.5
Trp	n.d.	n.d.

n.d. = not determined

To define further the carbohydrate structure of L-CAM, a series of digestions with three glycosidases, endoglycosidase F, endoglycosidase H, and neuraminidase were performed. First, the 81,000 dalton L-CAM fragment was exhaustively digested with each of the three enzymes. As can be seen in figure 15, 24 hour digestions were complete, as the addition of fresh enzyme for a further 24 hours caused no further increase in mobility on SDS-PAGE.

Neuraminidase and endoglycosidase H both caused small shifts in electrophoretic mobility, equivalent to approximately 1500 daltons of polypeptide, while endoglycosidase F caused a much larger shift, corresponding to approximately 6000 daltons. Because endoglycosidase F is reported to cleave all asparagine-linked oligosaccharides while endoglycosidase H cleaves only high-mannose asparagine-linked oligosaccharides, this result suggests that L-CAM contains multiple oligosaccharide chains, at least one of which is of the high-mannose type. This approach was taken one step further using a method suggested by Elder and Alexander (1982). Serial three-fold dilutions of endoglycosidase F were made, added to equal aliquots of the trypsin fragment of L-CAM and the digestion mixtures incubated at 37°C for 22 hours. The digests were then separated on SDS-PAGE; the results are shown in figure 16. A series of partial digestions was obtained; as the enzyme concentration was decreased, mixtures of partial cleavage products were generated, with fewer oligosaccharide chains removed by lower concentrations of enzyme. The partial digestion products have different electrophoretic mobilities. By comparing adjacent lanes on the electrophoresis gel, it can be seen that there are five bands,

Figure 15 - Digestion of the 81,000 dalton affinity purified tryptic fragment of L-CAM with endoglycosidase F (Endo F), endoglycosidase H (Endo H), and neuraminidase (Neur).

Digestions were performed on equal aliquots of affinity purified 81,000 dalton material, the digests were analysed by SDS-PAGE on a 6.5% acrylamide gel, and stained with Coomassie Blue. Each panel is labelled with the enzyme used. Lane a, untreated material. Lane b, material incubated 48 hours, 37°C, as a mock digestion for control. Lane c, material digested 24 hours with enzyme. Lane d, material digested 48 hours with enzyme.

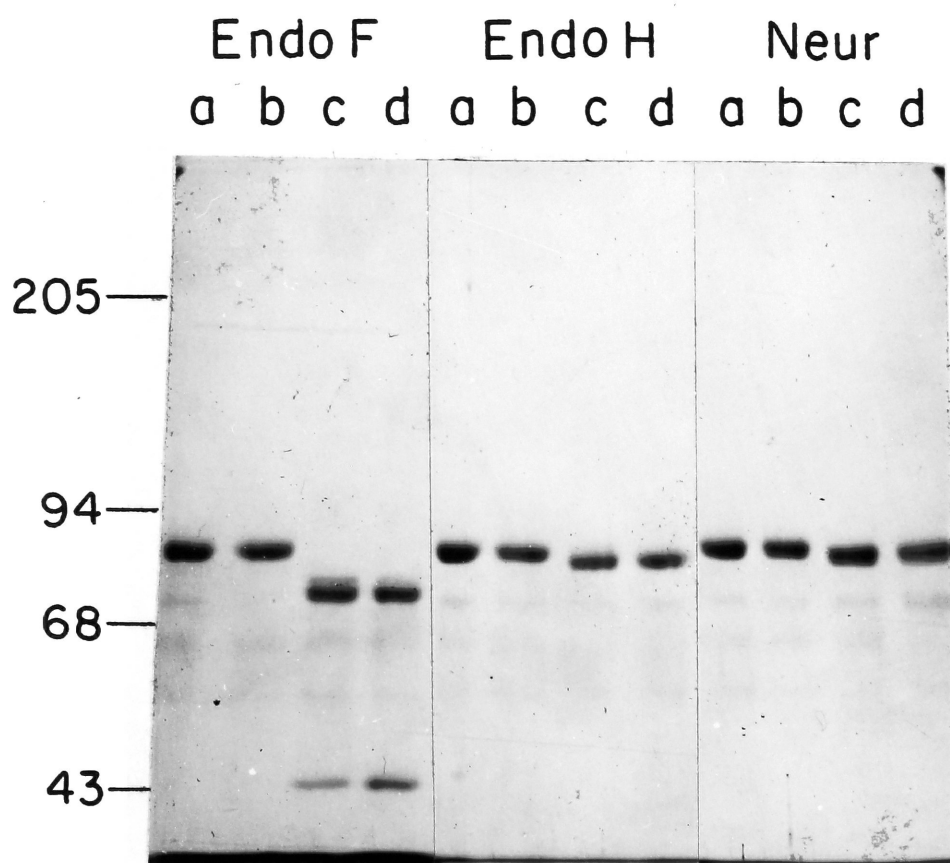


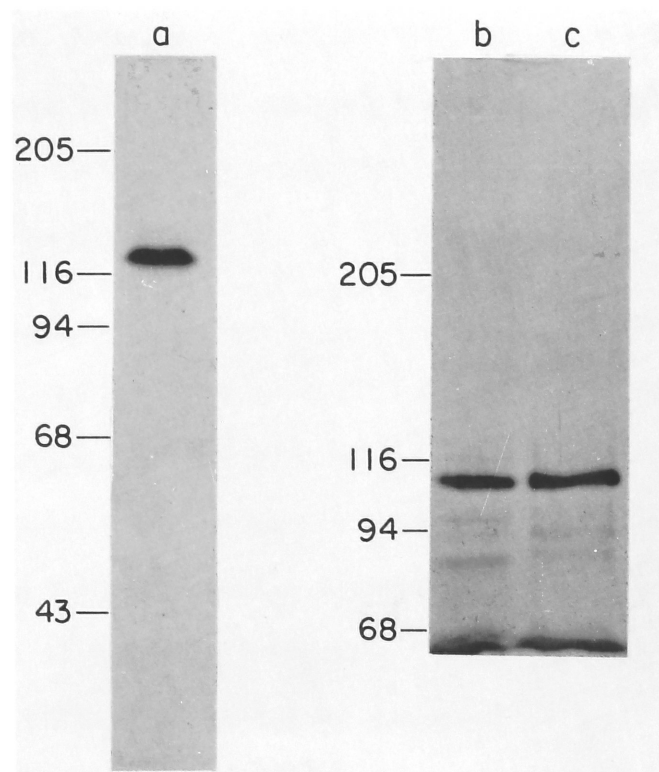
Figure 16 - Partial digestion of 81,000 dalton fragment of L-CAM with endoglycosidase F. Equal aliquots of the affinity purified material were digested with decreasing amounts of enzyme as described in Materials and Methods. Digestion was terminated by the addition of SDS-PAGE sample buffer. Samples were analyzed on 6.5% acrylamide gels and stained with Coomassie blue. Lane a, untreated material. Lane b, 48 hour digestion with two aliquots of undiluted enzyme. Lane c, 24 hour digestion with one aliquot of undiluted enzyme. Lanes d through n, 24 hour digestion with 1:3 serial dilutions of enzyme. The five closely spaced lines on the right of the panel indicate the positions of the five discrete bands. The line marked X indicates a band that is present in the enzyme solution.



ranging from undigested to totally digested material. This result suggests that there are four endoglycosidase F sensitive oligosaccharide side-chains on the 81,000 dalton trypsin fragment. The increment of increase in electrophoretic mobility for the removal of each chain corresponds to approximately 1500 daltons of protein. This change is similar to the increase in mobility caused by endoglycosidase H, suggesting that there is only one high-mannose carbohydrate chain and three complex carbohydrate chains.

To date, we have been unable to isolate the 124,000 dalton L-CAM molecule free of contaminants in amounts large enough for comparable analysis. As a result, all studies on the intact L-CAM have been carried out using radioactively labelled material. The question of how many oligosaccharide attachment sites are present on L-CAM was addressed using labelled material from cultured hepatocytes. Cells were cultured under two conditions, one with tunicamycin, an antibiotic that prevents the transfer of oligosaccharides to asparagine residues, in the medium, and a control culture with no drug. Tritiated leucine was added to the cultures after the tunicamycin had sufficient time to block glycosylation. The cells were harvested in boiling 1% SDS, conditions which prevent post-extraction modifications of the extracted material by endogenous enzymes. L-CAM was isolated by immune precipitation. The material that was immune precipitated from the culture grown without tunicamycin appears as a single band of 124,000 daltons, further evidence that this is the unmodified form of L-CAM. An aliquot of the material from the control culture (lacking tunicamycin) was digested with endoglycosidase F and SDS-PAGE was performed on the

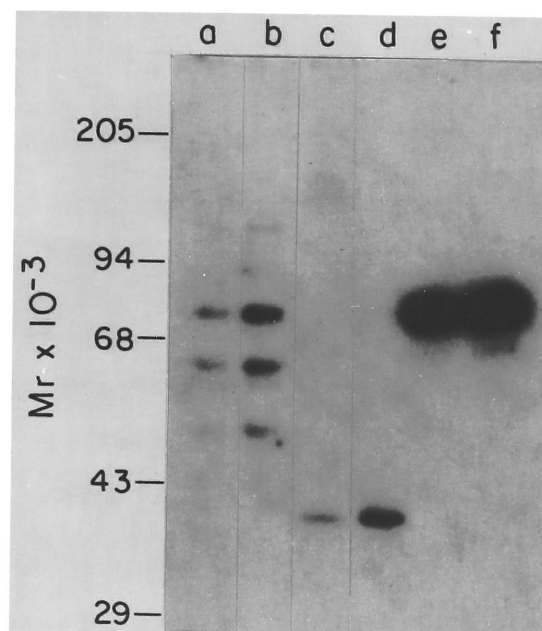
Figure 17 - Tritium labelled intact L-CAM Lane a, immune precipitation of 124,000 dalton intact L-CAM from SDS extract of cells labelled with ^3H -leucine. Lane b, immune precipitated material from cells treated with tunicamycin. Lane c, immune precipitated material from a digested with endoglycosidase F Material in a was separated on 7.5% acrylamide gel, material in b and c was separated on a 5% acrylamide gel. The fixed and stained gels were impregnated with sodium salicylate and exposed for autoradiography at -70°C using Kodak SB-5 film. Rabbit 623 antibody was used for these immune precipitations.



samples. As shown in figure 17, endoglycosidase F treated L-CAM comigrated with the material isolated from cells treated with tunicamycin, suggesting that endoglycosidase F removes all the asparagine-linked oligosaccharide chains from L-CAM. A digestion of labelled L-CAM (124,000 daltons) with serial dilutions of endoglycosidase F was performed. As in the case of the 81,000 dalton trypsin fragment, five bands could be detected in the digests (data not shown), consistent with the interpretation that the 81,000 dalton tryptic fragment of L-CAM contains all of the carbohydrate of the intact L-CAM molecule.

L-CAM-mediated cell adhesion requires calcium, and calcium ions appear to stabilize the structure of L-CAM. When membranes were digested with trypsin in the presence of 1 mM EGTA, L-CAM activity was released, as measured by the neutralization assay. When immunoblots were prepared with this material a fragment of $M_r=40,000$ was detected, but no fragment of $M_r=81,000$ (Fig 18). Similarly, when membranes were extracted with EDTA as described by Bertolotti et. al. (1980), several low molecular weight fragments were detected by immunoblotting (Fig. 18). Thus it appears that calcium ions make parts of the L-CAM molecule somewhat resistant to trypsin digestion, as well as to digestion by proteases present in the liver membrane preparations.

Figure 18 - L-CAM released from membranes by different extraction procedures. Membranes were prepared without (a,c, and e) and with (b,d, and f) PMSF and iodoacetamide present. Lanes a and b, material released with 50 mM EDTA (Bertolotti et. al., 1980); lanes c and d, material released with trypsin in 1 mM EGTA; lanes e and f, material released by trypsin in the presence of 1.2 mM Ca^{2+} . Samples were separated by SDS-PAGE on 7.5% acrylamide gels and analysed by immunoblotting, using rabbit anti-L-CAM (623) to visualize the L-CAM fragments. Lanes e and f were intentionally overexposed to demonstrate the presence of the lower amounts of material released as detectable fragments in the absence of calcium



SUMMARY

In this section I have described the purification of the chick embryo L-CAM. The assay for following the purification was based on an approach previously developed for the detection of a slime mold adhesion molecule and for the isolation of the chicken N-CAM. Optimal amounts of L-CAM were obtained in soluble form by trypsinization of liver membranes in the presence of calcium. A three-step purification yielded a single protein of $M_r=81,000$ that neutralized antibodies that inhibited aggregation of liver cells. F_{ab}' fragments of antibodies raised against this molecule inhibited cell-cell adhesion of isolated embryonic hepatocytes.

Specific polyclonal antibodies were raised in rabbits against the 81,000 dalton material after purification on an SDS preparative polyacrylamide gel. Five monoclonal mouse antibodies were produced that depleted the L-CAM activity from trypsin extracts. The rabbit antibodies and a monoclonal mouse antibody were used in immunoblotting experiments to demonstrate that the L-CAM exists in vivo as a protein of $M_r=124,000$ that is subject to fragmentation to species of $M_r=94,000$ and 81,000 during the isolation of membranes.

A one-step affinity purification was developed which yielded approximately 2.5 mg of 81,000 dalton L-CAM fragment from 1,800 14-day embryo livers. This material contained non-protein contaminants (possibly nucleic acids) which could be removed by ion-exchange chromatography without loss of activity or the 81,000 dalton protein.

L-CAM is a glycoprotein, containing approximately 12% carbohydrate present as four asparagine-linked oligosaccharide chains each of which contributes an increment of electrophoretic mobility equivalent to approximately 1500 daltons of polypeptide. One of these chains appears to be of the high mannose type, the other three of the complex type. In contrast to N-CAM, only a small amount of sialic acid is present.

Calcium ions are required for the L-CAM mediated adhesion mechanism to function in vitro and play some role in maintaining the structure of L-CAM. L-CAM extracted in the absence of calcium ions is degraded to smaller fragments than in the presence of calcium ions.

APPEARANCE OF L-CAM ON EMBRYONIC AND ADULT TISSUES

INTRODUCTION

When liver cells are plated on a tissue culture dish and incubated under appropriate conditions the cells stick down on the plate and form compact colonies. These cultured hepatocytes have been used as a model system to study the synthesis of enzymes in the heme biosynthesis pathway (Granick, 1966; Sassa and Granick, 1970) and of several plasma proteins (Liang and Grieninger, 1981). It has also been shown that colonies formed by dissociated adult rat hepatocytes have a polarized structure that will take up fluorescein diacetate (FDA) from the medium, hydrolyse it to fluorescein, and secrete it into the intercellular spaces within the colonies (Barth and Schwarz, 1982); this in vitro process thus mimics the clearance of FDA from the bloodstream into the bile of the intact rat. All of these studies suggest that this simple in vitro culture system may also be of use in defining physiological functions of the L-CAM molecule.

I therefore begin this section by describing experiments that show that anti-L-CAM interferes with cell-cell adhesion in these cultures and causes a reversible disordering of the compact colonies. The presence of L-CAM on the surface of these cultured cells is demonstrated by immunofluorescent staining.

The fact that anti-L-CAM disrupts the formation of histotypic liver structures in vitro suggests that L-CAM plays a role in cellular interactions during histogenesis. To define further the role that L-CAM may play in organogenesis, it is important to determine its spatial and temporal distribution in the developing animal. Such information could suggest possible roles for the molecule in development and suggest directions for future investigation.

N-CAM, initially isolated as a neural cell adhesion molecule, has been shown to have a temporally and spatially complex pattern of appearance and disappearance during embryogenesis (Thiery et. al., 1982). It appears on axial inducing centers very early during development, prior to the formation of many organ rudiments. It also appears on the obvious neural cell precursors of the neural tube and the neural crest. In later embryos, its distribution becomes restricted to muscle (Grumet et. al., 1982) and nerve, and in the adult to nervous tissue exclusively.

Preliminary experiments to determine the localization of L-CAM in the thirteen day chick embryo and in the adult animal are described in this section. Surprisingly, L-CAM was found to be widely distributed throughout the chicken, but within each tissue is localized in very specific areas. L-CAM was found in skin (an ectodermally derived tissue), kidney and the female reproductive tract (mesodermally derived tissues), and the digestive tract and organs originating as outgrowths of the embryonic gut (endodermally derived tissues). Thus, it appears that L-CAM is not liver specific, nor does it appear on cells arising

from only one germ layer, but instead occurs on epithelial cells with a number of physiological functions, primarily secretory or absorptive.

Immunoblotting experiments showed that the molecular species of L-CAM present in these various organs were the same, demonstrating that this generality is not due to different molecules with a common antigenic site, but is a genuine distribution of the L-CAM molecule. Moreover, L-CAM in adult tissues appeared identical on SDS-PAGE to that in embryonic tissue; this result is in marked contrast to N-CAM, which has electrophoretically distinguishable adult and embryonic forms, that differ only in sialic acid containing structures of the molecule (Rothbard et. al., 1982).

MATERIALS AND METHODS

Preparation of liver cell cultures for immuno-fluorescence microscopy- Cells were grown for 16 hours in culture. The medium was removed and the cells were fixed 20 minutes in 3.7% formaldehyde in PBS, followed by a 5 minute incubation in 0.1 M glycine in PBS. The cells were then washed with PBS. The fixed cells were incubated 45 minutes with a 0.1 mg/ml solution of anti-L-CAM IgG in PBS-10% calf serum and washed five times for 5 minutes each with PBS. The dishes were then incubated 45 minutes with rhodamine labelled goat-anti-rabbit IgG or rabbit-anti-mouse Ig (Miles) diluted 1:100, then washed five times for five minutes each with PBS. The cells were mounted in 10% PBS-90% glycerol-.1% p-phenylenediamine (Johnson and Nogueira Araujo, 1981), and viewed under phase-contrast or epifluorescence optics with a

Zeiss universal microscope equipped with Zeiss III RS fluorescence optics.

Sectioning-Organs or pieces of organs from older embryos and adult chickens were snap frozen in a dry ice-isopentane bath, mounted in Lipshaws M-1 medium, and sectioned. 10 micrometer sections were picked up on plain glass slides, air dried and stored frozen until used for staining.

Fixation- Sections of tissue on plain slides were fixed for 15 minutes in 3.7% formaldehyde in PBS at room temperature. Any free reactive groups were quenched by incubating in 0.1 M glycine in PBS for 15 minutes at room temperature.

Immunofluorescent staining-Fixed sections of embryonic or adult organs were incubated 45 minutes in 5% goat serum-0.1 M Na-K-PO₄, pH 7.2, to block non-specific binding. Sections were then incubated with the first antibody, 50 ug/ml rabbit anti-L-CAM (Rabbit 623) in 1% goat serum-phosphate buffer, for four hours at room temperature. Slides were washed 5 times for 5-10 minutes each time with 1% goat serum-phosphate buffer, and incubated for 1 hour with a 1:200 dilution of either rhodamine or fluorescein labelled goat-anti-rabbit IgG (Miles), at room temperature. The sections were washed five times, as above and mounted in 10%PBS-90%glycerol-0.1%p-phenylenediamine.

Control sections were stained using IgG prepared from pooled sera of unimmunized rabbits. These consistently showed only low levels of diffuse background fluorescence.

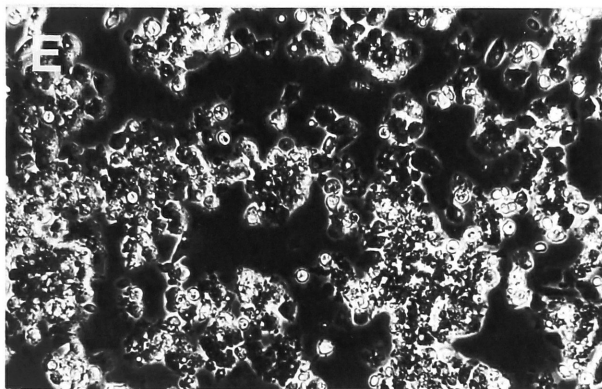
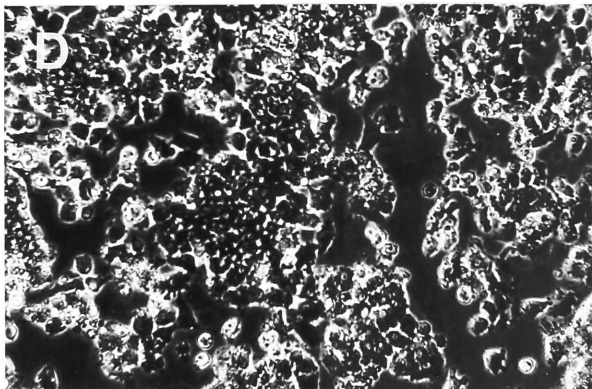
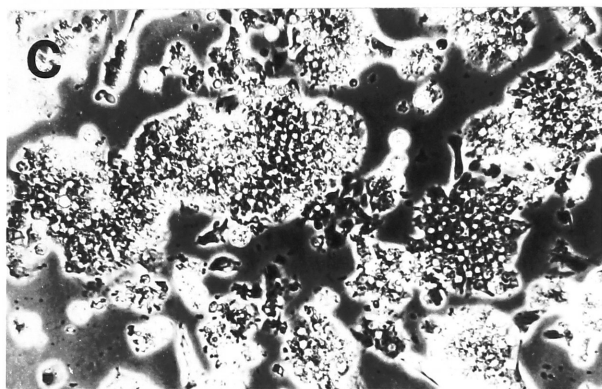
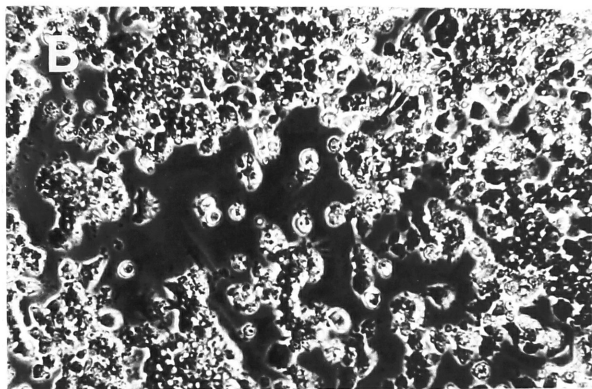
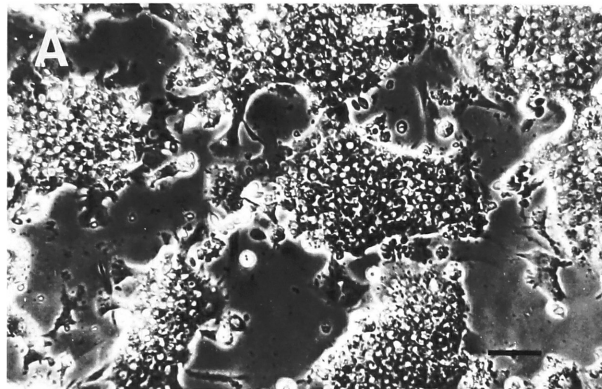
Photography- Sections were observed using phase optics or epillumination fluorescence optics (Zeiss III RS). Photographs were taken on Kodak Tri-X film. The organs and structures described below were identified by comparing sections with information in standard textbooks of fowl histology (Hodges, 1974) and embryology (Romanoff, 1960).

RESULTS

Effect of anti-L-CAM on liver cell cultures-Figure 19a shows the appearance of 11-day embryonic liver cells plated and grown in culture for 16 hours. The hepatocytes pull together in tight colonies with very sharply delimited boundaries. The open spaces between the colonies have some flattened fibroblastic cells growing on them. If the cultures are plated in the presence of anti-L-CAM F_{ab}' fragments, the compactness of the colonies is absent (Fig. 19b). The boundaries of the colonies become much less sharp, the cells within the colonies are more distinctly separate, and as the F_{ab}' concentration is increased, fewer of the hepatocytes are found in colonies at all - the majority of cells plate down as individual cells or as small clumps of a few cells.

When cells are plated for 5.5 hours in medium without F_{ab}' present, they form colonies no different from those observed after 16 hours. When anti-L-CAM F_{ab}' is added to these cultures the colonies are disrupted after 16 hours (Fig. 19 c,d). Conversely, when cells are plated in the presence of sufficient anti-L-CAM F_{ab}' to inhibit colony formation, allowed to adhere to the dish for 6.5 hours and then rinsed

Figure 19 - Embryonic liver cells grown in culture in the absence (A,C and F) and presence (B,D, and E) of anti-L-CAM F_{ab}' fragments. A) cells cultured in DMEM-10% calf serum for 22 hours. The hepatocytes are in tight colonies with sharply delineated edges. Some flat fibroblastic cells grow on the clear substrate outside the colonies. B) cells cultured as in A, with 50 ul of anti-L-CAM F_{ab}' in the culture medium. The cells do not form colonies with sharp boundaries, single hepatocytes or small clumps are observed, and the cells in the large groups appear to be only loosely associated with each other. C) cells cultured for 5.5 hours under conditions as in A. Colonies are well formed at this point. D) the culture in C was rinsed and fresh medium containing 50 ul of anti-L-CAM F_{ab}' was added. Photographed 16 hours later. The colonies have lost their sharp edges and the cells are only loosely associated. E) cells cultured as in B for 5.5 hours. Compared to the culture shown in C, these cells have not formed colonies, and the cells are loosely associated. F) the culture shown in E was rinsed after 5.5 hours and fresh medium without anti-L-CAM was added. The culture was photographed 16 hours later. The cells have now pulled together into tight colonies with sharp boundaries. All panels are the same magnification. Scale bar=50 micrometers.



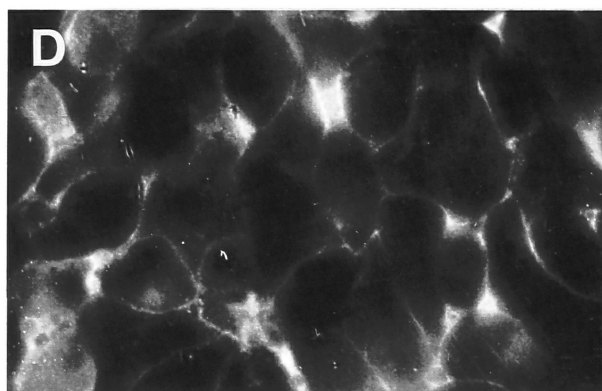
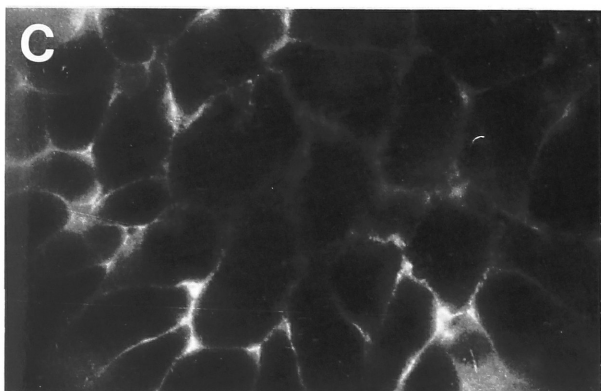
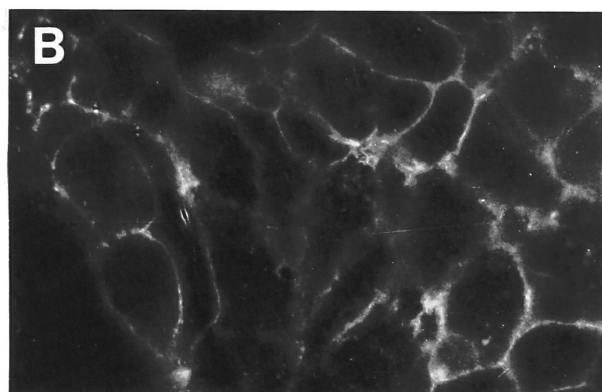
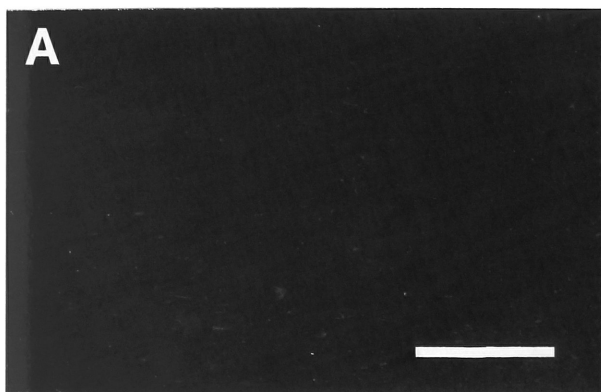
and incubated in fresh medium, without F_{ab}' present, the cells aggregate into colonies. After 16 hours these colonies are morphologically indistinguishable from colonies formed by cells plated directly into normal medium (Fig. 19 e,f). Thus, the L-CAM mediated colony formation in vitro is reversible, and so are the effects of the anti-L-CAM F_{ab}' on the colony formation. The results indicate that the cultures will be a useful system for studying the role of cell-cell interactions in a number of physiological functions.

To localize L-CAM in the intact colonies, the cells were fixed and stained with anti-L-CAM IgG followed by rhodamine-labelled second antibody (goat-anti-rabbit Ig or rabbit anti-mouse Ig). Anti-L-CAM antibodies stained the periphery of all cells in the colonies (Fig 20), while the flat cells outside the colonies were unstained. These photographs show only one plane through the colony, but by focussing through the thickness of the colony it is obvious that the whole cell surface is stained, including the surfaces on the edge of the colony, which are not in contact with other cells, and the bottom surface, which is in contact with the substrate. The description of any finer localization of L-CAM on the surface of cultured cells will require experiments at the electron microscopic level.

Appearance of L-CAM in organ rudiments and in adult organs

Organ rudiments generally arise in the embryo as concentrations of cells pulled together from a discontinuous mass, or as buds evaginated from sheets of cells. For most organs, a discrete rudiment can be detected at around 3 to 4 days of incubation. The rudiments are clearly

Figure 20 - Immunofluorescent staining of cultured embryonic hepatocytes. A) cells stained with Ig from a non-immune rabbit. B) cells stained with rabbit anti-L-CAM. C) cells stained with monoclonal antibody to L-CAM (7D6). D) cells stained with monoclonal antibody to L-CAM (4G4). All panels are the same magnification. Scale bar=20 micrometers.



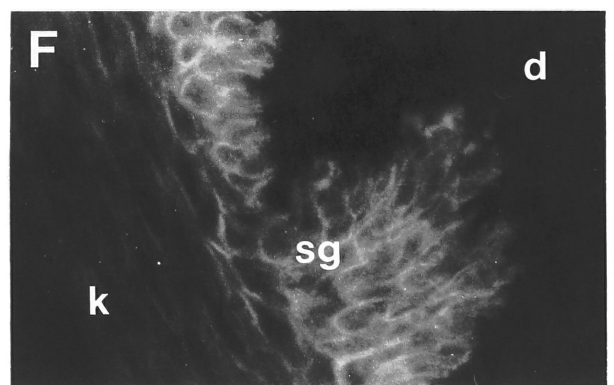
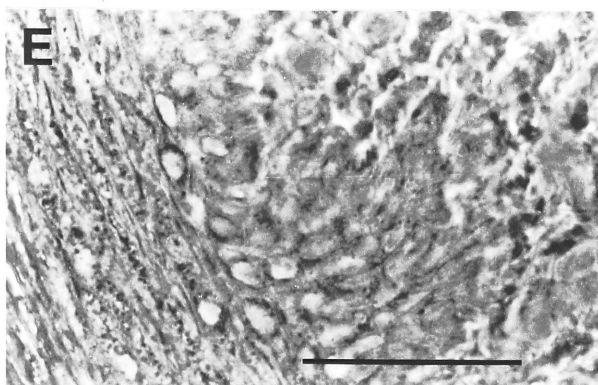
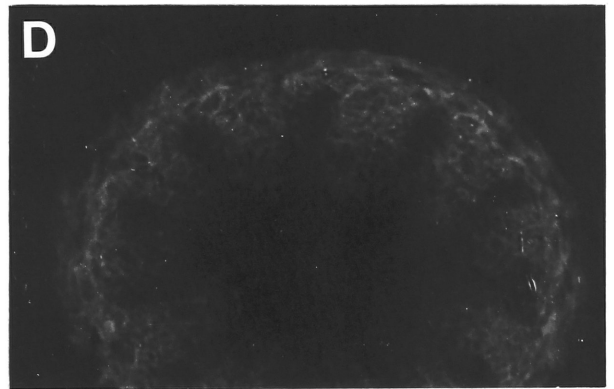
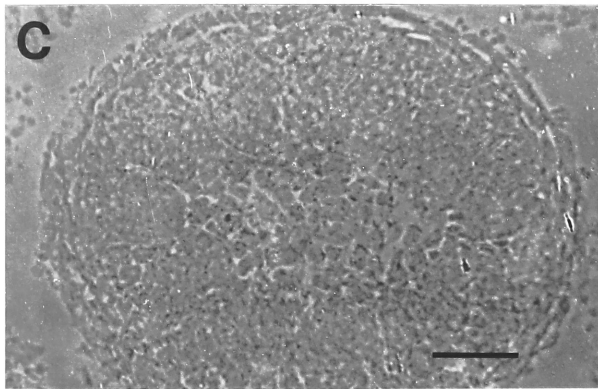
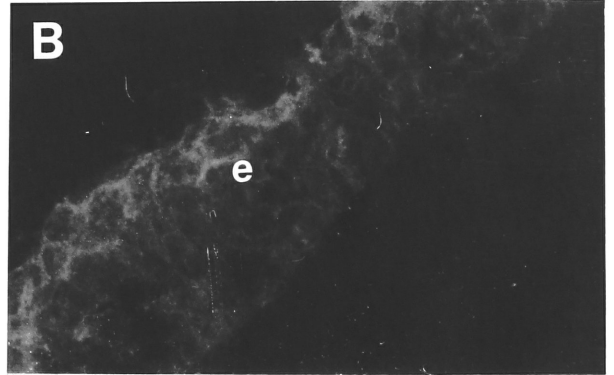
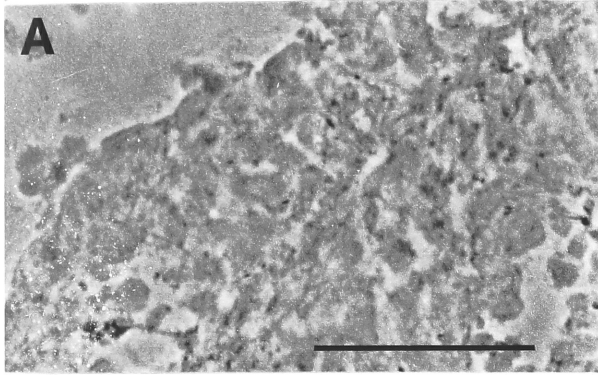
a part of the body plan that will develop into the adult, although they are not yet well differentiated. I will describe our preliminary findings of the location of L-CAM in several developing organs that contain the molecule. Experiments described here were performed on organs from 13 day embryos and adult chickens. At 13 days, most organs are well formed but lack the rigid definition and extensive connective tissue of the adult organs. Experiments using earlier embryos are in progress.

Many tissues appear to contain L-CAM. The results are presented here starting with the exterior surface, then tracing through the gut, then discussing the organs that arise as outgrowths of the gut during organogenesis, and ending with the urogenital system.

Skin

By the thirteenth day of incubation, when a well-defined embryo has developed, there is clear L-CAM staining on the outermost layer of cells of the skin (Fig 21 a,b). The feather rudiments have also started developing and L-CAM is seen on the feather quills, lining the pits that will give rise to the feather barbules (Fig 21 c,d). In the mature chicken, the L-CAM staining is present on the stratum germinativum, the layer of the epidermis which gives rise to the terminally differentiated keratinized epithelial cells (Fig 21 e,f). This staining is seen on the skin from all over the body, but is best observed in the thick skin pad on the plantar surface of the foot. There is no staining in the dermis, or in the blood vessels investing

Figure 21 - Immunofluorescent staining of skin with anti-L-CAM. A),C), and E) are phase contrast pictures of the same fields as B),D), and F) respectively. B) Skin of the 13 day embryo. The epidermal layer (e) is stained, the underlying dermis is unstained. D) Feather rudiment of the 13 day embryo. The surface cells of the rudiment and the cells lining the pits are stained, the central mesenchyme is unstained. E) Skin from the plantar foot surface of the adult. The cells of the stratum germinativum (sg) are stained on their surfaces. Staining intensity is progressively lower in the more differentiated keratinized squamous cells (k), and is totally absent in the cells of the dermis (d). Scale bars are all 50 micrometers.



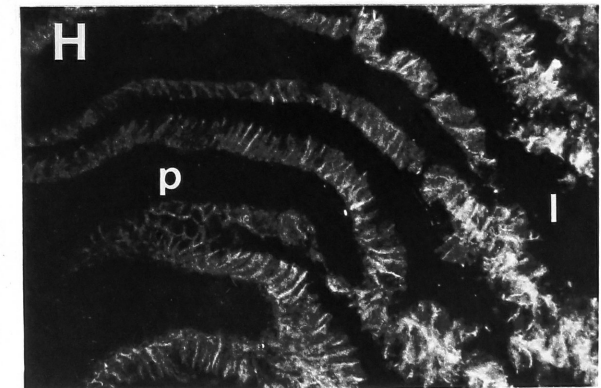
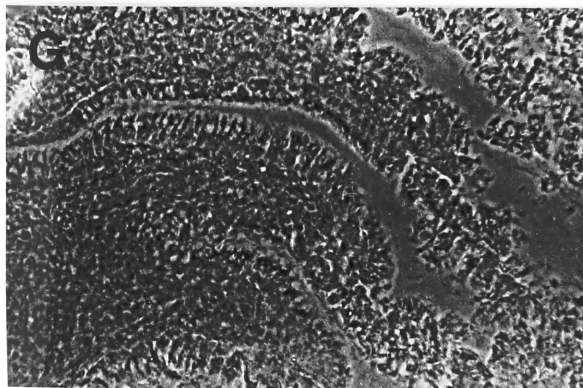
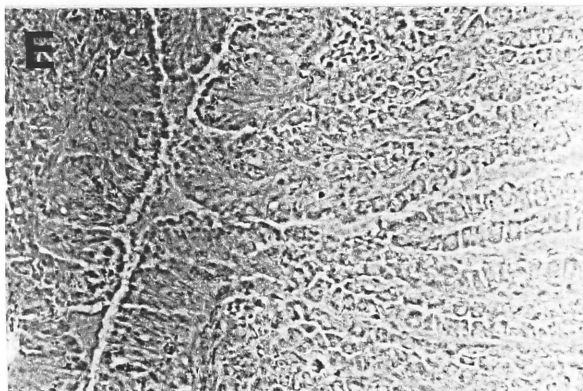
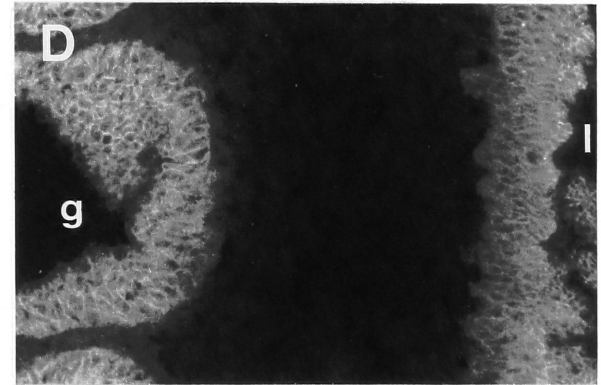
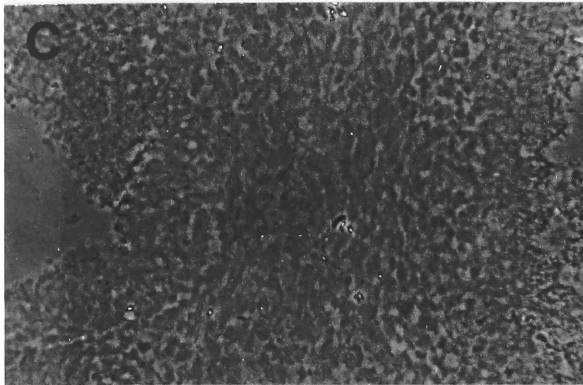
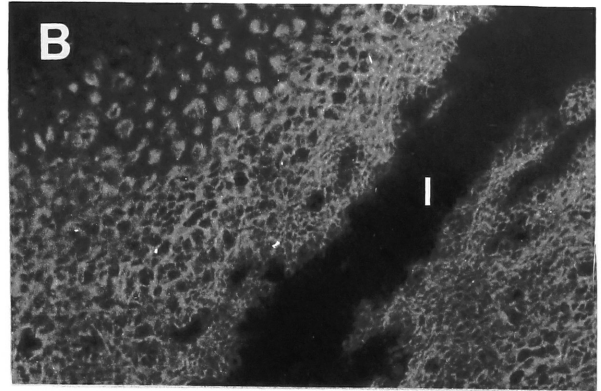
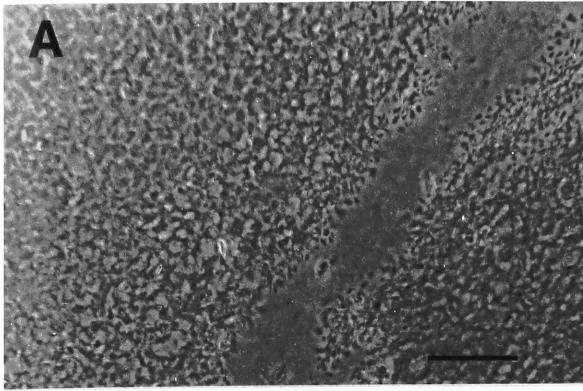
the dermis.

Proventriculus

The simple closed gut tube of the embryo differentiates along its length to form the various digestive organs. In the chicken, the functions of the stomach are separated into two organs. The proventriculus is the glandular stomach, which secretes acid and pepsinogen into the lumen of the gut as the food passes through it. The gizzard is the muscular stomach, a heavy knot of muscle encircling a lumen lined with an acellular substance secreted by the glands that line it. In the gizzard, food soaked in the proventricular secretions and gravel particles ingested by the bird are ground together to break up the hard-shelled grains that are a major part of the chicken's diet.

The proventriculus differentiates from a section of simple gut tube. At thirteen days of incubation, the organ is a thick spindle-shaped bulb at the posterior end of the esophagus. Anti-L-CAM staining shows two populations of cells with L-CAM on their surface. One population is the cells lining the lumen of the organ (Fig 22 a-d). The cells are present as a columnar epithelium, with staining uniform on the cell surface. The other population is the cells of the forming glands, in the wall of the gut (Fig 22 c,d). These are present as open spaces in the wall of the gut, lined with a layer of epithelium that stains strongly with anti-L-CAM. The surrounding cells have no L-CAM present on their surface. It is not clear from our pictures whether these precursors to the glands arise as condensations within the gut wall that will later open into the lumen of the gut, or whether they

Figure 22 - Immunofluorescent staining of L-CAM in the proventriculus. A),C),E), and G) are phase contrast pictures of the fields in B),D),F), and H) respectively. B) Section of the proventriculus from the 13 day embryo, cutting the lumen (1). Only the epithelial cells lining the lumen are stained; the developing connective tissue shows no staining. D) Cross section of part of the wall of the proventriculus of the 13 day embryo. Epithelial cells lining the lumen (1) are stained, epithelial cells lining the developing gland (g) are stained, but the cells of the intervening tissue are unstained. F) Section of the differentiated proventricular gland of the adult. The basal surfaces of the cells of the glandular alveolus (a) are stained, the apical surfaces are unstained. The columnar epithelium lining the collecting ducts (d) is stained uniformly on the cell surface. Connective tissue and blood vessels are unstained. H) Section of the lumen of the adult proventriculus. The epithelial cells lining the plicae (p) and facing the lumen (1) are stained on the surface. All the cells of the tunica propria and the blood vessels that form the core of the plicae are unstained. All panels are the same magnification. Scale bar=50 micrometers.



are evaginations of endoderm that bud into and invade the wall of the gut. The second alternative is more likely, as other glands in the digestive tract arise as evaginations of the gut, but more detailed studies are required to rigorously prove it.

In the proventriculus of the adult, the glands and luminal lining have fully developed and taken on their functions. Immunofluorescence staining shows L-CAM is concentrated on the basal surfaces of the gland cells. The apical surfaces of the gland cells, facing the lumen, are unstained (Fig 22 e,f). The lumen of each gland leads into a tertiary collecting duct. These tertiary ducts are lined with columnar epithelium which is stained uniformly on the cell surface with L-CAM. These ducts merge into secondary ducts, which in turn merge into primary ducts which then empty into the lumen of the proventriculus. All of these collecting ducts appear to be lined with columnar epithelium uniformly stained on the cell surface with L-CAM. The lumen of the proventriculus is thrown up into folds called plicae. The plicae consist of a central core of lamina propria, with connective tissue and blood vessels in it, covered on the luminal surface with columnar epithelium. Only the luminal columnar epithelium of the plicae is stained with anti-L-CAM, once again uniformly on the surface (Fig 22 g,h).

Gizzard

The gizzard of the 13 day embryo is a round white ball of loose cells which are in the process of differentiating into the thick muscular layer of the adult organ, with a lumen lined by endodermally

derived columnar epithelium. L-CAM staining of the gizzard at this stage shows that only the cells lining the lumen are stained (Fig 23 a,b). None of the loose mesenchyme of the rest of the organ is stained. In the adult organ, the lumen is lined by a tough flexible layer of acellular secretion produced by gland cells lining the lumen. Sections of the adult gizzard show that anti-L-CAM stains the gland cells of the lining of the gizzard, but not the lining material or the tunica propria at the center of the glandular protuberances (Fig 23 c,d). None of the mature smooth muscle, the connective tissue, or the blood vessels or blood cells stain.

Intestine

The intestine of the 13 day embryo has already started folding into the compact pattern of the mature intestine. This part of the gut is like all of the others: concentric layers of smooth muscle, connective tissue, and endodermally derived epithelium. In the 13 day embryonic intestine, a layer of cells lining the lumen, one or two cells thick, is stained with anti-L-CAM (Fig 24 a-d). The beginnings of villi are seen as this lining is starting to be thrown up into folds in the luminal space.

In the adult intestine staining is seen on the columnar epithelium lining the villi and the crypts of Lieberkuhn (Fig 24 e-g). All connective tissue, smooth muscle, and blood cells are negative for L-CAM.

Figure 23 - Immunofluorescent staining of L-CAM in the gizzard. A) and C) are phase contrast photographs of the fields B) and D), respectively. B) Section through the inner lining of the gizzard of the 13 day embryo. The epithelial cells facing the lumen (1) are stained, the underlying mesenchymal cells are unstained. D) Section through the glands of the lining of the adult gizzard. The cells of the glands (g) that secrete the acellular lining of the organ are stained. The connective tissue and smooth muscle that form the rest of the organ are unstained. All panels are the same magnification. Scale bar=50 micrometers.

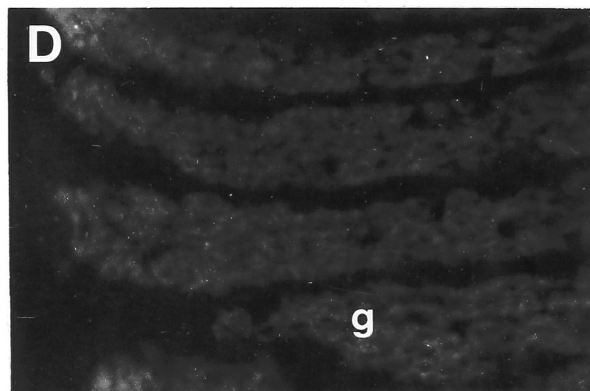
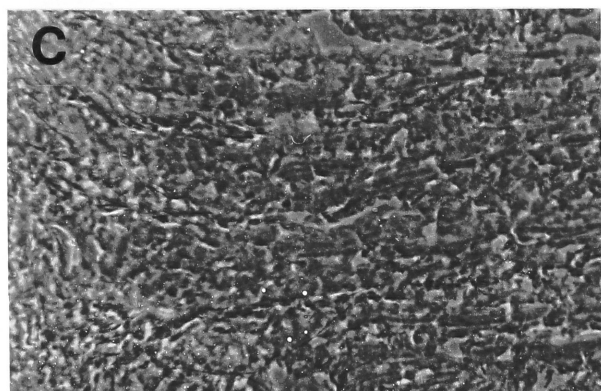
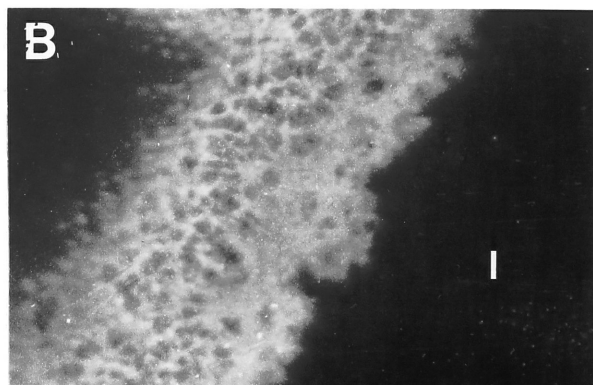
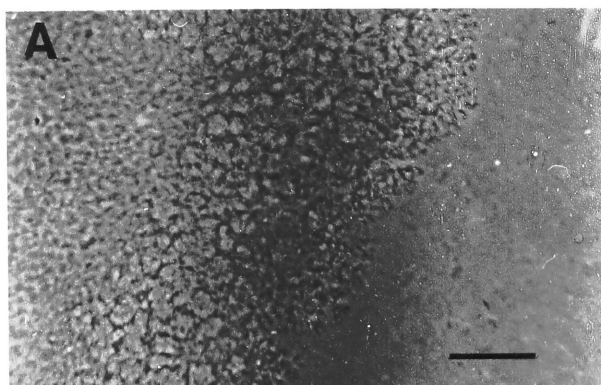
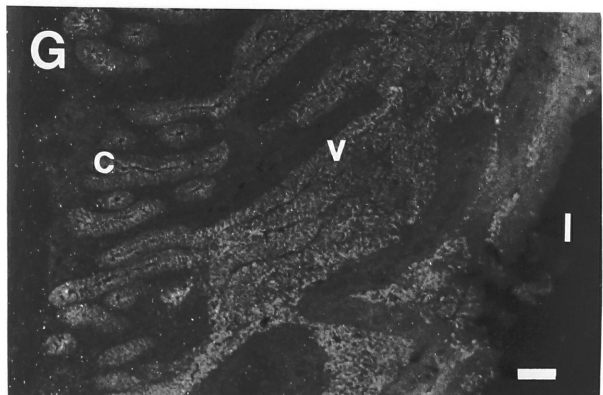
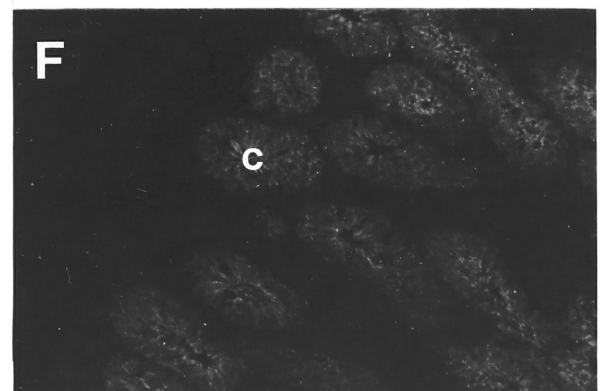
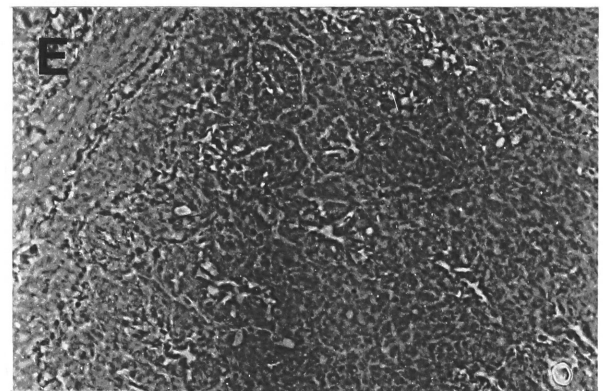
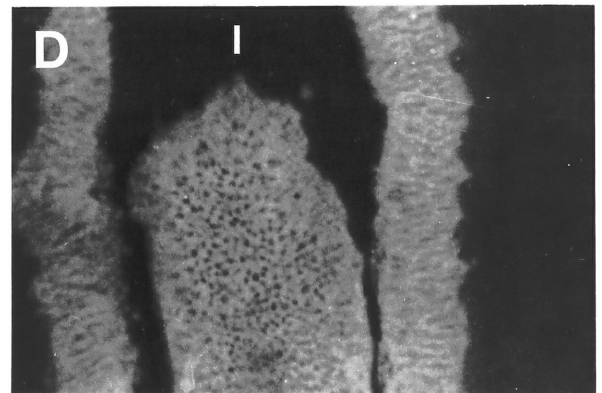
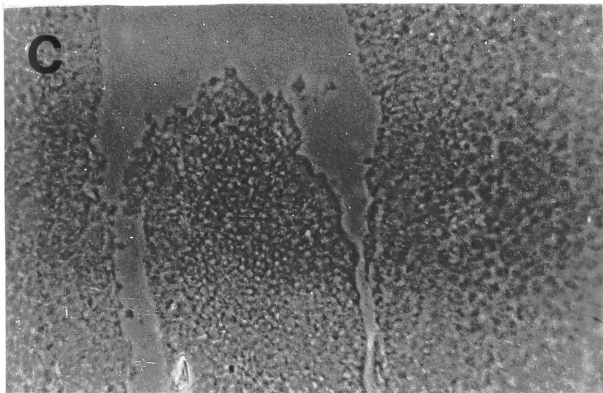
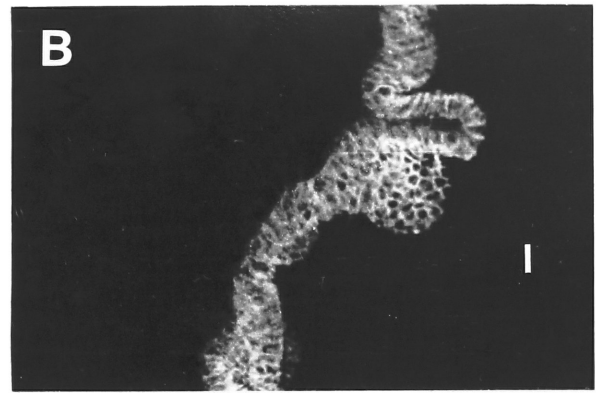
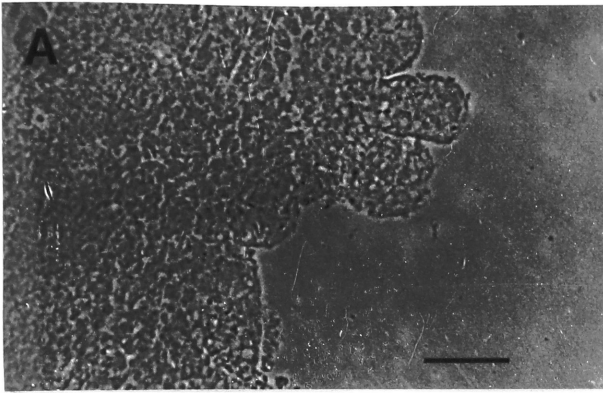


Figure 24 - Immunofluorescent staining of L-CAM in the intestine A),C), and E) are phase contrast photographs of the fields B),D), and F), respectively. B) Cross section of the intestine of the 13 day embryo. Only the epithelial cells lining the lumen (l) stain. D) Tangential section through the intestine of the 13 day embryo. Staining is only seen on cells lining the lumen (l). The beginning of a convolution that will form a villus is seen at center. F) Section through the base of the absorptive layer of the adult intestine. The crypts (c) are stained, while the surrounding connective tissue and smooth muscle are unstained. G) Section through the complete absorptive layer of the intestine. The villi (v) facing the lumen are stained on the luminal surface, the crypts (c) are stained, and the connective tissue forming the core of the villi and the lamina propria is unstained. Panels A)-F) are the same magnification, panel G is a lower magnification. Scale bars=50 micrometers.



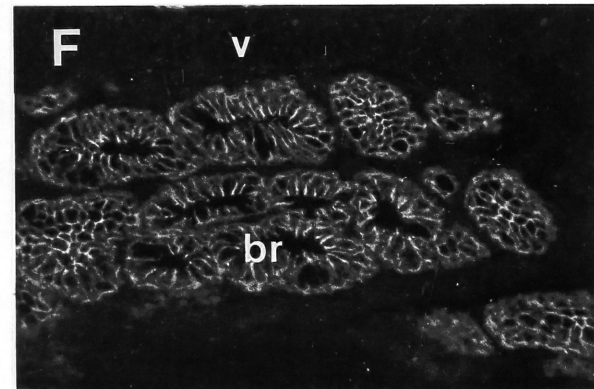
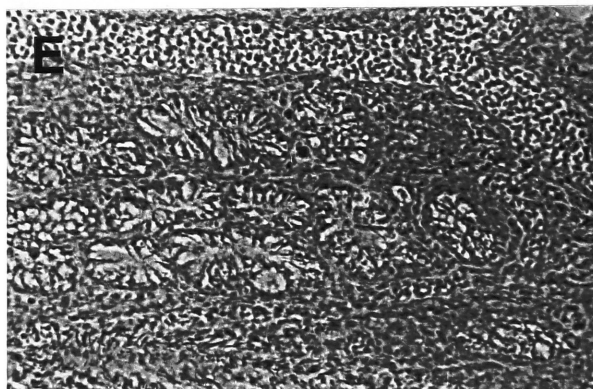
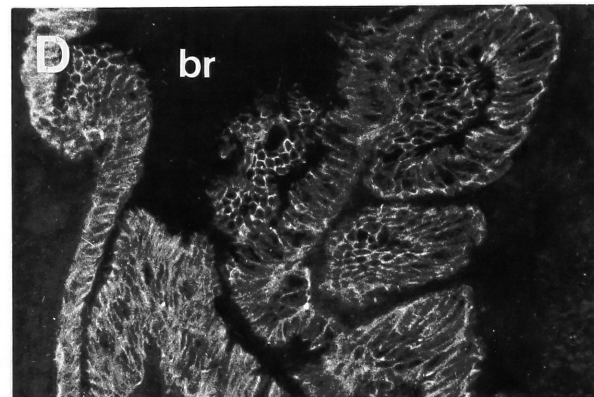
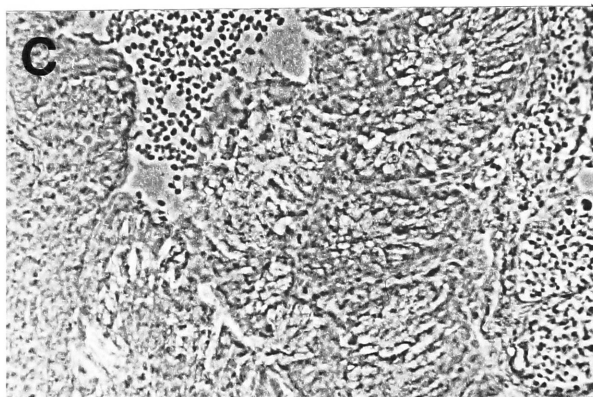
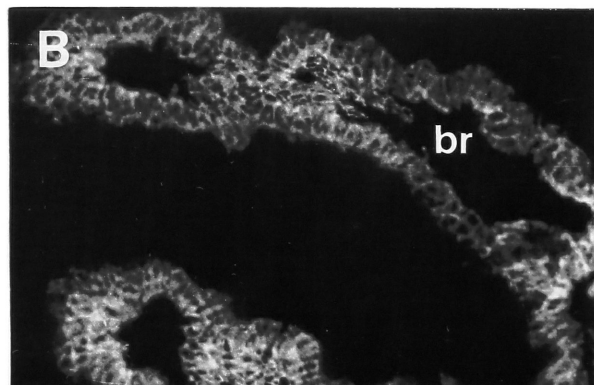
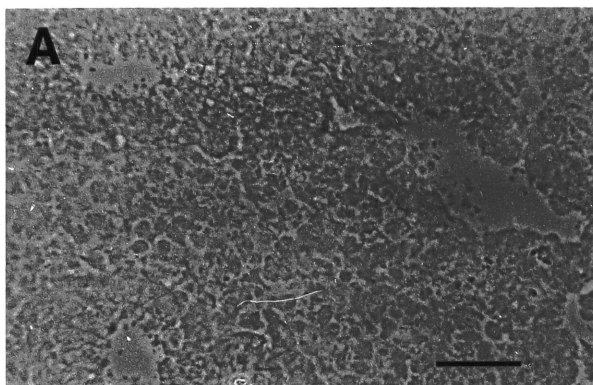
Lung

The lungs, along with the liver and the pancreas, arise as buddings from the primitive gut tube. The lungs arise from the tracheal outgrowth in the pharynx, which splits into two bronchi and invades the surrounding mesenchyme, continuing to subdivide into primary, secondary and tertiary bronchioli, which in turn differentiate into the atria, the actual sites of gas exchange in the lung. Unlike the mammals, chickens do not have sac-like alveoli, but instead have highly ramified structures along the bronchioli. The bronchioli form a large network of anastomosing tubes, as opposed to the dead end structure of the mammalian bronchioli.

In the 13 day embryo, the lung has the appearance of a developing gland. There are none of the terminal differentiations that are characteristic of the lung, just a series of epithelium-lined tubes branching through a rather loose mesenchyme. Anti-L-CAM staining shows that the epithelium lining these tubes contains L-CAM, whereas the mesenchyme does not (Fig 25 a,b). The cells of the epithelium are stained over their whole surface.

In the adult lung, the majority of the mass is connective tissue and the multiple blood vessels of the pulmonary circulation. None of these cells are stained with anti-L-CAM. The columnar epithelium lining the air passages is stained by anti-L-CAM (Fig 25 c,d), as are the cells of the tertiary bronchioli which form the atria (Fig 25 e,f).

Figure 25 - Immunofluorescent staining of L-CAM in lung. A),C), and E) are phase contrast photographs of the fields in B),D), and F). B) Section through the lung of the 13 day embryo. The endodermally-derived epithelium lining the developing bronchioles (br) is stained. The loose mesenchyme that is being invaded is unstained. D) Section through a bronchiole of the adult chicken. The epithelium lining the bronchiole (br) is stained. The connective and vascular tissue, and the blood cells are unstained. F) Section through tertiary bronchioles of the adult chicken. Cells lining the bronchioles (br), that form the atria, are stained. Connective tissue and cells associated with the blood vessel (v) are unstained. All panels are the same magnification. Scale bar=50 micrometers.



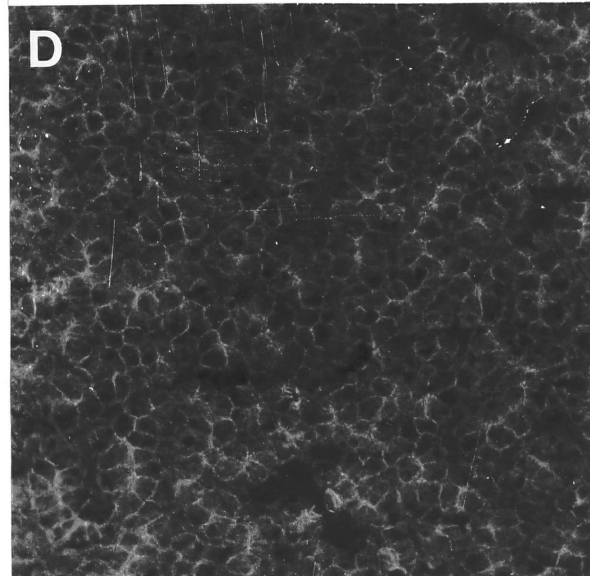
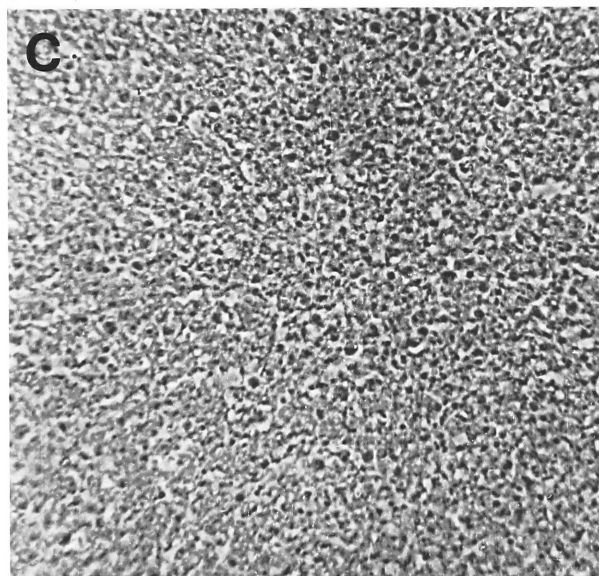
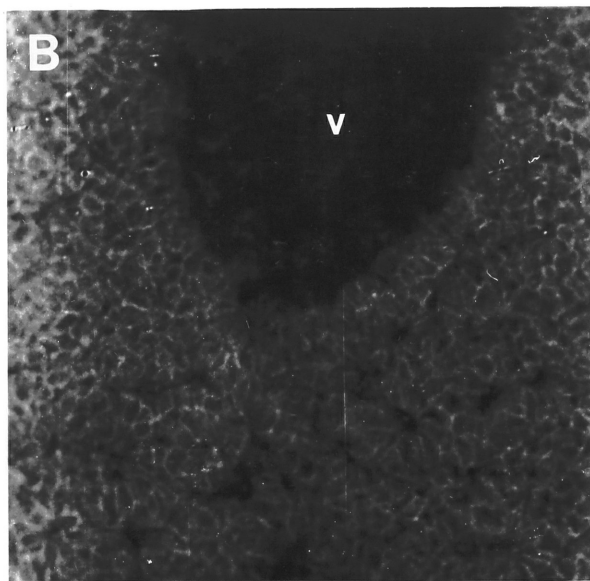
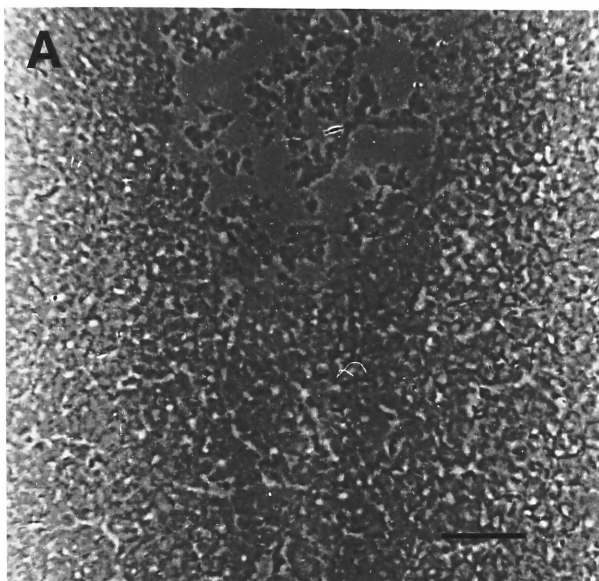
Liver

The liver arises as a diverticulum from the anterior intestinal portal of the closing gut at nearly the same time as the pancreas does. Its cells invade the hepatocardiac mesenchyme and the ductus venosus, as described in the Introduction.

The liver of the 13 day embryo, although far from being terminally differentiated, has histology typical for adult liver. As might be expected from the fact that L-CAM was isolated from the liver, all the hepatocytes are stained by anti-L-CAM. The staining is uniform on the surface, and apparently restricted to the hepatocytes (Fig 26 a,b). Blood vessels and connective tissue are not stained. The resolution of these sections is not sufficient to see staining or lack of it in the sinusoidal endothelial cells or the Kupffer cells, but from the results in culture, and the fact that blood vessel endothelial cells are not stained, we expect that L-CAM is restricted to the parenchymal cells.

Sections of adult liver show a staining pattern very similar to that of the embryonic liver (Fig 26 c,d). The cells are somewhat larger, and it appears that the L-CAM staining may be concentrated on one face of the hepatocytes, but the difference in intensity is not so striking as to allow an unequivocal statement to that effect. The circulatory system remains unstained. Some sections contain small ducts lined with epithelium which is stained with anti-L-CAM. These appear to be the ducts that carry the bile from the bile canaliculi to the gall bladder and intestine.

Figure 26 - Immunofluorescent staining of L-CAM in liver. A), and C) are phase contrast photographs of the fields in B) and D), respectively. B) Section through the liver of the 13 day embryo. The surfaces of all hepatocytes are uniformly stained. Cells lining the blood vessel (v) and blood cells within the vessel are unstained. D) Section through the liver of the adult. The hepatocytes are stained on the whole cell surface. It appears that there may be a higher amount of staining on some surfaces than on others. All panels are the same magnification. Scale bar=50 micrometers.



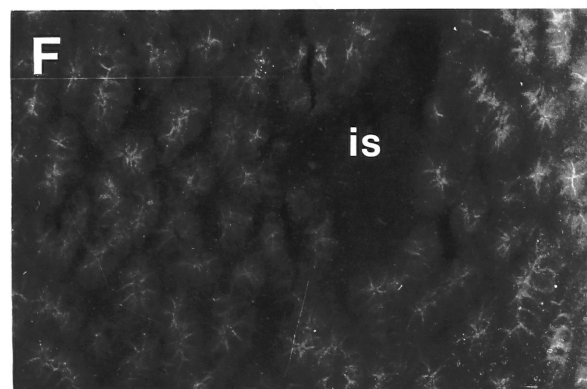
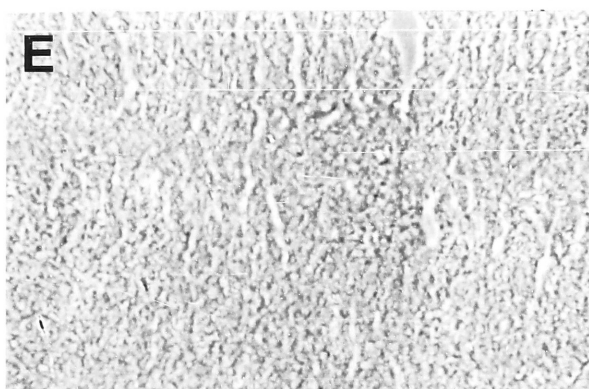
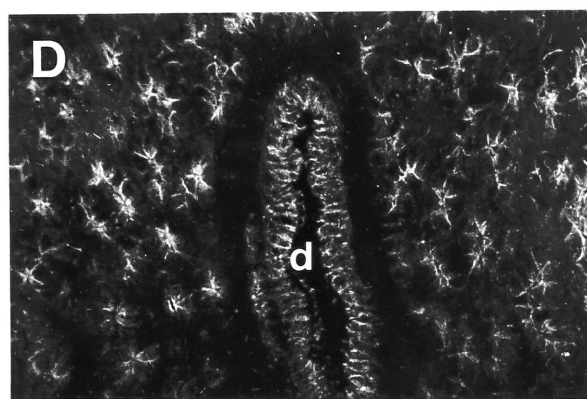
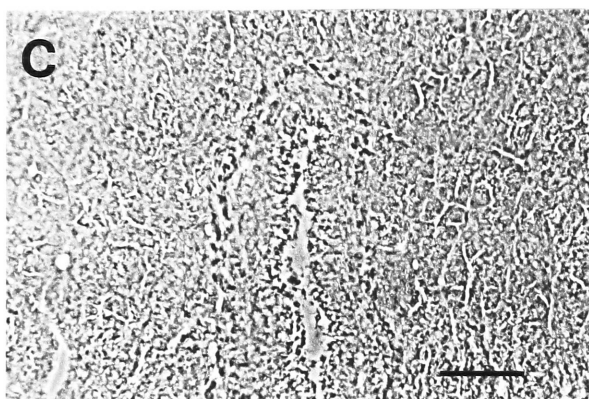
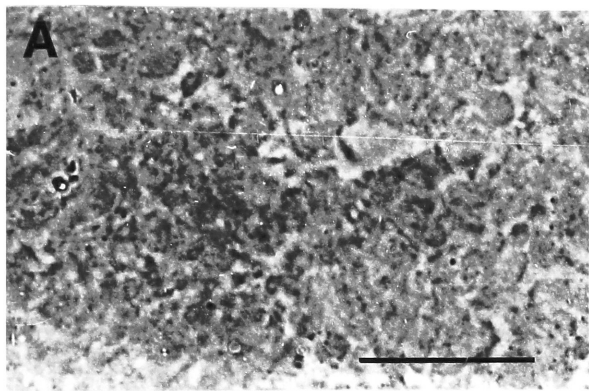
Pancreas

The pancreas arises as a budding off of some of the endodermally derived epithelium of the gut from the anterior intestinal portal as the gut is closing. These endodermal buds grow out into the mesenchyme which will develop into the mesentery, holding the pancreas within the first loop of the duodenum. When this loop from a 13 day embryo is sectioned, the cells of the endodermal buds stain uniformly over their surface, while the loose mesenchyme that they are invading remains unstained (Fig 27 a,b).

In the adult pancreas, the exocrine cells are formed into acinar glands, with their basal surfaces facing the capillaries investing the pancreas, and their apical surfaces facing the lumen into which they secrete digestive enzymes as inactive precursors. These acinar glands empty into ducts which carry the digestive enzymes to the intestine.

The exocrine pancreas cells show L-CAM present on only part of their cell surface, the lateral faces of the apical end of the cells (Fig 27 c-f). There are also ducts lined with cuboidal epithelium, the cells of which are stained on the whole cell surface (Fig 27 c,d). The blood vessels and connective tissue of the pancreas are unstained, as are the islets, the endocrine cell clusters of the pancreas (Fig 27 e,f).

Figure 27 - Immunofluorescent staining of L-CAM in pancreas. A),C), and E) are phase contrast photographs of the fields in B), D), and F), respectively. B) Section through the distal pancreas of the 13 day embryo. Endodermally derived cells that have budded out from the gut and invaded the mesenchyme of the abdomen are stained. The mesenchymal cells are unstained. D) Section through the adult pancreas. The lateral-apical surfaces of the glandular acinar cells are stained. The epithelium lining one of the pancreatic ducts (d) is stained. The connective tissue surrounding the duct is unstained, as are the blood vessel cells around the basal surfaces of the acini. F) Section through the adult pancreas. The acinar gland cells are stained on the lateral-apical surfaces. The cells of the endocrine islet (is) are not stained. Magnification of A) and B) is 2.5 times that of C) through F). Scale bar=50 micrometers.



Urogenital system

The morphogenetic events that lead to the formation of the urogenital system are different from those leading to the formation of the other organs containing L-CAM. The organs of the urogenital system are mesodermally derived, and arise by the condensation of loose cells into compact masses which then form tubules and other structures. This is different from the migrations of whole sheets of cells and budding out from these sheets that are typical for the formation of endodermally and ectodermally derived organs.

Three phases of kidney development are typically described: the pronephros, a kidney structure similar to that of the teleost fishes; the mesonephros, similar to the kidney of elasmobranches and amphibia; and the metanephros, which is the functional kidney of the amniotes. Torrey (1965) has suggested that these structures be considered collectively as the holonephros, a continuum of structures that comprise the developing kidney.

The primary collecting duct of the urinary system originates as the pronephric duct and grows caudally, becoming the mesonephric or Wolffian duct. The Wolffian duct will develop into the ureter and in the male will also form the genital ducts. The Wolffian duct also buds out into the metanephrogenic mesenchyme and induces this tissue to differentiate into the structures of the mature adult kidney. The Wolffian duct also induces the formation of the Mullerian duct, a parallel duct which, in the female gives rise to the oviduct, and which degenerates in the male.

Preliminary localization of L-CAM in the the 13 day metanephric kidney and the corresponding adult organ show very similar structures and distributions of L-CAM. L-CAM is present on all tubular structures observed (Fig. 28). This presumably includes the proximal and distal tubules, the chicken equivalent of the loop of Henle, and the collecting tubules. The parietal cells of Bowmans capsule, the outer layer of the glomerulus, are also stained, but the visceral cells are not. The cells of the blood vessels and capillaries are not stained, and neither are the stromal cells of the kidney (Fig 28).

The shell organ of the adult female is the posterior segment of the oviduct, just anterior to the vagina. It is a tube of muscular and connective tissue lined with large folds of tissue containing glands, and lined with columnar epithelium. At various levels of the oviduct these folds have different shapes and sizes, but they all have a roughly similar structure, like a tree with a central trunk of connective tissue and blood vessels, with branches of connective tissue and glands, and an outer coating of epithelium which lines the lumen of the oviduct. In the anterior portions of the oviduct, the glands secrete the substance of the egg white, primarily ovalbumin and lysozyme. In the lower oviduct the shell membrane and the shell are deposited.

L-CAM is present on the columnar epithelium lining the lumen of the oviduct, and on the lateral surfaces of the gland cells (Fig 29 a-d). Once again it is not present on connective tissue, endothelial cells or blood cells.

Figure 28 - Immunofluorescent staining of L-CAM in the kidney. A) and C) are phase contrast photographs of the fields in B) and D), respectively B) Section through the metanephric kidney of the 13 day embryo. The parietal cells of the glomerulus (gl) are stained, the visceral cells of the glomerulus and cells of the capillary plexus are unstained. The cells of the epithelia that form all tubular structures are stained. D) Section through the mature kidney of the adult. Staining is the same as it is for the 13 day embryo. All panels are the same magnification. Scale bar=30 micrometers.

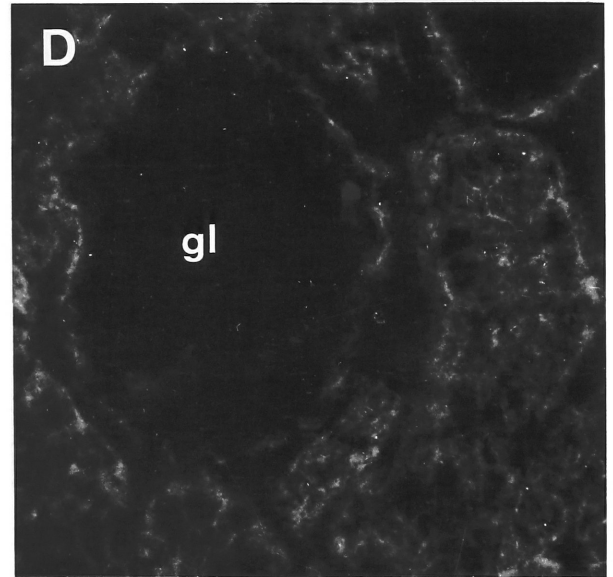
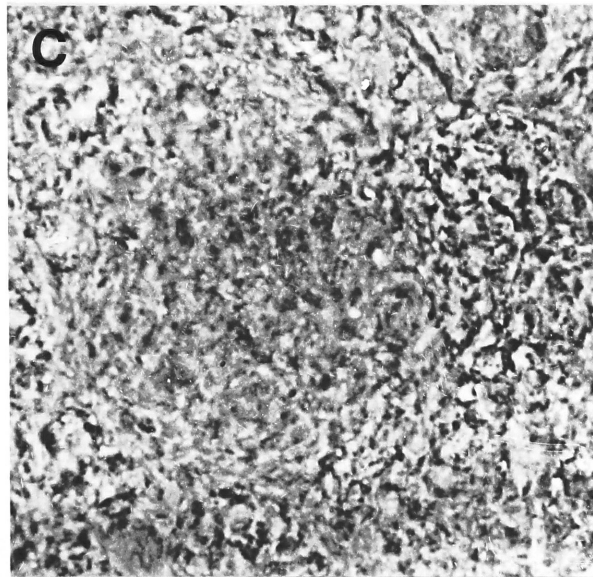
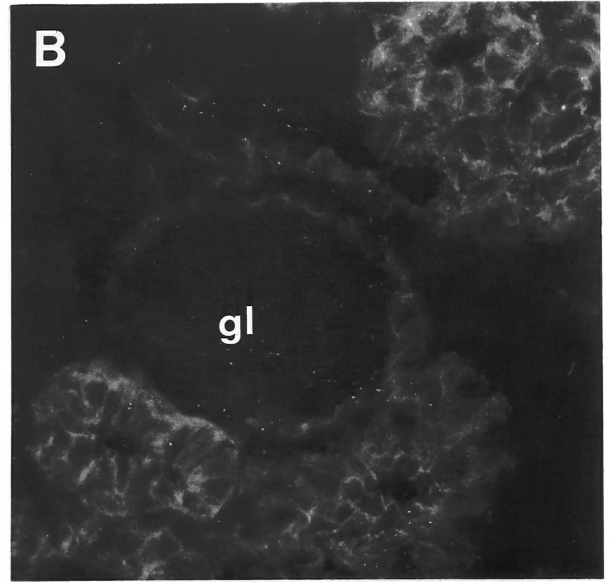
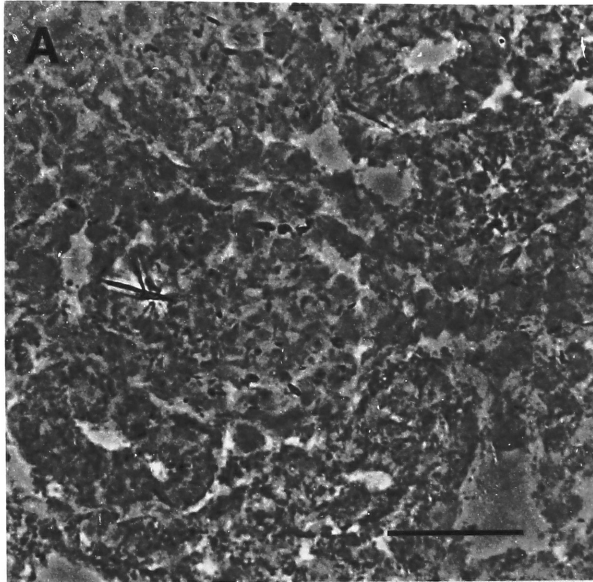
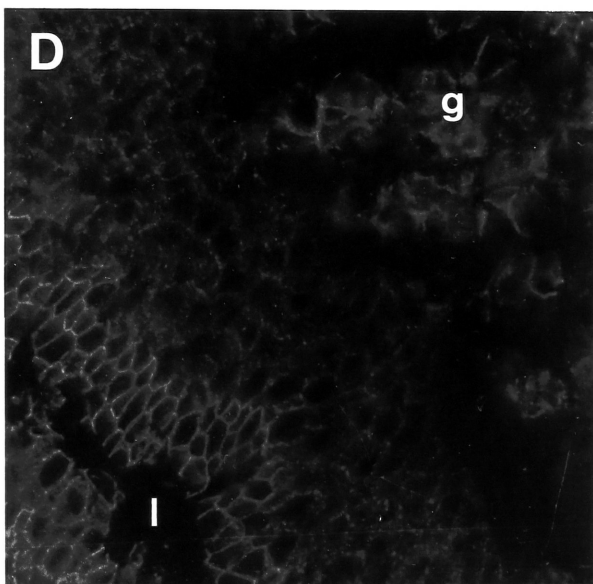
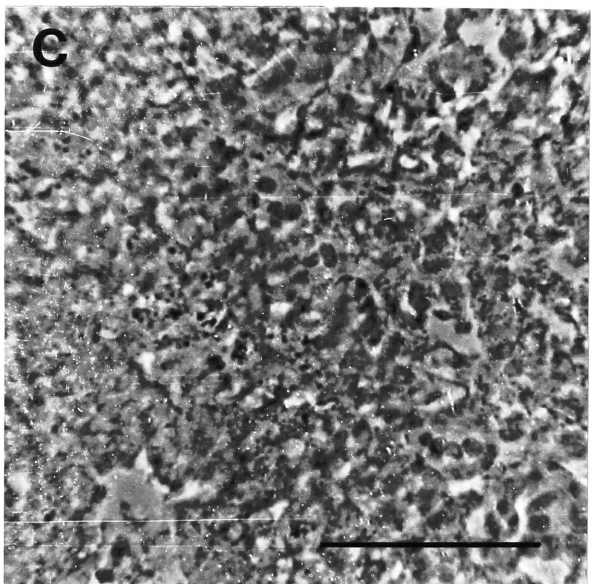
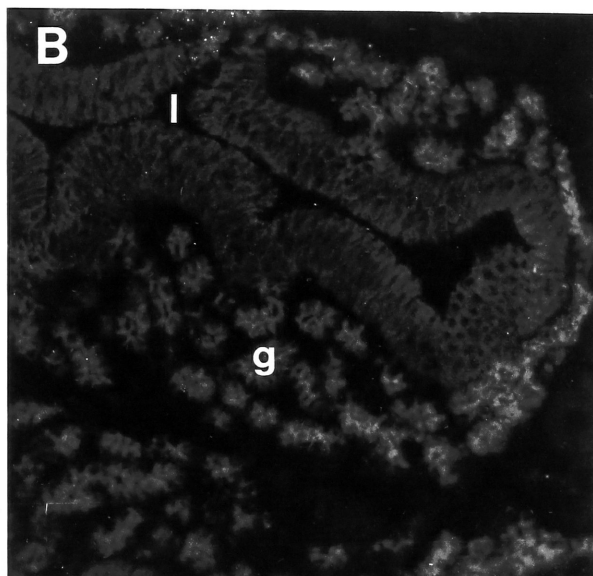
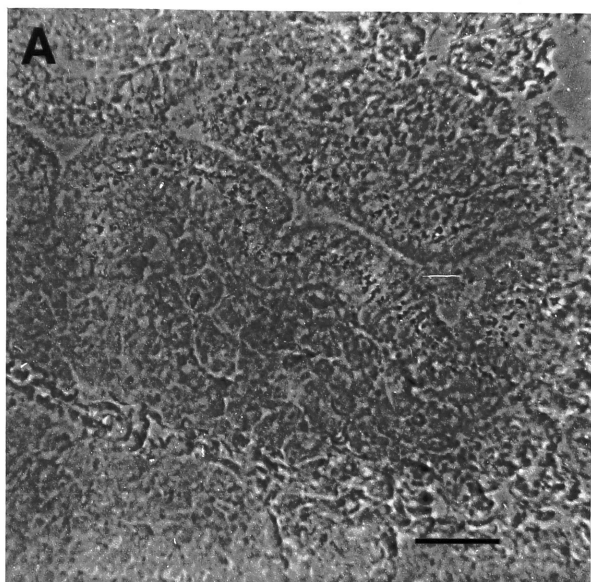


Figure 29 - Immunofluorescent staining of L-CAM in the female reproductive tract. A) and C) are phase contrast photographs of the fields in B) and D), respectively. B) Section through the basal area of the glandular structures of the adult shell organ. Cells of the glands (g) and the epithelium lining the lumen (l) are stained. The cells of the blood, blood vessels, and connective tissue are unstained. D) Higher magnification of structures shown in (B). Staining appears restricted to the lateral surfaces of the gland cells. The epithelium lining the lumen appears to be unstained on the luminal surface. Staining on the deeper layers of the luminal epithelium appears to be in dots. Scale bars=50 micrometers.



From these extensive but still fragmentary results, the tentative conclusion is that L-CAM is present on the epithelial cells of the urogenital system of the chicken. A more detailed scan is, of course, necessary to confirm this for the rest of the oviduct, the male genital tract, and the ureter.

Structure of L-CAM on different organs

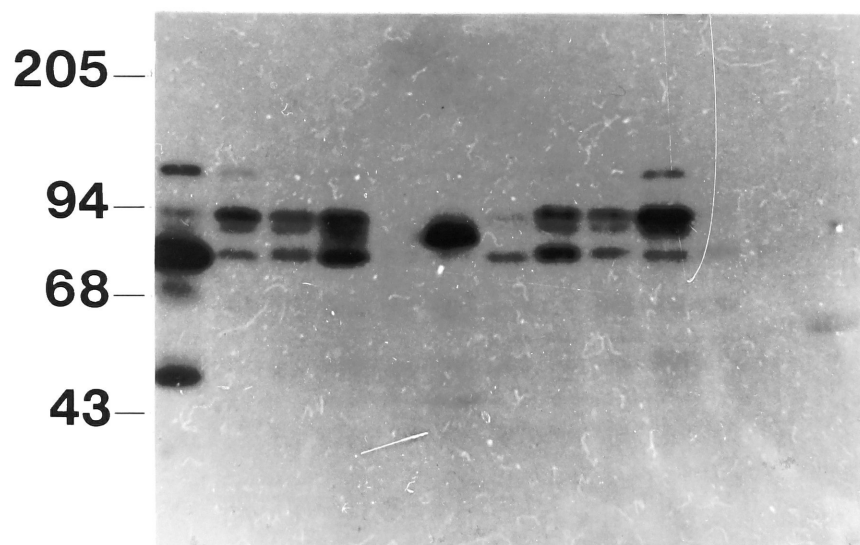
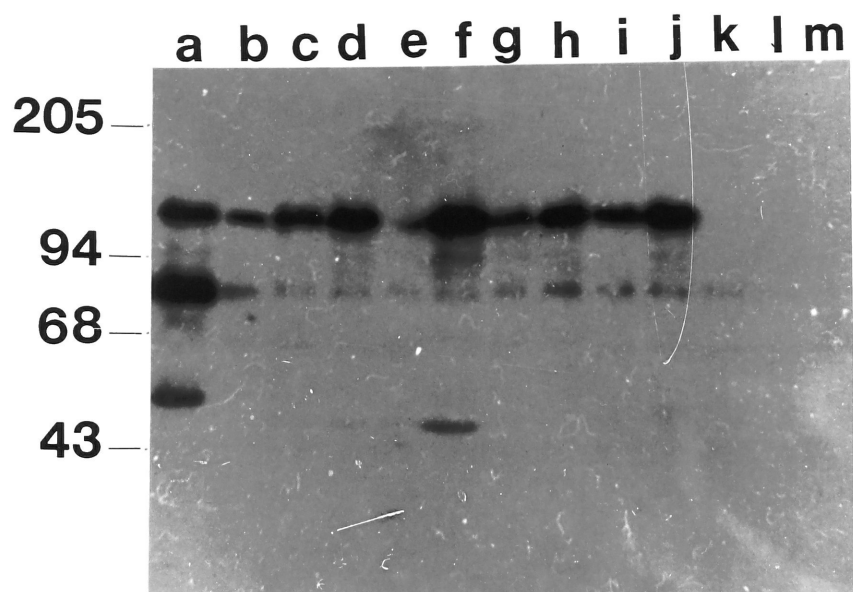
One possible explanation for this widespread distribution of staining with anti-L-CAM in the organs of the chicken is that a common antigen present on a number of different molecules is being detected. This is the case with some carbohydrate blood group antigens, which can be detected on glycolipids and glycoproteins.

To check this possibility, detergent extracts of organs were prepared, separated on 7.5% acrylamide SDS-PAGE, transferred to nitrocellulose, and visualized with rabbit anti-L-CAM and ^{125}I -labelled protein A. Two kinds of extractions were performed, one with boiling 1% SDS, to minimize degradation of L-CAM, the other with 1% NP-40, which allows a partial degradation of the molecule in the extract.

As can be seen in figure 30 (upper panel), the organs containing L-CAM all show predominantly a 124,000 dalton band. In figure 30 (lower panel), the partially degraded L-CAM can be seen to be at several molecular weights between 80,000 and 124,000 daltons. The bands all seem to be common, although they vary in relative amounts, presumably because this kind of degradation by intrinsic proteases is not easily controlled from organ to organ. L-CAM is not detected by this method

Figure 30 - L-CAM in detergent extracts of whole organs from 13 day embryos as detected on immunoblots. Top panel - 15 μ l of SDS extracts of each organ (200 mg organ/ml of extract) separated by SDS-PAGE on 7.5% acrylamide gel. Bottom panel - 15 μ l of NP-40 extracts of each organ (200 mg organ/ ml of extract) separated by SDS-PAGE on 7.5% acrylamide gel.

Lanes: a) gall bladder b) extraembryonic membrane
c) intestine d) kidney e) spleen f) liver g) gizzard
h) proventriculus i) lung j) skin k) breast muscle l) heart
m) brain Numbers to the left of the panels indicate molecular weight standards, $\times 10^{-3}$.



in brain, heart, breast muscle, or spleen.

Similar extractions performed on adult organs gave essentially the same results as were obtained with embryonic tissue. All corresponding adult organs have L-CAM that is indistinguishable from the L-CAM from embryos when evaluated by SDS-PAGE. There were no detectable differences in molecular species between the different adult organs.

SUMMARY

The colonies formed by embryonic liver cells in culture, which have structural features similar to the intact liver, can be reversibly disrupted by the presence of F_{ab} ' fragments of anti-L-CAM, both during the formation of colonies, and after morphologically recognizable colonies have formed. The hepatocytes in these cultures stain uniformly on the surface with anti-L-CAM in immunofluorescence experiments, while the flat cells outside the colonies do not stain with the same antibodies. These flat cells are probably cells from the blood vessels or connective tissue of the liver.

L-CAM is present in a wide variety of organs of the chicken, including tissues arising from all three germ layers. It appears on only some tissues, usually epithelial tissues, within a given organ. While it is present in embryonic and adult organs in some cases its distribution on the cell surfaces of adult organs is more restricted than on the cell surfaces of embryonic organs.

L-CAM on all organs, in both the adult and the 13 day embryo, appears to be the same molecular species, although small differences in structure cannot be ruled out by the techniques used so far.

DISCUSSION

Adhesion between cells appears to be one of the essential processes of the complex phenomenon of organogenesis. A large body of information exists describing simple in vitro systems demonstrating various processes of histogenesis (see Monroy and Moscona, 1979) but rigorous definition of the molecular elements mediating cell-cell adhesion and histogenesis has only begun in the last few years. Several molecules have been described that affect cell adhesion. Glycoproteins mediating the adhesion of sponge cells have been isolated, characterized, and their role in cell-cell adhesion defined (Humphreys, 1963; Henkart et.al., 1973). The sponge glycoproteins bear little structural or functional similarities to cell-cell adhesion molecules described so far in higher organisms in that they are proteoglycans, and are very large molecules, consisting predominantly of carbohydrate.

Two molecules, ligatin (Jakoi and Marchase, 1979) and retinal cognin (Hausman and Moscona, 1975), that have direct effects on cell-cell adhesion in vertebrates have been isolated and partially characterized, but descriptions of the role of these molecules have not been extended beyond the simple in vitro systems in which they were first characterized.

The first cell adhesion molecule to be purified and related to development of organ structures in vertebrates is N-CAM, a large glycoprotein that mediates calcium-independent cell-cell adhesion

between neural cells. N-CAM has been shown to have a role in the normal development of the retina, (Buskirk et. al., 1980), binding of nerves to muscle in culture (Grumet et. al, 1982) and the normal development of the brain (Edelman and Chuong, 1982). The distinguishing structural feature of N-CAM is the unusually large amount of sialic acid that it contains, apparently as polysialic acid (Hoffman et. al., 1982). N-CAM is modulated in two ways during development. The structure of the molecule is altered from embryonic to adult organs by a decrease in the amount of sialic acid (Rothbard et. al., 1982; Edelman and Chuong, 1982), and the amount of N-CAM expressed on the cell surface varies on cells at different developmental stages (Thiery et. al., 1982).

Edelman (1983) has suggested that the wide distribution of N-CAM during the development and the modulation of structure and prevalence of the molecule imply that only a small number of different kinds of adhesion molecules are necessary to account for the complex processes of embryogenesis. A single molecule could mediate specific processes through a number of different mechanisms. This notion requires that much of the specificity for development arises in the cellular physiology of the cells rather than in a large amount of simply encoded genetic information. This is a specificity of process rather than of structure. A necessary consequence of this theory is that each of the primary adhesion molecules in the embryo will have wide spatial distribution and variation in temporal pattern of appearance.

The goals of the experiments described in this thesis were: 1) to isolate a molecule mediating adhesion between embryonic liver cells 2) to begin to use antibodies to the molecule as probes for studying embryonic processes and 3) to compare the molecule to the N-CAM and to other cell-adhesion molecules. A molecule (L-CAM) that is involved in the initial binding of liver cell to liver cell in a rotating culture assay has been identified and isolated.

L-CAM is an intrinsic membrane glycoprotein with an apparent molecular weight on SDS-PAGE of 124,000 daltons. The molecule is not eluted from cell membranes by salt solutions, but requires detergents or proteolysis to be released from the membranes. When isolated liver membranes are treated with low concentrations of trypsin in the presence of calcium an 81,000 dalton fragment containing all of the carbohydrate is generated as a soluble entity. The carbohydrate is distributed as four approximately equal sized oligosaccharide chains linked to asparagine. One of the oligosaccharides appears to be of the high mannose type, the other three to be of the complex type. A small amount of sialic acid is also present, although its distribution on the molecule is not known.

Amino acid analysis of the 81,000 dalton fragment indicates that it contains approximately 11 methionine residues, suggesting that cyanogen bromide digestion should yield twelve peptides. There are 81 aspartate residues and 91 glutamate residues. At least four of the aspartates must be present in the protein as asparagine, since there are four asparagine-linked oligosaccharides. The acidic pI of the

81,000 dalton fragment suggests that many of the acidic residues detected in amino acid analysis are present in the protein as acids rather than amides. No cysteine or derivatives of cysteine were detected in the amino acid analysis, suggesting that disulfide bonds are not a prominent feature of this molecule. This may mean that the molecule has an extended, rather than a globular structure, and it certainly implies that there are other processes maintaining the tertiary structure of the molecule.

The L-CAM mediated adhesion process, unlike the N-CAM mediated adhesion, requires the presence of calcium. Extraction of liver membranes in the absence of calcium, either with added trypsin or with unidentified intrinsic proteases, yields small amounts of peptides with molecular weights lower than 81,000 daltons. These facts imply a structural role for calcium ions in this molecule, maintaining a tertiary structure that is necessary for the adhesion mechanism to function, and that also protects a number of potential trypsin cleavage sites from proteolytic degradation. More detailed studies, including amino acid sequence analysis, carbohydrate analysis, and calcium binding studies are necessary to obtain a clearer insight into the nature of the structure-function relationship for L-CAM.

One of the basic questions about the L-CAM mediated adhesion mechanism is the nature of the cellular ligands. It is fairly clear that one of the ligands is L-CAM. Does L-CAM on one cell bind to L-CAM on another cell, a homophilic mechanism as is suggested for N-CAM (Edelman, 1983), or is there some other ligand that binds to L-CAM,

i.e. a heterophilic mechanism? The only evidence at hand that addresses this question is the nearly complete neutralization of anti-hepatocyte activity in blocking cell adhesion by highly purified L-CAM (see Figure 11). If there were an L-CAM binding ligand, and it was immunogenic, then the antibodies against that molecule would be able to block adhesion and would not be neutralized by L-CAM. The fact that no such antibodies appear to exist in polyspecific sera implies either that there is no L-CAM binding molecule, or that such a molecule is not immunogenic in the procedures employed in these studies.

One approach to deciding between the homophilic and heterophilic mechanisms is to use an assay of binding liver cells in suspension to a liver cell monolayer. If the L-CAM mediated adhesion is homophilic, then treatment of either the suspension or the monolayer cells with anti-L-CAM F_{ab} should inhibit binding, whereas, in the case of heterophilic interactions, the hypothetical L-CAM ligands would remain unblocked and could mediate the adhesion by interaction with the L-CAM on the untreated cells.

Another approach to the problem is to develop an assay for the binding of soluble L-CAM to cells, as has been done in the case of N-CAM (Rutishauser et. al., 1982). This assay would allow the testing of the ability of various antibodies to the cell surface to block the binding of L-CAM to the cell, thus defining the membrane molecules to which L-CAM binds. This binding assay could also be used to define the molecular domains responsible for binding of L-CAM to cells, as has been done for N-CAM (Cunningham et. al, in press).

In summary, L-CAM is an acidic intrinsic membrane glycoprotein of $M_r=124,000$. The absence of half-cystines suggests that the molecule may exist in an extended form. The fact that calcium is required for L-CAM mediated cell adhesion, and confers resistance to proteolysis on the molecule, suggesting that the L-CAM molecule contains calcium ions.

As indicated above, the best characterized cell adhesion molecule is N-CAM, a glycoprotein that mediates calcium-independent adhesion between cells of the nervous system. Intact N-CAM appears as a region of continuous staining on SDS-PAGE of $M_r=200,000$ to $250,000$ daltons. Neuraminidase treatment generates two lower molecular weight species ($M_r=170,000$ and $140,000$), detected as discrete bands. These observations are consonant with the chemical analysis, which indicates that N-CAM is 30% sialic acid when isolated from embryonic brain. In the adult N-CAM appears as two bands ($M_r=180,000$ and $150,000$), the difference apparently being entirely due to lower sialic acid content (10%). L-CAM shows no similarity to N-CAM either in size, sialic acid content, presence of adult and embryonic forms, or calcium dependence of binding phenomenon. There is no immunological cross reactivity between these two molecules as tested by immunofluorescence, ELISA binding assays, immunoblotting, or activity in the neutralization assays used for defining the activities of the two molecules.

L-CAM does not appear to have any substrate adhesion activity. Sufficient F_{ab} to inhibit cell adhesion in the rotating culture assay and to totally disrupt colony formation has no detectable effect on the adherence of cells to tissue culture dishes in the presence of serum.

This finding does not rule out L-CAM mediated binding to other substrates, such as collagen or laminin; if such binding occurs, it would require two adhesion functions for the same molecule.

Ligatin is a protein of $M_r=10,000$ daltons found on the plasma membrane of rat ileum cells. It mediates the binding of beta-acetylhexosaminidase to the luminal epithelial cells, acting as a baseplate for attaching the enzyme to the membrane. It appears to be present as a filamentous structure which is depolymerized to the 10,000 dalton monomer by the removal of calcium ions. Ligatin, or a related molecule has also been isolated from the plasma membranes of chick retinal cells (Jakoi and Marchase, 1979), and has been shown to inhibit the aggregation of those cells in a rotating culture assay (Marchase et. al, 1981). L-CAM is not present on retinal cells, does not form large polymers or aggregates in the presence of calcium ions, and does not have any direct inhibitory effect on cell adhesion (see Figure 11). The two molecules are probably unrelated.

Retinal cognin is a glycoprotein isolated by Hausman and Moscona (1975) from tissue culture medium in which retinal cells have been grown. Cognin is a 50,000 dalton glycoprotein that increases the size of cell aggregates when added to 24 hour rotating cultures of retinal cells. The most pure cognin causes maximal enhancement at a concentration of 0.2-1.0 ug/ml. The fact that the molecule is isolated from tissue culture medium suggests that it may be a fragment of a membrane protein. Cognin differs from L-CAM in many essential respects. Although both have an acidic pI (cognins pI=3.8-4.1), their

amino acid compositions and molecular weights are significantly different. Cognin activity is demonstrated using a 24-hour culture, suggesting that it may not be involved in the initial adhesion events, but instead may mediate stabilization of adhesion, allowing larger aggregates to form in the long term rotating cultures. Also, there is no detectable L-CAM on the neural cells from which cognin is isolated.

After the initial reports of this laboratory on L-CAM (Bertolotti et.al., 1980), Ocklind and Obrink (1982) reported the isolation of a cell adhesion molecule from hepatocytes of juvenile rats, using an approach similar to the one described in this thesis. They reported a glycoprotein of $M_r=105,000$ as the intact species of the molecule. This molecule (designated as hepatocyte cell-CAM 105) neutralizes the ability of anti-hepatocyte antibodies to inhibit cell aggregation, and an antibody that appears to be monospecific for this species will inhibit liver cell aggregation. Since the molecule was isolated using the same approach as reported here, from the homologous organ in rat, the two molecules are probably related. Further structural information will be needed to explain the difference in molecular weight, and to rigorously check the homology of the two molecules.

Hyafil and his colleagues (1980) isolated an 84,000 dalton glycoprotein trypsin fragment from murine embryonal carcinoma cells that appears to mediate the adhesion of these cells to each other, and to mediate the cell adhesion involved in the compaction of the morula stage mouse embryo into the blastula. F_{ab}' fragments of antibodies to this molecule (which has been named uvomorulin) are effective in

preventing compaction and in decompacting later stage embryos. The effect of the F_{ab}' fragments can be neutralized by purified uvomorulin. The F_{ab}' fragments also inhibit cell-cell adhesion of the embryonal carcinoma cells that it is isolated from (PCC4 Aza R1). This adhesion system is calcium dependent, as is the L-CAM system.

Yoshida and Takeichi (1982) isolated a similar activity from F9 teratocarcinoma cells. They, however, released their activity with trypsin in the absence of calcium, and obtained a 34,000 dalton fragment that had neutralization activity. In comparison, L-CAM can be extracted from liver membranes by trypsin in the absence of calcium, in low yields, and appears to be present as a fragment of approximately 40,000 (Fig 18 c,d).

Antibodies to active material prepared by Yoshida and Takeichi immunoprecipitated a 140,000 dalton protein from detergent extracts of intact cells, a molecular weight more closely similar to the 124,000 daltons observed for chicken L-CAM. Ogou et. al. (1982) report that this antibody will also decompact mouse embryos. This calcium dependent cell adhesion is different from that defined in fibroblasts by Takeichi et. al. (1981). (L-CAM is not present on fibroblasts).

It appears that Hyafil and his colleagues and Takeichi's group have isolated a similar or identical molecule that mediates cell adhesion in early mouse embryos and teratocarcinoma cells. More important to the present discussion, these molecules have similar properties to chicken L-CAM.

Damsky and her colleagues (1982) recently reported the isolation of a glycoprotein of approximately 80,000 daltons from culture medium of mammary tumour cell lines of human and mouse. The molecule was assayed by its ability to neutralize the inhibition of cell-cell contact in monolayer cultures of tumour cells by anti-whole cell F_{ab}' . Antibodies to the purified molecule inhibited the cell-cell adhesion of the target cells without blocking binding of the cells to the substrate. These antibodies were also reported to decompact embryos. These results suggest that the mammary cells, which are epithelial cells, contain a cell adhesion molecule similar to uvomorulin, and possibly similar to L-CAM.

Currently all the antibodies that we have prepared against chicken L-CAM have not reacted with molecules from mouse or frog liver. Until more general immunochemical reagents can be prepared the comparison of the adhesion molecules from different species must be by criteria of homology of structure and of function.

An independent approach to the study of cell adhesion of epithelial cells is the characterization of junctional complexes by electron micrographic techniques. That these structures are involved in cell adhesion has been demonstrated by correlating treatments that disrupt cell adhesion, such as removal of calcium, with disruption of these junctions. Cells are close enough at these junctional contacts to make molecular contact, which is not true for most other parts of the cell surface, and by adding soluble reagents, such as horseradish peroxidase to the medium surrounding the cells it can be shown that the

junctional complexes form a tight seal on the cell surface that will not allow protein molecules to diffuse through.

There is a strong correlation between the general location of epithelial cell junctional complexes and immunofluorescent staining for L-CAM. This finding raises the possibility that the cell adhesion molecules defined in functional adhesion assays may be components of tight junctions or desmosomes.

Desmosomes have been isolated and specific polyclonal and monoclonal antibodies have been prepared against the major protein components of the structure (Franke et. al., 1983; Cohen et. al., 1983; Cowin and Garrod, 1983). Franke and his colleagues prepared antibodies to the desmoplankins, two proteins of $M_r=250,000$ and $215,000$ daltons. They used these antibodies to stain tissue sections and showed a fine dotted cell surface staining in epithelial tissue.

Cohen and his colleagues have prepared antibodies to three groups of immunologically related desmosomal glycoproteins of $M_r=150,000$, $97,000-118,000$ and $22,000$. Immunofluorescent staining of epidermis using antibodies against the $150,000$ dalton family shows uniform cell surface staining of epidermal cells, with some oblique sections through the cell showing staining of bright spots, probably individual desmosomes. Cowin and Garrod prepared guinea pig antibodies against the same material, and obtained essentially the same staining results.

No experiments were described by any of these groups as to the ability of these antibodies to inhibit cell-cell adhesion. Further

comparisons will be necessary to find whether L-CAM is a component of desmosomes, is unrelated to desmosomes, or perhaps mediates an adhesion process that is then stabilized by desmosomes. Electron microscopic immunocytochemical approaches should enable us to determine whether L-CAM is associated with junctional structures.

One approach towards studying the role of L-CAM in the physiology of multi-cellular structures is to use in vitro histotypic cell culture systems as models for the organ of interest. The preliminary experiments described in this thesis demonstrate the role of L-CAM in the formation and maintenance of structured colonies of liver cells grown on a solid substrate. Anti-L-CAM was shown to prevent the formation of liver cell colonies and to disrupt the structure of already formed colonies. Conversely, cells plated with enough anti-L-CAM F_{ab}' to prevent colony formation, when washed free of the F_{ab}' formed colonies indistinguishable from those formed by cells cultured in the absence of anti-L-CAM. This reversible non-toxic disruption of cell-cell interactions in a histotypic structure makes it possible to study the role of cell-cell contacts in a number of physiological functions. Such a system could be used to test the surface modulation assembly hypothesis (Edelman, 1976) in a model system comparable to a solid organ. Among straightforward processes to be tested are the synthesis of proteins, their expression (i.e. do they appear on the cell surface), and the clearance of dyes from the medium (Barth and Schwarz, 1982).

L-CAM has been detected in a number of organs in the late embryo and the adult chicken. Generally speaking, it is present on all epithelial cells that line the body surfaces of the chicken. The skin on the outer surface of the bird, the cells lining the gut, the lungs and the exocrine surfaces of the liver and pancreas, and the cells lining the urogenital tract, all were shown to contain L-CAM by immunofluorescent staining. That the material detected was L-CAM, and not some antigen common to L-CAM and other unrelated molecules, was demonstrated by performing immunoblotting experiments on the organs of the chicken. L-CAM clearly persists in the adult, as does N-CAM. Although no modulation of the structure of L-CAM has been detected, the distribution of L-CAM on the surface of cells in adult tissues is sometimes considerably more restricted than in the corresponding embryonic organ. The two most striking examples of this phenomenon are on the cells of the proventricular glands (Figure 22 f) and on cells of the exocrine acinar glands of the pancreas (Figure 27 d,f). Whether cell adhesion molecules serve the same function throughout development, or whether they have different functions at different developmental stages is an open question at this point.

It is clear that L-CAM is generally distributed in the late embryo and in the adult, that it is neither specific to liver, nor to cells originating from any single germ layer. It appears to be on most, if not all, epithelial tissues. These facts suggest that the specificity for organogenesis does not arise from the molecules mediating adhesion, but instead arises in a complex modulation of cellular events (Edelman, 1983).

Experiments to determine the sites and time of appearance of L-CAM in the early embryo are in progress. When more information is available a correlation of L-CAM appearance with known steps of induction and morphogenesis should indicate which developmental processes will be most fruitfully pursued using L-CAM as an entree to the problem. The data on the distribution of L-CAM and N-CAM are sufficient to support the proposal (Edelman, 1983) that there need not be a large array of organ-specific or tissue-specific cell adhesion molecules in embryos. Instead, a small collection of molecules can be modulated through modification of structure, amounts of each molecule expressed on the cell surface, and localization of expression on the cell surface, combinations of which would generate highly specific organogenic processes.

Two exciting directions for future experimentation are 1) the use of anti-L-CAM as an agent for interfering with development, as has been done already for histotypic liver cultures (described here) and for the retina, using N-CAM (Buskirk et. al., 1980), and 2) the study of the control of L-CAM expression in the developing embryo, and the effect of anti-L-CAM on the control of synthesis of other embryological markers. The availability of appropriate reagents and the accumulated experience with L-CAM make such experimentation both feasible and interesting.

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