AN IMMUNOLOGICAL ANALYSIS OF A CELL SURFACE ANTIGEN IN OOCYTES AND EMBRYOS OF THE MUD SNAIL, ILYANASSA OBSOLETA

Erich M. Schmedt
Department of Biology
McGill University, Montreal
March, 1984

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Masters of Science

Title to appear on binding of thesis:

AN IMMUNOLOGICAL ANALYSIS OF A CELL SURFACE ANTIGEN IN ILYANASSA OBSOLETA

FACULTÉ DES ÉTUDES AVANCÉES ET DE LA RECHERCHE

Date _____

NOM DE L'AUTEUR: _____

DÉPARTEMENT: _____ GRADE POSTULE: _____

TITRE DE LA THÈSE: _____

_____

_____

1. Par la présente, l'auteur accorde à l'université McGill l'autorisation de mettre cette thèse à la disposition des lecteurs dans une bibliothèque de McGill ou une autre bibliothèque, soit sous sa forme actuelle, soit sous forme d'une reproduction. L'auteur détient cependant les autres droits de publication. Il est entendu, par ailleurs, que ni la thèse, ni les longs extraits de cette thèse ne pourront être imprimés ou reproduits par d'autres moyens sans l'autorisation écrite de l'auteur.

2. La présente autorisation entre en vigueur à la date indiquée ci-dessus à moins que le Comité exécutif du conseil n'ait voté de différer cette date. Dans ce cas, la date différée sera le _____.

_____

Signature de l'auteur

Adresse permanente:

_____

_____

_____

_____

Signature du doyen si une date figure à l'alinéa 2.

(English on reverse)

ABSTRACT

The embryonic cell surface of the mud snail, Ilyanassa obsoleta, was examined with a monoclonal antibody (IVel0). The antibody was prepared against an embryonic membrane protein spectrum. Immunocytochemical studies have revealed that the IVel0 antigen first appears during oocyte maturation (artificially induced) and remains in the embryo throughout larval development. In the oocyte, egg, and early embryo it is located at the cell surface and within the cytoplasm. By the veliger stage the IVel0 antigen is found within the larvae at discrete locations. Immunoblot analysis has shown that the IVel0 monoclonal antibody reactes with three prominant bands designated IEA-1, IEA-2, and IEA-3. The IEA-3 antigen is prominent in mature oocytes and is diluted out or degraded as development proceeds. IEA-1 and IEA-2 both appear during oocyte maturation and remain throughout embryogenesis. The IVel0 antigens are not polar lobe specific nor are they affected by polar lobe removal. This work has established the techniques necessary for future attempts at examining the Ilyanassa cell surface and is part of a broader search for surface antigens that are affected by delobing embryos.

RESUME

La surface des cellules embryonnaires de l'escargot, Ilyanassa obsoleta, a été étudiée avec l'aide d'un anticorps monoclonal(IVel0). L'anticorps à été préparé en utilisant l'ensemble des protéines membranaires. Les études immunocytochimiques ont démontré que l'antigène IVel0 apparaît d'abord pendant la maturation de l'ovocyte (artificiellement induit) et se maintient dans l'embryon pendant le developpement larval. Dans l'ovocyte, l'oeuf, et le jeune embryon l'antigène IVel0 est localisé à la surface cellulaire ainsi qu'à l'intérieure du cytoplasme. Une fois à l'étape "vilager" l'antigène IVel0 est precisément localisé à l'intérieur de la larve. Des analyses immunologiques (immunoblot) ont démontré que l'anticorps monoclonal réagit avec trois bandes importantes désignées IEA-1, IEA-2, et IEA-3. L'anticorps IEA-3 est proéminent dans les ovocytes matures et se dilue ou se désintègre lors de la poursuite du développement. IEA-1 et IEA-2 tous deux apparaissent pendant la maturation de l'ovocyte et se maintiennent pendant l'embryogénése. L'anticorps IVel0 n'est pas spécifique aux lobes polaires et n'est pas affecté par l'ablation de ces derniers. Ce travail a démontré les techniques necessaires pour l'étude des surfaces cellulaires de l'Ilyanassa et fait partie d'une plus grande recherche d'antigènes de surface qui sont affectés par l'ablation des lobes embryonnaire

Translation by: Francis Ouellette

ACKNOWLEDGEMENTS

I would like to thank Dr. Kenneth Newrock for his invaluable counsel during the course of this work. I would also like to thank my committee of Dr. Lawrence Kleiman and Dr. Barid Murkerjee for their guidance and input. A special thanks goes to Dr. David McClay of Duke University for producing the monoclonal antibody used in this work.

I thank the past members of our laboratory, Anne Flenniken, Mark Lesk, and Ehud Isacoff for their help, support, and friendship. I would particularly like to thank Becky Dolan for her assistance in the immuno-cytochemical aspects of this work and also for her friendship. To the many friends I have come to make over the course of my studies I would also like to give thanks for their support and helpful discussions.

I thank my family for their constant love and support both emotionally and financially over the years. Most importantly I would like to thank Linda Hougan whose limitless love and support have made it all worthwhile.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The egg contains information which guides the events of early embryogenesis. This information is produced and stored during oogenesis and inhomogeneously distributed to cells during the early cleavage divisions. The result is the establishment of blastomeres with different developmental potentials. Little is known about the molecular nature or mode of action of this maternal information. It is, however, believed to be involved in establishing differential gene expression among cell lineages and in directing the early morphogenetic events of the embryo.

The embryo of the mud snail, Ilyanassa obsoleta, provides an elegant system for examining the nature of this maternal information. At first cleavage it forms an anucleate cytoplasmic protrusion, the polar lobe, resulting in a tripartite embryo. If the polar lobe is removed at this stage a disorganized embyro develops indicating that it contains information, likely provided during oogenesis, which is required for proper embryonic organization. This information could reside in the cell surface, chromatin, or other cellular locations. This thesis is concerned with the cell surface.

Historically the cell surface has been viewed as an organelle possessing information to direct embryonic organization. Dissociation experiments of Wilson (1907) and Holtfreter (1944) , on sponges and amphibians

respectively, revealed that cell types have selective affinities for one another. Numerous other investigations probing the nature of the cell surface have been undertaken (reviewed by Frazier and Glaser, 1979). The cells of embryos have been shown to express a variety of cell surface components which change qualitatively and quantitatively during development (Frazier and Glaser, 1979). These components are likely necessary for recognition phenomona.

It has been proposed that early morphogenetic events are timed by an inter-relationship between the cell surface and new gene expression (Mangiarotti et al, 1982, 1983; Blumberg et al, 1982). Cell surface differentiation would thus result from differential gene expression producing new cell surface phenotypes. These phenotypes would enable cells of specific types to associate and change again, thus allowing morphogenesis to proceed in a step-wise manner.

In Ilyanassa it is possible that there is sequestered within the polar lobe maternal information (i.e. produced and stored during oogenesis) which influences the composition of the embryo cell surface. This stored information could be cell surface constituents or information which directs or controls the synthesis of surface components. If this is the case, the morphological disorganization observed in delobed embryos could be due to the loss of surface components.

In the experiments presented in this thesis the Ilyanassa cell surface has been examined with a monoclonal antibody (IVel0) prepared against an embryonic membrane

antigen. This is part of a broader search for surface antigens affected by delobing. IVel0 first appears during oocyte maturation and remains present in the embryo throughout larval development. It is located in the oocyte, egg, and early embryo on or in the cell surface, as well as in an intracellular location. It is not found on freshly deposited unfertilized eggs (in reality immature oocytes). Polar lobe removal has no affect on the appearance of the IVel0 antigens(s) as it is present before first cleavage. This biochemical and microscopic study represents an initial study of the Ilyanassa cell surface. It provides the groundwork for future studies of this kind in Ilyanassa .

LITERATURE RFVIEW

The Effects of Polar Lobe Removal on Ilyanassa Development

The mud snail, Ilyanassa obsoleta , is one of a number of annelids and molluscs which form a protrusion known as the polar lobe during first cleavage. Within this anucleate protrusion is information, commonly referred to as mophological determinants, which is essential for normal development. This is envisioned by the removal of the polar lobe. When this is done an aberrant embryo develops. It is important to determine what in the polar lobe is necessary to development (reviewed by Dohmen, 1983; Collier, 1983a).

The Ilyanassa egg is approximately 170 um in diameter. Fertilization occurs prior to oocyte maturation (1° oocyte stage). In fact, several polar lobes form during cleavage. During the first and second meiotic divisions a polar lobe appears at the vegetal pole of the egg. The third polar lobe, the one of concern here, appears as a swelling at the vegetal pole of the embryo during first cleavage. As the lobe enlarges and attains full size (1/3 of the embryos total volume) cleavage commences with the cleavage furrow first appearing at the animal pole. As the cleavage furrow develops the polar lobe remains attached to one of the two blastomeres resulting in a tripartite structure referred to as a trefoil. Upon completion of the first cleavage division the polar lobe becomes reabsorbed into the blastomere it

remains attached to, now termed the CD blastomere. As a result two cells of unequal size are formed. There is no evidence that one of the initial two blastomeres is predetermined to receive the polar lobe. A fourth polar lobe appears at the second cleavage which gets reabsorbed in the D cell, making it larger that the other three blastomeres (A, B, and C). Subsequently in the following cleavage divisions the contents of the polar lobe become distributed among the cells of the D cell lineage (Clement, 1952).

Crampton (1896) removed the polar lobe from trefoil stage embryos. Lobeless embryos developed which were viable and which developed for several days. However the spiral cleavage pattern was altered and the embryo failed to produce mesenchyme. Later studies showed that there was also a loss of bilateral symmetry (Clement, 1952). Thus the embryo becomes radialized. That is, the differences in cell sizes, which exist in normal embryos between corresponding cells from different quadrants, is abolished and radial symmetry results. In normal development the cells in the D quadrant of the embryo cleave at a faster rate (Clement, 1952). Polar lobe removal also results in cleavage becoming synchronous in the four quadrants.

The thorough work of Clement (1952, 1956) and Atkinson (1971) revealed a more complex lobeless phenomenon. Removal of the polar lobe results in grossly abnormal larvae, lacking most mesodermally derived and induced structures such as eyes, velum, foot, apical tuft, intestine, tentacles, and heart. Atkinson's detailed study

of Ilyanassa organogenesis and histogenesis showed that although these structures were lacking, most of the cell types for these organs were, in fact, present. The possibility that abnormal development is simply due to removing a large part of the mass of embryos of Ilyanassa embryos was illiminated by allowing AB and CD cells to develop seperately. The CD blastomere is capable of developing into a normal veliger, although smaller than normal, whereas the AB blastomere resembles a lobeless larva (Clement, 1956).

To gain furthur information on the role of the polar lobe in the determination of larval organs, Clement (1962) removed the D macromere, which receives the polar lobe material, at successive stages of development and compared development of these altered embryos. Removal of the D macromere at the four cell stage produced larvae similar to lobeless embryos. Removal of the D macromere after the formation of the third micromere quartet deleted only those structures which arise from the mesentoblast cell. After the formation of the fourth micromere quartet, removal resulted in no qualitative effect other than the embryo being smaller than normal. Clement concluded that the morphogenetic influence of the polar lobe is exerted during the period of formation of the first four quartets of micromeres and that not all lobe dependant larval features were determined at the same time. Similar results were obtained in Dentalium (Cather and Verdonk, 1979). The intestine and heart have been shown to originate from the D quadrant, and thus are

directly dependant on polar lobe material. The eyes and tentacles, however, arise from other quadrants whose cells are dependant on an inductive influence from cells of the D quadrant (Cather and Verdonk, 1979). Cather (1967) revealed the inductive nature of the polar lobe in regards to shell gland formation and also revealed a possible inhibitory role for the polar lobe. Thus the A and B quadrants are capable of forming shell gland material in the absence of the polar lobe where normally they do not. Cather (1971) has suggested that the D quadrant, with its polar lobe material, acts as a primary organizer during early molluscan development.

Localization of the Morphological Information in the Polar Lobe

Morphological determinants are localized in the egg such that there is regionalized information. The polar lobe is an example of such regionalization. An approach to examining localization has been to subject eggs to low speed centrifugation. This results in the stratification of cytoplasmic components such as yolk granules, mitochondria, and lipid droplets. Centrifugation of Ilyanassa eggs has no effect on subsequent development implying no displaceable regionalization, prior to first cleavage. Since the cortex is not redistributed by this treatment the relevant factors may well be cortical.

Clement (1968) was able to separate the properties of the vegetal cortex and soluble cytoplasm. He obtained nucleated animal and nucleated vegetal halves by centrifugation under apprppriate conditions. Allowing these fragments to develop he found that the animal halves never formed polar lobes or lobe dependent structures whereas the vegetal halves did. Centrifugation thus did not displace the polar lobe information from the vegetal half of the egg. This work also suggested that the determinants of the Ilyanassa polar lobe are localized in the egg cortex and not the soluble cytoplasm. In contrast, a structure termed the vegetal body has been identified in Bithynia which is believed to contain morphogenetic information. It is displaced by heavy centrifugation and has been shown to be very rich in RNA (von Dam et al, 1982).

Molecular Characterization of Polar Lobe Localized Morphological Information

Investigators have tried to detect molecular differences between normal and delobed embryos in the hope of identifying the nature of the polar lobe determinants (reviewed by Collier 1983a). Nucleic acid synthesis (RNA synthesis in particular) has been extensively examined in normal and delobed embryos by Collier and coworkers (1975, 1976, 1977).

In normal Ilyanassa embryos ribosomal RNA (rRNA)

synthesis occurs until the veliger stage (Collier, 1976). When rRNA synthesis commences in the embryo is not clear. Collier (1976) detected uridine incorporation into rRNA as early as the four cell stage of development. Others have detected rRNA synthesis only by late cleavage (Newrock and Raff, 1975; Naus and Kidder, 1976). It is clear, however that the first major increase in total RNA accumulation that is attributable to rRNA synthesis accurs at day four of development (Collier, 1975). This indicates that the Ilyanassa egg provides sufficient numbers of ribosomes to support development for the first three days.

Synthesis of transfer RNA (tRNA) has also been detected as early as the four cell stage of normal Ilyanassa development (Collier, 1976). Collier found the molar ration of tRNA to rRNA to change from 20:1 in the egg to 10:1 in the post-gastrular embryo. The ratio changes after the first major increase in total RNA (day 4) and remains constant for the duration of embryogenesis.

Messenger RNA (mRNA) synthesis begins during the early cleavage stages in normal Ilyanassa embryos, somewhere between the four to sixteen cell stages, as shown by autoradiographic determination of radioactivity in pulse labelled embryos (Collier, 1976; Koser and Collier, 1976). The best evidence for mRNA synthesis at these early stages is from polyadenylation studies of nascent RNA from the Ilyanassa embryo (Collier, 1975). These studies showed that 40% of newly synthesized RNA during early cleavage was polyadenylated as compared to 10% at later stages of

development (values are per cent of total nascent RNA that was polyadenylated).

In the delobed embryo the first major increase in total RNA synthesis is delayed until day 6 of development as opposed to day 4 in the normal embryo. After day 6 the rate of accumulation of total RNA is the same as in the normal embryo. Measuring the absolute rates of RNA synthesis, Collier (1976) demonstrated that at the 25 cell and gastrula stages the synthesis of total RNA was less in the delobed embryo. However, the proportion of polyadenylated RNA synthesized was not affected. He detected no significant differences in RNA synthesis between the normal and delobed embryo during early cleavage. A more detailed study, in which RNA extracted from normal and delobed embryos was labelled with ^{3}H-uridine for 24 hours and examined electrophoretically, revealed similar patterns of RNA synthesis prior to gastrulation (Koser and Collier, 1976). After gastrulation, however, there was an increase in 12-16S RNA and a decrease in 34S RNA in delobed embryos. The increase in 12-16 S RNA was attributed to an increase in untranslated message and the decrease in 34S RNA synthesis to a transcriptional defect (Koser and Collier, 1976).

The unfertilized egg of Ilyanassa contains polysomes active in protein synthesis (Mirkes, 1970). Within fifteen minutes following fertilization the uptake and incorporation of labelled amino acids increases. By the 8 cell stage the number of active polysomes has increased by 80% and by the veliger stage there is a five-fold increase in protein

synthesis. Protein synthesis in the normal Ilyanassa embryo, thus, steadily increases for the first three days of development, levels off during organogenesis, and decreases during the later stages (Collier and Schwartz, 1969). Stage specific changes in protein synthesis between the early cleavage and mesentoblast stage of development have been demonstrated by two dimensional electrophoresis (Brandhorst and Newrock, 1981; Collier and McCarthy, 1981). Changes involve the disappearance and appearance of particular proteins and changes in the relative rate of accumulation of proteins.

Since Collier (1961) demonstrated that the delobed embryo had a reduced level of amino acid incorporation into proteins, extensive investigations have been undertaken examining protein synthesis in normal and delobed embryos. Isolated polar lobes can also be examined since they are capable of incorporating amino acids into protein (Clement and Tyler, 1967). Initial studies used one dimensional electrophoresis to examine the proteins of the normal and delobed embryo; the affects of actinomycin D treatment on these two types of embryo; and the proteins of isolated AB and CD blastomeres (Tietelman, 1973; Newrock and Raff, 1973; Donahoo and Kafatos, 1973). Each study detected differences in protein synthesis between the normal and delobed embryo. However these conclusions are somewhat limited due to the poor resolving power of one dimensional gels. Experiments using two dimensional gel electrophoresis (isolelectric focusing-SDS), on the other hand, have revealed in normal

and delobed embryos, and isolated polar lobes, identical sets of ^{35}S-labelled proteins during early cleavage (Brandhorst and Newrock, 1981; Collier and McCarthy, 1981). However it may well be that the two dimensional systems resolve different sets of proteins. In fact it has been shown by Flenniken and Newrock (manuscript in preparation) using one dimensional gels that the complement of H1 histones in normal and delobed embryos of early stages of development are not identical. There may be other differences involving proteins resolved by SDS polyacrylamide gel electrophoresis which are not detectable when used with isoelectricfocusing.

As mentioned, two dimensional gel electrophoresis has shown that at the mesentoblast stage (24 hours of development) stage specific changes in proteins occur in normal embryos. In delobed embryos there are quantitative but no qualitative differences in the protein profiles when compared to the normal. At 24 hours isolated polar lobes synthesized a subset of those proteins detected in normal and delobed embryos. These results indicated that the polar lobe does not contain a unique set of localized mRNAs. Brandhorst and Newrock (1981) suggest that the selective activation and inactivation of mRNAs resulted in the stage specific disappearance of some proteins in the mesentoblast embryo and 24 hour isolated polar lobe. They furthur suggest that there are cytoplasmic factors in the isolated polar lobe that are capable of activating and deactivating mRNAs which are present as ribonucleic protein particles (mRNPs). This in turn is evidence for translational control of mRNAs by some

maternal components, since the polar lobe undergoes a subset ot the changes found in the normal embryo. Of the 350 polypeptides detected by Collier and McCarthy (1981), 98% were insensitive to actinomycin D (AMD) and AMD did not qualitatively change the mesentoblast stage specific proteins. These data reveal that early protein synthesis in Ilyanassa embryos is from maternal RNA. It may exist as mRNPs which become preferentially activated and deactivated. This is furthur substantiated. At AMD concentrations which block RNA synthesis Ilyanassa embryos develop normally until gastrulation (Newrock and Raff, 1975). Tubulin synthesis is normal even in the presence of AMD indicating it was being synthesized on maternal templates (Raff, Newrock, and Secrist, 1975).

Recently Collier (1983b) labelled proteins during late and early organogenesis of Ilyanassa embryos and showed by two dimensional electrophoresis that lobeless and normal embryos produce the same sets of proteins. There were quantitative differences however and the greatest differences were among the most abundant proteins. He suggested that the differences in the abundant proteins were significant and that the polar lobe had both a negative and positive effect, since some of the proteins were more abundant in the delobed embryo while others were more abundant in the normal.

Studies on acid soluble precursors, have provided interesting results and speculations. The polar lobe contains a higher concentration of acid soluble precursors, especially ATP, than other parts of the embryo (Collier and

Garone, 1975). Whether this is morphologically significant is debatable since delobed embryos are capable of similar rates of nucleic acid and protein synthesis as are normal embryos. Nonetheless, Collier (1975, 1983a) has proposed a stem cell hypothesis. He suggests that ATP from the polar lobe is transfered to specific micromeres via gap junctions which provides them with sufficient energy to increase their rates of cleavage. This increased rate of cleavage would facilitate the production of sufficient numbers of stem cells for the differentiation of lobe specific organs. In the delobed embryo this energy requirement is not fullfilled and not enough stem cells are produced for normal differentiation to proceed. Although this model is feasible, it has not been substantiated. Much has been learned in regard to protein and nucleic acid synthesis in Ilyanassa embryos, yet nothing concrete has been revealed in terms of the nature of the determinants which reside in the polar lobe. Differences observed between normal and lobeless embryos have been interpreted as being the result of abnormal development rather than the cause.

Immunological Approaches towards examining the Cell Surface

The cell surface is involved in cell recognition, adhesion, communication, and motility. It mediates the morphogenetic events of embryos, in which cell recogniton and adhesion are integral aspects. The examination of the nature of the embryonic cell surface is thus important for an understanding of early development. One aspect of morphogenesis is selective cell-cell adhesion. This was first demonstrated in sponges and amphibians with cell sorting experiments (Wilson, 1907; Townes and Holtfreter, 1955). Wilson (1907) showed that a mixture of dissociated sponge cells of two species was capable of sorting out, in a species specific manner, to form discrete organisms. Embryonic cell suspensions of mixed cell types also sort out in a tissue specific manner (Moscona, 1967).

This sorting behaviour suggests that cell surfaces have the means to recognize and bind to other cell surfaces. It is believed that cell surface macromolecules function as intercellular ligands. The specificity of cellular interactions is thus dictated by the specificity among complex molecules. Since glycoproteins are a major cell surface component, protein-carbohydrate interactions may be one of the mechanisms involved in cell adhesion (reviewed by Harrison and Chesterton, 1980).

Numerous cell and tissue specific membrane antigens have been identified (see Frazier and Glaser, 1979). Cell

adhesion has been studied by dissociating embryonic cells and allowing them to reaggregate (McClay, 1971). Purified plasma membranes have been shown to enhance reaggregation of dissociated cells (Gottlieb et al, 1974). The cell surface has been altered with enzymes to provide information concerning the chemical nature of the cell surface (Marchase, 1977). Lectins have been extensively used to identify cell surface molecules, specifically glycoproteins, which may be involved in cellular adhesion (Monsigny et al, 1979).

Cultured cell supernatents contain aggregating promoting factors which have been isolated and used in adhesion experiments. This approach has provided a wealth of information with regard to cell surface molecules involved in cell-cell interactions. In particular work with embryonic neuronal retinal cells has identified a glycoprotein referred to as N- CAM (neuronal cell adhesion molecule). It is one of the more characterized cell surface molecules implicated in cellular adhesion (reviewed by Edelman 1983, 1984). Of paricular importance is the identification of embryonic and adult forms of this molecule which correspond with changes in adhesive behaviour during development (Rutishauser et al, 1976; Thiery et al, 1977; Rothbard et al, 1982).

One of the most powerful approaches for examining the cell surface, and the aprroach taken in this work, is immunological; the use of antibodies directed against cell surface constituents. In combination with other techniques antibodies provide a means by which the roles of cell surface

antigens in cellular adhesion can be examined. In review we will examine the range of applications of antibodies in three systems; the slime mold, the sea urchin, and in mouse teratocarcinoma cells.

Cellular Adhesion in the Slime Mold

The slime mold, usually Dictyostelium discoideum, has been a model system for addressing questions regarding the cell surface and its involvement in intercellular adhesion. In addition, many of the immunological techniques developed and used on D. discoideum have been applied to other developmental systems.

The feature that makes D. discoideum such an ideal system is that during its life cycle vegetative single cell amoebae aggregate when starved and form a multicellular slug. Changes in cell adhesion are part of the aggregation process. Cells, attracted into streams by pulses of cAMP, assemble into aggregates in which cells eventually form stable EDTA resistant contacts. Both processes (streaming and aggregation) require the elaboration, during the preaggregation phase, of cell surface molecules. Once aggregated the amoebae differentiate giving rise to two distinct cell types; spore and stalk cells.

D. discoideum has a dual adhesion system utilizing two classes of adhesion sites. One is intitially present on growth phase cells and is responsible for irregular cell

assembly. The initial adhesion of cells is side by side and EDTA sensitive (Gerisch, 1961). The other class appears at the cell surface when cells acquire aggregation competancy, resulting in the formation of EDTA resistant end to end contacts. EDTA resistant contacts are species specific, indicating that selective cell recognition occurs (Springer and Barondes, 1978; Gerisch, 1980).

The first evidence of changes in membrane antigenicity was provided by the agglutination studies of Gregg and Trygstad (1958) who showed that certain antisera raised against aggregating cells increased the agglutinability of cells from the growth to the aggregation stage. Gerisch and coworkers (Beug et al, 1973a) have described a set of cell surface antigens unique to aggregation competent cells by using Fab fragments of different specificities to block adhesion. Monovalent antibody fragments made from antisera against growth phase or vegetative cell membranes completely inhibit adhesion of growth phase cells. In aggregating cells the same Fab fragments inhibit side by side adhesion but not the EDTA resistant end to end adhesion. These Fabs are thought to bind to cell surface molecules termed Contact sites B (Muller and Gerisch, 1978).

Fab fragments prepared from antisera made against membranes of aggregation competant cells on the other hand, block adhesion at both stages completely. If these Fabs are preabsorbed with growth phase cells, only the end to end adhesion in aggregation competant cells is blocked (Beug et al, 1973b; Muller and Gerisch, 1978). These preabsorbed Fab

fragments are thus specific for the EDTA sensitive sites which are referred to as Contact sites A. Similar immunological approaches have revealed two such contact sites in another species of slime mold, P . pallidum (Bozzaro and Gerisch, 1978). The adhesion blocking ability of Contact site A specific Fabs is species specific, whereas Contact B specific Fabs are not (Jaffe and Garrod, 1977; Bozzaro and Gerisch, 1978).

Contact sites A are believed to be responsible for EDTA resistant cell adhesion in D . discoideum . Along with the evidence provided by the work with Fab fragments, immunofluorescent studies on mutant D . discoideum cells incapable of forming EDTA resistant cell contacts have shown that Contact sites A are absent (Beug et al, 1973a). In revertants they are again detectable. Contact sites A are developmentally regulated in that they appear when cells require them and disappear at later stages (Gerisch, 1980). Once aggregates are formed there is no furthur synthesis of these contact sites (Parish et al, 1977). Contact sites B, on the other hand, are present from the growth to the aggregation competant stage (Gerisch, 1980).

When Contact A specific Fabs are preabsorbed with detergent or n-butanol extracts of aggregation competent cell membranes, the ability of these fragments to block adhesion is removed (Muller and Gerisch, 1978). This allowed for the purification of this material from the extracts via DEAE cellulose chromatography followed by sucrose gradient centrifugation. When run on SDS polyacrylamide gels a single

glycoprotein, which binds concanavalin A, is detected with a molecular weight of 80,000 (gp80). Gp80 is glycosylated and phosphorylated and has been shown to be an integral membrane protein (Muller et al, 1979; Coffman et al, 1981; Stadler et al, 1982). It is believed to be the glycoprotein of Contact site A responsible for adhesion. It is the most predominantly ^{35}(S-met)- labelled protein of the plasma membrane. The label incorporated into the carbohydrate portion of the molecule (Stadler et al, 1982). Purified gp80 when incubated with Contact site A specific Fabs neutralized their ability to block the contact sites.

Furthur support that gp80 is important in EDTA resistant cohesion has come from the work of Finney and coworkers (1983). By causing amoebae to dedifferentiate and then stimulating them to redifferentiate, they demonstrated a corresponding loss and resynthesis of gp80. By monitoring the presence of gp80 by immunostaining, they showed that loss and reaquisition of gp80 corresponded with the loss and requisition of EDTA resistant cohesion.

Monoclonal antibodies have been produced against gp80 to furthur analyze the developmental control of and role of gp80 protein (Ochai et al, 1982; Murray et al, 1983). These antibodies could not detect gp80 on growth phase cells but did detect it on the cell surface simultaneously with the ability of the cells to form EDTA resistant contacts. Both groups, independantly raising their monoclonal antibodies, found cross reactivity between gp80 and other proteins with their antibodies. In all cases gp80 was the predominant

species and they concluded that the other reactive proteins must share the same antigenic site. Murray and coworkers (1983) have suggested that the antigenic determinant recognized by their antibody is associated with a post-translational modification since it is present on a number of proteins with different developmental expressions.

The monoclonal antibodies produced in both laboratories failed to prevent adhesion. Yet when these antibodies were used to purify gp80, the purified protein neutralized the ability of the rabbit serum against gp80 to block adhesion. The failure of monoclonal antibodies made against gp80 to prevent adhesion and the observation that other proteins may share the same antigenic site makes it difficult to rule out the possibillity that gp80 may not be an adhesion molecule but may simply share the antigenic site of the actual physiological molecule. However it seems more likely that the monoclonal antibodies are directed against a different antigenic site than that of the rabbit serum, one which does not interfere with cellular adhesion. Recent work by Murray and coworkers (1984) has shown, in fact, that the determinant on gp80 recognized by their monoclonal antibody is not necessary for adhesion, since mutants of this determinant exhibit Contact site A mediated cell adhesion.

A number of other proteins have been identified and implicated in cellular adhesion in the slime mold. Geltosky and coworkers (1976) used concanavilin A as a probe to examine cell surface glycoproteins and found 15 Con A binding proteins associated with the cell surface. One of these was

a developmentally regulated glycoprotein with a molecular weight of 150,000 termed gp150. It is expressed at low levels in growth phase cells and increases in concentration during periods of cellular adhesiveness. Immunoelectron microscopy demonstrated that in 16 hour aggregates the concentration of gp150 was highest in regions of cell-cell contact, suggesting it may be involved in cellular adhesion.

Furthur evidence to support the role for gp150 was provided when Fab fragments from antisera directed against partially purified gp150 prevented aggregation of aggregation competant cells (Geltosky et al, 1979). The specificity of the adhesion blocking antibody to gp150, however, was not analyzed in this study with purified proteins (such as gp150 or gp80). Thus the possibility that it reacted with gp80 was not eliminated. It was recently shown, however, that the gp150 antiserum contained antibodies to both gp150 and gp80 and that the antiserum's ability to block aggregation was neutralized by purified gp80 alone (Loomis et al, 1983). This indicated that the blocking activity of the antiserum was due to the presence of anti-gp80 activity. The antibodies specific for gp150 were not neutralized by purified gp80 and were concluded to be extraneous to the blocking activity of the serum. It is possible that gp150 and gp80 share antigenic determinants which are recognized by the adhesion blocking antibody.

A 95k glycoprotein, identified by antibodies made from slug plasma membranes, is believed to take over the function of cell adhesion from gp80, which disappears from the cell

surface at later stages. The synthesis of gp95 commences at the tip stage of development and it remains throughout the rest of development (Steinemann and Parish, 1980). When slug cells are disaggregated, Fabs prepared from gp95 antiserum prevent EDTA resistant reaggregation whereas gp80 Fabs do not. The gp95 Fabs have no blocking affects on cells from earlier stages.

Carbohydrate binding proteins or lectins have also been implicated in the involvement of amoeboid aggregation into multicellular masses (Rosen et al, 1974; 1979; Simpson et al, 1974). In D . discoideum they have been termed the discoidins. They are developmentally regulated soluable cytoplasmic proteins and are antigenically different than Contact sites A (Frazier et al, 1975). Cell-cell contact has been shown to be the developmental signal to deactivate discoidin 1 gene expression (Berger and Clark, 1983). Evidence implicating these lectins as being cell adhesion molecules was provided by cell surface labelling and immunoprecipitation on aggregation competant cells (Chang et al, 1975; Siu et al, 1976). Genetic evidence regarding their role in cellular adhesion was provided by a mutant which was deficient in lectins and noncohesive (Raje et al, 1975). Revertants recovered their aggregation competance presumably as a result of discoidin 1 activity. However these results have been thrown into doubt.

Recent evidence shows that the D . discoideum lectins are not involved in cellular adhesion. Antibodies prepared against purified lectins do not affect cell adhesion

(Bozzaro and Gerisch, 1978; Barondes et al, 1983). Erdos and coworkers (1983) performed a thorough investigation using indirect immunoferritin labelling and direct immunolabelling and detected no discoidin on the surfaces of cells whatsoever. They suggested that the role of the discoidins be reexamined since they are not present on the cell surface and that previous results so indicating were artifactual due to the presence of lysed cells. Two mutants have also been recently isolated which are capable of aggregation yet do not express the lectin genes (Alexander et al, 1983). Recent results thus suggest that the discoidins are not involved in cellular adhesion.

The Cell Surface of Early Sea Urchin Embryos

Echinoderm embryos, specifically those of the sea urchin, provide a good system for examining cellular interactions. Embryos can be dissociated into single cells by removing divalent cations from the sea water. When the cells are returned to normal sea water they readhere, resulting in the formation of morula-like structures which are capable of developing until the pluteus stage (Giudice, 1962; Spiegel and Spiegel, 1975). The recognition pattern in reaggregates is species specific and the reaggregates of dissociated gastrula cells sort out into the three germ layers (McClay and Hausman, 1975; Noll et al, 1981). Reaggregation requires the presence of cell surface proteins

inasmuch as the ability to reaggregate is abolished if these proteins are removed from the cell surface with n-butanol or if they are blocked with Fab fragments (Noll et al, 1981). Of course, sorting out of sea urchin cells from a mixture has lead workers to conclude that differences exist at the cell surface.

Along with the ability of dissociated sea urchin cells to reaggregate, gastrulation in the sea urchin allows for furthur examination of the cell surface during development. It provides a chance to look at what occurs in intact embryos undergoing development. During gastrulation three primary germ layers are established from a single layer of cells at blastula. Gustafson and Wolpert (1967) suggested that specific cell movements and specific cellular interactions are responsible for gastrulation. It is believed that molecular changes occur at the cell surface which alter cell-cell interactions and allow for gastrulation to proceed.

McClay and coworkers (1977) used aggregation collection methods on normal and hybrid (Lytechinus variegatus X Tripneustes esculentus) sea urchin embryos to examine cell surface changes at gastrulation. They showed that prior to gastrulation hybrid cells recognized cells of the maternal phenotype only, whereas at gastrulation they recognized cells with both maternal and paternal phenotypes. The appearace of new cell surface antigens of paternal specificity correlated temporally to changes in adhesiveness at gastrulation and lead McClay to suggest that these new cell surface antigens may be involved in adhesion. This was

substantiated by indirect immunfluorescence on hybrid embryos with antibodies made from membranes of the paternal species at mid-gastrula (McClay and Chambers, 1978). The paternal specific antibody did not react with embryos from the maternal species and in hybrids the antigen was detectable only at gastrulation and not prior to this stage. These data provided evidence that there were cell surface changes at gastrulation which influenced cellular recognition. Also agglutination titres and immunofluorescence revealed that there were antigenic differences between ectoderm and endoderm cells (McClay and Marchase, 1979). Immunofluorescence showed that there were qualitative differences in the antigens of the two germ layers.

The relationship between new cell surface antigens and the changes in adhesion at gastrulation was shown furthur by the use of Fab fragments (McClay et al, 1977). Fab fragments from paternal mid-gastrula antibodies prevented adhesion of paternal cells but not maternal cells, indicating a species specificity. The blocking effect of Fabs could only be removed if they were preabsorbed with paternal gastrula cells.

Noll and coworkers (1979; 1981) showed that reaggregation competence of dissociated sea urchin (Paracentrotus lividus) cells could be abolished if cell surface proteins were removed from the cell with 2.5% butanol. If these extracted proteins were readded, reaggregation and embryonic development could be restored. These protein extracts were not species specific in inducing

reaggregation. Fab fragments made from antibodies against these purified membrane preparations from blastula cells prevented aggregation and acted in a species specific manner. This effect could be removed by absorbing the Fabs to soluble protein extracts of purified membranes or with intact cells.

Another example of changes at the cell surface of sea urchin cells at gastrulation is provided by cellular interaction with the hyaline layer. The hyaline layer is an extraembryonic layer which appears shortly after fertilization. It provides a substrate to which cells remain attached (Spiegel and Spiegel, 1975). Removal of the hyaline layer results in cellular dissociation . The major constituent of this layer is the protein hyalin and by producing an antibody against this antigen McClay and Fink (1982) were able to examine its distribution throughout development and its role as a substrate. They found that as the embryo developed it became less dependant on the hyaline layer for its integrity. There were also germ layer affinities which changed with time. Prior to gastrulation all three germ layer types had affinities for hyalin, but at gastrula the primary mesenchyme cells lost their affinity. This correlated to the time at which these cells begin their movement into the blastocoel. The ectoderm and endoderm cells maintained their affinity for hyalin.

A number of other antibodies have been produced using membrane preparations and the extracellular matrix of gastrula stage embryos as immunogens (for review see McClay, 1983). After screening the monoclonal antibodies for germ

layer specificity by Elisa and immunofluorescence, McClay (1983) looked for patterns of expression of antigens during sea urchin development. He has identified antigens which are germ layer specific and appear at the time of germ layer delineation, antigens which appear initially on the egg cell surface and become compartmentalized with development, and antigens which are highly localized spatially and temporally in development. What remains to be done is to determine if any of these antigens are involved in cellular interactions during sea urchin development.

The Cell Surface of Mouse Preimplantation Embryos

Immunological approaches have been used extensively to study mouse preimplantation development. Antisera have been prepared using a wide variety of immunogens such as fetal germ cells, eggs, blastocysts, sperm, spleen cells, and teratocarcinomas. The result has been the development of a number of antibodies which define cell surface antigens.

Investigations at the cellular level in early stages of mammalian embryonic development have been facilitated by using the mouse teratocarcinoma as a model system (Jacob, 1975, 1978; Graham, 1977). In particular the use of this system has made possible the study of cell surface properties during the early stages of embryonic development (Artzt et al, 1973; Stern et al, 1975). Teratocarcinomas contain embryonal carcinoma cells (EC cells) which share many characteristics with the cells of the early embryo. EC cells can be maintained in culture and certain cell lines are capable of differentiation. There is extensive evidence that EC cells are very similar to pluripotent cells of early embryos (Artzt et al, 1973; Mintz and Illmensee, 1975)

Antisera obtained by using EC cells as immunogens have made it possible to identify and study cell surface antigens of early embryos. Edidin and coworkers (1971) made xenogeic antiserum against a 129 strain murine teratocarcinoma 402AX which reacted with the inner cell mass (ICM) of mouse blastocysts. Futhur studies demonstrated

that the antiserum detected at least three specificities, termed antigens 1, 2, and 3. Antigen 1 was shown to be physically associated with the H2 antigen at the cell surface of mouse L cell fibroblasts (Gooding and Edidin, 1974). It is present on fertilized eggs through blastula, the ICM of hatched blastocysts, the adult ovary, and numerous cell lines including human teratocarcinoma lines (Edidin et al, 1978). Antigen 2 reacted with trophoblast cells prior to implantation and after implantation was located on the ICM. Antigen 3 was absent from all embryonic cell surfaces.

The most highly characterized mouse cell surface antigen is the F9 antigen. It is defined by a syngeneic antiserum which was raised against the EC cell line F9 (Artzt et al, 1973, 1974). By complement mediated cytotoxicity tests and indirect immunofluorescence it has been found on all EC cell lines but is absent from the differentiated derivatives of these cell lines. It is not found on any somatic cells from the adult mouse but is expressed on sperm and during spermatogenesis (Gachelin et al, 1976). In the embryo F9 antigen is detected shortly after fertilization and increases in amount until morula, disappearing at about nine days post fertization (Buc-Caron et al, 1978). Immunofluorscent localization studies on sectioned blastocysts indicate it is expressed on the ICM and trophectoderm (Babinet et al, 1975). Not only is F9 found on all mice tested, its antiserum reacts with the morulae and sperm of all animals tested (Buc-Caron et al, 1974).

To examine the effect F9 antiserum has on <u>in vitro</u>

development of preimplantation mouse embryos, Fab fragments from rabbit anti-F9 IgG were added to embryos in culture (Kemler et al, 1977; Babinet et al, 1977). The conventional syngeneic anti-F9 serum was not used because it does not inhibit mouse preimplantation development (Jacob, 1977). In the presence of Fab fragments cleavage procedes normally until morula when the blastomeres fail to compact. As a result blastocysts do not form and the cells eventually die. The fragments have similar effects if embryos are treated at morula, however once the blastocoel starts forming treatment with monovalent antibodies has no affect.

The inhibition of morula compaction with Fab fragments is identical to the effect obtained if the calcium concentration is decreased in the culture medium of mouse embryos (Kemler et al, 1977). This ion is involved in intracellular adhesion by complexing glycoproteins of opposing cell surfaces with a divalent cation intermediate (for review see Takeichi et al, 1982). Since a decrease in calcium concentration and treatment of early embryos with Fab fragments have similar effects, it is possible that the interaction between calcium and the surface structure recognized by the anti-F9 serum is required for blastomere adhesion.

In attempts to determine the molecular nature of the F9 antigens, anti-F9 serum was used to immunoprecipitate radiolabelled antigens from detergent solubilized F9 cell lysates (Viletta et al, 1975; Muramatsu et al, 1979). Routinely two proteins with molecular weights of 22K and 44K

were precipitated. The conventional syngeneic F9 antiserum contains two components, an IgG and IgM. It is the IgG component which is responsible for precipitating the 22K and 44K antigens.

Both of the components of the conventional serum are responsible for the observed immunofluorescence in embryos, however the IgM component is the only one responsible for the cytotoxic effect (Morello et al, 1980; Damonneville et al, 1979). Attempts at characterizing the IgM antigen has met with conflicting results, although there is a concensus that the antigenic determinant is a carbohydrate. McCormick and associates (1982) were able to isolate molecules which inhibit the anti-F9 cytotoxicity from the culture medium of undifferentiated teratocarcinoma cells. They were very similar to the molecules isolated recently by performing immunoprecipitations on NP40 cell lysates with the IgM component of the conventional F9 antiserum and with an F9 monoclonal antibody designated ECMA-3 (Iwakura et al, 1983). In both cases a class of high molecular weight polysaccharides were precipitated which were strongly labelled with radioactive galactose and fucose. After extensive pronase digestions they still maintained molecular weights greater than 80k and were still capable of inhibiting the cytotoxic effect of F9 antibodies. Buc-Caron (1980) reported on the other hand that a glycopeptide of 2-3K obtained after extensive papain digestion of F9 cell surface antigens contained the target structure recognized by the F9 IgM.

A number of monoclonal antibodies have been made against F9 cells, one of them being IIC3 whose antigenic specificity is different from that of the conventional F9 antisera and other F9 monoclonals (Marticorena et al, 1983) Indirect immunofluorescence with this antibody revealed a temporally variable antigenic expression on trophoblast cells. The antigen is initially detected on compacted morulae and early blastula stage cells. It is strongly expressed on the primary endoderm and the trophoblast of expanded blastocysts. Immunoprecipitation results in the detection of a 70K molecule which is composed mainly of polysaccharides. Binding of IIC3 is enhanced when cells are treated with neuraminidase, which removes siliac acid. The authors have suggested that neuraminidase masks the antigenic site of the antigen and provides a means by which cell adhesion can be regulated.

Solter and Knowles (1978) detected a stage specific embryonic antigen (SSEA-1) defined by a monoclonal antibody made against F9 teratocarcinoma cells. The antigen is initially expressed at the eight cell stage and persists on ICM cells through the blastocyst stage. Upon development to the egg cylinder stage, its expression becomes restricted to the embryonic ectoderm and visceral endoderm (Fox et al, 1981). Maximal binding as determined by the radio-immune assay (RIA) occurs at morula and decreases on blastocysts. Indirect immunofluorescence, cytotoxicity, and RIA all reveal that the antibody reacts with antigens from all mouse and human teratocarcinoma cell lines and sperm.

SSEA-1 can be extracted with chloroform-methanol and immunoprecipitates run in front of the tracking dye on SDS polyacrylamide gels indicating that it is a glycolipid. Inhibition studies with oligosaccharides revealed that the antigenic determinant resides with a branched lacto-N-glycosyl type 2 carbohydrate chain, very similar if not identical to that of the human blood group antigen 1 (Gooi et al, 1981; Knowles et al, 1982).

Another stage specific antigen, SSEA-2, is found on murine preimplantation embryos and is maximally expressed at the four and eight cell stages (Shevinsky-Hamburger, 1981). It is found in reduced quantities at morula and on blastocysts. The antigen, whose molecular weight is unknown, is expressed on teratocarcinoma cells, sperm, and some SV40 transformed cell lines. A third stage specific antigen, SSEA-3, is defined by monoclonal antibodies produced against whole mouse embryos (Hamburger et al, 1982). The antigen is found on unfertilized eggs and mouse embryos up until the blastocyst stage. During early post implantation it is restricted to the endoderm and in the adult it is localized to the kidney.

Much of the work with monoclonal antibodies in this mouse system has been descriptive in nature. Specific roles in cell adhesion for the above mentioned antigens are yet to be established.

MATERIAL AND METHODS

Maintaining Snails and Obtaining and Handling Embryos

Mud snails, Ilyanassa obsoleta , were obtained from the Marine Biological Laboratories, Woods Hole, Massachusetts. Hibernating snails were maintained at 4°C in tanks containing Instant Ocean Salt (Aquarium Systems Inc.) made to a density of 1.024 in deionized water, or in natural sea water obtained from the Marine Biological Laboratories. To induce breeding hibernating snails were periodically placed into 10 gallon all glass tanks in a 14° C incubator and exposed to a 14-10 hour light-dark cycle. Breeding tanks were filtered with an over-the-side filter containing glass wool, activated charcoal and a gravel layer to remove nitrogenous wastes. The bottom of the tanks had a substantial gravel layer. Ilyanassa embryos are layed fertilized in jelly filled capsules containing 50-200 synchronous embryos. Strings of capsules are deposited on the side walls of the breeding tanks.

To facilitate culturing, embryos were released from capsules into petri dishes containing Millipore filtered sea water (MPFSW) and antibiotics (100ug/l each of penicillin and streptomycin (Sigma)) at room temperature. Release of embryos from the capsules was achieved by cutting the capsule open at one end with corneal scissors and gently forcing the embryos out. Once released from the capsules embryos were

transferred by gravity transfer from a wide mouthed pipette. This has the advantage of exchanging the sea water. Abnormal, asynchronous, and unfertilized eggs were removed with a hand drawn breath operated micropipette. Embryos were cultured in a 16°C incubator with daily changes of MPFSW.

Synchronization of Embryos

When synchronous embryos were required egg capsules were allowed to develop in individual petri dishes (Corning) at room temperature in capsules until they reached the 2-cell stage. They were then opened and placed at 6°C to retard development. In this manner embryos from a large number of capsules can be synchronized. After synchronization embryos were placed at 16°C and allowed to develop to the desired age.

When a full developmental spectrum of embryos was required laying snails were allowed to deposit capsules over a fifteen day period on the sides of tanks. Capsules were then harvested and opened at random. When such a preparation was made care was taken to ensure that uncleaved eggs were represented.

Removal of the Polar Lobe

Delobed embryos were obtained by releasing fertilized eggs from a single capsule into MPFSW, allowing them to develop to the trefoil stage, and removing the polar lobe by

rotary agitation (150 rpm) on a New Brunswick rotary shaker (Newrock and Raff, 1975). Delobed embryos were collected immediately with a breath operated micropipette fashioned from a pasteur pipette and transferred to fresh MPFSW at 6°C. After delobing was completed the embryos were placed at 16°C until the desired age was attained.

Obtaining Oocytes and the Induction of Maturation

Snails which have not mated after hibernation will lay unfertilized eggs (Mirkes, 1972). In Ilyanassa , as in many invertebrates, maturation occurs after fertilization. Thus, unfertilized eggs are equivalent in many ways to oocytes. To get such oocytes, snails, freshly removed from hibernation, were placed in "isolation vials" fashioned from scintillation vials glued by their caps to the sides of a 10 gallon breeding tank. The vials had 5 mm holes in their sides to allow for the passage of water and were placed far enough apart so that the snails could not mate through the holes. Capsules layed inside the vials contained unfertilized eggs.

We have found that maturation, as visualized by polar lobe formation, is induced when oocytes obtained as described above are released from the capsules into sea water. Oocytes stimulated in this manner will form polar lobes at both reduction divisions. Not all oocytes from a capsule will successfully be induced. Oocytes which showed such stimulation were pooled for experimental purposes and were

cultured at 16°C until the desired time point was reached.

Membrane Isolation

Embryos were homogenized by 20 strokes of a Dounce homogenizer (B pestle) in Z medium (50 mM glycine; 10 mM Tris maleate, pH 7.0; 5 mM MgCl2; 10 mM B mercaptoethanol; 0.1 mM PMSF (Boehringer Mannheim)). The homogenate was then centrifuged for 30 min at 10,000 rpm and the pellet was resuspended by homogenization in 2 ml of 22% (w/v) sucrose (Sigma) in Z medium. Aliquots of 250 ul were layered over a discontinuous sucrose step gradient prepared in a 5 ml polyallomer tube (Beckman) containing from the bottom up, 1.5 ml 60% sucrose-Z medium, 1.0 ml 50% sucrose-Z medium, 0.67 ml of 45% sucrose-Z medium, and 0.67 ml of 36% sucrose-Z medium. This was then centrifuged for 90 min at 30,000 rpm in a Beckman SW 50.1 rotor. Following centrifugation the 36-45% and 45-50% interfaces, which contained the membrane fraction, were collected, pooled, and diluted 1:1 in Z medium and centrifuged through 2.0 ml of 22% sucrose for 60 min at 20,000 rpm. The pellet, containing the membrane fraction was used as antigen and for electrophoretic analysis. The amount of protein in the preparation was determined by the method of Lowry (1951).

Monoclonal Antibody Production

A membrane fraction prepared as described above was

utilized by Dr. David McClay and Ms. Gail Cannon at Duke University for production of monoclonal antibodies (as described by Wessel et al, 1984). The method used was that of Galfre et al (1977). Fusions were done between HAT sensitive P3X63Ag8 myeloma cells and spleen cells from Balb C mice that had been immunized with our Ilyanassa membrane fraction. The mice were injected intraperitonially with antigen and their spleens were obtained by sterile disection. Hybridomas were raised in HAT media according to Littlefield (1964) and supernatents of hybrid clones were screened for antibody using an ELISA assay (Engvall and Perlmann, 1972) or by immunofluorescence, using the original immunogen.

The fusion produced 39 clones six of which were positive by an ELISA assay. These were designated IVe1Ø, VIe5, IVf1Ø, IVf2, IIId6, and IIIe9. Clone IVe1Ø, the main topic of this study, was cloned by limit dilution.

Polyacrylamide Gel Electrophoresis under Denaturing conditions

Membrane proteins were visualized by discontinuous tris-glycine SDS polyacrylamide gel electrophoresis according to the method of Laemlli (197Ø). Gels were cast 1 mm thick and measured 15 cm in length. The sample wells were 6 mm in width. The 1 cm stacking gel contained 6% (w/v) acrylamide and Ø.4% (w/v) bis and was buffered to pH 6.8. Resolving gels were 14 cm and contained either 1Ø% (w/v) acrylamide, Ø.3% (w/v) bis pH 6.8 or 7.5% (w/v) acrylamide, Ø.2% bis

as indicated in the figure legends. All electrophoresis chemicals were purchased from Biorad. The running buffer was tris-glycine pH 8.3. Gels were run at a constant 150V until the tracking dye ran off or at 75V for 1300 V/hr, as indicated in the figure legends.

Samples were prepared for electrophoresis by either dissolving by heating for 1 min in 1X SDS sample buffer (0.064M Tris, pH 6.8; 2% SDS; 2% sucrose; 5% B-mercaptoethanol; 0.001% bromophenol blue) or, if already in solution, by adding an equal volume of 2X sample buffer. Samples were spun in an Eppindorf centrifuge at full speed for 30 sec prior to loading. Proteins were visualized by staining the gels with 0.1% Coomassie Brilliant Blue R-250 (1 g/l in methanol:water:acetic acid; 5:16:1) and destained in 5:16:1 over several changes. Molecular weight markers were from Biorad.

Glycoprotein Staining of Polyacrylamide Gels

Glycoproteins were detected on polyacrylamide gels by the dansyl hydrazine method of Eckhardt et al (1976). Unstained gels were placed overnight in 55% H2O:40% ethanol:5% glacial acetic acid and then oxidized for two hours in 0.7% periodic acid (w/v) (BDH Chemicals) at room temperature. After rinsing with ddH2O for 0.5 hr. the gels were treated for 1.5 hr. with 0.5% sodium metabisulphite in 5% acetic acid and rinsed again several times in ddH2O. The gels were then incubated for 2 hr. in acidic DMSO:dansyl

hydrazine (1/1) (Fisher) in a water bath maintained at 60° C. The dansyl hydrazine:DMSO solution was then removed and the gels put into $NaBH_4$ in DMSO (20 mg/ml DMSO) for 30 min., rinsed with ddH2O, and placed overnight in 1% aqueous acetic acid. Stained glycoproteins were visualized as fluorescent bands on a Fotodyne UV light box and photographed with a polaroid camera fitted with a Kodak Wratten 16 filter. To avoid erroneous results from the natural fluorescence of proteins controls ommitting the periodate oxidation step were performed.

Protein Transfer from Polyacrylamide Gels to Nitrocellulose Paper

Proteins from gels were transferred to nitrocellulose sheets (Biorad, 0.45 u.) by the method of Towbin et al (1979). Immediately following electrophoresis gels were equilibrated in blot transfer buffer (20 mM Tris base; 150 mM glycine; 20% methanol) for one half hour. Scotch Brite padding, filter paper, and the nitrocellulose paper (all cut to the size of the gel) were also soaked in transfer buffer prior to the assembly of the sandwich. The blotting sandwich consisted of the gel upon which the nitrocellulose paper was layered surrounded by filter paper and the scotch brite pads. The nitrocellulose side of the sandwich was placed inside the blotting apparatus facing the anode. The transfer was at a constant voltage of 10V overnight or 40V for 6 hours.

Immunofluorescent Staining of Nitrocellulose Blots

Nitrocellulose sheets containing blotted proteins were incubated in 5% BSA (Sigma) (5 g/100ml of Tris saline, pH 7.4) for 90 min., rinsed briefly in Tris saline, and incubated overnight in the appropriate antibody diluted 1:50 in 5% BSA. Following incubation with the antibody the sheet was rinsed with Tris saline three times (10 min. each) and then incubated for 90 min. in fluorescein conjugated goat-anti-mouse anti serum (IgG and IgM) (Boehringer-Mannheim). The sheet was then washed three times (10 min. each) with Tris saline and photographed under blue light with a polaroid camera. Nonspecific staining was controlled for with P3X ascites fluid in place of the primary antibody and by deletion of the primary antibody from this procedure.

Lectin Binding to Nitrocellulose Blots

Membrane proteins were transferred to nitrocellulose filters as described above and then stained with lectins according to the modified method of Clegg (1982). Nitrocellulose blots were first incubated in 2.5% BSA in phosphate buffered saline (PBS) pH 7.4 for one hour with intermitant agitation to saturate the protein binding sites of the nitrocellulose.

For Con A (Miles-Yeda) binding, after the nitrocellulose was saturated, it was incubated in Con A (25

ug/ml in PBS containing Ø.5% Triton-X-1ØØ) for one hour at room temperature. It was then washed (5 X 5 min) in the same buffer and incubated in FITC-conjugated anti-Con A (5Ø ug/ml) (Miles-Yeda) for one hour. Excess Con A was washed off (5 X 5 min) and the nitrocellulose was examined and photographed under blue light. The control reaction contained α-methyl-D-mannose, a competative inhibitory sugar of Con A.

For staining with Wheat Germ Agglutinin (WGA) and Soybean Agglutinin (SA), the blots were incubated with either 2Ø ug/ml of FITC-conjugated WGA or 2Ø ug/ml of FITC-conjugated SA in PBS (CalBiocem Behring). Following 5 washes (5 min each) in PBS the transfers were examined and photographed under blue light. Contols for non-specific binding were the sugars Ø.2M N-acetyl-glucosamine and Ø.1M D-galactose which are competitive for WGA and SA respectively.

Embedding and Sectioning of Embryos

Embryos were fixed in Bouin's fixative (75 ml picric acid; 25 ml 37% formaldehyde, prepared from paraformaldehyde; 5 ml glacial acetic acid) for 24 hr to 48 hr and passed through a graded alcohol series for dehydration. Embryos were then embedded in paraplast by first exchanging the alcohol with xylene, then 1:1 xylene:paraplast (overnight on a hot plate) and finally equilibtated in 1ØØ% paraplast (3 changes over Ø.5 hr). Embryos in paraplast were then

pipetted into the wells of microtitre plates and the parplast was hardened on an ice slurry. Prior to addition of the embryos into the wells, three drops of melted paraplast were put into the bottom of the wells.

Slides washed with ethanol and water and coated with Mayer's albumin (egg white:glycerol, 1:1) were used. Single or serial sections were cut with a Spencer 820 microtome and were 10 u thick. Cut sections were floated onto flooded slides which were allowed to dry on a slide warmer.

Immunofluorescent Staining of Sections

IVe10 antigen was visualized in sectioned embryos by indirect immunofluorescence. Parafin was removed from the sections with xylene (2 X for 10 min) and rehydrated through a graded alcohol series. Sections were then washed (2 X 2 min) with phosphate buffered saline containing 0.05% Tween 20 (PBS-TW-20) and incubated overnight in a petri dish containing the appropriate antibody or control supernate in a humid chamber. Antibodies were diluted 1:50 in PBS, pH 7.4. After incubation in antibody, slides were rinsed three times with PBS-TW-20 and 20 ul of rhodamine-conjugated rabbit anti-mouse IgG (Miles-Yeda), diluted 1:50 with PBS, was placed on each section. The slides were then placed in a humid chamber for 90 min. Following another PBS-TW-20 wash, coverslips were floated over the sections and the stained sections were examined under the fluorescent microscope.

Immunofluorescence was assessed and photographed with a Leitz fluorescent microscope equipped for epifluorescence. Rhodamine fluoresces under green light, whereas the natural fluorescence of yolk in Ilyanassa embryos occurs under blue light. We were thus able to distinguish natural flourescence from that due to presence of bound antibody. Non-specific binding was controlled for with P3X ascites fluid, or primary antibody and secondary antibody alone.

RESULTS

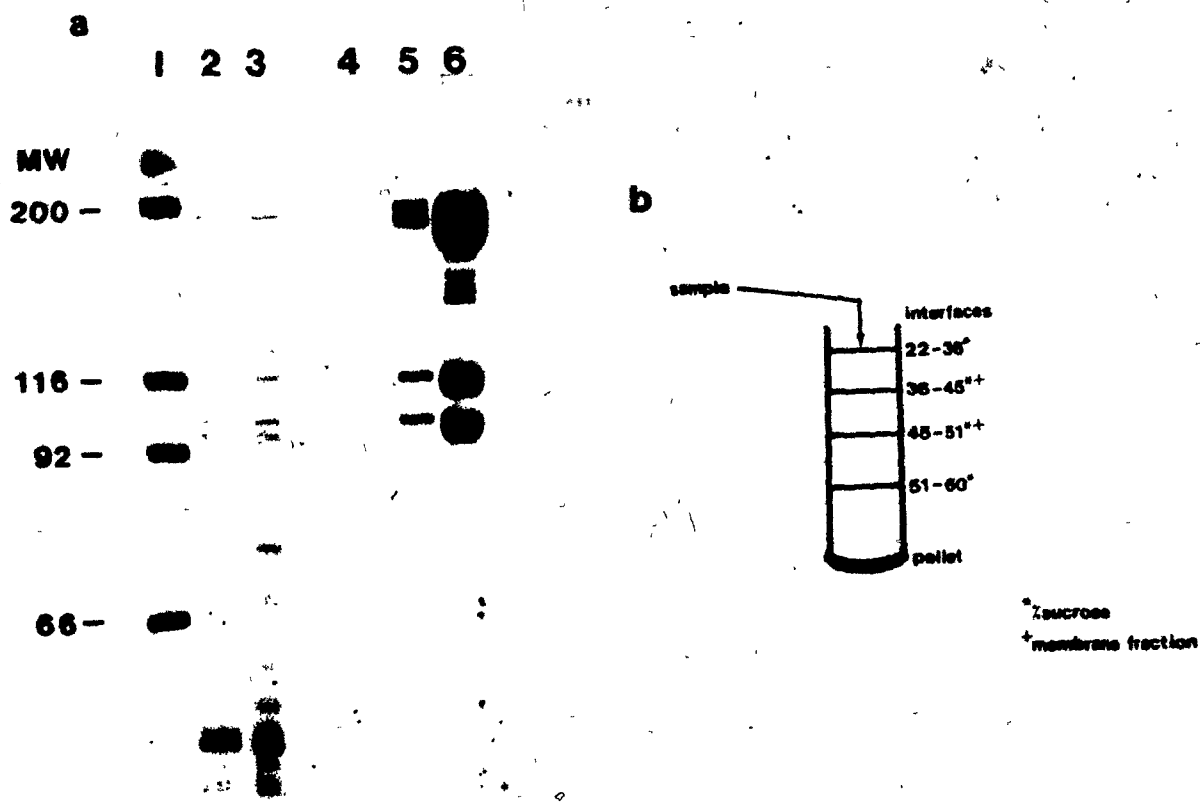
Production and Screening of Monoclonal Antibodies

Monoclonal antibodies to embyonic membrane proteins which could be used to study cell surface antigens were produced. The Ilyanassa membrane fractions used as immunogen, for screening of monoclonals, and for electrophoretic analysis were obtained as described in materials and methods. Centrifugation of Ilyanassa embryo homogenate through a sucrose step gradient (illustrated in figure 1b) facilitated the extraction of the relevant membrane antigens. The Membrane fractions used in this study sedimented at the 36-45% and 45-51% sucrose interfaces. These interfaces were pooled. When the components of these interfaces were electrophoresed on SDS polyacrylamide gels the profile observed in figure 1a (lanes 2 and 3) resulted. The pellet obtained from the gradient contained nuclei and yolk (figure 1a, lane 5).

For the production of monoclonal antibodies a membrane fraction was isolated from a mixture of embryos of all stages and used as immunogen. Approximately 100,000 embryos ranging in age from egg to veliger yielded 1.7 mg of protein, as determined by the Lowry assay, in the membrane fraction. This preparation was sent to Dr. David McClay's laboratory at Duke University for the production of monoclonal antibodies.

Of the 39 hybrids produced, 6 proved positive when

Figure 1. A typical membrane fractionation as prepared from a mixture of Ilyanassa embryos aged from egg to veliger larvae. Proteins were electrophoresed on 7.5% polyacrylamide gels in the presence of SDS for 1300 V/hr and visualized with Coomassie Brilliant Blue. The protein profiles of the various interfaces from the sucrose gradient are shown in 1a. Lane 1 represents molecular weight markers; lane 2, 25 ug from the 36-45% sucrose interface; lane 3, 25 ug from the 45-50% sucrose interface; lane 4, 20 ug from the 22-36% sucrose interface; and lanes 5 and 6 the pellet (25 and 75 ug respectively). The molecular weight markers are myosin (220K), B-galactosidase (116K), phosphorylase B (92K), and BSA (66K). Figure 1b represents the sucrose gradient from which the samples were obtained. The 36-45% and 45-50% interfaces are where the membrane fractions sediment and are routinely pooled. Yolk proteins, visualized in lanes 5 and 6 were obtained from the pellet.

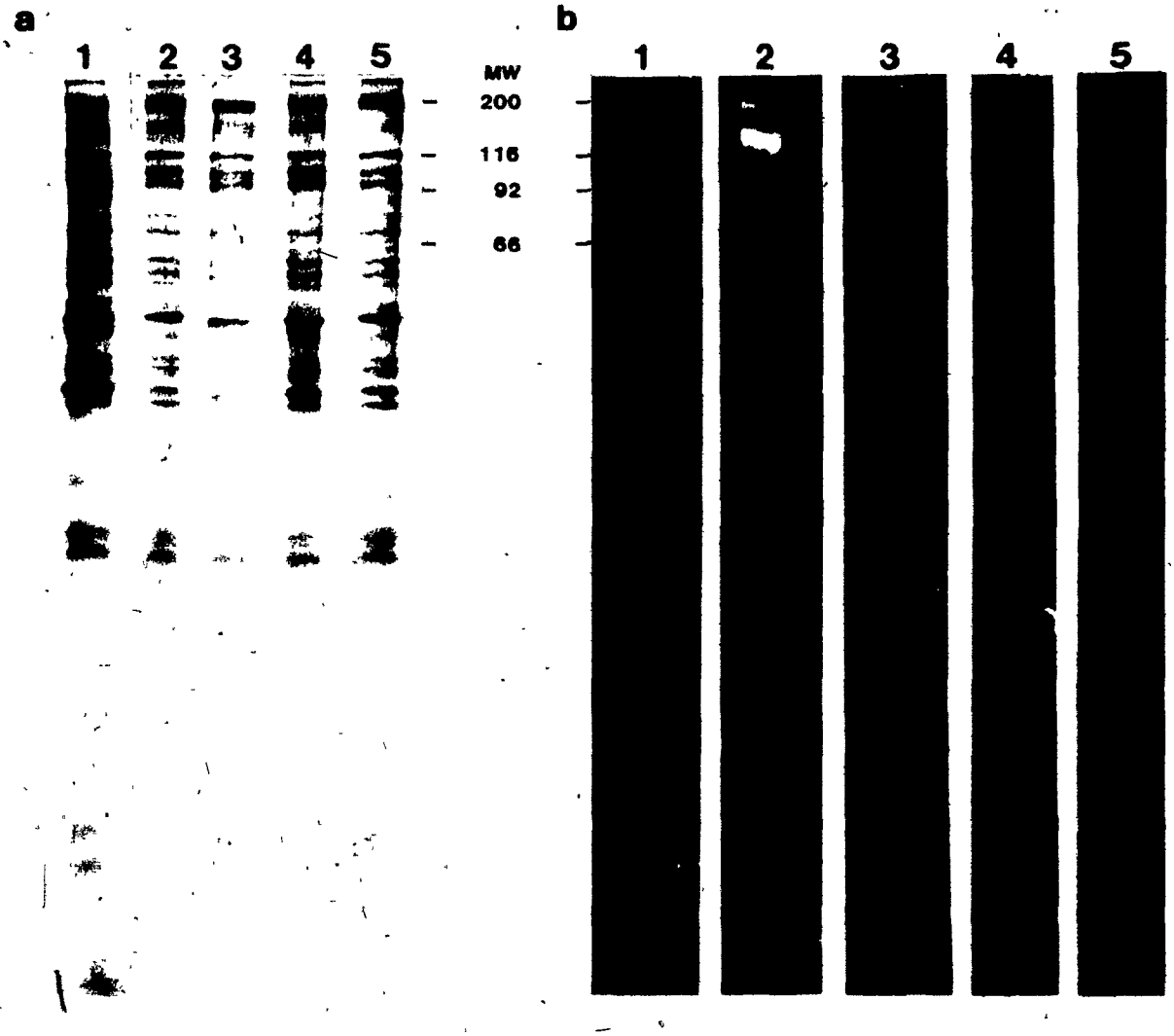

a
1 2 3 4 5 6
MW
200 –
116 –
92 –
66 –
b
sample
interfaces
22-36*
36-45*+
45-51*+
51-60*
pellet
*%sucrose
+membrane fraction

screened by ELISA against the original membrane preparation. The six positive monoclonals were designated IVf2, IIId6, IVf1Ø, VIe5, IIIe9, and IVe1Ø. The latter four were the most potent. These were furthur screened by indirect immunofluorescent staining of parafin embedded sections of embryos and by immunofluorescent staining of proteins electroblotted onto nitrocellulose. In both these screening procedures a mixture of embryos of all stages was used. As can be seen in figure 2 (lane 2) only IVe1Ø gave a positive reaction when screened by immunoblotting. The other three antibodies (lanes 1, 3, and 4) and the P3X control (lane 5) were negative. The stained sections gave similar results. Because of this IVe1Ø was chosen to work with. The temporal and topological expression of the IVe1Ø antigen(s) is the focus of the remainder of the work in this study.

Identification and Characterization of the Antigen(s) reactive with IVe1Ø

In the initial screening of fusions by immunoblotting 25-3Ø ug of protein was seperated on 1Ø% SDS polyacrylamide gels run only until the dye front had run off the end of the gel. This assured that no reactive protein had been lost (figure 2a). However IVe1Ø reacted only with proteins of high molecular weight. Thus, to achieve better seperation, proteins were electrophoresed on lower concentration gels (7.5%) and run twice as long. This facilitated seperation of

Figure 2. The screening of monoclonal antibodies by immunoblotting. Ilyanassa membrane proteins were transferred to nitrocellulose after SDS gel electrophoresis. The membrane preparation was obtained as outlined in materials and methods and contained membranes from egg to 14 day Ilyanassa embryos. Membrane extracts (30 ug) were subjected to 10% SDS PAGE at 150V until the tracking dye ran off the front of the gel and stained for protein with Coomassie Brilliant Blue (figure a, lanes 1-5) or blotted onto nitrocellulose and labelled by indirect immunofluorescence (figure b) with monoclonal antibodies VIe5 (lane 1), IVe10 (lane 2), IVf10 (lane 3), IIIe9 (lane 4). Lane 5 is a similar blot incubated in P3X, the naive culture fluid. The dilution of antibody was 1:50 in PBS, as was the FITC-conjugated secondary antibody.


a
1
2
3
4
5
MW
200
116
92
66
b
1
2
3
4
5

high molecular weight proteins and resulted in better resolution of the proteins reactive with IVel0 (figure 4).

On immunoblots of a membrane spectrum IVel0 reacts with two bands with molecular weights of approximately 190K and 130K (figure 3, lane 2). They are designated Ilyanassa embryonic antigen 1 (IEA-1) and Ilyanassa embryonic antigen 2 (IEA-2) respectively. In addition to recognizing IEA-1 and IEA-2, IVel0 reacted strongly with a third band of membrane fractions isolated from artificially activated twenty hour oocytes (figure 3, lane 1). This antigen designated IEA-3 has a molecular weight of 160K.

In all cases when blotted proteins were incubated in the P3X supernatent no immunofluorescent labelling was observed. If only secondary antibody was used and IVel0 was ommitted a similar result was obtained. The specificity of IVel0 to Ilyanassa membrane proteins was verified by its negative reaction to a membrane preparation from sea urchin (S . purpuratus) blastula (figure 5, lane 2). IVel0 antigens are also absent in adult snail membrane (figure 5, lane 1).

An initial concern was that these antigens were yolk proteins, rather than membrane proteins, since their molecular weights are similar to high molecular weight yolk proteins (figure 4). The data shown in figure 4 shows that IVel0 does not react with yolk protein.

Occasionally faint staining species with molecular weights greater than 200K are seen. The fact that IVel0 recongizes several Ilyanassa membrane proteins shows that

Figure 3. One IVe10 antigen is enriched in the Ilyanassa oocyte. A membrane preparation from 20 hr oocytes was electrophoresed on a 7.5% SDS polyacrylamide gel for 1300 V/hr, immunoblotted and stained with monoclonal antibody (lane 1). Lane 2 represents the stained immunoblot of an Ilyanassa embryonic membrane spectrum (egg to 14 days old). The oocyte enriched antigen is designated IEA-3. IEA-1 and IEA-2 are the two major IVe10 antigens which are present throughout development. These 20 hour oocytes are artificially activated oocytes which have been allowed to "develop" for 20 hours.

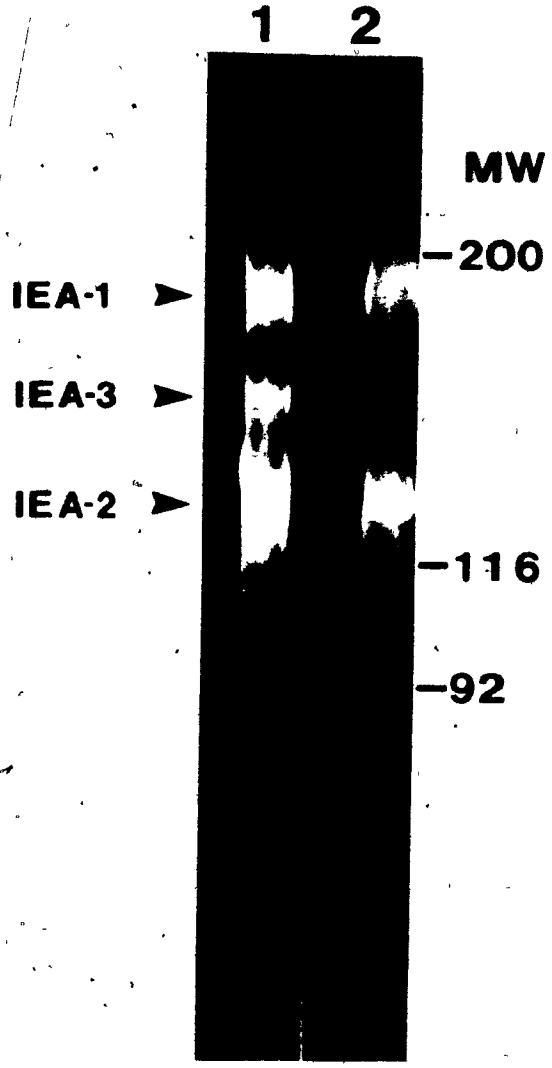

1
2
MW
200
IEA-1
IEA-3
IEA-2
116
92

Figure 4. Monoclonal antibody IVe10 does not react with yolk protein. Ilyanassa membrane fractions obtained from egg through 16 day embryos and yolk proteins obtained from the sucrose gradient pellet of the same preparation were seperated on 7.5% SDS polyacrylamide gels for 700 V/hr and stained with Coomassie Brilliant Blue (figure 4a). Lane 1 represents a mixture of membrane and yolk proteins (25 ug of each); lane 2 is the membrane spectrum (25 ug); lane 3 contains the yolk proteins (25 ug); and lane 4 contains molecular weight markers. When transferred to nitrocellulose and stained for IVe10 reactivity by indirect immunofluorescence (figure 4b) only those lanes containing membrane fraction were positive (lanes 2 and 3). Lane 1 contains the mixture and lane 4 is the P3X control. The molecular weight markers are myosin (220K), B-galactosidase (116K), phosphorylase B (92K), and BSA (66K). The dilution of antibody was 1:50 in PBS, as was the FITC-conjugated secondary antibody.

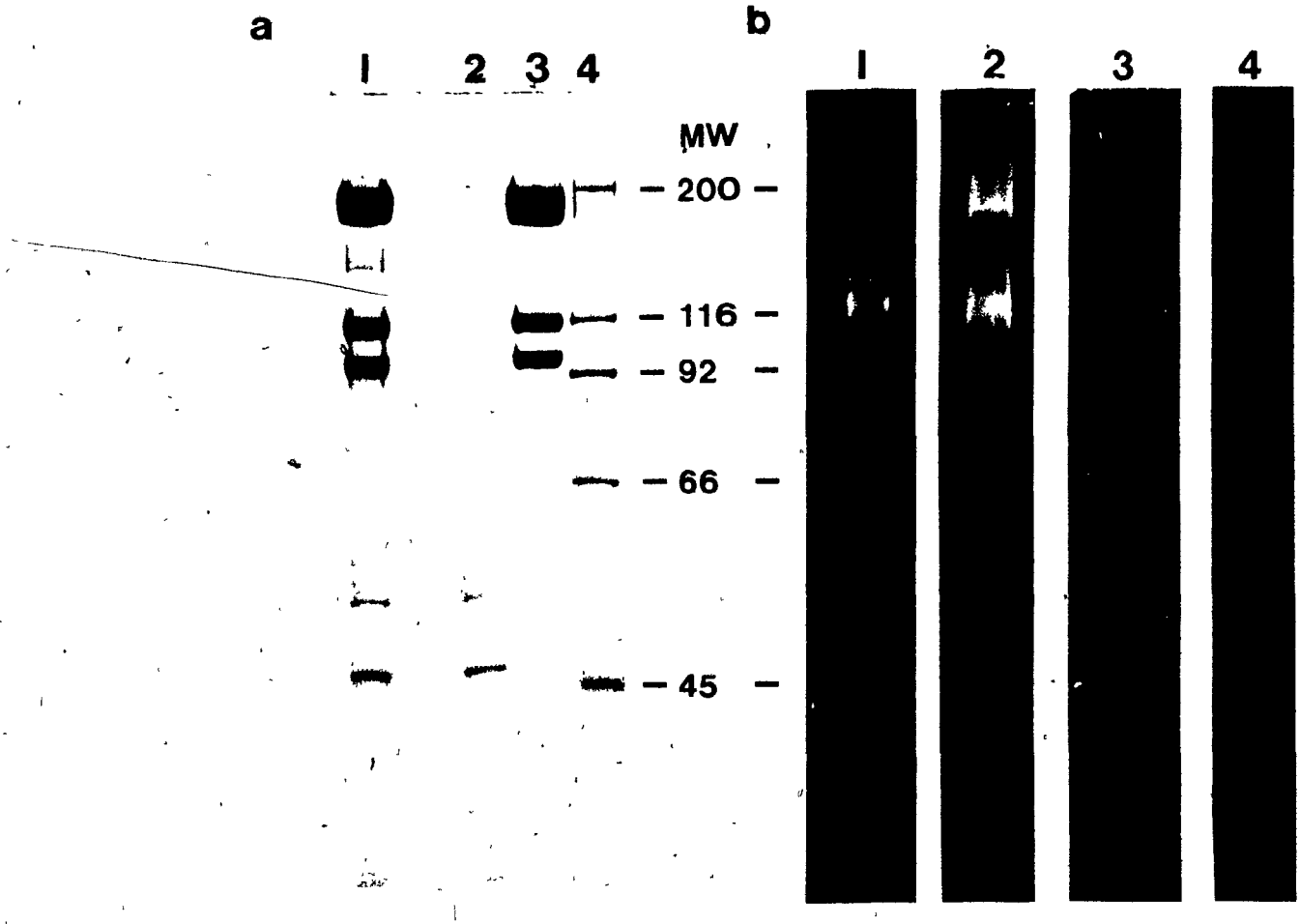
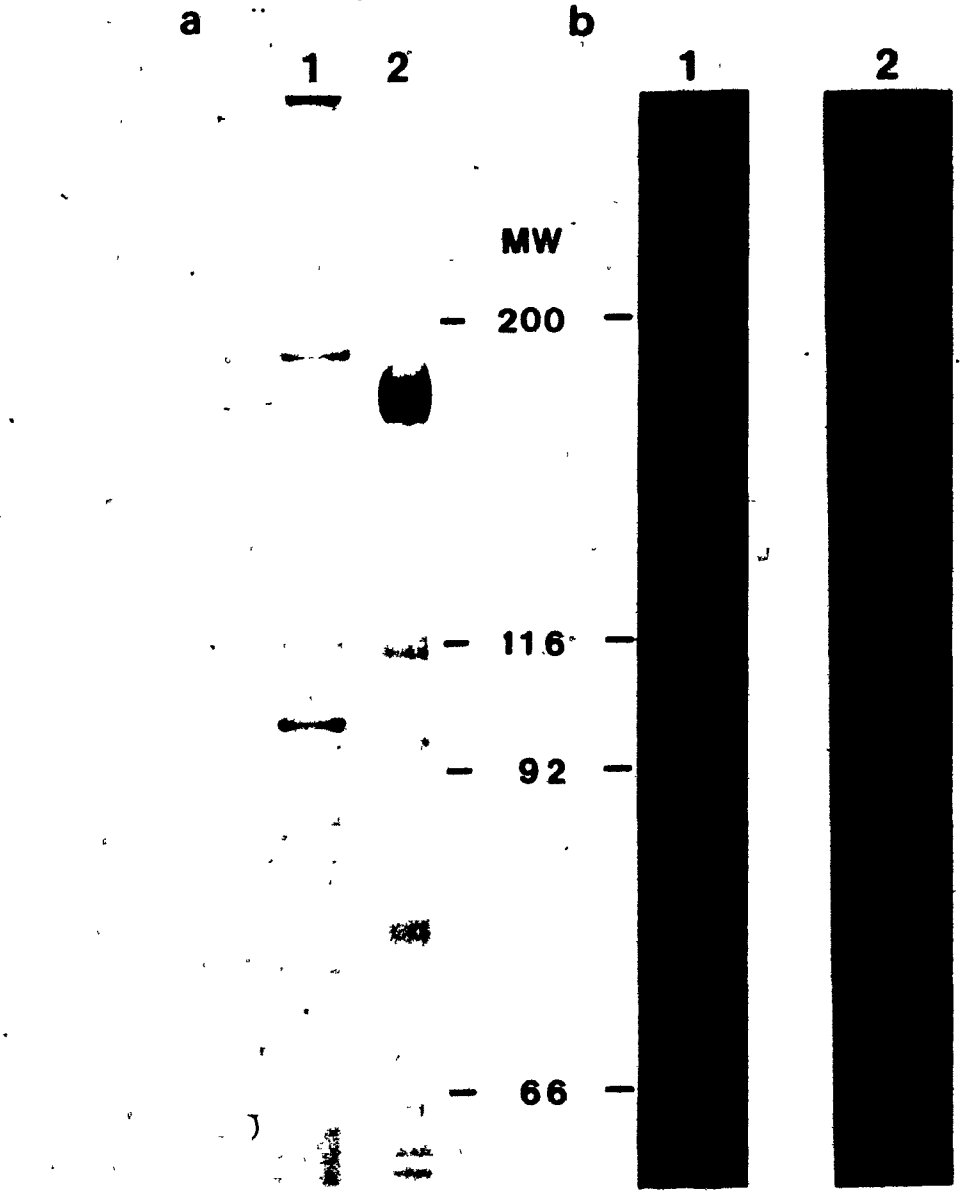

a
1
2
3
4
MW
200
116
92
66
45
b
1
2
3
4

Figure 5. Monoclonal antibody IVe10 does not react with adult snail membrane nor sea urchin embryonic membrane. Lanes 1 and 2 of figure 5a represent membrane fractions from adult Ilyanassa and sea urchin (S. purpuratus) blastula embryos respectively (30 ug of each) seperated on 7.5% SDS PAGE for 1300 V/hr and visualized with Coomassie Brilliant Blue. When transferred to nitrocellulose and stained by indirect immunofluorescence with IVe10 antibody both the adult (figure b, lane 1) and the sea urchin blastula (figure b, lane 2) fractions show no reactivity. The dilution of primary and secondary antibody was 1:50 in PBS.

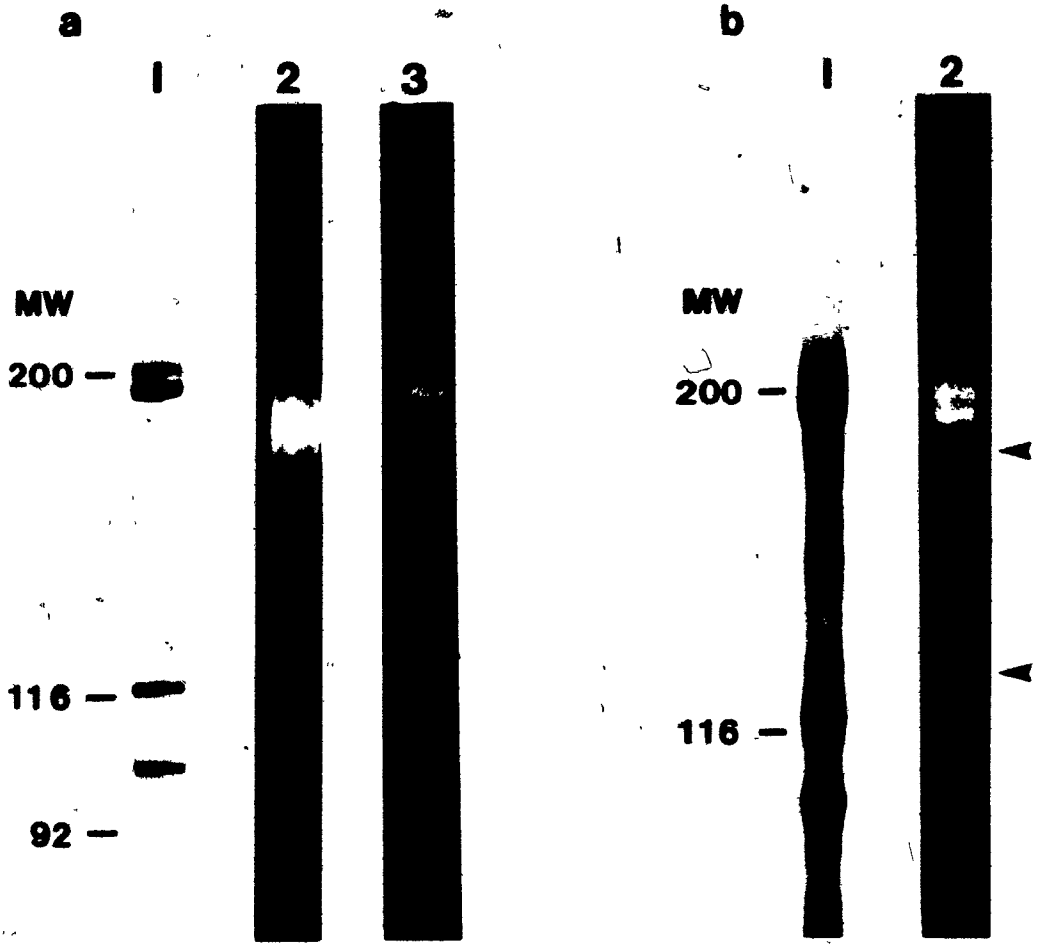

a
1
2
b
1
2
MW
200
116
92
66

these proteins share the same antigenic site. How similar these proteins are is not clear at this time. Although the approximate molecular weights of the IVe1Ø antigens has been established, it has not been possible to determine which bands on Coomassie Blue stained gels are the IVe1Ø antigens. Although there are protein bands at the 19ØK and 13ØK to 16ØK regions on the stained gels these bands have a different "shape" than do the bands visualized by immunological staining, being discrete bands and not regions of staining as seen by immunofluorescence. Furthur characterization will require enrichment of the IVe1Ø antigen(s).

Such wide bands are characteristic of glycoproteins. Glycoproteins are also suspected given that the antigens are high molecular weight membrane proteins. Attempts to obtain direct evidence as to whether the IVe1Ø antigens are glycoproteins has not been successful. Glycoprotein staining by the dansyl hydrazine technique failed to show bands in the exact molecular weight regions of the IVe1Ø antigens with the appropriate shapes (figure 6b).

Lectins are useful probes for identifying and isolating cell surface glycoproteins. Three lectins were tried for the purpose of determining whether or not the IVe1Ø antigens were glycoproteins. These were Concanavalin A (Con A), Soybean agglutinin (SA), and Wheat Germ agglutinin (WGA). Con A is a glucose/mannose binding haemagglutinating isolate from jack bean. Soybean agglutinin binds to N-acetyl-galactosamine containing molecules and less strongly to galactose resisdues, whereas Wheat Germ agglutinin binds

Figure 6. Lectin binding studies on Ilyanassa membrane proteins. Figure 6a, lane 3, shows the fluorescent Con A staining of membrane proteins from a developmental age spectrum (egg to 14 days) of Ilyanassa embryos resolved by 7.5% SDS PAGE and blotted onto nitrocellulose. Fluorescent Con A staining was performed as outlined in materials and methods. Lane 1 is the corresponding Coomassie Brilliant Blue stained gel containing 25 ug of protein and lane 2 is the corresponding immunoblot stained with IVe10 antibody. Figure 6b, lane 2, is the same membrane preparation seperated by 7.5% SDS PAGE and stained for glycoprotein by the dansyl hydrazine technique. Arrows indicated where the IVe10 antigens run. Lane 1 is the corresponding Coomassie Brilliant Blue stained gel intentionally overloaded with 60 ug of membrane protein. Both Con A staining and the dansyl hydrazine staining failed to detect the IVe10 antigens.


a
1
2
3
MW
200
116
92
b
1
2
MW
200
116

to N-acetyl-B-D-glucosamine residues and less strongly to sialic acid residues. These three lectins were used as fluorescent conjugates to stain electroblotted membrane fractions. No detectable binding to IVel0 antigens was seen (figure 6a). Con A and WGA did bind to some membrane proteins whereas SA did not bind to any of them.

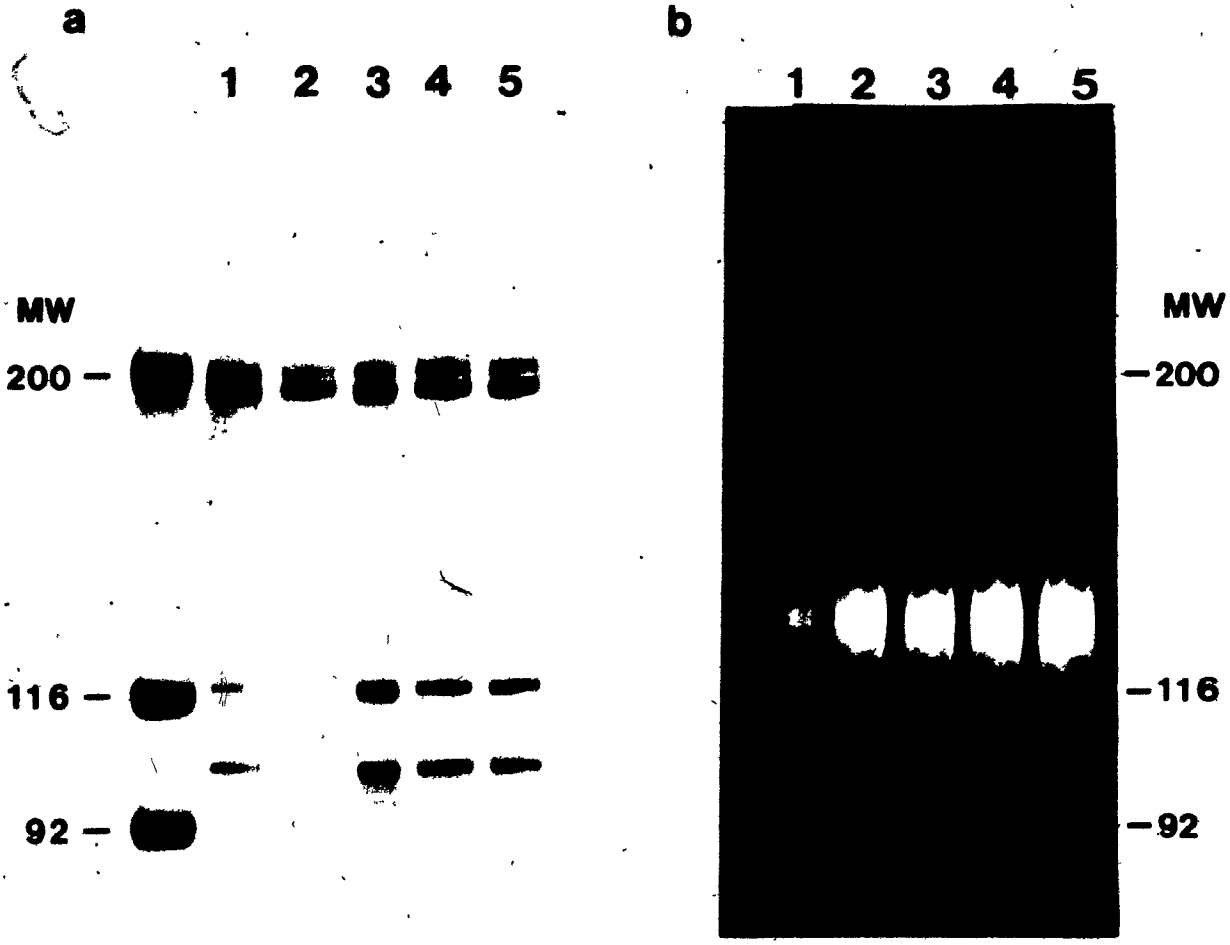
Developmental Studies

The remainder of the work concentrated on two areas. The first involved a more detailed analysis of the presence or absence of the antigens throughout development utilizing immunoblot procedures. The second approach was to locate the antigens in the embryo by indirect immunofluorescence of parafin embedded and sectioned embryos. First the immunoblot studies will be discussed.

Figure 7 shows the immunofluorescent staining of membranes from different age embryos. IVel0 antigens are present at all stages tested, from early cleavage stages to the 20 day fully developed veliger. At all ages the same antigens are present with the same characterisitic staining patterns. As the IVel0 antigens are not present in the adult snail (figure 4, lane 1), the antigen(s) is therefore embryonic.

A membrane fraction was also prepared from oocytes which had been undergoing artificial activation for 20 hrs. Immunoblot analysis of this preparation (figure 3, lane 1)

Figure 7. Indirect immunofluorescent staining of Western blots of Ilyanassa membrane proteins from successive stages of embryonic development. Membrane extracts (30 ug) were seperated on 7.5% SDS polyacrylamide gels for 1300 V/hr and stained with Coomassie Brilliant Blue (a) or transferred to nitrocellulose, reacted with IVe10 antibody, and visualized by indirect immunofluorescence (b). Lanes 1 through 5 represent early cleavage stage (2 cell to 32 cell); 1 to 3 days old; 3 to 5 days old; 11 to 13 days old; and 18 to 20 days old embryos respectively. Primary and secondary antibody dilutions were 1:50 in PBS.

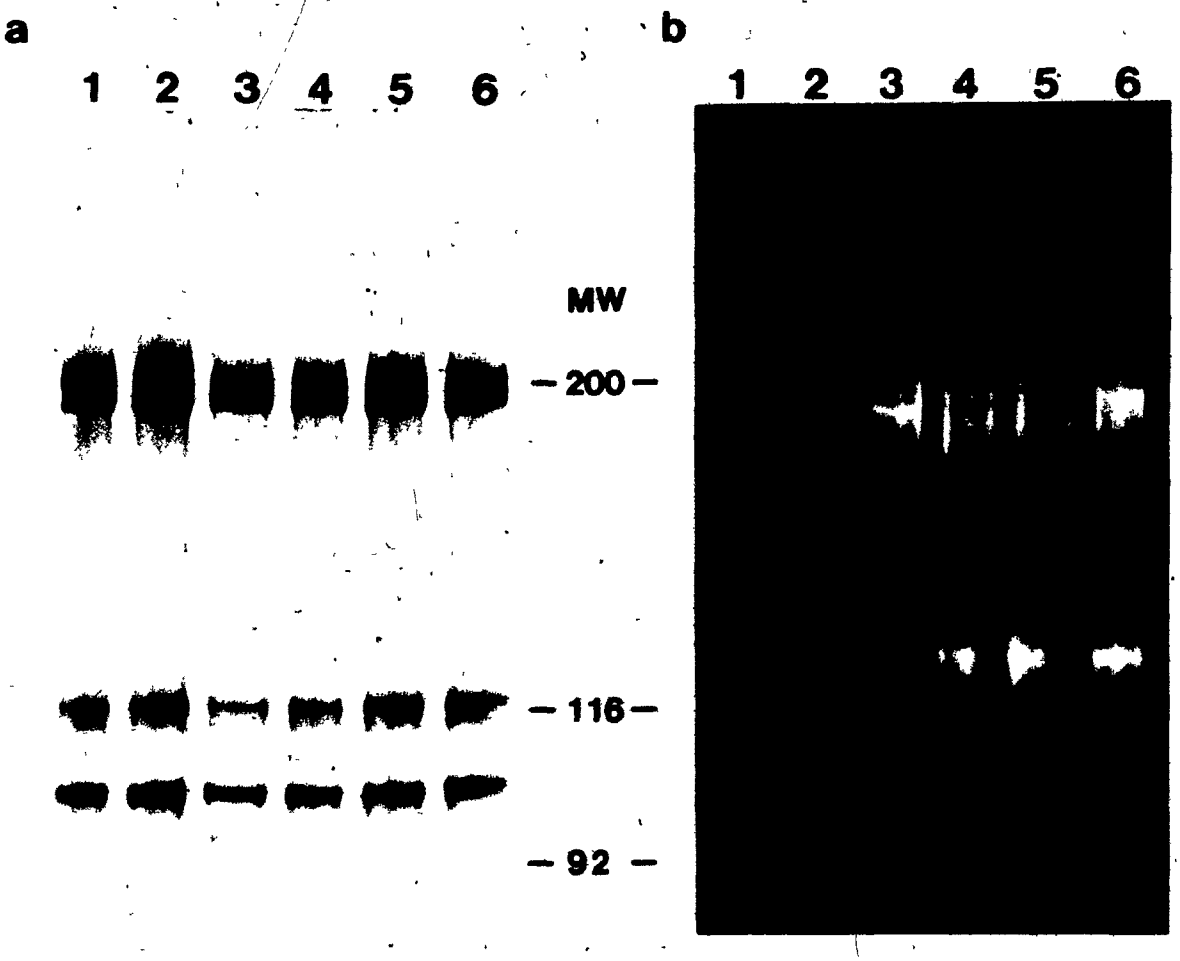

a
1 2 3 4 5
MW
200
116
92
b
1 2 3 4 5
MW
200
116
92

revealed IEA-1 and IEA-2 antigens as found in the embryo. It also revealed an intensely staining region at the approximate molecular weight of 160K designated IEA-3. This band is intense in the 20 hr oocyte but not in the embryo. When detectable at all in the embryo its intensity is greatly reduced relative to IEA-1 and IEA-2 (figure 5, lane 2).

Earlier oocyte maturation times were examined to determine if the IVe10 antigens are present before maturation. Induction of maturation was monitored under the disecting microscope. First polar lobe formation indicated successful induction had occured. Only those oocytes which extruded a polar lobe were used in these membrane prepartations, except for the "0" hour preparation. Zero hour oocytes were harvested and processed immediately. The first polar lobe usually appeared within half hour after exposure to sea water and roughly 60% of the exposed oocytes underwent induction.

At "0" hour only IEA-1 could be detected (figure 8, lane 1) and this at an intensity which is much lower than that seen in embryos and older oocytes. Four hour oocytes showed the same staining pattern as that of 0 hr oocytes (figure 8, lane 2). By eight hours IEA-1 had increased in staining intensity to that detected in the embryo and IEA-2 became detectable for the first time, but at an intensity which was lower to that of the embryo or fertilized egg. IEA-3 has also become faintly detectable in the 8 hour oocyte. By 11 hours post artificial maturation induction all three antigens are detectable at their maximum level. This

Figure 8. Appearance of IVe10 antigens during artificial oocyte maturation. Antigens were visualized by immunolabelling of Western blots. Membrane extracts (25-30 ug) were subjected to 7.5% SDS PAGE and stained for protein with Coomassie Brilliant Blue (a) or transferred to nitrocellulose and labelled by indirect immunofluorescence with the IVe10 monoclonal antibody (b). Lanes 1 through 5 in both a and b represent 0 hr, 4 hr, 8 hr, 11 hr, and 20 hr oocyte membrane extracts respectively. Lane 6 contains membrane proteins from the fertilized egg.


a
1 2 3 4 5 6
MW
200
116
92
b
1 2 3 4 5 6

level is similar to the 20 hour oocyte and the fertilized egg (figure 8, lanes 5, and 6). The accompanying coomassie stained gel in figure 8 shows that identical amounts of protein were loaded and that the same staining patterns were produced for all of the oocyte stages. The immunoblot analysis of oocyte membrane preparations reveals that the IVel0 antigens appear during oocyte maturation. What this represents in molecular terms is not yet clear.

In summary, IEA-3 appears to be developmentally regulated. It is present in the mature oocyte and decreases in prominence as development proceeds. It is barely detectable in newly deposited eggs and is absent in non-activated oocytes, appearing with maturation. IEA-1 and IEA-2, on the other hand, are present in both the oocyte and all other stages of the developing embryo (figure 7).

Immunocytological Localization of the IVel0 Antigens in Ilyanassa Oocytes and Embryos

The patterns of antigen distribution were examined throughout oocyte maturation and embryogenesis. Ilyanassa embryos contain an abundance of yolk. Yolk fluoresces under blue excitation, the excitation wavelength of FITC. To avoid this interference a rhodamine conjugated secondary antibody, which fluoresces under green excitation, was used in place of the FITC-conjugated secondary antibody which was used in the preliminary screenings of the monoclonals (see figure 9). All analyses of this type were controlled for by using: 1) no

primary antibody; 2) P3X fluid with secondary antibody; 3) no secondary antibody; 4) and no primary or secondary antibody. A representative series of controls is shown in figure 9. Under green excitation faint fluorescence can be observed for the controls using P3X with secondary antibody (figure 9d) and secondary antibody only. This fluorescence resembles that seen under blue excitation (figures a, c, and e). We believe this is due to a low level of non-specific binding of the secondary antibody to yolk. Controls using IVel0 only or no primary or secondary antibody were too dark to be photographed under green excitation (figure 9b) .

Zero hour and 20 hour (of maturation) ooctyes were examined first (figure 10). At both ages two types of fluorescence were evident; a cytoplasmic, "granular" flourescence, which was found in all fluorescing oocytes; and a patchy membrane fluorescence at the oocyte surface, seen in some but not all fluorescing oocytes. Rountinely 90-95% of the oocytes examined from both age groups exhibited some fluorescence. At both ages there was no fluorescence within the germinal vesicle. Although oocytes of the two ages exhibited no differences in the pattern of fluorescence, the "older" oocytes (20 hr) fluoresced more intensely than the younger ones (0 hr) (figure 10). The cytoplasmic fluorescence was, in particular, more intense at 20 hours, taking on a more uniform distribution throughout the ooplasm as opposed to the spotty distribution seen at 0 hours (figure 10). Also, more of the older oocytes exhibited the patchy membrane fluorescence (28% as opposed to 18% at 0

Figure 9. Representative controls for IVe10 indirect immunofluorescent staining. Staining was performed on parafin embedded sections (10 u thick) prepared as outlined in materials and methods. Sections in this figure are of 20 hr oocytes which have been artificially induced to mature. Frames a and b represent fluorescence observed in the presence of IVe10 antibody only under blue and green excitation respectively. There is yolk fluorescence in a but not in b. C and d are stained in the presence of P3X culture medium and the rhodamine conjugated secondary antibody (blue and green excitations respectively). Frames e and f are blue and green excitations respectively of oocytes reacted with IVe10 followed by rhodamine conjugated secondary antibody. The fluorescence present in f but absent from e is due to bound antibody. Fluorescence under blue excitation (frames a, c, and e) is due to the yolk and that observed in frame d is due to the non-specific binding of the rhodamine conjugated secondary antibody to yolk. Controls with nothing added to the sections are identical to that seen in the presence of IVe10 only (a and b). Controls with secondary antibody only are identical to those using the P3X fluid with the secondary antibody (c and d). (X400).

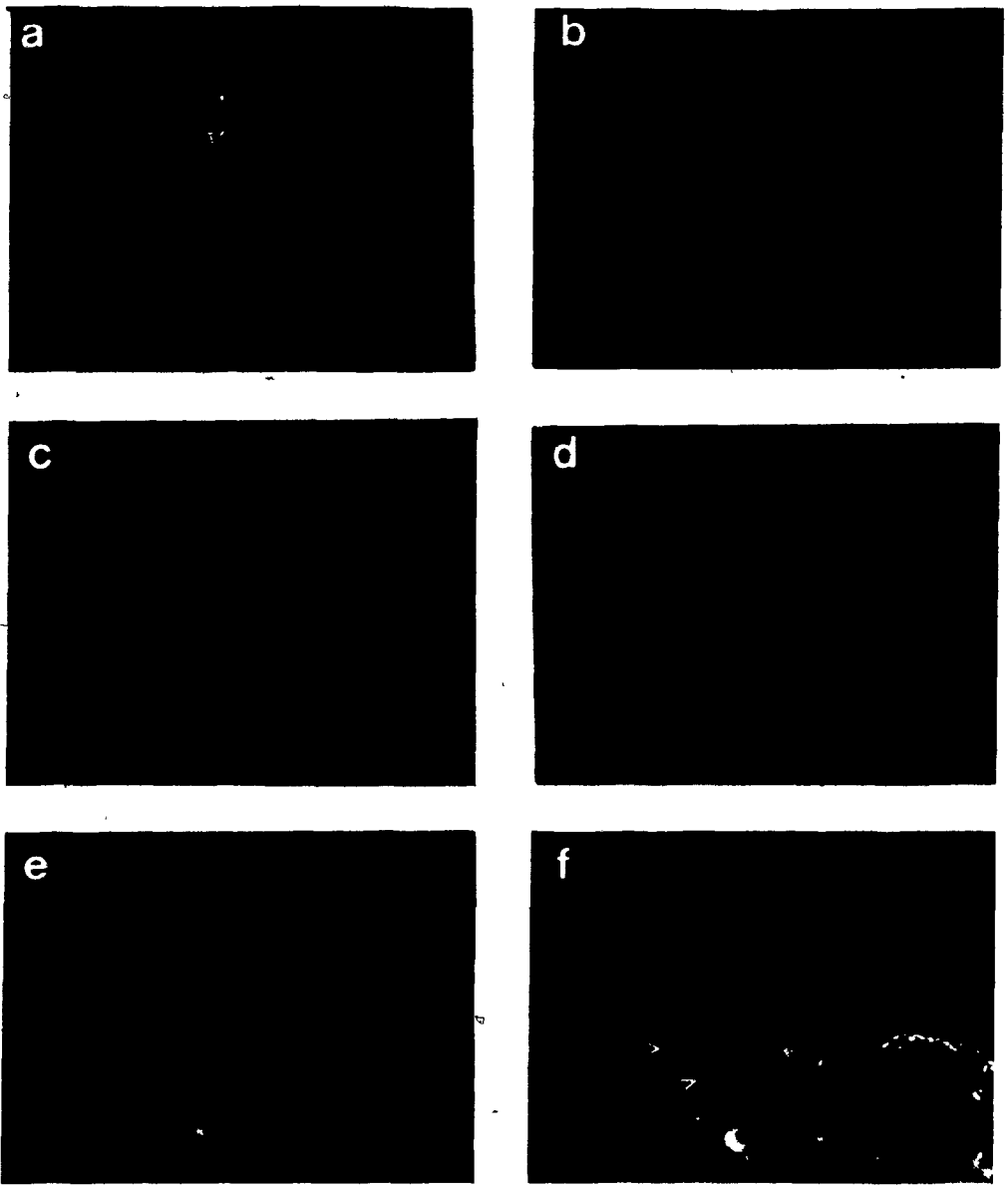
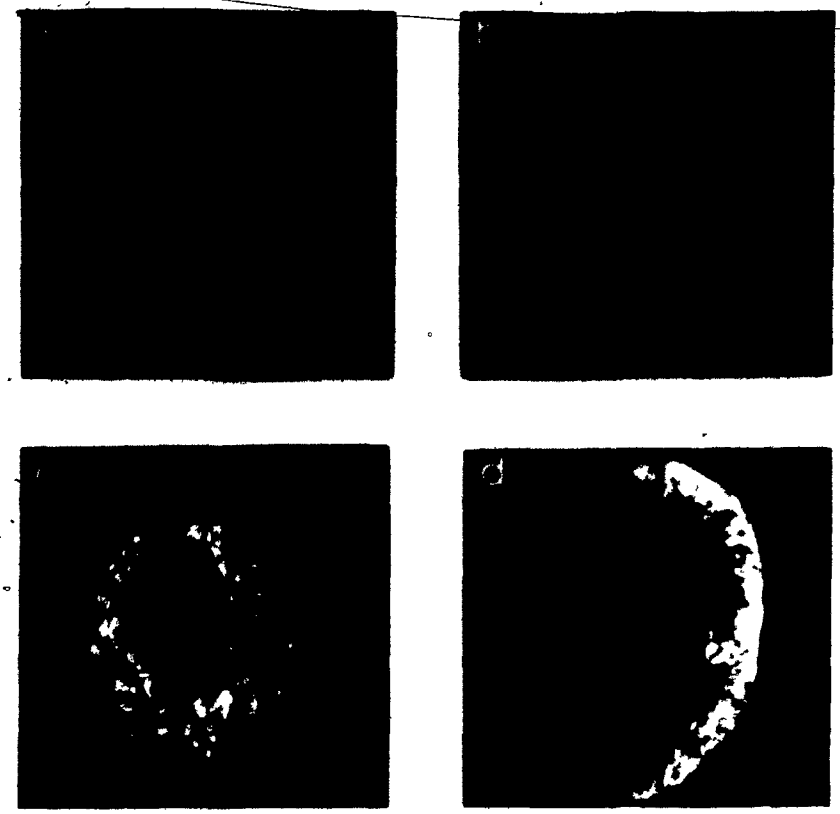

a
b
c
d
e
f

Figure 10. Indirect immunofluorescence of oocytes which have undergone artificial maturation for 0 and 20 hrs. They are stained with IVe10 antibody and rhodamine conjugated secondary antibody (diluted 1:50). Frames a and b are 0 hr oocytes and c and d are 20 hr oocytes. Arrows indicate the membrane fluorescence and the cytoplasmic fluorescence. Note the increased intensity of fluorescence in the older oocytes. X1000.



hour). Thus, there is an increase in fluorescence with increasing times of maturation, both in cytoplasmic fluorescence intensity and the number of oocytes with surface fluorescence. This agrees with the immunoblot data on oocytes which revealed the appearance of more IVe1Ø antigen(s) with increasing oocyte age. However the immunoblot data did not indicate that the antigen(s) was present as early on during artificial oocyte maturation as is indicated by the immunocytochemical data.

The fertilized egg first undergoes maturation, then pronuclear fusion. Thus, the first stages after fertilization would be expected to be similar to what is seen during artificial maturation. This is in fact so. Newly deposited fertilized egg-oocytes show little fluorescence if any (figure 11, frame a). As the germinal vesicle breaks down, fluorescence appears at the surface and internally (figure 11, frame b). By the two cell stage the fluorescence is extensive. All fertilized eggs which have matured exhibit cytoplasmic and membrane fluorescence (figure 12). Figure 13 shows a comparison between a mature egg and an immature and likely unfertilized oocyte. In the oocyte only the cytoplasmic fluorescence is evident whereas the egg stains at the surface and in the cytoplasm.

Throughout early cleavage stages both types of fluorescence persist in the embryo (figure 14). At the two cell stage (figure 14a and b) IVe1Ø antigen(s) is found on the cell surface of both blastomeres (figure 14a) and, as seen in figure 14b between the two blastomeres at regions of

Figure 11. The pattern of IVe10 fluorescence during maturation and pronuclear fusion and after first cleavage. Sections were stained with IVe10 followed by rhodamine conjugated secondary antibody (diluted 1:50). Frames a and b are during germinal vesicle breakdown; frame c is during the formation of the second palar body; frame d during pronuclear fusion; and frame d is at the two cell stage. Note the increased cytoplasmic fluorescence as maturation and pronuclear fusion proceeds and the appearance of cell surface fluorescence. X500.

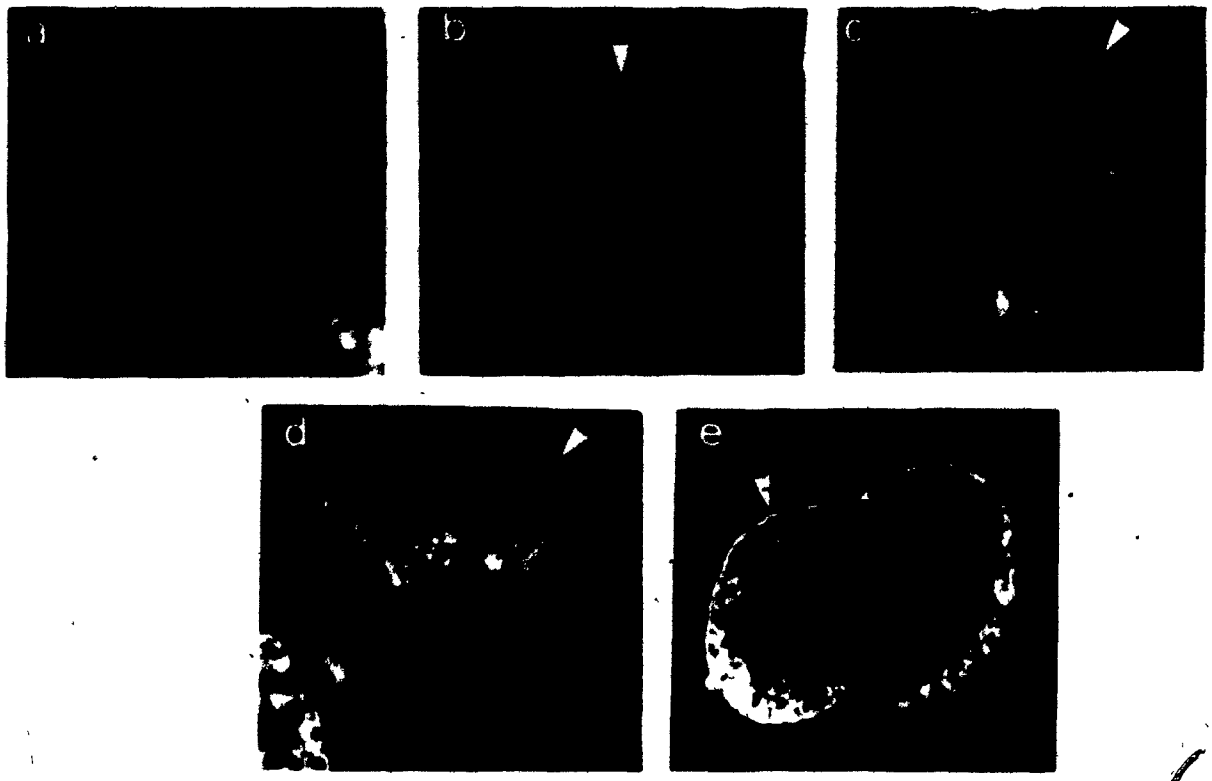

a
b
c
d
e

Figure 12. Indirect immunofluorescence of the Ilyanassa fertilized egg stained with IVe10 followed by rhodamine conjugated secondary antibody (both diluted 1:50). Frame a is the light micrograph; frame b shows the fluorescence under blue excitation; and frame c shows the fluorescence under green excitation, the excitation wavelength of rhodamine. Notice the pronounced membrane fluorescence in frame c. X4000.

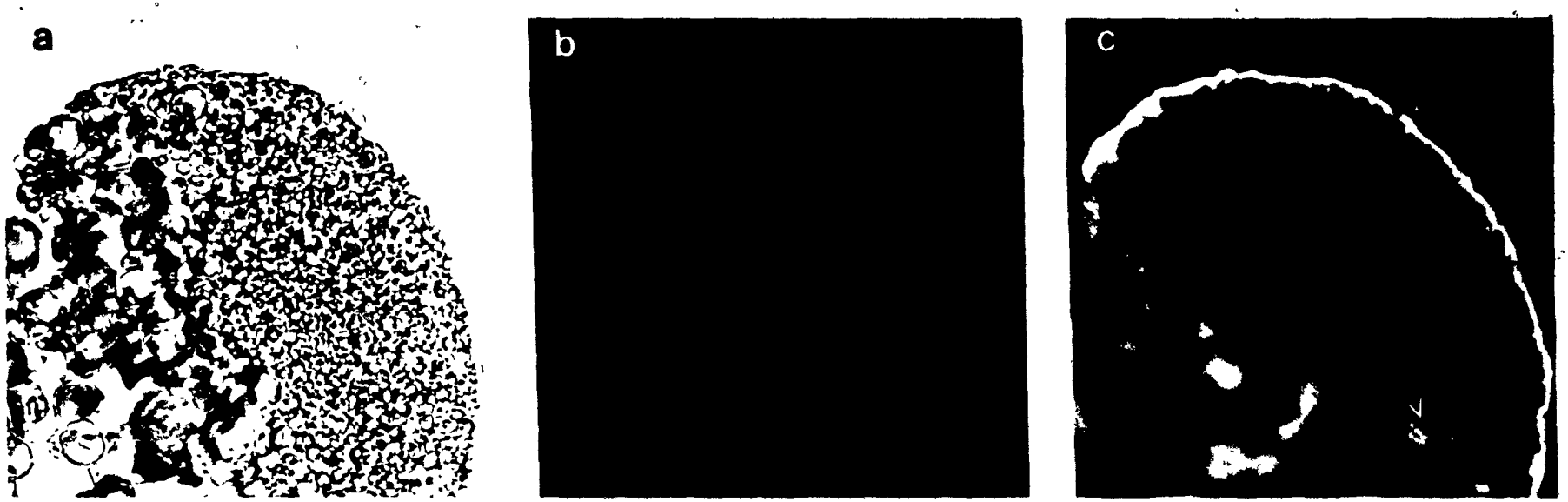

a
b
c

Figure 13. The comparison of fluorescence between the oocyte and egg of Ilyanassa using IVe10 monoclonal antibody and rhodamine conjugated secondary antibody. Frame a is the light micrograph; frame b shows the fluorescence under blue excitation; and frame c shows the fluorescence under green excitation. oo-oocyte; eg-egg; y-yolk; GV-germinal vesicle. X400.

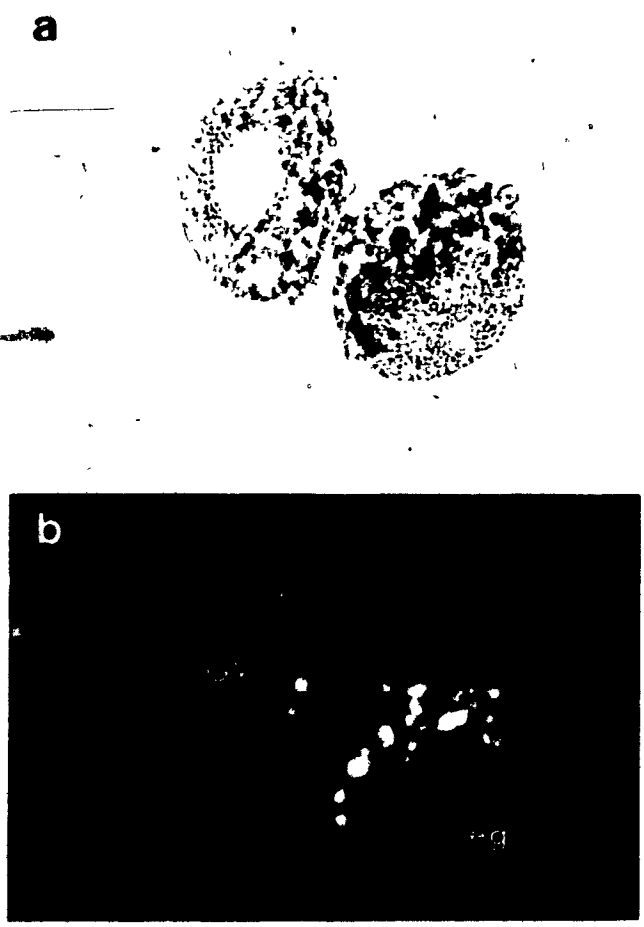
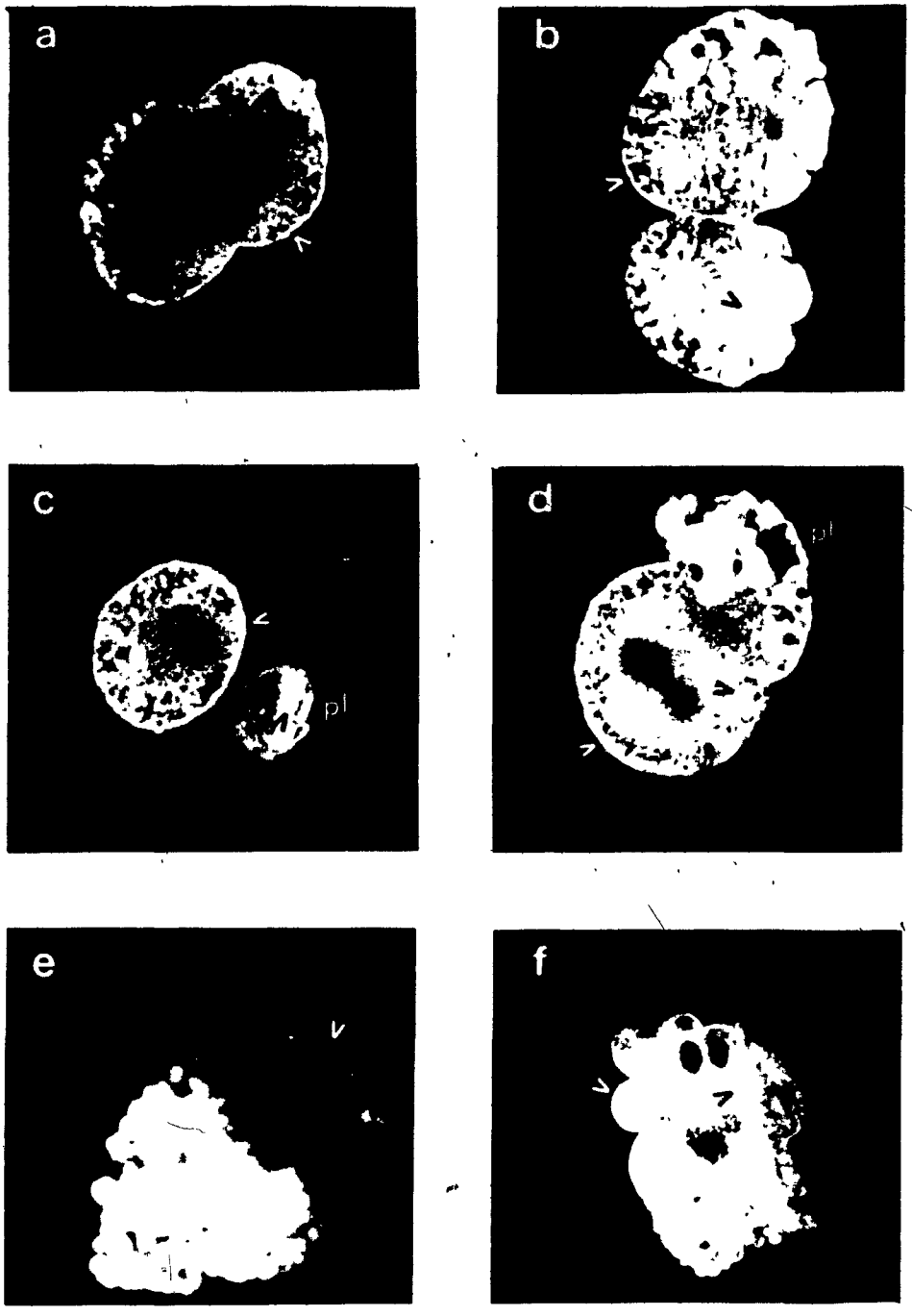

a
b
c

Figure 14. Fluorescent micrographs of early cleavage stage Ilyanassa embryos stained with IVe10 monoclonal antibody and rhodamine conjugated secondary antibody (both diluted 1:50). At the two cell stage (frames a and b) fluorescence occurs on both blastomeres and within their cytoplasm. Although not classic trefoil profiles, frames c and d reveal that at this stage no membrane fluorescence is detectable on the polar lobe (pl). Frames e and f are 16 and 32 cell embryos respectively. Arrows point to fluorescence due to bound IVe10 antibody. X500.


a
b
c
pl
d
pl
e
f

cell contact. At the trefoil stage cytoplasmic fluorescence is found in both blastomeres and the polar lobe. Membrane fluorescence is only seen on the the two blastomeres (figures 14c and 14d). Frames 14e and 14f show 16 and 32 cell stage embryos respectively and both exhibit cell surface fluorescence along with the cytoplasmic staining.

In 1, 2, and 3 day old embryos it is very difficult to differentiate individual cells. However at one day IVel0 antigens are at the embryo surface and within the embryo (figure 15). Two and 3 day embryos show similar staining. By 14 days of development the IVel0 antigens have become regionalized within the veliger larva (figure 15d). Gone is both the fluorescence which surrounds the embryonic surface and the cytoplasmic fluorescence. The same is true for the 20 day veliger larvae.

The Delobed Embyro.

The affects polar lobe removal has on the IVel0 antigen(s) distribution was examined. Immunoblot and indirect immunofluorescence on sectioned delobed embryos revealed that polar lobe removal had no dramatic affect on the IVel0 antigens. On immunoblots the band pattern is the same as that for normal embryos (figure 16). At early embryonic stages the staining of sections is identical to normal embryos. By the veliger stage (figure 17), as in normal embryos, the staining became internalized. A

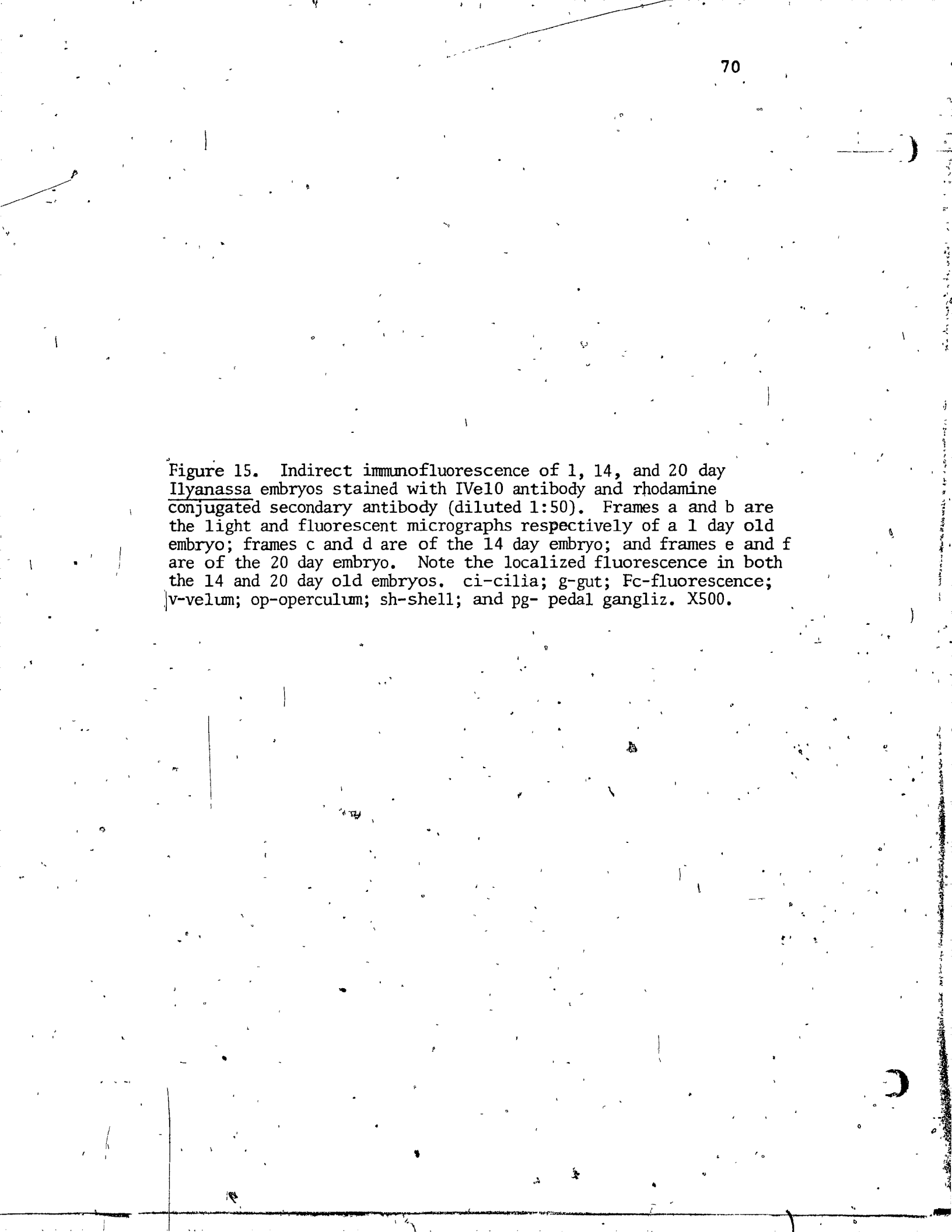


Figure 15. Indirect immunofluorescence of 1, 14, and 20 day Ilyanassa embryos stained with IVe10 antibody and rhodamine conjugated secondary antibody (diluted 1:50). Frames a and b are the light and fluorescent micrographs respectively of a 1 day old embryo; frames c and d are of the 14 day embryo; and frames e and f are of the 20 day embryo. Note the localized fluorescence in both the 14 and 20 day old embryos. ci-cilia; g-gut; Fc-fluorescence; v-velum; op-operculum; sh-shell; and pg- pedal gangliz. X500.

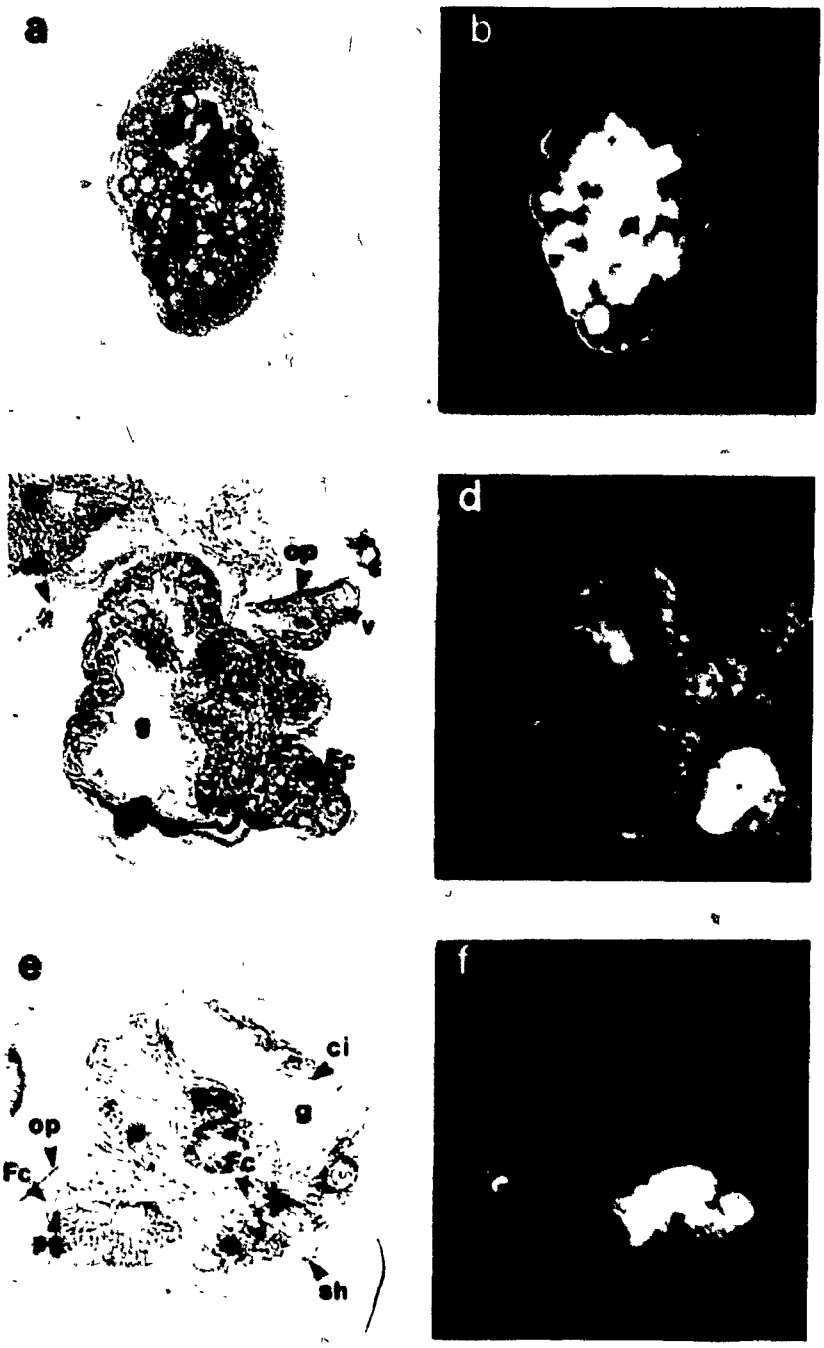

a
b
op
v
Fc
d
e
ci
g
op
Fc
sh
f

Figure 16. The IVel0 antigens are present in delobed Ilyanassa embryos. Embryos were delobed as outlined in materials and methods. A membrane preparation from 1 to 13 day delobed embryos was subjected to 10% SDS PAGE at 150V until the dye front ran off the gel, and stained with Coomassie Brilliant Blue (lane 1) or blotted onto nitrocellulose and labelled by indirect immunofluorescence (lane 2).

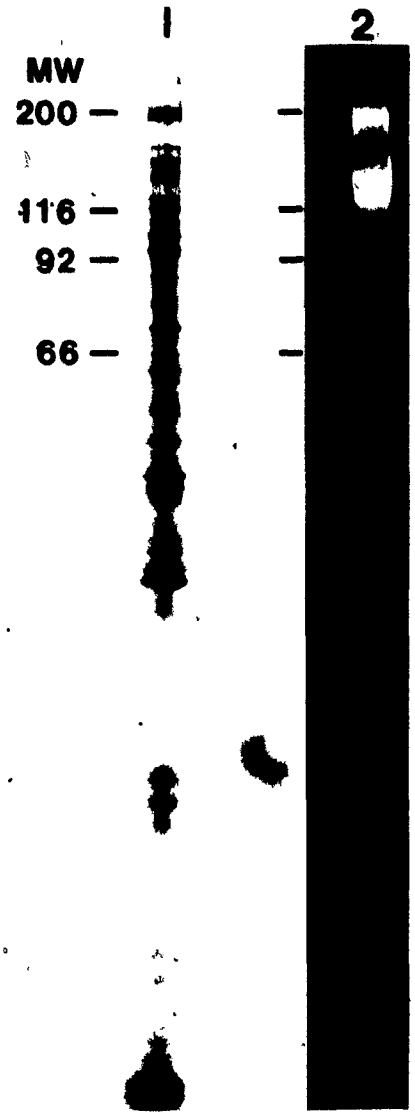
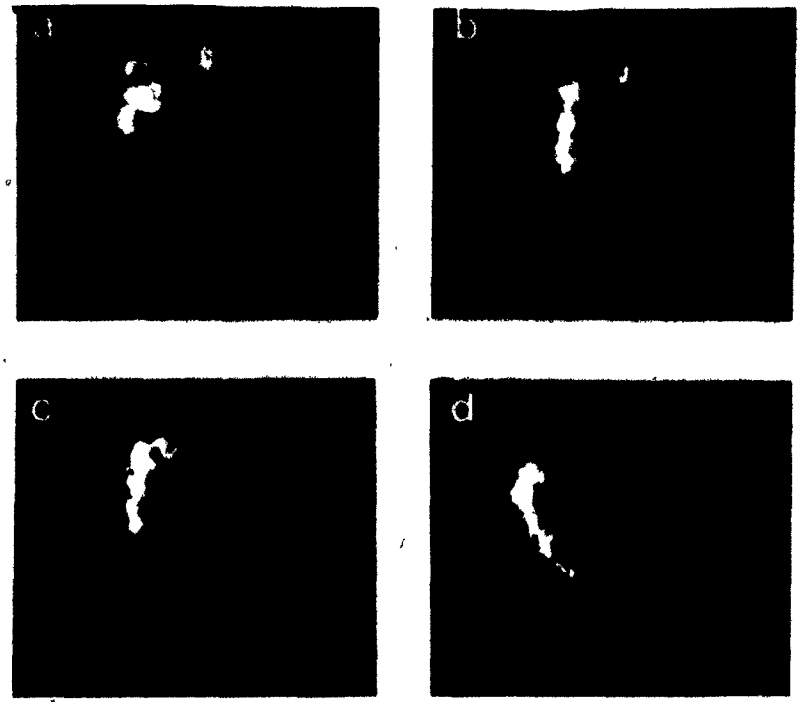

1
2
MW
200
116
92
66

Figure 17. Indirect immunofluorescence of a 13 day delobed Ilyanassa embryo stained with IVe10 monoclonal antibody and rhodamine conjugated secondary antibody (1:50 dilution). Frames a-d represent serial sections through the embryo. The delobed embryo at this stage is yolk-free. X400.


a
b
c
d

difference between the two types of embryos is that the staining in delobed embryos is more diffuse. The reason for this is not clear.

DISCUSSION

The monoclonal antibody IVel0 recognizes three prominent bands on immunoblots. These have been designated IEA-1, IEA-2, and IEA-3. The antigen(s) first become detectable by immunoblot during oocyte maturation and remain in the embryo throughout embryogenesis. Some antigen is detected in oocytes by immunocytochemistry at an earlier stage than can be detected by immunoblotting. Otherwise the immunobot and immunocytochemical data are in good agreement with one another. In the oocyte and the early embryo they are found on the cell surface and within the cytoplasm. It is not clear where the antigen(s) is localized in the cytoplasm ie. a particular structure or organelle. By the veliger stage the antigens are found within the larvae at discrete locations. Of the three predominant IVel0 antigens, one is enriched in oocytes (IEA-3) as determined by immunoblotting. This antigen is abundant in mature oocytes but not in immature ones. As development proceeds it is diluted out or degraded. Antigens EA-1 and EA-2 appear during artificial oocyte maturation and remain throughout Ilyanassa embryogenesis. They show no apparent developmental regulation.

This work represents the first attempt at examining the cell surface of the Ilyanassa embryo, although extensive work has been done on other systems (for review see Frazier and Glaser, 1979). Like many of the other

studies using antibodies to examine the cell surface this is a preliminary descriptive study. The function of the IVel0 antigens remains unknown. The description of the temporal and topographical expression of the antigen, however, is an important prerequisite to the investigation of a functional role.

It is not yet determined if the embryo actively synthesizes these antigens or if they are made during oogenesis and stored. Examination of antigen synthesis will reveal if it is stored or newly made. If it is stored it will be important to determine the information responsible for the creation of the antigenic site.

That IVel0 recognises several antigens is not unusual for a monoclonal antibody (Murray et al, 1982). The bands recognised by IVel0 must share the same antigenic site. The three major IVel0 antigens may also be related in other undetermined, possibly functional, ways. Furthur relationships among the antigens can only be determined upon their isolation and characterization. Enrichment of the antigens by either immunoprecipitation or immunoaffinity columns will allow for more precise characteriazations of the antigens.

There is little doubt that the IVel0 antigen(s) is a cell surface antigen. It is not possible to state which one, or, if all the IVel0 antigens are localized to either the cell membrane and/or the cytoplasm. Furthurmore whether one or more of the bands are reponsible for the cytoplasmic fluorescence seen in oocytes and early embryos. These

questions will be answered only when polyclonal or monoclonal antibodies to each of IEA-1, IEA-2, and IEA-3 are available such that the antigens can be examined seperately.

Another important question is whether or not there is a relationship between membrane and cytoplasmic fluorescence. Perhaps the antigen(s) in the cytoplasm, moves to the cell surface. In support of this possibility is what occurs in the maturing oocyte. At 0-4 hours of artificial maturation only IEA-1 is detectable and the oocyte exhibits mainly the cytoplasmic fluorescence. In older oocytes however IEA-2 and IEA-3 are also present and there is an increase in the number of oocytes which exhibit membrane fluorescence. Perhaps this represents a sequence in which the antigenic site first appears in the cytoplasm and then moves to the surface.

By the veliger stage the IVel0 antigens are localized within the larvae. A precise identification of the structures stained by the IVel0 antibody is required. This could be done by combining immunological staining with histological. Immunoelectron microscopy may also facilitate more precise identification. This technique can also be used to localize more clearly the cytoplasmic fluorescence of early embryos. The high degree of localization seen in veliger larva as opposed to the general cytoplasmic and cell surface staining of embryos is of particular interest.

If the polar lobe influences the composition of the cell surface in a developmentally meaningful way its removal will result in the removal of this information. This could result in the aberrant development observed in delobed

embryos. The IVelØ antigens are not polar lobe specific nor are they affected by polar lobe removal. Nonetheless this work has established the techniques necessary for future attempts at examining the Ilyanassa cell surface.

This work has identified a set of antigens, the IVelØ antigens, which are cell surface antigens of the Ilyanassa oocyte and early embryo and which become localized in veliger stage embryos. Not only has is provided the background for furthur such studies using Ilyanassa , but is has also raised a number of new and important questions with reguard to the identity, behaviour, and function of the IVelØ antigens.

BIBLIOGRAPHY

1) Alexander, S., Shinnick, T. M., and R. A. Lerner (1983). Mutants of Dictyostelium discoideum blocked in expression of all members of the developmentally regulated discoidin multigene family. Cell 34: 467-475.

2) Artzt,K., Dubois, P., Bennett, D., Condamine, H., and F. Jacob (1973). Surface antigens common to mouse embryos and primitive teratocarcinoma cells in culture. Proc. Natl. Acad. Sci. USA. 70: 2988-2992.

3) Artzt,K., Bennett, D., and F. Jacob (1974). Primitive teratocarcinoma cells express a differentation antigen specified by a gene at the T-locus in the mouse. Proc. Natl. Acad. Sci. USA. 71: 811-814.

4) Atkinson, J. W. (1971). Organogenesis in normal and lobeless embryos of the marine prosobranch gastropod Ilyanassa obsoleta . J. Morph. 133: 339-352.

5) Babinet, C., Condamine, H., Fellous, M., Gachelin, G., Kemler, R., and F. Gacob (1975). In Teratoma and Differentiation (M. I. Sherman and D. Solter, eds.). pp. 101-107. Acad. Press, New York.

6) Babinet, C., Kemler, R., Dubois, P., and F. Jacob (

1977). Les fragments monovalents d'immunoglobulin de lapin anti-F-9 empechent la formation de blastocystes chez la. C.R. Acad. Sci. Paris 284: 1919-1922.

7) Barondes, S. H., Cooper, D. N., and P. L. Haywood-Reid (1983). Discoidin I and Discoidin II are localized differently in developing Dictyostelium discoideum . J. Cell Biol. 96: 291-296.

8) Berger, E. A., and J. M. Clark (1983). Specific cell-cell contact serves as the developmental signal to deactivate Discoidin I gene expression in Dictyostelium discoideum . Proc. Natl. Acad. Sci. USA. 80: 4983-4987.

9) Beug, H., Katz, F. E., and G. Gerisch (1973a). Dynamics of antigenic membrane sites relating to cell aggregation in Dictyostelium discoideum . J. Cell Biol. 56: 647-658.

10) Beug, H., Katz, F. E., Stein, A., and G. Gerisch (1973b). Quantitation of membrane sites in aggregating Dictyostelium cells by use of tritiated monovalent antibodies. Proc. Natl. Acad. Sci. USA. 70: 3150-3154.

11) Blumberg, D. D., Margolskee, J. P., Chung, S., Barklis, E., Cohen, N. S., and H. F. Lodish (1982). Specific cell-cell contacts are essential for induction of gene expression during differentiation of Dictyostelium

discoideum . Proc. Natl. Acad. Sci. USA. 79: 127-131.

12) Bozzaro, S., and G. Gerisch (1978). Contact sites in aggregating cells of Polysphondylium pallidum . J. Mol. Biol. 120: 265-279.

13) Brandhorst, B. P., and K. M. Newrock (1981). Post translational regulation of protein synthesis in Ilyanassa embryos and isolated polar lobes. Dev. Biol. 83: 250-254.

14) Buc Caron, M. H., Gachelin, G., Hofnung, M., and F. Jacob (1974). Presence of a mouse embryonic antigen on human spermatozoa. Proc. Natl. Acad. Sci. USA. 71: 1730-1733.

15) Buc Caron, M. H., Condamine, H., and F. Jacob (1978). The presence of F9 antigen on the surface of mouse embryonic cells until day 8 of embryogenesis. J. Embryol. Exp. Morph. 47: 149-160.

16) Buc Caron, M. H., and M. H.Dupuouey (1980). On the nature of F9 antigenic determinants. Mol. Immunol. 17: 655-664.

17) Cather, J. N. (1967). Cellular interaction in the development of the shell gland of the gastropod Ilyanassa . J. Exp. Zool. 166: 205-223.

18) Cather, J. N. (1971). Cellular interaction and the regulation of development in annelids and molluscs. Adv. Morph. 9: 67-84.

19) Cather, J. N., and N. H. Verdonk (1979). Development of Dentalium following removal of D-quadrant blastomeres at successive cleavage stages. Wilhelm Roux Arch. Dev. Biol. 187: 355-366.

20) Chang, C. M., Reitherman, R. W., Rosen, D. D., and S. H. Barondes (1975). Cell surface location of discoidin, a developmentally regulated carbohydrate-binding protein from Dictyostelium discoideum . Exp. Cell Res. 95: 136-142.

21) Clegg, J. C. S. (1982) Glycoprotein detection in nitrocellulose transfers of electrophoretically seperated protein mixtures using Con A and peroxidase: Application to arenavirus and flavivirus proteins. Anal. Biochem. 127: 389-394.

22) Clement, A. C. (1952). Experimental studies on germinal localization in Ilyanassa I. The role of the polar lobe in determination of the cleavage pattern and its influence in later development. J. Exp. Zool. 121: 593-625.

23) Clement, A. C. (1956). Experimental studies on germinal localization in Ilyanassa II. The development of isolated blastomeres. J. Exp. Zool. 132: 427-445.

24) Clement, A. C. (1962). Development of Ilyanassa following removal of the D macromere at successive cleavage stages. J. Exp. Zool. 149: 193-215.

25) Clement, A. C. (1968). Development of the vegetal half of the Ilyanassa egg after removal of the yolk by centrifugal force compared with development of animal halves of similar visible comparison. Dev. Biol 17: 165-186.

26) Clement, A. C., and A. Tyler (1967). Protein synthesis activity of the anucleate polar lobe of the mud snail, Ilyanassa obsoleta . Science 158: 1457-1460.

27) Coffman, D. S., Leichtling, B. H., and H. V. Rickenberg (1981). Phosphoproteins in Dictyostelium discoideum . J. Supramol. Struct. Cell Biochem. 15: 369-385.

28) Collier, J. R. (1961). Nucleic acid and protein metabolism of the Ilyanassa embryo. Exp. Cell Res. 24: 320-328.

29) Collier, J. R. (1975). Nucleic acid synthesis in the normal and lobeless embryo of Ilyanassa obsoleta . Exp. Cell Res. 95: 254-263.

30) Collier, J. R. (1976). Nucleic acid chemistry of the Ilyanassa embryo. Amer. Zool. 16: 483-500.

31) Collier, J. R. (1977). Rates of RNA synthesis in normal and lobeless embryos of Ilyanassa obsoleta . Exp. Cell Res. 106: 390-394.

32) Collier, J. R. (1981). Protein synthesis in the polar lobe and lobeless egg of Ilyanassa obsoleta . Biol. Bull. 160: 366-375.

33) Collier, J. R. (1983a). The biochemistry of molluscan development . In The Biology of Molluscan Development (N. H. Verdonk and J. A. M. van den Biggelaar, eds.) Acad. Press; New York.

34) Collier, J. R. (1983b). Protein synthesis during Ilyanassa organogenesis. Dev. Biol. 100: 256-259.

35) Collier, J. R., and L. M. Garone (1975). The localization of ATP in the polar lobe of the Ilyanassa egg. Differ. 4: 195-196.

36) Collier, J. R., and M. E. McCarthy (1981). Regulation of polypeptide synthesis during early embryogenesis of Ilyanassa obsoleta . Differ. 19: 31-46.

37) Collier, J. R., and R. Schwartz (1969). Protein synthesis during Ilyanassa embryogenesis. Exp. Cell Res. 54: 403-407.

38) Crampton, H. E. (1896). Experimental studies on gastropod development. Arch Entwicklungsmech. Org. 3: 1-26.

39) Damonneville M., Morello, D., Gachelin, G., and M. Stanislawski (1979). Antibody response to embryonal carcinoman cells in syngeneic mice. Eur. J. Immunol. 9: 932-937.

40) Dohmen, M. R. (1983). The polar lobe in eggs of molluscs and annelids: Structure, composition, and function. In Time, Space, and Pattern in Embryonic Development (W. R. Jeffery and R.A. Raff, eds.) pp. 197-220, Liss, New York.

41) Eckhardt, A. E., Hayes, C. E., and I. J. Goldstein (1976). A sensitive fluorescent method for the detection of glycoproteins in polyacrylamide gels. Anal. Biochem. 73: 192-197.

42) Edelman, G. M. (1983). Cell adhesion molecules. Science 219: 450-457.

43) Edelman, G. M. (1984). Cell adhesion and morphogenesis: The regulator hypothesis. Proc. Natl. Acad. Sci. USA. 81: 1460-1464.

44) Edidin, M., Patthey, H. L., McGuire, E. J., and W. D. Sheffield (1971). In Proceedings of First Conference and

Workshop on Embryonic and Fetal Antigens in Cancer (N. G. Anderson and J. H. Coggin, eds.) pp. 239-248. Oak Ridge Natl. Lab., Oak Ridge Tenn.

45) Edidin, M., Ostrand-Rosenberg, S., and P. F. Bartlett (1978). In Cell Differentiation and Neoplasia (G. F. Sanders, ed.) pp. 67-79. New York.

46) Engvall, B. E., and P. Perlamann (1972). Enzyne-linked immunoabsorbant assay III. Quantitation of specific antibodies by enzyme labelled anti-immunoglobulin in antigen coated tubes. J. Immunol. 109: 129-135.

47) Erdos, G. W., and D. Whitaker (1983). Failure to detect immunocytochemically reactive endogenous lectin on the surface of Dictyostelium discoideum . J. Cell Biol. 97: 993-1000.

48) Finney, R. E., Mitchell, L. H., Soll, D. R., Murray, B. A., and W. F. Loomis (1983). Loss and resynthesis of a developmentally regulated membrane antigen (gp80) during dedifferentiation and redifferentiation in Dictyostelium . Dev. Biol. 98: 502-509.

49) Fox, N., Damjanov, I., Martinez-Hernandez, H., Knowles, B. B., and D. Solter (1981). Immunochemical localization of early embryonic antigen (SSEA-1) in postimplantation mouse embryos and fetal and adult tissues.

Dev. Biol. 83: 391-398.

50) Frazier, W. A., and L. Glaser (1979). Surface Components and Cell Recognition. Ann. Rev. Biochem. 48: 491-523.

51) Frazier, W. A., Rosen, S. D., Reitherman, R. W., and S. H. Barondes (1975). Purification and comparison of two developmentally regulated lectins from Dictyostelium discoideum . J. Biol. Chem. 250: 7714-7721.

52) Gachelin, G., Fellous, M., Guenet, J. L., and F. Jacob (1976). Developmental expression of an early embryonic antigen common to mouse spermatazoa and cleavage embryos and to human spermatazoa. Its expression during spermatogenesis. Dev. Biol. 50: 310-320.

53) Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W., and S. C. Howard (1977). Antibodies to major histocompatability antigens produced by hybrid cell lines. Nature (London) 266: 550-552.

54) Geltosky, J. E., Siu, C. H., and R. A. Lerner (1976). Glycoproteins of the plasma membrnae of Dictyostelium discoideum during development. Cell 8: 391-398.

55) Geltosky, J. E., Weseman, J., Bakke, A., and R. A. Lerner (1979). Identification of a cell surface

glycoprotein involved in cell aggregation in Dictyostelium discoideum . Cell 18: 981-988.

56) Gerisch, G. (1961). Cell functions and change in cell function in the development of Dictyostelium discoideum : V. Stage-specific cell contact formation and its quantitative evaluation. Exp. Cell Res. 25: 535-554.

57) Gerisch, G. (1980). Univalent antibody fragments as tools for the analysis of cell interaction in Dictyostelium . Curr. Top. Dev. Biol. 14: 157-270.

58) Gooding, L. R., and M. Edidin (1974). Cell surface antigens of a mouse testicular teratoma: Identification of an antigen physically associated with H2 antigen on tumor cells. J. Exp. Med. 140: 61-78.

59) Gooi, H. C., Kapadia, A., Feizi, T., Knowles, B. B., Solter, D., and M. J. Evans (1981). Stage-specific embryonic antigen involves 1-3 fucosylated type 2 blood group chains. Nature (London) 292: 156-158.

60) Gottlieb, D. I., Merrell, R., and L. Glaser (1974). Temporal changes in embryonal cell surface recognition. Proc. Natl. Acad. Sci. USA. 71: 1800-1802.

61) Graham, C. F. (1977). In Concepts in Mammalian Embryogenesis (M. I. Sherman, ed) pp. 315-394. MIT Press,

Cambridge, Mass.

62) Gregg, J. H., and C. W. Trygstad (1958). Surface antigen defects contributing to developmental failure in aggregating variants of the slime mold Dictyostelium discoideum . Exp. Cell Res. 15: 358-369.

63) Guidice, G. (1962). Restitution of whole larvae from disaggregated cells of sea urchin embryos. Dev. Biol. 5: 402-411.

64) Gustafson, T., and L. Wolpert (1967) Cellular movement and contact in sea urchin morphogenesis. Biol. Rev. 442: 442-498.

65) Hamburger, A., Shevinsky, L., Knowles., B. B., Damjanov, I., and D. Solter (1982). Monoclonal antibody to murine embryos defines a stage-specific embryonic antigen expressed on mouse embryos and human teratocarcinoma cells. Cell 30: 697-705.

66) Harrison, F. L., and C. J. Chesterton (1980). Factors mediating cell-cell recognition and adhesion: Galaptins, a recently discovered class of bridging molecules. Febs Lett. 122 #2: 157-165.

67) Holtfreter, J. (1944). Experimental studies of the development of pronephros. Rev. Canad. de Biol. 3: 220-250.

68) Iwakura, Y., McCormick, P., Arzt, K., and D. Bennett (1983). A class of large polysaccharides contain the antigenic determinants for the cytotoxic antibodies in conventional syngeneic anti-F9 as well as monoclonal antibody prepared against F9 cells. Cell Differ. 13: 41-48.

69) Jacob, F. (1975). In The Early Development of Mammals . pp. 233-241. Cambridge University Press, London and New York.

70) Jacob, F. (1977). Mouse teratocarcinoma and embryonic antigens. Immunol. Rev. 33: 3-32.

71) Jacob, F. (1978). Mouse teratocarcinoma and mouse embryo. Proc. R. Soc. London (Biol) 201: 249-270.

72) Jacob, F. (1979). Cell surface antigens and early stages of mouse embryogenesis. Curr. Top. Dev. Biol. 13: 117-137.

73) Jaffe, A. R., and D. R. Garod (1979). Effect of isolated plasma membranes on cell cohesion in the cellular slime mold. J. Cell Science 40: 245-246.

74) Kemler, R., Babinet, C., Eisen, H., and F. Jacob (1977). Surface antigens in early differentiation. Proc. Natl. Acad. Sci. USA. 74: 4449-4452.

75) Knowles, B. B., Rappaport, J., and D. Solter (1982). Murine embryonic antigen (SSEA-1) is expressed on human cells and structurally related human blood group antigen I is expressed on mouse embryos. Dev. Biol. 93: 54-58.

76) Koser, R. B., and J. R. Collier (1976). An elecrophoretic analysis of RNA synthesis in normal and lobeless Ilyanssa embryos. Differ. 6: 47-52.

77) Laemlli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 226: 680-685.

78) Littlefield, J. W. (1964). Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. Science 145: 709-710.

79) Loomis, W. F., Murray, B. A., Yee, L., and T. Jongens (1983). Adhesion blocking antibodies prepared against gp150 reacte with gp80 of Dictyostelium . Exp. Cell Res. 147: 231-234.

80) Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and R. J. Randall (1951). Protein measurement with the Folin reagent. J. Biol. Chem. 193: 265-275.

81) Mangiarotti, G., Lefebvre, P., and H. F. Lodish (1982

). Differences in stability of developmentally regulated mRNAs in aggregated and disaggregated Dictyostelium cells. Dev. Biol. 89: 82-91.

82) Mangiarotti, G., Bozzaro, S., Landfear, S., and H. F. Lodish (1983). Cell-cell contact, cyclic AMP, and gene expresseion during development of Dictyostelium discoideum . Curr. Top. Dev. Biol. 18: 117-154.

83) Marchase, R. B. (1977). Biochemical investigations of retinotectal adhesive specificity. J. Cell Biol. 75: 237-257.

84) Marticorena, P., Hogan, B., DiMeo, A., Arzt, K., and D. Bennett (1983). Carbohydrate changes in pre- and peri-implantation mouse embryos as detected by a monoclonal antibody. Cell Diff. 12: 1-10.

85) McClay, D. R. (1971). An autoradiographic analysis of the species specificity during sponge cell reaggregation. Biol. Bull. 141: 319-330.

86) McClay, D. R., and R. E. Hausman (1975). Specificity of cell adhesion: Differences between normal and hybrid sea urchin cells. Dev. Biol. 47: 454-460.

87) McClay, D. R., Chambers, A. F., and R. H. Warren (1977). Specificity of cell-cell interactions in sea urchin

embryos. Dev. Biol. 56: 343-355.

88) McClay, D. R., and A. F. Chambers (1978). Identification of four classes of cell surface antigens appearing at gastrulation in sea urchin embryos. Dev. Biol. 63: 179-186.

89) McClay, D. R., and R. B. Marchase (1979). Seperation of ectoderm and endoderm from sea urchin pluteus larvae and demonstration of germ layer specific antigens. Dev. Biol. 71: 289-296.

90) McClay, D. R., and R. D. Fink (1982). Sea urchin hyalin: Appearance and function in development. Dev. Biol. 92: 285-293.

91) McClay, D. R., Cannon, G. W., Wessel, G. M., Fink, R. D., and R. B. Marchase (1983). Patterns of antigenic expression in early sea urchin development. In Time, Space, and Patterns of Embryonic Development (W. R. Jeffery and R. A. Raff, eds.) pp. 157-169. Liss, New York.

92) McCormick, P. J., Dimeo, A., Neuner, E., and K. Arzt (1982). Characterization of the F9 antigen(s) isolated form teratocarcinoma cell culture medium. Cell Differ. 11: 135-140.

93) McKay,S., and K. M. Newrock (1982). Histone subtypes

and switches in synthesis of histone subtypes during Ilyanassa development. Dev. Biol. 93: 430-437.

94) Mintz, B., and U. Ilmensee (1975). Normal genetically mosaic mice produced from malignant teratocarcinoma cells. Proc. Natl. Acad. Sci. USA. 72: 3585-3589.

95) Mirkes, P. E. (1972). Polysomes and protein synthesis during development of Ilyanassa obsoleta . Exp. Cell Res. 74: 503-507.

96) Monsigny, M., Kieda, C., and A. C. Roche (1979). Membrane lectins. Biol. Cell. 36: 289-300.

97) Morello, D., Condamine, H., DeLarbre, C., Babinet, C., and G. Gachelin (1980). Serological identification and cellular distribution of three F9 antigen components. J. Exp. Med. 152: 1497-1502.

98) Moscona, A. A. and M. H. Moscona (1967). Comparison of aggregation of embryonic cells dissociated with trypsin and versene. Exp. Cell Res. 45: 239-243.

99) Muller, K., and G. Gerisch (1978). A specific glycoprotein as the target site of adhesion blocking Fab in aggregating Dictyostelium cells. Nature (London) 274: 445-449.

100) Muller, K., Gerisch, G., Fromme, I., Mayer, H., and A. Tsugita (1979). A membrane glycoprotein of aggregating Dictyostelium cells with the properties of contact sites. Eur. J. Biochem. 99: 419-426.

101) Muramatsu, T., Gachelin, G., Damonneville, M., Delarbre, C., and F. Jacob (1979). Cell surface carbohydrate of EC cells: Polysaccharide side chains of F9 antigens of receptors of two lectins, FBP and PNA. Cell 18: 183-191.

102) Murray, B. A., Niman, H. L., and W. F. Loomis (1983). Monoclonal antibody recognizing gp80, a membrane glycoprotein implicated in intercellular adhesion in Dictyostelium discoideum . Mol. Cell. Biol. 3: 863-870.

103) Murray, B. A., Wheeler, S., Jongens, T., and W. F. Loomis (1984). Mutations affecting a surface glycoprotein, gp80, of Dictyostelium discoideum . Mol. Cell. Biol. 4: 514-519.

104) Newrock, K. M., and R. A. Raff (1975). Polar lobe specific regulation of translation in embryos of Ilyanassa obsoleta . Dev. Biol. 42: 242-261.

105) Noll, H., Matranga, V., Cascino Domenico R., and L. Vittorelli (1980). Reconstruction of membranes and

embryonic development in dissociaated blastula cells of the sea urchin by reinsertion of aggregating promoting membrane proteins extracted with n-butanol. Proc. Natl. Acad. Sci. USA 76: 288-292.

106) Noll, H., Matranga, V., Palma, P., Cutrono, F., and L. Vittorelli (1981). Species specific dissociation into single cells of live sea urchin embryos by Fab fragments against membrane components of Paracentrotus lividus and Arbacia lixula. Dev. Biol. 87: 229-241.

107) Ochai, H., Schwartz, H., Merkl, R., Wagle, C., and G. Gerisch (1972). Stage specific antigens reacting with monoclonal antibodies against contact site A, a cell surface glycoprotein of Dictyostelium discoideum . Cell. Diff. 11: 1-13.

108) Parish, S. W., Schmidlin, S., and U. Muller (1977). The effects of proteases on proteins and glycoproteins of Dictyostelium discoideum plasma membranes. Exp. Cell Res. 110: 267-276.

109) Raff, R. A., Newrock, K. M., and R. D. Secrist (1975). Regulation of microtubule protein synthesis in embryos of the mud snail Ilyanassa obsoleta . Dev. Biol. 44: 369-376.

110) Rosen, S. D., Simpson, D. L., Rose, J. E., and S. H. Barondes (1974). Carbohydrate binding protein from

Polysphondylium pallidum implicated in intracellular adhesion. Nature (London) 252: 128-130.

111) Rosen, S. D., Kaur, J., Clark, D. L., Pardos, B. T., and W. A. Frazier (1979). Purification and characterization of multiple species (isolectins) of a slime mold lectin implicated in intracellular adhesion. J Biol. Chem. 254: 9408-9411.

112) Rothbard, J. B., Brackenbury, R., Cunningham, B. A., and G. M. Edelman (1982). Differences in the carbohydrate structures of neural cell adhesion molecules from adult and embryonic chicken brains. J. Biol. Chem. 257: 11064-11069.

113) Rutishauser, U., Thiery, J-P., Brackenbury, R., Sela, B. A., and G. M. Edelman (1976). Mechanisms of adhesion among cells from neural tissues of the chick embryo. Proc. Natl. Acad. Sci. USA. 73: 577-581.

114) Shevinsky, L. H., Knowles, B. B., Howe, C. C., Aden, D. P., and D. Solter (1981). A marine stage-specific embryonic antigen (SSEA-2) is expressed on some murine SV-40 transformed cells. J. Immunol. 127: 632-636.

115) Simpson, D. L., Rosen, S. D., and S. H. Barondes (1974). Discoidin, a developmentally regulated carbohydrate binding protein from Dictyostelium discoideum . Purification and characterization. Biochem. 13: 3487-3483.

116) Siu, C. H., Lerner, R. A., Ma, G., Firtel, R. A., and W. I. Loomis (1976). Developmentally regulated proteins of the plasma membrane of Dictyostelium discoideum . The carbohydrate binding protein. J. Mol. Biol. 100: 157-178.

117) Solter, D., and B. B. Knowles (1978). Monoclonal antibody defining a stage specific mouse antigen (SSEA-1). Proc. Natl. Acad. Sci. USA. 75: 5565-5569.

118) Spiegel, M. and E. Spiegel (1975). The reaggregation of dissociated embryonic sea urchin cells. Amer. Zool. 15: 583-606.

119) Springer, W. R., and S. H. Barondes (1978). Direct measurement of species specific cohesion in cellular slime molds. J. Cell. Biol. 78: 937-942.

120) Stadler, J., Bordier, C., Lottspeich, F., Henschen, A., and G. Gerisch (1982). Improved purification and N-terminal amino acid sequence determination of the contact site A glycoprotein of Dictyostelium discoideum . Physiol. Chem. 363: 771-776.

121) Steinemann, C., and R. W. Parish (1980). Evidence that a developmentally regulated glycoprotein is the target of adhesion blocking Fab in reaggregating Dictyostelium . Nature (London) 286: 621-623.

122) Stern, P. L., Martin, G. R., and M. J. Evan (1975). Cell surface antigens of clonal teratocarcinoma cells at various stages of differentiation. Cell 6: 455-465.

123) Takeichi, M., Atsumi, T., Yoshida, C., and S. Ogou (1982). Molecular approaches to cell-cell recogniton mechanisms in mammalian embryos. In Teratocarcinoma and Embryonic Interactions (T. Muramatsu et al., eds) pp. 283-293. Japan Sci. Soc. Press, Tokyo.

124) Tietelman, G. (1973). Protein synthesis during Ilyanassa development: Effect of the polar lobe. J. Embryol. Exp. Morph. 29: 267-274.

125) Thiery, J-P., Brackenbury, R., Rutishauser, U., and G. M. Edelman (1977). Adhesion among neural cells of the chick embryo II Purification and characterization of a cell adhesion molecule from neural retina. J. Biol. Chem. 252: 6841-6845.

126) Towbin, H., Staechlin, T., and J. Gordon (1979). Electrophoretic transfer of protein form polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA. 76: 4350-4354.

127.) Townes, P. L., and J. Holtfreter (1955). Directed movements and selective adhesion of embryonic amphibian

cells. J. Exp. Zool. 128: 53-120.

128) Vitteta, E. S., Arzt, K., Bennett, D., Boyse, E. A., and F. Jacob (1975). Structural similarities between a product of the T/t locus isolated from sperm and teratoma cells and H-2 isolated from spleenocytes. Proc. Natl. Acad. Sci. USA. 72: 3215-3219.

129) Von Dam, W. I., Dohmen, M. R., and N. H. Verdonk (1982). Localization of mophogenetic determinants in a special cytoplasm present in the polar lobe of Bithynia tentacalata (Gatropoda). W. Roux Arch. Dev. Biol. 191: 371-377.

130) Wessel, G. M., Marchase, R. B., and D. R. McClay (1984). Ontogeny of the basal lamina of the sea urchin embryo. Dev. Biol. 103: 235-245.

131) Wilson, G. (1907). On some phenomena of coalescence and regeneration in sponges with four figures. J. Exp. Zool. 5:245-258.