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EMBRYONIC CELL SURFACE ANTIGENS

DETECTED BY AN ANTISERUM AGAINST MOUSE BLASTOCYSTS

by

Lincoln V. Johnson

B.A., University of California Riverside 1971
M.A., University of California San Francisco 1978

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ABSTRACT

Cell surface proteins expressed during murine preimplantation development have been analyzed by lactoperoxidase labeling and gel electrophoresis. Major qualitative changes in surface protein composition occur following fertilization and coincident with blastocyst formation. Stage-specific cell surface antigens expressed during preimplantation development and detected by a rabbit antiserum prepared against mouse blastocysts (A-BL) have been characterized by serological and biochemical techniques. Immunofluorescence, immunoradiolabeling and complement-mediated cytoxicity assays reveal the expression of A-BL surface antigens beginning at the 4-cell stage and reaching a maximum at the 8-cell to morula stage. Earlier stages do not express detectable A-BL antigens and there is an apparent decline in expression at the blastocyst stage. Treatment of embryos with β-N-acetylglucosaminidase inhibits the binding of A-BL antibodies. No antibody reactivity is detected against adult tissues or teratocarcinoma cell lines. The presence of A-BL antibodies during *in vitro* embryo culture interferes with normal development. Immunoprecipitation and electrophoretic analyses of A-BL antigens reveals specific antibody activity against two proteins with molecular weights of 69,000 and 71,000 daltons (gpe 69/71) which may show charge heterogeneity. This report is the first molecular characterization of stage-specific embryonic antigens synthesized during murine preimplantation development.

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INTRODUCTION

It is now well established that the cell surface plays an important role during development and differentiation. Components of the cell surface are involved in intercellular recognition, adhesion and communication, cell motility, cell division and the functional integration of cells into tissues (see Moscona, 1974; Poste and Nicolson, 1976). Reactions at the cell surface involving extracellular factors or adjacent cells can trigger changes in intracellular metabolism and may affect gene expression. Such changes, in turn, may affect the properties of the cell surface by stabilizing existing surface characteristics or by altering the composition and/or organization of surface components. Sequences of reciprocal interactions between intracellular and extracellular factors mediated at the cell surface may function to direct embryonic cells along specific pathways of differentiation and development.

During mammalian development, evidence suggests that cell membrane components may be of developmental significance as early as the preimplantation period. While there is as yet no direct evidence for the participation of specific membrane components during early mammalian embryogenesis, alterations in cell surface characteristics do occur during preimplantation development as has been indicated by temporal variations in (1) surface morphology, (2) surface biochemistry, (3) surface charge, (4) lectin interaction, (5) antigenic reactivity with specific antisera, (6) alloantigen expression and by (7) stage-specific, embryo-lethal mutations which have been proposed to involve cell surface abnormalities. It is the purpose of the research described here to

further such observations by characterizing specific molecular species which show stage-related changes in their surface expression and which may be functionally significant during development.

The presently available evidence for developmentally associated cell surface alterations during mammalian preimplantation development and the possible functional significance of these surface characteristics are discussed below (see also reviews by Edidin, 1976a,b; Jenkinson and Billington, 1977; Solter, 1977; Wiley, 1979). The involvement of cell surface componenents in the fertilization process (Gwatkin, 1976) and implantation-related changes in the blastocyst surface (Enders, 1972; Sherman and Wudl, 1976) have been considered elsewhere and are not dealt with in detail.

1. Surface Morphology

Scanning electron microscopic investigations (Calarco and Epstein, 1973; Calarco, 1975) have revealed that the preimplantation embryo has a microvillus exterior surface at all stages. However, several morphological changes in the cell surface have been noted during this period. Prior to fertilization there is a non-microvillus (i.e. smooth) region of membrane which overlies the second metaphase spindle in the mouse (Eager et al., 1976; Nicosia et al., 1977; also see Lectin Interaction below). Another smooth membranous region, overlying the fertilizing sperm nucleus, reportedly enlarges to form the incorporation cone in the rat (Phillips et al., 1978). During cleavage of mouse embryos, increasing interdigitation of microvilli, smooth membrane where blastomeres are apposed, and thin cellular processes which spread over adjacent cells have been observed. Such morphologically identifiable surface alterations may be involved in intercellular adherence, especially during compaction of the 8-cell embryo. In blastocysts, cells of the inner cell mass and the surfaces of trophectoderm cells facing the blastocoel are composed exclusively of smooth membrane (Calarco and Epstein, 1973; Calarco, 1975). Investigations of the surface of the rat embryo during the preimplantation period have also revealed increasing surface area and increasing complexity of cellular projections (Burgos et al., 1976).

Ducibella et al. (1977) have reported that during compaction of the 8-cell embryo, when intercellular adhesion is increasing, a reorganization of the blastomere surface is observed in which microvilli become localized apically and in a basal zone of intercellular contact.

These surface changes and coordinated changes in cortical distribution of microtubules and mitochondria are suggested to be the first indications of the development of cellular polarity and to indicate that cell membrane changes associated with trophoblast differentiation appear as early as the 8-cell stage (Ducibella, et al., 1977).

The establishment of intercellular junctions during development, and differences in the types of junctions formed within and between the inner cell mass (ICM) and the trophectoderm of mouse blastocysts have also been described (Enders and Schlafke, 1965; Calarco and Brown, 1969; Enders, 1971; Nadijcka and Hillman, 1974; Ducibella and Anderson, 1975; Ducibella et al., 1975; Magnuson et al., 1977). Gap junctions and focal tight junctions are present between blastomeres beginning at the 8-cell stage. The formation of zonular tight junctions begins between presumptive trophectodermal cells of 16 to 32-cell morulae and are extensive between trophectodermal cells of blastocysts. These tight junctions are likely to be involved in the establishment of a permeability barrier required for blastocoel formation. The point in development at which this barrier is complete has been reported to be as early as the morula stage (Magnuson et al., 1978) or as late as the mid to late blastocyst stage (McLaren and Smith, 1977). Desmosomes also appear at the blastocyst stage and are located proximal to the tight junctions.

Morphological studies thus demonstrate several changes occurring at the cell surface during preimplantation development. The smooth membrane overlying the mataphase II spindle may function to prevent

sperm attachment in the area of polar body extrusion. The absence of microvilli on internal cells (ICM) and internal cell surfaces (trophectoderm) may be a response to differences in internal and external environments and/or a reflection of cellular differentiation. The apical localization of microvilli allowing apposition of smooth membrane, and the thin cellular process of one cell stretched across adjacent cells would appear to be involved in increasing intercellular adhesion during the compaction process. On a morphological basis it therefore appears that the embryonic cell surface is involved in a number of developmental events, however, the molecular basis for the observed morphological alterations remain to be explored.

2. Surface Biochemistry

The investigations of Pinsker and Mintz (1973) have shown that trypsin digests of metabolically radiolabeled (glucosamine) surface glycoproteins from embryos at or near the blastocyst stage are enriched in forms of higher molecular weight when compared to digests from 4- to 8-cell embryos. Since these changes in surface glycoproteins are similar to those demonstrated by cultured cells following viral transformation, these investigators have suggested that the early biochemical differentiation of the embryonic cell surface may be related to the stage-specific capacity of trophoblast to attach to the uterine wall and become invasive. However, the possibility that newly appearing, blastocyst-specific surface glycoproteins with increased molecular weight are directly involved in embryo-uterine attachment, implantation or invasiveness has yet to be pursued.

Ultrastructural cytochemistry has demonstrated changes, both

temporal and spatial, in the expression of a number of membrane-associated enzymes during mouse preimplantation development. Vorbodt et al. (1977) have examined the presence of alkaline phosphatase (present from the 4- to 8-cell stage and on outer membrane of trophectodermal cells), 5'-nucleotidase (present from the 4- to 8-cell stage, highest activity in trophectoderm and ICM), Mg^{++}-ATPase (present in unfertilized eggs and all preimplantation stages), transport (Na^{+}-K^{+})-ATPase (present only on apposed membranes of morulae and blastocysts), cyclic AMP-phosphodiesterase (present from 2-cell stage, highest activity on outer membrane of trophectoderm cells), and adenylate cyclase (present as early as the zygote, only enzyme detected on cytoplasmic side of membrane). A report by Mulnard and Huygens (1978) on the expression of non-specific alkaline phosphatase differs from that above in that activity is detected primarily in the ICM and on the inner surfaces of trophectodermal cells. Developmental changes during the preimplantation period have also been described for membrane transport systems involved in the uptake of metabolites, nucleic acid precursors, and amino acids (reviewed by Epstein, 1975; Biggers and Borland, 1976).

Biochemical studies therefore suggest that there are both spatial and temporal patterns of expression for specific membrane-associated enzymes and transport systems during early mammalian development. In general, more membrane systems become active as development progresses and this is accompanied by an increase in the molecular weight of trypsin-sensitive surface proteins. Specific developmental functions for such membrane-associated enzyme activities and surface proteins remain to be elucidated.

3. Surface Charge

A number of histochemical studies have examined the presence of charged surface groups on preimplantation mammalian embryos. Cooper and Bedford (1971) have shown that an increase in the binding of positively charged iron colloid to the rabbit egg surface occurs following fertilization indicating an increase in negatively charged residues. Analysis following neuraminidase treatment and KOH saponification suggests that these negative surface charges are due to carboxyl radicals of sialic acid. However, both the zona pellucida and the plasma membrane of the hamster egg possess negatively charged residues but show no apparent change in the density or distribution of bound colloidal iron hydroxide following fertilization (Yanagimachi et al., 1973). This species difference in post-fertilization surface charge alteration parallels that described for the binding of the lectin concanavalin A (Gordon et al., 1975; Yanagimachi and Nicolson, 1976; see below) and may be related to differences in the site of the block to polyspermy: the zona pellucida of the hamster egg (Yanagimachi and Nicolson, 1976) the plasma membrane of the rabbit egg (Austin, 1961).

At the blastocyst stage in the mouse, electron histochemical studies also using colloidal iron to label negatively charged surface groups have revealed a decrease in surface negative charge with activation and implantation (Nilsson et al., 1973, 1975; Jenkinson and Searle, 1977). Protein-bound sialic acid residues may be responsible for the negative surface charge since colloidal iron binding can be reduced by treatment with neuraminidase, pronase or trypsin (Holmes and Dickson, 1973; Jenkinson and Searle, 1977). However, other investigators have

reported the contradictory observation that colloidal iron, identified by Prussian blue staining of whole-mounts, binds to the blastocyst surface after, but not before, the activating estrogen surge (Holmes and Dickson, 1973). In addition, Enders and Schlafke (1974) have shown that ruthenium red and colloidal thorium dioxide, both of which interact with acidic carbohydrate moieties, bind to the blastocyst and uterine epithelium of the mouse on days 4 and 5 of normal pregnancy, and on day 7 of delayed implantation indicating an increase in negatively charged surface residues. Consistent with the histochemical demonstrations of negative blastocyst surface charge is the observation that the zone pellucida-free rat blastocyst behaves as a negatively charged body on electrophoresis (Clemetson et al., 1970, 1971).

Changes in histochemically identified surface charge in conjunction with fertilization and implantation indicate that there are alterations in the molecular composition of the cell membrane coincident with these events. In both cases, surface negative charge is apparently due in large part to the presence of sialic acid moieties linked to protein. An increase in surface negative charge at fertilization appears to be species dependent and may be involved in a block to polyspermy. The data concerning blastocyst surface charge is contradictory and inconclusive. However, a change in surface charge at the time of implantation might be associated with changes in adhesiveness, invasiveness and/or antigenicity that accompany implantation. Whether molecular alterations at the cell surface involve newly synthesized and inserted membrane components, the selective loss of specific components, or a molecular alteration (conformational and/or enzymatic) remains unclear.

4. Lectin Interaction

Lectins with varying carbohydrate specificities have been reported to bind to the zona pellucida of mammalian eggs and early embryos. The zona pellucida of the unfertilized egg binds a variety of lectins (Oikawa et al., 1974; Nicolson et al., 1975), and there appear to be species differences in the oligosaccharide lectin receptors of rat, mouse and hamster zonae (Oikawa et al., 1975). Treatment of zona-intact hamster (Oikawa et al., 1973, 1974) and mouse (Parkening and Chang, 1976) eggs with specific lectins blocks *in vitro* fertilization. Most lectins, however, do not block the attachment of sperm to the zona pellucida but rather inhibit sperm penetration. Oikawa and coworkers (1974) have suggested that this effect may be due to lectin-induced cross-linking of adjacent polysaccarides such that zona lysins of spermatozoa are incapable of depolymerization of the zona material. In hamster eggs, however, the binding of sperm to the zona surface can be inhibited by relatively high concentrations (250 µg/ml) of wheat germ agglutinin (WGA) suggesting that N-acetyl-D-glucosamine and/or N-acetyl neuraminic acid residues may be involved in sperm-zona interaction (Oikawa et al., 1974). However, the cortical reaction of hamster eggs can be induced by lectins (and a number of other surface-active agents as well) and this reaction, which is likely to be involved in the block to polyspermy, may be responsible for the observed lectin-mediated inhibition of fertilization (Gwatkin et al., 1976).

It appears, therefore, that carbohydrate moieties in the acellular zona material are of importance in the attachment and penetration of sperm. However, lectins probably lack the specificity required for the

identification of "sperm-receptor" molecules (i.e. more than a single molecular species may contain receptor sites for a certain lectin and lectins may have more than a single carbohydrate specificity) and the possibilities that lectin-induced inhibition of normal sperm-zona interaction may be due to strictly steric factors, non-specific secondary (e.g. conformational) effects, and/or the cortical reaction have not as yet been excluded.

Receptor sites for WGA, Ricinus communis agglutinin 1 (RCA_1) and concanavalin A (Con A) have also been detected on the plasma membranes of the unfertilized eggs of mice, hamsters and rats (Oikawa et al., 1975; Nicolson et al, 1975). During egg maturation and preimplantation development in hamster embryos maximal binding of fluorescent Con A, RCA_1 and WGA is seen when eggs reach full maturity (metaphase II) and binding decreases during the later stages of preimplantation development (Yanagimachi and Nicolson, 1976). Zona pellucida-free eggs are agglutinated by numerous lectins except those recognizing L-fucose-like residues. Changes in lectin-mediated agglutination and fluorescein-labeled lectin binding to hamster eggs immediately following fertilization are not dramatic, however, post-fertilization increases in Con A binding have been noted in the rabbit (Gordon et al., 1975). The lack of a change in lectin binding at the time of fertilization in the hamster may be related to the observation that in this species the block to polyspermy is not a function of the plasma membrane, but rather of the zona pellucida (Barros and Yanagimachi, 1971), the rabbit egg, however, is apparently protected from polyspermic fertilization by a modification of the plasma membrane (Austin, 1961).

The investigations of Johnson et al. (1975) have revealed membrane mosaicism in Con A receptor distribution in the unfertilized egg and zygote of the mouse. Unfertilized eggs are diffusely stained by fluorescein-labeled Con A except for an unstained area consistently associated with the underlying second metaphase spindle. Subsequent ultrastructural examination of fertilized and unfertilized mouse eggs indicates that the presence or absence of staining with fluorescent Con A correlates with the presence or absence of microvilli on the egg surface but, in contrast, the density of ferritin-labeled Con A binding to smooth-membrane surfaces is not significantly different from that on microvillous surfaces (Eager et al., 1976). Eager et al.(1976) have, therefore, proposed that the apparent density of Con A binding to the smooth-membrane area of unfertilized eggs is reduced due to the absence of surface "corrugations" (microvilli) and not due to quantitative differences in lectin binding. It has been suggested that the mosaicism of Con A staining may reflect membrane specialization which prevents sperm penetration at a site at which polar body extrusion might be impaired or extrusion of the fertilizing sperm head might occur (Johnson et al., 1975). In fact, the smooth-membrane area associated with the meiotic spindle in unfertilized eggs is apparently incapable of binding capacitated epididymal spermatozoa (Nicosia et al., 1977).

Newly fertilized eggs at pronuclear and earlier stages show patchy staining with fluorescent Con A over the entire membrane while the polar body is usually unstained. The degree of patchiness of Con A receptors correlates with egg agglutinability and suggests an increase in receptor mobility following fertilization (Johnson et al., 1975). In contrast

Johnson and Edidin (1978), utilizing a photobleach recovery technique, have detected a significant reduction in the diffusion of membrane lipids and proteins following fertilization in the mouse.

Pienkowski (1974) has shown that mouse unfertilized eggs and preimplantation embryos up to the 6- to 8-cell stage have a similar binding capacity for radioiodinated Con A and that this capacity is quantitatively similar to that of somatic cells. However, Wu and Chang (1978) have reported a ten-fold increase in the binding of ^{3}H-Con A between the early and late blastocyst stages. Preimplantation embryos (zygote, 2-, and 6- to 8-cell stages) and embryos derived from parthenogenetically activated eggs are agglutinated by Con A at a concentration of 10-20 µg/ml, but unfertilized eggs are not (Pienkowski, 1974, Siracusa et al., 1978). Con A (free or Sepharose-bound) also inhibits embryonic development in vitro without apparent cytotoxicity.

More extensive studies by Rowinski et al. (1976) have described decreasing Con A agglutinability during preimplantation development. Early cleavage stage mouse embryos (zygote to 8-cell stage) are agglutinated by Con A at a concentration of 10 µg/ml, agglutination of morulae requires 100 µg/ml, while blastocysts are not agglutinable with Con A at concentrations as high as 5000 µg/ml. Isolated inner cell masses agglutinate readily at a Con A concentration of 10 µg/ml. Rector and Granholm (1978) have recently shown that the decrease noted in Con A-mediated agglutination between the 8-cell and morula stages actually occurs during the compaction process in the 8-cell embryo. The results of the Con A agglutination studies by Rowinski et al. (1976) have been confirmed by Magnuson and Stackpole (1978) except for the fact that the

Con A-mediated agglutination of inner cell masses could not be distinguished from non-specific agglutination. These investigators also reported that the lectins WGA and RCA agglutinate embryos strongly throughout the preimplantation period and do not show an age-related decline in agglutinating ability as does Con A (Magnuson and Stackpole, 1978).

These results indicate a rapid reduction in membrane fluidity or the induction of other, possibly cytoskeletal, restraints on the mobility of Con A-reactive membrane components during compaction and morula formation. The decrease in Con A-mediated agglutinability of blastocysts may reflect the loss of aggregation competence by late morula (Burgoyne and Ducibella, 1977) and the tendency of microsurgically isolated trophoblastic vesicles not to aggregate while isolated ICM's do so readily (Gardner, 1972). Ultrastructural studies have shown that the amount and distribution of Con A receptors on mouse embryos change during the preimplantation period and indicate that increased clustering of Con A binding sites with development parallel the described increase in Con A-mediated agglutinability (Konwinski, et al., 1977). Changes in agglutinability may also reflect changes in surface molecules bearing Con A receptor site(s). Webb et al. (1977) have observed developmental changes in the electrophoretic mobility of immunoprecipitated Con A-bound surface glycoproteins during the preimplantation period. The functional significance of reduced Con A-mediated agglutinability at the blastocyst stage remains to be determined, however, it may be related to polyploidization and cessation of cell division by trophectoderm cells (Barlow et al., 1972), cell surface changes required

for blastocyst formation or implantation, or provide a mechanism which prevents fusion of embryos after hatching in vivo.

The binding of fluorescein-labeled lectins to mouse embryos has also been examined at the morula and blastocyst-stage (Brownell, 1977). Con A, WGA, RCA_1 and RCA_2 all bind with no apparent differences in density or distribution between morulae and blastocysts. However, an L-fucose specific lectin shows no detectable binding at either stage. These observations are consistent with those of Yanagimachi and Nicolson (1976) who observed the absence of binding of fucose-specific lectins while others bound readily to fertilized and unfertilized hamster eggs. Incubation of mouse embros in the presence of the lectins Con A, RCA_1 and RCA_2 at 37°C (but not at 4°C) induces the aggregation of lectin receptors into patches indicating lateral mobility of these receptors within the cell membrane. WGA, however, does not induce patch formation under similar conditions (Brownell, 1977).

While blastocyst-stage embryos are apparently not agglutinable by Con A, Sobel and Nebel (1976) have described differences in Con A-mediated hemagglutination between early (day 4) and late (day 5) in vivo mouse blastocysts. Erythrocytes adhere randomly to the trophectoderm of Con A-coated day 4 blastocysts while adhering only to mural and not polar trophectoderm of day 5 blastocysts. Interestingly, this regional pattern of Con A hemadsorption is not observed on day 4 uterine blastocysts cultured for 24 hours in vitro (Sobel and Nebel, 1978). Failure of polar trophectoderm to bind erythrocytes is suggested to be due to alterations in physical properties of the cell surface (e.g. surface charge or membrane rigidity) which prevent close contact between

trophectoderm cells and erythrocytes since there is apparently no decrease in the number of Con A receptor sites in this region (Enders and Schlafke, 1974; Sobel and Nebel, 1976). However, the possibility that alterations in the mobility of Con A receptors may be responsible for changes in hemagglutinating capacity cannot be excluded.

It is clear, then, that preimplantation mammalian embryos possess a variety of carbohydrate specificities (with the possible exception of L-fucose) which are recognizable by lectins. However, there is no available evidence in the mammalian system indicating specific developmental roles for any of the carbohydrate moieties apparently present on the embryonic cell surface. In terms of quantitative lectin-binding there is apparently little change during preimplantation development at least through the 8-cell stage. However, lectin studies do indicate that there are physical changes in the embryonic surface membrane which affect some but not all of the lectin receptor-bearing molecules. Thus, Con A, but not WGA or RCA, shows a developmentally related decline in its ability to cause embryo agglutination, and Con A and RCA induce patching of lectin-receptor molecules within the membrane while WGA does not. Such phenomena are likely to be related to changes in membrane fluidity and cytoskeletal interaction with membrane components. In addition, the surface components which contain carbohydrate moieties recognized by lectins may vary (at least in molecular weight) during development (Webb et al., 1977).

5. Antigenic Reactivity with Specific Antisera

Stage-specific differences in the expression of cell surface antigens during mouse preimplantation development have been detected

by a number of antisera. These are discussed below under headings denoting the immunogen utilized in antiserum preparation and are summarized in Table 1.

(a) Mouse Fetal Germ Cells - Heath (1978) has described a rabbit antiserum prepared against fetal mouse (13.5 days post coitum) germ cells which reacts with fetal germ cells, adult male germ cells and embryonal carcinoma cells of teratocarcinoma cell lines. No reactivity with preimplantation-stage embryos is detected with the possible exception of the inner cell mass from 3.5 day blastocysts.

(b) Mouse Unfertilized Egg - A guinea pig antiserum to pronase-treated mouse unfertilized eggs which is cytotoxic to syngeneic and allogeneic eggs and preimplantation-stage embryos has been described by Moskalewski and Koprowski (1972). Inner cell masses of late blastocysts were shown to be slightly less susceptible to immune lysis than trophectodermal cells. Trophoblast cultures and 6- to 7- day egg cylinders are non-reactive with this serum but SV-40-transformed mouse fibroblasts do cross-react (Baranska et al., 1970; Koprowski, et al., 1971). A rabbit antiserum prepared by Glass and Hanson (1974) against mouse cumulus-oocyte masses also detects egg antigens but these as yet have not been defined as components of the cell surface.

(c) Mouse Blastocyst - A heterologous rabbit antiserum prepared by Wiley and Calarco (1975) against zona pellucida-free mouse blastocysts (A-BL serum) has been shown, by immunofluorescence, to detect embryonic cell surface antigens maximally expressed on 8- to 12-cell mouse embryos. This antiserum is less reactive with other preimplantation stages and unreactive against in vitro blastocyst outgrowths, tissues of 16- to 18- day mouse fetus, a number of adult tissues and two teratocarcinoma

TABLE 1. REACTION OF SPECIFIC ANTISERA WITH SURFACE ANTIGENS EXPRESSED BY PREIMPLANTATION EMBRYOS

IMMUNOGEN	ASSAY	UE	ZYG	2-C	4-C	8-C	MOR	BLASTOCYST ICM	BLASTOCYST TRPH	OUTGROWTH ICM	OUTGROWTH TRPH
MOUSE FETAL GERM CELLS											
Heath (1978)	IIF[1]	-	-				-	-	-		
	CTX[2]	-	-				-	±	-		
MOUSE UNFERTILIZED EGG											
Moskalewski & Koprowski (1972)	CTX	+		+	+	+	+	±	+		
MOUSE BLASTOCYST											
Wiley & Calarco (1975)	IIF	±	±	+	++	++++	+	+	+	-	-
	CTX			-		+					
Johnson (1979)[3]	IIF	-	-	-	+	++++	++++	+++	+++	±	±
	IIR[4]	-	-	+		++++	++++		++++		
	CTX	-		+	++++	++++	++++		++++		
MOUSE ECTOPLACENTAL CONE											
Searle & Jenkinson (1978)	IIP[5]	-	-			++	++++	+	++++		++++
MOUSE TERATOCARCINOMA											
402 AX Edidin et al. (1971)	(I) IIF	+			+	+	+		±	+	-
Edidin & Gooding (1975)	(II) IIF	±			-	-	-		+	+	-
Gooding et al. (1976)	(III) IIF	-			-	-			-	-	-

F9 Artzt et al. (1973, 1974)	IIF	-	-	+		+	++	++	++
Babinet et al. (1975) Kemler et al. (1976)	IIP	-	+	+		++			
	CTX		-	+		++			
Solter & Knowles (1978)	IIF	-	-	-	-	±	++++	++++	++++
	RIA[6]	-	-	-	-	++	++++	++	+
	CTX			-		-	+++	+	+
SIKR Stern et al. (1975)	IIF				+	+	+		
ENDO Artzt et al. (1976)	CTX					+			
PCC4 Gachelin (1976) Gachelin et al. (1976)	IIF	-	-		-	-	-	+	-
PCC4-AZA-1 Calarco & Linney[7]	IIF	-	-	-	-	-	-	+	-
OTT 6050 Dewey et al. (1977)	IIF	-		++		+++		+++	++
N1 Calarco & Banka (1979)	IIF	+	+	+	+	+	+	+	+
MOUSE SPERM									
NORMAL Menge & Fleming (1978) (IgG)[8]	IIF	+	+	+	+	+			+
(IgG-OA)[8]	IIF	-	-	-	+	+			+
(IgA)	IIF	-	-	-	+	+			+
T/t MUTANTS Kemler et al. (1976)									
t^{w5}, t^{0}, t^{w18}	IIF					+[9]			
t^{w32}	IIF					+[9]	+[9]		

MOUSE PLACENTA											
Kometani et al. (1973)	IIF	-	-	+	+	+	+		+		
Wiley & Calarco (1975)	IIF	++++	++++	++++	++++	++++	++++		++++	-	+
MOUSE CEREBELLUM											
Solter & Schachner (1976)	CTX	+	++	+	++	++		++++	+++		
Zimmerman et al. (1976)	IIF			+	+	+	+		+		
MOUSE SPLEEN											
Solter & Schachner (1976)	CTX	+	++	++	++++	++++		++++	++++		
Willison & Stern (1978)	IIF	-	-	-	-	-	±	+	+		
MOUSE L-CELL											
Wiley (1979)	IIF	-	-	-	-	-	+	+	+		
MOUSE INTRACISTERNAL A PARTICLES											
Huang (1978)	IIF	±	±	++	++	++	-		-		
	CTX	-	-	-	-	-	-		-		
HUMAN CHORIONIC GONADOTROPIN											
Wiley (1974)	IIF	-	-	±		+	++++		±	-	-
	CTX						-				

HUMAN COLD INSOLUBLE GLOBULIN (LETS)

Zetter & Martin (1978)	IIF	-	-	-	-	±	-

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[1]IIF - Indirect immunofluorescence

[2]CTX - Cytotoxicity

[3]Results presented here

[4]IIR - Immunoradiolabeling

[5]IIP - Indirect immunoperoxidase

[6]RIA - Radioimmunoassay

[7]Unpublished results

[8]IgG-OA - Ovary absorbed IgG

[9]Only embryos presumed to carry the specific t mutation react positively.

cell lines (PCC-Aza-1, Nulli-SCC-1). A functional role in early embryogenesis is indicated for A-BL-detected antigens by evidence demonstrating an inhibitory effect on blastocyst formation in vitro by the A-BL serum (as compared to non-immune serum) at concentrations as low as 2.5% in the absence of complement. This evidence suggests that the blocking, redistribution, phagocytosis and/or shedding of A-BL-detected surface components which may be brought about by antibody-binding may interfere with normal development.

A second antiserum, similarly prepared against mouse blastocysts, has been utilized in the investigations presented here to characterize stage-specific cell surface molecules which may be of functional significance during preimplantation development.

(d) Mouse Ectoplacental Cone - Searle and Jenkinson (1978) have recently described a rabbit antiserum raised by immunization with 7.5 day mouse ectoplacental cone. Utilizing an indirect immunoperoxidase assay for antibody binding these investigators detect no labeling on unfertilized eggs or zygotes but patches of peroxidase staining are observed on the outer aspects of 8-cell embryo blastomeres. Staining increases on morulae and trophectoderm of blastocysts forming a continuous, heavy layer. Immunosurgically isolated inner cell masses are only lightly stained, however. Antibody binding activity is retained by trophoblast giant cells and extraembryonic ectoderm in the post-implantation embryo. It is suggested that antigens recognized by this anti-ectoplacental cone serum are confined to trophoblastic cell populations and that such trophoblast-specific antigens may be potential targets for immunological fertility control.

(e) Mouse Teratocarcinoma - 402AX - Edidin et al. (1971) have developed rabbit antisera against embryoid bodies of a multipotential ascites subline (402AX) of mouse strain 129 teratocarcinoma. The antisera are reported to be cytotoxic to pronase-treated unfertilized mouse eggs and SV-40-transformed mouse fibroblasts, and to localize on the inner cell mass of blastocysts and embryonic endoderm of day 6 embryos. Since these antisera only react with cells of the embryonic portion of the conceptus which later express major histocompatibility (H-2) antigens, it has been suggested that the teratocarcinoma antigens detected may be characteristic of surface antigens present on embryonic cells prior to the appearance of major adult histocompatibility antigens (Edidin et al., 1971; Edidin, 1976a). Immunofluorescent characterization of such antisera after specific absorptions has revealed the presence of two different surface antigens on mouse preimplantation embryos both of which react with a number of tumor cell antigens (Gooding and Edidin, 1974; Edidin and Gooding, 1975; Gooding et al., 1976). Antigen I is physically associated with H-2 antigens on L-cell plasma membranes and found on many transplantable mouse tumors. It is also expressed by mouse unfertilized eggs, cleavage-stage embryos, and post-implantation (in vitro) inner cell mass. Antigen II, found on teratocarcinoma and hepatoma cells, is absent from cleavage-stage embryos and is first expressed on trophectoderm cells of blastocysts. The ICM portion of in vitro blastocyst outgrowths is positive for antigen II but trophoblast giant cells are not. A third antigen III identified by the serum is apparently teratocarcinoma-specific and does not react with embryonic material.

F9 - Syngeneic antisera against a pseudo-nullipotential embryonal car-

cinoma cell line (F9) prepared by Artzt and coworkers (1973) (reviewed by Jacob, 1977) have been shown in immunoperoxidase and cytotoxicity assays to react with cleavage-stage embryos and male germ cells but not with other adult mouse tissues (normal or malignant) or differentiated derivatives of the original tumor of the immunogen. Characterization of the anti-F9 serum has been extended to demonstrate maximal expression of F9 surface antigens on 16-cell embryos and their presence on both inner cell mass and trophectoderm of sectioned blastocysts (Babinet et al., 1975). F9 antigens have also been demonstrated on male germ cells and their precursors during postnatal development in the mouse (Artzt et al., 1974; Gachelin et al., 1976) and human spermatoza (Buc-Caron et al., 1974; Gachelin et al., 1976).

The presence of syngeneic mouse anti-F9 serum or xenogeneic rabbit anti-F9 serum does not inhibit mouse preimplantation development in vitro (Kemler et al., 1977), although anti-F9 antibodies have been shown to induce redistribution and capping of F9 antigens on blastomeres (Jacob, 1977). However, Fab fragments prepared from rabbit anti-F9 IgG cause a reversible inhibition of compaction and the formation of "grape-like" structures composed of an apparently normal number of uncompacted blastomeres (Babinet et al., 1977; Kemler et al., 1977). Since the effects of rabbit anti-F9 Fab are similar to those observed when embryos are exposed to media low in Ca^{++} (Wales, 1970; Ducibella and Anderson, 1975), it has been suggested that F9 antigens may be involved in calcium interaction and intercellular junction formation and that this function is blocked by Fab fragments (Kemler et al., 1977). Interestingly, Fab from the syngeneic mouse anti-F9 serum does not have a similar effect

even though preincubation of embryos with mouse anti-F9 blocks the binding of rabbit anti-F9 as assayed by immunofluorescence. The relationship between the antigenic specificities of the xenogeneic serum and the better characterized syngeneic anti-F9 remains to be determined.

There are two reports of monoclonal antibodies prepared against the F9 cell line which react with preimplantation-stage embryos. Such reagents have the advantage over xenogeneic and syngeneic antisera in that they are monospecific and allow the characterization of specific antigenic molecular species. Solter and Knowles (1978) have described monoclonal antibody derived by the fusion of mouse myeloma cells with spleen cells from a mouse immunized with F9 cells. This antibody detects (by radioimmunoassay, cytotoxicity and immunofluorescence) a stage-specific embryonic antigen (SSEA-1) which first appears at the 8-cell stage. Antibody binding increases at the morula stage, decreases on blastocysts and increases on isolated inner cell masses through 72 hours of culture. Immunofluorescence assays show that most 8-cell embryos are negative but some have both positively and negatively staining blastomeres indicating asynchrony among embryos and among blastomeres of single embryos. At later stages the number of positively reacting embryos increase, however, there are always a group of embryos (10-30%) which are completely negative.

Five embryonal carcinoma monoclonal antibodies (ECMA 1-5) prepared by fusion of lymphocytes from an F9-immunized rat with mouse myeloma have been described by Kemler et al. (personal communication). These antibodies have been tested against preimplantation-stage embryos by immunofluorescence and have been shown to react with varying specifi-

cities: ECMA-1 (positive on blastocyst and ICM), ECMA-2 and 3 (positive on 2-cell through blastocyst and ICM), ECMA-4 (negative on morula through blastocyst and ICM), ECMA-5 (negative on blastocyst).

It has been suggested that F9 surface antigens may be specified by the normal haplotype of the developmentally significant $\underline{t^{12}}$ mutation (Artzt et al., 1974; see below). It has also been proposed that a reciprocal relationship between the developmental expression of H-2 and T/t antigens may exist (Artzt et al., 1974; Artzt and Bennett, 1975) and that the expression of the F9 embryonic antigen and the absence of adult H-2 antigens may provide a mechanism by which germinal cells are isolated from other differentiating tissues during embryogenesis (Babinet et al., 1975).

SIKR - Stern et al (1975) have described a syngeneic antiserum against a pluripotent teratocarcinoma cell line (SIKR) which reacts with mouse embryos of 4-32 cells (stage-specificity not determined), a variety of embryonal carcinoma cells, sperm, kidney, and brain, but not with liver, thymus, a variety of differentiated cell lines, or endoderm cells of embryoid bodies.

Endo - Artzt et al., (1976) have also described a hyperimmune mouse antiserum against a teratoma-derived endodermal carcinoma (Endo) which defines surface antigens on 8-cell mouse embryo, embryonic liver, sperm, endodermal tumor cells and a small proportion of non-endodermal tumors.

PCC4 - A syngeneic antiserum to another pluripotent mouse teratocarcinoma (PCC4) has been described by Gachelin and coworkers (1976, 1977). It detects antigens on adult sperm and the ICM of newly implanted blastocysts but not on cells of mouse preimplantation stages. Interestingly,

inner cell masses from in vitro grown blastocysts do not show similar reactivity in immunofluorescence tests. Calarco and Linney have prepared a heterologous rabbit antiserum to a pluripotent, 8-azaguanine resistent derivative of the PCC4 teratocarcinoma stem cell line (PCC4-Aza-1). Preliminary investigations utilizing immunofluorescence show that this serum also reacts with immunosurgically isolated inner cell mass (from in vitro blastocysts), but not with any other mouse cleavage stage (Calarco and Linney, personal communication).

OTT 6050 - Dewey et al (1977) have described a syngeneic antiserum prepared against multipotential embryoid bodies of the ascites form of OTT 6050 transplantable mouse teratoma. Immunofluorescence tests have shown this antiserum to detect cell surface antigens on preimplantation-stage mouse embryos from the 2-cell through blastocyst stages. Both the inner cell mass and the trophectoderm of blastocysts are antigen-positive (the ICM slightly more so), but unfertilized eggs are negative. Tests of cleavage-stage embryos from matings of $+/t^{12}$ heterozygotes are uniformly positive. This serum also cross reacts with a number of normal adult tissues, including ovary and testis, but not with sperm.

N1 - A rabbit antiserum to a clonally derived nullipotential teratocarcinoma cell line (Nulli-SCC-1) described by Calarco and Banka (1979) has been shown by immunofluorescence to react with mouse follicular oocytes, unfertilized eggs, zygotes, and all preimplantation stages. Immunosurgically isolated inner cell mass and all morulae and blastocysts from matings of $+/t^{12}$ heterozygotes also stain positively. Anti-N1 serum reacts with 15.5 day primary oocytes but not with 15.5 day spermatogonia nor with mature spermatozoa. Anti-N1 serum inhibits

blastocyst formation in vitro in a concentration-dependent manner when present in culture medium without complement. The serum, however, is not cytotoxic to preimplantation embryos in complement-mediated cytotoxicity tests.

(f) Mouse Sperm - Menge and Fleming (1976, 1978) have reported that both serum IgG antibodies and IgA antibodies from the colostrum of rabbits immunized with mouse sperm detect surface antigens on early embryos. Immune serum detects surface antigens on oocytes, unfertilized eggs, zygotes and cleavage stages through the blastocyst. Absorption of this serum with mouse ovarian tissue removes activity against all except the 4- to 8-cell and blastocyst stages. IgA colostrum antibodies react only with the 4- to 8-cell and blastocyst stages.

In earlier investigations of rabbit anti-sperm isoantibodies Menge et al. (1974) had shown by immunofluorescence that rabbit blastocysts bind secretory IgA antibodies from uterine fluid but not uterine IgG nor serum IgA or IgG. Additionally, only uterine IgA inhibits in vitro blastocyst formation. Transplantation antigens are apparently not involved since absorption with paternal lymphocytes fails to remove the anti-embryo effects.

Antisera prepared against specific bacterial cell wall mannans which cross-react with surface carbohydrates of mammalian sperm also detect surface antigens on rabbit blastocysts (Sarkar and Menge, 1977). It is suggested from absorption studies that the cross-reactive antibodies recognize terminal N-acetylglucosamine linked to mannose residues.

Antisera have also been developed against sperm from mice carrying mutations at the T/t complex (t^{w32}, t^{w5}, t^{0}, t^{w18}) (Kemler et al., 1976).

Following absorptions designed to make these antisera specific for t antigens, each shows reactivity with 8-cell mouse embryos (presumably +/t^x) when assayed by immunofluorescence (see discussion of T/t antigens below).

(g) Mouse Placenta - The immunoglobulin fraction of an antiserum to mouse placenta prepared by Kometani and coworkers (1973) has been reported in immunofluorescence tests to recognize antigens on mouse embryos from the 2-cell stage through day 9 of gestation but not on unfertilized eggs or zygotes. A rabbit antiserum prepared against mouse placental homogenate (A-PL serum) has been shown by Wiley and Calarco (1975) to detect cell surface antigens on unfertilized eggs and mouse embryos at all preimplantation states. A-PL detected antigens decrease on the ICM portion of in vitro blastocyst outgrowths, are present only on the synctiotrophoblast of mouse placenta and are absent from mouse fetal tissues. A-PL serum also cross reacts with mouse teratocarcinoma cell lines (PCC4-Aza-1, Nulli-SCC-1), transformed mouse cell lines (JLS-VII and C_3H Gross virus transformed) and a cell line producing B viruses (MT 29240) (Calarco and Wiley, personal communication). While this antiserum is cytotoxic in the presence of complement for 2- and 8-cell embryos, it does not affect preimplantation development in vitro in the absence of complement.

(h) Mouse Cerebellum - Solter and Schachner (1976) have described an antiserum (anti-NS-4) prepared against 4-day-old mouse cerebellum which reacts with sperm, unfertilized and fertilized eggs, and cleavage-stage embryos, with maximal reactivity against ICM and trophectoderm cells of blastocysts. Anti-NS-5 antiserum, also prepared against 4-day-old mouse cerebellum and described by Zimmerman et al (1976), binds to pre-

implantation embryos from the 2-cell through the blastocyst stage. Mature spermatozoa and postnatal day 4, but not adult, kidney cells also express antigens detected by anti-NS-5. Another antiserum against 6- to 8-day-old mouse cerebellar cells (Seeds, 1975) has been shown to have similar reactivity with mouse preimplantation stages (Solter and Schachner, 1976).

(i) Mouse Spleen - Solter and Schachner (1976) have also demonstrated that a rabbit antiserum to mouse spleen cells shows increasing cytotoxic activity on mouse embryos between the zygote and blastocyst stages.

A monoclonal antibody preparation against mouse spleen cells which detects a Forssman-like specificity on teratocarcinoma stem cells (Stern, et al., 1978) also reacts with preimplantation mouse embryos (Willison and Stern, 1978). Antibody binding is first detected on a proportion of late morulae and increases to a maximum on the trophectoderm of early (3-3.5 day) blastocysts. Smaller proportions of late (3.5-4 day) and hatched (4-5 day) blastocysts are reactive while inner cell masses are antigen positive at all stages. Immunofluorescent staining often is not detected on all trophectodermal cells, however no consistent distribution of positive and negative cells (with respect to the position of the inner cell mass for example) is apparent. This observation is consistent with that obtained by Solter and Knowles (1978) utilizing a monoclonal anti-F9 antibody. Immune precipitation data indicates that the antigenic specificity recognized by the anti-spleen monoclonal antibody on embryonal carcinoma cells is carried by a glycolipid (Stern et al., 1975), but it remains to be determined if the same is true of the embryonic antigen. Willison and Stern (1978)

have, however, suggested the possibility that the molecule bearing the Forssman-like specificity on trophectodermal cells may be involved in embryo-hormone interaction as has been shown for other glycolipids.

(j) Mouse L-cells - Wiley (1979) has recently described antiserum against mouse L-cell fibroblasts (NCTC 929) which, following absorption with an embryonic mouse liver cell line (1469) retains activity against preimplantation embryos at the morula and blastocyst stages. Both ICM and trophectoderm of blastocysts react positively. Cross reactivity is detected with adult and fetal connective tissue, smooth muscle, bone and cartilage but not brain or sperm.

(k) Mouse Intracisternal A Particles - Antisera prepared against purified intracisternal A particles detect the stage-specific expression of embryonic surface antigens with a peak occurring at the 2-cell stage. This antiserum is not cytotoxic in the presence of complement (Huang, 1978), nor does its presence interfere with normal in vitro development.

(l) Human Chorionic Gonadotropin - Immunofluorescent assays described by Wiley (1974) have shown that a commercially available rabbit antiserum to human chorionic gonadotropin (A-HCG serum) recognizes surface antigens during mouse preimplantation development with maximal expression at the morula stage. Neither A-HCG serum nor its immunoglobulin fraction is cytotoxic to mouse morulae but in vitro development is arrested at the morula stage in the presence of A-HCG serum (but not A-HCG Ig) (Wiley and Calarco, personal communication). The possible presence of an HCG-like molecule on the surface of preimplantation embryos has been suggested to be of importance in protecting the conceptus from maternal rejection on implantation or in initiating

steroidogenesis (Wiley, 1974).

(m) Human Cold Insoluble Globulin (LETS) - Zetter and Martin (1978) have examined the presence of a high molecular weight cell surface glycoprotein (LETS, fibronectin) which is "transformation-sensitive" and has been implicated in cell-substratum adhesion and maintenance of cellular morphology (see Vaheri et al., 1978). Using an antiserum against human cold insoluble globulin (a soluble form of LETS found in blood plasma) and an indirect immunofluorescence assay, the expression of LETS is not detected on preimplantation mouse embryos except for the isolated inner cell mass. The intensity of staining is significantly increased on the inner cell masss of expanded (day 4) blastocysts compared to early (day 3) blastcysts. With the additional examination of a number of teratocarcinoma cell lines it is suggested that the LETS protein is a product of the embryonic ectoderm, but may also be synthesized by some types of embryonic endoderm (Zetter and Martin, 1978). Other investigators (Wartiovaara et al., 1978; Wolf et al., 1979) have shown LETS to be present at the late egg cylinder stage between endoderm and embryonic and extraembryonic ectoderm and associated with endoderm-like derivatives of teratocarcinoma cell lines.

It is clear from the numerous reports that the preimplantation mammalian embryo expresses cell surface antigenic specificies detectable by antisera prepared using a variety of immunogens. These antigens differ in their temporal expression on preimplantation stage embryos and can be placed in 3 classes: (1) those which are present during the entire preimplantation period, (2) those which appear and subsequently decline or disappear during this period (stage-specific), and (3) those which appear at a particular stage and remain expressed throughout

the remainder of this period. These surface antigens are also likely to differ in their relationship with the cell membrane (i.e. integral membrane components versus membrane-associated molecules), in their distribution (mobility?) on the cell surface, and in their developmental functions (if any). Evidence also indicates that the antigens may be glycolipids, as well as glycoproteins, and may include certain viral antigens. However, since there has been little direct comparison of the antigenic specificities detected by the various sera, their relationships remain to be determined. With the exception of recent studies which utilize monospecific reagents, the antisera which have been shown to be embryo-reactive are complex and are likely to contain multiple specificities. Such multi-specific reagents compound the problem of attempting to identify and characterize developmentally significant cell surface molecules. In fact, the evidence that antibodies can interfere with specific developmental events is minimal.

6. Alloantigen Expression

Due to the interest in the immunologic relationship between the fetus and the mother during mammalian development, numerous studies have been directed toward determining the extent to which histocompatibility antigens are expressed by the early embryo. This area of research has been the subject of recent reviews by Edidin (1976a,b) and is dealt with only briefly here with a summary of experimental results presented in Table 2.

Most investigators have been unable to detect major histocompatibility antigens (H-2) on mouse preimplantation embryos (Heyner et al., 1969; Palm et al., 1971; Gardner et al., 1973; Searle et al.,

TABLE 2. ALLOANTIGEN EXPRESSION BY PREIMPLANTATION EMBRYOS

ALLOANTIGEN	ASSAY	UE	ZYG	2-C	4-C	8-C	MOR	BLASTOCYST ICM	BLASTOCYST TRPH	OUTGROWTH ICM	OUTGROWTH TRPH
H-2 (MAJOR HISTOCOMPATIBILITY											
Heyner et al. (1969)	CTX[1]					-	-		-		
Palm et al. (1971)	IIF[2]	-		-					-		
Gardner et al. (1973)	HAG[3]		-	-	-	-	-		-		
Heyner (1973)	IIF								-	+	-
Jenkinson & Billington (1974)	CTX								-		
Searle et al. (1974)	TPL[4]								-		
Hakansson et al. (1975)	IIR[5]								+[8]		
Muggleton-Harris & Johnson (1976)	IIF			-	-		-	-	-		
Searle et al. (1976)	IIP[6]					-			+	+	-
Krco & Goldberg (1977)	CTX					+					
Billington et al. (1977)	IIP	-			-	-			+	+	-
NON-H-2 (MINOR HISTOCOMPATIBILITY											
Palm et al. (1971)	IIF			+[9]							
Searle et al. (1974)	TPL								+		

Hetherington & Humber (1975)	TPL							+[10]			
Muggleton-Harris & Johnson (1976)	IIF	+	+		+	+	+		+		-
Billington et al. (1977)	IIP		+	+	+		+		+		-
H-2 & NON-H-2											
Kirby et al. (1966)	TPL	+	+	+			+				
Simmons & Russell (1966)	TPL				+						
Olds (1968)	HAG	+									
Heyner et al. (1969)	CTX			+	+		+				
James (1969)	TPL						+				
Palm et al. (1971)	IIF	+									
Vandeputte & Sobis (1972)	TPL						+				
James & Yoshida (1972)	TPL						+				
Searle et al. (1974)	TPL						+				
Carter (1976)	HAD[7]								+		
Hunt & Avery (1976)	TPL							+[11]			
Muggleton-Harris & Johnson (1976)	IIF	+	+		+	+	+				
Searle et al. (1976)	IIF			+	+		+				
Billington et al. (1977)	IIP		+	+	+		+		+		-

[1]CTX - Cytotoxicity

[2]IIF - Indirect Immunofluorescence

[3]HAG - Hemagglutination

[4]TPL - Transplantation

[5]IIR - Indirect Immunoradiolabeling

[6]IIP - Indirect Immunoperoxidase

[7]HAD - Hemadsorption

[8]Experimentally delayed blastocysts.

[9]H-3 and H-6 antigens.

[10]H-3 antigens.

[11]H-2 and H-3 antigens.

1974; Muggleton-Harris and Johnson, 1976; Billington et al., 1977). It has been reported that H-2 antigens do not appear until after implantation at which time they are confined to inner cell mass-derived tissues (Heyner, 1973; Patthey and Edidin, 1973; Searle et al., 1975, 1976; Billington et al., 1977). However, several more recent reports indicate that H-2 antigens may be expressed at low levels on preimplantation mouse embryos. Krco and Goldberg (1977) have reported the results of cytotoxicity assays which indicate the presence of H-2 antigens (including paternal H-2 antigens) at the 8-cell stage of embryogenesis. Häkansson et al. (1975) have detected paternal H-2 antigens on blastocysts experimentally delayed from implanting. Hormonal activation of delayed blastocysts results in disappearance of these antigens coincident with the onset of implantation. Immunohistochemical studies at the electron microscopic level by Searle et al. (1976) have also shown that trophectoderm cells express low levels of H-2 antigens which are lost at the time of implantation. B_2-microglobulin, a subunit of major histocompatibility and other cell surface antigens, has also been detected on mouse blastocysts (Häkasson and Peterson, 1976). Antigen loss by the trophoblast at the time of implantation may be important in the prevention of maternal immune rejection.

Immunological studies by Webb and coworkers (1977) confirm that the synthesis of H-2 antigens by preimplantation mouse embryos does not occur until the late blastocyst stage. Immune precipitation reveals that the synthesis of proteins with H-2 antigenic specificities occurs primarily in the inner cell mass and not the trophoblast. These observations are consistent with earlier reports of H-2 antigen expression on inner cell mass-derived tissues of post-implantation embryos in vivo

and in vitro (Heyner, 1973; Patthey and Edidin, 1973; Searle et al., 1975, 1976; Billington et al., 1977).

It has also been shown (Ostrand-Rosenberg, et al., 1977) that cell lines derived from 4-day-old mouse blastocysts (Sherman, 1975) express H-2 antigens but certain H-2 specificities deviate from the expected expression based on genotype and the H-2 specificities of adult lymphocytes. These results may indicate the presence of incomplete or modified H-2 antigens on the embryonic cell surface and may relate to a suggestion by Edelman (1974) that a hierarchy of variable histocompatibility products may be involved in cell recognition during embryonic development.

Minor histocompatibility antigens (e.g. H-3, H-6) have, however, been detected on the cells of cleavage-stage embryos, including trophectoderm and inner cell mass of blastocysts, by serological (Palm et al., 1971; Muggleton-Harris and Johnson, 1976; Billington et al., 1977) and allograft (Searle et al., 1974; Hetherington and Humber, 1975) techniques. The appearance of these antigens on trophoblast cells is evidently transient since in vitro blastocyst outgrowths show reactivity to non-H-2 antisera only on the presumed inner cell mass cells and not on trophoblastic giant cells (Muggleton-Harris and Johnson, 1976) and postimplantation ectoplacental cone trophoblast apparently lacks histocompatibility antigens (Jenkinson and Billington, 1974). However, Carter (1976) has reported the detection of both maternal and paternal antigens on in vitro trophoblast outgrowths, and these have subsequently been identified as non-H-2 specificities (Sellens, 1977).

The presence of the male (H-Y) antigen has also been reported to occur as early as the 8-cell stage of mouse preimplantation development

(Krco and Goldberg, 1976), however, these results have been questioned (Erickson, 1977).

Undifferentiated teratocarcinoma cells also show a lack of H-2 determinants (Artzt and Jacob, 1974; Edidin and Gooding, 1975; Nicolas et al., 1975; Stern et al., 1975). They do, however, incite a rejection response in grafts between strains differing at multiple histocompatibility loci indicating the presence of minor histocompatibility antigens as in early embryos (Edidin et al., 1974).

These results suggest that significant levels of H-2 antigens are not present during the preimplantation period. Although low levels of H-2 antigens are detectable with sensitive techniques, they disappear at implantation. Minor histocompatibility antigens are present, however. The absence of H-2 antigens of preimplantation embryos has been suggested to be reciprocally related to high levels of antigens (e.g. F9) detected by antisera against undifferentiated teratocarcinomas (Edidin et al., 1971; Artzt and Bennet, 1975).

7. Stage-Specific Embryo-Lethal Mutations

The results of studies of lethal mutation in the T/t complex of the mouse have been cited as evidence for the participation of cell surface components in mammalian development (see Bennett, 1975; Klein and Hammerberg, 1977; Sherman and Wudl, 1977). Each of a series of recessive t mutations in this gene complex is defined by its lethality at a specific point in embryonic development when present in the homozygous state. The wild-type alternatives to each of the mutant haplotypes in the T/t complex have been postulated to specify the expression of cell surface components which function at the developmental

stages identified by abnormal development in the homozygous mutants (Bennett etal., 1972; Artzt and Bennett, 1975). A number of mutant haplotypes at the T locus have been shown to be expressed as serologically detectable surface antigens on sperm (Bennett et al., 1972; Yanagisawa et al., 1974). The hypothesis that the normal function of the T/t complex may be in the specification of the sequential expression of cell surface components is attractive. However, investigations by Kemler and co-workers (1976) has demonstrated the presence of preimplantation (t^{w32}) and postimplantation (t^{w5}, t^{0}, t^{w18}) lethal t antigens on cleavage stage (8-cell) embryos. Therefore, although different t haplotypes block development at various stages of development, their respective antigenic specificities are apparently not expressed sequentially during development.

One of the T/t complex haplotypes (t^{12}, t^{w32}) is lethal in homozygous mutant embryos at the morula to early blastocyst stage. The t^{12} and t^{w32} mutants are classified in the same complementation group and may be identical, however, there is evidence that they may act at slightly different points in development (Hillman and Hillman, 1975). Death of homozygous t^{12}/t^{12} embryos typcially occurs at the morula stage, but morphological abnormalities can be identified in such embryos prior to this point in development (Calarco and Brown, 1968; Hillman et al., 1970). Biochemical investigations suggest that homozygous t^{12} mutant embryos cease macromolecular synthesis at the morula stage (Wudl and Sherman, 1976).

A product of the wild-type allele at the t^{12} locus (i.e. $+t^{12}$) has reportedly been identified by an anti-F9 teratocarcinoma serum on the surfaces of sperm and normal embryos with maximal expression at the

morula stage (Artzt et al., 1974; Babinet et al., 1975). Sperm from $+/t^{12}$ males are only half as effective as those from normal (+/+) males in absorbing anti-sperm activity from anti-F9 serum. On this basis it has been suggested that the death of t^{12}/t^{12} embryos at the morula stage may be due to the lack of a surface antigen normally expressed by the wild haplotype $+t^{12}$. In fact, evidence shows that 8-cell embryos presumably homozygous for the t^{w32} mutation do fail to express the F9 antigen. However, embryos presumably homozygous for t^{w5} also fail to produce antigens recognizable by the anti-F9 serum (Kemler et al., 1976). These results indicate that the F9 antigen is probably not a specific product of the wild type $+t^{12}$ haplotype, but may be a product of gene(s) in the region of the T/t complex which segregates as expected for a product of the wild t haplotype (Kemler et al., 1976).

The contention that genes in the T/t complex specify functionally significant cell surface molecules is further supported by the analogies between the T/t complex and the major histocompatibility complex of the mouse (Artzt and Bennett, 1975; Hammerberg and Klein, 1975). The histocompatibility complex is important in the detemination of cell surface structures which function in a number of biologic phenomena including allograft rejection and immune response. The expression of histocompatibility antigens may also be important in cell interactions involved in morphogenesis (Bennett et al., 1971; Bodmer, 1972, Hakansson et al., 1975; Bach and Van Rood, 1976; Jacob, 1977, Ohno, 1977). These two gene complexes are linked on mouse chromosome 17 and their products may be similar in molecular weight and subunit structure. It has also been reported that both are associated with a

beta-2 microglobulin-like moiety on the cell surface (Vitetta et al., 1975), however, more recent studies have shown these moieties to be immunologically dissimilar (Dubois et al., 1976). Additionally it has been shown that t mutants are associated with certain H-2 haplotypes (Hammerberg and Klein, 1975) and that t mutants of the same complementation group fail to stimulate a mixed lymphocyte reaction and, therefore, appear to share the same H-2 haplotype (Levinson and McDevitt, 1976).

The developmental expression of H-2 and T/t antigens may be reciprocal. H-2 antigens appear to be present on all adult cells and absent from early embryos while antigens specified by the T/t locus are apparently present on teratocarcinoma and embryonic cell surfaces, and absent from adult somatic tissues (Artzt et al., 1973). A possible exception to this reciprocality may be the endodermal cells in embroid bodies of a differentiating teratocarcinoma cell line which evidently possess neither H-2 or teratocarcinoma antigens (Stern et al., 1975).

The role of the T locus in events mediated by the cell surface during preimplantation development is unclear, but some generalizations can be made: 1) There appears to be a reciprocal expression of H-2 and T locus antigens on embryos and teratocarcinomas, 2) Death of homozygous t^{12} embryos at the morula stage does not appear to be due only to the absence of the F9 antigen, 3) At least four different antigens may be present as early as the 8-cell stage.

8. Summary

The preimplantation mammalian embryo expresses a variety of cell surface components that are detectable by a number of experimental

techniques. Additionally, it is clear that the characteristics of the embryonic cell surface change considerably, probably both qualitatively and quantitatively, during the preimplantation period. Available evidence also suggests that cell surface molecules are of functional significance during the period of preimplantation development in mannals.

While functions for such cell surface molecules have not yet been identified experimentally, there are a number of roles that might be envisaged for embryonic cell surface components.

(a) Surface molecules may serve as receptors for extracellular factors, including other cells, which upon interaction can induce intracellular events such as hormone and enzyme synthesis, complex developmental changes and/or mitosis. Such receptors may function to mediate reciprocal interactions between intracellular and extracellular factors of significance in the process of differentiation. Evidence indicates that blastomere position in the early embryo is important in the determination of developmental fate (see Herbert and Graham, 1974). It might be predicted that surface receptors are involved in the differentiation (and/or maintenance of the differentiated state) of inner cell mass and trophectoderm, possibly in response to differences in the mircoenvironments to which internal and external cells are exposed.

(b) Embryonic cell surface molecules are also likely to function in intercellular adhesion during the preimplantation period. Adhesive components could function in a non-specific homologous adhesion which would serve to bind cleavage stage blastomeres to one another and/or in selective adhesion involving the recognition and aggregation of

specific cell types, such as inner cell mass and trophectoderm cells during blastocyst formation.

(c) Cell surface molecules present on the early mammalian embryo may be related to the development of the stage-specific capacity of trophectoderm cells to attach to the uterine wall and become invasive. The reported similarities between the surface characteristics of embryonic cells and those of transformed and teratocarcinoma cells support this possibility.

(d) The presence or absence of components of the embryonic cell surface may be involved in the protection of the fetus from immunologic attack by the mother. For example, H-2 antigens appear to be absent from the trophectoderm cells of the implanting blastocyst.

(e) Alternatively, some of the surface components detected during the preimplantation period in mammals may merely reflect developmental changes secondarily (e.g. the appearance or activation of new membrane transport systems) and may have no direct role in the developmental process at the cell surface.

RATIONALE

It is clear from the above that cell surface components undergo both qualitative and quantitative changes during preimplantation mammalian development. The observation of such changes implies that surface components may be functionally significant during this period. However, relatively little information is available concerning the actual molecular characteristics of the embryonic cell surface and the molecular basis for the observed cell surface changes which occur with development. It has thus far been impossible to assign specific developmental roles to specific cell surface molecules or classes of molecules. The studies

described here have attempted to extend the characterization of cell surface molecules expressed during preimplantation development toward the goal of defining such specificities. Stage-specific cell surface antigens that are expressed during mouse preimplantation development and which may have a developmental role during this period have been examined by a number of serological and biological approaches.

MATERIALS AND METHODS

1. Antisera

The studies described here utilized a xenogeneic antiserum prepared by the immunization of a male New Zealand White rabbit with zona pellucida-free SWR mouse blastocysts. The details of the preparation of this anti-blastocyst (A-BL) serum have been described previously (Wiley, 1975; Wiley and Calarco, 1975), however, this antiserum is different from that described by Wiley and Calarco (1975) and has not been previously characterized.

Prior to experimental use the antiserum was routinely heat inactivated (56°C, 30 minutes) and absorbed twice (45 minutes, on ice) with equal volumes of a crude membrane homogenate prepared from adult mouse liver, kidney and spleen. Completeness of absorption was verified by indirect immunofluorescence assays (see below) on live mouse spleen cells. Following absorption the antiserum was dialyzed extensively against 10 mM phosphate-buffered saline (PBS, pH 7.0-7.2) filter sterilized (Millipore, 0.22 μm) and stored in aliquots at -40°C.

The immunoglobulin G (IgG) fraction of the A-BL serum was isolated by precipitation with 40% saturated ammonium sulfate followed by separation on DEAE Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway,NJ) (Fahey and Terry, 1973). Fab fragments were prepared from A-BL IgG by papain digestion and fractionation on carboxymethyl cellulose (Whatman CM-52) (Porter, 1959; Dr. S. Rosen, personal communication). The presence of Fab fragments in such preparations was verified by polyacrylamide gel electrophoresis with which a single 45,000-dalton band was demonstrated. The retention of anti-embryo activity by Fab preparations was verified by indirect immunofluorescence.

In all cases pre-immune or normal rabbit serum (NRS) from a non-immunized animal was treated identically and utilized in experimental controls.

Other antisera utilized in these studies included anti-F9 teratocarcinoma (syngeneic, # 7592, provided by Dr. K. Artzt; monoclonal, # II C3, provided by Drs. T. Magnuson and K. Artzt), rabbit anti-mouse alpha-fetoprotein (Miles Research, Elkhart, ID), goat anti-Rausher leukemia virus (NIH # 78S-225) and goat anti-feline leukemia virus gp70 (NIH # 76S-455) (provided by Biological Carcinogenesis Branch, NIH).

2. Preimplantation Embryos

Preimplantation embryos were flushed from the oviducts of superovulated pregnant female ICR mice (Simonson Labs, Gilroy, CA) and cultured from the 2-cell stage in 25-50 µl drops of embryo culture medium (modified from Biggers et al., 1971 by Dr. A. Spindle, personal communication) under paraffin oil at 37°C in a humidified atmosphere of 5% CO_2 in air. Embryos allowed to develop in vivo were flushed from the oviducts or uterine horns at appropriate times following mating. Follicular cells were removed from unfertilized eggs by treatment with hyaluronidase (75 U/ml, Sigma Chemical Co., St. Louis, MO). Zonae pellucidae were removed either mechanically by pipetting or enzymatically by brief treatment with 0.5% pronase (22 U/ml, Calbiochem, La Jolla, CA). Following removal of the zona pellucida, embryos were typically allowed to recover for two to five hours in culture medium prior to experimental use.

3. Immunofluorescence

Indirect immunofluorescence (IIF) assays were performed on live preimplantation embryos or inner cell masses isolated by immunosurgery (Solter and Knowles, 1975). Zona pellucida-free embryos were typically utilized in IIF assays since the zona pellucida reacts strongly with A-BL serum and its fluorescence obscures that of the embryo proper. Embryos were first incubated for 30 minutes in 20 µl drops of antisera (diluted 1/5), IgG (1.0 mg/ml), or Fab (1.0 mg/ml) in embryo culture medium. Following several rinses in normal culture medium to remove unbound antibody, embryos were incubated for 15 minutes in fluorescein- or rhodamine-labeled goat anti-rabbit IgG serum (diluted 1/5 in PBS; Antibodies Inc., Davis, CA, Cappel Laboratories, Cochranville, PA) and subsequently rinsed again to eliminate excess second antibody. The binding of mouse or goat antiserum was assayed using fluorescein-labeled rabbit anti-mouse immunoglobulins (Miles) or rabbit anti-goat immunoglobulins (Antibodies, Inc.). These manipulations were generally carried out at 37°C, however, results were identical when the temperature was maintained at 0-4°C. IIF samples were examined with a Zeiss fluorescence microscope and photographed on GAF 500 daylight color slide film (ASA 500).

Live monolayer cultures or embryoid bodies of the teratocarcinoma cell lines F9, Nulli-SCC-1, PSA-1 and PCC 4-Aza-1 (provided by Drs. G. Martin and E. Linney) and the type-C virus-infected cell lines 1356 (mouse embryo fibroblast, AKR, ecotrophic MuLV) and Dog C 57L (dog kidney fibroblast, spontaneous NZB xenotrophic MuLV) (provided by Dr. J. Levy) were similarly treated.

Cryostate sections of adult mouse tissues (male and female)

including liver, kidney, spleen, brain, skeletal muscle, ovary and testis, and live spleen cells, thymus cells and sperm were also similarly tested for reactivity with A-BL antibodies.

4. Immunoradiolabeling

IgG from a goat antiserum against rabbit IgG (GAR, Miles) was radioiodinated (^{125}I) by the chloramine T technique (Greenwood et al., 1963) and purified on Sephadex G-25 (Pharmacia). Indirect immunoradiolabeling (IIR) was carried out on live zona pellucida-free preimplantation embryos. Samples containing 25-50 embryos were incubated in A-BL or NRS IgG (1.0 mg/ml) for 45 minutes at 37°C, rinsed in embryo culture medium, incubated in ^{125}I-labeled goat anti-rabbit IgG (^{125}I-GAR, 1.4 mg/ml, 0.19-0.23 mCi/mg for 45 minutes at 37°C, rinsed in PBS containing 1.0 mM potassium iodide (PBS-KI), transferred to a disc of glass fiber filter paper (Whatman GF/C, presoaked in PBS-KI containing 1.0 mg/ml unlabeled GAR) and rinsed under suction with 3 ml PBS-KI. Radioactivity retained by the filters was determined by gamma counting (facilities provided by Dr. T. Hayashida). Controls included embryos incubated in antibody-free medium prior to ^{125}I-GAR to assess non-specific binding of ^{125}I-GAR, and embryos incubated in unlabeled GAR prior to ^{125}I-GAR for verification that the radioactivity retained by embryos was due to specific binding of ^{125}I-GAR. Non-specific binding of ^{125}I-GAR, which typically accounted for 10-30% of the embryo-associated activity, was subtracted from the corresponding embryo-containing samples. Additionally, for each sample a volume of the final PBS-KI rinse equal to that in which the embryos were transferred to the filter paper was treated similarly to assess background

counts contained in the rinse solution and those non-specifically retained by the glass fiber filter. These background counts were also subtracted from those of the corresponding embryo-containing samples and the counts normalized in relation to the calculated decay based on a half-life for ^{125}I of 60 days. Each embryonic stage was assayed for the binding of A-BL or NRS antibodies or for the non-specific binding of ^{125}I-GAR in a minimum of two (more typically three) separate experiments.

5. Immunodiffusion and Immunoelectrophoresis

Double immunodiffusion assays (Ouchterlony, 1948) utilized commercial immunodiffusion plates (Hyland Immuno-Plate D, Miles) or immunodiffusion plates prepared with 1% agarose (SeaKem-ME, Miles) containing 0.1% sodium lauryl sulfate (SDS, British Drug House) or 0.5% Nonidet P-40 (Particle Data Laboratories, Elmhurst, IL) to inhibit reaggregation of detergent-solubilized proteins. For use in immunodiffusion assays preimplantation embryos were solubilized at varying concentrations in PBS alone or 3 M KCl, 0.1% SDS or 0.5% NP-40 in PBS with repeated freeze-thawing and Vortex mixing and tested against serial dilutions of A-BL serum or IgG. NP-40-soluble extracts of adult mouse tissues (liver, kidney, spleen, skeletal muscle, brain, ovary, testis) prepared by homogenization in 0.5% NP-40 in PBS and centrifugation (50,000 x g, 60 minutes) to remove insoluble components were similarly tested.

Immunoelectrophoresis was performed in 1% agarose containing 0.5% NP-40 in 0.1 M sodium barbital buffer (pH 8.6). One hundred 8-cell embryos were solubilized in 10 µl 0.5% NP-40 and 3 µl aliquots electro-

phoresed for two hours at a constant voltage of 100 volts. A-BL and NRS serum were added to the longitudinal basins and diffusion was allowed to take place at 4°C overnight.

6. Cytotoxicity

The binding of A-BL antibodies to surface antigens of preimplantation-stage embryos was also assayed in complement-mediated cytotoxicity tests. Samples containing 10-20 zona-intact embryos were incubated in A-BL or NRS serum or IgG at varying concentrations in culture medium for 45 minutes at 37°C. The embryos were subsequently transferred to a solution containing embryo culture medium, the appropriate antiserum or IgG dilution, and guinea pig or rabbit complement (1:1:2) and incubated for an additional 45 minutes at 37°C. The degree of toxicity was assessed by determining the number of dye-filled embryos following five minutes in 0.5% trypan blue. Controls included embryos initially incubated in antibody-free culture medium to determine the extent of non-specific complement toxicity, and embryos incubated in a rabbit anti-mouse L cell serum (proveded by Drs. L. Wiley and A. Spindle) as a positive control for complement function. Each developmental stage was assayed in a minimum of two separate experiments.

7. In Vitro Culture

The effect of A-BL antibodies on preimplantation development was examined by *in vitro* culture in varying concentrations of A-BL or NRS serum, IgG or Fab. Samples containing 10-25 embryos were cultured from the 2-cell stage for three days during which time their development was monitored daily and at the end of this period they were scored for

blastocyst formation. These studies were typically carried out in duplicate in a minimum of two separate experiments. The stage-specificity and reversibility of the effects of A-BL antibodies was analyzed by placing 2- or 8-cell embryos, morulae or blastocysts into A-BL-containg culture medium for 24 hours with subsequent culture in antibody-free medium.

8. Enzyme Treatment

In an attempt to determine the molecular characteristics of the surface antigens detected by A-BL antibodies, zona pellucida-free embryos at the 8-cell to morula stage were treated with proteases, glycosidases, lipases and nucleosidases prior to assays for A-BL binding. The enzymes utilized included: (a) Proteases - chymotrypsin (α,B,γ; bovine pancreas), Kallikrein (pancreatic), thrombin (bovine plasma), trypsin (bovine pancreas), pronase (S. griseus), pepsin (hog stomach mucosa), bromelain (pineapple stem), papain (papaya latex), collagenase (Cl. histolyticum), protease V (S. griseus), protease VIII (B. subtilis); (b) Glycosidases - α-amylase (bacterial), β-amylase (sweet potato), α-galactosidase (A. niger), β-galactosidase (bovine liver), α-mannosidase (C. ensiformis), β-N-acetylglucosaminidase, glucose oxidase (A. niger), neuraminidase (Cl. perfringens), α-glucosidase (yeast), β-glucosidase (almond), lysozyme (egg white), endoglycosidase H (S. griseus), endoglycosidase D (D. pneumoniae), mixed glycosidases (C. lampus); Lipases - phospholipase A_2 (porcine pancreas), phospholipase C (Cl. welchii), phospholipase D (peanut), lipase (C. cylindracea); (d) Nucleosidases - deoxyribonuclease I (bovine pancreas), ribonuclease A (bovine pancreas)(all of the above obtained from Sigma

with the exception of endoglycosidases D and H and mixed glycosidases obtained from Miles). Embryos were exposed to enzymes solubilized in PBS or albumin-free embryo culture medium at concentrations ranging from 10-1000 µg/ml for periods ranging from 30 minutes to two hours at 37°C. Following enzyme treatment, embryos were rinsed in enzyme-free culture medium and tested in standard immunofluorescence or immunoradiolabeling assays as described above.

9. Blocking Experiments

Zona pellucida-free 8-cell to morula stage embryos were treated with lectins of varying carbodydrate specificities prior to immunofluorescence assay in an attempt to observe a block to A-BL binding and determine if carbohydrate moieties on the cell surface comprise a part of the antigenic sites(s) recognized by A-BL antibodies. The lectins utilized included concanavalin A (Man,Glu), wheat germ agglutinin (GluNAc), Ricinus communis agglutinin-1 (Gal), peanut agglutinin (Gal-Gal-GalNAc), Dolichos biflurus agglutinin (GalNAc), soybean agglutinin (GalNAc, Gal) and Ulex eropus agglutinin (Fru) (all obtained from E-Y Laboratories, San Rafael, CA). Embryos were treated with lectins at a concentration of 500-1000 µg/ml in PBS for 60 minutes at 37°C, rinsed in lectin-free PBS and tested by immunofluorescence as described above.

Since antisera prepared against the F9 teratocarcinoma cell line show anti-embryo activity similar to that of the A-BL serum (Artzt et al., 1973; Babinet et al., 1975), the ability of anti-F9 antibodies to block the binding of A-BL antibodies was tested by pretreatment of embryos with syngeneic or monoclonal anti-F9 antibodies followed by immunofluorescence assay of the binding of A-BL antibodies.

10. Hapten Inhibition

A number of sugars (mono- and polysaccaride) and glycoproteins with known carbohydrate composition were tested for their ability to reduce the binding of A-BL antibodies to embryos. A-BL IgG (1.0 mg/ml) was incubated for two hours at room temperature in the presence of the absorbing hapten at concentrations ranging from 0.01-100 mM (McKenzie et al., 1977). The antibody-hapten mixture was then utilized in standard immunofluorescence or cytotoxicity assays as described above. The haptens utilized in these studies included: (a) Monosaccharides- D-mannose, D-glucose, D-galactose, L-fucose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine; (b) Glycosides - thiodigalactoside, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside; (c) Oligosaccharides-sucrose, stachyose, melibiose, lactose; (d) Glycoproteins-yeast invertase, yeast mannan, horse radish peroxidase (all of the above obtained from Sigma), asialofetuin (obtained from Dr. S. Rosen).

11. Antibody Absorption

In an attempt to determine the extent of cross-reactivity between A-BL antibodies reacting with the embryo surface and those detecting antigenic sites on the zona pellucida, A-BL serum was absorbed with a crude homogenate of mouse ovarian tissue. Such absorption removed anti-zona pellucida activity without altering the anti-embryo reactivity as assayed by immunofluorescence.

To test possible activity against a Forssman-like blood group specificity (Willison and Stern, 1978), A-BL IgG was absorbed with sheep red blood cells and then tested by immunofluorescence for anti-

embryo activity. This procedure did not alter the binding pattern of A-BL antibodies on preimplantation-stage embryos.

12. Metabolic Inhibitors

In an attempt to determine if the expression of A-Bl-detected antigens requires protein synthesis, 2- or 8-cell embryos were incubated for 24 hours in the presence of an inhibitor of protein synthesis, cycloheximide or puromycin (Sigma), at 1.0, 5.0 or 10.0 µg/ml in culture medium. These concentrations of inhibitors have been shown to inhibit cleavage without apparent cytotoxic effects (Thomson and Biggers, 1966). Following treatment embryos were rinsed and assayed for antigen expression by immunofluorescence.

13. Embryo Radiolabeling

Embryo-synthesized proteins were radiolabeled by incubating zona pellucida-free embryos at 37°C for 5-6 hours in 5-10 µl drops of embryo culture medium containing ^{35}S-methionine (7-8 mCi/ml, 900-1100 Ci/mmole, Amersham, Arlington Heights, IL). Following the labeling period, embryos were rinsed several times in culture medium, solubilized in immunoprecipitation or electrophoresis sample buffer (see below) and frozen at -40°C if not utilized immediately.

Lactoperoxidase-catalyzed iodination of exposed surface proteins utilized techniques described by Hubbard and Cohn (1972, 1977) and Dr. H. Rodriquez (personal communication). Zona pellucida-free preimplantation embryos were rinsed three times in embryo culture medium containing 10 mg/ml polyvinylpyrrolidone (PVP-40, Sigma) in place of bovine serum albumin (PVP-medium). Embryos were then transferred to

a 20 µl drop of freshly prepared lactoperoxidase glucose oxidase reaction mixture containing ^{125}I (10 mCi/ml, 13-17 mCi/µg, Amersham), lactoperoxidase (22.5 µg/ml, 58 U/mg, Sigma) and glucose oxidase (0.9 µg/ml, 90 U/mg, Calbiochem) in PVP-medium containing 5.5 mM D-glucose. The reaction was allowed to proceed at 37°C for 30 minutes following which the embryos were rinsed five times in PBS containing 1.0 mM KI and solubilized in immunoprecipitation or electrophoresis sample buffer (see below). If not utilized immediately samples were stored at -40°C. TCA-precipitable counts from labeled embryos were determined from 1 µl samples as described by Epstein and Smith (1973). Lactoperoxidase labeling by these techniques typically yielded 1000-2000 counts per minute per embryo, of which 10-25% was TCA precipitable. In the absence of either lactoperoxidase or glucose oxidase in the reaction mixture the labeling of embryos did not exceed background. Additionally, embryos exposed to a complete reaction mixture which had been incubated at 37°C for 30 minutes and the reaction then stopped by the addition of 2mM L-cysteine and 5 mM KI were not significantly labeled. Thus labeled proteins from lactoperoxidase-labeled embryos probably do not represent self-labeled components of the reaction mixture which have bound to the embryo surface.

14. Immunoprecipitation

A-BL-detected embryonic antigens were immunoprecipitated from NP-40 extracts of radiolabeled preimplantation embryos utilizing Stapholoccus aureus as a precipitating agent (Kessler, 1975; Jones, 1977). Typically 100-500 embryos were solubilized in 25 µl immunoprecipitation sample buffer (0.5% NP-40 in PBS containing 100 U/ml

aprotinin to inhibit proteolysis). Non-soluble components were removed by centrifugation (50,000 x g, 60 minutes). To 10 µl aliquots of the NP-40 extract (50,000 x g supernatant) were added A-BL or NRS IgG (2.5-5 µl) to a final antibody concentration of 0.4 mg/ml and the mixture was incubated on ice for a minimum of one hour with periodic mixing. In an additional experiment designed to confirm the presence of A-BL antigens at the cell surface (Gupta et al., 1977), live 8-cell embryos previously labeled with ^{35}S-methionine were treated with A-BL IgG (1.0 mg/ml, 30 minutes) and rinsed to remove unbound antibody prior to solubilization and precipitation with S. aureus. Precipitation was carried out by the addition of 25-30 µl of a 10% (w/v) suspension of heat-killed, formalin-fixed S. aureus (Cowan I) (provided by Dr. J Sawicki and M. Fraher) and an additional incubation period of at least one hour on ice with frequent mixing. The bacteria-antibody-antigen complexes were isolated by low speed centrifugation and rinsed three times with PBS containing 2 mM methionine or KI, depending on the radiolabel utilized. For one-dimensional gel analysis precipitated antigens were solubilized from the precipitating bacteria by the addition of 5-10 µl electrophoresis sample buffer containing 2.3% SDS, 5% β-mercaptoethanol, 10% glycerol and 100 U/ml aprotinin in 0.0625 M Tris-HCl, pH 6.8 (O'Farrell, 1975) followed by heating to 95-100°C for 1.5-2 minutes and removal of the bacteria by centrifugation. Samples for two-dimensional gel analysis were solubilized in isoelectric focusing sample buffer (O'Farrell, 1975): 9.5 M urea, 2% NP-40, 2% Ampholines (1.6% pH 5-7, 0.4% pH 3-10) and 5% β-mercaptoethanol. If not utilized immediately, samples were stored at -40°C prior to electrophoretic analysis.

15. Gel Electrophoresis

One-dimensional electrophoretic analysis of embryonic proteins and immunoprecipitates was carried out on SDS-polyacrylamide slab gels utilizing 10% separating and 4.5% stacking gels (Laemmli, 1970; Martin et al., 1978). Samples were applied to 15 cm x 16.5 cm x 0.8 mm gels in a total volume of 10-25 µl and electrophoresis carried out at a constant current of 20 mA for 2-2.5 hours. For rapid analysis of immunoprecipitates, 1 µl samples were applied to microslab gels (50 mm x 65 mm x 0.1 mm) and electrophoresed at a constant current of 2 mA for 20-30 minutes. Molecular weight standards were run simultaneously on each gel and included: phophorylase a (94,000), bovine serum albumin (68,000), ovalbumin (43,000), chymotrypsinogen A (25,700), myoglobin (17,000) and cytochrome C (12,000) (all obtained from Sigma). Apparent molecular weights were calculated using a plot of mobility versus the logarithm of the molecular weights of the standards (Ferguson, 1964; Dunker and Ruekert, 1969).

Two-dimensional electrophoresis utilized techniques described by O'Farrell (1975) and Jones (1977) and was carried out by Dr. J. Sawicki. The first dimensional separation based on isoelectric point employed a 4% acrylamide gel containing 9 M urea, 2% NP-40 and 2% Amopholines (1.6% pH 5-7, 0.4 pH 3.5-10) for a total of 5200 volt-hours. Molecular weight separation in the second dimension utilized a 10% polyacrylamide gel with a 4.5% stacking gel as described above.

Gels were fixed and stained for 30 minutes in 50% TCA containing 0.1% Coomassie brilliant blue R-250, destained in 7% acetic acid and dried onto Whatman 3MM chromatography paper or Whatman 50 filter paper. Kodak X-Omat R X-ray film was utilized for autoradiography.

Fluorographs were prepared by impregnation of gels with 2,5-diphenyloxazole (PPO) as described by Bonner and Laskey (1974). Autoradiographs were typically exposed for 3-30 days and processed in Kodak X-ray developer for four to eight minutes.

RESULTS

1. Reactivity of A-BL Antibodies

(a) Immunofluorescence - The expression of A-BL-detected surface antigens by mouse preimplantation embryos is first detectable by immunofluorescent staining on 4-cell embryos and increases to a maximum on 8-cell to morula stages (Figs. 1,2). Antral oocytes, ovulated unfertilized eggs, zygotes (1-cell fertilized eggs), and 2-cell embryos are not stained. Following the morula stage, the intensity of fluorescent staining decreases slightly on the trophectoderm of early (day 4) and late (day 5, hatched) blastocysts. The staining of inner cell masses isolated by immunosurgery parallels that of the trophectoderm. The zona pellucida is intensely stained throughout the preimplantation period, however, absorption with ovarian tissue removes the anti-zona activity without altering anti-embryo reactivity. The stage-specific pattern of immunofluorescent staining consistently observed on embryos developing in vitro from the 2-cell stage and those allowed to develop in vivo. The staining pattern is similar whether A-BL serum, IgG or Fab is utilized as the first antibody in the immunofluorescence assay. Anti-embryo activity apparently is not contained in the IgM fraction of A-BL serum since no binding of A-BL antibodies can be detected at any stage when rhodamine-conjugated goat anti-rabbit IgM is utilized as the second antibody in the reaction.

The distribution of immunofluorescent staining is diffusely punctate at all stages which react positively suggesting the existence of small clusters of A-BL antigens (Fig. 3a). No evidence of antibody-induced patching or capping of surface antigens is observed when immunofluorescence assays are carried out at 0 or 37°C. Addition-

ally, when embryos are treated with A-BL serum and then maintained in culture at 37°C for up to six hours no change in the distribution of A-BL-detected antigens is observed. However, treatment of embryos with certain enzymes or metabloic inhibitors can induce formation of large patches of reactive antigens detectable by immunofluorescence (see below). During the compaction process, staining becomes localized to the apical portions of blastomere surfaces with unstained areas adjacent to apposed membranes (Fig. 3b). This redistribution of A-BL-detected antigens associated with compaction is apparently not antibody-induced since it can be observed using A-BL IgG or Fab at 0 or 37°C.

Immunofluorescence assays of the binding of A-BL antibodies to surface antigens of teratocarcinoma cell lines (F9, Nulli-SCC-1, PSA-1, PCC4-Aza-1) and C-virus infected cell lines (1356, Dog C 57L) are without exception negative. Likewise, no reactivity against frozen sections of adult tissues (liver, kidney,spleen, skeletal muscle, brain, ovary, testis) or live spleen cells, thymus cells or sperm can be detected by immunofluorescence.

Immunofluorescence assays for the activity of anti-mouse alpha-fetoprotein, anti-Rausher leukemia virus gp 69/71, and anti-feline leukemia virus gp 70 sera against surface antigens expressed by 8-cell to morula stage embryos are also negative.

(b) Immunoradiolabeling - The binding of A-BL antibodies to preimplantation-stage embryos assayed by immunoradiolabeling parallels that detected by immunofluorescence (Fig. 4). Binding to unfertilized eggs is low (5.1 counts/minute/egg) but a significant increase ($\rho < 0.5$, Mann-Whitney U test) in binding can be detected at the 2-cell stage

FIGURE 1. Indirect immunofluorescence assay of the binding of A-BL antibodies to preimplantation mouse embryos, (a) unfertilized egg, (b) zygote, (c) 2-cell, (d) 4-cell, (e) 8-cell, (f) morula, (g) blastocyst, (h) inner cell mass. (X 350 except b and d X 250).

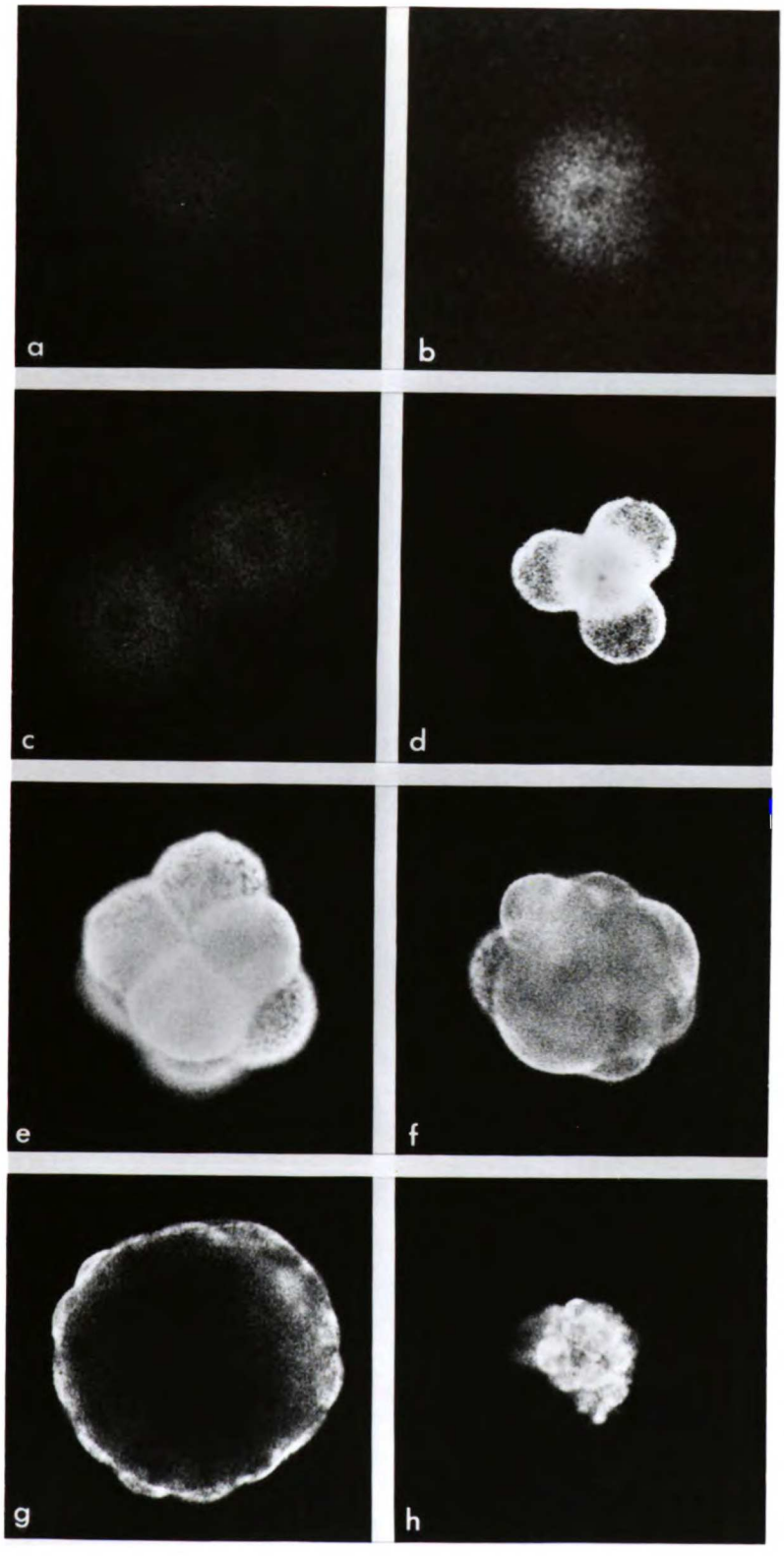


FIGURE 1

FIGURE 2. Indirect immunofluorescence assay of the binding of normal rabbit serum antibodies to preimplantation mouse embryos (a) unfertilized egg, (b) zygote, (c) 2-cell, (d) 4-cell, (e) 8-cell, (f) morula, (g) blastocyst, (h) inner cell mass. (X 350 except b and d X 250).

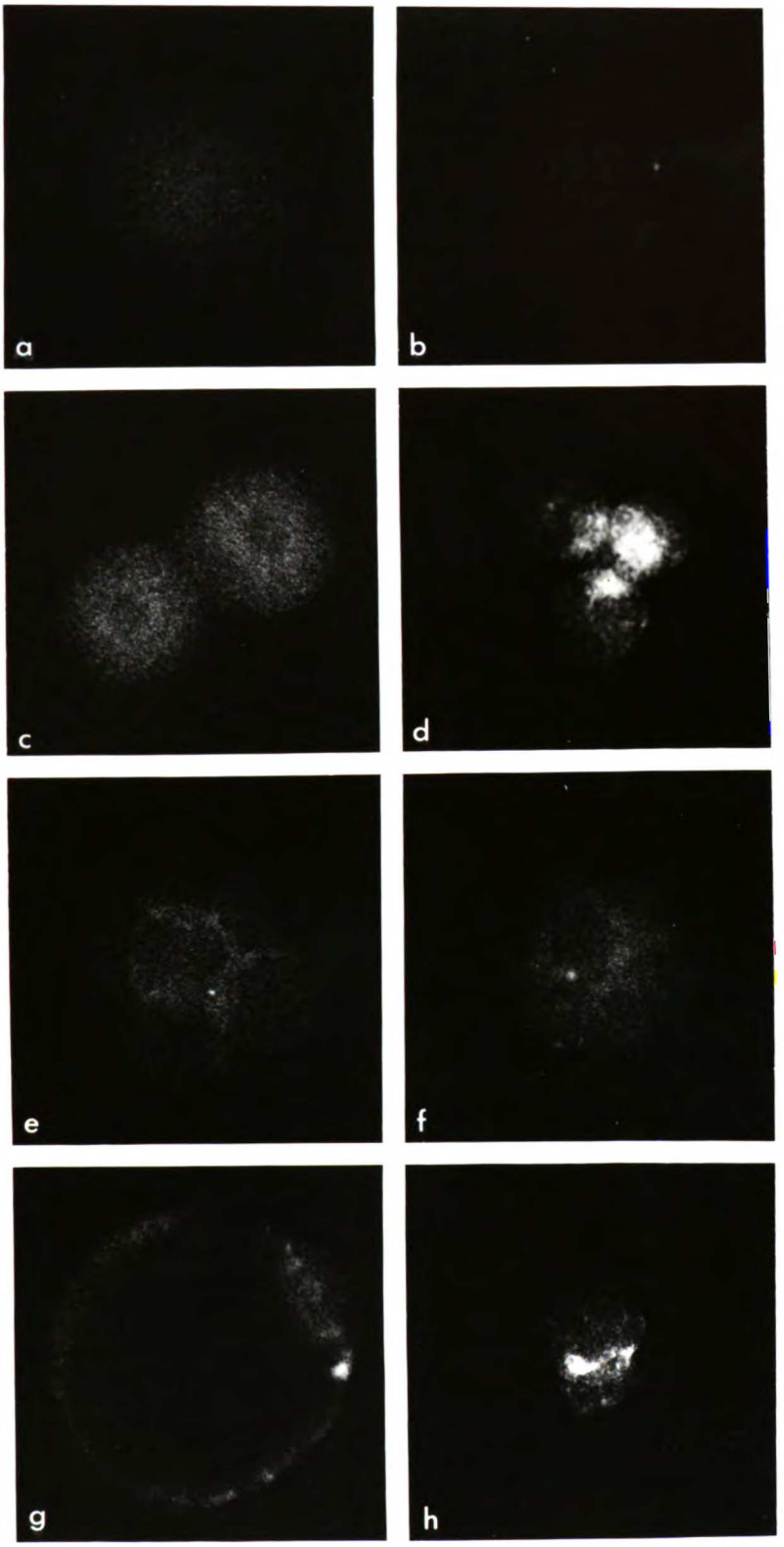


FIGURE 2

FIGURE 3. Indirect immunofluorescence assay of the binding of A-BL antibodies to preimplantation mouse embryos. (a) 8-cell blastomere showing punctate distribution of A-BL staining (X 2800). (b) compacting 8-cell embryo showing redistribution of A-BL antigens (X 350). (c) 8-cell embryo treated with 1 mg/ml pronase for 30 minutes prior to immunofluorescence assay (X 350). (d) Untreated control and (e) β-N-acetylglucosaminidase-treated (500 μg/ml, 2 hours) 8-cell embryos (X 350).

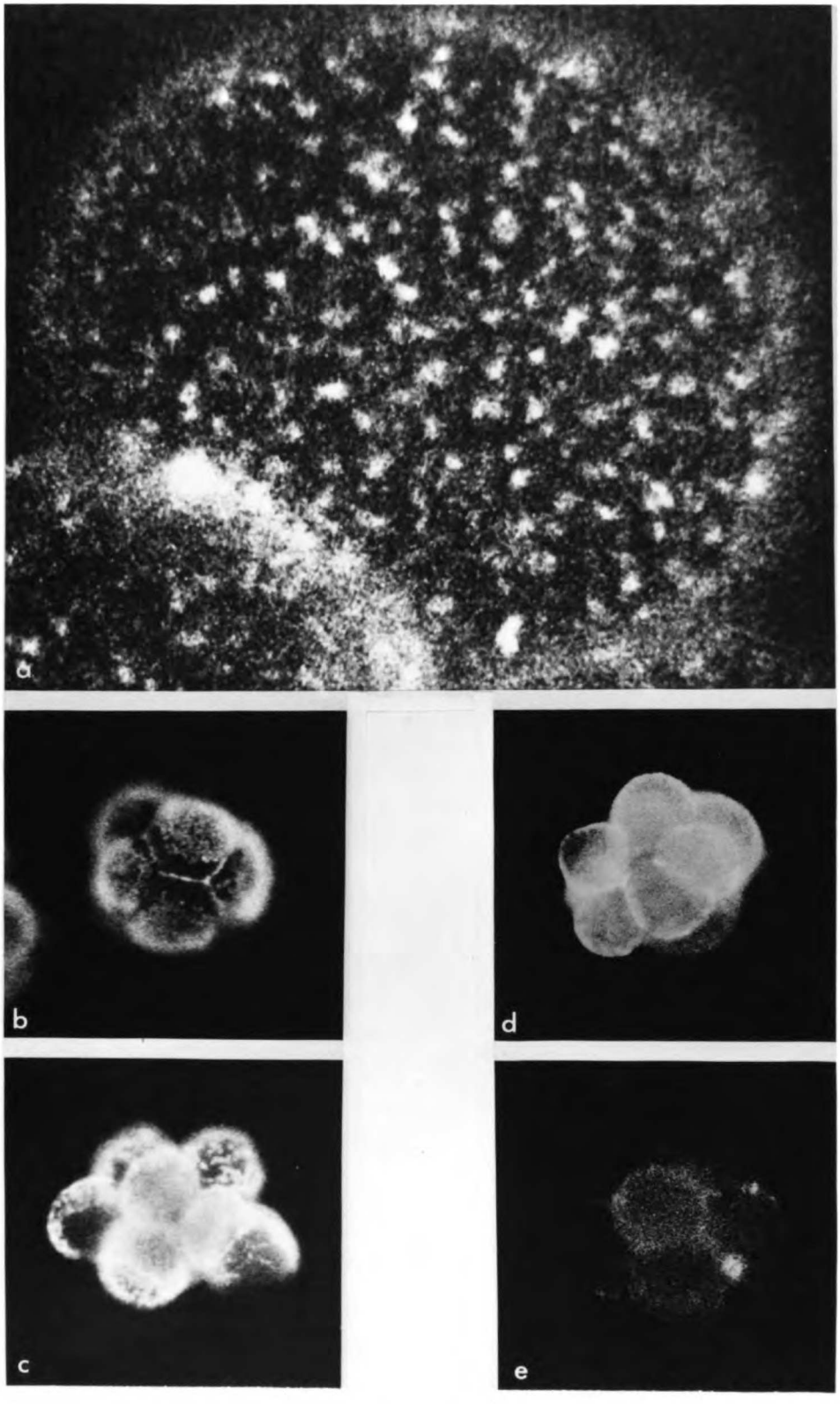


FIGURE 3

FIGURE 4. Immunoradiolabeling assay for the binding of A-BL and normal rabbit serum antibodies to preimplantation mouse embryos. Each point represents the mean of three separate experiments ± standard error. Non-specific background counts have been subtracted. UE (unfertilized egg), 2C (2-cell), 8C (8-cell), MR (morula), BL (blastocyst).

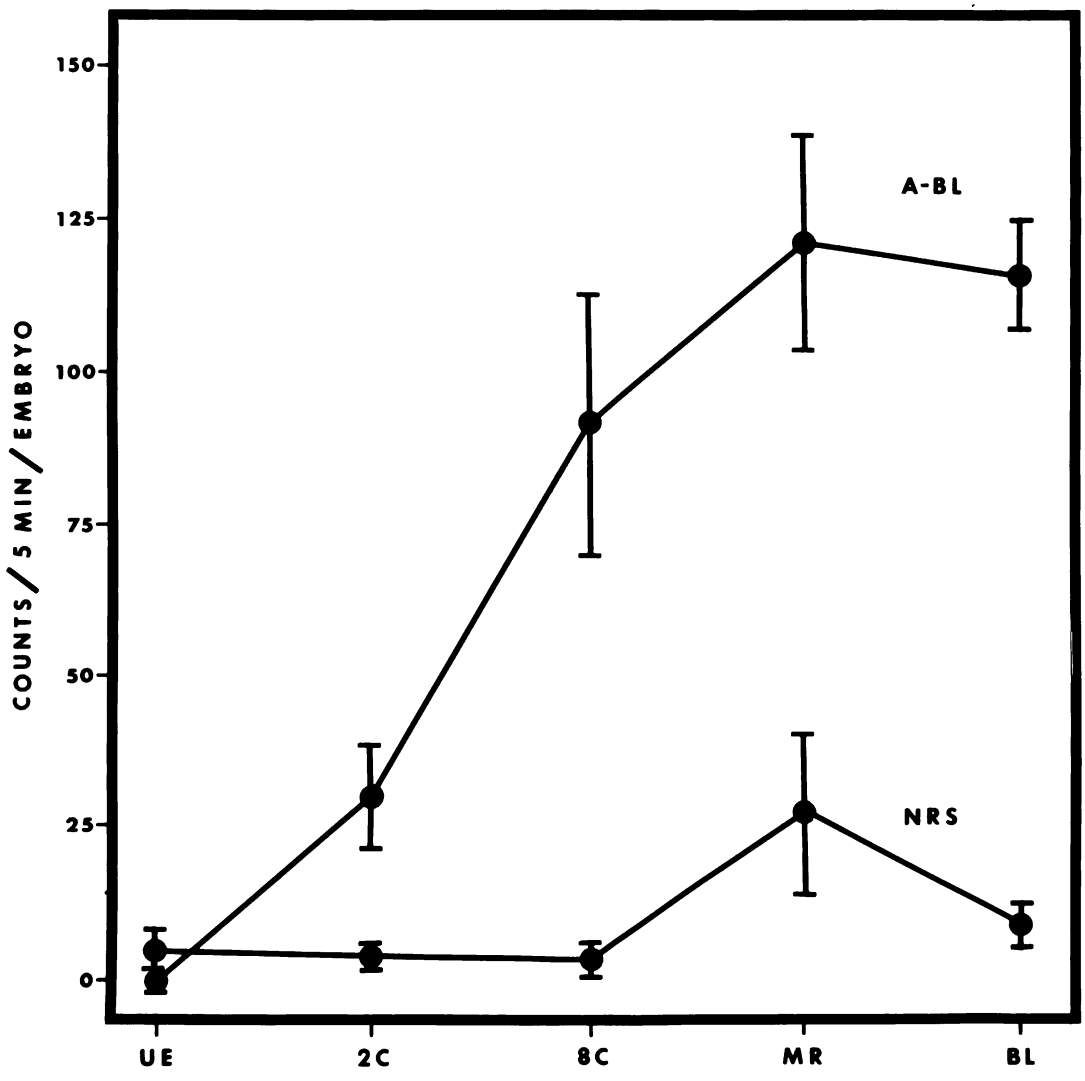


FIGURE 4

(31.6 C P 5M/embryo). A significant increase in antibody binding is also detected between the 8-cell (91.3 C P 5M/embryo) and morula (132.5 C P 5M/embryo) stages ($\rho < 0.05$), but not between the morula and blastocyst (155.4 C P 5M/embryo) stages ($\rho < 0.20$). However, if the increased non-specific binding of ^{125}I-GAR at the morula and blastocyst stages in considered, there is not significant difference in labeling between the 8-cell and blastocyst stages ($\rho < 0.20$-0.50). The apparent decrease in the immunofluorescent staining of blastocysts may be the result of increased surface area or an optical effect of the blastocoel cavity. In control experiments, the binding of NRS remains low through the 8-cell stage but significant increases in the binding of NRS antibodies are detected at morula (38.0 C P 5M/embryo, ($\rho < 0.05$) and blastocyst (47.9 C P 5M/embryo, $\rho \leq 0.10$) stages; however, increases in the non-specific binding of ^{125}I-GAR in the absence of first antibody are also observed at these stages. The labeling of embryos treated with unlabeled GAR prior to ^{125}I-GAR is indistinguishable from background. Analysis of significance utilizing the students t test verified that determined by the Mann-Whitney U test.

(c) Cytotoxicity - The sensitivity of preimplantation embryos to complement-mediated cytotoxicity in the presence of A-BL antibodies reflects the increase in antibody-binding from the 2-cell stage onward detected by immunofluorescence and immunoradiolabeling (Fig. 5a,b). No cytotoxic activity can be detect against unfertilized eggs or zygotes by A-BL serum at dilution as low as 1/2 or IgG at concentrations as high as 1000 µg/ml. Two-cell embryos are sensitive only to very high concentrations of serum (dilutions between 1/2 and 1/8) and only 10%

of 2-cell embryos are lysed by complement following exposure to A-BL IgG at 1000 µg/ml. Later stages (4-cell through blastocyst) are sensitive to lower concentrations of antibody (serum dilutions between 1/40 and 1/120, 100-400 µg/ml IgG) suggesting a higher level of antibody binding at these stages. Neither normal rabbit serum or IgG is cytotoxic in the presence of complement at any preimplantation stage, nor can non-specific toxicity of guinea pig or rabbit complement be detected.

(d) Immunodiffusion and Immunoelectrophoresis - Regardless of the solubilization procedure utilized, no precipitin reaction can be visualized between A-BL and preimplantation embryos in double immunodiffusion (Ouchterlony) assays. This is true even when detergent-containing agarose is used to inhibit reaggregation of detergent-solubilized proteins. Neither can a precipitin reaction be detected when A-BL is tested against NP-40 extracts of adult tissues. By immunoelectrophoresis in the presence of NP-40, however, a single precipitin arc is formed between A-BL and NP-40-solubilized 8-cell embryos (Fig. 6). The lack of a visible reaction in simple immunodiffusion tests may reflect a lack of adequate solubilization and perhaps residual association with membrane components such that A-BL antigens do not diffuse through the agarose matrix. The precipitin arc formed in immunoelectrophoresis assays indicates that even on electrophoresis A-BL antigens migrate very little suggesting a relatively large molecular species (again possibly due to aggregation or persistent association with membrane components and/or relatively little charge at the pH utilized (8.6).

FIGURE 5. Cytotoxicity assay for A-BL antibody activity against preimplantation mouse embryos. (a) Serum, (b) IgG. Each point represents the mean of two or more separate experiments.

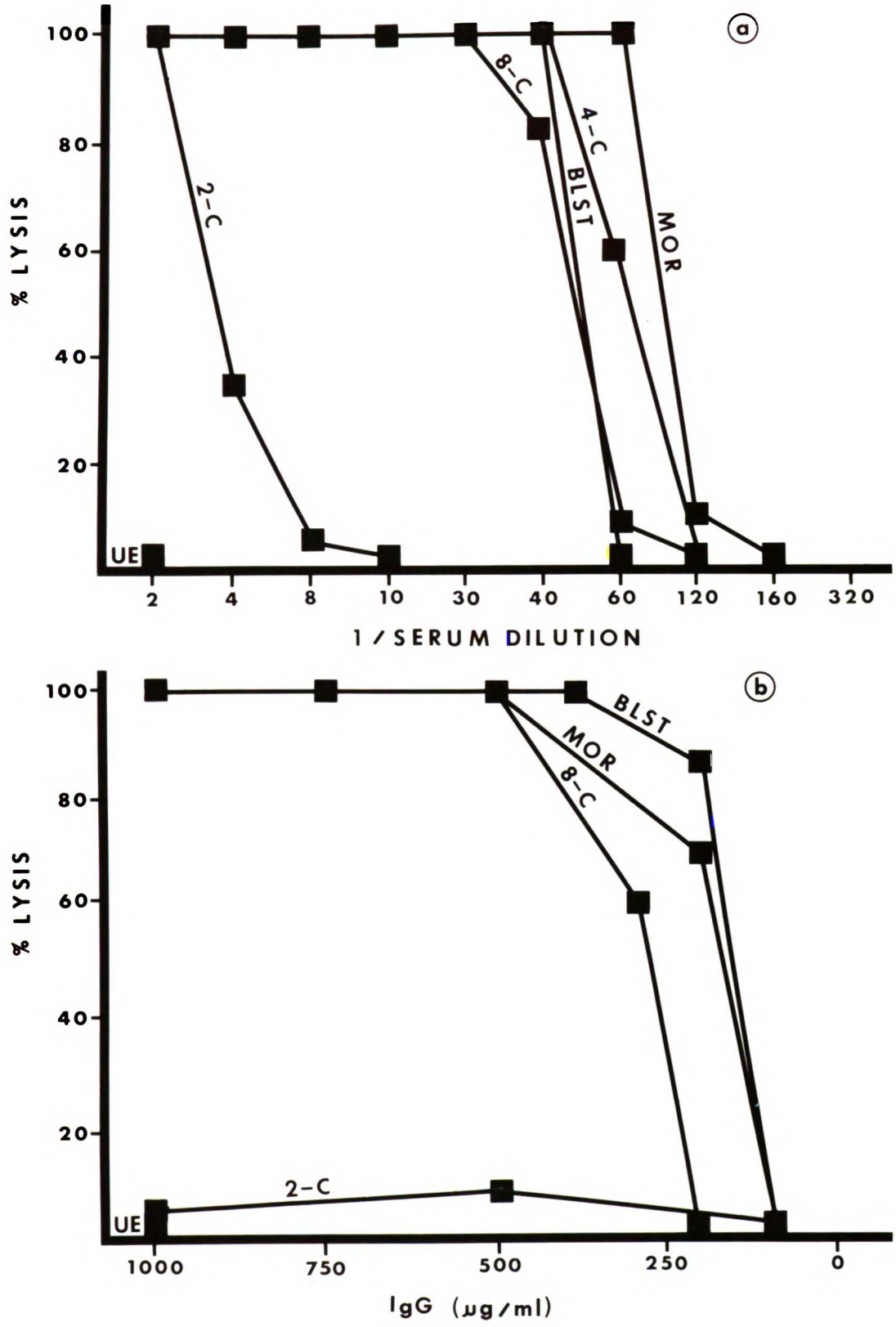

a
100
80
60
40
20
% LYSIS
2-C
8-C
4-C
BLST
MOR
UE
2
4
8
10
30
40
60
120
160
320
1 / SERUM DILUTION
b
100
80
60
40
20
% LYSIS
BLST
MOR
8-C
2-C
UE
1000
750
500
250
0
IgG (µg/ml)

FIGURE 5

FIGURE 6. Immunoelectrophoretic assay of the activity of A-BL and normal rabbit serum (NRS) antibodies against 8-cell mouse embryos. A single precipitin arc is formed between NP-40-solubilized 8-cell embryos and A-BL serum but not by normal rabbit serum.

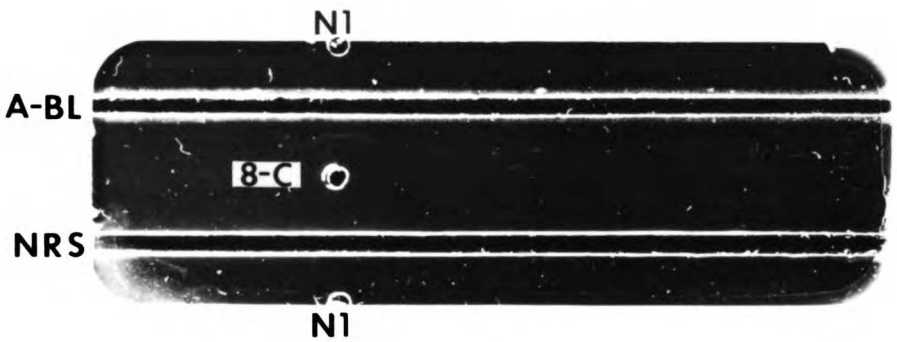


FIGURE 6

Immunodiffusion assays of the reactivity between A-BL and fetal mouse serum or amniotic fluid (tested because of their high alpha-fetoprotein content) and purified human alpha-fetoprotein were negative.

2. A-BL Antibody Effects on In Vitro Development

The presence of A-BL serum or IgG in the culture medium interferes with the normal development in vitro of embryos cultured from the 2-cell stage (Fig. 7). A-BL serum causes a decrease in the proportion of embryos reaching the blastocyst stage at dilutions as high as 1/160 while no embryos reach this point in development at a serum dilution of 1/20. The effects of A-BL IgG at concentrations ranging from 100-1000 µg/ml parallel those of A-BL serum. The presence of A-BL serum or IgG in culture typically causes a cessation of cell division at the 4-cell to morula stages without apparent cytotoxicity (as determined by trypan blue exclusion), however, at higher concentrations (serum dilution $\leq$1/40, IgG $\geq$ 500 µg/ml) embryo death and disintegration are often observed. The results of tests of the stage-specificity and reversibility of the effects of A-BL antibodies on development are preliminary but suggest a critical period for antibody effect between the 2- and 8-cell stages. Embryos exposed to A-BL antibodies for 24 hours beginning at the 2- or 8-cell stage typically develop poorly on removal to normal embryo culture medium, while embryos exposed as morulae fare better and there is no apparent effect on established blastocysts.

Since multivalent antibodies may induce effects due to redistribution, phagocytosis or shedding of surface antigens, monovalent Fab

FIGURE 7. Effects of *in vitro* culture in the presence of A-BL or normal rabbit serum (NRS). Each point represents the mean of at least two separate experiments.

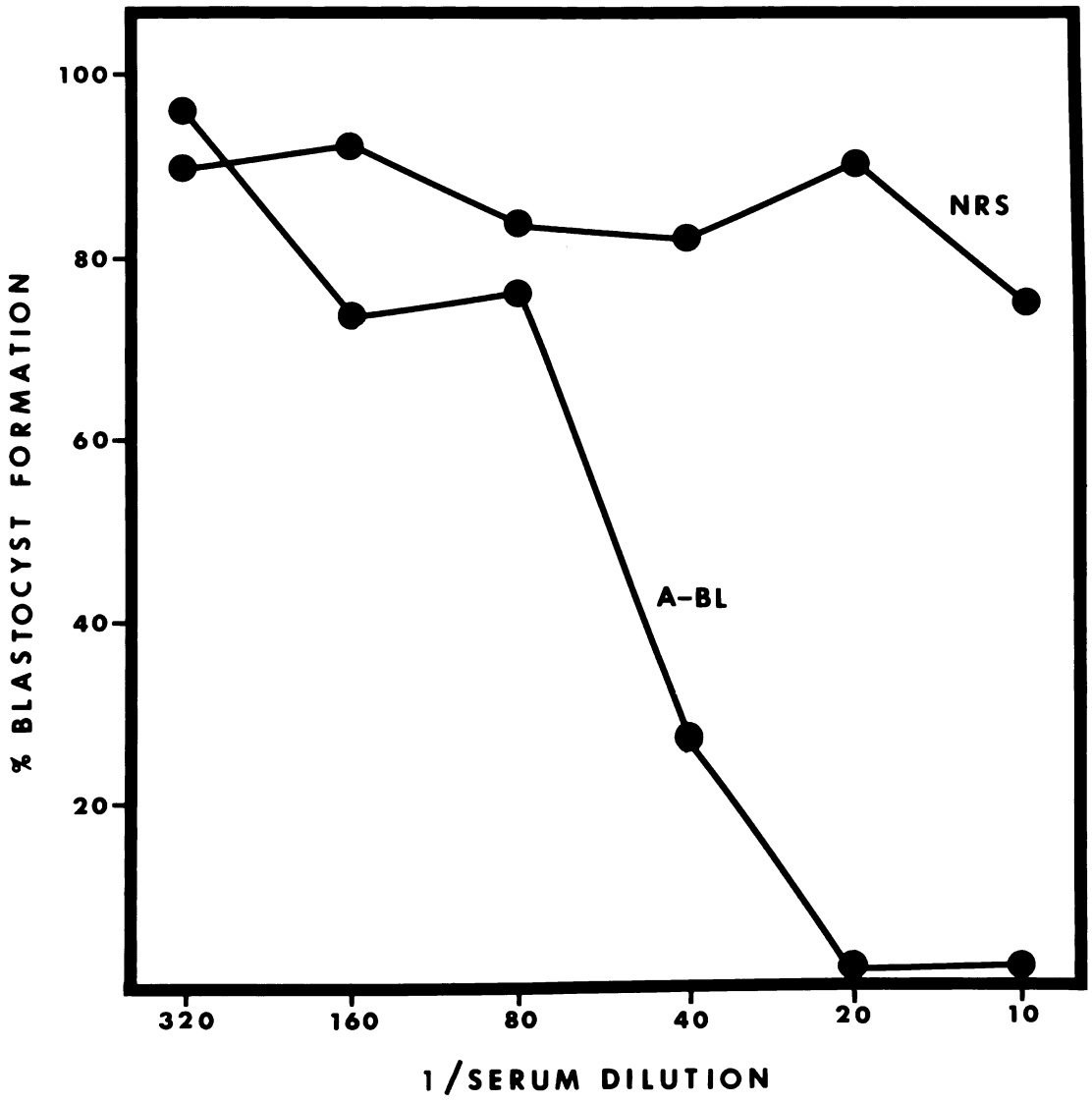

100
80
60
40
20
% BLASTOCYST FORMATION
NRS
A-BL
320
160
80
40
20
10
1/SERUM DILUTION

FIGURE 7

fragments which do not have similar effects are often utilized in studies of the functional aspects of cell surface antigens. However, the results of studies of the effects of A-BL Fab on preimplantation development have thus far proved inconclusive. Immunofluorescence assays show that A-BL Fab has similar anti-embryo activity to that observed for intact A-BL IgG and that Fab prepared from normal rabbit serum IgG is consistently non-reactive. However, Fab preparations from both A-BL and normal rabbit serum have non-specific toxic effects on embryonic development in vitro. It is suspected that reagents used in the preparation of Fab fragments may be contaminating and causing non-specific toxicity. Efforts to produce Fab reagents which will be useful in the investigation of possible functional roles for A-BL antigens are continuing.

3. Molecular Characterization of A-BL Antigens

(a) Immunoprecipitation of NP-40 extracts of 8-cell embryos labeled with ^{35}S-methionine shows that A-BL antibodies react primarily with two protein species (gpe 69/71) which migrate as a closely spaced pair of bands on one-dimensional SDS-polyacrylamide gels with apparent molecular weights of 69,000 and 71,000 daltons (Fig. 9). These proteins are not, however, precipitated from 8-cell embryos by normal rabbit serum or from labeled unfertilized eggs (which do not express A-BL surface antigens) by A-BL antibodies. Neither are the gpe 69/71 proteins apparent in the A-BL immunoprecipitates from ^{35}S-methionine-labeled teratocarcinoma cells (Nulli-SCC-1) or from the C-virus-producing cell lines 1356 and Dog C 57L. The precipitation of gpe 69/71 by A-BL from 8-cell embryos but not from unfertilized eggs, and the

absence of any detectable species precipitated by normal rabbit serum are consistent findings having been confirmed in a minimum of six separate experiments.

Immunoprecipitates from 8-cell embryos with surface proteins radiolabeled by lactoperoxidase iodination do not contain sufficient protein-bound radioactivity to make gel electrophoresis and autoradiography feasible. These results are not suprising since the electrophoretic profiles of surface proteins of lactoperoxidase-labeled 8-cell embryos do not contain strongly labeled bands in the 70,000 dalton region (see below). Surface proteins often vary in their susceptibility to lactoperoxidase labeling (see Hubbard and Cohn, 1977), a fact which may be due to relatively low tyrosine content or the physical inability of the labeling enzyme to reach tyrosine residues either due to protein configuration or exclusion by carbohydrate residues. However, immunoprecipitates from ^{35}S-methionine-labeled embryos treated with A-BL antibodies while alive such that only surface antigens are available for antibody binding (Gupta et al., 1977) do contain the 69,000 and 71,000 dalton proteins confirming their surface expression. The autoradiographic intensity of these protein bands appears to be less than that in immunoprecipitates from NP-40-solubilized embryos. However, a quantitative estimate of the amounts of A-BL antigens present at the cell surface versus that present intracullularly has not been attempted. While it is possible in this type of experiment that antibodies bound to surface antigens may dissociate and recombine with intracellular antigens once the embryos are solubilized, it has been shown in an investigation of viral surface antigens that this occurence probably makes only a minimal contribution to the final immuno-

precipitate (< 10% even in the presence of a tenfold excess of unbound antigen; Gupta et al., 1977).

Preliminary evidence from the analysis of immunoprecipitates from 8-cell embryos by two-dimensional gel electrophoresis confirms the specificity of A-BL antibody reactivity revealing three pairs of spots in the 70,000 dalton region with different isoelectric points (Fig.10b). These pairs of spots, in addition to a large number of others, are clearly evident in the NP-40 extract of 8-cell embryos (Fig. 10a). The three pairs of immunoprecipitated spots are all located in the more basic region of the gel with the most basic pair having the highest autoradiographic grain density and the more acidic pairs lower densities. Five low-density spots present in the more acidic region of the gels are the only recognizable spots present on autoradiographs of gels of normal rabbit serum immunoprecipitates from 8-cell embryos (Fig. 10c). The above represent the results obtained in a single two-dimensional electrophoretic analysis; confirmatory analyses are currently in progress.

(b) Cell Surface Labeling - Electrophoretic analysis resolves a number of protein bands which are labeled by lactoperoxidase-catalyzed iodination of preimplantation embryos (Fig. 8, Table 3). It is apparent from such analyses that a major qualitative change in embryonic cell surface proteins occurs following fertilization, between the unfertilized egg and the 2-cell stage. The surface of the zygote appears to be transitional between those of the unfertilized egg and the 2-cell embryo possessing proteins characteristic of both stages. Of the 24 labeled protein bands resolved from unfertilized eggs, 16 are apparently egg-specific and not expressed at cleavage stages. The

majority of the proteins expressed at the 2-cell stage (17 of 19) continue to be expressed through the morula stage and 12 of these are also present on blastocysts. There also appear to be stage-specific protein bands expressed by 8-cell and morula-stage embryos. These observations are in general agreement with those made by one-dimensional (Epstein and Smith, 1974; Van Blerkom and Brockway, 1975; Martin et al., 1978) and two-dimensional (Levinson et al., 1978) gel analyses of mouse preimplantation development which show that the major qualitative changes in protein synthetic patterns occur early in development and that synthetic patterns remain relatively uniform from the 4-cell through the blastocyst stage. However, there is apparently no surface expression of a 36,000 dalton protein synthesized only at the 8-cell stage (Martin et al., 1978) although a protein of approximately this molecular weight does appear on the embryonic cell surface at the blastocyst stage.

An additional major qualitative shift in surface protein composition detectable by lactoperoxidase labeling is observed on blastocyst formation. At least six blastocyst-specific proteins (63, 35, 33, 21, 19, 17 x 10^3 daltons) appear at this stage while six proteins present on morulae are no longer labeled. Surface proteins which appear at the blastocyst stage are of relatively low molecular weight in contrast to the report of Pinsker and Mintz (1973) that surface glycoproteins are enriched in forms of higher molecular weight at the blastocyst stage. The 63,000 dalton blastocyst-specific surface protein corresponds in molecular weight to a trophectoderm-specific protein synthesized at morula and blastocyst stages (Martin et al., 1978). However, there is no evidence to suggest that these proteins are the same or

FIGURE 8. Autoradiograph of 10% polyacrylamide gel showing lactoperoxidase-labeled cell surface proteins of the unfertilized egg (UE), zygote (ZG), 2-cell (2C), 8-cell (8C), morula (MR), and blastocyst (BL)-stage mouse embryo. Each track represents 20-40,000 TCA-precipitable cpm ^{125}I from approximately 200 embryos.

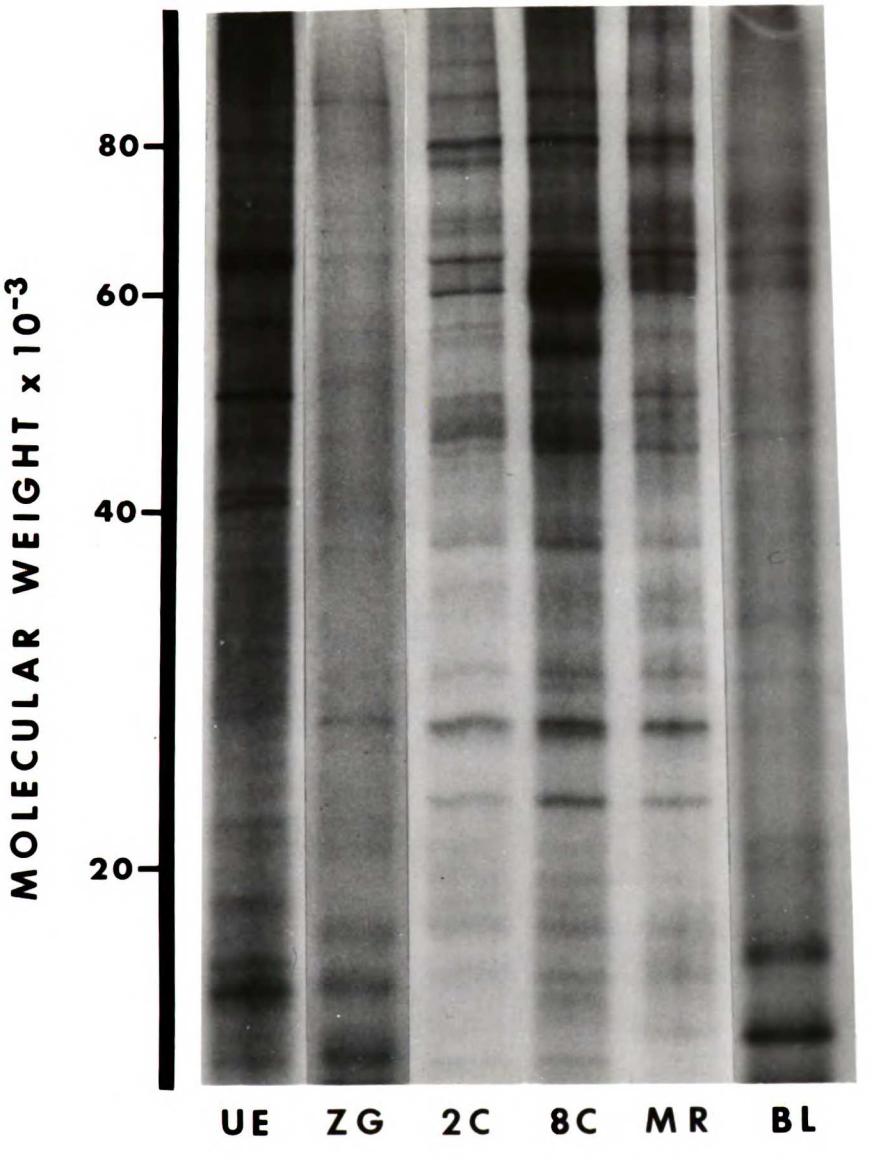


FIGURE 8

TABLE 3. Representation of cell surface proteins of preimplantation mouse embryos labeled by lactoperoxidase-catalyzed iodination. UE (unfertilized egg), ZG (zygote), 2C (2-cell), 4C (4-cell), 8C (8-cell), MR (morula), BL (blastocyst).

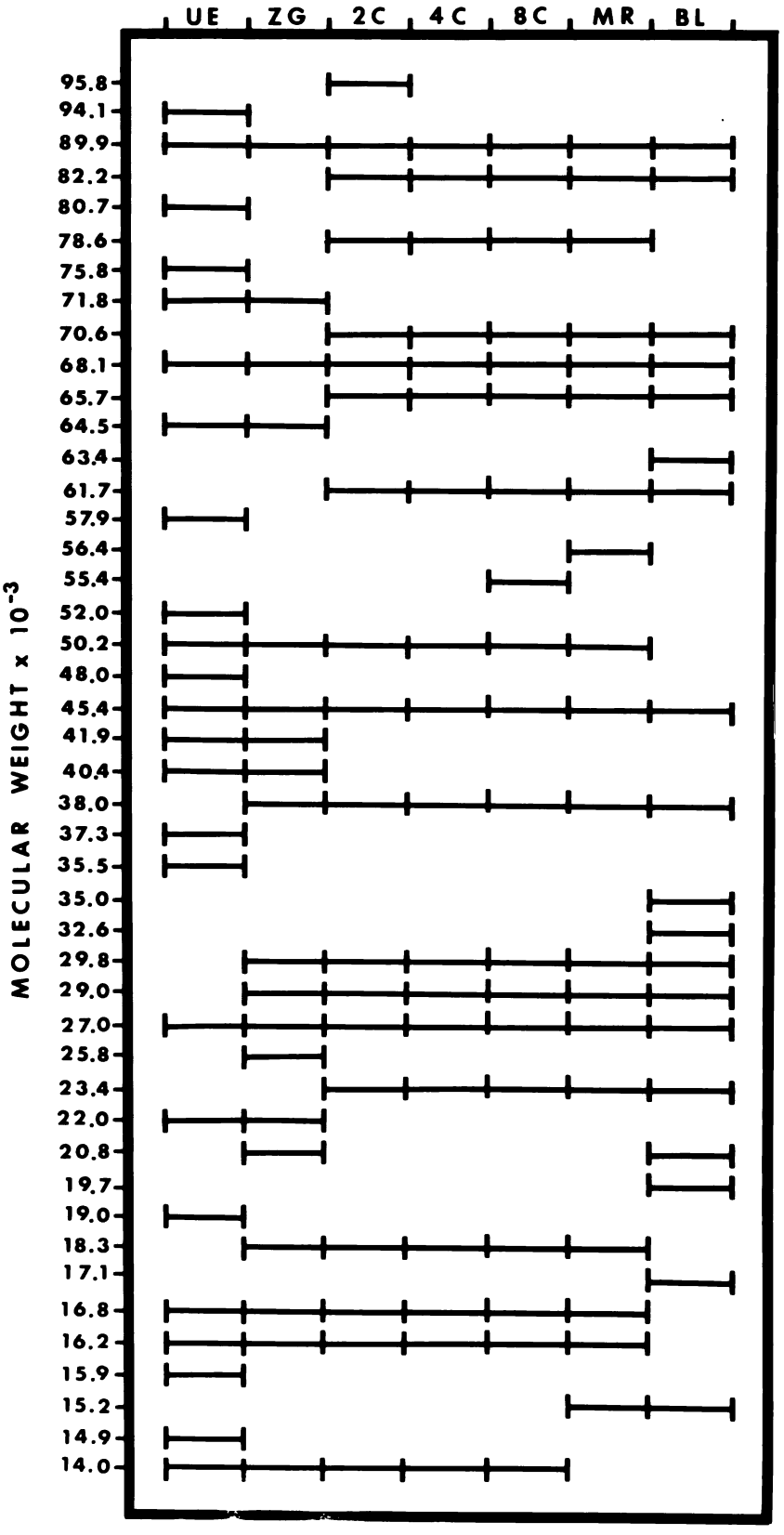
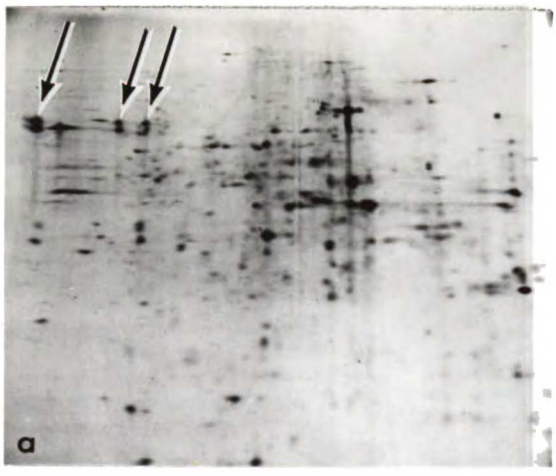

UE
ZG
2C
4C
8C
MR
BL
MOLECULAR WEIGHT x 10^{-3}
95.8
94.1
89.9
82.2
80.7
78.6
75.8
71.8
70.6
68.1
65.7
64.5
63.4
61.7
57.9
56.4
55.4
52.0
50.2
48.0
45.4
41.9
40.4
38.0
37.3
35.5
35.0
32.6
29.8
29.0
27.0
25.8
23.4
22.0
20.8
19.7
19.0
18.3
17.1
16.8
16.2
15.9
15.2
14.9
14.0

TABLE 3

FIGURE 9. Autoradiograph of 10% polyacrylamide gel showing the results of immunoprecipitation of ^{35}S-methionine-labeled 8-cell mouse embryos by A-BL or normal rabbit serum (NRS) IgG. The primary molecular species precipitated by A-BL antibodies (gpe 69/71) are indicated at 69,000 and 71,000 daltons.

FIGURE 9

FIGURE 10. Autoradiographs of two-dimensional gels with isoelectric focusing in the horizontal dimension (basic left) and molecular weight separation in the vertical dimension (origin top). (a) NP-40 extract from ^{35}S-methionine-labeled 8-cell mouse embryos. (b) A-BL immunoprecipitate from sample identical to (a). (c) Normal rabbit serrum immunoprecipitate from sample identical to (a).



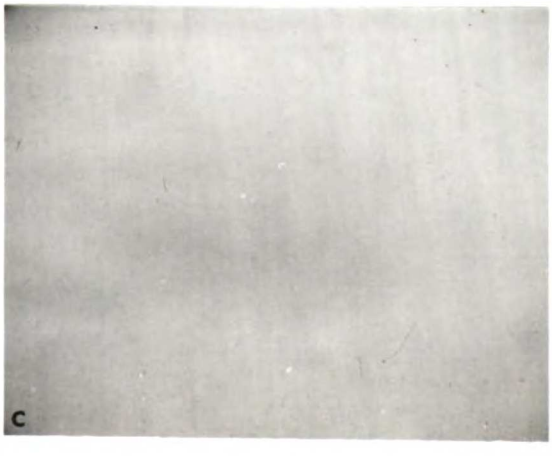


FIGURE 10

even related.

It is clear from these analyses that a number of embryonic cell surface proteins can be labeled by lactoperoxidase at all stages tested. The described electrophoretic profiles are, however, probably only a minimal representation of surface protein composition owing to the limitations of sensitivity of the system, the probable existence of higher and lower molecular weight proteins not resolved by the gel system utilized and the known variations in the susceptibility of surface molecules to lactoperoxidase labeling (Hubbard and Cohn, 1977).

(c) Enzyme Treatment - Of the enzymes tested, only B-N-acetyl-glucosaminidase affected the binding of A-BL antibodies. Treatment of 8-cell or morula-stage embryos with 500-1000 µg/ml B-N-acetylglucosaminidase for 1-2 hours prior to immunofluorescence assay reduced the fluorescent staining to control levels (Figs. 3d,e). Embryos treated with other glycosidases, proteases, lipases, and nucleosidases showed no reduction in the intensity of fluorescent staining. However, a number of proteases (trypsin, pronase, bromelain, and kallekrein) and phospholipase C all induced a change in the distribution of fluorescence from diffusely punctate to one in which large patches of fluorescence were evident (Fig. 3c), while the overall intensity of the fluorescent staining is not strikingly different from that of untreated controls.

(d) Hapten Inhibition - None of the sugars or glycoproteins utilized in hapten-inhibition studies regularly caused a reduction in the cytotoxic or IIF staining activity of A-BL antibodies. At times, high concentrations (100 mM) of D-mannose or N-acetyl-D-galactosamine

reduced the cytotoxicity of A-BL but these results were not consistent.

(e) Blocking Experiments - No change in the density of A-BL binding in immunofluorescence assays is observed following pretreatment of embryos with lectins. However, pretreatment with wheat germ agglutinin, a lectin specific for N-acetylglucosamine residues, results in a more diffuse, non-punctate distribution of fluorescent staining compared to non-lectin-treated controls.

Pretreatment of 8-cell to morula stage embryos with either syngeneic or monoclonal anti-F9 sera has no apparent effect on the binding of A-BL antibodies. The binding of anti-F9 antibodies was confirmed by immunofluorescence using fluorescein-labeled rabbit anti-mouse immunoglobulin.

(f) Metabolic Inhibitors - Cycloheximide and puromycin both inhibit cleavage of preimplantation embryos at concentrations as low as 1.0 μg/ml. However, a 24 hour exposure to these inhibitors of protein synthesis is not generally toxic in that embryos so treated appear normal and exclude trypan blue. Two-cell embryos assayed for A-BL binding following exposure to cycloheximide or puromycin remain negative while similarly treated 8-cell embryos continue to stain heavily in IIF assays. However, the distribution of fluorescent staining on puromycin- or cycloheximide-treated 8-cell embryos is patchy resembling that of emzyme-treated embryos described above (Fig. 3c).

DISCUSSION

The studies described here deal with the characterization of cell surface antigens expressed during preimplantation mouse development and detected by a rabbit antiserum directed against mouse blastocysts. The investigations have been focused in three major areas: (1) Reactivity of A-BL antibodies, (2) A-BL antibody effects on development, (3) Molecular characterization of A-BL antigens.

1. Reactivity of A-BL Antibodies

The results obtianed suggest that the cell surface antigens detected by A-BL antibodies may be embryo-specific. Thus, following absorption with adult mouse tissues, antibody activity against preimplantation-stage embryos remains while none is detected against adult somatic or germ-line tissues. It is acknowledged that not all adult tissues have been tested and therefore A-BL antigens can be considered embryo-specific only with the qualifacation that reactivity with untested tissues may exist. It is also possible that the embryonic antigens recognized by A-BL antibodies may go undetected on adult tissues due to their presence only at relatively low concentrations. Low-level antigenic expression by adult tissues has been described for a number of oncofetal or carcinoembryonic antigens which are present primarily during embryonic development and associated with malignant transformation (see Alexander, 1972; Costanza and Nathanson, 1974; Jerry et al., 1976).

The apparent embryo-specificity of the A-BL antigens described here is in agreement with that reported for the anti-blastocyst serum described by Wiley and Calarco (1975). Other antisera reported to recognize surface antigens of preimplantation-stage embryos (see

Introduction) have not been prepared directly against embryonic material and their reactivity appears to be less specific. Most show activity against one or more adult tissues (most notably brain and sperm), tumor cells (most notably teratocarcinoma cells) and/or a variety of normal and transformed cell lines. It appears therefore that early mammalian embryos share antigenic determinants with a number of different cell types but may also possess antigens with expression limited to the early embryogenic period. Such expression is suggestive of an embryo-specific function not required of adult cells or the existence of an antigenically different embryonic form of a normal cellular consistuent, such as has been shown for the enzyme alkaline phosphatase (see Fishman, 1974).

A-BL antibodies also fail to recognize surface antigens expressed by teratocarcinoma cell lines, both nulli- and milti-potential. These results seem somewhat inconsistent in light of the numerous reports of antisera prepared against teratocarcinaoma cell lines which recognize antigens expressed during preimplantation development (see Introcution) and the strong analogies between teratocarcinoma cells and normal embryonic cells (see Martin, 1975, 1978; Jacob, 1975, 1977, 1978; Hogan, 1977). The lack of A-BL-detected cross-reactivity between teratocarcinomas and embryos might be explained, however, by the fact that the biochemical composition of teratocarcinoma cells probably most closely resembles that of the embryonic ectoderm of the day six mouse embryo (Martin et al., 1978) rather than that of cleavage stages which show the most significant expression of A-BL antigens. The embryonic ectoderm first differentiates morphologically as part of the inner cell mass of the expanded (day 4) blastocyst while the primary expression

of A-BL-detected antigens apparently occurs 24-48 hours earlier at the 8-cell to morula stages and only a low level of A-BL binding is detected on the inner cell mass (i.e. its outer endodermal layer) or trophoblast of *in vitro* blastocyst outgrowths. In fact, electrophoretic evidence discussed below suggests that A-BL antigens may no longer be synthesized by the blastocyst stage. The extent of possible cross-reactivity of A-BL antibodies against the embryonic ectoderm has not been established, however, antigens which are detected by anti-F9 teratocarcinoma sera and show a peak in surface expression on embryos at about the same time as A-BL antigens have been shown to become localized to the embryonic ectoderm by the eighth day of gestation (Jacob, 1977). Since A-BL antibodies may be detecting virus-like antigens related to those of murine C-type viruses (see below), and alternative explanation for the lack of cross-reactivity between A-BL antibodies and teratocarcinoma cells may lie in the fact that such cells typically do not show viral expression (G. Martin, personal communication).

During the preimplantation period the expression of antigens recognized by A-BL antibodies is stage related. There is no detectable surface expression of A-BL antigens until the 2- to 4-cell stage. Immunofluorescence assays detect no expression until the 4-cell stage while low levels of antigenic expression are detectable at the 2-cell stage by immunoradiolabeling and cytotoxicity assays. Maximal immunofluorescent staining is observed at the 8-cell to morula stage with a slight decline apparent coincident with blastocyst formation. However, this apparent decline in antigen expression may be an optical artifact due to an increase in surface area without increased antigenic expres-

sion, increased embryo diameter and/or properties of the blastocoel cavity itself. A similar decline is not detected by immunoradiolabeling in which there is no significant difference between the labeling of morulae and blastocysts when values are corrected for nonspecific labeling. By cytotoxicity assays, there appears to be little change in sensitivity to complement-mediated lysis from the 4-cell through the blastocyst.

The stage-specificity of expression of A-BL antigens observed here is similar to that described for the anti-blastocyst serum of Wiley and Calarco (1975). A minor exception appears in the relative decline in immunofluorescent staining at the blastocyst stage; that described by Wiley and Calarco (1975) appearing to be greater than observed in these investigations.

The appearance of A-BL antigens at the cell surface correlates temporally with significant changes in the qualitative pattern of protein synthesis which occurs between the 2- and 8-cell stages and the increased antigen expression on 8-cell to morula stages correlates with the large increase in overall protein synthetic activity observed between the 8-cell and blastocyst stages (see Epstein, 1975; Johnson et al., 1977; Van Blerkom and Manes, 1977). Electrophoretic evidence (discussed below) indicates that A-BL antigens are in fact synthesized during this period indicating that they are not merely unmasked at the cell surface at this time (by a newly-synthesized or newly-activated enzyme for example). It also appears that A-BL antigens may not be synthesized following the morula (see below) stage and therefore a decline in expression might be expected at subsequent stages. As noted above, immunofluorescent staining does detect an apparent blastocyst-

associated decline. Additionally, results of immunofluorescence assays of A-BL antibody binding to in vitro blastocyst outgrowths indicate only low levels of antigen expression by both the inner cell mass and trophoblastic portions of outgrowths.

Interaction of A-BL antibodies with embryonic surface antigens does not induce patching or capping. Immunofluorescent staining is diffusely punctate even when assays are performed at 37°C and are extended to six hours. These results indicate that A-BL antigens have limited mobility within the membrane such that antibody interaction induces the formation of only small aggregates or that the normal distribution of A-BL antigens is in small clusters. Antibody-induced redistribution of surface antigens of preimplantation embryos has however been reported for anti-F9 sera (Jacob, 1977) indicating that A-BL antigens differ from F9 antigens even though they are coexpressed on cleavage-stage embryos.

Lectins with a variety of carbohydrate specificities have also been shown to induce patching of surface components of preimplantation embryos (Nicolson et al., 1975; Brownell, 1977). Treatment of embryos with wheat germ agglutinin (WGA) prior to exposure to A-BL antibodies in immunofluorescence assays results in a diffuse, non-punctate staining without an apparent decrease in the overall intensity of staining. WGA is a lectin specific for N-acetyl-D-glucosamine residues, does not induce patching of surface components on mouse morulae or blastocysts (as opposed to concanavalin A and Ricinus communis agglutinins which do so) (Brownell, 1977). Evidence presented here suggests that A-BL antigens contain N-acetylglucosamine residues as part of the antigenic determinant since treatment of embryos with N-acetylglucosaminidase

abolishes the binding of A-BL antibodies. Pretreatment of embryos with WGA may cause anchorage modulation (Edleman, 1976) of glucosamine containing molecules and inhibit the formation of small antigen clusters by A-BL antibodies thereby causing immunofluorescent staining to appear diffuse rather than punctate. The glucosamine residues recognized by WGA apparently differ from those involved in the antigenic site, or WGA interacts with a different portion of glucosamine molecules than A-BL antibodies, since WGA binding does not markedly reduce the binding of A-BL antibodies. It is also possible that WGA does not inhibit the mobility of A-BL antigens by binding directly but rather by the crosslinking of other glucosamine-containing surface molecules such that the mobility of A-BL antigens is physically hindered, while glucosamine-containing antigenic sites are unaffected.

A massive redistribution of A-BL antigens has however been observed during the compaction of 8-cell embryos. At this time, immunofluorescent staining becomes localized to the apical (peripheral) portions of blastomeres. This change in antigen distribution is apparently not antibody induced since it is observed at 0°C and in the presence of monovalent Fab. The redistribution parallels the compaction-related reorganization of microvilli to an apical region and a basal zone of intercellular contact and associated cortical changes observed by Calarco and Epstein (1973) and Ducibella et al. (1977). The segregation of A-BL antigens to the apical region of the blastomere surface may represent a selective segregation of these antigens to the outer (exposed) embryo surface or non-specific migration induced by microvillar and/or cortical changes initiated during the compaction process. Conversely, this apparent redistribution of A-BL antigens may

merely reflect differences in the relative density of plasma membrane per unit area, which is significantly increased by microvilli, rather than quantitative differences in the density of A-BL antigens. However, since such an antigenic redistribution has not been reported for other surface antigens which are expressed at this time (e.g. F9), nor have lectin-binding studies detected this occurence, the redistribution of A-BL antigens may be specific. If so, the restriction of A-BL antigens to the outer embryo surface and their possible exclusion from internalized surfaces may reflect positional (inside vs. outside) differentiation which has been suggested to function during this period of development (see Herbert and Graham, 1974). While the presence of A-BL antigens on the surface of the inner cell mass would argue against it, studies in which sectioned embryos are analyzed for A-BL expression would be required to confirm such a polarization of antigen expression.

Redistribution of A-BL antigens can also be induced by treatment with proteolytic enzymes and inhibitors of protein synthesis. Such treatment results in the aggregation of A-BL-detected antigens into large patches without an apparent decrease in the overall intensity of immunofluorescent staining. These results suggest that protease-sensitive proteins may be involved in maintaining the distribution of A-BL antigens, while the antigens themselves are apparently not protease-sensitive. The observations of protease-induced patch formation may also involve the disruption of submembrane cytoskeletal elements (Furcht and Wendelschafer-Crabb, 1978) which are involved directly or indirectly in establishing the pattern of surface expression of A-BL antigens. Since inhibitors of protein synthesis have similar effects on the distribution of A-BL antigens, continued synthesis of proteins

involved in the maintenance of surface antigen distribution may be required.

2. Antibody Effects on Development

The data presented here and that of Wiley and Calarco (1975) suggest that A-BL antigens may play a functional role during early development. The presence of A-BL antibodies blocks cell division and thus normal development whereas the presence of normal rabbit serum immunoglobulins does not. One might speculate that the observed inhibition of cell division and subsequent development is the result of interference with the normal function of A-BL surface antigens by bound antibodies. However, no specific developmental event appears to be disrupted, such as has been described for anti-F9 Fab by Kemler et al. (1977), and it is therefore difficult to infer a developmental role for A-BL antigens. At this point, it can merely be said that the inhibition of cell division and subsequent development caused by A-BL antibodies correlates with the primary period of antigen expression between the 4-cell and morula stages. Non-specific toxicity cannot be ruled out, however, normal development in the presence of similarly-treated normal rabbit serum would argue against it being a factor. In addition, a number of other antisera have been reported to adversely affect preimplantation development in vitro (Wiley, 1975; Wiley and Calarco, 1975; Kemler et al., 1977; Calarco and Banka, 1979) while others do not (Heyner et al., 1969; Wiley and Calarco, 1975). Unfortunately, attempts to utilize monovalent Fab fragments prepared from A-BL antibodies to differentiate between specific effects caused by antibody-interaction with functional molecular sites and non-specific

effects due to antibody-induced crosslinking, shedding or phagocytosis of reactive antigens have thus far failed. Therefore, the possible functional significance of A-BL antigens remains unclear. However, attempts to develop useful Fab reagents are continuing and should ultimately help decide this question.

3. Molecular Characterization of A-BL Antigens

The results of studies of the biochemical characteristics of A-BL antigens have revealed that the primary antibody activity in A-BL serum resides in the IgG fraction and is directed against two proteins (probably glycoproteins) which are similar in both molecular weight and isoelectric points. These findings are somewhat surprising in that it might be expected that a xenogeneic immunization would produce an antiserum with a wider range of specificities than that which is indicated for the A-BL serum. Perhaps preimplantation embryos, in fact, express only very few embryo-specific antigens not present in adult tissues such that a significant proportion of the antibodies in the immune serum (i.e. those directed against non-embryo-specific antigens) may be removed by the routine absorption with adult tissue. Alternatively, A-BL antigens may be the most highly immunogenic of embryonic molecules and therefore elicit the strongest immunological response (Humphrey and White, 1970). It remains to be determined if the two protein species identified by A-BL antibodies share antigenic determinants and thus could both be identified by a single antibody population or whether two separate antibody populations with different specificities are responsible for their identification. While it appears that the carbohydrate portion of the A-BL antigens detected by

immunofluorescence is responsible to some extent for establishing the molecular configuration of the antigenic determinants, this has not been confirmed for the antigen immunoprecipitated by A-BL antibodies.

Electrophoretic analysis of embryonic proteins precipitable by A-BL antibodies reveals that anti-embryo activity lies principally against a pair of proteins with apparent molecular weights of 69,000 and 71,000 daltons (gpe 69/71) as resolved on one-dimensional SDS-containing acrylamide gels. Minor components detected in the A-BL immunoprecipitate are likely due to subpopulations of antibodies with varying antigenic specificities and non-specific precipitates. Precipitating activity against gpe 69/71 cannot be detected in normal rabbit serum nor can gpe 69/71 be precipitated by A-BL antibodies from unfertilized eggs, which do not express A-BL surface antigens.

Initial analysis of the A-BL immunoprecipitate from 8-cell embryos by two-dimensional electrophoresis reveals that the gpe 69/71 proteins identified in one-dimensional gels consist of pairs of proteins with at least three different isoelectric points. This charge heterogeneity may represent the existence of biosynthetic intermediates which undergo intracellular modification, perhaps involving glycosylation, as has been described for H-2 antigens (Jones, 1977). The glycosylation of proteins with neutral sugars would be expected to produce small increases in molecular weight while the addition of charged sugars, such as sialic acid, would be expected to cause a shift to a more acidic isoelectric point as well as some increase in molecular weight. However, of the three pairs of autoradiographic spots which make up the A-BL immunoprecipitate the more basic pair appears to be larger and

and more dense than the other two. This would suggest that synthetic intermediates are modified such that they become more basic with the most basic species accumulating as the final product. Conversely, a large precursor pool may be synthesized from which more acidic species are derived by glycosylation with sialic acid. Pulse-chase experiments would be valuable in differentiating between these two alternatives. Whichever of the above may be the case, the molecular modifications which result in charge heterogeneity apparently do not involve the antibody binding site(s) since molecules with at least three different isoelectric points are apparently recognized by A-BL antibodies. Other explanations for the electrophoretic appearance of A-BL antigens might include the physical association of molecules with similar molecular weights but different isoelectric points such that one or more of the molecules can be non-specifically precipitated by antibody interaction with an associated molecule, or the presence of antibody activity against distinct molecules with varying isoelectric points but coincidently similar molecular weights. These latter two alternatives, however, seem less likely than the above.

The most basic of the peptide spots immunoprecipitated by A-BL antibodies appear to correspond to the stage-specific polypeptides numbered 23 and 24 by Levinson et al. (1978) in their two-dimensional electrophoretic analysis of murine preimplantation development (which utilized electrophoretic procedures similar to those employed here). The two more basic pairs of spots which are included in the A-BL immunoprecipitate are also clearly present in the gels presented by Levinson et al. (1978). These three pairs of spots show similar spatial relationships amongst themselves and in relation to their rela-

tive isoelectric points and molecular weights as those of the A-BL immunoprecipitate. Levinson (personal communication) has estimated the molecular weight in the region of the stage specific polypeptides 23 and 24 to be between 70,000 and 80,000 daltons. These two peptides are described as being synthesized only between the 2- and 8-cell stages, corresponding with the time of appearance of A-BL surface antigens, while no synthesis is detected at the blastocyst stage. Two-dimensional gels prepared in the laboratory of Dr. C. Epstein confirm the observations of Levinson et al. (1978) and further indicate that the three pairs of peptide spots which appear to correlate with the A-BL precipitated antigens are no longer synthesized at the morula stage. Autoradiographs of two-dimensional gels described by Dewey et al. (1978) and Braude (1979) also contain similar peptide spots which appear to be synthesized by morulae but not by blastocysts, inner cell masses or teratocarcinoma stem cells (OTT 6050).

The apparent lack of synthesis of these proteins by teratocarcinoma cells is consistent with the lack of reactivity between A-BL antibodies and such cell lines in immunofluorescence and immunoprecipitation assays. However, further investigations will be required to confirm molecular identity between the proteins which comprise the A-BL immunoprecipitate and the stage-specific proteins expressed during preimplantation development and detectable by two-dimensional electrophoretic analysis.

It appears likely that A-BL antigens are first synthesized at the 2-cell stage and are immunologically-identifiable on the embryonic cell surface in increasing amounts from the 2-cell through the 8-cell to morula stage. Synthesis of A-BL antigens apparently ceases at the

8-cell to morula stage and, therefore, the A-BL surface antigens detected on blastocysts and inner cell masses by immunofluorescence may be molecules reminaing in the cell membrane following synthesis at earlier stages. However, the possibility exists that the antibody population responsible for the cell surface reactions detected by immunofluorescence, immunoradiolabeling and cytotoxicity assays may differ in specificity from that involved in the immunoprecipitation of gpe 69/71. An experiment which could support a correlation between the specificities of the surface-active and immunoprecipitating antibodies would involve immunoprecipitation from live embryos (as described above) previously treated with the enzyme β-N-acetylglucosaminidase (such treatment has been shown to reduce the binding of A-BL antibodies to embryos by immunofluorescence). If gpe 69/71 cannot be precipitated following such enzyme treatment it would suggest that the cell surface antigens showing stage-specific expression are in fact gpe 69/71. A more conclusive experiment, but one of questionable technical feasibility, would be the absorption of A-BL antibodies with purified gpe 69/71 and a subsequent demonstration of a loss in activity against embryonic surface antigens.

It would be interesting to determine at what point the existence of A-BL antigens can be detected intracellularly. At the 2-cell stage rough endoplasmic reticulum is not identifiable and at the 4- to 8-cell stages ribosomes are observed adjacent to the plasma membrane (Calarco and Brown, 1969). Immunoelectron microscopic investigations might reveal whether these ribosomes are involved in the synthesis of surface components as suggested by Calarco and Brown (1969). Since A-BL antigens appear to be sensitive to glycosidic cleavage, also of

interest would be the determination of the site of possible glycosylation of A-BL antigens since the Golgi apparatus is not apparent morphologically during the preimplantation period (Calarco and Brown, 1969). Perhaps cell surface glycosyltransferases are involved as has been proposed by Roseman (1970; see also Shur and Roth, 1975).

Enzyme-treatment of embryos indicates that A-BL antigens are insensitive to proteolytic cleavage and glycosidic cleavage except by the enzyme N-acetylglucosaminidase. These results suggest that, as expressed at the embryo surface, A-BL antigens consist of a protein component which is either protease insensitive or protected from attack by proteolytic enzymes, perhaps by carbohydrate components, or by the majority of the protein component existing in an intramembrane configuration and thus being unavailable to exogenously applied enzymes. Proteins with apparent molecular weights of 69,000 and 71,000 daltons are only weakly labeled by lactoperoxidase-catalyzed iodination which also suggests a lack of availability to exogenously applied reagents but might also merely reflect a relatively low tyrosine content. It is also possible that the molecules containing the antigenic sites detected by A-BL antibodies are in fact cleaved by proteolytic enzymes but only at points distant to the antigenic site(s) which remains associated with the embryo surface. The sensitivity of A-BL antigens to treatment with N-acetylglucosaminidase indiates the involvement of N-acetylglucosamine residues in the molecular configuration of the antibody binding sites. However, the β-N-acetylglucosaminidase preparation utilized in these studies has low levels of contamination (< 0.1%) from α-N-acetylglucosaminidase, β-galactosidase, α-mannosidase and α-L-fucosidase. While the latter three enzymes apparently do not act

against A-BL antigens, confirmation of the specificity of β-N-acetylglucosaminidase against A-BL antigens will require competitive assays in which it is shown that N-acetylglucosamine-containing compounds can act as competitive substrates for enzyme activity against A-BL antigens.

A-Bl antibodies are not aborbed by N-acetylglucosamine or chitin (poly B(1→4) N-acetylglucosamine) suggesting that the antigenic determinants are complex and do not consist solely of N-acetylglucosamine. These results are consistent with the observation that antibodies with anti-polysaccharide activity typically have combining sites which span several moieties and which may also recognize tertiary structure of carbohydrate chains and associated protein components. The ability of monosaccharides to inhibit the activity of such antibodies is typcially low compared to that of tetra-, penta- and hexasaccharides (Kabat, 1966).

The moleculear species precipitated from embryos by A-BL correspond in molecular weight to the major envelope glycoproteins of mammalian type-C RNA tumor viruses (gp 69/71, also denoted gp 70) (see Strand et al., 1976). It has been suggested that the viral genome may play a necessary role during early embryogenesis (Huebner et al., 1970; Temin et al., 1970). The genomes of such viruses are present in all strains of mice (Todaro and Huebner, 1972) and morphologically identifiable C-type viruses have been observed budding from mouse blastocysts (Chase and Piko, 1973). Piko (1977) has also reported the nuclear expression of antigens which cross-react with antibodies directed against the major core protein (p30) of murine leukemia viruses by mouse oocytes and early embryos. Virus-infected cells show

surface expression of gp 69/71 and these are the major glycoproteins precipitated by sera of mice reacting against endogenous type C viruses. In addition to corresponding in molecular weight with A-BL antigens, the viral proteins also show charge heterogeneity on isoelectric focusing (Witte et al., 1977) and contains approximately 30% carbohydrate, of which over 25% is glucosamine in the form of N-acetyl derivatives (Marquardt et al., 1977). The primary antigenic determinants of the viral gp 69/71 have been shown to reside in the protein rather than in the carbohydrate portion of the molecules (Bologenesi et al., 1975).

However, in a previous study Calarco et al. (1975) were unable to detect the expression of murine leukemia virus group-specific antigens during mouse preimplantation period. Antisera against Rausher leukemia virus gp 69/71 and Feline leukemia virus gp 70 tested in the studies described here do not precipitate the gpe 69/71 doublet from 8-cell mouse embryos nor do they react with embryonic surface antigens in immunofluorescence assays. Likewise A-BL antibodies do not precipitate gp 69/71 from two C-virus (AKR and spontaneous NZB) producing cell lines nor do they react positively by immunofluorescence. Although preliminary evidence suggests that the gpe 69/71 immunoprecipitated by A-BL from 8-cell embryos may not be antigenically related to the gp 69/71 envelope glycoproteins of murine C-type viruses the possibility remains that the gpe 69/71 molecules are in fact virus-related but do not share antigenic determinants with viral proteins. Piko (1977) has shown that the p30 antigens expressed by early embryos are not cross-reactive with those of Rausher or Feline leukemia viruses, while those of the AKR, Gross, Kirsten and Maloney strains are.

The same may hold true for the envelope glycoproteins and, therefore, antisera directed against the gp 69/71 of these strains should be tested for possible cross-reactivity with A-BL antigens. (Such antisera are, however, less available than those already tested.) The limited number of anti-viral sera and virus-producing cell lines examined here and the known tendency for viral proteins and glycoproteins to possess multipule antigenic determinants which may vary within and between host species and between virus strains would make it premature to eliminate the intriguing possibility that virus-related molecules may be expressed during early mammalian development.

Mouse alpha-fetoprotein also has a molecular weight of approximately 70,000 daltons and may run as a closely spaced pair of bands in SDS-containing acrylamide gels (Dziadek and Adamson, 1978). However, alph-fetoprotein has not been identified as being synthesized by mouse embryos until the seventh day of gestation and results presented here show that antibodies directed against mouse alpha-fetoprotein do not identify surface antigens on cleavage-stage embryos. Furthermore, no reactivity between A-BL and fetal mouse serum or amniotic fluid, both of which are high in alpha-fetoprotein, or purified human alpha-fetoprotein is evident in double immunodiffusion assays. It appears therefore that the specificity of A-BL antibodies is not directed against alpha-fetoprotein despite its similarity in molecular weight to A-BL antigens and its fetal relationship.

4. Possible Developmental Roles for A-BL Antigens

While the existence of a necessary developmental function for A-BL surface antigens remains to be established, possible functions might be

predicted on the basis of the data presented here and known functions of cell surface molecules. There is a temporal correlation between the increased expression of A-BL surface antigens and the process of compaction at the 8-cell to morula stages. It might be expected that during this process cell surface molecules involved in intercellular adhesion and perhaps function formation would be required. Thus, A-BL surface antigens may participate in cell adhesion and/or junction formation, however, no specific compaction -related event has been shown to be blocked by the presence of A-BL antibodies. A number of other membrane-associated functions (e.g. transport of metabolites, amino acids and nucleic acid precursors) show increases coincident with increases in the surface expression of A-BL antigens between the 2- and 8-cell stages. While such functions may not be required for early development (i.e. embryos can develop in vitro in the absence of metabolites, amino acids and nuclei acid precursors), it cannot be excluded that such transport systems do function in vivo and, therefore, normally participate in development. Likewise, the existence of cell surface receptors for extracellular factors such as hormones and growth factors should not be ruled out, although little is known about the function of such factors during preimplantation development. A-BL surface antigens might also be involved in surface-related enzyme activities (e.g. alkaline phosphatase, 5'-nucleotidase) which first appear at the 4- to 8-cell stages. The ability of A-BL antibodies to block or alter such membrane-associated functions has not been examined but should be amenable to experimental tests.

An alternative possibility is that the increased surface expression of A-BL antigens between the 2- and 8-cell stages merely reflects

changing qualitative patterns of protein synthesis and quantitative increases in protein synthetic activity in the embryo and that a functional role for A-BL antigens does not exist until some later developmental period. In this regard, A-BL antigens might be involved in attachment, implantation and/or protection of the embryo from a maternal immune response. However, since there appears to be a decrease in the expression of A-BL surface antigens at the blastocyst and later states and A-BL antibodies interfere with development prior to the blastocyst stage, such functions would appear to be unlikely. However, there is no direct evidence to rule them out.

Therefore, if it is assumed that the period of peak expression of A-BL surface antigens coicides with a period of functional significance, it might be expected that A-BL antigens participate in a compaction-related event or any one of a number of surface-associated biochemical functions initiated between the 2-cell and morula stages. Conversely, A-BL surface antigens may reflect intracellular events and have no direct developmental role. However, the ability of A-BL antibodies to interfere with normal development would argue against this possibility.

5. Directions for Future Research

A detailed investigation of the cross-reactivity of A-BL antigens with viral antigens and the expression of virus-related proteins by early mammalian embryos is potentially the most significant avenue for future research based on the results described here. The identification of virus-related proteins during embryogenesis would lend credence to the proposals that such molecules may have normal, necessary developmental roles (Huebner et al., 1970; Temin, 1970).

A number of experimental approaches could add valuable information in this area. Peptide mapping of A-BL immunoprecipitated antigens and comparison with maps of viral gp 69/71 (Elder et al., 1977) would establish if the viral and embryonic proteins share similar structure. An additional approach would involve attempts to absorb A-BL antigens with purified virus preparations or virus-infected cells. The ability to remove or reduce the anti-embryo activity of A-BL antigens by such absorptions would establish antigenic cross-reactivity. However, negative results in such experiments would be inconclusive since viral and embryonic proteins could vary in antigenic specificity but be otherwise homologous. Likewise, the results of attempts to show anti-embryo activity in antisera directed against viral proteins would be subject to the same qualifications. Since viral proteins and glycoproteins possess multiple antigenic determinants which can vary within and between host species and between virus strains, serological approaches may not provide conclusive results. However, strictly biochemical approaches will likely be hindered by a lack of sufficient quantities of embryonic gpe 69/71 proteins.

A second area of research which is deserving of further examination, especially in light of the possible viral relationship of A-BL antigens, is the possible functinal significance of these antigens during development. This type of investigation is inherently difficult due to the extreme sensitivity of early mammalian embryos to perturbations of the in vitro culture system. Fab preparations have been utilized in a number of systems to examine the role of cell surface antigens without the potential non-specific effects of multivalent antibodies and this approach can likely provide useful information in

the mammalian system as well (e.g. see Kemler et al., 1977). However, the results of such experiments, while they are based on established techniques, may still involve effects not directly related to specific developmental events mediated by cell surface molecules. Other approaches to the identification of a possible function for A-BL antigens might involve the purification of gpe 69/71 from embryos (or possibly similar molecules of viral origin) and an examiniation of the developmental effects of these molecules either when present during in vitro culture or following their addition to embryonic cell membranes (possibly via liposomes). Membrane-associated functions (e.g. enzymatic and transport activities) might also be examined folloiwng the complexing or removal of A-BL antigens or the artificial insertion of A-BL antigens into plasma membranes. Possible enzyme activities of A-BL antigens might also be analyzed in cell-free assays. The most attractive system for the study of A-BL antigen function would utilize genetic mutants which fail to express A-BL antigens or express abnormal forms of A-BL antigens. However, this approach may not be technically feasible and could prove to be impossible if mutation in the gene (s) coding for A-BL antigens are lethal to early embryos, an effect which might in fact be predicted. Therefore, while it is clear that accurate analyses of the functions of specific cell surface molecules, such as A-BL antigens, are difficult, such investigations remain as some of the most worthy of future research efforts.

SUMMARY

1. A number of cell surface proteins detected by lactoperoxidase labeling are present throughout the preimplantation period with major qualitative changes in these proteins occurring following fertilization and coincident with blastocyst formation.

2. A-BL antibodies detect the embryo-specific and stage-specific expression of surface antigens increasing in concentration on the cell surface from the 2-cell through the 8-cell to morula stage of mouse preimplantation development.

3. A-BL surface antigens are relatively immobile within the cell membrane since antibody interaction does not induce patching or capping, however small antigen clusters may be present and compaction-related redistribution is observed.

4. A-BL surface antigens are insensitive to proteolytic cleavage, however, proteins may be involved in the maintenance of antigen distribution in the cell membrane.

5. The antigenic determinants of A-BL-detected surface antigens probably contain N-acetylglucosamine residues based on their sensitivity to N-acetylglucosaminidase.

6. A-BL antibody activity is primarily directed against a pair of proteins with apparent molecular weights of 69,000 and 71,000 daltons (gpe 69/71) which may show charge hetergeneity suggesting the possibility of various degrees of glycosylation.

7. A-BL antibodies can interfere with cell division and subsequent development but the specificity of this effect remains to be determined.

APPENDIX

Black Bottom Pie

1. Materials:

9 in. baked pie shell
½ c. sugar
2 tbl. cornstarch
½ tsp. salt
2 c. milk
2 eggs, separated
2 tsp. unflavored gelatin
3 tbl. cold H_2O
¼ c. rum
1 oz. melted unsweetened chocolate
¼ tsp. cream of tartar
1/3 c. sugar

2. Methods:

a) Stir together in sauce pan: ½ c. sugar, cornstarch and salt

b) Blend milk and egg yolks and stir into sugar mixture

c) Cook over medium heat stirring constantly until mixture boils

d) Remove from heat and reserve 1 c. of the custard mixture

e) Soften gelatin in cold H_2O and stir into remaining custard, then stir in rum

f) Chill in refrigerator until mixture mounds when dropped from a spoon

g) Add chocolate to reserved cup of custard and pour into pie shell

h) Beat egg whites with cream of tartar and 1/3 c. sugar until stiff

i) Fold in rum custard

j) Spread over chocolate in pie shell and refrigerate covered overnite (at least)

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