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Developmentally regulated antigens of early mouse embryos and teratocarcinomas

Author
Banka, Carole Lynn

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DEVELOPMENTALLY REGULATED ANTIGENS OF EARLY MOUSE EMBRYOS AND TERATOCARCINOMAS

by
Carole Lynn Banka

B.S., Rollins College, Winter Park, FL
M.S., Adelphi University, Garden City, NY

DISSERTATION

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Patricia L. Calarco

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Dedicated to my father, Don Lynn, who introduced me to the fascination of living things and my mother, Ruth Lynn, who taught me to balance the other important aspects of my life.

ABSTRACT

The identification and partial characterization of developmentally regulated antigens of early mouse embryos and teratocarcinomas was accomplished with a variety of immunological techniques. Eleven monoclonal antibodies (McAbs) recognizing cell surface antigens of the 8-cell mouse embryo were generated by fusion of mouse plasmacytoma cells with spleen cells from a rat immunized with the early embryos. The mose extensively studied McAb, 2-C_4C_3, detected antigens expressed first at the mid-4-cell stage and throughout the remaining preimplantation period including both the inner cell mass and trophectoderm of the blastocyst. The McAb was not cytotoxic in the presence of complement, had no effect on in vitro development and through a variety of techniques was determined to have a low avidity for the anitgen, rendering it an ineffectual reagent for biochemical analysis. These findings combined with the difficulties experienced in other laboratories attempting to generate anti-embryo McAbs led to the conclusion that the McAb approach to studies of preimplantation embryos is impractical in any but very large laboratories.

A rabbit antiserum (A-N_1) to nullipotential teratocarcinoma stem cells was found to cross-react with early embryos and proved to be an effective reagent. A-N_1 detects cell surface antigens on the female germ line, but not the male, and on all stages of preimplantation embryos. When embryos are cultured in the presence of A-N_1, their development is inhibited. Immunoprecipitation analyses have revealed a set of seven surface antigens in the

14,000 - 130,000 d range on N_1 cells and embryonic fibroblasts at least four of which are also present on blastocysts. None of these molecules is embryo specific. In addition, A-N_1 precipitates a heavily glycosylated protein, gp67, synthesized as early as the 2-cell stage and detected in pluripotential teratocarcinoma cells but not nullipotential teratocarcinomas or other cell types. The absence of embryo-specific gp67 in nullipotential teratocarcinoma cells may correlate with the inability of these cells to differentiate. This is the first description of a molecule common to preimplantation embryos and undifferentiated pluripotent teratocarcinoma cells but not nullipotent teratocarcinoma cells.

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CONTENTS

INTRODUCTION

The cell surface is the interface between a cell and its environment and, therefore, communicates information of developmental significance in both directions. Molecules inserted in the cell membrane or secreted through it can alter the extracellular environment or influence other cells. Conversely, control mechanisms involved in developmental processes such as differentiation and morphogenesis may operate externally at the cell surface. Molecules found on the cell surface include receptors, transport molecules, enzymes and junctional components. In addition, surface molecules are involved in cell recognition or specificity marking, cell adhesion, cell division, cell differentiation and cell motility. Surface interactions of some or all of these molecules may alter gene expression and, in so doing, may influence a cell toward a specific pathway of differentiation.

In the mammalian system the early embryo offers the unique opportunity of studying the cellular interactions involved in the transition of a single cell into a complex multicellular organism. In the mouse, development of the preimplantation embryo can be studied in an *in vitro* system which parallels *in vivo* development. During this period the embryo undergoes three cleavages from a zygote to an eight-cell embryo. The 8-cell embryo then undergoes a process of compaction during which the cells become closely adherent and individual cell boundaries become indistinct. Three further cell divisions, junction formation and the first cellular differentiation result in formation of a blastocyst, a fluid filled sphere of cells bounded by an epithelial trophectoderm with the

inner cell mass (destined to become the embryo proper) at one pole. It is at this stage that the embryo begins to implant in the uterine wall. Using this model system it should be possible to answer questions concerning the role of the cell surface in such processes as fertilization, cleavage, amino acid transport, protein synthesis, compaction, junction formation, cellular differentiation and implantation. While there is as yet little direct evidence for a developmental role of the cell surface in the preimplantation period, its importance is suggested by temporal alterations in surface morphology, biochemistry and antigenic profile, reviewed extensively by Edidin, 1976a,b; Solter, 1977; Jenkinson and Billington, 1977; Johnson and Calarco, 1980a; Wiley, 1980.

Two methodological approaches can be used to study the functions of cell surface molecules. One approach involves the design of an assay for a specific interaction mediated at the cell surface (such as adhesion) and use of the assay to identify the molecule(s) involved in the interaction. Examples of this approach include the study of lectin interactions in the adhesion of teratocarcinoma stem cells by Grabel and coworkers (1979) and the discovery of bindin, the molecule responsible for sperm and egg adhesion in the sea urchin (Vacquier and Moy, 1977). The second approach is an immunological one in which antibodies to a purified or complex immunogen are utilized to study the surface interactions of antigens. It is the purpose of the work presented here to further our knowledge of developmentally regulated embryonic surface antigens of the preimplantation mouse embryo through the use of this latter approach and it is with this immunological approach that we

will be concerned. Descriptive studies of the cell surface of the early embryo employing other techniques have been reviewed recently by Johnson and Calarco (1980a) and are summarized by Johnson (1981).

1. Immunologic Approaches to the Study of the Cell Surface

The specificity of antibody-antigen interactions has lead to the evolution of techniques for specific identification and isolation of molecules of developmental interest. Researchers studying cell surfaces in many biological systems have made the assumption that a given glycoprotein or glycolipid of functional significance should have a unique structural configuration conferring antigenicity. In fact, immunologic methods have proven useful in studying the cell surface interactions in aggregating slime mold amoebae (Rosen, et al., 1976) and the chick neural retina (Buskirk et al., 1980), developmentally regulated surface antigens in non-neuronal (Schachner, 1979; Schachner et al., 1981) and neuronal (Cohen et al., 1981) cells of the mammalian nervous system, and differentiation antigens of mammalian lymphocytes (Springer et al., 1978).

The antibodies used as reagents can be generated in a variety of ways. Syngeneic antisera will detect only antigens not present in the adult animal immunized and will have a limited number of specificities; thus, it is often difficult to generate an immune response with syngeneic immunization. Syngeneic sera have been employed to study teratocarcinoma cell surface antigens (Artzt et al., 1973; Stern et al., 1975). Allogeneic antisera will detect only those antigens which differ from one strain to another within a species. These antisera have been useful in the study of H-2

antigens in the mouse (Webb et al., 1977). Heterogeneic antisera usually have multiple specificities; however, they may be rendered very specific (as determined by antigen isolation) by appropriate absorptions (Larraga and Edidin, 1979; Johnson and Calarco, 1980d). Heterosera can also be used to identify a group of antigens one or more of which can then be isolated as an immunogen for a more specific serum. This has been effective in the study of neural retina adhesion molecules (Brackenbury et al., 1977; Thiery et al., 1977). Monoclonal antibodies (McAbs) have a single antigenic specificity and have been employed successfully in studies of cell surface antigens of mammalian sperm (Myles et al., 1981; Feuchter et al., 1981), mouse and human lymphocytes and thymocytes (Springer et al., 1978; Trucco et al., 1978; Kung et al., 1979), tumor cells (Nowinski et al., 1979; Woodbury et al., 1980) and mouse preimplantation embryos and teratocarcinomas (see below).

Once generated, antibodies can be utilized to answer diverse questions concerning cell surface antigens through a wide variety of experimental determinations including:

1. Cell marking or identification -- antibodies coupled wth fluorescent, enzymatic or ferritin tags can be used at the light or electron microscopic level to identify different cell types in a given population. This immunocytochemical method has obvious applications for the study of developmentally regulated antigens in differentiating cells.

2. Antigen localization -- the immunocytochemical methods described above also elucidate the topographical localization of antigens on a single cell.
3. Cell sorting -- a direct application of antibody marking of cell surfaces is the technology for separation of cells by virtue of surface antibody binding. This can be done by affinity binding of whole cells on columns or on beads with magnetic cores or with the technique of cell sorting. Pure viable cell populations can be recovered for further study in this manner. An alternate approach is to kill through immune cytolysis all cells bearing a common surface antigen leaving a viable cell population which do not express the antigen.
4. Antigen identification -- using antibodies to purified antigens, it is possible to ascertain the presence or absence of these known antigens on the surface of any cell type. It is also possible to study the interactions of two different surface antigens on the same cell using the respective specific antibodies with different fluorescent tags.
5. Cell surface modulation -- the binding of antibodies to the surfaces of living cells can result in modulation such as patching, capping or internalization of antigen revealing information about lateral mobility of antigen within the membrane, the fate of antibody-antigen complexes and the time course of antigen replacement on the surface in cases where antibody binding leads to

removal of antigen. Comparisons of bivalent antibody and monovalent Fab fragment effects can be particularly useful in these experiments.

6. Antigen function -- if antibodies bound to specific cell surface antigens of living cells cause physiological or morphological perturbations, one may gain insight into a functional role for the antigen. Here the comparison of bivalent and monovalent reagent effects is quite important for interpretation. Clearly, developmental or functional effects resulting from antigen-antibody binding must be carefully analysed through use of appropriate controls.
7. Antigen quantitation -- radiolabelled antibodies are useful tools for quantitating cell surface expression of antigens. Antibodies are usually labelled with ^{125}I; however, monoclonal antibodies can be metabolically labelled with a variety of radioisotopes for this purpose. Developmental regulation of antigen quantity can be explored with this technology.
8. Antigen isolation -- antibodies bound to chromatgraphic substrates can be used in antibody mediated affinity chromatography (on columns or in bulk) to isolate a specific antigen from a complex molecular mixture for further biochemical characterization. Alternatively, antigen-antibody complexes can be removed from a mixture by precipitation with a second antibody or by selective adsorption to Staphalococcus aureus. A sophisticated use of this technique has allowed for the bridge of the

gene-protein gap. Antibodies can be produced to synthetic peptides whose structure has been predicted from the nucleotide sequence of isolated genes. These antibodies can then be used to isolate the protein gene product carrying the peptide sequence (for review, see Lerner et al., 1981).

2. Historical Background

Many of the techniques described above have been applied to the study of preimplantation mouse embryo surface antigens and the information thus accumulated about cell surface antigen expression and developmental regulation is discussed below with emphasis on the preimplantation period. Implications for cell surface involvement in fertilization and implantation have been reviewed elsewhere (fertilization, Gwatkin, 1976; implantation, Enders, 1972; Sherman and Wudl, 1976) and will not be included here.

Whereas a marked increase in the quantity of total protein synthesis occurs at the 8-16 cell stage in the preimplantation mouse embryo (Epstein and Smith, 1973), the most marked alterations in the qualitative pattern of total protein synthesis appear by the 4-8 cell stage (Van Blerkom and Brockway, 1975; Martin et al., 1978). In contrast to the total qualitative pattern, ^{125}I-labelling of cell surface proteins indicates that the major qualitative changes in surface protein composition occur following fertilization and coincident with blastocyst formation (Johnson and Calarco, 1980b). The antigens of greatest interest on the surface of the preimplantation embryo are those which show stage-specific and/or tissue specific expression as these are most likely to serve a

functional role in development. In addition, study of the molecules with specific temporal expression may yield information concerning controls of protein synthesis or surface expression in the early embryo.

Historically the use of immunological studies of the embryonic cell surface can be divided into two phases, the descriptive phase and the more recent molecular phase. During the descriptive phase interest centered on the stage and/or tissue specificity of antigens. Therefore, in the following historical summary antigens will be discussed in the order in which they appear during preimplantation development. Functional correlates will be discussed in cases where they have been implicated. Finally, recent findings concerning the molecular nature of these antigens will be discussed.

Descriptive Studies

Anti-Intracisternal A Particle: A rabbit antiserum to a purified preparation of intracisternal A particles (IAP) detects cell surface antigens on the preimplantation mouse embryo which appear first on zygotes, are expressed maximally on 2- to 8-cell embryos and are not detectable on morulae or later stage embryos (Huang and Calarco, 1981a). The antiserum does not detect cell surface antigens on IAP-producing neoplastic cell lines. This is the only known viral antigen expressed on the preimplantation embryo cell surface.

Anti-402AX (I): A heterogeneic antiserum to the teratoma 402AX cell line (a partially differentiating line) detects antigens on embryos and SV-40 transformed cells (Gooding et al., 1976). Selective absorptions of this serum revealed three antigen specificities, two of which are present on embryos. Antigen I is present on

unfertilized ova and early cleavage stages. As early as 12-16 cells, the outer blastomeres appear to lose antigen I while inner blastomeres continue to express it. Following implantation (as tested on in vitro outgrowths) antigen I is present on ICM but not trophoblast giant cells, and disappears altogether after 8½ days (Edidin, 1976b). Antigen 402AX (II) will be discussed later.

Anti-SSEA-2: BALB/c mice immunized with a BALB/c x human hybrid cell line produce an antiserum with at least three antigen specificities, one of which (SSEA-2) appears on preimplantation ICR mouse embryos (Shevinsky et al., 1981). SSEA-2 is present on all cleavage stages with maximal expression at the 8-cell stage. The antigen is present at low levels, if at all, on trophectoderm of blastocysts but persists on the ICM and parietal endoderm. Several murine teratocarcinoma cell lines express SSEA-2 and it is present on sperm. The hybrid cells used as immunogen carry the SV40 genome integrated on human chromosome 7, the only human chromosome retained by the hybrid cells. SSEA-2 was detected on 7 of 10 SV40-transformed murine cell lines but not transformed cells of other species. The antigen is thought to be an oncofetal antigen, i.e., an embryonic antigen which is re-expressed on virally transformed cells.

Anti-F9: Perhaps the most extensively studied embryonic surface antigens are the F9 antigens detected by syngeneic antisera to F9 (a pseudo-nullipotential mouse teratocarcinoma stem cell line) developed by Artzt and her coworkers (1973). These antigens are present on sperm but not oocytes and appear on embryos 5-6 hours after fertilization (see Jacob, 1977). This early synthesis could

represent transcription of the embryonic genome or translation of stored maternal message which was not translated during oogenesis. Studies with inhibitors of RNA synthesis (i.e. actinomycin D or α-amanitin) might distinguish between these alternatives but have not been done. F9 antigen reaches maximum expression at the 8-cell to morula stages and is present on both trophoblast and inner cell mass (ICM) of the blastocyst although in decreasing amounts. The antigen is present on postimplantation embryos through day 8 but the tissue distribution has not been determined (Buc-Caron et al., 1978). Kemler and his coworkers (1977) have prevented compaction of the 8-cell embryo by culturing in the presence of Fab fragments from a rabbit antiserum to F9. This phenomenon is reversible in that embryos washed free of Fab will go on to compact and form blastocysts. Fab added to compacted embryos causes "decompaction". It is known that compaction is accompanied by the formation of macular tight junctions between blastomeres of the 8-cell embryo (Magnuson et al., 1977). While Kemler and his coworkers speculate that the molecule recognized by their anti-F9 may mediate Ca^{++} interactions necessary for intercellular adhesion, it is also possible that the molecule involved is a precursor of a tight junctional element. In fact, incubation in anti-F9 Fab causes teratocarcinoma cells to roundup and lose all gap and tight junction and the gap junctions are internalized (Dunia et al., 1979). Anti-F9 Fab also causes "decompaction" of teratocarcinoma clumps in culture (Hyafil et al., 1980).

Kemler et al. (1979) have produced monoclonal antibodies using spleen cells from rats immunized with F9 cells. Two clones produce

antibodies which react with all preimplantation stages from the 2-cell stage on. Maximal reactivity with indirect immunofluorescence (IIF) was seen at the morula stage. Antigens were detected on the early ICM and increased following endoderm differentiation. No further characterization of these antigens has been reported.

Other antigens which appear at the 2-cell stage about which little is known include antigen(s) detected by a rabbit anti-mouse placenta serum which persist on ectoplacental cones at day 7 of gestation (Kometani et al., 1973) and antigen(s) detected by a syngeneic serum against OTT6050 teratoma embryoid bodies which persist on day 6 embryonic endodem and ectoderm (Dewey et al., 1977). These antigens are not general tumor antigens as they were not detected on other tumor cells.

Several antigens appear at the 4-cell stage. Menge and Fleming (1978) developed a rabbit antiserum to mouse sperm which, when absorbed with ovarian tissue from PMS treated mice, stained embryos by indirect immunofluorescence beginning at the 4-cell stage and persisting through blastocyst formation.

Anti-Blastocyst: More extensively studied antigens which appear at the 4-cell stage are those detected by rabbit antiserum to zona pellucida-free mouse blastocysts. Two such antisera have been developed. A-BL_1 antigens appear at the 4-cell stage. Maximal expression of the antigens is seen at the 8-12 cell stage followed by decreasing expression through the preimplantation period. No antigens are detectable on blastocyst outgrowths. When embryos are cultured from the 2-cell stage in the presence of A-BL_1, there is a concentration dependent inhibition of cleavage and subsequent

blastulation not seen when embryos are incubated in normal (non-immune) rabbit serum. That blocking, redistribution or loss of the antigens detected by A-BL_1 interferes with normal cleavage and/or development of the embryo suggests a role for these surface moieties (Wiley and Calarco, 1975).

A-BL_2 detects antigens with a similar stage-specific distribution and both whole serum and IgG prepared from serum have similar in vitro inhibitory effects on cleavage and blastulation when compared with normal whole rabbit serum and IgG respectively (Johnson and Calarco, 1980c). A-BL_2 does not cross-react with teratocarcinoma stem cells. Further molecular characterizations of the BL_2 antigens will be described below.

Anti-Endo: An alloantiserum to an endodermal carcinoma line (Endo) derived from a teratoma detects embryonic antigens which appear at the 8-cell stage but their subsequent expression has not been determined (Arztz et al., 1976). If these antigens segregate to endodermal cells in the embryo, they would serve as useful markers for this tissue.

Anti-Ectoplacental Cone: Searle and Jenkinson (1978) produced a rabbit antiserum with mouse 7½ day ectoplacental cone as the immunogen. An indirect immunoperoxidase assay detected expression of antigens with this serum beginning at the 8-cell stage and increasing through the blastocyst stage where trophectoderm showed heavy staining but ICM showed relatively little. The antigens detected in this system segregate to trophoblast giant cells and extraembryonic ectoderm following implantation.

Anti-H-Y, H-3, H-6: The appearance of two other antigens at the 8-cell stage are of interest because they provide evidence of paternal genome expression at this stage. Krco and Goldberg (1976) reported the appearance of H-Y (histocompatability-Y) on approximately half of a population of 8-cell embryos based on a cytotoxicity study. Epstein and coworkers (1980) confirmed this observation by karyotyping the affected and unaffected embryos in a similar cytotoxicity approach. Of unaffected embryos karyotped at the blastocyst stage, 92% were female while only 6% of the affected embryos were female.

Muggleton-Harris and Johnson (1976) found that paternal non-H-2 alloantigens appeared at the 6- to 8-cell stage although maternal non-H-2 antigens were detectable at all stages in F_1 embryos.

Anti-SSEA-1: Recently, Solter and Knowles (1978) reported a monoclonal antibody to F9 cells which detected an antigen, Stage Specific Embryonic Antigen-1 (SSEA-1) on sperm and on preimplantation embryos beginning at the late 8-cell stage. Radioimmunoassay showed the morula to have the highest binding activity of the cleavage stages. Trophoblast of the blastocyst became negative 24 hours after blastocoele formation while the ICM became increasingly positive. The antigen is expressed by both endoderm and ectoderm of the ICM. During the early postimplantation period the antigen is restricted to embryonic ectoderm and visceral endoderm and can be found during later development in the brain, promordial germ cells, kidney tubules and the epithelial linings of various organs of the genital tract (Fox et al., 1981).

Although the authors suggest interesting implications for cell lineage phenomena during development, it is more likely that the SSEA-1 McAb is recognizing different antigens which share a small terminal carbohydrate chain. In fact, the antigenic determinant of SSEA-1 appears to be a terminal trisaccharide (see below).

Anti-hCG: Several rabbit antisera to human chorionic gonadotrophin (hCG) recognize antigens beginning at the 8-cell stage. These antigens persist through the preimplantation period, then disappear from the trophoblast but continue to be expressed on endoderm and ectoderm of the inner cell mass (Wiley, 1974, 1979). These antigens are not detected by an antiserum specific for the beta subunit of hCG (Calarco and Wiley, personal communication). The antigen(s) detected, therefore, may be one of the other trophic hormones which share a common alpha chain with hCG (luteinizing hormone or thyroid stimulating hormone) or a hormone specific to the early embryo. The relationships of the antigen detected remain to be explored. A subsequent study using a different commercially prepared anti-hCG detected surface expression first at the morula stage with restriction to the ICM on blastocysts and embryo outgrowths (Wiley, 1980). An interesting observation in this study was detection by IIF of antigens in the cytoplasm of fixed, permeabilized unfertilized oocytes and all cleavage stage embryos as well as trophectoderm cells none of which display surface fluorescence with the antibody.

Anti-β2-Microglobulin: A more convincing demonstration of temporal separation of molecular synthesis and surface expression comes from the studies of β2-microglobulin by Sawicki and coworkers (1981).

β2-microglobulin synthesis cannot be detected in the fertilized egg (zygote). Immunoprecipitation and 2-dimensional gel analysis reveal that maternal and paternal β2-microglobulin variants are synthesized in equal quantities from the 2-cell stage onward. Interestingly, β2-microglobulin cannot be identified by IIF on embryonic cell surfaces until the blastocyst stage (Sawicki and Maguson, personal communication; Hakansson and Peterson, 1976). This delay in surface expression may be related to the fact that β2-microglobulin is assocated with H-2 antigens in the membrane which are not expressed during the early preimplantation period.

Anti-H-2: The expression of histocompatability antigens (H-2) on preimplantation mouse embryos is controversial (for review see Johnson and Calarco, 1980a). Webb and coworkers (1977) have successfully demonstrated by immunoprecipitation the synthesis of antigens with H-2 specificities in the ICM of the late blastocyst but not in the trophectoderm. Immunohistochemical studies have identified low levels of H-2 antigens on the surface of the trophectoderm of the blastocyst which are lost at the time of implantation (Searle et al., 1976). This loss may be important in preventing immunological rejection of the embryo by the maternal system. This transient expression of H-2 on the blastocyst correlates also with the expression of β2-microglobulin.

Anti-Forssman: A monoclonal antibody recognizing embryonic surface antigen of the late preimplantation period has been investigated by Willison and Stern (1978). The antigen is first detected on the trophectoderm of the early blastocyst but disappears from these cells following hatching of the embryo from the zona pellucida. It

is present on the ICM and on primary endoderm of the ICM although it is not present on the endoderm of simple embryoid bodies formed by teratocarcinomas. The McAb appears to recognize a glycolipid with the species and tissue distribution of Forssman antigen (Stern et al., 1978). This antigen was initially detected on teratocarcinoma cells although the immune rat spleen cells employed in the fusion were derived from a rat immunized with mouse spleen. The antigen is not detected on differentiated derivatives of teratocarcinoma or other tumor cell types.

Anti-402AX (II): The second specificity of the differentially absorbed heterologous anti-teratoma serum mentioned previously, antigen II, appears on mural trophoblast of early blastocysts (Gooding et al., 1976) and segregates to the ICM of postimplantation embryos. It disappears from the embryo proper following day 8½ of gestation although it persists on yolk sac cells (Edidin, 1976b).

Anti-F9 (McAb): Among the set of monoclonal antbodies produced by Kemler et al. (1979) against F9 cells (see above) is one McAb which does not detect antigen until the blastocyst stage. The antigen is detected primarily on ICM although a few trophectoderm cells are also positive.

Anti-PCC4: A syngeneic serum to PCC4 (a pluripotential teratocarcinoma cell line) recognizes antigens only on the ICM of late blastocysts (Gachelin et al., 1977). The authors were unable to determine whether the activity was specific for endodem or ectoderm of the ICM.

Anti-SIKR: An antiserum with which stage specificity has not been defined is the syngeneic antiserum to a pluripotential

teratocarcinoma cell line (SIKR) described by Stern and coworkers (1975). Anti-SIKR reacts with 4- to 32-cell embryos but other stages have not been examined. The antigen(s) detected was present on SIKR stem cells, but not on endoderm of embryoid bodies formed by the SIKR cells suggesting disappearance coincident with differentiation.

Several antisera detect embryonic antigens with no stage- or tissue-specificity. These include a guinea pig serum to mature mouse oocytes (Moskalewski and Koprowski, 1972), a rabbit serum against mouse placenta (Wiley and Calarco, 1975), a rabbit serum against OTT6050 embryoid bodies (Webb, 1980) and a rabbit serum to a nullipotential teratocarcinoma (L55770) (Johnson et al., 1979).

Thus we see that each of the early stages of embryonic development is characterized by the appearance and/or disappearance of specific cell surface antigens as determined serologically. The appearance and disappearance of these molecules must be responsible in part for the qualitative changes in total protein synthetic patterns during early embryogenesis. However, initiation of synthesis and surface expression are not necessary correlated temporally (see discussion of β2-microglobulin and hCG). The relation of the rabbit anti-F9 antigen(s) to gap and tight junction integrity and embryo compaction has been described. Other theories concerning the role of specific cell surface antigens have appeared in the literature during the last decade which warrant a brief summary.

Functional Hypotheses

A variety of functional correlations have been suggested by investigators working with anti-teratocarcinoma sera. It was suggested that the F9 antigen was a product of the wild-type allele at the $\underline{t}^{12}$ locus in mice, when Artzt et al. (1974) reported that $\underline{+}/\underline{t}^{12}$ and $\underline{T}/\underline{t}^{12}$ sperm expressed approximately 50% less F9 antigen than other sperm. $\underline{t}^{12}$ is one of a series of recessive mutations in the $\underline{T}/\underline{t}$ complex in mice, most of which are stage-specific embryonic lethals in the homozygous condition. Thus the hypothesis evolved that $\underline{t}^{12}/\underline{t}^{12}$ embryos ceased development at the morula stage due to the lack of F9 antigen which was necessary for cell-cell interactions at this stage. It was further hypothesized that the wild-type product for each of the $\underline{t}$ alleles is a cell surface molecule which functions in vital cell interactions at the developmental stage when lethality occurs in the mutants. However, Kemler et al. (1976) developed antisera to sperm of several $\underline{t}$ haplotypes and found that stage specificity of $\underline{t}$ antigen expression did not correlate with stage of lethality in these embryos. This finding argues against stage-specific functions of the wild-type products and this theory is no longer widely held.

Artzt and Bennett (1975) have also hypothesized that F9 antigen(s) is the embryonic analogue or evolutionary precursor of the adult major histocompatibility complex (the H-2 complex in the mouse). Genetic evidence supporting this hypothesis is the linkage of the H-2 complex and the $\underline{T}/\underline{t}$ complex on chromosome 17 in the mouse and the suppression of recombination between the two regions (see Artzt and Bennett, 1975). Developmental expression of the H-2 and

F9 antigens seem to be reciprocal; as F9 antgen(s) (as defined by the syngeneic antiserum) decreases and disappears in the postimplantation embryo, H-2 antigens increase in quantity (see Jacob, 1977). A similar reciprocal relationship on embryos has been documented for H-2 antigens and antigen I detected by anti-402AX sera (see Edidin, 1976b). Disappearance of F9 antigen also correlates with appearance of H-2 antigens as teratocarcinoma stem cells undergo differentiation (see Jacob, 1977). The genetic linkage of H-2 and F9 presupposes that F9 is a product of the T/t complex. The reciprocal expression of these antigens may be circumstantial.

It has been reported that H-2 and F9 antigens are similar in molecular weight and subunit structure and are both associated with a β2-microglobulin-like subunit in the cell membrane (Vitetta et al., 1975). Attempts to confirm the presence of β2-microglobulin on F9 cells by other investigators have been unsuccessful (Dubois et al., 1976; Fellous et al., 1978). Although it is appealing to think of F9 or other teratocarcinoma-defined antigens as wild-type products of the T/t complex which are embryonic precursors of H-2 antigens with a functional role in cellular recognition, the hypothesis is falling into disfavor primarily due to the comparisons made with anti-t sperm sera by Kemler and his coworkers (1976), and the finding that the carbohydrate portion of F9 antigens differs strikingly from that of H-2 antigens (Muramatsu et al., 1979a). Clearly, different antisera to F9 cells are detecting different antigens as indicated by the difference in stage specificities of syngeneic and rabbit anti-F9. Investigations with monoclonal

antibodies to F9 have revealed several specificities (Solter and Knowles, 1978; Kemler et al., 1979). The relatedness of the antigens on embryos and embryonal carcinoma cells (teratocarcinoma stem cells) defined with different reagents can best be determined by molecular characterization of these antigens. This approach may also aid in clarification of the possible regulatory roles of these molecules. Some molecular characterizations have begun and this is clearly the direction of the immunological approach to the study of development in the next decade.

Molecular Studies:

A variety of molecular characterizations have employed the anti-F9 antibodies. Immunoprecipitation of ^{125}I and ^{3}H-leucine labelled F9 cells with sygeneic anti-F9 revealed proteins or glycoproteins which electrophoresed under reducing conditions in the range of 44,000 22,000 and 12-14,000 daltons (d) (Vitetta et al., 1975). Similar studies with embryos were not done. Muramatsu and coworkers (1979a) also using a syngeneic anti-F9 precipitated a variety of molecules from F9-41 cells labelled with various radioactive sugars. The precipitates contained rather large amounts of fucose, galactose and glucosamine but only small amounts of mannose. Approximately 20-25% of galactose-labelled material was found to be lipid (only 4% of fucose label was extracted in lipid). The majority of glycoproteins precipitated had an mr (molecular weight) of 40,000-43,000 with a small component of 22,000. Furthermore, the majority of labelled sugars in the glycoproteins were found to be in large glycopeptides (>6000) following protein digestion. High molecular weight glycopeptides are synthesized in

quantity by mouse blastocysts and embryonal carcinoma cells (ECC) including F9-41, but not by the differentiated derivatives of ECC (Muramatsu et al., 1978). The decrease in large glycopeptides accompanying differentiation correlates with the loss of syngeneically defined F9 antigen (Jacob, 1977). The F9 antigens containing large glycopeptides are assumed to be on the surface as the main source of these entities in F9 cells is membrane proteins (Muramatsu et al., 1979b). No attempt to inhibit antibody binding to embryos or ECC with any of these molecular preparations was made. Therefore, it is unclear whether all the glycolipids and glycoproteins are specific antigens defined by the syngeneic antiserum.

A larger glycoprotein than those described by Muramatsu et al. (1979a) has been immunoprecipitated from PCC4 Aza R1 EC cells with the rabbit anti-F9 described above (Kemler et al., 1977; Dunia et al., 1979) by Hyafil and coworkers (1980). The size of the intact membrane antigen has not been determined, but a glycoprotein of 84,000 d can be purified from a trypsin digest of a crude membrane preparation. This molecule is precipitated by rabbit anti-F9 IgG and inhibits the "decompaction" activity of anti-F9 Fab on preimplantation embryos and PCC4 Aza R1 embryonal carcinoma cells. The gP84 binds Concanavalin A and contains galactose, fucose, mannose and N-acetylglucosamine.

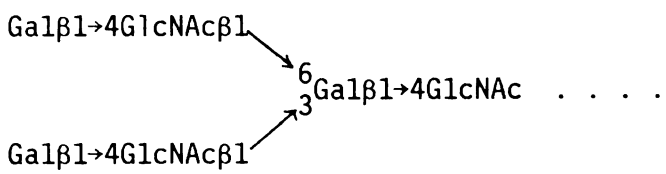
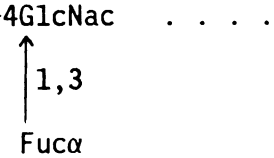
Using the anti-teratoma 402AX with antigen I specificity, Gooding (1976) immunoprecipitated radiolabelled protein/glycoprotein peaks of 110,000 d and 40,000 d from biosynthetically labelled mouse L cells which cross-react with anti-402AX. Larraga and Edidin

(1979), on the other hand, found the major component to be a complex mixture of glycolipids in immunoprecipitates from surface labelled Ter C cells (a line derived from 402AX) with an anti-402AX serum. A protein/glycoprotein peak of approximately 40,000 d was also present in the immunoprecipitates. Presumably this antiserum was identical to that used by Gooding (1976) although this point was not clarified. Lymphocytes coated wth an extract of glycolipids from the Ter C cells absorbed up to 80% of the antibody reactivity to Ter C cells. It is unclear how many of the glycolipids brought down are specific antigens for the antiserum and how many were contaminants complexed in micelles with the specific antigens.

A specific antigenic derminant on glycolipid has been identified on embryonal carcinoma cells with the anti-Forssman McAb (Stern et al., 1978) which cross-reacts with late preimplantation embryos (Willison and Stern, 1978). The antigenic determinant (Forssman) on several cell types is known to be a glycosphingolipid. ^{14}C-galactose labelled material was immunoprecipitated from Nulli-SCC-1 (nullipotential teratocarcinoma) cells with the McAb while no surface (^{125}I) or internally (^{35}S-methionine) labelled material could be precipitated. The ^{14}C-labelled antigens migrated with the dye front in SDS-polyacrylamide gels. No immunoprecipitations of embryo material have been done with this McAb; however, it is possible that the molecules bearing the Forssman antigenic determinant are identical on teratocarcinoma cells and embryonic cells. Interestingly, an antibody to globoside reacts with F9 Teratocarcinoma cells and preimplantation embryos from the 2-cell stage through the blastocyst, including ICM and trophectoderm, with

TABLE I

Terminal saccharides of known carbohydrate antigens detected on embryos:

Globoside	GalNAcβ1→3Galα1→4Galβ1→4Glc . . . . .
Forssman	GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glc . . . . .
i antigen	Galβ1→4GlcNAcβ1→3Galβ1→4GlcNac . . . . .
I antigen	 . . . .
Blood Group H antigen	Fucα1→2Gal . . . .
SSEA-1	 . . . .

peak expression at the morula stage (K. Willison, personal communication). Globoside is structurally identical to the Forssman glycolipid with the exception of its terminal sugar (globoside bears a terminal β-N-acetyl-galactosamine, Forssman has an additional α-N-acetyl-galactosamine) (Nowinski et al., 1980) (see Table I). Thus, there is the suggestion that two different antigenic determinants with very different stage specific distributions on preimplantation embryos are saccharides borne on glycolipids. The antigenic determinants differ by one sugar residue. Both are present on teratocarcinoma stem cells and may be present in the complex mixture of glycolipids precipitated from Ter C cells by Larraga and Edidin (1979).

A third possible glycolipid antigen with yet a different stage specific distribution on preimplantation embros is the SSEA-1 antigen identified by Solter and Knowles (1978). Because early attempts to immunoprecipitate a glycoprotein from F9 cells with anti-SSEA-1 resulted instead in ^{125}I-labelled surface material which migrated ahead of the dye front on SDS polyacrylamide gels, the antigen was suspected to be a glycolipid (Solter and Knowles, 1978). The terminal saccharides of SSEA-1 have now been identified through a series of experiments inspired by the identification of blood group antigens on embryos and teratocarcinoma stem cells.

The oligosaccharide chains of several blood group antigens have recently been identified on post-implantation embryos and teratocarcinoma cells (Kapadia et al., 1981). The i blood group antigen consists of a linear sequence of N-acetyllactosamine residues and is converted to the I blood group antigen by the terminal branching of

two N-acetyl lactosamine units (see Table I). Embryonal carcinoma cells (PSA4) express I antigen but not i. In post-implantation embryos as well as embryoid bodies differentiating from teratocarcinoma clumps, primary endoderm expresses i as well as I. Embryonic and extraembryonic ectoderm show a decrease in the amount of I expressed and never contain i. Transitional and squamous epithelium differentiated from embryonic ectoderm in embryoid bodies after 7 weeks in culture do not express I antigen; however, anti-blood group H antibody detects antigens on these epithelia. Blood group H antigens are oligosaccharides with terminal fucose residues which have Ii oligosaccharides as their core. Thus, the I antigens of embryonic ectoderm may be altered during the differentiation of the epithelia. Unfortunately, no studies of the expression of these blood group antigens on preimplantation embryos were done.

Based on these findings experiments were designed using immunodiffusion and plate binding assays to determine the reactivities of anti-SSEA-1 with a number of purified human erythrocyte membrane glycolipids (Nudelman et al., 1980). SSEA-1 was determined to have a similar structure to a blood group H component, H_4-a, with a close resemblance to I antigen. Subsequent studies (Gooi et al., 1981) using chemically synthesized and natural oligosaccharides proved Ii antigen sequences were inactive as inhibitors of anti-SSEA-1. In fact, the most active inhibitor was an oligosaccharide with a terminal N-acetyllactosamine with an $\alpha 1 \rightarrow 3$ fucosylation of the N-acetylglucosamine. It is proposed that the SSEA-1 determinant is formed by a fucosylation of the I or i antigens. The Ii antigens

can be carried on glycoproteins or glycolipids (see Kapadia et al., 1981). Therefore, the identification of the terminal trisaccharide of the SSEA-1 antigen tells us nothing of the nature of the whole molecule although the information from immunoprecipitation studies points to a glycolipid. The important concept which arises from the studies of blood group antigens and SSEA-1 on embryos is the idea that simple changes in branching of oligosaccharides (as in the transition of I expression to i in endoderm), or fucosylation of oligasaccharides (as in the transition of I to H expression in epithelia or the formation of SSEA-1 in early embryos) may be the basis for stage-specific expression of cell surface antigens during development. These simple alterations in glycosylation may, therefore, be the basis for significant regulatory changes operating at the cell surface. However, any regulatory mechanisms invoked for SSEA-1 must account for the appearance of the antigenic determinant on a variety of post-implantation and adult mouse cell types as discussed earlier (Fox et al., 1981). Thus the appearance and disappearance of many of the stage-specific antigens discussed above may reflect alterations in terminal saccharides.

The first report of stage-specific antigens immunoprecipitated directly from preimplantation embryos was that of Johnson and Calarco (1980d) with anti-blastocyst serum (A-BL_2). A-BL_2 brings down a pair of molecules in the 65,000- to 70,000 d region from 2-cell, 8-cell and morula stage embryos. The antigens are not detectable in immunoprecipitates from unfertilized eggs or blastocysts. However, immunoprecipitates from blastocysts contain a 45,000 d protein/glycoprotein which may represent an altered form of

the BL_2 antigens (a nonglycosylated form or cleavage fragment) or may be actin brought down nonspecifically. BL_2 antigens are not found in precipitates from Nulli-SCC-1 teratocarcinoma cells (Johnson, 1980). Two-dimensional electrophoretic analysis of the BL_2 molecules reveals pairs of proteins with three isoelectric points which may represent varying degrees of glycosylation during intracellular synthesis or following insertion in the membrane (Johnson and Calarco, 1980d). The BL_2 antigens are glycoproteins as they can be metabollically labelled with ^{3}H-glucosamine and are reduced to a single species of approximately 60,000 d when embryos are grown in the presence of tunicamycin (an inhibitor of the formation of asparagine-linked oligosaccharides) (Johnson and Calarco, 1980c). The surface expression of the BL_2 antigens is not totally synchronous with their synthesis. Although synthesized at the 2-cell stage, BL_2 antigens are not detectable by IIF on the cell surface at this stage. They are detected at low levels on the blastocyst surface although no longer synthesized.

Intracisternal A particle (IAP) associated antigens have also been precipitated from preimplantation embryos with rabbit anti-IAP serum (Huang and Calarco, 1981b). Five molecular species are present in the precipitates from 2- to 8-cell embryos, the stages which show physical presence of IAP. Three of these bands with apparent molecular weights of 67,000, 69,000 and 73,000 d are present only during these stages. Two other antigens with apparent molecular weights of 75,000 and 77,000 are also synthesized by morulae and blastocysts. The 73,000 d antigen probably represents the core protein of the IAP (a p73 core protein has been identified

in IAP from neoplastic cells). The other four antigens may be processing intermediates, cross-reacting species or physically associated with other IAP antigens in the embryo. Presumably one or more of these molecules represents the antigens seen on the surface with anti-IAP although this has not been determined due to limited amount of embryonic material.

From these last two studies we see that "miniaturization" of immunological analytical techniques has made them more feasible for studies of the early embryo; however, the limited quantity of antigen available for biochemical analysis remains a significant if not deterring factor. For this reason the majority of studies described above have employed the teratocarcinoma system as a model for early mammalian embryogenesis. Several lines of evidence indicate that teratocarcinomas are of embryonic origin and that differentiating embryonal carcinoma cells recapitulate early embryonic development (for review see Martin, 1980). However, morphological (Martin et al., 1977) and biochemical (Martin et al., 1978) studies have shown that differentiating teratocarcinomas are probably equivalent to the embryonic ectoderm of the post-implantation embryo and are, therefore, not appropriate models for the study of early cleavage stage embyos. In addition, we have see that there are at least two groups of surface antigens on preimplantation embryos, the BL_2 antigens (Johnson and Calarco, 1980c,d) and the IAP antigens (Huang and Calarco, 1981a,b) which are not found on teratocarcinoma cells.

The work to be presented here represents the results of immunological studies of early embryonic cell surface antigens.

These studies combined the heteroserum and monoclonal antibody approaches as well as the early embryo and the teratocarcinoma model system. Monoclonal antibodies were generated directly to preimplantation embryos in an attempt to identify other cleavage stage antigens not expressed by teratocarcinomas. The 8-cell embryo was chosen as the immunogen because it represents a qualitative intermediate in the profile of surface antigen expression during the preimplantation period. Therefore, it is conceivable that one might generate monoclonal antibodies to surface antigens which disappear during early cleavage stages as well as antigens which appear later in preimplantation development. A rabbit heteroserum was developed using nullipotential teratocarcinoma stem cells (Nulli-SCC-1) in the hopes of identifying antigens common to these cells and early embryos which could then be purified from the teratocarcinoma cells in quantities necessary for biochemical analysis. This reagent was then used to identify and partially characterize surface and cytoplasmic antigens of various stages of preimplantation embryos, nullipotent and pluripotent teratocarcinoma cells and several differntiated cell types.

MATERIALS AND METHODS

1. Collection and Handling of Preimplantation Mouse Embryos:

Female ICR (random-bred) mice (Simonson Laboratories, Gilroy, CA) were superovulated by administering 5 i.u. pregnant mare's serum (Gonadotropin, Sigma Chemical Co.) intraperitoneally (i.p.) followed 48 hr later by an i.p. injection of 2.5 i.u. HCG (A.P.L., Ayerst Laboratories). The females were caged with ICR males and mating was confirmed by checking for vaginal plugs on the following morning. Two-cell mouse embryos were flushed from the oviducts approximately 36 hr after mating. The embryos were cultured to desired stages in modified embryo culture medium (ECM) (Biggers et al., 1971; A. Spindle, personal communication) under paraffin oil at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. Eight-cell embryos were obtained after 24 hr in culture, morulae at 48 hr and blastocysts at 72 hr. For most studies the zonae pellucidae were removed from the embryos with a brief incubation in 0.5% pronase (Sigma Chemical Co.) in ECM followed by several ECM rinses. The embryos were allowed to "recover" in culture for 4 to 8 hr following pronase digestion to permit replacement of surface molecules possibly removed by enzyme treatment.

When mature oocytes from unmated females or zygotes were required, cumulus masses were removed from the oviducts by dissection the morning following mating. Cumulus cells were dispersed by brief incubation in 75 U/ml hyaluronidase (Sigma Chemical Co.) in ECM. Oocytes and zygotes were used immediately unless zonae were removed as described above.

Inner cell masses (ICMs) were removed from chimeric blastocysts by immunosurgery (Solter and Knowles, 1975) using an anti-mouse L-cell serum (kindly provided by Drs. R. Pedersen and A. Spindle) and guinea pig (GIBCO) or Low-Tox rabbit (Cedarlane Laboratories, Ltd., Londin, Canada) complement. Chimeras were made by aggregating three zona-free 8-cell embryos in 200 µg/ml phytohemagglutinin (Bacto Phytohemagglutinin P, Difco) in PBS (0.01 M phosphate buffered saline, pH 7.1) at 37°C. Chimeras were rinsed in warmed PBS and cultured to the blastocyst stage in ECM. The ICMs from these embryos were larger and more easily handled than ICMs from single blastocysts.

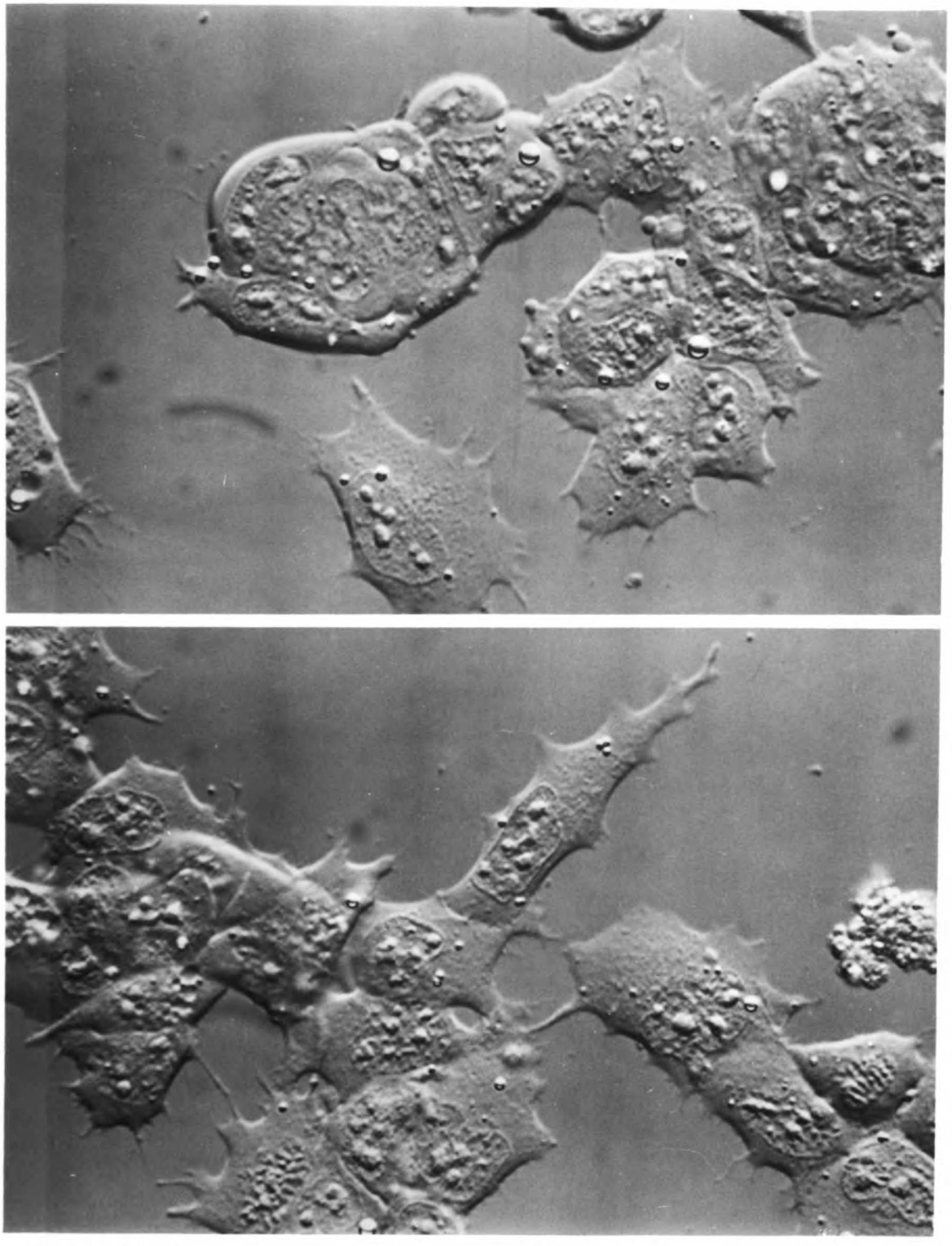
Blastocyst outgrowths were grown by placing Day 4 blastocysts in modified Eagle's Medium (A. Spindle and R. Pedersen, personal communication) with 10% Hyclone calf serum (Sterile Systems, Logun, Utah) in 8-chamber tissue culture Chamber Slides (Lab-Tek Products, Naperville, Ill.) (Glass et al., 1979).

2. Preparation of antisera:

A rabbit heteroserum was prepared to Nulli SCC-1 (N_1), a nondifferentiating teratocarcinoma stem cell line clonally derived from a spontaneous testicular tumor (#402, Dr. L.C. Stevens, Jackson Laboratory) (Martin and Evans, 1975). The cells were kindly provided by Dr. G.R. Martin. (see Figure 1).

N_1 cells which had reached confluency were removed mechanically from culture dishes. In general, 1 ml of packed cells which had been rinsed in PBS was mixed with Freund's adjuvant (complete for the first two injections, incomplete for subsequent injections) and injected subcutaneously suprascapularly into a male New Zealand

Figure 1: Photomicrograph of nullipotential teratocarcinoma stem cells (embryonal carcinoma cells) Nulli-SCC.1. Note large irregular nuclei and numerous prominent nucleoli in each cell.



NULLI-SCC.1 CELLS (Nomarski X3200)

rabbit at 4 weekly intervals. After a rest period of one month, the animal was boosted with an injection of 2-4 times the usual amount of immunogen in Freund's incomplete adjuvant. The rabbit was bled beginning ten days after the boosting injection and at weekly intervals thereafter. The animal was boosted periodically. Several preimmune bleeds were taken for use as control sera. Control sera also included antisera absorbed 2 or 3 times with equal volumes of packed N_1 cells. Normal rabbit sera (NRS) collected from nonimmune rabbits was processed as described. All antisera were heat inactivated (56°C, 30 min), absorbed with mouse tissue (see below), filtered (Millipore, 0.22 um) and stored at -40°C in small aliquots.

3. Absorption of Antisera:

All antisera were absorbed to remove any activity to general mouse molecules. Sera were absorbed two or more times with an equal volume of pelleted homogenate of mouse livers, kidneys and spleens (LKS) which had been rinsed extensively with PBS to remove blood and cytoplasmic components. Each absorption was for 60 min at room temperature or overnight at 4°C. Completeness of absorption was routinely determined by indirect immunofluorescence (IIF) on live mouse lymphocytes or liver cells. All sera were dialyzed for 5 days against several changes of PBS following tissue absorption.

In vivo absorption of antisera was also employed to ensure removal of specificities for adult mouse molecules (Hilgers et al., 1972). This was accomplished by injecting 1 ml of immune serum intraperitoneally (i.p.) into a female mouse. The mouse was bled 16-24 hours later and serum collected and processed as described above. These sera gave comparable results to the tissue homogenate

absorbed sera which were adopted for routine use. All tissue or *in vivo* absorbed sera were tested by IIF on the immunogen (N_1 cells) for retention of specific immune reactivity. It should be kept in mind that *in vitro* tissues protected by blood barriers such as the testis, brain and cumulus-enclosed oocyte.

Differential absorptions of $A'N_1$ were done to distinguish between cell surface and cytoplasmic antigens recognized by the antiserum. Whole cell absorption, method 1: N_1 cells were removed mechanically from two semi-confluent 100 mm plates with Ca^{++}, Mg^{++}-free PBS containing 10^{-4} EDTA. A 100 µl aliquot of $A\text{-}N_1$ IgG (2mg/ml) was placed on a pellet of cells of equal volume and allowed to stand on ice for 45 min. Cells were spun down and the antiserum transferred to a second pellet of cells for 45 min. Again, cells were removed by centrifugation. Whole cell absorption, method 2: A 150 µl aliquot of $A\text{-}N_1$ IgG (2 mg/ ml) was absorbed on monolayers of live N_1 cells by placing it for 15 min. each on 12 semi-confluent cultures in a MultiWell culture plate. Each culture was rinsed twice with PBS immediately before antiserum was transferred to the well. This absorption was done at 37°C. At the termination of the absorption, any floating cells were removed by centrifugation. Cell lysate absorption: N_1 cells on one confluent 100 mm plate were washed thoroughly with PBS and lysed with 175 µl of lysis buffer containing 0.5% Nonidet P40 (Particle Data Laboratories, Elmhurst, Ill.), 2 mM Benzamidine (Sigma Chemical Co.) in PBS. $A\text{-}N_1$ IgG (4mg/ml) was diluted 1:1 with cell lysate and allowed to stand for 24-36 hr at 4°C. The lysate absorbed serum was centrifuged for 70 min at 50,000 x g. If the absorbed preparations of $A\text{-}N_1$ were not

for immediate use, they were stored in the presence of 10 mM Benzamidine at 4°C.

4. Production of Monoclonal Antibodies:

Fusion #1

A. Immunization for production of immune spleen cells:

Two 4-week-old Sprague Dawley virgin female rats (Simonson Laboratories) were bled from the tail twice at weekly intervals. One ml samples were collected at each bleeding to serve as pre-immune controls. The rat which proved easier to handle and a better bleeder was chosen for immunization and the second rat was retained as a source of further non-immune serum. Rat #1 was immunized by injecting 200 live, whole, zona pellucida-free 8-cell embryos (100 injected i.p. and 100 injected subcutaneously) at weekly intervals. The initial injection contained complete Freund's adjuvant and subsequent injections contained incomplete Freund's. The rat was bled one week after each immunization and tested for surface reactivity with 8-cell embryos by indirect immunofluorescence following heat inactivation of complement in the serum (30 min at 56°C). Later bleeds were homogenate or in vivo absorbed as described above before testing on embryos to determine specificity.

B. Fusion of immunized spleen cells with myeloma cells:

The techniques for generating hybrid cells producing monoclonal antibodies were developed by Kohler and Milstein (1975, 1976). The protocol used in the first fusion was a modification of that of Kennett et al. (1978) (see Figure 2). The spleen was removed from the anesthetized rat under sterile conditions and immune spleen

Figure 2: Protocol for production of monoclonal antibody-producing hybridomas:

(1) Rat is immunized with 8-cell embryos

(2) Spleen cells are perfused from rat

(3) Mouse plasmacytoma cells (P3-X63-Ag8) are collected during log growth phase

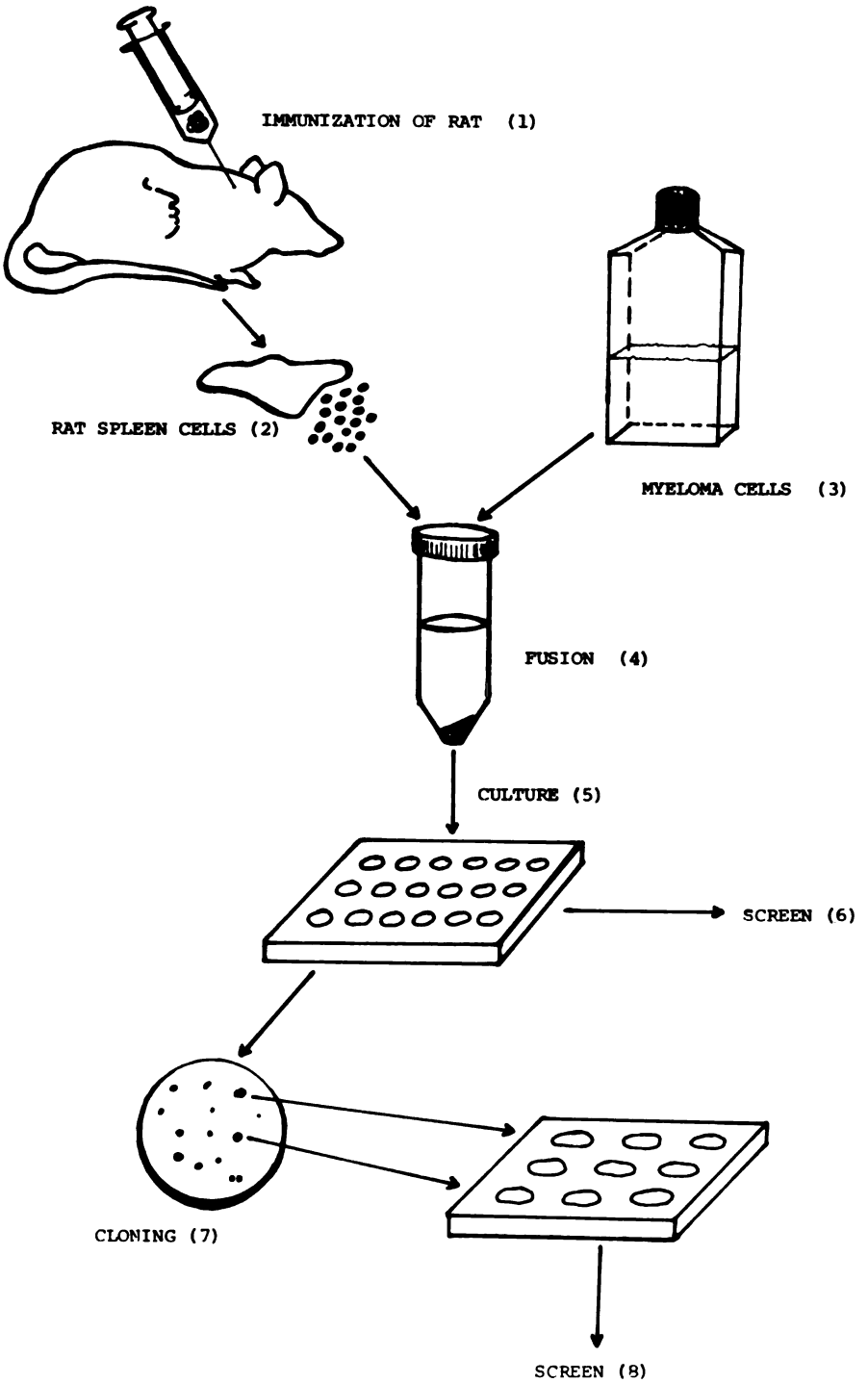
(4) Spleen and plasmacytoma cells are fused in the presence of PEG

(5) Fusion products are cultured in the presence of HAT

(6) Screening is begun at two weeks by RID and IIF

(7) Positive primary cultures are cloned in soft agar

(8) Clones are screened by RID and IIF and clonal lines expanded


IMMUNIZATION OF RAT (1)
RAT SPLEEN CELLS (2)
MYELOMA CELLS (3)
FUSION (4)
CULTURE (5)
SCREEN (6)
CLONING (7)
SCREEN (8)

cells collected by gentle perfusion. The spleen cells were placed in ice cold 0.17 M NH_4Cl for 10 min on ice to lyse erythrocytes, washed in serum-free medium and combined with P3-X63-Ag8 plasmacytoma cells (Kohler and Milstein, 1975) at a ratio of 3:1 (3×10^7 spleen cells, 10^7 plasmacytoma cells) in the presence of 40% polyethelene glycol 1500 (PEG) (Aldrich Chemical Co.) in serum-free medium. The cells were centrifuged for 6 min in the PEG and the pellet allowed to remain undisturbed for 5 min. The PEG was then very gradually diluted and the cells gently resuspended, washed and distributed to two 96-well Microtest II plates (Falcon 3040).

Fusion #2

A. In vitro immunization for production of immune spleen cells:

The procedure for immunizing rat spleen cells in culture was adapted from that of Luben and Mohler (1980) in which mouse spleen cells were immunized.

Rat thymocytes were obtained from 6 Sprague Dawley rats which were 14 days old. The cells were washed and resuspended at a density of 5×10^6 cells/ml in normal thymocyte medium (see below). The cells were cultured for 48 hr and then removed by centrifugation. The conditioned medium was filter sterilized before use in the immunization procedure.

Spleen cells were collected by perfusion from the spleen of a non-immune mature female Sprague Dawley rat. Red blood cells were removed as described and the spleen cells cultured at a density of 10^7 cells/ml in 50% Eagle's MEM supplemented with 10^{-4} M

2-mercaptoethanol (Sigma Chemical Co.) and 4mM glutamine and 50% conditioned thymocyte medium. The immunogen, blastomeres from 600 zona-free 8-cell embryos, was added to the culture system. Blastomeres were prepared by brief incubation in Ca^{++}, Mg^{++}-free PBS followed by a 20 min fixation in 2% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4. The embryos were rinsed in PBS for several hours before being added to the spleen cell culture. Following this preparative procedure approximately 50% of the blastomeres were dissociated as single cells, the remainder in groups of three to four blastomeres. Spleen cells and immunogen were cultured together for four days in a horizontal T-25 flask (Falcon) which was gently agitated several times each day to permit as many spleen cells as possible to contact embryo blastomeres.

B. Fusion of immunized spleen cells with myeloma cells:

The protocol for the second fusion was adapted from Fazekas de St. Groth and Scheidegger (1980). 5×10^5 spleen cells recovered from the in vitro immunization and 10^7 P3K plasmacytoma cells (Kearney et al., 1979) were washed in saline, combined and centrifuged for 10 min at 200 G. The supernatant was removed, the pellet loosened and 1.0 ml fusing solution (50% PEG, 5% DMSO in water) added over 1 min with constant agitation of the tube followed by 90 sec of further agitation of the tube in a $37^{o}C$ water bath. Saline was then added gradually to dilute the PEG and the tube allowed to remain undisturbed for 5 min. The cells were then spun, washed once in medium and distributed to nine 24-well MultiWell plates (Falcon, 3008). Each well also contained 2.5×10^4 mouse peritoneal macrophages as feeder cells.

5. Cell Lines and Media Preparation:

All media described below contained 2 mM glutamine and 100 U/ml each of streptomycin and penicillin unless otherwise specified. All basic media, calf and fetal calf sera, glutamine, penicillin and streptomycin were prepared and provided by the Cell Culture Facility, University of California, San Francisco. Monolayer cultures were grown on Lux 100 mm tissue culture plates (Flow Laboratories) and suspension cultures in vertical T-25 flasks (Falcon 3013). All cells were cultured at $37^{o}C$ in a moist atmosphere of 7% CO_2 in air.

Nulli SCC-1 - N_1 cells (see description above) were grown on plates precoated with 0.1% gelatin (Swine Skin Gelatin I, Sigma Chemical Co., Batch #83C-2570). The medium used was Dulbecco's Modified Eagle's Medium with 4.5g/l glucose (DME-H21) supplemented with 10% calf serum (CS). Cells are grown from 6 X 10^6 cells/100 mm plate to confluency and then passed.

PSA1 - PSA1 is a clonally derived mouse teratocarcinoma stem cell (embryonal carcinoma) line which forms embryoid bodies in vitro when kept in suspension (Martin et al., 1977). The cells were kindly provided by Dr. G.R. Martin. The undifferentiated cells, normally maintained on STO feeder layers (see below), were passaged off feeders to gelatin coated plates for metabolic labelling. Embryoid bodies were generated by maintaining cell clumps in suspension in bacteriological petri plates (Falcon 1001). These were metabolically labelled on Day 3 + 4 when a complete layer of endoderm was visible on all embryoid bodies (see Martin et al.,

1977). All PSA 1 cultures were grown in DME-H21 supplemented with 10% CS.

STO - STO is a line of thioguanine-resistant and oubain-resistant fibroblasts derived from a continuous line of SIM late embryonic mouse fibroblasts (Ware and Axelrad, 1972). They were kindly provided by Dr. G.R. Martin. STOs were grown in DME-H21 supplemented with 10% fetal calf serum (FCS). When used as feeder cells, STOs were rendered unable to divide by a two hr treatment of confluent monolayers with freshly prepared mitomycin C (Sigma Chemical Co.) at 10 μg/ml, followed by replating at the desired density.

Macrophages - Primary cultures of peritoneal macrophages were prepared by flushing the peritoneal cavity of ICR mice with 6 ml 0.34 M sucrose warmed to 37°C. The cells were collected under sterile conditions. Macrophages were maintained in DME-H21, 10% CS or added to other cell cultures as feeder cells.

N115 - N115 is a mouse neuroblastoma cell line. The line was kindly provided by Dr. M. Kirschner. N115 cells were grown in DME-H21 supplemented with 10% CS.

P3-X63-Ag8 (P3)- This plasmacytoma line (myeloma) was derived from a Balb/c MOPC 21 and secretes IgG1 heavy chain and kappa light chain (Kohler and Milstein, 1975). The cells were obtained from the Salk Institute Cell Distribution Center. The cells were seeded at 10^5 cells/ ml in suspension in DME-H21, 10% FCS and diluted 1/10 in fresh medium every 3-5 days. Cells in log phase were obtained for fusion by diluting a confluent culture (2 X 10^6 cells/ml) 1/10 3 days before fusion followed by a 1/2 dilution late on the day

before fusion (P. Goodfellow and J. Levinson, personal communication).

X63-Ag8.653 (P3K) - This plasmacytoma line is a derivative of P3-X63-Ag8 which produces no heavy or light antibody chains (Kearney et al., 1979). The cells were kindly provided by Dr. A. Blumenthal. The cells were cultured and prepared for fusion as described for P3-X63-Ag8 (see above).

Hybridomas - Hybrid cells from the first fusion were cultured in growth medium (GM) which consisted of DME-H21 supplemented with 20% FCS, 20% medium NCTC 109, glutamine, penicillin, streptomycin, 4.5×10^{-4}M sodium pyruvate (Sigma Chemical Co.), 0.2 IU/ml bovine insulin (Sigma Chemical Co.), 10×10^{-4}M hypoxanthine (Sigma Chemical Co.), 10^{-3}M aminopterin (methotrexate hydrate, Aldrich Chemical Co.) and 1.6×10^{-5}M thymidine (Sigma Chemical Co.). The latter three components comprise HAT selection medium (Littlefield, 1964). After two weeks, aminopterin was removed from the GM. After two months, hypoxanthine and thymidine were removed.

The fusion products from the first procedure were plated in 96 well Microtest II plates without feeders. Medium was drawn off and replaced every other day. Screening was begun when growth became obvious in the wells, at approximately 10 days post fusion (see Screening of Hybridoma Cultures below). Cultures shown in the screening procedure to be making antibodies of interest were cloned and the positive clones (those producing monoclonal antibodies to embryonic surface antigens) expanded and frozen. Clones producing negative antibodies, rat antibodies with no anti-embryo activity, were expanded and frozen to be used as a source of control McAbs.

Hybridomas from the second fusion were grown in RPMI 1640 supplemented with 15% FCS, 4mM glutamine, penicillin, streptomycin, 4.5 X 10^{-4}M sodium pyruvate, and 5 X 10^{-3}M 2-mercaptoethanol. The medium contained HAT for the initial 2 weeks after which aminopterin was removed.

The fusion products were distributed in nine MultiWell plates to decrease the necessity of subsequent cloning. Each well also contained 2.5 X 10^4 mouse peritoneal macrophages. The cultures remained undisturbed for 7 days. The cells were then fed by replacing 1 ml of medium on days 7, 10 and every other day thereafter. Screening was begun at 14 days.

Cloning - Initial cloning of hybridoma cultures was done in soft agar according to the method of Kennett et al. (1978). 5 X 10^4 STO feeders were plated in 35 mm dishes and overlayed with 1.8 ml GM containing 0.24% Seakem Agarose (Microbiolical Associates). Hybridoma cells were removed from one Microtest II well mechanically or with trypsin (STV, provided by the Cell Culture Facility) and suspended as single cells in 1.8 ml GM with 0.24% agarose. This was dispensed over the first layer of agarose and the agarose allowed to set. These plates were fed weekly by overlaying the agarose with 1 ml of GM. Clones were picked when they became visible to the naked eye. This was done under a dissecting microscope using a finely pulled sterile glass pipette controlled by mouth pressure. The clones were transferred to MultiWell plates containing STO feeders. Clones were screened and the positive ones expanded and frozen. When a clonal line was subcloned, it was done by limiting dilution. Cells were counted and diluted such that, on average, every third

well in a MultiWell plate would contain a single cell. This was done in the presence of feeder cells.

Freezing of Cells - Hybridoma clones producing antibodies of interest were frozen in multiple aliquots as soon as possible to prevent loss of antibody production. Hybridomas were frozen in DME-H21, 20% FCS and 10% dimethyl sulfoxide (DMSO, Sigma Chemical Co.). Ampoules were insulated with cotton balls to allow for slow freezing, placed initially in a -70^{o}C freezer and transferred to liquid nitrogen for storage. Frozen amoules were thawed quickly in a 37^{o}C water bath and the cells cultured in the presence of feeder cells (STO or macrophage) until the culture was well established. All other cell lines were frozen and thawed as described but did not require feeder cells when thawed.

6. Handling of Culture Supernatants:

Supernatants containing monoclonal antibody (McAb) were removed from culture dishes under sterile conditions and centrifuged to remove debris. Supernatants were used neat in some experiments. For others the supernatants were concentrated 5-10X in a minicon A25 unit (Amicon Corporation) or with Lyphogel (Gelman). Large volumes of supernatant collected for IgG preparations were stored in sterile bottles at 4^{o}C or as ammonium sulfate precipitates in the cold (see below).

7. Production of Monoclonal Antibodies in Nude Mice:

Nude mice (nu/nu) were purchased from the colony at the Animal Care Facility, University of California, San Francisco, when they were 4-6 wks old. 2 X 10^{6} cells of one clonal hybridoma line or the P3-X63-Ag8 plasmacytoma parent were injected i.p. into each mouse.

The animals were housed in a sterile room designed for this purpose. When abdominal swelling became obvious (10-15 days after injection), ascites were removed from the peritoneal cavity using a syringe with a 25 gauge needle. The mice were tapped in this manner at one or two day intervals for several weeks after which the animals succumbed to tumors which grew at the site of injection. Volumes of 0.5 to 5.0 ml were collected each time. The ascites were centrifuged to remove cells and stored in small aliquots at $4^{o}C$ or $-70^{o}C$ neat or diluted 1:1 with PBS. Some aliquots were quick frozen in liquid nitrogen before storing at $-70^{o}C$.

8. Screening of Hybridoma Cultures:

Because of the limited availability of immunogen (8-cell embryos), culture supernatants were initially screened for the production of rat antibody, the antibody contribution from immunized spleen cells. This was done by radial immunodiffusion (RID). When supernatant from a given culture had twice produced a ring in the RID test it was tested for surface reactivity with 8-cell embryos by indirect immunofluorescence (IIF).

Supernatants from clones were screened by the same two step procedure. However, after transfer of a clone from agarose to medium, two medium changes preceded the first RID test as McAb which had diffused into the agarose from other clones resulted in false positives.

9. Radial Immunodiffusion:

A 1/300 or 1/400 dilution of rabbit anti-rat IgG (heavy and light chain) (Cappel Laboratories, Inc.) was made in PBS containing 1% Seakem Agarose at $56^{o}C$. Ten ml of this solution was spread

evenly on a level 75 X 95 mm plastic RID plate (kindly provided by Dr. A. Good). Forty-five wells (1.5 mm diameter) were punched in the solidified agarose and filled with culture supernatants. Concentration of supernatants was not required. Immune rat serum at varying dilutions was placed in several wells as a control. RID plates were incubated 24-36 hr in a moist chamber at 37°C. The plates were screened for precipitin rings using indirect light in a darkened room. The plates were then washed for 10-24 hr in PBS, dipped in 1-2% glycerol and dried overnight under filter paper. Staining was carried out for 3-5 min in 0.25% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories), 10% acetic acid, 25% isopropyl alcohol. Plates were destained in 5:1:5 isopropyl alcohol: acetic acid:water.

10. Indirect Immunofluorescence

Double indirect immunofluorescence (2X IIF) was carried out by incubating live embryos in control or immune rabbit serum or the equivalent IgG preparations for 30 min. The dilutions of antisera used routinely were 1:5 or 1:10 and IgG was used at a concentration of 1 mg/ml. All dilutions of rabbit sera were made in embryo medium. All incubations were carried out in microdrops under paraffin oil in an atmosphere of 5% CO_2 in air. The first incubation was followed by 3 rinses in embryo medium and a 15 min incubation in goat anti-rabbit IgG conjugated with fluorescein (Antibodies, Inc.) diluted 1:5 with PBS. Following 3 final rinses (total 10-15 min), embryos were mounted in a drop of medium on a slide, covered with a coverslip raised on supports and photographed under UV illumination on GAF 500 film using a Zeiss microscope

equipped with an Osram mercury lamp, Exciter filters BG 38, KP 500 and Barrier filters 53/44.

Triple indirect immunofluorescence (3X IIF) was employed to enhance the sensitivity for screening McAbs. This involved a first incubation of 45 min in concentrated supernatant or McAb IgG (0.5 - 1.0 mg/ml), a second incubation of 20 min in rabbit anti-rat IgG and a third incubation of 15 min in goat anti-rabbit IgG conjugated with fluorescein. As in 2X IIF, 3 rinses in embryo medium followed each incubation and mounting and photography were as described for 2X IIF. Controls for 3X IIF included P3 supernatant or IgG fraction, substitution of medium alone in the first incubation to test for nonspecific binding activity in the second and third antibody preparation and supernatant or IgG prepared from clones which produced rat antibody with no reactivity with embryos ("negative" McAbs). Serum from the immunized rat served as a positive control.

IIF on cells was done using cells grown on coverslips or handling them as cell suspensions in which case rinses were done by centrifugation.

Titration studies were done by placing embryos in a doubling dilution series of antibody in embryo culture medium. The titer is the reciprocal of the highest dilution at which fluorescence is detected.

Gonads removed from 15.5 day ICR mouse embryos were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.1) for 15 min and rinsed in PBS for 30 min. The ovaries and testes were then frozen and 10 μm sections cut and mounted on coverslips previously dipped in a solution of 0.5% gelatin and 0.5% chromium potassium

sulfate (Pappas, 1971). Sections were then incubated, mounted and photographed as described above.

11. Immunoglobulin G Purification:

Immunoglobulin G (IgG) was prepared from all antibody sources (heterosera or McAb supernantant). The immunoglobulin fraction was precipitated from the starting material by dropwise addition of saturated ammonium sulfate (pH 7.4) to a final concentration of 40%. In cases where the McAb was later identified as having an IgM component, precipitation was accomplished by 5 day dialysis against frequent changes of distilled H_2O in the cold (Garvey et al., 1977). Protein was redissolved in 5-10 ml 0.1 M phosphate buffer (pH 7.4). A maximum of 250 mg of protein was separated on a column of DEAE Sephadex A-50 (Pharmacia Fine Chemicals) with a bed volume of approximately 90 ml. The column was equilibrated and the IgG eluted with 0.1 M phosphate buffer, pH 7.4 (Fahey and Terry, 1973). IgG was concentrated in an ultrafiltration cell fitted with an XM50 Diaflo membrane (Amicon Corp.) followed by 5 day dialysis in several changes of PBS. IgG was stored at $4^{o}C$, $-40^{o}C$ or $-70^{o}C$ in small aliquots at a concentration of 10-30 mg/ml. The purity of each IgG preparation was determined by polyacrylamide gel electrophoresis.

Fab fragments were prepared from A-N_1 IgG by papain digestion and fractionation on carboxymethyl cellulose (Whatman CM-52) (Porter, 1959). The presence of Fab fragments in the preparations was verified by the presence of a 45,000 dalton band on polyacrylamide electrophorectic gels and by IIF on embryos.

12. Embryo Culture Studies:

The effect of various antibodies on preimplantation development was determined by culturing embryos from the 2-cell stage in microdrops of embryo culture medium containing varying concentrations of antiserum or IgG fractions. The microdrops were incubated under paraffin oil at 37°C in an atmosphere of 5% CO_2 in air. The stage of embryonic development achieved was scored daily for 3 days. The blastocyst stage is normally reached between 48-72 hr of culture and the cultures were terminated after 72 hr. Duplicate antibody and control drops containing 12-15 embryos/drop were observed in each experiment. To determine that the antibody in the culture drops remained active after 3 days, the embryos from each drop were rinsed and placed directly into the second incubation of a 2X IIF or 3X IIF procedure and observed and photographed as described above. In some experiments embryos were transferred to fresh antibody drops each day during the culture period.

13. Cytotoxicity:

In the standard assay, embryos were placed in microdrops of antibody (serum or IgG at 10 mg/ml) diluted 1:1 with embryo culture medium for 45 min at 37°C under humidified 5% CO_2 in air. Subsequently they were transferred to a mixture of antibody, complement and embryo culture medium (1:2:1) for a second 45 min. Viability was assayed by placing the embryos in 0.5% trypan blue in 0.85% NaCl (freshly filtered) for 5 min and counting the number of blue embryos or blastomeres. Sources of complement included guinea pig serum, normal rabbit serum, and "low tox" rabbit complement (Accurate Chemical and Scientific Corp). Controls for this

procedure included: 1) incubation in complement alone (complement source and embryo culture medium, 1:1); 2) first incubation in heat inactivated normal rabbit serum or negative McAb instead of experimental antibody; 3) first incubation in anti-mouse L-cell serum (a positive control). The anti-L-cell serum was diluted 1:20 and was not cytotoxic in the absence of complement. Each experimental and control drop contained 10-15 embryos and the experiments were repeated 3 times.

14. Radiolabelling of Embryos and Cell Lines:

Embryos of all stages were metabolically labelled in 5-10 µl drops of embryo culture medium containing 7-8 mCi/ml ^{35}S-methionine (900-1100 Ci/mole, Amersham). The isotope was removed from carrier solution by evaporation under vacuum and redissolved in an equal volume of ECM. Incubation was done under paraffin oil in a moist atmosphere of 5% CO_2 in air at 37°C. Early stages of embryos were labelled for 3 hr, 8-cell to blastocyst stages for 3-4 hr. Early experiments were done with zona-free embryos; however, this step was subsequently shown to be unnecessary and was omitted. Following the labelling period, embryos were rinsed 3X in ECM and solubilized in electrophoresis sample buffer or immunoprecipitation lysis buffer (see below). Samples were stored at -70°C until use.

Monolayers of cells were labelled overnight in 0.5 ml methionine-free DME-H21 with or without serum containing 7.5-10 µl ^{35}S-methionine (final concentration 150-200 µCi/ml). The cells were rinsed gently 3X with DME-H21 at 37°C following the labelling period and lysed *in situ* with electrophoresis sample buffer or immunoprecipitation lysis buffer. Samples were stored as described.

15. Immunoprecipitation:

The initial method used in the characterization of antigens recognized by A-N_1 and McAbs was immunoprecipitation with Staphylococcus aureus (Staph A) as described by Kessler (1975) and Jones (1977). Because only certain classes of rat and mouse immunoglobulins bind Staph A (Medgyesi et al., 1978; Goudswaard et al., 1978), it was necessary to determine which of the McAbs could be bound directly by Staph A. Hybridoma cells were labelled with ^{35}S-methionine as described above. Two 35 µl aliquots of supernatant were collected from each labelled clone. To one aliquot was added rabbit anti-rat IgG (final concentration 0.4 mg/ml) followed 30 min later by addition of 25 µl of a 10% solution of Staph A (IgGsorb, The Enzyme Center, Inc., Boston). Staph A alone was added to the second aliquot. The quantity of radiolabelled McAb precipitated by the two methods was compared by polyacrylamide gel electrophoresis and autoradiography (see below). This technique also permitted analysis of the heavy and light chain components of the McAbs.

The standard immunoprecipitation technique for antigens detected by the experimental antibodies employed 100-500 ^{35}S-methionine labelled embryos or 25-50% of labelled cells from a 16 mm culture well in 25-50 µl of immunoprecipitation lysis buffer (LB) consisting of 0.5% NP40, 2mM methionine (Sigma Chemical Co.) and 2mM benzamidine. Pre-precipitation was carried out by incubation of the lysate with normal rabbit serum IgG (NRS) for A-N_1 or rabbit anti-rat IgG for McAbs (final concentration 0.4 mg/ml) followed by incubation on a resuspended pellet of 50 µl of 10% Staph A. This

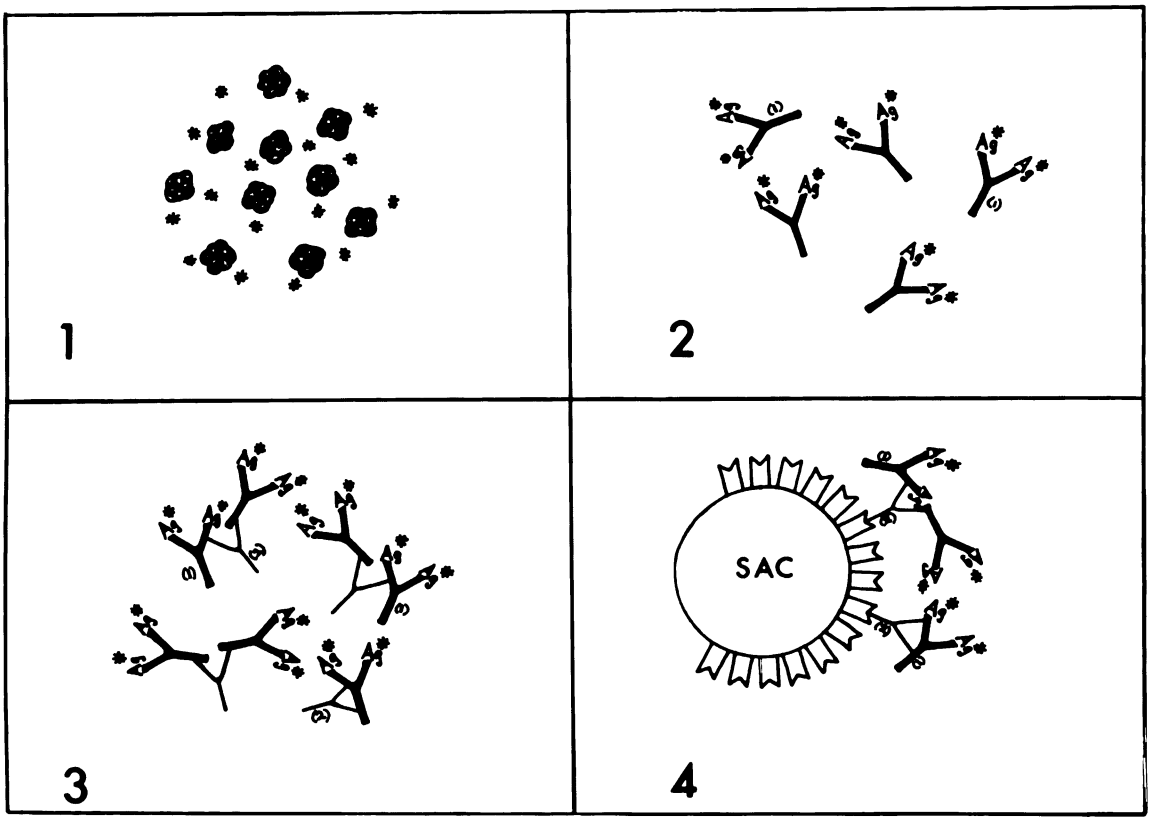
step removed all proteins bound non-specifically by control sera or Staph A. Staph A was removed by centrifugation and the lysate divided equally into experimental and control samples. For the heteroserum precipitations, A-N_1 IgG and NRS IgG were added to lysate aliquots to a final concentration of 0.2-0.4 mg/ml. In some cases differentially absorbed A-N_1 IgG replaced NRS as a control. Following the IgG incubations, 25 µl of 10% Staph A was added to each sample. All incubations were carried out on ice for 45 min. During incubation with Staph A, tubes were agitated frequently. Following the final incubation, the Staph A was rinsed 3X with lysis buffer and suspended in 25 µl of the appropriate electrophoresis sample buffer.

Immunoprecipitation with McAbs differed from that with heteroserum (see Figure 3). Following the preincubation, lysate samples were incubated in the presence of 0.35 mg/ml experimental or control McAb IgG. IgG from the parental plasmacytoma P3 or the negative clone 2-G_2C_1 were used as controls. An incubation with rabbit anti-rat IgG (0.4 mg/ml) followed. The final step, again, was the Staph A incubation as described above.

An alternate method, "reverse Staph A" immunoprecipation, (C. Poodry, personal communication) consisted of preincubation of Staph A in a excess of experimental or control IgG, washing the Staph A in lysis buffer and adding it to the radiolabelled antigen lysate. Again an extra step was introduced when working with McAbs. Staph A was preincubated in rabbit anti-rat IgG followed by McAbs and then added to the antigen mixture.

Figure 3: Protocol for immunoprecipitation with monoclonal antibodies

1. Embryos are metabolically labelled with ^{35}S-methionine (*) *in vitro* and lysed
2. Monoclonal antibody (1) is added to embryo lysate, binds antigen
3. Rabbit anti-rat IgG second antibody (2) is added to lysate; binds monoclonal antibody
4. *Staphyloccocus aureus* (SAC) is added to lysate, binds second antibody


1
2
3
4
SAC

A final method for precipitating antigens with McAbs was the double antibody technique which has been effective in other laboratories (Springer et al., 1978; Myles et al., 1981). This technique circumvents the problem that many McAbs do not bind Staph A. The technique employed was that of Springer and coworkers (1978). The concentration of rabbit anti-rat IgG required to precipitate a known concentration of each McAb was determined by the precipitin ring (interfacial) test (Garvey et al., 1977). 10-25 µl McAb (1 mg/ml) was added to antigen lysate. After 2 hr at 4°C, the appropriate concentration of rabbit anti-rat IgG was added and allowed to precipitate overnight at 4°C. Pellets were washed gently and prepared for electrophoresis as described. In some cases normal rat serum was added to the incubation mix with the McAb to enhance precipitation. A preprecipitation using normal rat serum followed by overnight precipitation with rabbit anti-rat IgG was also done in some cases to remove nonspecifically precipitating proteins. Controls included negative McAb, P3 McAb and normal rat serum.

16. Gel Electrophoresis

One-dimensional analysis of radiolabelled McAbs and antigens precipitated by the experimental antibodies was carried out on SDS-polyacrylamide slab gels (Laemmli, 1970). The gels were 0.3 mm thick and measured 15 cm x 16.5 cm. Separating gels of 8, 10 and 12% were utilized to study proteins and glycoproteins with a wide range of molecular weights. The stacking gel was 4.5%. Samples were electrophoresed for 3-4 hr at a constant current of 25 mA/gel. Gels were fixed and stained in 0.1% Coomassie Brilliant Blue, 50% trichloracetic acid for 20 min and destained in 7% acetic acid.

Gels were dried on filter paper on a slab gel dryer (Hoefer Scientific Instruments). High molecular weght standards (Biorad Laboratories, Richmond, CA) were run on each gel and included: myosin (200,000 d), β-galactosidase (116,250 d), phosphorylase B (92,500 d), bovine serum albumin (66,200 d) and ovalbumin (45,000 d). Apparent molecular weights of the unknowns were calculated using a linear regression of mobility of protein versus the logarithm of the apparent molecular weights of the standards (Ferguson, 1964; Dunker and Ruekert, 1969).

Two-dimensional electrophoresis utilized isoelectric focusing in the first dimension and SDS polyacrylamide electrophoresis in the second, as described by O'Farrell (1975) and Jones (1977). Isoelectric focusing was carried out on 4% acrylamide tube gels (13 cm long, 2 mm in diameter) containing 9 M urea, 2% NP-40 and 2% Ampholines (1.6% pH 5-7, 0.4% pH 3.5-10) (LKB Bromma, Sweden) for a total of 10,000 volt-hours. Molecular weight separation in the second dimension was carried out as described above using a 12% separating gel and a 4.5% stacking gel. Gels were fixed and stained as described above. Kodak X-Omat R X-ray film was utilized for autoradiography of all gels.

17. Antibody Overlay of Electrophoretic Gels:

The technique of gel overlay described by Burridge (1976) was employed to identify antigens recognized by experimental antibodies. This technique does not depend on incorporation of methionine into the antigens and, therefore, will identify any antigen of a protein or glycoprotein nature in the electrophoresis system.

Six hundred 8-cell embryos were lysed in electrophoresis sample buffer, divided equally between two lanes on a 10% SDS slab gel and electrophoresed as described above. The gel was fixed for 6 hr in 50% ethanol, 10% acetic acid and neutralized for 1 day in 50 mM Tris-Cl (pH 7.5), 100 mM NaCl. The gel was then washed for 3 days in 10 mM Tris-Cl (pH 7.5), 140 mM NaCl, 0.1% gelatin (Difco). The gel was cut in half and each half was incubated for 12 hr with 150 µl of McAb in 100 ml of 10 mM Tris-Cl, gelatin buffer. The experimental McAb was 2-C_4C_3 ascites; the control, McAb ascites from the P3 plasmacytoma parent line. This incubation was followed by 6 days of washing in 10 mM Tris-Cl, gelatin buffer. The gels were each overlayed with 1 ml of 10 mM Tris-Cl, gelatin containing 1.5 µg affinity purified ^{125}I-goat anti-mouse IgG (5 x 10^6 dpm/µg) (kindly provided by Dr. J. Grainger) for 12 hr followed by 6 days of washing in 10 mM Tris-Cl, gelatin buffer and 1 day of washing with 10 mM Tris-Cl buffer without gelatin. All buffers contained 10 mM NaN_3. The gels were refixed and stained for 6 hr in 0.1% Coomassie blue, 47.5% ethanol, 10% acetic acid and destained in 12.5% ethanol, 5% acetic acid. The gels were dried as described above. Autoradiography was done on Kodak X-Omat R X-ray film using a Cronex intensifying screen (Dupont, Wilmington, DE).

18. Immunoradiolabelling:

Immunoradiolabelling (IRL) was carried out on live zona-free 8-cell embryos as a method for quantitating the binding of McAbs. Embryos were incubated in McAb ascites (1/20 in ECM) for 45 min followed by three rinses in ECM. Embryos were then transferred to

drops of ^{125}I-rabbit anti-mouse IgG (7 µg/ml in 5% CS, 5 x 10^5 dpm/µg) for 45 min, rinsed in PBS containing 1.0 mM potassium iodide and 5% CS, transferred to a disc of glass fiber filter paper (Whatman GF/C, presoaked in PBS-KI containing 1.0 mg/ml bovine serum albumin) and rinsed under suction with 10 ml PBS-KI. The filters were then counted on a gamma counter (Facilities provided by Dr. T. Hayashida). In general each sample contained 50 embryos. An incubation of 50 embryos in the ^{125}I-rabbit anti-mouse IgG was carried out as described to determine what portion of the radioactivity was due to non-specific binding of the second antibody. In addition, for each sample a volume of the final embryo rinse equivalent to the amount of rinse solution in which the embryos were transferred to the filters was also counted.

19. Tunicamycin Studies

Embryos and N_1 cells were cultured in the presence of tunicamycin to determine the effects of blocking glycosylation of glycoproteins in each cell type. Tunicamycin (Calbiochem) was prepared in ECM using 1N NaOH to solubilize. Embryos were placed in various concentrations of tunicamycin at the two-cell stage and monitored throughout the culture period. 0.5 µg/ml was the lowest concentration which prevented the embryos from blastulating. This concentration was in no other way damaging to the embryos and was used for all further studies. Two-cell embryos were cultured overnight in the presence of tunicamycin and used for experimentation the following day. Several embryos from each group were kept in tunicamycin for two more days to monitor the effectiveness of the tunicamycin treatment. Antigen expression and

alteration in embryos thus treated were monitored by immunofluorescence and immunoprecipitation respectively. N_1 cells were cultured overnight in the presence of various concentrations of tunicamycin (added at the time of replating the cells) and monitored with phase microscopy for deleterious effects. In fact N_1 cells grown in concentrations ranging from 0.1 - 50 µg/ml appeared healthy and a concentration of 5 µg/ml was chosen for further studies. Following overnight culture in the presence of tunicamycin the N_1 cells were labelled for 4-5 hr in ^{35}S-methionine for immunoprecipitation.

RESULTS

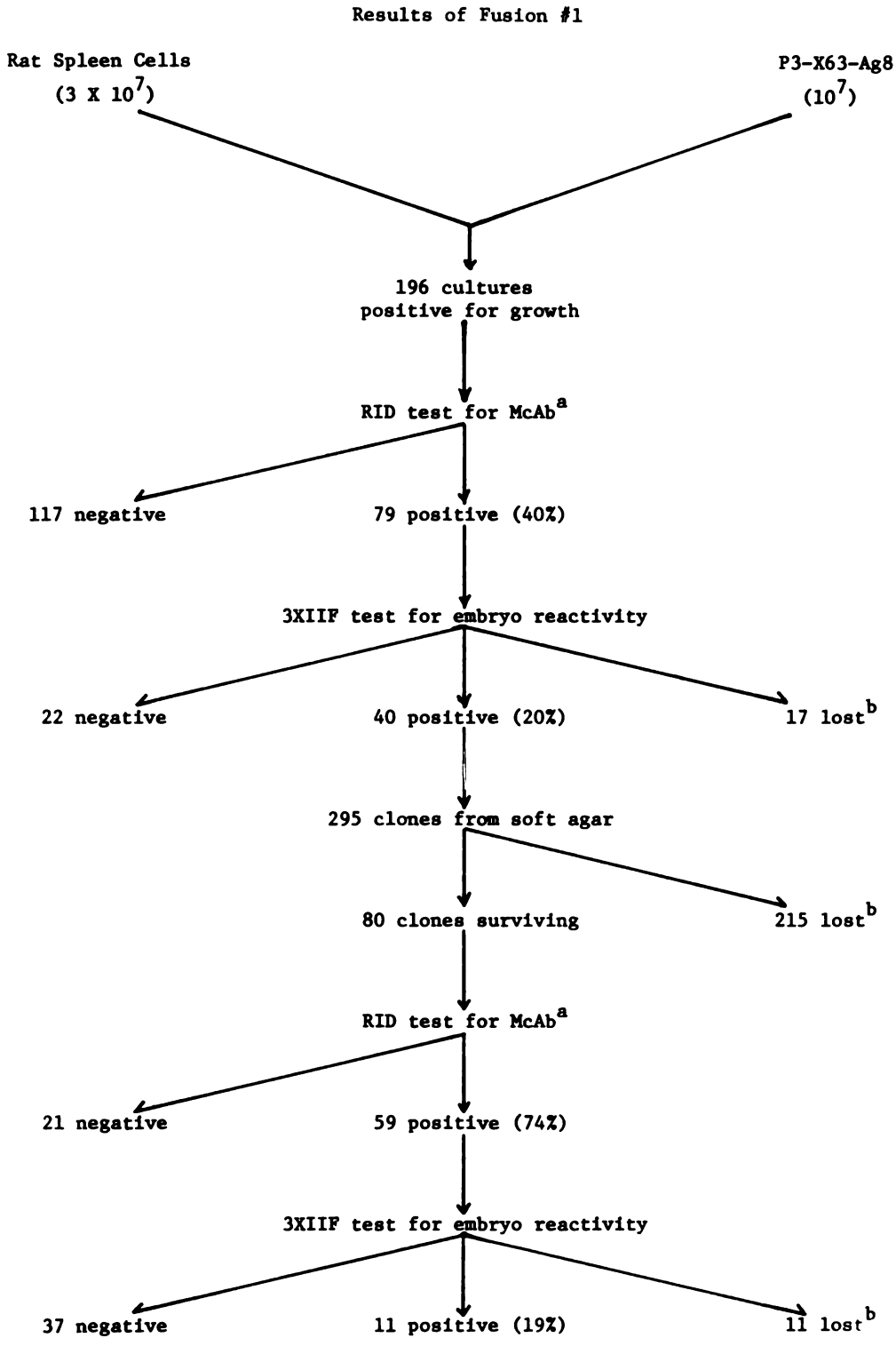
Monoclonal Antibody Production:

Fusion #1

1. Immunization of Rat: One week following the first immunization, the rat had mounted a considerable antibody response to 8-cell embryos as determined by indirect immunofluorescence (2X IIF). Preimmune serum showed no anti-embryo activity. Subsequent immune bleeds demonstrated high embryo reactivity following absorption with mouse liver, kidney and spleen (LKS) indicating the presence of embryo specific antibodies in the serum. In vivo absorption of serum taken one week after the final boost (sixth weekly embryo injection) failed to remove the antibody activity in 2X IIF and the rat was judged to be sufficiently immunized to proceed with the fusion. A final antigen boost was administered by tail vein injection and the spleen removed 3 days later.

2. Hybridoma Production: The results of the first fusion are summarized in Figure 4. Growth was detectable in all 192 Microtest II wells at two weeks after fusion. Supernatant from each culture was tested twice (with an intervening medium change) in radial immunodiffusion (RID) for the presence of secreted rat antibody. Supernatants from 79 cultures (40%) formed precipitin rings in two consecutive RID tests (see Figure 5). These cultures were expanded and 66 survived. Supernatants from the expanded cultures were concentrated 10X and tested for anti-embryo activity on the 8-cell stage using the "triple sandwich" IIF (3X IIF). Forty of the 66 showed surface reactivity with 8-cell embryos. This represents 61%

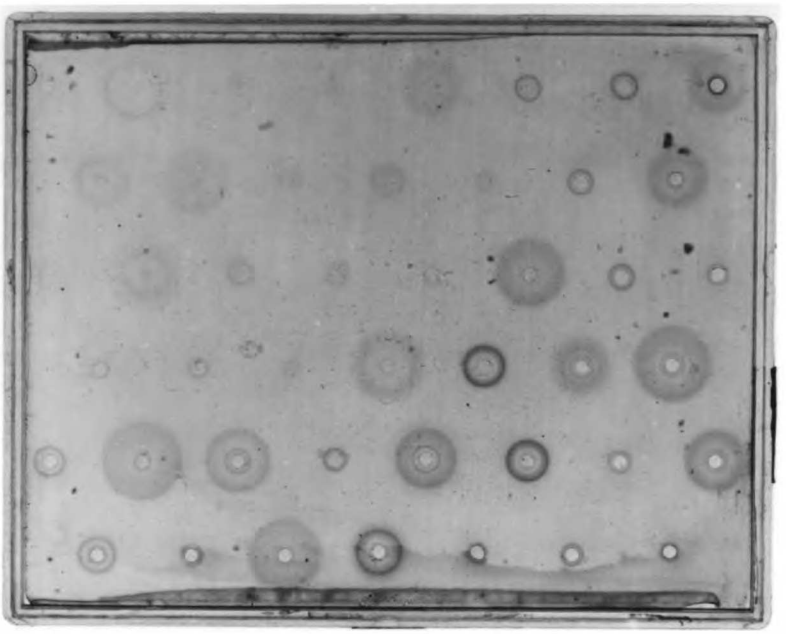
Figure 4: Summary of results of spleen cell/plasmacytoma fusion



a. Abbreviations: RID - radial immunodiffusion; McAb - monoclonal antibody

b. Cultures lost through growth failure, contamination or inappropriate gas mixture

Figure 5: Photograph of a radial immunodiffusion plate used to test hybridoma culture supernatants for the presence of rat antibody. Rabbit anti-rat IgG was diluted 1/400 in agarose before the plate was poured. Culture supernatants from 45 primary cultures were placed in the wells. Plate was rinsed, dried and stained with Coomassie Blue. Rings around the wells represent positive reactions, i.e. the presence of rat antibody in the supernatant.



of the antibody-producing cultures tested and 20% of the original hybridoma cultures. The cultures producing antibodies were cloned in soft agar. Colonies became visible at 1-2 weeks and a total of 259 were picked. The first clones were transferred to Microtest II wells without feeders (Kennett et al., 1978). These did not survive. After testing several types of plastic culture plates and a variety of culture conditions, it was determined that survival of the clones depended upon the presence of feeder cells. Therefore, subsequent clones were transferred to MultiWell culture plates with STO (mouse fibroblast) feeder cells. Some clones were lost due to yeast infection; some failed to proliferate. Of the 80 clones which survived, 59 (74%) demonstrated monoclonal antbody (McAb) production in RID testing and were then assayed for reactivity with 8-cell embryos in 3X IIF. Eleven of 48 clones tested (23%) produced McAb which recognized surface antigens of the 8-cell embryo. The McAb producing clones were expanded and frozen immediately after screening. Those which did not react with embryos were reserved for use as negative controls.

Fusion #2

This fusion involved the use of rat spleen cells which had been immunized *in vitro* for four days in thymocyte conditioned medium (Luben and Mohler, 1980) in the presence of fixed 8-cell blastomeres. Following the *in vitro* immunization, the spleen cells appeared healthy and the embryo blastomeres were intact. 5×10^5 spleen cells were recovered from culture and fused with P3K plasmacytoma cells. The hybridomas were distributed to 207 wells of MultiWell plates with mouse macrophage feeders. At 2 weeks growth

was apparent in 23 wells (11%) and none appeared thereafter. Although use of the larger wells should eliminate the need for cloning (Fazekas de St. Groth and Scheidegger, 1980), multiple colonies were obvious in 15 of the wells. In addition, fibroblasts were found to be proliferating in many of the wells. The orgin of these cells was unknown; they most likely originated from the rat spleen when lymphocytes were collected as no fibroblasts have ever been found associated with macrophages collected as feeder cells from mice. Due to the multiple colonies of hybridomas growing in the wells and the threat of losing them through overgrowth by fibroblasts, the original cultures were cloned before screening for antibody production was done. Five of the original cultures did not proliferate to the extent required for cloning. Seven grew no colonies when cloned. A total of 61 clones were derived from 11 primary cultures, 39 (64%) of which produced McAb as determined by RID. When tested in 2X IIF against 8-cell embryos, none of the 39 was positive. Three clones which evidenced possible reactivity in the 2X IIF assay were tested with 3X IIF on embryos; one ($6\text{-}D_5C_8$) was positive. The aim of the second fusion was to produce a McAb with greater anti-embryo activity than those resulting from the first fusion. As there was no reason to believe that this one McAb which did not show reactivity in a 2X IIF assay would be a better reagent than those from the first fusion which were already well characterized, the products of this fusion were not pursued further.

3. Characterization of Monoclonal Antibodies

The P3-X63-Ag8 (P3) parental plasmacytoma line produces a mouse IgG_1 (kappa) antibody. Therefore, the McAbs produced by cell fusion

should contain a mouse gamma 1 heavy chain and kappa light chain as well as a heavy and light chain contributed by the immune rat spleen cell. Supernatants of hybridoma clones metabolically labelled with ^{35}S-methionine were analyzed by 1-D polyacrylamide gel electrophoresis to determine number of antibody chains and class of the rat antibody component. Figure 6b shows an autoradiograph of electrophoretically separated antibody secreted by the P3 mouse plasmacytoma line and a rat x mouse monoclonal antibody. The P3 mouse component consists of a gamma 1 heavy chain of approximately 53,500 d and a kappa light chain of approximately 24,000 d. The rat component is an IgG consisting of a gamma chain of approximately 57,000 d and a light chain of approximately 23,000 d. This is in agreement with the reported molecular weights of rat IgG_1, IgG_{2a} and IgG_{2c} heavy chains (Bazin et al., 1974) and rat light chain (Bazin et al., 1973). The light chain is assumed to be a kappa chain as the lambda chain is very rare in the rat (Querinjean, et al., 1973). Of the 11 positive McAbs, 9 were determined to have a rat IgG component and 2 a rat IgM component. Immunoglobulin chains from the 11 positive clones, one negative clone and the P3 parental plasmacytoma, are illustrated in Figure 6. All positive IgG McAbs had the rat species specific heavy and light chains described above. The 2 IgM McAbs had μ heavy chains of approximately 68,000 d and light chains of approximately 23,500 d. That these 2 McAbs were rat IgM was verified in Ouchterlony immunodiffusion and precipitin ring (interfacial) tests using a rabbit anti-rat IgM (Miles Laboratoreis, Elkhart, Ind.) which showed no cross-reactivity with the rat IgG McAbs. The only McAb containing a rat component different from the

Figure 6: One-dimensional electrophoretic analysis of the monoclonal antibodies of interest. The mu chains are approximately 68,000; the gamma chains are approximately 53,000 - 55,000 d; the kappa chains are approximately 21,000 - 23,000 d. a,b and d - autoradiographs; c - Coomassie Blue stained gel.

A. 2-C_4C_5

B. 2-F_6C_2

C. 2-G_1C_4

D. 1-$F_{12}C_2$

E. 2-D_4C_4

F. 2-F_5C_5

G. 2-C_4C_4

H. 2-C_4C_1

I. P3-X63-Ag8

J. 2-$F_{10}C_4$

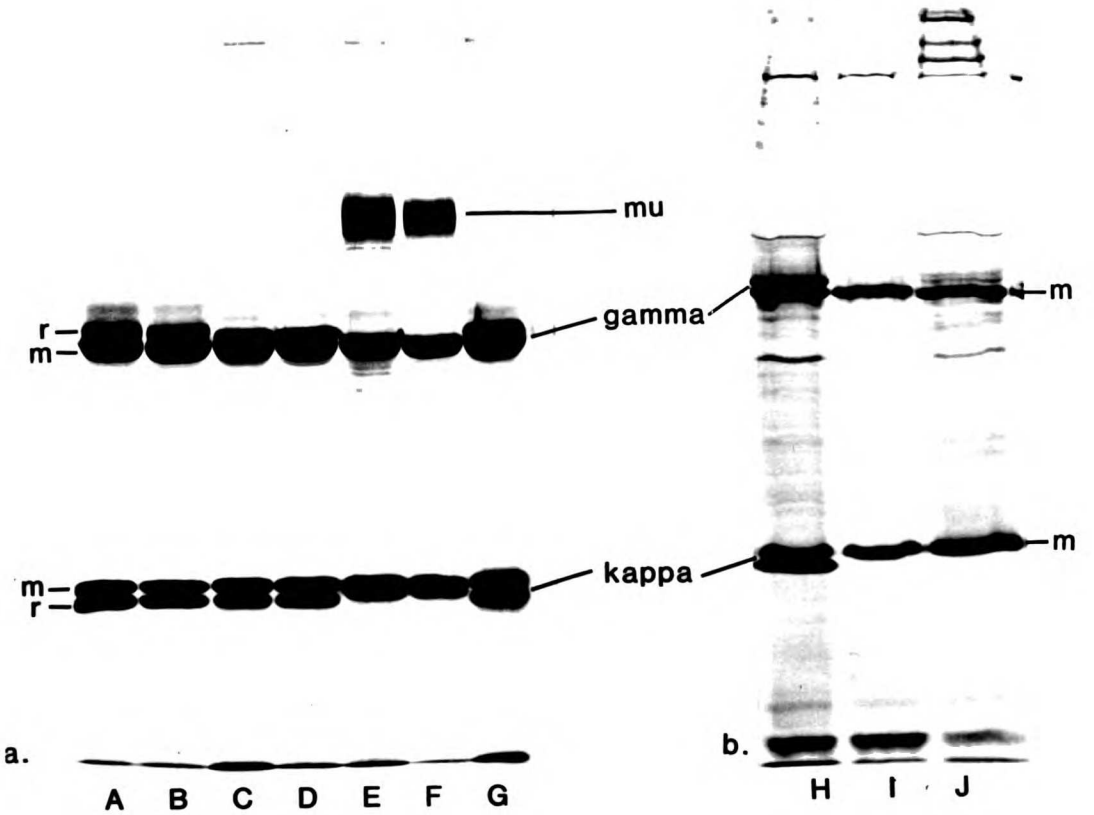
K. C_6E_5

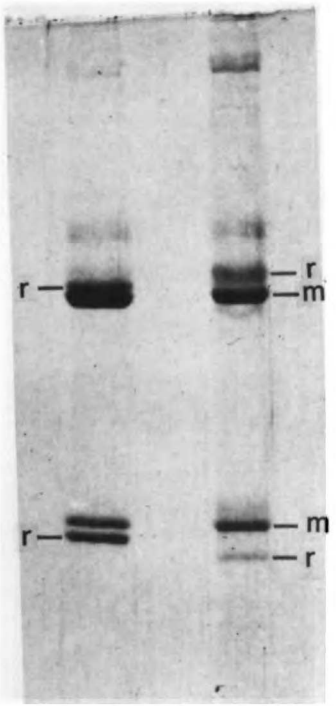
L. 2-G_2C_1

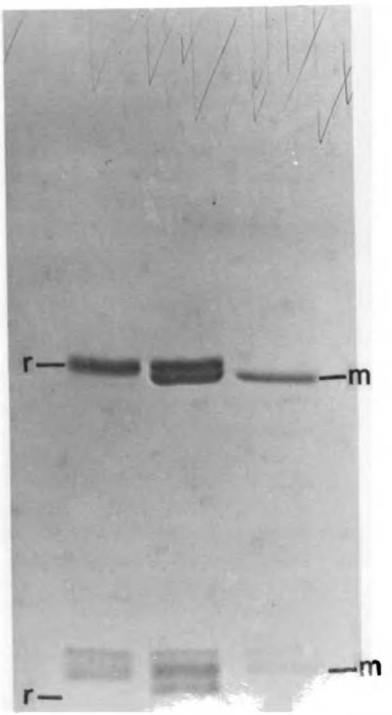
M. 2-$C_4C_{3.2}$

N. 2-C_4C_3

O. P3-X63-Ag8


mu
gamma
kappa
r
m
m
r
m
m
a.
b.
A B C D E F G
H I J


r
r
m
r
m
m
r
c.
K L


r
m
m
r
d.
M N O

species specific chains expected was a negative clone, 2-G_2C_1 (see Figure 6c). The rat heavy chain component of 2-G_2C_1 had an mr of approximately 58,000 and the light chain an mr of approximately 21,500. The molecular weight of the heavy chain is typical of that of the rat IgA molecule (Bazin et al., 1974); however, the small light chain can only be explained as a mutation.

Isoelectric focusing or SDS polyacrylamide electrophoresis have been used in some laboratories to show homogeneity of McAb; however, a better demonstration of homogeneity combines the two techniques in a two dimensional separation (see Melchers et al., 1978; Oi et al., 1978). When the most basic (unmodified) molecular species of heavy and light chains of numerons McAbs are compared, the isoelectric points vary significantly (Oi et al., 1978). Figure 7 illustrates an autoradiograph of a 2-D separation of metabolically labelled P3 antibody and the McAb, 2-C_4C_3, the experimental McAb used for the embryo studies described below. Following 2-D separation, 2-C_4C_3 can be seen to contain a single mouse heavy and light chain identical to those of P3 as well as a single rat heavy and light chain.

Because hybrid cells, particularly hybrids between two species, are often unstable and segregate chromosomes, antibody-producing hybridomas may loose a heavy or light chain due to chromosome loss (Kohler, 1980). For this reason it was necessary to monitor chain composition of McAbs at regular intervals when the hybridomas were cultured for long periods. Of the 12 McAb clones carried in culture, only one showed changes in antibody production. Within the first two months of culture, 2-$F_{10}C_4$, a clone originally producing

Figure 7: Autoradiograph of 2-dimensional electrophoretic analysis of radiolabelled monoclonal antibody, 2-C_4C_3, and antibody secreted by the parental plasmacytoma, P3-X63-Ag8.

pH 6.8 4.1

2.C4C3

P3-X63-Ag8

anti-embryo McAb, lost both the rat heavy and light chains (see Figure 6b). All other clones retained four antibody chains.

Staphylococcus aureus (Staph A) binding properties were determined for each McAb by comparing the amount of radiolabelled McAb brought down by Staph A with that brought down by Staph A and rabbit anti-rat IgG. Figure 8 illustrates an autoradiograph from a typical Staph A binding study. None of the McAbs tested, including the eleven positive clones and the negative clone, 2-G_2C_1, bound Staph A directly.

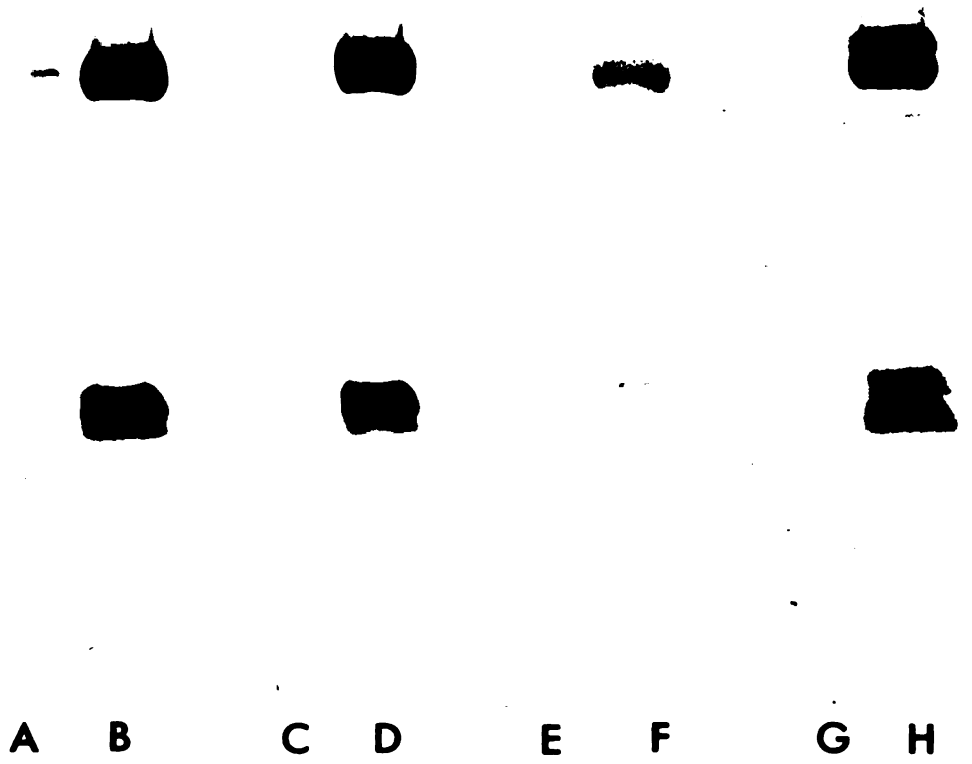
4. Subcloning of Hybridomas: In an attempt to increase the specificity of McAb 2-C_4C_3, the major experimental antibody used in the embryo studies, subcloning was done with the aim of identifying a subclone which had lost the P3 mouse Ig chains through chromosome segregation. Using limited dilution cloning, 34 subclones were generated. McAb from each subclone was metabolically labelled with ^{35}S-methionine and analyzed electrophoretically. One subclone had lost the rat heavy chain and was, therefore, of no interest. A second subclone, 2-$C_4C_{3.2}$, had lost the mouse heavy chain and rat light chain (see Figure 6d). The remaining 32 subclones retained both mouse and rat chains.

5. Immunoglobulin Preparation

The protein fraction precipitated from culture supernatants was separated by ion exchange chromatography on DEAE A50 cellulose. The IgG McAbs eluted from the column as a single peak in the void volume. The IgM McAbs eluted in the first two peaks and these were combined. The yields of McAb for each of 13 clones ranged from an average of 9.4 µg/ml to 100 µg/ml of supernatant. Fractions

Figure 8: Autoradiograph of electrophoretic gels demonstrating lack of binding of monoclonal antibodies to Staphylococcus aureus. A,C,E and G - McAbs brought down by Staph A alone. B,D,F and H - McAbs brought down by Staph A when rabbit anti-rat IgG is introduced as a linker.

A,B - 2-C_4C_3; C,D - 1-$F_{12}C_2$; E,F - 2-$F_{10}C_4$; 2-F_6C_2.


A
B
C
D
E
F
G
H

comprising the antibody peak were combined, concentrated and the proteins analyzed for purity by polyacrylamide electrophoresis. The electrophoretic analysis of the two typical IgG preparations is illustrated in Figure 6c. In general, the preparations were found to have very little contaminating protein.

6. Stability of Clones and Monoclonal Antibodies

The McAbs developed for this project were discovered to be very unstable, a problem not addressed in the technical literature. In early studies one or two clones were expanded rapidly in culture and the McAb stored for 7-10 days as an $(NH_4)_2SO_4$ precipitate of supernatant before IgG preparations were made. McAb IgG was then stored at -70°C in small aliquots of 8-30 mg/ml after verification of anti-embryo activity with IIF. Within three months the frozen IgG preparations had lost all antibody activity. Alternate methods of storage were then tested with six of the anti-embryo McAbs. The methods and results are summarized in Table II. Purified IgG stored at 4°C was compared with IgG stored at -70°C. IgG stored at 4°C lost activity after 3 mos and sterility was a problem in these samples. Supernatant was stored neat or a $(NH_4)_2SO_4$ precipitate at 4°C for 6 to 12 weeks. IgG preparations made following either of these storage methods had no anti-embryo activity. Based on these results, the routine procedure adopted involved the study of one McAb at a time which allowed rapid expansion in culture and purification of IgG before loss of antibody activity occurred. In addition, the use of nude mice (nu/nu) was adopted (see below).

Clone 2-C_4C_3 was well stabilized in culture (as evidenced by the subcloning results descrbed above), having retained anti-embryo

activity following more than a year in continuous culture, but was subject to the storage problems described. Therefore, early freezes of these clonal cells were cultured and stability of anti-embryo activity tested. In fact, the quantity of McAb isolated per unit volume of supernatant decreased at this point and the IgG purified retained activity no longer than other preparations. At no time during these studies of stability was there any indication of gross alterations in the antibody molecules, as determined by RID and electrophoretic separation of labelled IgG from the clones studied.

One possible cause of decreased antibody production or lack of stability of the molecule was mycoplasma contamination. This contamination of all cultures was suspected several months following the fusion based on phase contrast microscopic observations. The presence of mycoplasmas was confirmed by a mycoplasma colony assay kindly performed by P. Sumner, Animal Care Facility, U.C.S.F. Two methods were adopted to eliminate mycoplasmas from the cultures: culturing in the presence of thioglycollate-activated mouse macrophages (Schimmelpfeng et al., 1980) and growing the McAb clones as ascites in nude mice. Macrophage treatment controlled, but did not eliminate, the mycoplasma contamination; mycoplasma would reappear several seeks after termination of treatment. The treatment had no effect on antibody production or stability.

Positive clones 2-C_4C_3, 1-$F_{12}C_2$, subclones 2-C_4C_{3-1} (identical to 2-C_4C_3 in chain types), 2-C_4C_{3-2} (expressing only rat heavy and mouse light chains), negative clone 2-G_2C_1 and parent plasmacytoma line P3-X63Ag8 were grown as nude mouse ascites. Ascites of all positive clones were shown by IIF to have anti-embryo activity at

time of removal from the mice while 2-G_2C_1 and P3 were negative. Ascites, which contained an estimated 20-25 mg/ml McAb, were stored neat or diluted 1:1 with PBS as described in Table II. None of the storage techniques permitted retention of anti-embryo activity beyond 2-3 weeks.

Cells were placed in culture after passage through the nude mice and electrophoretic analysis of the antibody products showed no gross alteration of the immunoglobulin chains due to culture as ascites. These cells, which had been passed through nude mice, were completely cured of mycoplasma contamination.

Table I

Method of Storage	Retention of Activity[1]
IgG preparation (8-30 mg/ml)	
Stored at -70°C	3 months
Stored at 4°C	3 months
Culture supernatant	
Stored neat at 4°C	6-8 weeks
Stored as $(NH_4)_2SO_4$ precipitate at 4°C	6-12 weeks
Ascites from nu/nu mice	
Stored neat at 4°C	2-3 weeks
Stored neat at -70°C	2-3 weeks
Stored 1:1 with PBS at 4°C	2-3 weeks
Stored 1:1 with PBS at -70°C	2-3 weeks
Stored at -70°C following quick freezing with liquid nitrogen	2-3 weeks

[1]Anti-embryo activity as measured by IIF on 8-cell embryos (preparations tested at an antibody concentration of 1 mg/ml). Periods are approximate as there was some variation between samples.

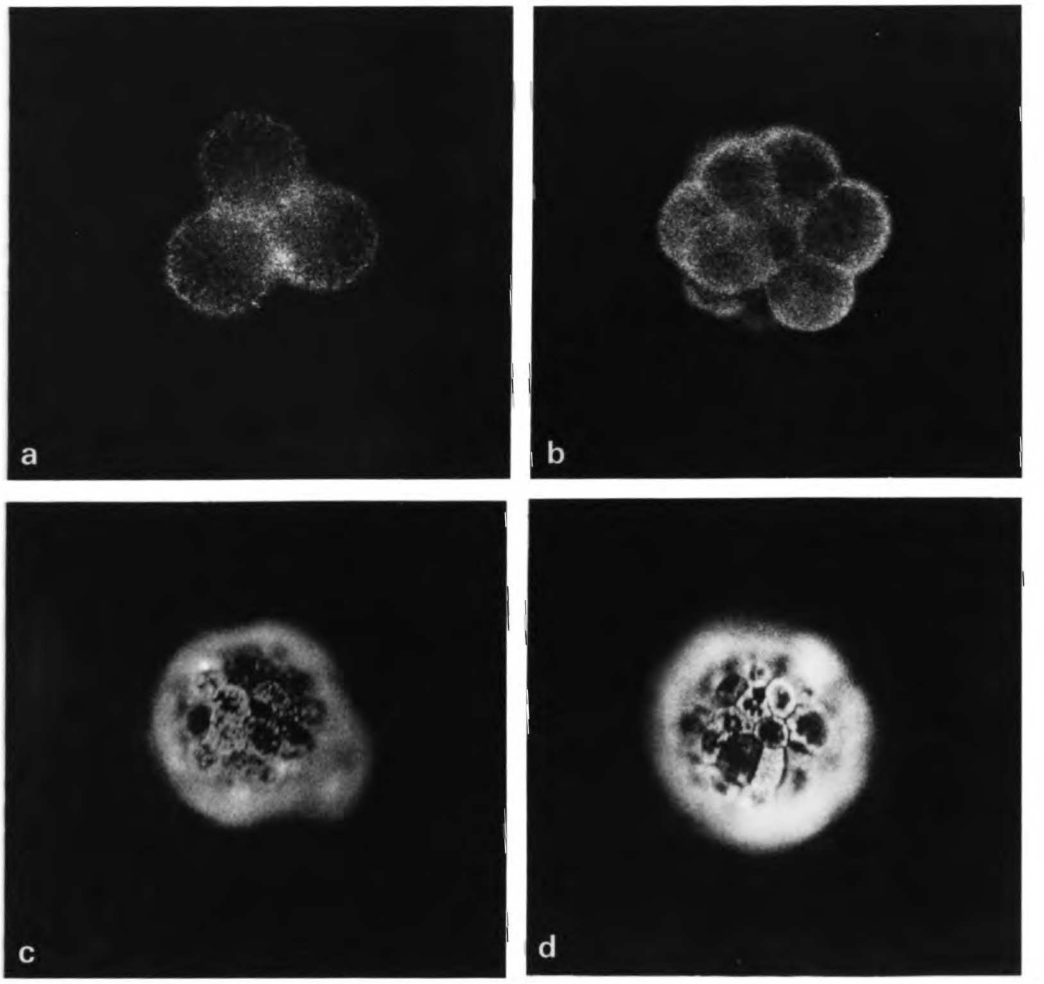
Monoclonal Antibody Analysis of Embryonic Surface Antigens

1. Immunofluorescence

As the McAbs were generated from a fusion of immune rat spleen cells with plasmacytomas, they might represent subsets of the circulating antibodies in the serum of the immunized rat. Therefore, IIF studies were done with anti-8-cell embryo rat serum. The immune rat serum (Rt-1) titered to 512 on 8-cell embryos before absorption and to 64 following *in vivo* absorption in a female mouse (assuming a 1/5 dilution during *in vivo* absorption). Thus the majority of antibody activity was directed toward antigens present in the adult, but one or more antibodies present in the serum recognized embryo specific antigens. *In vivo* absorbed Rt-1 was also positive on Nulli SCC-1 teratocarcinoma cells (N_1), the only cell line studied. It is also possible that these antibodies detected sperm or brain antigens, as *in vivo* absorbed serum is not exposed to these cell types.

With 2X IIF there was evidence of slight patching of antigens by Rt-1 on early stage embryos. The degree of patching increased as development progressed, being maximal on trophectoderm cells of the blastocyst. The 3X IIF assay with Rt-1 intensified the degree of antigen patching particularly on later stage embryos. At the blastocyst stage this procedure resulted in the sweeping of antigens to the periphery of some trophectoderm cells leaving central unstained areas on the cell surface. This resulted in a patchwork effect on the surface of the blastocyst. Figure 9 illustrates the developmental progression of antigen patching with Rt-1 serum on the 4-cell, 8-cell and blastocyst stage. Eight-cell embryos undergoing compaction also displayed localization of fluorescence to an apical

Figure 9: Photomicrographs showing results of triple indirect immunofluorescence (3X IIF) with adult tissue-absorbed Rt-1 antiserum on whole, live mouse embryos. a. 4-cell embryo; b. 8-cell embryo; c,d. blastocysts. Note the progression from even distribution of antigens on the early stages to variable patterns of redistribution on individual trophectoderm cells of the blastocysts.



indirec
-absorbe
a. 4-ce
Note the
on the
ution of

cap on each blastomere. This phenomenon is a characteristic of membrane events during compaction and is not antigen capping mediated by divalent ligand. In fact, the redistribution of antigens parallels a compaction-related reorganization of microvilli to an apical cap on each blastomere (Ducibella et al., 1977). Redistribution of microvilli may also explain the variable staining patterns seen on blastocysts.

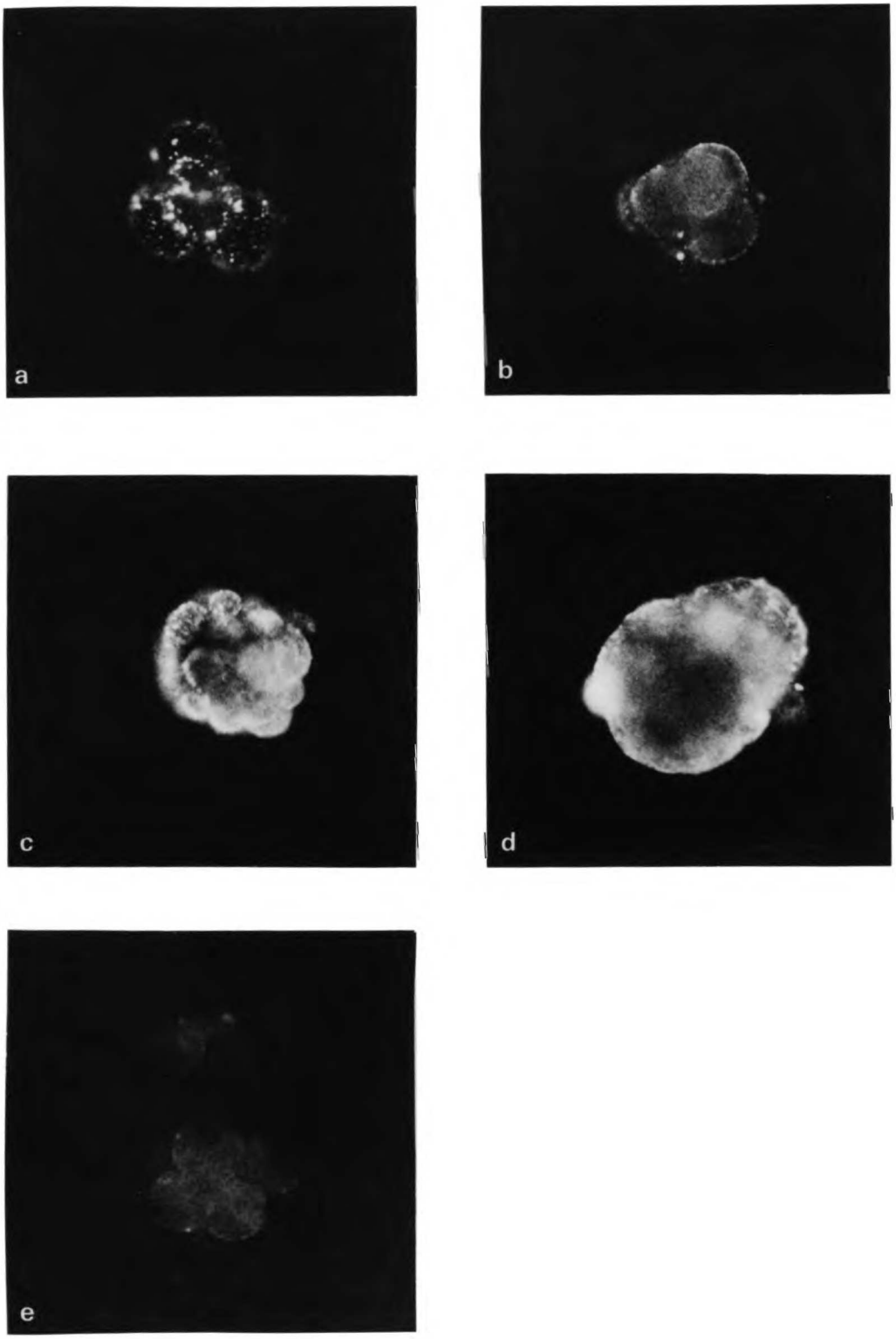
Table III summarizes the immunofluorescence data for all McAbs studied. Studies were done using Ig preparations at concentrations of 0.5-1.0 mg/ml in the 3X IIF assay. Following initial studies on one or two embryonic stages with each McAb, two antibodies, 2-C_4C_3 and 2-C_4C_1, were chosen for extensive studies as they evidenced the greatest reactivity with preimplantation embryonic surface antigens in the IIF testing. Fluorescent staining of the embryo surface with 2-C_4C_3 was observed first at the 4-cell stage at which time approximately 50% of the embryos were positive. The positive embryos showed uniform staining of all blastomeres. When the embryos reached the 8-cell stage, all embryos were positive with staining again uniformly distributed on all blastomeres. Fluorescence was seen on all subsequent preimplantation stages including the morula, early and late blastocyst stage and the ICM. The intensity of fluorescence appeared to increase with developmental stage, maximal fluorescence appearing on trophectoderm cells of the blastocyst. Patching of antigens was not observed at any stage, the fluorescence having a smooth, rimming appearance (see Figure 10).

Table III

McAb	Rat Ig Class	IIF Results[a] 2-cell	4-cell	8-cell	Morula	Blast	ICM	Cytotoxicity 8-cell	Blast
$2.C_4C_1$[b]	IgG	--	+	+	+	+	+	--	--
$2.C_4C_3$	IgG	--	+/-	+	+	+	+	--	--
$2.C_4C_4$	IgG	--	+/-	+	+	+	NT	NT	NT
$2.C_4C_5$	IgG	--	+	+	+	+	NT	NT	NT
$1.F_{12}C_2$	IgG	NT	NT	+	NT	+	NT	NT	NT
$2.F_6C_2$	IgG	--	--	+	+	+	NT	NT	NT
$2.D_4C_4$	IgM	NT	--	+	NT	+	NT	NT	NT
$2.F_5C_5$	IgM	NT	NT	+	NT	+	NT	NT	NT
$2.G_1C_4$	IgG	NT	NT	+	NT	+	NT	NT	NT
$2.F_{10}C_4$*	IgG	NT	NT	+	NT	NT	NT	NT	NT
C_6E_5	IgG	NT	NT	+	NT	NT	NT	NT	NT
$2.G_2C_1$	IgA?	--	--	--	--	--	--	NT	NT

a. Each stage tested 2 or more times in 3X IIF assay. b. The first portion of McAb designation (i.e., $2.C_4$) refers to the primary culture; the second portion of the designation (i.e., C_1) refers to the clone from the primary culture.
+ = positive, -- = negative, +/- = positive on some, but not all embryos, ICM = inner cell mass, NT = not tested, *$2.F_{10}C_4$ tested before loss of rat antibody chains.

Figure 10: Photomicrographs showing results of triple indirect immunofluorescence (3X IIF) with monoclonal antibody, 2-C_4C_3, on whole, live mouse embryos. a. 4-cell; b. 8-cell; c. morula, d. blastocyst; e. control monoclonal antibody, 2-G_2C_1, on 4-cell embryos. Figure a. shows slight rimming of fluorescence with large non-specific patches of staining occassionally seen with the McAbs.


a
b
c
d
e

Nude mouse ascites generated with clone 2-C_4C_3 was an effective reagent in the 2X IIF assay whereas the sensitivity of the 3X IIF assay was required to demonstrate activity of the IgG isolated from 2-C_4C_3 supernatant. The ascites titered beyond 50 (representing a concentration of approximately 0.4 mg/ml) when tested on 8-cell embryos with 2X IIF.

The earliest detectable surface fluorescence seen with McAb 2-C_4C_1 was also at the 4-cell stage. However, unlike the results with 2-C_4C_3, all 4-cell embryos stained with 2-C_4C_1. All subsequent stages were positive in the 3X IIF assay and increased intensity of fluorescence was observed at the blastocyst stage. Patching was not observed with this McAb. 3X IIF with McAbs on N_1 cells was difficult to interpret. The sequential exposure to 3 reagents with intervening rinses appeared to be quite harsh for the cells. Filling of the dead cells with the fluorescein conjugated third antibody made assessment of cell surface staining difficult. Under these conditions the experimental antibodies did not appear to bind to cell surfaces to a greater extent than the control antibodies which included the negative McAb, 2-G_2C_1, and the plasmacytoma antibody, P3. Further attempts to determine cross-reactivity of the McAbs with N_1 or other cell lines employed immunoprecipitation (see below).

2. Cytotoxicity

Neither 2-C_4C_3 nor 2-C_4C_1 were cytotoxic to 8-cell embryos. Negative results were obtained with concentrations of IgG as high as 5 mg/ml. In all cases the positive control sera, anti-mouse L-cell and Rt-1, were cytotoxic. Complement alone was not cytotoxic.

Because IIF studies had shown a possible increase in antigen expression at the blastocyst stage with both McAbs, cytotoxicity was also studied at this stage. Again both antibodies were negative even at high concentrations.

3. Culture Experiments

Culture of embryos from the 2-cell stage to the blastocyst in the presence of 2-C_4C_1 or 2-C_4C_3 antibody concentrations ranging from 0.25 - 5.0 mg/ml had no significant effect on embryo development. Each antibody was tested in three separate experiments with thirty embryos cultured in each antibody concentration. Although deleterious effects on embryo development were observed in 5 mg/ml of the positive McAb 2-C_4C_3, the number of embryos affected did not differ from that observed in 5 mg/ml of the negative McAb 2-G_2C_1. Pilot culture studies with 1-$F_{12}C_2$ and 2-C_4C_4 demonstrated no effects on embryo development.

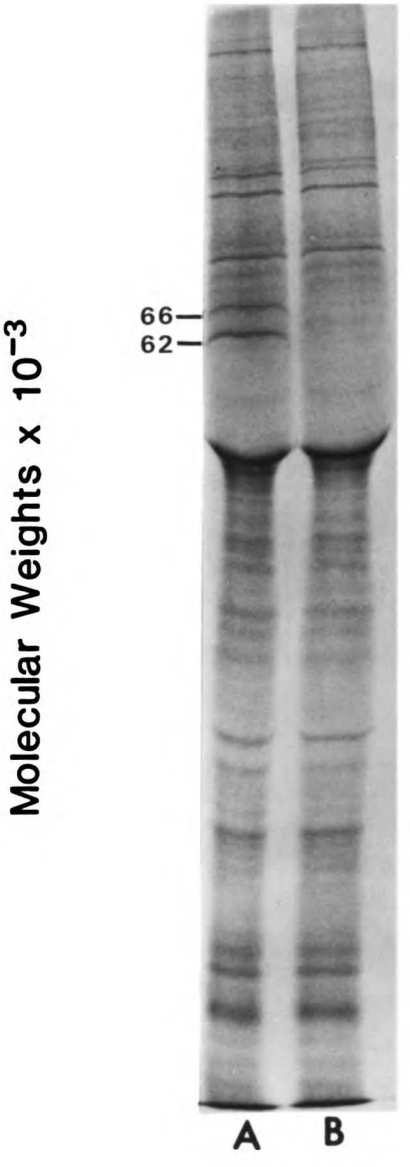
Embryos were also cultured from the 2-cell stage in the presence of 2-C_4C_3 and rabbit anti-rat IgG as a cross-linker of the first antibody to determine whether increased patching of surface antigens would alter development. Analysis revealed no significant differences between the experimental and control groups.

4. Immunoprecipitation

Immunoprecipitation of metabolically labelled 8-cell embryos, blastocysts, N_1 cells and STO cells were attempted with clones 2-C_4C_1, 2-C_4C_3, 2-C_4C_4, 2-C_4C_5, 1-$F_{12}C_2$, 2-F_5C_5, 2-D_4D_4 and 2-$F_{10}C_4$ (before chain loss in this clone had been determined). All clones were tested on embryos, not all on both cell lines. The negative clone 2-G_2C_1 or P3 were used as controls. Although specific bands

appeared to be precipitated with many of the McAbs in the initial studies, no two precipitations with the same McAb/antigen combination were identical. When the long term stability of the McAbs became suspect, this was thought to be the explanation for the nonreproducible results. Thereafter, the McAbs were tested on embryos with IIF immediately before each immunoprecipitation. This did not improve the results. Very few counts were brought down in precipitates from embryos (a McAb precipitation from 150 ^{35}S-methionine labelled 8-cell embryos yielded an average of 6000 to 7500 cpm). Counts brought down by experimental and control McAbs did not differ in any given precipitation. In spite of the limited counts precipitated, the gels showed a great many background molecules (for example see Figure 11). This suggested that perhaps molecules specifically precipitated were being masked on the gels by nonspecific bands. Because the complexing of rabbit anti-rat IgG with McAb and that of Staph A with rabbit anti-rat IgG occurred in the presence of labelled embryonic molecules, one might expect nonspecific trapping of labelled molecules within the complexes. Therefore the "reverse Staph A" procedure was attempted. Because the Staph A is precoated with rabbit anti-rat IgG followed by McAb, only the McAb/antigen interaction takes place in the presence of labelled embryonic proteins and the background should be reduced. In fact, only a slight reduction of background was observed. Although molecules apparently specific for the experimental McAb were present in the precipitate, the results could not be duplicated on further attempts with this technique.

Figure 11: Autoradiograph of electrophoretically separated proteins in a typical immunoprecipitate from 8-cell embryos with monoclonal antibody, 2-C_4C_3. Note numberous background bands. The apparently specific bands of 62,000 and 66,000 d were not found in subsequent immunoprecipitations. A. 2-C_4C_3; B. 2-G_2C_1.


Molecular Weights x 10^{-3}
66
62
A
B

The double antibody technique of immunoprecipitation might also be expected to reduce the background as the Staph A/antibody binding step is eliminated. The technique was applied with a variety of McAb/antigen combinations but reproducible results were never obtained.

5. Gel Overlay Studies

The Burridge technique of gel overlay was adopted to examine the possibility that the antigen(s) detected by McAbs were not labelled with ^{35}S-methionine. This would be the case if synthesis did not occur during the period of exposure to radioisotope or if the antigen(s) lacked detectable quantities of methionine residues. This procedure depends upon labelled antibody rather than labelled antigen. The procedure was carried out with 8-cell embryos as the antigen source and 2-C_4C_3 and P3 ascites as the positive and negative McAbs respectively. Surprisingly, after a 7 day exposure (longer than that usually required with this technique) the autorad revealed "specific" binding to several molecular weight markers with both 2-C_4C_3 and P3. 2-C_4C_3 bound heavily to phosphorylase B and a breakdown product of phosphorylase B and less markedly to β-galactosidase and ovalbumin. There was no apparent binding to myosin or bovine serum albumin. P3 showed less intense staining of the same 3 molecular weight markers but no staining of the breakdown product of phosphorylase. Due to limited quantity of antigen a control consisting of overlay with second antibody (^{125}I-rabbit anti-mouse IgG) alone was not done. However, Dr. J. Grainger (personal communication) performed several overlay studies with the same second antibody reagent and found no binding other than that

specific to his experimental sera. The difference in degree of binding seen with the two McAbs also suggests that the phenomenon observed was due to binding of the McAbs rather than the second antibody which would be expected to bind equally to both molecular weight samples. This result cast considerable doubt on the specificity of embryonic antigen recognition by the McAb 2-C_4C_3.

6. Immunoradiolabelling

The immunoradiolabelling technique was adopted for two purposes. First was the desire to quantitate antigen binding on different stage embryos. Second was the desire to titer the antibody binding to determine whether the process displayed the titration characteristics of a true antibody/antigen interaction. Initial attempts to immunoradiolabel directly with metabolically labelled McAbs (^{35}S-methionine) were unsuccessful presumably due to lack of sufficient radioactivity incorporated in to the antibodies. Indirect radiolabelling with an ^{125}I-goat anti-mouse IgG was used to test the binding of McAb IgG and ascites to embryos. Several experiments with each antibody source looked promising but results were not reproducible. Because stability of the McAbs had become suspect by this time, several radiolabelling experiments were carried out with the same 2-C_4C_3 ascites preparation within a 3 day period. Although embryos displayed fluorescent binding in IIF with the antibody preparation chosen, the immunoradiolabelling results were again irreproducible. At this point the quality of the McAb reagents became highly suspect and the procedure was abandoned.

Reactivity of Anti-Nulli SCC-1

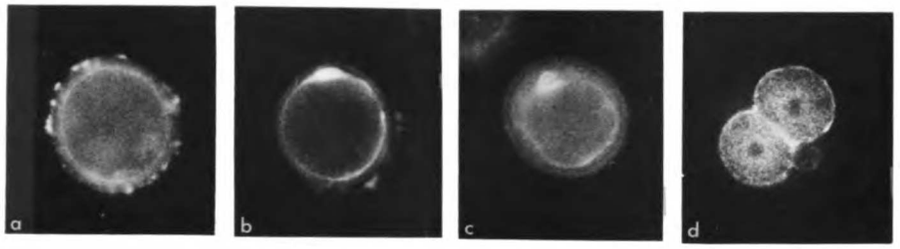
1. Immunofluorescence

All cleavage-stage embryos, unfertilized eggs and primary oocytes removed from antral follicles express antigens detected by A-N_1 in 2X IIF assays (Figure 12) (Calarco and Banka, 1979). A-N_1 (whole serum) titers in the range of 32-64 on zygotes through morulae, increases to a titer of 128 in the ICM and reaches 256 on the trophectoderm of the late blastocyst stage. The zona pellucida is negative before and after fertilization. A-N_1 retains anti-embryo activity following in vivo absorption in adult mice indicating that one or more of the antigens recognized is embryo specific.

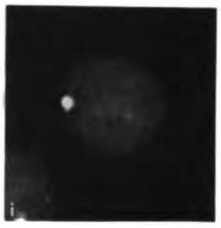
The presence of antigens on cells of the embryonic germ line was also examined in frozen sections of embryonic ovaries and testes. Indirect immunofluorescence indicates antigens are present on primary oocytes in 15.5 day ovaries but not spermatogonia in 15.5 day testes. This is supported by the deservation that in vivo absorption of A-N_1 on a volume/weight basis in a female mouse reduced fluorescent staining on 8-cell embryos to a greater extent than in vivo absorption in a male mouse. Whether this decrease in activity can be attributed entirely to antibody absorption by oocytes is questionable, however.

Experiments designed using IIF to study surface modulation reveal a similar distribution of antigens on N_1 cells and embryos. Surface antigens on N_1 cells show a high degree of patching at 37°C. Incubations of 2-cell embryos in A-N_1 for increasing intervals (5 min - 1 h) at 37°C reveal increasingly patchy antigen distribution but no capping is apparent. The intensity of fluorescent staining

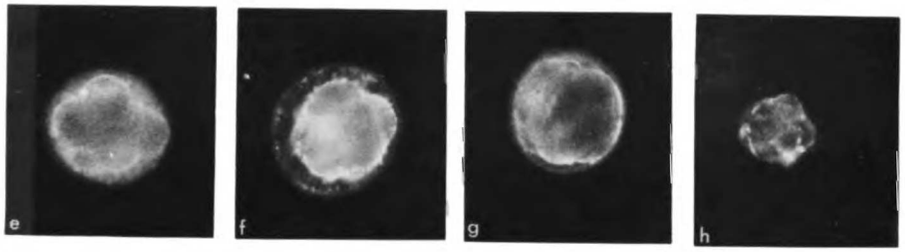
Figure 12: Photomicrographs of mouse oocytes and preimplantation embryos showing surface fluorescence following indirect immunofluorescence with A-N_1 antiserum. The zona pellucida is present on all but the 2-cell embryo. The 8-cell control was incubated in normal rabbit serum as the first antibody. All embryos X 175.



OVARIAN OOCYTE | UNFERTILIZED EGG | ZYGOTE | 2-CELL



8-CELL CONTROL



8-CELL | MORULA | BLASTOCYST | ICM

on embryos varies inversely with the duration of the assay. The strongest fluorescence is seen in short incubations in A-N_1 (5 min). When embryos are placed in ECM between the first and second antibody incubations for increasing intervals (5 min - 12 h), fluorescence diminishes after 60 min in medium and remains constant at the diminished level over 12 h.

Differentially absorbed A-N_1 IgG has been studied with IIF. Absorption of A-N_1 with mechanically harvested N_1 cells eliminates fluorescence on N_1 cells and all embryo stages. Whole cell absorption of A-N_1 (on monolayers of N_1 cells or with N_1 cells harvested in Ca^{++}, Mg^{++}-free PBS) reduces surface fluorescence on N_1 cells to background level while a diminished level of fluorescence persists on 8-cell embryos. Lysate absorbed A-N_1 could not be analysed using IIF on live cells due to the detergent present in the lysate.

In addition to strong surface reactivity with N_1 cells, IIF studies with LKS absorbed A-N_1 have revealed slight cross reactivity with other cell lines including F9 (another nullipotential embryonal carcinoma obtained from Dr. G. Martin), P815 (mouse mastocytoma obtained from Cell Culture Facility, U.C.S.F.), mouse rhabdomyosarcoma cells obtained from Dr. J. Dahlberg, NIH, and STO mouse fibroblasts. Other cell line cross reactivities were studied by immunoprecipitation (see below).

2. Cytotoxicity

A-N_1 whole serum dilutions ranging from 1:2 to 1:10 are not cytotoxic to embryos or oocytes in the presence of complement from guinea pig serum, normal rabbit serum or "low tox" rabbit

complement. Rabbit anti-L cell serum (used as a positive control) was cytotoxic to embryos at all stages in the presence of each of the 3 complement sources.

Functional Studies:

Embryos cultured from 2-cell to blastocyst (3 days) in 1.25%, 5%, 10% and 20% A-N_1 antiserum (LKS absorbed) show dose related developmental inhibition: 5%, 13%, 55% and 80%, respectively, fail to reach the blastocyst stage as compared to N_1 cell absorbed A-N_1 (Figure 13). A-N_1 delays cleavage slightly and some embryos arrest at the 4- to 8-cell stage. By 72 h, the majority of embryos which have not reached blastocyst are degenerating. Embryos in 5% 10% and 20% A-N_1 continue to express surface antigens detectable by IIF after 3 days in culture indicating the antiserum is still active after 3 days in the culture system.

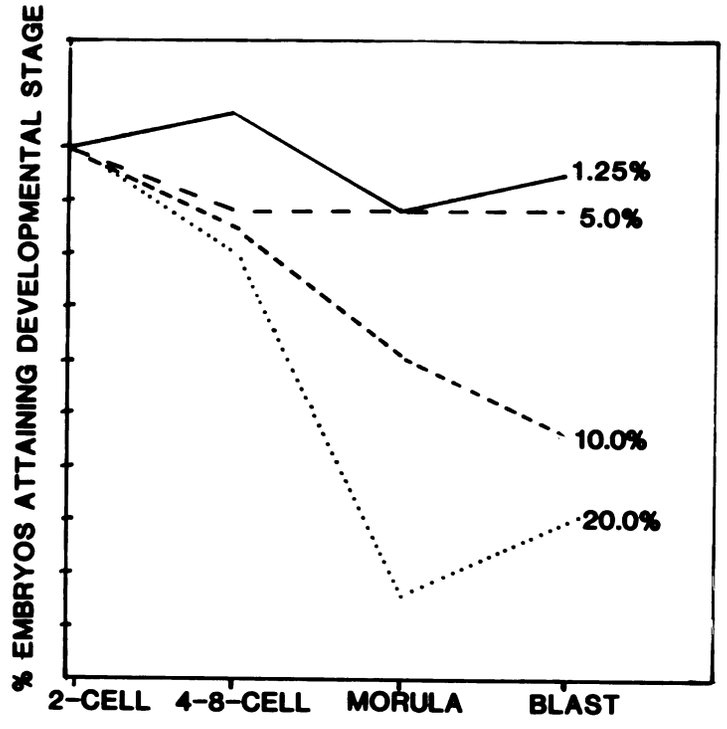
Embryos cultured from 2-cell to blastocyst in the presence of A-N_1 IgG fractions (1.5, 1.0 and 0.5 mg/ml) show no inhibition of development when compared with embryos cultured in 1.5, 1.0 and 0.5 mg/ml NRS. The highest concentration of IgG should be roughly comparable to the antibody concentration in 10% antiserum. The most likely explanation for the apparently contradictory results with whole serum and IgG is a difference in immune reactivity in different bleeds from the immunized rabbit.

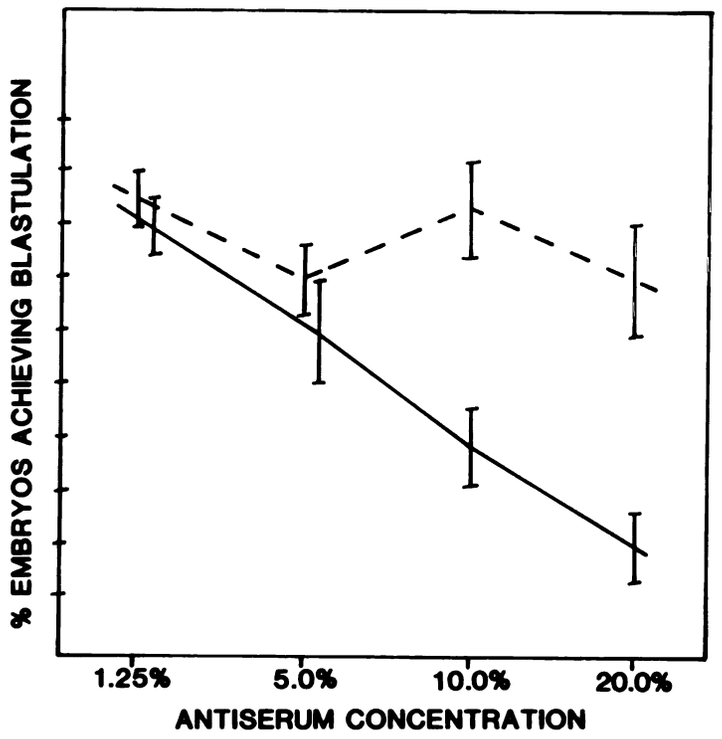
Culture in the presence of A-N_1 Fab (2.75 and 0.75 mg/ml) had no effect on embryo development as compared with culture in the presence of identical concentrations of NRS Fab. Culture in the presence of Fab and goat anti-rabbit IgG was carried out to determine whether cross-linking of the Fab would induce

Figure 13: Results of culturing preimplantation embryos from the 2-cell stage in the presence of varying concentrations of A-N_1 antiserum.

Upper panel: The embryos were scored at 24 h intervals for stage of development attained. The data has been normalized for controls which consisted of culturing embryos from the 2-cell stage in the same concentrations of A-N_1 absorbed 3 times with N_1 cells.

Lower Panel: The percentage of embryos achieving blastulation when cultured from the 2-cell stage in the presence of increasing concentrations of A-N_1; ——— Embryos in the presence of A-N_1; ----- Embryos in the presence of A-N_1 absorbed 3 times with N_1 cells. Error bars represent the standard error of the mean for three experiments with two samples of fifteen embryos each in each antiserum concentration.


% EMBRYOS ATTAINING DEVELOPMENTAL STAGE
1.25%
5.0%
10.0%
20.0%
2-CELL
4-8-CELL
MORULA
BLAST


% EMBRYOS ACHIEVING BLASTULATION
1.25%
5.0%
10.0%
20.0%
ANTISERUM CONCENTRATION

developmental inhibition but, again, experimentals did not differ from controls.

Molecular Characterization of N_1 Antigens:

Molecular characterization of N_1 antigens was accomplished by Staph A mediated immunoprecipitation. Initial immunoprecipitates from NP-40 lysates of N_1 cells were shown by slab gel electrophoretic analysis to contain a high number of background bands. Experimentation with all variables in the procedure (varying concentration of antibody, varying quantity of Staph A, preincubation of Staph A with N_1 cell lysate) failed to reduce the background. However, the background was reproducible with a variety of control sera including normal rabbit serum (NRS), normal mouse serum (NMS) and A-N_1 absorbed with N_1 cells. Results of the "reverse" Staph A precipitation technique were identical to those with the Kessler (1975) technique and the latter was used routinely.

Differential absorption of A-N_1 with whole, live N_1 cells or N_1 cell lysate made it possible to identify surface and cytoplasmic antigens in immunoprecipitates. Results of this approach are summarized in Figure 14 and several autorads are shown in Figures 15, 16, 17 and 18. Bands which appear only in precipitates with A-N_1 and not in precipitates with whole N_1 cell absorbed A-N_1 (A-N_1/wc) nor in precipitates with N_1 lysate absorbed A-N_1 (A-N_1/lys) are assumed to be on the cell surface of N_1 cells. There are six reproducible surface molecules precipitated from N_1 cells with apparent molecular weights of 126,000, 61,000, 38,600, 20,000, 19,000 and 16,000 (Figures 15 and 18). All six are also found on STO cells (Figure 16). Four of the bands have been identified on

Figure 14: Summary of the expression of surface and cytoplasmic antigens detected by immunoprecipitation from a variety of cells with A-N_1. BLAST = blastocyst; PSA = PSA1 undifferentiated pluripotent embryonal carcinoma cells; BODS = day 3 + 4 embryoid bodies differentiated from PSA1 cells; N_1 = NULLI-SCC.1 nullipotent embryonal carcinoma cells; STO = fibroblast line derived from late mouse embryos; MACRO = mouse primary macrophages; nt = not tested.

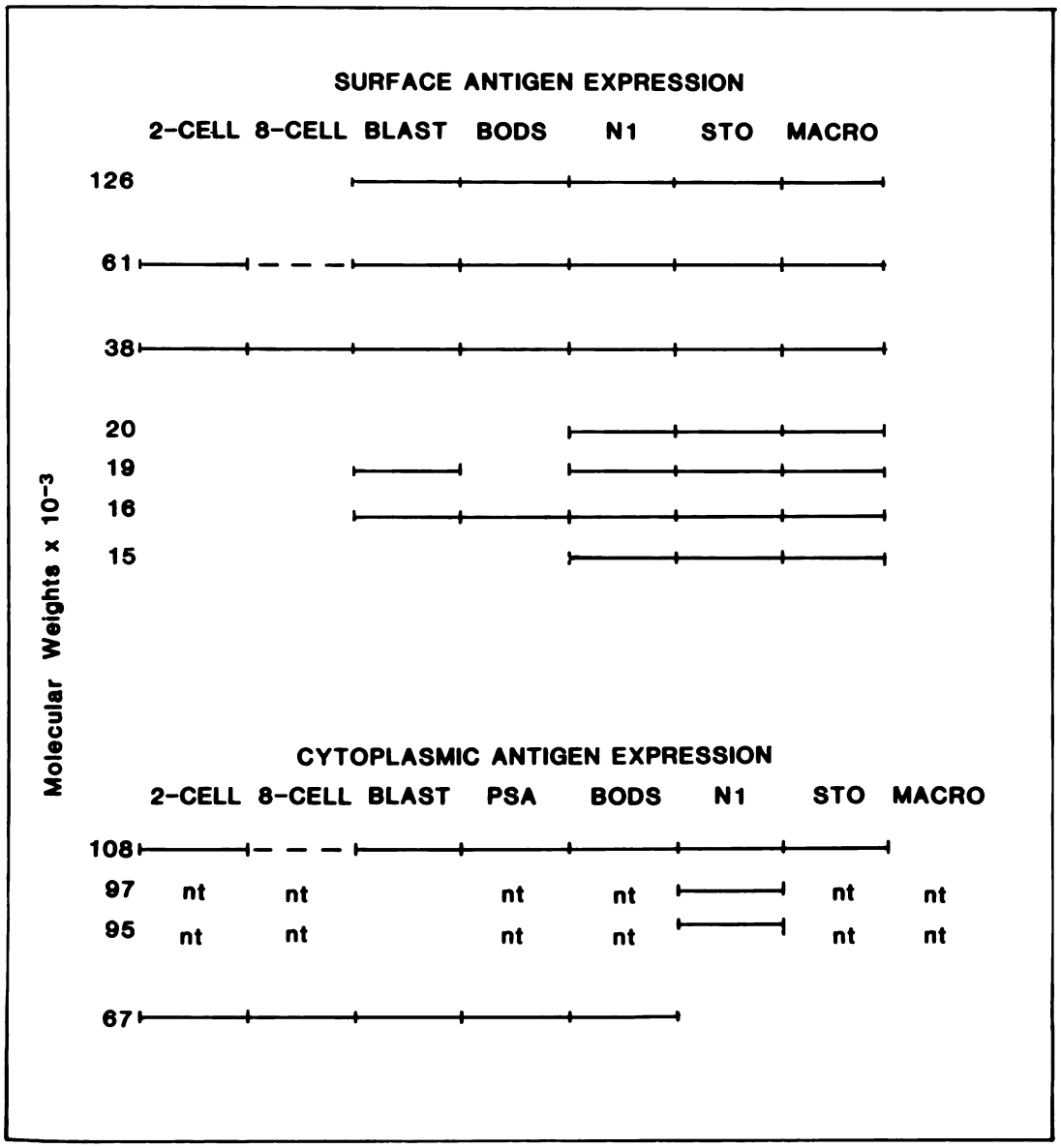

SURFACE ANTIGEN EXPRESSION
2-CELL
8-CELL
BLAST
BODS
N1
STO
MACRO
126
61
38
20
19
16
15
Molecular Weights x 10⁻³
CYTOPLASMIC ANTIGEN EXPRESSION
2-CELL
8-CELL
BLAST
PSA
BODS
N1
STO
MACRO
108
97
95
67
nt

Figure 15: Autoradiographs of immunoprecipitates from N_1 cells, blastocysts and day 3+4 PSA1 embryoid bodies with A-N_1 analysed on 12% SDS-polyacrylamide gels. A-N_1 = IgG preparation of A-N_1 which has been absorbed with adult tissues; WC = A-N_1 absorbed with whole, live N_1 cells; Lys = A-N_1 absorbed with NP40 lysate of N_1 cells. A-N_1 lanes should contain all molecules specifically precipitated by the antiserum. WC lanes should contain only those molecules not found on the surface of N_1 cells (i.e., cytoplasmic molecules). Lys lanes should contain none of the molecules specifically precipitated by A-N_1.

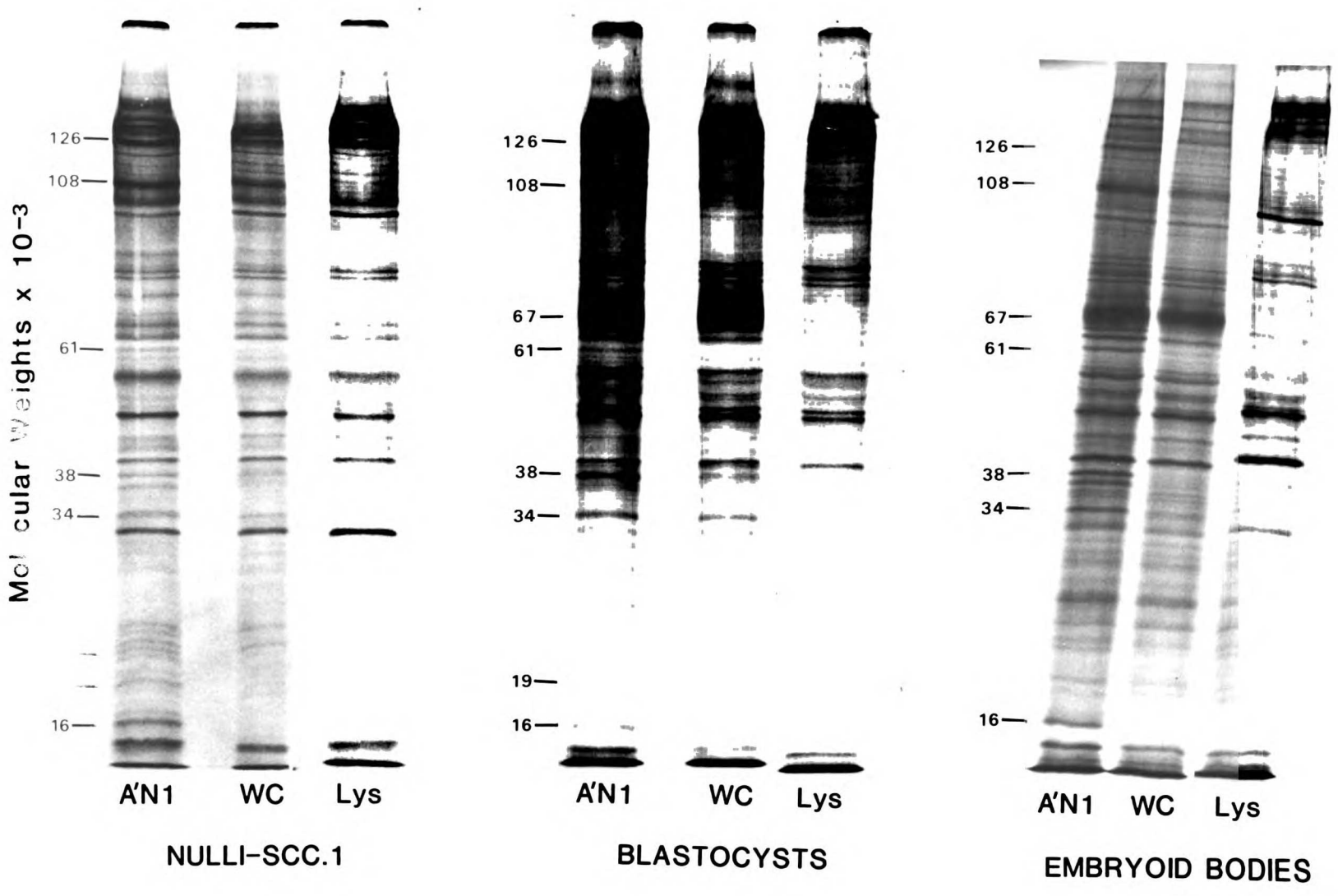
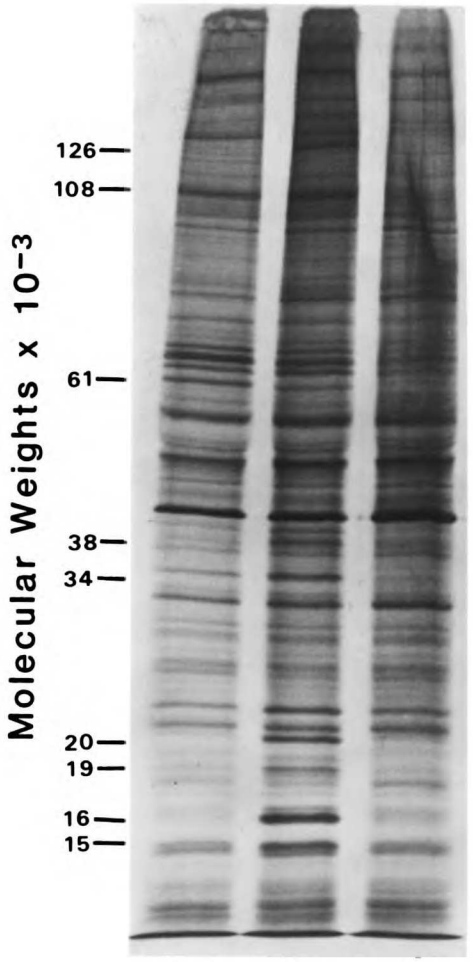

Molecular Weights x 10-3
126
108
61
38
34
16
A'N1
WC
Lys
NULLI-SCC.1
126
108
67
61
38
34
19
16
A'N1
WC
Lys
BLASTOCYSTS
126
108
67
61
38
34
16
A'N1
WC
Lys
EMBRYOID BODIES

Figure 16: Autoradiograph of immunoprecipitates from STO fibroblasts with A-N_1 analyzed on a 12% SDS polyacrylamide gel. A-N_1 = IgG preparation of A-N_1 which has been absorbed with adult mouse tissues. WC = A-N_1 absorbed with whole, live N_1 cells. Lys = A-N_1 absorbed with NP40 lysate of N_1 cells.



STO FIBROBLASTS

Figure 17: Autoradiographs of immunoprecipitates of N_1 cells and blastocysts with A-N_1 analyzed on 8% SDS polyacrylamide gels. A-N_1 = IgG preparation of A-N_1 which has been absorbed with adult mouse tissues; WC = A-N_1 absorbed with whole, live N_1 cells; Lys = A-N_1 absorbed with NP40 lysate of N_1 cells.

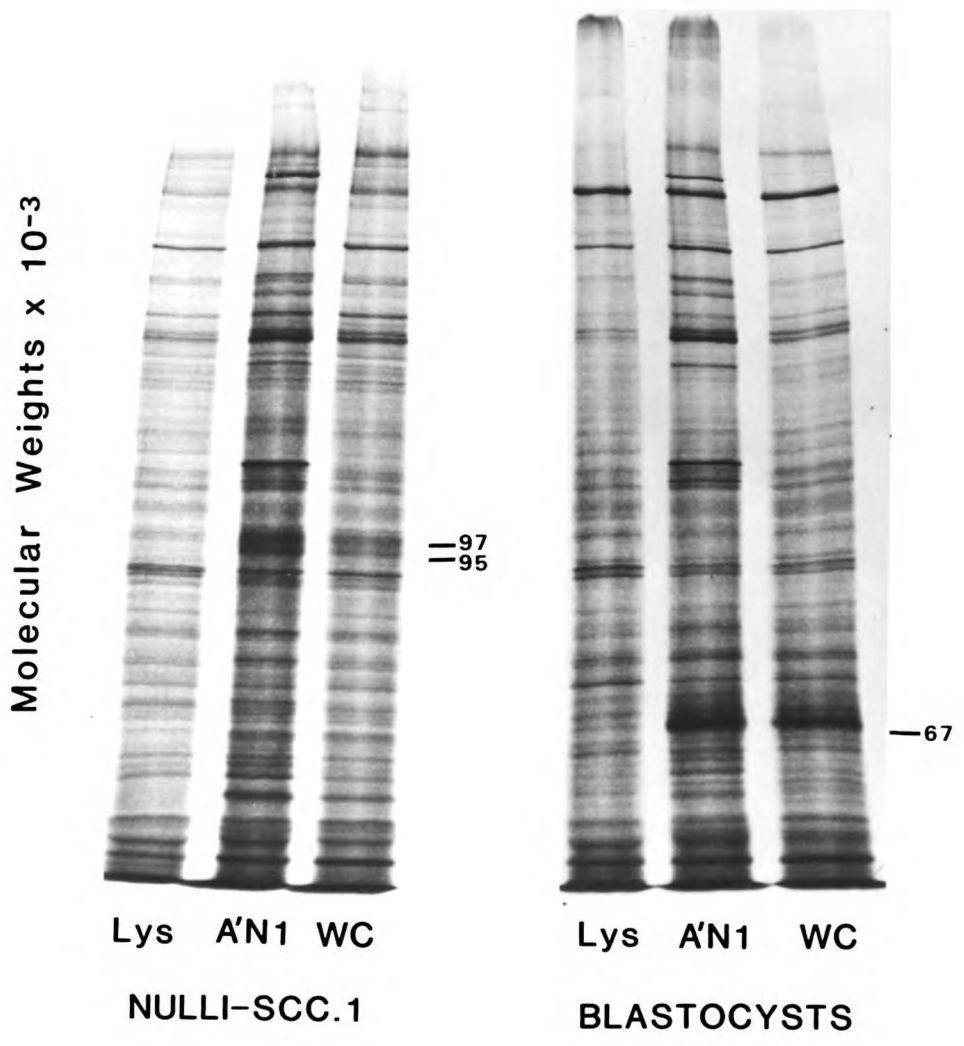
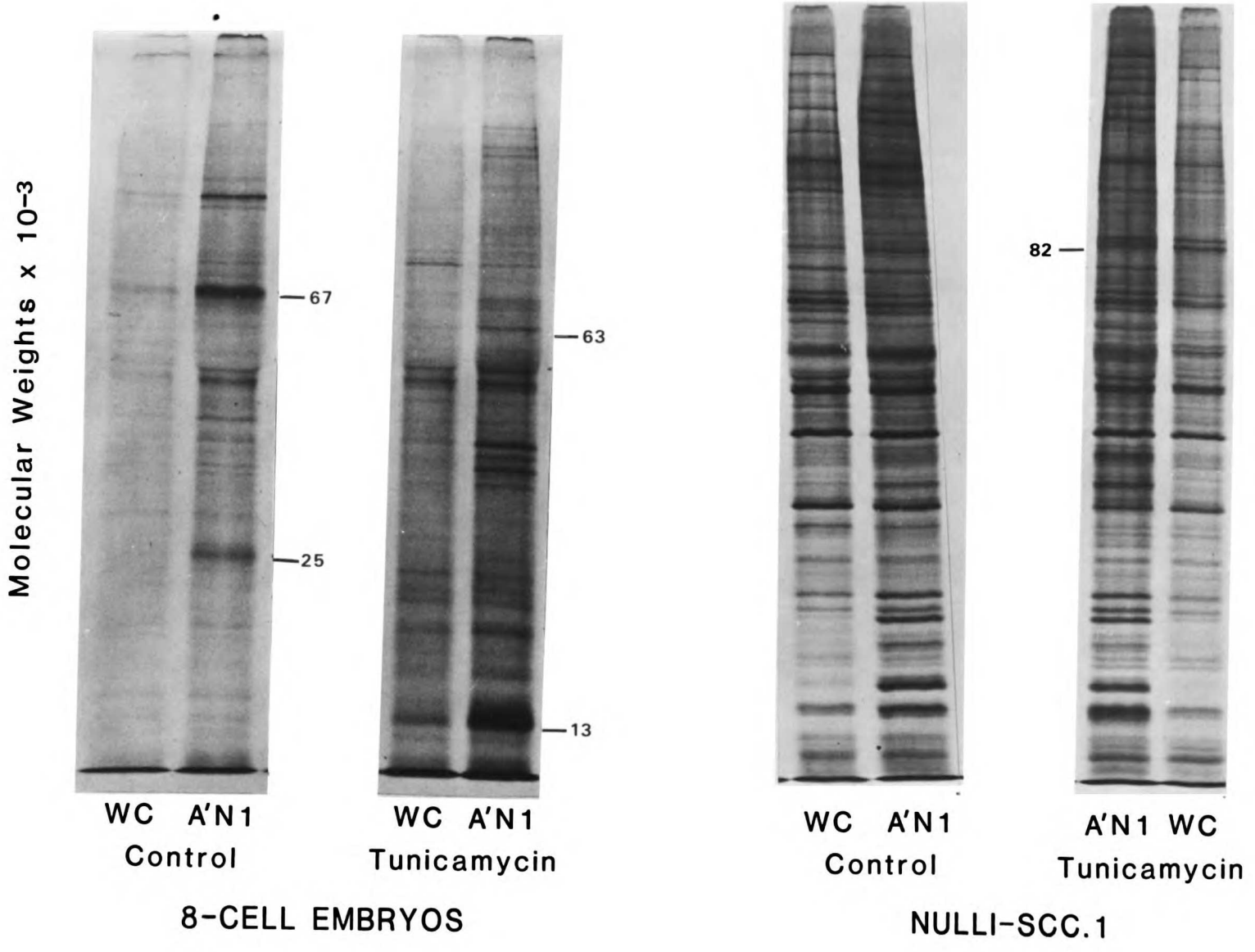

Molecular Weights x 10-3
97
95
67
Lys A'N1 WC
NULLI-SCC.1
Lys A'N1 WC
BLASTOCYSTS

Figure 18: Autoradiograph of A-N_1 immunoprecipitates of control and tunicamycin treated 8-cell embryos and N_1 cells analyzed on 12% SDS polyacrylamide gels. A-N_1 = IgG preparation of A-N_1 which has been absorbed with adult mouse tissues. WC = A-N_1 absorbed with whole, live N_1 cells. Those bands which disappear with tunicamycin treatment are indicated on control lanes. Those which appear with tunicamycin treatment are indicated on tunicamycin lanes.


Molecular Weights x 10-3
67
25
63
13
82
WC A'N1
Control
WC A'N1
Tunicamycin
8-CELL EMBRYOS
WC A'N1
Control
A'N1 WC
Tunicamycin
NULLI-SCC.1

blastocysts and PSA1 embryoid bodies (Figure 15). The 20,000 and 19,000 d molecules have not been identified on embryos or embryoid bodies. However, they appear in an area on 12% gels that is not well resolved and their presence on these cell types can not be ruled out. A 34,000 d molecule has been identified on all cell types, but classification as surface or cytoplasmic has not been possible due to variation in the gels. A molecule of 15,000 d is found on the surface of N_1 and STO cells but it is often obscured by molecules running immediately ahead of it on the gels (Figures 16 and 18). Thus it may be present but undetectable in the one dimensional analysis on the other cell types. No differences are seen in the electrophoretic profiles of precipitates with A-N_1 absorbed with mechanically harvested (CA^{++}-, Mg^{++}-free PBS) N_1 cells or A-N_1 absorbed on monolayers of N_1 cells. Trypan blue exclusion studies of the cells used for these absorptions show minimal cell death as a result of exposure to the A-N_1 IgG during absorption.

Molecules which appear in immunoprecipitates with A-N_1 and A-N_1/wc are assumed to be cytoplasmic and not expressed on the surface of N_1 cells. Only one cytoplasmic band (108,000 d) was identified in all cell types (Figures 14, 15 and 16). A major cytoplasmic band of 67,000 d is present in 8-cell embryos, blastocysts, undifferentiated PSA1 cells and PSA1 embryoid bodies, but not in N_1, STO cells or macrophages (Figures 15, 17 and 18). When immunoprecipitates were analyzed on 8% polyacrylamide gels, two cytoplasmic molecules, 95,000 and 97,000 d, were identified in N_1 cells but not in blastocysts (Figure 17).

Due to the similarity in molecular weights of the 67,000 d molecule and the BL_2 antigens, a comparison of the profile of these two antigen types on blastocysts was made. A-N_1 precipitates the 67,000 d molecule from blastocysts at a stage when BL_2 antigens are no longer being synthisized and can not be precipitated by A-BL_2. In addition, Johnson (1979) could not precipitate BL_2 antigens from N_1 cells.

When N_1 cells are cultured for 24 h in the presence of 5 µg/ml of tunicamycin, six of the seven surface bands in the A-N_1 immunoprecipitates are unaltered. The 61,000 d band is no longer present but a band appears at 53,000 d. One additional surface band appears with an apparent molecular weight of 82,000 (Figure 18).

Embryos cultured from the 2-cell stage in the presence of 0.5 µg/ml of tunicamycin for 24 h lack the 67,000 d band as demonstrated by immunoprecipitation with A-N_1 (Figure 18). Other alterations in the gel profile include the appearance of a major band at 38,000 d.

Control precipitations have shown that neither Staph A alone nor NRS followed by Staph A specifically precipitate any of the surface or cytoplasmic bands. In fact, the profile of bands from NRS precipitations closely resembles that of A-N_1 following N_1 cell lysate absorption.

Immunoprecipitations of N_1 cells and embryos employing <u>in vivo</u> absorbed A-N_1 whole sera (sera from mice in which A-N_1 has been absorbed) have failed to provide the predicted information concerning embryo specificity of cell surface molecules. In some cases no differences are seen in the electrophoretic profiles of

immunoprecipitates with *in vivo* absorbed serum and serum taken from the mouse prior to injection of A-N_1. None of the surface bands detected with LKS absorbed A-N_1 are seen in immunoprecipitates with *in vivo* absorbed A-N_1. The presence or absence of specific bands does not correlate with the presence or absence of surface binding of the sera in IIF tests.

DISCUSSION

1. Anti-Embryo Monoclonal Antibodies

The generation of monoclonal antibodies to embryo cell surface molecules was undertaken for a variety of reasons, some related to the antibody and some related to the immunogen:

a. Monoclonal antibodies recognize a single antigenic determinant and are felt to be much more specific reagents than heterosera.

b. Because each monoclonal antibody is generated by the fusion of a single immune cell, antibodies can be isolated which recognize very minor antigenic components in a complex immunogen. This minor subset of antibodies would probably go undetected in a complex antiserum with which only the most antigenic species or the antigens present in greatest quantity would be identified. Thus with a collection of McAbs one might expect to identify fever immunogenic determinants as well as very minor components of an antigen mixture.

c. At the time this project was undertaken only two McAbs recognizing cell surface antigens of the preimplantation embryo had been identified. Neither had been generated using embryos as the immunogen. Anti-Forssman had been isolated from a set of McAbs to mouse spleen (Stern et al., 1978; Willison and Stern, 1978). Anti-SSEA-1 was produced by immunization with teratocarcinoma cells (Solter and Knowles, 1978). As mentioned previously, teratocarcinomas serve as an excellent model for peri-and post-implantation mouse embryogenesis, but not for the cleavage stages of preimplantation development. For example, two sets of surface antigens, the BL_2 antigens (Johnson and Calarco, 1980c,d) and the IAP antigens (Huang and Calarco, 1981a,b) have been identified on

preimplantation embryos which do not appear on nullipotential teratocarcinomas. Therefore, the ideal approach for generating McAbs to stage-specific antigens of the preimplantation embryos was to use embryos themselves as the immunogen.

The 8-cell embryo was chosen as the immunogen because it represents an intermediate stage in the preimplantation period and thus should express antigens characteristic of early and late preimplantation development. Live, whole embryos were used for immunization in hopes that the predominant response of the host would be to surface components. The rat was chosen for immunization as this type of immunization was expected to generate a greater immune response than syngeneic immunization.

Clearly, the results described above indicate that the immunized rat mounted a significant response to molecules on the embryonic surface. A portion of the antibodies generated in this serum (Rt-1) were specific for embryo cell surface antigens as they were not absorbed by any adult tissues as judged by in vivo absorption. It should be mentioned that this absorption does not exclude the possibility of antibody cross-reactivity with brain or testes due to blood barriers. In fact, two antisera to mouse cerebellum cross-react with early embryos (Solter and Schachner, 1976; Zimmerman et al., 1976), and antisera to mouse sperm are also cross-reactive with embryos (Menge and Flemming, 1978). Cross-reactivity of Rt-1 with these tissues was not explored as this serum itself was not to be used as an experimental reagent, but rather as a simple measure of anti-embryo immune response of the animal.

The first fusion of immune and plasmacytoma cells was quite successful in that a great number of hybridomas secreting McAbs were generated. Half the primary cultures producing McAbs showed reactivity with embryonic surface antigens (40/79). The number of clones producing anti-embryo McAbs was smaller than expected (19% of all clones producing McAb) on the basis of the primary culture results. This was probably due to the small sample size. The number of clones picked from any primary culture represented less than 10% of the total. In addition, only 80 of 295 clones survived to be screened.

The eleven positive McAbs clearly demonstrated reproducible binding in the 3XIIF assay. The requirement for the degree of sensitivity conferred by the 3X assay was interpreted initially as an indication that the antigenic determinants were very minor components on the cell surface. The ability to detect minor components of an antigen mixture has been mentioned previously as an attribute of the McAb system. The "minor component" theory could also be invoked to explain the lack of complement-mediated cytotoxicity of the two McAbs tested. The lack of effect of the 2 McAbs tested in culture experiments might also be predicted if the components being blocked by antibody binding are minor components. Neither of the McAbs generated to F9 cells (Solter and Knowles, 1978; Kemler et al., 1979) are reported to have the "decompaction" effects of the anti-F9 heteroserum.

The inability to detect a specific antigen in immunoprecipitations with McAbs might also be explained if the antigen detected by each McAb were a minor component of the cell

surface and its presence were masked by background molecules on the gels. Three different methods of immunoprecipitation were used in an attempt to reduce the background and rule out this possibility, but no specific antigen was detected. In addition, the antibody overlay of electrophoretically separated embryo components should overcome the problem of background material and this technique failed to reveal specific binding of embryonic antigen with clone $2\text{-}C_4C_3$.

Other explanations for the inability to detect antigens recognized by the McAbs in the immunoprecipitation/electrophoresis system include: 1) antigens of molecular weights too high or too low to be resolved in the electrophoretic analysis employed; 2) lack of incorporation of radioisotope into the molecule if no synthesis occurs during the period of exposure of embryos to isotope; 3) lack of incorporation of radioisotope into the molecule if the antigen lacks detectable numbers of methionine residues; 4) inappropriate analytical system for the molecules precipitated, for example glycolipids would not be resolved in the electrophoretic analysis, although they would travel at the dye front; 5) inappropriate detergent combination or concentration for solubilizing the membrane antigens.

Experiments were designed to rule out as many of these interpretations as possible. The use of 8, 10 and 12% polyacrylamide gels permitted resolution of proteins and glycoproteins with apparent molecular weights in the range of <14,000 to >300,000. Analysis of much larger or smaller molecules would require gel filtration techniques which would not be practical

with the limited quantity of embryo material available. The lack of incorporation of methionine was eliminated as a causative factor in failure to identify the 2-C_4C_3 antigen with the Burridge antibody overlay procedure. This technique involves unlabelled antigen and detection depends upon binding of a radioiodinated second antibody. No binding to any embryonic molecule occurred. It may be that antigens were distorted by SDS in the gel system. This analysis was also limited in that molecules too large to enter a 10% polyacrylamide gel would not be detected. However, small glycopeptide or glycolipid antigens, although they would not separate in this system, would be present and available for antibody binding at or ahead of the dye front. The fact that no antibody binding to embryonic material was seen in this region of the gel suggests the 2-C_4C_3 antigen is not a glycolipid or glycopeptide. In addition, electrophoretic analysis of a number of McAb precipitates from embryos and cells revealed no differences in the amount of labelled material at the dye front between experimentals and controls. The possibility that antigens specific for the McAbs were not solubilized has not been explored. The lysis buffer used throughout consisted of 0.5% NP-40 in PBS (Jones, 1977) which has been used successfully in the immunoprecipitation of the BL_2 antigens (Johnson and Calarco, 1980d). Ter C antigens (Larraga and Edidin, 1979) and Concanavalin A precipitated proteins from mouse embryos Magnuson and Epstein, 1981). Solubilization of the IAP antigens required 1mM DTT and 0.1% SDS in addition to 0.5% NP-40 presumably due to the intracisternal localization of the particles necessitating solubilization of both the plasma membrane and the

endoplasmic reticulum (Huang and Calarco, 1981b). In the McAb experiments embryos and cells were subjected to a freeze/thaw cycle, vortexing at high speed and centrifugation at 50,000 x g for 1 hr following the solubilization step, ensuring release of soluble cytoplasmic components. Clearly, the question of solubility was an avenue which might have been explored had not other data been accumulating which suggested that the difficulties in identifying antigen determinants lay not in the experimental system but with the McAbs as reagents.

The problem of limited stability of the McAbs upon storage was surprising as there are no reports of similar problems with McAbs in other laboratories. Rat and mouse antibodies are considered to be less stable than rabbit antibodies (A. Good, personal communication) and antibodies with carbohydrate antigenic determinants are less stable than those to protein deteminants (A. Good, personal communication). Antisera to H-Y, prepared syngeneically in mice, are known to have low titers and to be very unstable (Wachtel et al., 1974; Epstein et al., 1980). The instability of the McAbs was a problem of considerable practical significance. It necessitated continual growth of expanded cultures, frequent preparations of McAbs including column chromatography followed by electrophoretic and IIF analysis. Before use in any experiment, the McAb aliquot to be used was tested for activity in the 3X IIF assay on embryos, a tedious and time consuming prodedure. Because embryos were available only on selected days further limitations were imposed on experimentation. When nude mice were adopted for production of McAbs, procedures were simplified as no isolation of McAb was

required. However, the ascites used as experimental reagents also proved to be unstable and required pretesting with IIF before each experiment. Once the stability problem had been identified and procedures designed to deal with it, only McAbs reactive in the IIF assay were employed in other experiments and the stability of the McAb was assumed to have no direct effect on experimental results (i.e. in vitro, cytotoxicity and immunoprecipitation studies). However, the lack of stability of the McAbs may have been indicative of a more significant problem, that of avidity of the McAbs for their respective antigens. That is, very little distortion of the binding site of an antibody with low avidity would be required to destroy reactivity of the antibody.

Titration of McAb binding to embryos with the immunoradiolabelling (IRL) technique was felt to be the best approach for determining avidity of the McAbs. In addition, IRL would provide data concerning quantitative antigen expression at each stage of preimplantation development. The lack of reproducible differences in binding between experimental McAbs and controls (P3 and radiolabelled second antibody alone) in the IRL system made necessary a serious reevaluation of all experimental results obtained with the McAbs. The IIF assays had provided the only reproducible proof of McAb binding to embryonic surface antigens and, except with ascites, this had required the sensitive 3X IIF assay. Using ascites in the 2XIIF assay, the titer was determined to be low. Five McAbs, 2-C_4C_1, 2-C_4C_3, 2-C_4C_4, 2-C_4C_5 and 2-F_6C_2, demonstrated remarkable similarities in stage specificity of antigen expression. These were the McAbs which had been tested on all

embryonic stages. No significant differences were seen with the other six positive McAbs on the embryo stages tested. In fact, none of the McAbs tested reacted with 2-cell embryos. The antigens detected by all McAbs tested appeared between the 4- and 8-cell stage. All persisted throughout the remainder of the preimplantation period. Except for 2-C_4C_3 and 2-C_4C_4 which stained approximately 50% of 4-cell embryos, the McAbs reacted with all embryos of a given stage and with all blastomeres within an embryo. This differs from the results with two other McAbs (anti-SSEA-1 and anti-Forssman) which cross-react with embryos. Not all embryos nor all blastomeres within an embryo are positive in fluorescent studies with either anti-SSEA-1 or anti-Forssman (Solter and Knowles, 1978; Willison and Stern, 1978). The McAbs described here may differ from these two in that they may recognize antigens which are expressed on all blastomeres of all embryos from the 8-cell stage onward. It is possible that all McAbs generated by immunization with 8-cell embryos recognize antigens appearing at the 4- to 8-cell stage. The selection process, IIF on 8-cell embryos, may increase this possibility. This is particularly plausible for the four McAbs in the 2-C_4- series as these clones derived from the same primary culture and, therefore, may produce the same McAb. If these four McAbs are identical, we are dealing with a set of seven (rather than eleven) positive McAbs. Nothing in the cell surface analysis of Johnson and Calarco (1980b) or the body of information concerning stage specificities of whole sera or McAbs from other laboratories predicts that seven McAbs to preimplantation embryos would all recognize antigens appearing at the 4- to 8-cell stage, although

this is the most obvious interpretation of the IIF data based on the immunogen used. This finding might also reflect an alteration in the cell surface of the 4- to 8-cell embryo conferring a nonspecific "stickiness" to the membrane. Augmented by the low avidity binding of the McAbs and low density of surface antigens, this would explain the negative results in the cytotoxicity, IRL and culture effect studies and the lack of reproducibility in immunoprecipitation studies. In retrospect, the assumption that the lack of stability of the McAbs was of little theoretical importance may have been incorrect. Antibodies with low avidity for antigens would be expected to lose activity on storage much more readily than antibodies with high affinity for their antigens.

The most puzzling results with the McAbs are the binding of 2-C_4C_3 and P3 to phosphorylase B, β-galactosidase and ovalbumin but not to any antigens in the 8-cell embryo in the Burridge gel overlay studies. It is possible that these McAbs recognize an antigenic determinant common to these three molecules as well a molecules on the surface of preimplantation embryos which were not present in quantities sufficient for detection. This interpretation requires consideration of the fact that P3 appears to recognize the same antigenic determinants. Preliminary IIF results with embryos reacted with P3 and a fluorescein conjugated rabbit anti-mouse IgG show no binding of P3 to 8-cell embryos. However, it is possible that P3 binding to embryos went undetected in the earlier experiments due to the use of an inappropriate second antidoby (rabbit anti-rat IgG) and that underlying the reactivity of all the positive McAbs was a low avidity interaction of the mouse components

of the McAbs. Unfortunately, Solter and Knowles (1978) do not report any studies of the embryo reactivity of the P3 parental plasmacytoma antibody, a seemingly important control for studies of anti-SSEA-1 activity. This complicating factor will not be a problem for future workers in the field as the majority of McAbs being produced now are resulting from fusions with plasmacytoma lines which do not synthesize mouse antibody.

To summarize this portion of the project, eleven monoclonal antibodies were generated which showed reproducible binding to the cell surface of early embryos. However, it appears that the avidity of these antibodies for their respective antigens was low. Thus they were not reasonable reagents for the functional and biochemical studies designed. At the time this project was undertaken there was a widespread feeling that McAbs would prove to be the ideal reagents for the study of developmentally regulated molecules, cell surface or otherwise, for all systems in which immunological techniques are useful. In the ensuing years, however, a more critical analysis of the technology has been made in many laboratories. At the Workshop on Embryonic Cell Surface Antigens held at the W. Alton Jones Cell Science Center, Lake Placid, N.Y., in October, 1980, many workers conveyed disappointment with the lack of progress in generating McAbs useful for the study of cell surface antigens of the early embryo. Several McAbs have been generated using teratocarcinomas as the immunogen (Solter and Knowles, 1978; Kemler et al., 1979; Goodfellow et al., 1979). Not all of these cross-react with early embryos and, in fact, only one, anti-SSEA-1, has proven useful for detailed analysis of embryonic surface antigen expression. One

useful McAb, anti-SSEA-3, has apparently been selected using mouse embryos as the immunogen (D. Solter, personal communication). This McAb was found as a result of more than a dozen fusions and screening of more than 2500 clones. This is extremely labor intensive and expensive and requires a large laboratory organization. This degree of difficulty also suggests that embryos are not highly immunogenic, perhaps enabling the embryos to circumvent immune rejection by the maternal system, but making it quite difficult for those of us striving to develop immunological reagents. However, an embryo-specific immune response has been generated successfully in a rat (Rt-1 described here) and a rabbit ($A\text{-}BL_2$, Johnson and Calarco, 1980c). Thus, the inability to generate a useful McAb reagent through embryo immunization may be due to the random screening of small numbers of clones.

The other McAb which has provided useful information concerning the expression of early embryonic cell surface antigens, anti-Forssman, was generated by immunization with mouse spleen cells and screened initially on teratocarcinoma cells (Stern et al., 1978; Willison and Stern, 1978). This protocol was not restricted by limited numbers of embryos. The discovery of common antigenic determinants on embryos and adult cell types such as the Forssman antigen, blood group antigens (Kapadia et al., 1981) and globoside (K. Willison, personal communication) suggests a more practical approach to the identification of McAb reagents useful for the study of embryonic development.

Other problems with McAbs have surfaced in laboratories studying other systems. Our experience with immunoprecipitation is

not an isolated one. Several other groups have been unable to precipitate antigens with seemingly specific, high titer McAbs (M. Eddy, personal communication; M. Kirschner, personal communication). This may be due, in part, to the fact that many McAbs do not bind Staph A, necessitating the use of an intermediate linking antibody.

One further drawback of McAbs may be their extreme specificity. Many McAbs may recognize an antigenic determinant represented only once in a given molecule. These McAbs are not useful in any techniques which depend upon antibody/antigen precipitation; RID, Ouchterlony tests, precipitin ring (interfacial) tests, etc. Heterosera rendered specific by differential absorption or by immunization with a purified antigen are often useful where McAbs would not be. An excellent example fo this is found in the tunicamycin studies of the BL_2 antigens (Johnson and Calarco, 1980c). Following tunicamycin treatment of embryos, A-BL_2 binds embryos in IIF to a reduced degree indicating that a portion of the antibodies in the heteroserum have glycosyl groups as their antgenic determinants. The fact that some of the A-BL_2 antibodies also recognize the protien portion of the BL_2 molecules made it possible to immunoprecipitate the antigens even when glycosylation had been blocked. Thus the protein portion of the molecules could be analyzed. Ideally, immunological studies in any system should utilize both heterosera and McAbs.

In conclusion, the unsatisfactory nature of the monoclonal reagents tested necessitated a different approach to surface antigen analysis.

2. Anti-Nulli-SCC1 Analyses:

A heteroserum was prepared to the mouse nullipotential teratocarcinoma stem cells, Nulli-SCC1 (N_1). This line was chosen because it is a clonal line, does not undergo differentiation and, therefore, should be homogeneous. Embryonal carcinomas provide a model system for peri-and postimplantation mouse development and might, therefore, be expected to share common surface antigens with preimplantation mouse embryos. Indeed this has been the case with the F9 teratocarcinoma Artzt et al., 1976; Jacob, 1977). The quantities of cells available for immunological analysis confer an advantage to this system over that of embryo immunization.

The rabbit serum generated by immunization with N_1 cells detects cell surface antigens on the female germ line and all stages of preimplantation embryos as well as the immunogen, N_1 cells, following absorption with adult mouse tissues. Titration studies with whole serum suggest an increased expression of antigens on the ICM and trophectoderm of the late blastocyst. Assuming multiple specificities of the heteroserum due to use of a complex immunogen, the presence of several stage-specific antigens with overlapping temporal expressions during preimplantation development would be masked by the immunofluorescence (IIF) technique and would require detection by a more sophisticated molecular analysis (see below).

A loss of antigens from the embryonic cell surface as a consequence of antibody binding was suggested by the diminished fluorescence with the increased antibody incubation and rinse times observed in IIF assays. The lack of cytotoxicity of A-N_1 might also suggest that some antigens are loosely associated with the membrane

or are shed in response to antibody binding (Nicolson and Poste, 1976).

The dose related inhibition of embryonic development in the presence of A-N_1 serum suggested that N_1 antigens might play a developmental role during the preimplantation period. The lack of developmental effects of A-N_1 IgG or Fab do not support this suggestion. Several other antisera to embryonal carcinoma cells have been shown to inhibit normal development by preventing compaction. Divalent rabbit anti-F9 prepared by Kemler and coworkers (1977) has no effect on development whereas Fab fragment of the same antibody specifically block compaction. Divalent antibodies from two other rabbit anti-F9 sera do block compaction (Ducibella, 1980). Fab fragments of these antibodies were not tested. Interestingly, 0.3 mg/ml of purified IgG was more effective in blocking compaction than a 1/10 dilution of the whole serum. A rabbit antiserum to another nullipotent teratocarcinoma stem cell line (LS5770) has similar effects on compaction (Johnson et al., 1979). Purified IgG or Fab from this antiserum were not studied. In light of these findings in other laboratories, our results are surprising. Three explanations for the difference in effects of whole serum and IgG are possible. First, the deleterious effects of the whole serum may be due to a serum component other than antibody. This is unlikely as one control in the culture experiments was A-N_1 serum absorbed with N_1 cells. Second, the component active in inhibition of development may be IgM antibodies which do not co-purify with the IgG fraction in our preparative procedure. This possibility could be investigated by purifying the IgM fraction and testing it in the

culture assay. Third, the difference in effects may result from variations between bleeds of the A-N_1 rabbit. The rabbit was bled weekly for two years with infrequent immunogen boosts, thus variation between bleeds might be expected. The whole serum culture studies were done with early bleeds; later bleeds were pooled for purification of the IgG and Fab used in culture studies. Experiments are under way comparing whole sera and IgG preparations from different bleeds to determine whether this explanation underlies the divergent results seen with the different immune reagents.

The electrophoretic profile of A-N_1 immunoprecipitates from N_1 cells reveals ten labelled bands between 14,000 and 130,000 d which are not present in NRS immunoprecipitates. The number of specificities detected in this antiserum is surprising when compared to specificities of other anti-teratocarcinoma (TC) sera. For example, Gooding (1976) identified only a 110,000 d and a 40,000 d protein in immunoprecipitates with anti-402AX while Larraga and Edidin (1979) identified a 40,000 d protein and a complex mixture of glycolipids with the same antiserum (see Introduction). However, none of the other anti-TC immune precipitates have been analyzed by slab gel electrophoresis; rather, they have been separated on tube gels which are sliced and the slices analyzed for radioactivity content. Many of the ten antigens detected by A-N_1 appear to be minor components on slab gels which would very likely go undetected against background in the tube gel analysis. Interestingly, Webb (1980) using two rabbit antisera to teratocarcinoma OTT6050 detected five to eight different antigens on various cell types using the

tube gel analysis. Thus, the large number of antigens detected with A-N_1 is probably a measure of the sensitivity of the detection system.

Further analysis of the N_1 antigens reveals that absorption of A-N_1 with whole, live N_1 cells removes activity for seven of these antigens. Absorption of A-N_1 with N_1 cell lysate removes all ten. This suggests that seven of the molecules detected by A-N_1 are cell surface antigens and the other three are cytoplasmic. However, because some cell death does occur during the whole cell absorption, other interpretations must be considered. Leakage of soluble cytoplasmic antigens from dead cells might result in absorption of antibodies specific for those antigens. Thus, the whole cell absorption may remove antibodies specific for soluble cytoplasmic proteins which are present in large quantity, but not minor soluble cytoplasmic components. The N_1 lysate absorption would then remove antibodies specific for the minor soluble and insoluble cytoplasmic antigens. In fact, the N_1 cell lysate appears to remove all antibody activity from A-N_1 as the background profile of immunoprecipitates with the lysate absorbed serum does not differ from that of NRS immunoprecipitates. On the other hand, the whole N_1 cell absorption may not be sufficient to remove all cell surface specificities from the antiserum. Thus, the classification of the antigens detected by A-N_1 as N_1 surface or cytoplasmic antigens must be guarded. Three other methods would provide direct evidence for the surface localization of antigens. Live cells can be incubated in the presence of antibody then lysed and the antibody/surface antigen complexes isolated from the lysate with Staph A. Although

this method has been successful for some plasma membrane antigens (Sen Gupta et al.), it has not been effective in the A-N_1 system. When whole, live, ^{35}-methionine labelled N_1 cells in monolayers were exposed to A-N_1, lysed with NP40 and spun at 50,000 x g for 1 h, the antigen/antibody complexes generated on the surface of the cells were found in the pellet and, thus, could not be precipitated from the supernatant by Staph A exposure. A second approach to the identification of surface antigens is that of immunoprecipitation from cells which have been labelled by surface iodination. Attempts to immunoprecipitate from iodinated N_1 cells have been unsucessful to date, perhaps due to lack of sufficient labelling, but further experiments with iodinated N_1 cells and embryos are in progress. The third method for the identification of cell surface antigens would involve immunoprecipitation from a plasma membrane preparation. As cytoplasmic contamination could not be ruled out easily with this technique, this avenue has not been pursued. Bearing in mind that the classification of antigens as surface or cytoplasmic on the basis of the differential absorptions is provisional, these designations will be applied throughout the remainder of this discussion.

While the most important characterizations with A-N_1 were those of the N_1 cells and embryos, other cell types were studied to provide information concerning embryo specificity of the antigens detected (see below). All seven of the surface proteins/ glycoproteins identified in N_1 precipitations are also present in STO fibroblast immunoprecipitates (mr 126,000, 61,000, 38,000, 20,000, 19,000, 16,000 and 15,000). The 126,000, 61,000, 38,000 and 16,000 d

surface bands have been identified on embryonic cells (blastocysts and embryoid bodies), however, the presence of the other three molecules has not been ruled out.

Interestingly, major differences are seen in the profiles of those molecules classified as cytoplasmic between N_1 and embryonic cells. The 108,000 d molecule has been identified on all cell types studied. The 67,000 d protein/glycoprotein is found in 8-cell embryos, blastocysts, undifferentiated PSA1 embryonal carcinoma cells and embryoid bodies but not in N_1 cells or STO fibroblasts. Two larger cytoplasmic bands, 95,000 and 97,000 d, are found in precipitates from N_1 cells but not blastocysts.

The 67,000 d band is the major component of the immune precipitates from embryos and embryoid bodies. The absence of this band in N_1 immune precipitates suggests that some other molecule synthesized by N_1 cells bears an antigenic determinant common to the embryonic molecule, as N_1 was the immunogen for antiserum preparation. Alternatively, the 67,000 d embryonic molecule may be present in N_1 cells but not synthesized during exposure to ^{35}S-methionine. This is unlikely as the overnight labelling period for these cells is 14-16 h, the approximate doubling time of N_1 cells in culture (G. Martin, personal communication). There is no evidence as to which N_1 molecule may share an antigenic determinant with the 67K embryonic molecule. The 95,000 and 97,000 d molecules in N_1 cells may be related to the embryonic molecules, antigenic determinants may be shared by any of the other eight species precipitated by A-N_1 or a related molecule in N_1 cells may not have been detected in the analytical system used due to size or lack of

methionine residues. Determination of the relation between the 67K embryonic antigen and N_1 antigens awaits further experimentation.

An equally interesting observation concerning the 67,000 d molecule is its presence on undifferentiated PSA1 pluripotential embryonal carcinoma cells. This difference between nullipotential and pluripotential EC cells may be related to differentiation capacity. One other study of protein patterns in pluripotential vs. nullipotential EC cell lines has compared cell surface proteins (Howe and Solter, 1981). The pluripotential EC cell line, PSA4, expresses a set of cell surface proteins in the 40,000 d range which are not found on F9 cells or other nullipotential EC cells. No other study has been made of proteins synthesized by nullipotential EC cells. In the variety of one dimensional (Martin et al., 1978; Johnson and Calarco, 1980b) and two dimensional analyses (Van Blerkom and Brockway, 1975; Dewey et al., 1977; Levinson et al., 1978; Braude, 1979; Howe and Solter, 1981; Magnuson and Epstein, 1981) of total proteins or cell surface proteins of embryos and EC cells, there is no striking demonstration of a 67,000 d molecule which would correspond to the molecule identified by A-N_1. However, comparisons of two dimensional gels from one laboratory to another are difficult. Additionally, the immunoprecipitation procedure is greatly enriching the 67K molecule which might otherwise be lost in the background on gels of total cell proteins. The significance of the 67K molecule found in early embryos and pluripotential EC cells but not nullipotential EC cells awaits further study.

Studies of tunicamycin treated embryos indicate that the 67K embryonic molecule is a glycoprotein. Tunicamycin (which blocks the

N-asparagine linkage of oligosaccharides in glycoproteins) inhibits compaction and blastulation in preimplantation embryos (Surani, 1979). Embryos cultured from the 8-cell stage in tunicamycin show no significant reduction in A-N_1 binding with IIF. However, the 67K band is no longer present in A-N_1 immune precipitates of these embryos. A 25,000 d protein band also disappears with tunicamycin treatment. A small band of 63,000 d and a heavily labelled band of 13,000 d appear in immunoprecipitates from tunicamycin treated embryos.

The glycosylated state of gp67 suggests that the molecule may be a secretory glycoprotein. The observation that whole N_1 cells do not absorb the A-N_1 reactivity for this molecule does not rule out the possibility that gp67 is a cell surface molecule of embryos and PSA1 cells. Further experiments are underway to determine the cell localization of this molecule.

Tunicamycin treatment has little effect on the surface and cytoplasmic antigens of N_1 cells. The A-N_1 specific surface bands are unaltered. An additional surface band specific to A-N_1 appears at 82,000 d. The lack of effect of tunicamycin treatment on the six surface molecules is surprising as surface molecules are assumed to be glycosylated. However, Surani (1979) has reported that tunicamycin inhibits incorporation of ^{3}H-glucosamine into blastocysts by only 28%. Inhibition of N-asparagine linkage of oligosaccharides may be incomplete in embryonic cells or the oligosaccharides may be linked to other amino acids in embryonic glycoproteins. In fact, studies of the carbohydrate portions of porcine zona pellucida glycoproteins has shown them to be atypical of N-asparagine linked

oligosaccharides (J. Hedrick, personal communication). Thus, other linkages of oligosaccharides may be typical of embryonic cells rendering glycosylation of the glycopeptides not susceptible to tunicamycin inhibition.

The 67K glycoprotein is not related to the BL_2 antigens which are similar in molecular weight (69/70K). A-N_1 precipitates gp67 from mid- to late blastocysts when A-BL_2 no longer precipitates BL_2 from the same pooled blastocyst sample. The gp67 is also found in undifferentiated pluripotential and differentiated EC cells while BL_2 antigens have not been detected in nullipotential or pluripotential EC cells (Johnson, 1979).

The question of embryo specificity of the antigens detected by A-N_1 has been difficult to answer due to inconsistent results with fluorescence and immunoprecipitation with <u>in vivo</u> absorbed A-N_1. Thus, immunoprecipitation of other cell types with A-N_1 has been used to determine embryo specificity. In fact, all surface antigens detected on N_1 cells and embryos are also present on STO fibroblasts. STO fibroblasts were derived from late mouse fetuses and may, therefore, represent an intermediate cell type between embryonic and adult cell types. Thus we may conclude that the seven surface antigens are not specific to N_1 or embryonic cells and are present on at least one differentiated cell type. Other adult cell types are being studied for presence of the N_1 antigens. The 67,000 d glycoprotein is specific to embryonic cell types including pluripotential and differentiating EC cells. It is not found in nullipotential EC cells, STO fibroblasts or macrophages.

To date the characterization of N_1 antigens on preimplantation embryos has been confined primarily to blastocysts, although the 67K glycoprotein has been identified in 2-cell and 8-cell embryos. Further analysis of immunoprecipitates of oocytes, zygotes, 2-cell and 8-cell embryos are ongoing in the laboratory and should yield information concerning the stage specificity of embryonic expression of the N_1 antigens.

In conclusion, an antiserum has been developed to nullipotential EC cells which detects antigens on the female germ line and all preimplantation stage embryos. The antiserum is not cytotoxic to embryos in the presence of complement. A subset of the antigens detected appear to be loosely associated with the cell surface and are perhaps shed as a consequence of antibody binding. Whole A-N_1 serum inhibits embryonic development in vitro while purified IgG and Fab fragments do not, perhaps reflecting differing immune states at different times during the immunization of the rabbit. A-N_1 detects a set of seven surface antigens on N_1 cells and STO fibroblasts at least four of which are also present on blastocysts. None of these molecules appears to be embryo specific. In addition, A-N_1 precipitates one cytoplasmic molecule (108,000 d) common to all cell types, 95,000 and 97,000 d cytoplasmic molecules found in N_1 cells but not blastocysts and a glycoprotein, gp67 specific to embryos and pluripotential EC cells but not synthesized by nullipotential EC cells or other cell types. The antigenic determinant in N_1 cells responsible for the immune reaction to gp67 has not been identified. The absence of gp67 in N_1 cells may correlate with the inability of these cells to differentiate. This is the first description of a

molecule common to embryos and undifferentiated pluripotent EC cells but not nullipotent EC cells.

Future studies of the N_1 antigens should include comparisons of inhibitory effects of A-N_1 serum and IgG preparations to ascertain whether the developmental effects initially described for whole serum can be attributed to A-N_1 antibodies. Further studies of embryonic stage specific expression of the surface and cytoplasmic antigens detected by A-N_1 are under way and should yield interesting information. Experiments with surface iodinated N_1 cells and embryos are ongoing and should provide stronger evidence for the classification of N_1 antigens as surface or cytoplasmic entities. Absorption of A-N_1 with PSA1 cells may yield conclusive information on the cell surface or cytoplasmic localization of gp 67. Perhaps the most exciting avenue of exploration in this system is further characterization of the gp67. Two dimensional analysis of the gp67 is currently being done. The presence of gp67 in PSA1 cells should provide the quantity of material necessary to isolate the molecule by immunoprecipitation with the existing A-N_1 serum and prepare a more specific antiserum for further analysis. Experiments could then be designed to determine whether correlations exist between presence or absence of gp67 and the ability of embryos and EC cells to differentiate. The more specific antiserum would also be useful in detecting molecules of N_1 cells which share antigen determinants with gp67.

This body of work suggests that the use of monoclonal antibodies may not be practical in all systems. In addition, it has been shown that careful analysis of antigens detected by heterosera permits

identification and characterization of antigens of developmental interest. The number of specificities of the A-N_1 heteroserum described here illustrates the caution that must be applied when analyzing purely descriptive studies with whole sera such as descriptions of stage specificities done with immunofluorescence. Future immunologic studies of embryonic antigens should be directed toward molecular characterizations of antigens.

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