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**THE DETECTION OF HISTOCOMPATIBILITY-2 ANTIGENS ON
PREIMPLANTATION MOUSE EMBRYOS**

Iowa State University

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**The detection of histocompatibility-2
antigens on preimplantation mouse embryos**

by

Karen Marie Cozad

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

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LIST OF ABBREVIATIONS

β_2^m	β_2 -microglobulin
BSA	bovine serum albumin
Ci	curie
CML	cell-mediated lympholysis
con A	concanavalin A
cpm	counts per minute
^{51}Cr	chromium 51
<u>D</u>	region of the major histocompatibility complex
DB	dextran buffer
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetate
γ	gamma radiation
hCG	human chorionic gonadotropin
HBSS	Hank's balanced salt solution
<u>H-2</u>	histocompatibility-2 complex
<u>H-Y</u>	histocompatibility Y antigen
<u>I</u>	region of the major histocompatibility complex
Ia	I region associated surface antigens
ICM	inner cell mass
i.p.	intraperitoneal
I.V.	intravenous

<u>K</u>	region of the major histocompatibility complex
MELIA	mixed embryo lymphocyte interaction assay
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MW	molecular weight
NBCS	GG-free newborn calf serum
N	normal
NMS	normal mouse serum
NRS	normal rabbit serum
PBS	phosphate buffered saline
PHA	phytohemagglutinin
PMS	pregnant mare serum
POPOP	p-bis [2-(5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
sp. act.	specific activity
Staph aureus	<u>Staphylococcus aureus</u>
TB	tuberculosis
T cell	thymus derived lymphocyte
TCGF	T-cell growth factor
³ H	tritium

I. INTRODUCTION

The preimplantation stages of mammalian development involve the in vivo fertilization of an ovum to form a zygote which then undergoes a series of cleavage divisions to give rise to a blastocyst embryo in about four days. The hollow, spherical blastocyst embryo is equivalent in size to the original zygote (about 0.1 mm in diameter), but is composed of 30-40 cells. Three-fourths of these cells comprise the surface monolayer of trophoctoderm, while the remaining one-fourth of cells comprise the inner cell mass (ICM). The trophoctoderm surrounds the blastocoel and the inner cell mass which is located at one pole of the blastocoel. The trophoctoderm is surrounded by the mucoproteinous zona pellucida. Prior to implantation, between four to five days after fertilization, the blastocyst embryo hatches from the zona pellucida and invades the uterine endometrium.

In man and laboratory rodents, implantation initiates the development of the hemochorial placenta. The trophoctoderm of the embryo becomes very invasive, exhibiting phagocytic-like properties. The mural trophoblast cells, adjacent to the blastocoelic cavity cease dividing, but continue to replicate their DNA to form primary giant cells. The polar trophoblast cells, overlying the inner cell mass, continue to divide and form a conical mass of cells, the ectoplacental cone. The peripheral cells of this cone transform into secondary giant cells that are indistinguishable from those of the mural trophoblast. Nongiant cells of the

ectoplacental cone produce villi which penetrate the endometrial cell layer and invade maternal capillaries. The inner cell mass divides to form the egg cylinder which differentiates to form the chorion, allantois, yolk sac, amnion, and the embryo (Gardner and Johnson, 1975). A parabiotic relationship is thus established between the embryo and the mother. The embryo is nourished through the fetal stages of development until birth terminates the relationship.

The preimplantation stages of mammalian development involve a series of observable morphological changes, as described above. An understanding of the molecular events during this time of development has been more difficult to obtain due to limitations on availability of materials. Therefore, most ideas concerning early mammalian embryogenesis have been obtained from experiments on amphibians and echinoderm embryos--both of which are large in size and can be obtained in large quantities. Generally speaking, many conclusions about early embryogenesis in amphibians and echinoderms do not apply to mammals. Externally developing eggs, from amphibians and echinoderms, inherit from the maternal organism the machinery for protein synthesis that is necessary for development through the cleavage stages. The embryo may synthesize mRNA during cleavage, but it is not essential since embryos appear to be insensitive to actinomycin D treatment. Synthesis of tRNA and rRNA begins at the late blastula and gastrula stages of development (Gross, 1968).

With the development of microanalytic techniques, micromanipulative procedures, and chemically defined culture media, the feasibility of examining mammalian embryos has been improved (Sherman, 1979). Most

work on early mammalian embryogenesis has been done with the mouse because of its short gestation period (about 20 days), the availability of genetic markers, and the ability to successfully culture mouse embryos from the two-cell stage to the blastocyst stage in simple, chemically defined Whitten and Biggers (1968) medium.

Molecular events (i.e., protein synthesis, RNA synthesis, and DNA synthesis) have been investigated in mouse embryos in order to determine when the embryonic genome is first expressed during the preimplantation stages of development and how the expression is regulated. The expression of the embryonic genome into cellular phenotypes, requires the synthesis of messenger RNA (mRNA) molecules and their subsequent translation into protein. Protein synthesis was initially studied by examining enzyme activity during the early stages of development. The first report of enzyme activity in preimplantation mouse embryos was made by Brinster (1965). He found that lactate dehydrogenase decreased in activity from the two-cell through the blastocyst stages of development. The activity of several other enzymes during preimplantation development have been reported and are briefly reviewed. The activities of glucose-6-phosphate dehydrogenase (Brinster, 1966; Epstein et al., 1969), guanine deaminase (Epstein et al., 1971) and glucose phosphate isomerase (Chapman et al., 1971) decrease from the fertilized egg to the blastocyst stage of development, while the activities of hypoxanthine-guanine phosphoribosyl transferase, adenine phosphoribosyl transferase, and hexokinase increase during these same stages of development (Brinster, 1968; Epstein, 1970).

It should be mentioned that enzyme activity studies do not necessarily confirm embryonic gene expression. Increases and decreases in enzyme activities could be attributed to the unmasking of certain maternal mRNA's and the degradation of other maternal mRNA's. Chapman et al. (1971) were the first to clearly demonstrate that early mammalian embryos translated embryonic mRNA's as opposed to maternal mRNA's. The investigators detected the presence of the paternal isozymic form of glucose phosphate isomerase in late mouse blastocyst embryos. Brinster (1973) later demonstrated that expression of the paternal glucose phosphate isomerase occurred by the 8-cell stage. These findings with mouse embryos are in contrast to findings in other species in which paternal alleles are not active until the hatching stage in Drosophila (Wright and Shaw, 1970), the tailbud stage in frogs (Wright and Subtelny, 1971) and the primitive streak stage in Japanese quail (Ohno et al., 1968).

Another independent set of data which supports the necessity of the embryonic gene expression during early mouse development involves the T-locus (reviewed by Bennett, 1975; McLaren, 1976; Klein and Hammerberg, 1977). The T-locus is located on chromosome 17 and has been identified by sets of dominant and recessive mutations which affect embryonic development in the mouse embryo. One mutation, the \underline{t}^{12} , when present in the homozygous condition, allows the embryo to develop to the morula (16-cell) stage, but no further--the embryo dies. Several biochemical hypotheses have been proposed to explain the lethality of the \underline{t}^{12} homozygous recessive condition. Defects in ribosomal RNA production, lipid

production and aerobic metabolism have been shown, so it is clear that the embryonic genome is not functioning properly.

Generally speaking, there appears to be no net increase in the protein content of the preimplantation mouse embryo. Epstein and Smith (1973) examined the problem of amino acid uptake and endogenous pools in relation to protein synthesis. They concluded that there was a significant increase in the rate of protein synthesis in mouse embryos between the 8-cell and blastocyst stages of development, but the protein synthetic rate remained fairly constant when examined on a per cell basis. This study suggested that protein synthesis during preimplantation development was a dynamic process and supported the appearance of different enzymes during development.

Electrophoretic profiles on 2-D gels have demonstrated that a variety of proteins are produced throughout the preimplantation stages of development in the mouse. A large population of proteins is constitutive (present throughout the entire period), while other proteins appear to be stage specific. Proteins have been detected in the fertilized egg through the second cleavage division, then disappear. Proteins that were absent during early cleavage were detected from the 8-cell stage through the blastocyst stage of development (Van Blerkom et al., 1976; Levinson et al., 1978). The 2-D gel method is very sensitive. It is capable of detecting protein synthesized at rates as low as 0.05% of the total synthesis (Solter and Knowles, 1979). The 2-D gel method does have limitations. Levinson et al. (1978) were unable to detect any differences in protein patterns of embryos from several

different inbred strains of mice, although the strains differed in several isozymes synthesized during the period examined. Also, post-translational modifications of proteins at certain stages of development may alter their electrophoretic profile and, consequently, give the impression that they are stage specific.

Stage-specific glycoproteins have been examined by in vitro incubation of preimplantation mouse embryos with D-[³H]-or D-[¹⁴C]-glucosamine (Pinsker and Mintz, 1973). The embryos were treated with pronase to remove the zona pellucida, then disrupted with detergent, and fractionated by gel filtration. It was found that mouse embryos of the 4- to 8-cell stage and morula to blastocyst stage of development were able to convert glucosamine to galactosamine, N-acetyl glucosamine, and glucose with blastocyst embryos showing the most label incorporation. It was postulated that these glycoproteins may be involved in providing the receptors for the embryo to implant into the uterine wall (Pinsker and Mintz, 1973; Surani, 1979). A more complete discussion of cell surface glycoproteins on preimplantation mouse embryos is given later.

The demonstration of quantitative and qualitative changes in protein synthesis does not in itself furnish conclusive evidence for activity of the embryonic genome. Through the use of isozymic markers, as mentioned earlier, and the use of inhibitors of RNA synthesis, the expression of the embryonic genome can be studied. In the mouse, there is a negligible increase in the net RNA content from fertilization to the early blastocyst stage of development (Olds et al., 1973). The following classes of RNA have been identified in the preimplantation mouse

embryo: 4s RNA and 5s RNA by the 2- or 4-cell stage and ribosomal RNA by the 4-cell stage (Clegg and Piko, 1975).

Monesi et al. (1970) incubated preimplantation mouse embryos in the presence of 1 μ g/ml actinomycin D. The rate of 3 H-leucine incorporation decreased to about 50% of control values over a period of 12 hours, then remained relatively constant. These results suggested that the embryonic genome was impaired in its de novo production of a class of mRNA's, which are turned over fairly rapidly, then replaced. The nonspecific effects of actinomycin D on tRNA's and rRNA's complicate the validity of this conclusion. Later studies with actinomycin D and the more specific, RNA polymerase inhibitor, α -amanatin, have been used to show that compaction of blastomeres is relatively resistant to inhibitor at the 8-cell stage, but identical or lower doses of inhibitor interfere with cavitation when added to morula stage embryos (Golbus et al., 1973; Warner and Versteegh, 1974; Levey et al., 1977). These results suggest that endogenous mRNA's support the initial stages of preimplantation development, but the morula stage requires the synthesis of new mRNA. This could also mean that post-transcriptional control is important in the earliest stages of development, whereas, transcription control is important in the morula and later stages of development. Studies involving enzyme activities, paternal isozyme expression and inhibitors of RNA synthesis leave little doubt that the embryonic genome is expressed during preimplantation development in the mouse.

Several studies have been performed to characterize DNA synthesis in preimplantation mouse embryos according to duration of cell cycles,

quantity of DNA present, and DNA polymerase activity. The first cell division in the mouse zygote occurs 24 hours after fertilization. The time required for subsequent cell cycles diminishes as cleavage progresses (McLaren, 1972). DNA synthesis begins four to six hours after fertilization (Luthardt and Donahue, 1973). The initial S phase following fertilization lasts for about four hours. In the second and subsequent cell cycles, DNA replication requires six to seven hours (Luthardt and Donahue, 1975; Mukherjee, 1976; Sawicki et al., 1978). The shortening of the cell cycle time is probably due to a decrease in the duration of the G_2 phase. It has been reported that the 2-cell mouse embryo has a 12-15 hour G_2 phase, while the G_2 phase in the 4- to 8-cell transition period is only 0.5 hour (Sawicki et al., 1978).

The cell cycle is believed to vary within the cells of the same embryo, starting at the morula (16-cell) stage of development. The inner cells of morula and blastocyst embryos have been shown to have higher ^3H -thymidine labeling indices than the outer cells (Barlow et al., 1972). This suggests that a shorter cell cycle is present in the inner cells as opposed to the outer cells of the embryos at these stages of development.

Olds et al. (1973) determined the quantity of DNA present in each stage of preimplantation mouse development by perchloric acid precipitation and fluorometry. They found the mean values of DNA to be 23.8, 29.3, 41.1, 155, and 439 pg for the oocyte, 1-cell, 2-cell, 8-cell, and blastocyst (about 32 cells) stages of embryonic development, respectively. On a per cell basis, DNA content decreases from the oocyte (24 pg/cell)

through the blastocyst stage (13 pg/cell) of development. The "extra" DNA in early stages was attributed to polar body DNA, with three polar bodies persisting to the 2-cell stage and one polar body persisting to the 16-cell stage. The point that needs to be emphasized, is though the embryo has retained the same size throughout the preimplantation stages of development, total DNA content has increased almost 20 times.

The onset of DNA synthesis infers the existence of the necessary enzymatic machinery for DNA replication. Kiessling and Weitlauf (1979, 1981) have detected DNA polymerase activity in preimplantation mouse embryos. Their detected enzyme exhibited properties similar to DNA polymerase- γ and the activity increased markedly when the embryos differentiated into inner cell mass and trophectoderm. Mammalian cells contain three types of template directed DNA polymerizing enzymes designated DNA polymerases α , β and γ (Weissbach et al., 1975). DNA polymerase- α is believed to be involved in replication, while DNA polymerase- β is believed to be involved in DNA repair. DNA polymerase- γ has had no function ascribed to it at this time, though it has been hypothesized to have a very specialized role (Huberman, 1981). Therefore, Kiessling and Weitlauf's report of DNA polymerase- γ -like activity during embryonic development when differentiation into two cell types occurs is of special interest for defining a role for DNA polymerase- γ and for further investigation into the details of the mechanisms which control DNA synthesis in preimplantation mouse embryos.

A discussion of DNA synthesis in preimplantation mouse embryos cannot be concluded without mentioning the role of mitochondrial DNA. The

mouse oocyte contains round mitochondria with concentrically arranged cristae. During early embryogenesis, there is a gradual elongation of the mitochondria to an adult-like form. Pikò and Chase (1973) examined the mitochondrial activity in mouse embryos of the 2- to 4-cell stage through the blastocyst stage of development. They used a DNA inhibitor, ethidium bromide, and a protein inhibitor specific to the mitochondria, chloramphenicol. They found that mitochondrial ribosomal and transfer RNA syntheses occurred from the 8- to 16-cell stage through the blastocyst stage of development. A mitochondrial protein synthesizing system also operated during these stages of development. However, they concluded that embryo development and cellular differentiation up to the blastocyst stage were not dependent on mitochondrial genetic activity. Cascio and Wassarman (1981) used an inhibitor of cytoplasmic protein synthesis, emetine, and confirmed Pikò and Chase's earlier work with regard to 8-cell mouse embryos. In addition, Cascio and Wassarman (1981) found that proteins of mitochondrial origin comprise only one to two percent of the total protein produced during this stage.

In summary, the preimplantation mouse embryo genome is biochemically active with regard to protein synthesis, RNA synthesis and DNA synthesis. The rest of this introduction will deal with one particular class of proteins found on early mouse embryos: cell surface proteins.

A large part of this dissertation involves the examination of cell surface proteins on 8-cell and blastocyst mouse embryos. Special effort

was placed on the detection of Histocompatibility-2 (H-2) antigens on these embryonic stages of development. Therefore, a brief account of the history, structure and function of these proteins will be given.

In 1937, Peter Gorer described a set of molecules that he had observed in the mouse (Gorer, 1937). These molecules could be recognized by: (1) making antiserum against them and then studying them serologically and (2) transplanting cells or tissues carrying one type of molecule into a mouse containing corresponding molecules which were different; the introduced cells were destroyed (rejected) by the recipients. The molecules were designated histocompatibility-2 (H-2) antigens by Gorer, Lyman and Snell (1948). Later research proved that the H-2 antigens were coded for by a series of linked genes which were then denoted as the H-2 complex. Other species exhibit similar complexes: e.g., HLA in the human, Rt-1 in the rat, B in the chicken, and SLA in the pig. The major histocompatibility complex (MHC) serves as the generic name for these complexes and also reflects the fact that there are many "minor" histocompatibility systems which appear to play a lesser role in the rejection phenomenon (Klein, 1975).

The H-2 complex is located in the middle portion of chromosome 17 (see Figure 1a). Chromosome 17 also bears three other groups of loci: (1) loci controlling embryonic differentiation (T, t, Kb, Fu, and qk); (2) loci controlling hair growth (tf and thf); and (3) loci coding for isozymes (Pgk-2, Ce-2, Ap-1, Glo-1, and Map-2) (Klein, 1975, 1979).

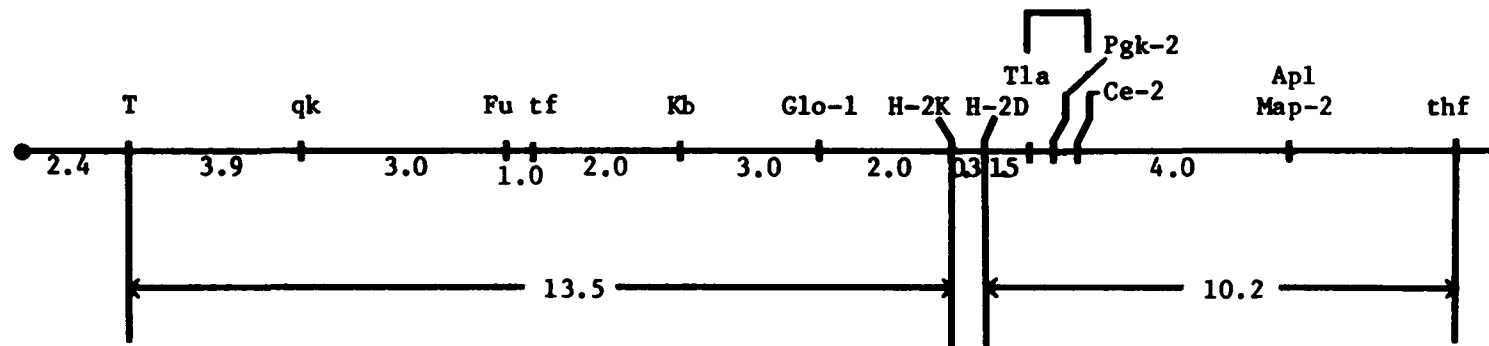


Figure 1a. Abbreviated genetic map of chromosome 17 (Klein, 1975, 1979). The loci from left to right are centromere (●), brachyury (T), quaking (qk), fused (Fu), tufted (tf), knobby (Kb), glyoxylase-1 (Glo-1), histocompatibility-2K (H-2K), histocompatibility-2D (H-2D), thymus-leukemia antigen (Tla), phosphoglycerate kinase-2 (Pgk-2), kidney catalase (Ce-2), acid phosphatase-liver (Apl), α -mannoside processing-2 (Map-2), thin fur (thf). Brackets indicate the order of loci is not known

The H-2 complex is diagrammed in Figure 1b. The position of each H-2 locus was determined by typing recombinants derived from heterozygous parents in which two chromosome 17s have undergone crossing-over in a particular position within the H-2 complex.

The H-2 loci can be grouped into at least three classes based on phenotypic expression. Class I loci (H-2K and H-2D) code for membrane-bound glycoproteins (the H-2 antigens) with a molecular weight of 45,000. The Class I molecules (H-2K and H-2D) consists of a single polypeptide chain of about 350 amino acids with two associated carbohydrate side chains. In the membrane, the Class I molecule is noncovalently associated with β_2 -microglobulin which is a shorter polypeptide chain (about 100 amino acids) and 12,000 MW. β_2 -Microglobulin is not encoded by the H-2 complex, but has recently been mapped to the H-3 region of chromosome 2 (Michaelson, 1981). Class I molecules are detectable on all lymphoid cells and most other cells of the organism. Class I molecules can be detected serologically by producing antibodies against them or by generating lymphocytes that are cytotoxic for H-2K and H-2D bearing target cells.

Class I molecules are characterized by private and public specificities. These specificities are serologically detectable and are used to describe the haplotype of a mouse strain. The set of serologically detected proteins produced by the H-2 complex of an inbred strain of mice comprises the haplotype for that strain of mice and is designated by a small Roman letter (e.g., a, b, d, or k). The private

Region	K	I	S	G	D	TL
Locus	<u>H-2K</u>	<u>Ir-1</u>	<u>Ss</u>	<u>H-2G</u>	<u>H-2D</u>	<u>Tla</u>
Products	Cell Membrane Molecules	Cell Membrane Molecules	Serum Proteins	Erythrocyte Membrane Molecules	Cell Membrane Molecules	Cell Membrane Molecules
Functions	Marker for cytotoxicity against nonself components	Immune response control cell- cell inter- actions	Complement components	Complement component	Marker for cytotoxicity against nonself components	Unknown

Figure 1b. Enlarged H-2 complex (Shreffler, 1979; Klein et al., 1981)

specificity is exhibited by only the K or only the D protein product of a particular inbred strain of mice, whereas, the public specificities are detectable in either the K or D protein product of one inbred strain of mice or may be detectable in the K or D protein product of a different inbred strain of mice.

Class II molecules (Ia antigens) are membrane-bound glycoproteins consisting of two noncovalently associated polypeptide chains, α (35,000 MW) and β (28,000 MW), and an unknown number of carbohydrate chains. A minimum of four polypeptide chains A_α , A_β , $A_e(E_\beta)$, and E_α are encoded by the I region. The chains are synthesized separately in the cytoplasm, but combine to form heterodimers before they integrate into the plasma membrane. The A_α combines with A_β to form an A molecule and the A_e combines with E_α to form an E molecule (Klein et al., 1981).

Class II molecules are expressed on lymph node and splenic lymphocytes, but are very weakly expressed on thymocytes. The Class II molecules are detectable on macrophages, fetal liver cells, epidermal cells, bone marrow cells, and spermatozoa, but are absent from erythrocytes, brain tissue, kidney cells, and liver cells. The Class II molecules were originally detected serologically with antibodies and were later shown to be responsible for the activation of lymphocytes in a mixed lymphocyte reaction (reviewed by Cullen et al., 1976 and David, 1976).

Class III molecules are the serum protein Ss (serum serological) and Slp (sex-limited protein). Both proteins have a molecular weight of 200,000 and consist of three covalently linked polypeptide chains,

α (87,000 MW), β (78,000 MW) and γ (33,000 MW). These molecules comprise the C4 component of the classical complement pathway (Klein, 1979).

Class I and Class II glycoproteins are involved in immune responsiveness, immune suppression, T-B cell interaction, and the restriction of T-cell specificity. The role of the H-2 products appears to be to provide a context for antigen recognition. This recognition capability may enable the organism to distinguish self from nonself. The simultaneous recognition of antigen and H-2 molecule restricts the specificity of the T lymphocyte. The Class I and Class II molecules are all heterodimers and consist of one highly variable polypeptide chain (K, D, A β , and E β) and one relatively constant polypeptide chain (β_2 -microglobulin, A α , and E α) (Klein et al., 1981). The polymorphism of the resulting heterodimers would provide the potential for an array of H-2 antigens which could specifically interact with cells within the same individual and against nonself cells or organisms.

Mammalian viviparity has produced a situation which has puzzled transplantation biologists for at least the last three decades. The embryo, an allograft exhibiting a genetically different set of antigens than the mother, escapes the immune surveillance system of the mother and will implant in the endometrium and will be successfully nourished to term by the mother.

Medawar (1953) proposed that there were three possible reasons why the fetus did not provoke an immunological response from the mother:

(1) there was an anatomical separation of the fetus from the mother; (2) the mother was immunologically inert during pregnancy; and (3) the fetus was antigenically immature. It is well-established that immunoglobulins are transferred from the mother to the fetus during pregnancy (Brambell, 1970). Therefore, there is no anatomical barrier to an immunological response in the post-implanted embryo.

The immunological inertness of the mother has been shown to be unlikely. Bernard et al. (1977) demonstrated with sheep Fab anti-mouse immunoglobulins, labeled with peroxidase, that maternal immunoglobulins were present in the blastocoel and on the trophectodermal cells of day 5 implanted mouse blastocysts. Maternal immunoglobulins were also found in the visceral yolk sac and embryonic gut of nine-day embryos. In a later study, Bernard et al. (1981) characterized the isotype and concentration of immunoglobulin in the mouse uterus from two to six days post-conception. They found that IgA plasma cells gradually concentrated around uterine glands and in the uterine lumen. IgG concentration increased in the uterine stroma, while the decidua contained no detectable immunoglobulins.

The cell-mediated response of the pregnant female has been reported to be depressed during pregnancy, but there also have been reports which clearly establish that the mother's cellular immune system can be sensitized against paternal and embryonic antigens (reviewed by Bernard, 1977).

Maternal hormonal changes may be responsible for suppressing some cellular responses during pregnancy. Estradiol has been shown to prolong first and second set skin grafts in mice and to involute the thymus

in adult mice. Estradiol, progesterone and diethylstilbestrol at 10-50 $\mu\text{g/ml}$ reduce lymphocyte proliferative response to mitogenic plant lectins PHA and con A (Rocklin et al., 1979). Most hormonal suppressions of immune systems in vitro involve hormone concentrations that are 10^2 to 10^3 times higher than normal physiological concentrations.

Another suppressor of cellular responses during pregnancy is alpha-fetoprotein which is produced by the fetus and is present in the amniotic fluid and fetal serum of many species. The role of alpha-fetoprotein is uncertain, but it appears to activate suppressor cells (Goidl et al., 1979).

The fetus is believed to express antigens which are detectable by the mother, as can be demonstrated by the occurrence of hemolytic disease of the newborn (erythroblastosis fetalis), and by the variety of antibody specificities detectable in multiparous female serum. It has been postulated that multiparous serum contains blocking or enhancing antibodies which may act (1) by masking the foreign antigens on the embryonic cell surface, or (2) by leading to the shedding of antigens by the cell, thus preventing recognition by effector cells, or (3) by forming antigen-antibody complexes which are then unable to be processed by maternal lymphocytes. The exact role that maternal immunoglobulins play is difficult to ascertain since some evidence for each of the three postulates have been observed in animal systems (reviewed by Bernard, 1977 and Rocklin et al., 1979). However, there is general agreement that the postimplantation embryo expresses antigens detectable by the maternal immune system (Kirkwood and Billington, 1981).

The role of the preimplantation embryo in escaping maternal immune surveillance is not understood. There is no physical barrier between the mother and the preimplantation embryo except for the zona pellucida, which is permeable to immunoglobulins (Sellens and Jenkinson, 1975). It is possible that the preimplantation embryo is not present long enough to be recognized by the maternal immune system and responded against. This seems unlikely, especially with reference to Bernard et al. (1981), who found that immunoglobulin concentrations increased in the uterus during the preimplantation stages of pregnancy. The exact target of these immunoglobulins is difficult to pinpoint. The immunoglobulins may be against proteins of the zona pellucida, which is shed prior to implantation. Nevertheless, the presence of increased immunoglobulin concentrations in the uterus may suggest that the preimplantation embryo expresses antigenic determinants. The identification and characterization of these determinants have been the subject of a great deal of investigation (reviewed by Johnson and Calarco, 1980; Jenkinson and Billington, 1977).

Studies involving preimplantation stages of mouse development have shown that preimplantation mouse embryos express teratoma-associated antigens (Artzt et al., 1973; Gooding et al., 1976); stage-specific antigens (Levinson et al., 1978; Solter and Knowles, 1978); the F9 antigen (Artzt et al., 1973); sperm antigens (Menge and Fleming, 1978); PCC4 antigens (Gachelin et al., 1977); and H-Y antigens (Krco and Goldberg, 1976).

Of special interest in the phenomenon of maternal tolerance of the preimplantation embryo is whether or not H-2 antigens are expressed on the embryo. Disparity between H-2 antigens is responsible for tissue graft rejection. Therefore, their absence may explain the inability of the mother to reject the genetically different embryo. H-2 antigens have been identified on post-implantation mouse embryos, seven to nine days old (Edidin, 1972a; Pathey and Edidin, 1973). The presence of H-2 antigens on preimplantation mouse embryos has proven to be more controversial due to questions involving the specificity of antisera and the sensitivity of detection methods.

H-2 antigens are detectable on sperm and unfertilized mouse ova (Johnson and Edidin, 1972; Edidin, 1972b). Several reports have failed to demonstrate H-2 antigens on embryos between the 1-cell and blastocyst stages (day 1 through day 5) of development (Palm et al., 1971; Edidin, 1972a; Muggleton-Harris and Johnson, 1976; Sellens, 1977). Transplantation experiments in which blastocyst embryos were treated with anti-H-2 serum or in which the recipient, allogeneic females were preimmunized with H-2 antigens prior to the transfer of the blastocyst embryos, have shown that the blastocyst embryos fail to develop (Kirby et al., 1966; Kirby, 1968; James, 1969). Searle et al. (1976) demonstrated the presence of H-2 antigens on the trophectoderm of blastocyst embryos by using a sensitive electron microscope-immunoperoxidase technique and specific anti-H-2 sera produced in congenic strains of mice. Webb et al. (1977) used ¹²⁵I-lactoperoxidase and anti-H-2 sera to attempt to label and isolate H-2 antigens from the 2-cell to the blastocyst stage of preimplantation mouse

development. Their method was able to detect H-2 antigens only on the inner cell mass of the late blastocyst embryo. They were unable to detect H-2 antigens on earlier stages of development or on the trophoblast of the blastocyst where Searle et al. (1976) had detected H-2 antigens. This discrepancy could be explained by the relative insensitivity of the ^{125}I -lactoperoxidase procedure. Krco and Goldberg (1977) have reported the presence of H-2 antigens on 8-cell embryos, though no confirmation of this report has appeared in the literature. They treated inbred and congenic 8-cell mouse embryos with pronase to remove the zona pellucida, then with anti-H-2 sera obtained from the National Institutes of Health (Bethesda, Maryland), followed by an incubation with guinea pig complement. Embryos were examined with a light microscope and scored positively for H-2 if at least one of the 8-cells was lysed. It can be concluded that H-2 antigens are probably expressed on the blastocyst embryo and maybe on the 8-cell embryo. Data presented in this dissertation will support such a conclusion.

As mentioned previously, Ia antigens are found on only select tissues throughout the body, as compared to wide distribution of H-2K and H-2D antigens. Examination of Ia antigens on mouse embryos with anti-Ia sera in conjunction with immunoprecipitation and immunofluorescence has shown that embryonic cells do not exhibit Ia antigens until 11 days postconception (Delovitch et al., 1978). Jenkinson and Searle (1979) used a sensitive electron microscopic immunoperoxidase technique and specific anti-Ia sera on 3.5 day blastocyst embryos and the 7.5 day post-implantation conceptus. They were unable to detect Ia antigens on

either stage of development. Therefore, it appears as though Ia antigens are not expressed as preimplantation embryos nor early post-implantation embryos.

In order to successfully detect H-2 antigens on mouse embryos, a sensitive assay system needed to be developed. Searle et al. (1976) used an immunoperoxidase electron microscopic system and Webb et al. (1977) used an immunoprecipitation system with an ^{125}I -label. Both research groups demonstrated the presence of H-2 on mouse blastocyst embryos. A third type of assay system was employed to generate the data for this dissertation--the complement-dependent cytotoxicity assay. Complement-dependent cytotoxicity assays are commonly used to demonstrate antibodies to cell surface antigens. The complement-dependent cytotoxicity assay has been successfully applied to lymphoid cells where cytotoxicity is commonly measured by (1) release of ^{51}Cr (Sanderson, 1964; Wigzell, 1965) or (2) the uptake of trypan blue dye (Gorer and O'Gorman, 1956). In the ^{51}Cr release cytotoxicity assay, target lymphocytes are labeled with ^{51}Cr -chromate during a short incubation period. Chromate enters the cells by an energy-independent process. The intracellular chromate reduces to chromic ion which hydrates to form a hexaquo ion. This maintains a concentration gradient for chromate and allows for very high levels of uptake. The cells are then washed and incubated with antiserum and complement. A cytotoxic index is determined by the activity released by the test sample divided by the total activity released by control (normal serum-tested) cells.

In the cytotoxicity assay involving the uptake of trypan blue dye, the cytotoxic effects of antibody and complement are measured by the failure of dead cells to exclude the vital dye. Quantitation of cells is determined by microscopic examination of test samples.

These cytotoxicity assays have both advantages and disadvantages which should be considered. Cytotoxicity assays involving the release of ^{51}Cr have the advantage of being objective, but have the disadvantage of requiring prelabeling and washing of cells. And, of course, ^{51}Cr is a γ -emitter, so that prolonged use could be a health hazard. Dye uptake cytotoxicity assays have the advantages of being inexpensive, fast and require few manipulations. Disadvantages are the possibility of operator subjectivity, a limit to the number of samples that can be analyzed by a single operator and the impossibility of evaluating samples when a large percentage of dead cells from the preparation procedure may be present.

Objectivity and reproducibility have been obtained with cytotoxicity assays which take advantage of the fact that live cells will incorporate small molecular weight radioactive precursors into high molecular weight products, whereas dead cells will not. Klein and Perlmann (1963) first reported a cytotoxicity assay system in which ^3H -thymidine was incorporated into the DNA of ascites tumor cells which had been treated with antiserum (or normal serum) and complement prior to ^3H -thymidine treatment. Later, Knudsen et al. (1974) automated the assay, using a MASH (multiple automated sample harvester). The only limitation of the DNA synthesis cytotoxicity assay procedure is that the tested cells must be

undergoing mitosis, which requires a mitogenic stimulation of lymphoid cells or the testing of tumor cells in their log phase of growth. It will be shown in this dissertation that a complement-dependent cytotoxicity assay, employing ^3H -thymidine, is feasible for use on preimplantation mouse embryos since they are a rapidly dividing tissue and need not be exogenously induced to divide. The use of ^3H -thymidine uptake and cell harvesting provides an objective and automated procedure for the collection of data. Visual observation of trypan blue dye uptake by embryos treated with antiserum and complement was not feasible due to the three-dimensional morphology of the spherical embryos. Lysed cells could not be easily and objectively deciphered from live, nonlysed cells. Likewise, ^{51}Cr release was not possible to use since only one-two cpm per cell are taken up.

The major purpose of the work described in this dissertation was to develop a reproducible and reliable assay procedure to examine H-2 antigens on preimplantation mouse blastocyst and 8-cell embryos. First, the ability of rapidly dividing embryonic cells to incorporate ^3H -thymidine was examined and parameters for optimal ^3H -thymidine incorporation were developed. The effect of various compounds on ^3H -thymidine incorporation by embryos was then examined. The ability of embryos to incorporate ^3H -thymidine was then used as a viability measurement after embryos were treated with anti-H-2 sera and complement in a cytotoxicity assay. Embryos were then screened for potential H-2 antigens using a variety of antisera.

II. EXPERIMENTAL PROCEDURES

A. Experimental Animals

1. Mice

CF1 mice were obtained from Charles River Breeding Laboratories, Wilmington, Massachusetts. The inbred and congenic mouse strains listed in Table 1 were obtained from the Jackson Laboratory, Bar Harbor, Maine. The mice were housed in a day (14h)-night (10h) cycled room with food and water ad libitum. The room was light from 7 a.m. to 9 p.m. daylight savings time, or 6 a.m. to 8 p.m. standard time. Therefore, the clock was never changed throughout the year. Female mice were kept in polycarbonate cages in groups of eight. Male mice were kept in metal cages with one male per cage.

2. Superovulation of mice

Female mice, two-six months old, were superovulated with 5 IU PMS (pregnant mare serum, Organon, Pinebrook, New Jersey) at 4 p.m. (daylight savings time) or 3 p.m. (standard time) followed 48 hours later with 10 IU hCG (human chorionic gonadotropin, ICN Nutritional Biochemicals, Cleveland, Ohio). Each female was placed with a single male immediately after hCG injection and checked for the presence of a vaginal plug the following morning.

Table 1. H-2 haplotypes of mouse strains used in this study

[illegible]

B. Buffers, Solutions, and Culture Media

The various buffers, solutions and culture media used can be found in Table 2.

C. Preparation of Embryos

1. Collection of embryos

Embryos were collected as described by Rafferty (1970). Plug positive females were killed by cervical dislocation at 64 to 71 hours post-hCG injection to obtain eight-cell embryos, or 88 to 96 hours post-hCG injection to obtain blastocysts. A mid-line abdominal incision was made to expose the peritoneal cavity. The intestines were moved to the side to expose the bicornate uterus. For eight-cell embryos, the oviduct along with one-fourth of the upper uterine horn was excised and placed in a culture concavity slide (Carolina Biological Supply, Burlington, North Carolina) containing about 0.2 ml of Whitten and Biggers medium. The second oviduct was collected in the same slide. For blastocysts, the oviduct and entire uterine horn, excised at the vaginal junction, was placed in a culture concavity slide containing about 0.2 ml of Whitten and Biggers medium. The second uterine horn was collected into the same slide.

A disposable 1 ml TB syringe (Becton-Dickinson, Rutherford, New Jersey) was filled with Whitten and Biggers media. For eight-cell embryos, a nondisposable blunted 30 gauge needle (Popper and Sons, New Hyde Park, New York) was attached to the syringe. The needle was then inserted into

the ostium of the oviduct as viewed through a Bausch & Lomb dissecting microscope (Fischer, Fair Lawn, New Jersey) and 0.2 ml of medium was flushed through the oviduct. For blastocysts, a disposable 30 gauge needle was attached to the syringe. The needle was then inserted into the uterine lumen through the apical end of the uterine horn, near the oviduct and 0.5 ml of medium was flushed through the horn. Flushings from one mouse were collected in one concave slide, which was then placed in 5% CO₂ in air (Matheson, analyzed, Joliet, Illinois) and allowed to settle before embryos were collected, sorted, counted, and pooled. The pooled embryos were washed with three changes of Whitten and Biggers medium, then used.

2. Pronasing of embryos

Embryos were treated with pronase to remove their zona pellucida, as described by Biggers et al. (1971). A 1 to 2 ml aliquot of pronase was thawed, then passed through a 0.45 μ m millipore filter before diluting 1:2 with Whitten and Biggers medium without BSA. The 0.4 to 0.5 ml of diluted pronase was placed in a concave culture slide, then gassed with 5% CO₂ in air before adding any embryos. A micropipette was partially filled with Whitten and Biggers medium without BSA before picking up the embryos to be pronased. Twenty to fifteen embryos were treated at a time. Pronasing took 3 to 10 minutes. Pronase solution containing embryos was kept in 5% CO₂ in air until the zona pellucidae were swollen and began to disappear. The embryos were then retrieved from the pronase solution by using a micropipette which was partially filled

with Whitten and Biggers medium containing BSA. The embryos were washed by passage through three changes of Whitten and Biggers medium, then were incubated at 37°C-7% CO₂ for one to two hours before being assayed.

3. Immunosurgery

Immunosurgery was performed on CFl blastocysts according to the method of Solter and Knowles (1975). Blastocysts, collected from super-ovulated mice, were pronased, washed, and incubated at 37°C-7% CO₂ for three hours.

Rabbit anti-C57BL/10Sn serum was diluted 1:8 with Whitten and Biggers medium and 50 μ l was placed on a concave tissue culture slide. Five to ten embryos were then transferred to the antiserum and the slide was incubated. A normal rabbit serum slide was also prepared and 5 to 10 embryos were added to the 50 μ l drop and incubated. After 30 minutes, the embryos were retrieved with a micropipette from the slides and washed in three changes of Whitten and Biggers medium. The antiserum-treated embryos were pipetted and expelled several times to remove the swollen trophoblastic cell layer. The inner cell masses were then transferred to 50 μ l of Whitten and Biggers medium in the well of a microtiter plate. The normal serum treated blastocysts served as positive controls for ³H-thymidine incorporation. After a 10-15 minute period at 37°C-7% CO₂, 50 μ l of ³H-thymidine (8 μ Ci/ml) were added to all wells and the microtiter plate was reincubated for 24 hours, then the embryonic DNA was collected with a cell harvester. The filters were processed and were counted in a liquid scintillation counter.

Table 2. Buffers, solutions and culture media

<u>A. Embryo Culture Medium and Solutions</u>				
<u>1. Whitten and Biggers Culture Medium (as modified by our laboratory)</u>				
<u>Component</u>	<u>Mol. Wt.</u>	<u>g/l</u>	<u>mM</u>	<u>Milliosmols</u>
NaCl ^a	58.4	5.140	88.01	176.02
KCl	74.6	0.356	4.77	9.54
KH ₂ PO ₄	136.1	0.162	1.19	2.38
MgSO ₄ ·7H ₂ O	246.5	0.294	1.19	2.38
NaHCO ₃	84.0	2.106	25.07	50.14
Glucose	180.2	1.000	5.55	5.55
Ca lactate·5H ₂ O	308.3	0.527	1.71	5.13
Na pyruvate ^a	110.0	0.036	0.33	0.66
61% DL-Na lactate	112.1	2.245	20.03	40.06
		(in 3.68 ml syrup/l)		
Penicillin G, K salt (100 IU/ml)		0.075		
Streptomycin SO ₄		0.050		
1% phenol red ^b		1.000 ml		
Crystalline Bovine Serum Albumin ^a		3.000 g		
Distilled water ^c		to 1 liter		
Total				291.9

^aThe original formula of Whitten and Biggers (1968) consisted of NaCl--4.000 g/l, Na lactate--2.416 g/l and crystalline bovine serum albumin--4.00 g/l.

^bA 1% stock solution of phenol red was prepared, refrigerated and used as needed.

^cDouble strength medium was prepared by adding distilled water to 500 ml.

Table 2. (Continued)

2. Pronase Solution

Pronase (56 units/mg) (Calbiochem., LaJolla, California)	100 mg
Polyvinylpyrrolidone (GAF, New York, New York)	20 mg
Whitten and Biggers medium without BSA	20 ml

B. Solutions for Deoxyribonuclease Activity1. MgSO₄ Solution (0.05 M)

MgSO ₄ ·7H ₂ O	3.08 g
Distilled H ₂ O to	250 ml

2. Acetate Buffer (1.0 M) pH 5.0

17 N acetic acid	42 ml
0.29 M Na acetate	23.78 g
Distilled H ₂ O to	1000 ml

3. Stock Solution of Sodium Thymonucleate (2mg/ml)

DNA calf thymus Type V (Sigma, St. Louis, Missouri)	50 mg
Distilled H ₂ O to	25 ml

4. Enzyme Solution

DNase I (Worthington, Freehold, New Jersey)	1 mg
Distilled H ₂ O to	100 ml

5. Substrate Solutions

Sodium thymonucleate stock solution	2 ml
0.05 M MgSO ₄ ·7H ₂ O	10 ml
1.0 M acetate buffer	10 ml
Distilled H ₂ O	78 ml

Table 2. (Continued)

<u>C. Solutions Tested on Embryos</u>	
<u>1. Salts</u>	
<u>a. Phosphate Buffered Saline (PBS)</u>	pH 7.0
0.003 M $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	0.45 g
0.007 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	2.40 g
0.13 M NaCl	7.40 g
Distilled H_2O to	1000 ml
<u>b. Saline</u>	pH 7.0
0.15 M NaCl	8.50 g
Distilled H_2O to	1000 ml
<u>2. Known Inhibitors of DNA Synthesis</u>	
<u>a. α-Amanatin Stock Solution (400 $\mu\text{g}/\text{ml}$)</u>	
1 vial α -amanatin	2 mg
(Calbiochem., LaJolla, California)	
Distilled H_2O	2.5 ml
<u>b. Aphidicolin Stock Solution (2.5 mg/ml)</u>	
Aphidicolin	50 mg
(Imperial Chem., Cheshire, England)	
DMSO	15 ml
Distilled H_2O	5 ml
<u>c. Mitomycin C Stock Solution (500 $\mu\text{g}/\text{ml}$)</u>	
1 vial Mitomycin C	2 mg
(Sigma, St. Louis, Missouri)	
Whitten and Biggers medium	4 ml
<u>3. Known Inhibitors of Protein Synthesis</u>	
<u>a. Cycloheximide Stock Solution (2mM)</u>	
Cycloheximide	0.563 g
(Sigma, St. Louis, Missouri)	
Distilled H_2O to	1000 ml

Table 2. (Continued)

<u>b. Puromycin Stock Solution (0.1 mM)</u>		
Puromycin	0.0545 g	
(Sigma, St. Louis, Missouri)		
Distilled H ₂ O to	1000 ml	
<u>4. Other Compounds</u>		
<u>a. Human chorionic gonadotrophin (hCG) (MW 36,700)</u>		
(1.) hCG (lot CR-123; R. E. Canfield, Columbia University, New York, New York)		
in distilled water	5 mg/ml	
(2.) β -subunit (G.S. Cox, Iowa State University, Ames, Iowa)		
in distilled water	4.6 mg/ml	
<u>b. Theophylline Stock Solution (2 mM)</u>		
Theophylline	0.036 g	
(Sigma, St. Louis, Missouri)		
Distilled H ₂ O to	100 ml	
<u>D. Lymphocyte Separation Medium</u>		
<u>1. 9% Ficoll (w/v)</u>		
Ficoll	9 g	
(Pharmacia, Piscataway, New Jersey)		
Distilled water	100 ml	
<u>2. 34% Hypaque (w/v)</u>		
Hypaque	34 g	
(Winthrop Laboratory, New York, New York)		
Distilled water	100 ml	
<u>3. Ficoll-Hypaque Medium (specific gravity 1.080)</u>		
9% Ficoll	62.7 ml	
34% Hypaque	37.3 ml	

Table 2. (Continued)

<u>E. Solutions for Spermatocyte Cytotoxicity Assay</u>		
<u>1. 10% Buffered Formalin (Drake et al., 1972)</u>		
Na ₂ HPO ₄	0.65 g	
NaH ₂ PO ₄ ·H ₂ O	0.40 g	
Formaldehyde (37%)	10 ml	
0.2% trypan blue (GIBCO)	90 ml	
Stirred for two hours		
<u>2. CaCl₂ (0.1 M)</u>		
CaCl ₂	0.3675 g	
Distilled H ₂ O to	25 ml	
<u>3. 0.1% Deoxyribonuclease Solution</u>		
Deoxyribonuclease	1.0 g	
(Worthington, Freehold, New Jersey)		
Distilled H ₂ O to	100 ml	
PBS	1000 ml	
<u>4. Earle's Balanced Salt Solution (lacking calcium and magnesium) (GIBCO Catalogue 1978/1979)</u>		
	pH 7.0	
0.005 M KCl	0.40 g	
0.12 M NaCl	6.80 g	
0.03 M NaHCO ₃	2.20 g	
0.001 M NaH ₂ PO ₄ ·H ₂ O	0.14 g	
0.006 M Glucose	1.00 g	
Phenol red	0.01 g	
Distilled H ₂ O to	1000 ml	
<u>5. 0.4% Eosin in Buffer (Mayer et al., 1951)</u>		
	pH 7.4	
NaH ₂ PO ₄ ·H ₂ O	1.72 g	
Distilled H ₂ O to	100 ml	
Na ₂ HPO ₄	1.78 g	
Distilled H ₂ O to	100 ml	
0.12 NaH ₂ PO ₄ ·H ₂ O	20 ml	
0.13 Na ₂ HPO ₄	80 ml	
Eosin Y	0.4 g	
(Fisher, Fair Lawn, New Jersey)		

Table 2. (Continued)

6. Ethylenediaminetetraacetate (EDTA) (0.1 M)

EDTA	2.922 g
Distilled H ₂ O to	100 ml

7. NaCl (0.73 M)

NaCl	4.25 g
Distilled H ₂ O to	100 ml

8. PBS with 0.5% Fructose, 5% Fetal Calf Serum

pH 7.0

Fructose	0.005 g
Fetal calf serum	5.26 ml
PBS	100 ml

F. Solutions for the Haemagglutination Test1. Dextran Buffer Stock Solution

6% dextrose solution	
D-glucose	6 g
Distilled water to	100 ml
10% dextran solution	
dextran (MW 173,000)	10 g
6% dextrose to	100 ml

Autoclave, cool and freeze at -20°C

2. 0.5% Dextran Buffer

Dextran stock solution	5 ml
Fetal calf serum (GIBCO)	2 ml
NaN ₃	0.1 g
PBS to	100 ml

Table 2. (Continued)

<u>G. MLR, CML, TCGF, and MELIA Media and Solutions</u>		
<u>1. Buffered Ammonium Chloride Solution (Garvey, et al., 1977)</u>		
<u>a. Tris Buffer (0.05 M)</u>		pH 7.4
Trizma base		0.97 g
Trizma HCl		6.61 g
<u>b. NH₄Cl (0.14 M)</u>		
NH ₄ Cl		7.42 g
Distilled H ₂ O to		1000 ml
<u>2. Concanavalin A Stock Solution (1 mg/ml)</u>		
Concanavalin A		0.025 g
(Miles-Yeda, Israel)		
Distilled H ₂ O to		25 ml
<u>3. Hank's Balanced Salt Solution (HBSS)</u>		pH 7.4
(GIBCO Catalogue 1978/1979)		
0.0025 M CaCl ₂ •6H ₂ O		0.28 g
0.0054 M KCl		0.40 g
0.0004 M KH ₂ PO ₄		0.06 g
0.0005 M MgCl ₂ •6H ₂ O		0.10 g
0.0006 M MgSO ₄ •7H ₂ O		0.10 g
0.1369 M NaCl		8.00 g
0.0042 M NaHCO ₃		0.35 g
0.0055 M Glucose		1.00 g
Phenol red		0.01 g
Distilled H ₂ O to		1000 ml
<u>4. Mitomycin C Stock Solution (500 µg/ml)</u>		
1 vial mitomycin C		2 mg
(Sigma, St. Louis, Missouri)		
PBS (filtered with 0.45 µm filter)		4 ml

Table 2. (Continued)

5. 1% Phytohemagglutinin-P (PHA-P) Stock Solution (w/v)

1 vial PHA-P (Difco, Detroit, Michigan)	0.05 g
Distilled H ₂ O	5 ml

6. Protein Standardsa. Crystalline Bovine Serum Albumin (1 mg/ml)

BSA (Sigma, St. Louis, Missouri)	0.024 g
Distilled H ₂ O to	25 ml

b. Human Chorionic Gonadotrophin (0.2 mg/ml)

hCG (Calbiochem, LaJolla, California)	0.005 g
Distilled H ₂ O to	25 ml

c. Ribonuclease A (1.1 mg/ml)

Ribonuclease A (Worthington, Freehold, New Jersey)	0.028 g
Distilled H ₂ O to	25 ml

7. Saturated Ammonium Sulfate ((NH₄)₂SO₄ Table,
P. L. Biochem, Milwaukee, Wisconsin)

25°C, pH 8.0

(NH ₄) ₂ SO ₄	767 g
Distilled H ₂ O to	1000 ml

8. Sephadex G-100 for 2 x 80 cm column

Sephadex G-100 (Pharmacia, Piscataway, New Jersey)	18.9 g
0.9% NaCl (pH 7.4)	400 ml

9. Supplemental RPMI 1640 (Rosenberg et al., 1978a)

0.4 mM glutamine
1.0 μM sodium pyruvate
5 x 10 ⁻⁵ M 2-mercaptoethanol
100 units/ml penicillin
100 μg/ml streptomycin
10% heat-inactivated GG-free newborn calf serum

Table 2. (Continued)

<u>I. Lowry Solutions (based on Lowry et al., 1951)</u>		
<u>1. Reagent A</u>		
2.68% KNa tartrate $4\text{H}_2\text{O}$	2.68 g	
Distilled H_2O to	100 ml	
<u>2. Reagent B</u>		
1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	1.0 g	
Distilled H_2O to	100 ml	
<u>3. Reagent C</u>		
0.1 N NaOH	4 g	
Distilled H_2O to	1000 ml	
2% Na_2CO_3	2 g	
0.1 N NaOH	100 ml	
<u>4. Mix 1 part A + 1 part B + 100 parts C (stable for 5 hours)</u>		
<u>5. Folin Reagent</u>		
2 N Phenol		
(Fisher, Fair Lawn, New Jersey)		
Dilute to 1 N with distilled H_2O		
<u>6. Bovine Serum Albumin (BSA) Stock Solution (200 $\mu\text{g}/\text{ml}$)</u>		
BSA	0.005 g	
(Sigma, St. Louis, Missouri)		
Distilled H_2O to	25 ml	

Table 2. (Continued)

<u>J. Ehrlich-Lettre Cells Media and Solutions</u>		
<u>1. Acetic Alcohol</u>		
Acetic acid	1 part	
Absolute ethanol	2 parts	
<u>2. Colcemid Stock Solution (10 µg/ml)</u>		
1 vial (GIBCO, Grand Island, New York)		0.1 mg
Distilled H ₂ O		10 ml
<u>3. Culture Medium</u>		
90% NCTC 135		
(GIBCO, Grand Island, New York)		
10% GG-free newborn calf serum		
(GIBCO, Grand Island, New York)		
1% Antibiotic-Antimycotic solution		
(GIBCO, Grand Island, New York)		
0.5% Garamycin (10 mg/ml gentamycin)		
(Schering, Kenilworth, New Jersey)		
<u>4. Freezing Medium</u>		
90% culture medium		
10% dimethyl sulfoxide (DMSO)		
<u>5. 0.9% Sodium Citrate</u>		
Sodium citrate		0.9 g
Distilled H ₂ O to		100 ml
<u>6. 2% Trypsin Stock Solution</u>		
Trypsin (100,000 units/mg)		0.5 g
(GIBCO, Grand Island, New York)		
PBS (0.01 M phosphate) to		25 ml

D. Incorporation of ^3H -thymidine by Embryos

1. DNA synthesis assay

DNA synthesis in the embryos was assessed by the incorporation of ^3H -thymidine ([methyl- ^3H]-thymidine, 20 Ci/mmol, aqueous solution, New England Nuclear, Boston, Massachusetts). The ^3H -thymidine was diluted with an equal volume of double-strength Whitten and Biggers medium. Further dilutions were made with single-strength Whitten and Biggers medium. All assays were performed in 96-well flat-bottom tissue culture plates (Linbro; well capacity: 0.35 ml). For most assays, a solution of 8 $\mu\text{Ci/ml}$ of ^3H -thymidine was used. This was prepared by adding 200 μl of ^3H -thymidine in water to 25 ml of single-strength Whitten and Biggers medium. (See especially "Embryo Cytotoxicity Assay" later in dissertation.)

At the beginning of each assay, 50 μl of Whitten and Biggers medium were placed into each well of the microtiter plate and gassed for 10-15 minutes with 5% CO_2 in air. The appropriate number of embryos was added to the wells, using a finely drawn pasteur pipette. Either 50 μl of the desired strength of ^3H -thymidine were added immediately, or the plate was preincubated for a given time before the ^3H -thymidine was added. After the addition of ^3H -thymidine, the plates were incubated for the length of time dictated by the individual experiments. All incubations were at 37°C in a humid atmosphere of 7% CO_2 in air.

Each experiment was terminated by placing the tissue culture plate on ice. The contents of the wells were then harvested with distilled

water and 50-60 cm Hg vacuum (vacuum pump from Millipore, Bedford, Massachusetts) using a Titertek cell harvester (Flow Laboratories, MacLean, Virginia) as previously described by Thurman et al. (1973). Briefly, the filter papers were prewet with water for five seconds by pressing the "rinse" button with the suction head in an empty row on the microtiter plate. Then the sample row was harvested with water by pressing the "rinse" button, for 30-60 seconds. The filters were then dried for 10-15 seconds by pressing the "air" button. The water line to the cell harvester must not have any air bubbles to ensure proper water flow. The GF/C filter paper mats were then dried at 65°C for at least 20 minutes. The filter paper discs were punched from the mat and each disc was placed in a glass scintillation vial (Fisher, Fair Lawn, New Jersey) containing 10 ml of toluene fluor. The fluor contained 18.6 gm PPO, 2,5-diphenyloxazole (Fischer, Fair Lawn, New Jersey) and 0.94 gm POPOP, p-bis-[2-(5-phenyloxazolyl)]-benzene (Amersham/Searle, Des Plaines, Illinois) per 3.8 liters of toluene (Fischer, Fair Lawn, New Jersey). The vials were then counted in a liquid scintillation counter (Beckman, Fullerton, California).

2. Experimental protocols

To prove that ^3H -thymidine was being incorporated into the embryonic DNA; two types of experiments were designed. In the first type, mitomycin C, a known inhibitor of DNA synthesis, was tested on embryos prior to incubation with ^3H -thymidine. In the second type of experiment, embryos were first incubated with ^3H -thymidine, then were treated with DNase I (Worthington, Freehold, New Jersey).

a. Mitomycin C treatment of embryos Two milligrams of mitomycin C (Sigma, St. Louis, Missouri) were dissolved in 2 ml of Whitten and Biggers medium to give a 1000 $\mu\text{g/ml}$ stock solution which was then diluted with Whitten and Biggers medium to give mitomycin C concentrations of 500, 400, 300, 200, 100, 50, and 25 $\mu\text{g/ml}$. CF1 blastocysts were either treated with pronase to remove the zona pellucida or were left untreated. All embryos were then incubated for one-two hours at 37°C and 7% CO_2 in air. The embryos were placed into 0.5 ml of the designated concentrations of mitomycin C or Whitten and Biggers medium only for 0.5, 1, 1.5, or 2 hours at 37°C and 7% CO_2 in air. Then the embryos were removed and washed in three changes of 0.5 ml Whitten and Biggers medium. All incubations with mitomycin C and all washes were performed in 35 x 10 mm Falcon plastic tissue culture dishes (Becton, Dickinson and Co., Oxnard, California). After the third wash, the embryos were transferred to a 96-well microtiter plate. One embryo was placed per well containing 25 μl of Whitten and Biggers medium and ^3H -thymidine (sp. act. 20 Ci/mole) at a concentration of 4 $\mu\text{Ci/ml}$. A seven-hour incubation period at 37°C and 7% CO_2 in air was performed prior to cell harvesting and processing of filter papers.

b. DNase I treatment of embryos DNase I activity was determined by the spectrophotometric method of Kunitz (1950). Several 8 ml glass test tubes, each containing 3 ml of substrate solution, were placed in a water bath at 25°C for 10 minutes. One ml of H_2O was added to one of these tubes and served as the blank. A sample of 1 ml deoxyribonuclease solution (5 or 10 $\mu\text{g/ml}$) was added to each of the

other test tubes. The test tubes were gently vortexed (Clay Adams, Chicago, Illinois) and a stop watch was started. A sample from each tube was transferred to a cuvette (Fisher, Chicago, Illinois). Readings were taken at intervals of 0.5 to 1 minute for 5 minutes, using a Gilford 250 spectrophotometer (Oberlin, Ohio) set at 260 nm. All assays were performed in duplicate. The activity of the enzyme solution was expressed in terms of the mean slope of a plot of the optical density versus time (in minutes). The specific activity was then determined by dividing the mean slope by the concentration of the enzyme in milligrams of proteins per milliliter of the final digestion mixture.

In order to examine the effect of the Whitten and Biggers medium, pH, and Triton X-100 on DNase I activity, Kunitz's procedure was repeated with the following modifications. The substrate solution was diluted with 2X Whitten and Biggers medium and the pH was adjusted to 5.0 with 1 M HCl. Kunitz's procedure was also repeated with 0.1% Triton X-100 per test tube. The DNase I solution was used at a final concentration of 2.5 $\mu\text{g/ml}$ on CF1 embryos.

Either 1 CF1 blastocyst or 5 CF1 blastocysts were added to 25 μl of Whitten and Biggers medium in a well of a 96-well microtiter plate. After a two-hour incubation of 37°C and 7% CO₂ in air, 25 μl of Whitten and Biggers media containing 8 $\mu\text{Ci/ml}$ ³H-thymidine were added to all wells and the embryos were incubated for two hours. Fifty μl of either Whitten and Biggers medium or 1% Triton X-100, diluted with Whitten and Biggers medium were added to designated wells and the embryos were incubated for 30 minutes. Then 100 μl of either Whitten and Biggers

medium or DNase I (5 $\mu\text{g/ml}$ in Whitten and Biggers medium) adjusted to pH = 5.0 by adding 0.8 μl 1M HCl for every 100 μl of medium was added to designated wells and the embryos were reincubated for one hour. The microtiter plate was then placed on ice and treated as described previously.

3. Effect of compounds on embryos

The DNA synthesis assay for embryos was used to investigate the effects of PBS, saline, α -amanitin, aphidicolin, mitomycin C, cycloheximide, puromycin, hCG, and theophylline on CF1 blastocysts. For these experiments, 3-5 blastocysts were incubated in 50 μl of a designated concentration of compound diluted with Whitten and Biggers medium. All concentrations of compounds were tested in duplicate. Controls consisted of blastocysts incubated in Whitten and Biggers medium only. For the aphidicolin studies, the control consisted of Whitten and Biggers medium containing the appropriate concentrations of DMSO. After a seven-hour incubation at 37°C and 7% CO₂ in air, 50 μl of 8 $\mu\text{Ci/ml}$ of ³H-thymidine were added to all wells. The embryos were reincubated for an additional three hours, then the embryonic DNA was collected with a cell harvester and the filters were processed.

The effect of α -amanitin was further examined on CF1 8-cell and blastocysts embryos. Five blastocysts or ten 8-cell embryos were cultured in 50 μl of Whitten and Biggers medium either with or without a given concentration of α -amanitin. After a two-hour incubation at 37°C in a humid atmosphere of 7% CO₂ in air, 50 μl of an 8 $\mu\text{Ci/ml}$ solution

of ^3H -thymidine in culture medium were added to all wells and the embryos were reincubated for three to seven hours. The microtiter plate was then processed with a cell harvester and the filters were counted.

The effect of aphidicolin was further examined on CF1 8-cell embryos according to the blastocyst protocol except 10 embryos were incubated per 50 μl of designated aphidicolin concentrations.

The experimental data were expressed as:

$$\begin{aligned} \text{\% Inhibition of } ^3\text{H-thymidine Incorporation} = \\ 1.0 - \frac{\text{Experimental cpm}}{\text{Average control cpm}} \times 100. \end{aligned}$$

E. Sera

1. Rabbit anti-mouse serum

Rabbit anti-mouse serum was prepared by injecting 1×10^8 C57BL/10Sn spleen cells into the marginal ear vein of an eight-pound female New Zealand rabbit. Three injections were given on days 1, 10 and 20 with a test bleeding performed on day 30. Three more booster injections of 1×10^8 spleen cells were given about six weeks apart with a cardiac puncture performed two weeks after the final booster. The blood was allowed to clot at room temperature, then refrigerated overnight. The clot was removed and discarded. The remaining serum was centrifuged (Beckman Model TJ-6, Irvine, California) at 125 g and room temperature for about 15 minutes to sediment any cells. The supernatant was collected via a pasteur pipette and was transferred to a clean test tube. The serum was heat inactivated in a 56°C water bath for 30 minutes and

was then tested in a trypan blue cytotoxicity assay against CF1 spleen lymphocytes and also against C57BL/10Sn spleen lymphocytes. The antiserum from the final bleeding gave the highest percent killing of lymphocytes ($> 80\%$ at 1:64) and was, therefore, used for all experiments. Normal rabbit serum (GIBCO, Grand Island, New York) was heat-inactivated and used as the serum control. The antiserum was stored in 50 μ l aliquots at -70°C until used. The normal serum was stored in 100 μ l aliquots at -70°C until used.

2. Congenic antisera

Anti-H-2 sera were prepared using spleen cells from C57BL/10Sn mice and the B10 congenic strains B10.BR, B10.A and B10.D2 by a modification of the method of Batchelor (1973). The spleen cell suspension was prepared by injecting one spleen with a 1 ml disposable TB syringe (Becton-Dickinson, Rutherford, New Jersey) filled with RPMI 1640 (GIBCO, Grand Island, New York). The swollen spleen was then pressed through a 40-gauge stainless steel screen. The cells on the underside of the screen were gently washed into the resulting cell suspension. One mouse spleen per 1 ml of RPMI 1640 yielded a cell suspension containing about 10^7 cells per a 0.2 ml injection. Usually, two spleens were used to immunize eight mice. Prior to immunization, the mice were bled from the orbital venus sinus for normal control sera which was heat inactivated and stored at -70°C before being used. Each mouse received one injection per week for seven weeks. A test bleeding was made in which serum from each set of injected mice was pooled and heat-inactivated

at 56°C for 30 minutes. The antisera were then tested against the lymphocytes from the immunizing strain and also against the lymphocytes from the other strains. Injections were resumed the week after the test bleeding. Three more weekly injections of 10^7 cells followed. Another test bleeding was performed a week after the tenth injection. The mice were boosted once a month, then bled one week later. Five bleedings were taken with the fifth bleeding yielding the best cytotoxicity titers ($> 90\%$ killing at 1:4).

Table 3 lists the K, D and Ia alloantigens of the mouse strains used in this study. A list of the antisera, and their possible K and D specificities are shown in Table 4. Since the mice were immunized with lymphocytes, only K, I and D antigens should be recognized with K and D antigens showing immunodominance to I region antigens. This is the basis for listing only K and D antigenic specificities in Table 4.

3. NIH antisera

The antisera listed in Table 5 were obtained from the Transplantation and Immunology Division, NIH, Bethesda, Maryland. Single vials containing the lyophilized antiserum were reconstituted with 1.0 ml de-ionized distilled water and stored in aliquots of either 50 μ l or 100 μ l at -70°C.

Table 3. K, D and Ia alloantigens of inbred strains^{a,b,c}

Strain	H-2 Haplotype	K, D and Ia Alloantigens
C57BL/10Sn	b	K ^b 5, 28, <u>33</u> , 35, 36, 39, 53, 54 D ^b <u>2</u> , 6, 27, 28, 29, 56 Ia 3, 8, 9, 15, 20
B10.A	a	K ^k 1, 3, 5, 8, 11, <u>23</u> , 24, 25, 45, 49?, 52 D ^d 3, <u>4</u> , 6, 13, 27, 28, 29, 35, <u>36</u> , 41, 42, 43, 44, 49 Ia 1, 2, 3, 6, 7, 15, 17, 18, 19, 22
B10.BR	k	K ^k 1, 3, 5, 8, 11, <u>23</u> , 24, 25, 45, 49?, 52 D ^k 1, 5, <u>32</u> Ia 1, 2, 3, 7, 15, 17, 18, 19, 22
B10.D2	d	K ^d 3, 8, 28, 29, <u>31</u> , 34 D ^d 3, <u>4</u> , 6, 13, 27, 28, 29, 35, 36, 41, 42, 43, 44, 49 Ia 6, 7, 8, 11, 15, 16, 23

^aThis information was compiled from Demant's haplotype specificity tables (1979).

^bPrivate specificities are underlined.

^cAny superscripts within text of table refer to specific alloantigens; these are not footnote notations.

Table 4. Specificities of congenic alloantisera^{a,b,c}

Antiserum	Recipient-Donor	Possible K and D Specificities
X-B10	B10.BR anti-B10	K ^b 28, <u>33</u> , 35, 36, 39, 53, 54 D ^b <u>2</u> , 6, 27, 28, 29, 56
X-B10.A	B10 anti-B10.A	K ^k 1, 3, 8, 11, <u>23</u> , 24, 25, 45, 49?, 52 D ^d 3, <u>4</u> , 6, 13, 35, 36, 41, 42, 43, 44, 49
X-B10.BR	B10 anti-B10.BR	K ^k 1, 3, 8, 11, <u>23</u> , 24, 25, 45, 49?, 52 D ^k 1, 3, 5, <u>32</u>
X-B10.D2	B10 anti-B10.D2	K ^d 3, 8, 29, <u>31</u> , 34 D ^d 3, <u>4</u> , 13, 35, 36, 41, 42, 43, <u>44</u> , 49

^aThis information was compiled from Demant's haplotype specificity tables (1979).

^bPrivate specificities are underlined.

^cAny superscripts within the text of the table refer to specific alloantigens; these are not footnote notations.

Table 5. Specificities of NIH alloantisera^a

Antiserum	Recipient-Donor (Strains and Genotype)	Detected Specificities ^{b,c}
D-2	[B10.A(5R) x LP.RIII] anti-B10 (K ^b D ^d x K ^r D ^r) anti-K ^b D ^b	D ^b <u>2</u> , 56
D-23b	(B10.D2 x SJL) anti-B10.A (K ^d D ^d x K ^s D ^s) anti-K ^k D ^d	K ^k 11, <u>23</u> , 25, 52
D-32	[B10.A(2R) x C3H.5W] anti-C3H (K ^k D ^b x K ^b D ^b) anti-K ^k D ^k	D ^k <u>32</u>
D-31	(B10 x A) anti-B10.D2 (K ^b D ^b x K ^k D ^d) anti-K ^d D ^d	K ^d <u>31</u> , 34
Ia 1,2,3,7	A.TH anti-A.TL (K ^s I ^s D ^d) anti K ^s I ^k D ^d	Ia 1, 2, 3, 7 (15, 22?)

^aPrivate specificities are underlined.

^bThis information was obtained from the NIH catalog of Mouse Alloantisera (Snell, 1975; Ray, 1977).

^cAny superscripts within the text of the table refer to specific alloantigens; these are not footnote notations.

4. Guinea pig complement

Complement was obtained from reconstituted lyophilized guinea pig serum prepared in our laboratory. Guinea pigs were bled via the jugular vein into a 250 ml beaker. The blood was allowed to clot at room temperature for about one hour, then was refrigerated overnight. The clot was removed and the remaining supernatant was centrifuged at 100 g and room temperature for 15-20 minutes to sediment any suspended cells. The 150 ml of supernatant was collected with a pasteur pipette and placed in a 250 ml round bottom flask. The contents of the flask were frozen with dry ice and acetone. The flask was attached to a lyophilizer (Virtis Research Equipment, Gardner, New York) and a vacuum was induced. About 24 hours later, the resulting precipitate was weighed and aliquotted into 12 ml glass storage vials. The vials were stored at -70°C . The complement was reconstituted with 10 ml of distilled water and was then divided into 200 μl aliquots and stored at -70°C . The complement was tested for cytotoxic activity in a lymphocyte cytotoxicity assay with an antiserum exhibiting cytotoxicity of greater than 90% killing.

5. Rabbit complement

Rabbit serum was obtained by cardiac puncture of a New Zealand white rabbit. The blood was allowed to clot then set on ice for two hours to allow the clot to contract. The serum was collected after centrifugation at 100 g and room temperature for 20 minutes, then was stored at 4°C until absorbed within the next 24 hours.

Rabbit serum was absorbed according to the method of Boyse et al. (1970). Spleens, thymuses, spermatocytes and testes, obtained from C57BL/10Sn male mice were minced in 10 ml of a 0.1% deoxyribonuclease (Worthington, Freehold, New Jersey) solution. The tissue suspension was vortexed for two to three minutes at low speed in a Virtis Homogenizer (Gardner, New York). The suspension was then centrifuged (Beckman Model TJ-6, Irvine, California) at 125 g and room temperature for about five minutes. The supernatant was decanted and the cells were washed three times with Earle's medium lacking calcium and magnesium. One volume of 0.1 M EDTA was added to nine volumes of rabbit serum about 0.5 hour prior to addition of cells. Seven volumes of EDTA-treated serum were absorbed with one volume of packed cells. Absorption was performed on ice and in a cold room (4°C) for 45 minutes with frequent agitation of the cell-serum suspension. The serum was recovered after centrifugation (Beckman Model TJ-6, Irvine, California) at 125 g and 4°C for 15 minutes. Free divalent cations were restored by the addition of one volume of 0.1 M CaCl_2 . Absorbed serum was tested in a lymphocyte assay where the rabbit serum replaced the guinea pig serum as the source of complement.

6. Attempted production of H-Y antisera

Three different injection protocols for H-Y antisera production were followed. The first protocol was the same as that used for the production of congenic H-2 antisera, except male spleen cells of a strain were injected into female mice of the same strain. The second

protocol involved weekly inoculations of 25×10^6 C57BL/6 male spleen cells into C57BL/6 female mice as described by Krco and Goldberg (1976). Serum was obtained on day 10 after the seventh inoculation and was then aliquotted and frozen at -70°C . The third protocol was adapted from Shalev et al. (1978). Fifteen i.p. injections of $1-3 \times 10^6$ C57BL/6 male spleen cells in a 0.1 ml volume were injected into C57BL/6 female mice. Mice were bled on days 17, 24, 32, and 40 from the initial injection. Serum was tested within a couple of hours of collection.

7. Tests for H-Y antisera activity

H-Y antisera were tested by using at least one of the following methods.

a. Spermatocyte cytotoxicity assay The spermatocyte cytotoxicity assay of Goldberg et al. (1971) was followed. Sperm were obtained by mincing the epididymus in a concave culture slide containing a few drops of PBS with 0.5% fructose and 5% fetal calf serum. The sperm suspension was washed two times with PBS, then diluted to 4×10^6 cells/ml. The assay was performed by adding 50 μl of anti-H-Y sera of designated dilutions or normal mouse serum, 50 μl of sperm cell suspension, and 50 μl of absorbed rabbit complement diluted 1:4 with PBS to 12 x 75 mm disposable culture tubes (Fisher, Pittsburgh, Pennsylvania) then incubating at 37°C . After 45 minutes, 0.1 ml of a 1% trypan blue dye solution (GIBCO, Grand Island, New York) diluted 1:5 with a 0.73 M NaCl solution was added. The spermatocytes were examined for dye uptake after 10-15 minutes. Formalin fixed

spermatocytes served as dye inclusion controls. In a few experiments, eosin dye (0.4%) was substituted for trypan blue dye to enhance dye uptake.

Spermatocytes were fixed with formalin to provide a positive control which took up trypan blue dye. The procedure of Drake et al. (1972) was used. Mouse spermatocytes were collected in 0.15 M saline and washed once, then were resuspended at 10^7 cells/ml. An equal volume of methanol was added and the cell suspension was vortexed (Cyclomixer, Clay-Adams, Chicago, Illinois) at low speed for one minute, then centrifuged at 125 g and room temperature for five minutes. The supernatant was removed with a pasteur pipette. The spermatocytes were then fixed in 10% buffered formalin in 25 times the cell volume. The cells were suspended in solution and were fixed overnight at room temperature. Cells were stored in the 10% buffered formaline at 4°C.

b. Staph aureus "rosetting" procedure The Staph aureus "rosetting" procedure of Tokuda et al. (1977) was followed. Staphylococcus aureus (Cowan I, strain) was obtained from Dr. P. Patee (Bacteriology Department, ISU) and was cultured in a shaker in Todd-Hewitt Broth (Difco, Detroit, Michigan) at 37°C for 18 hours. A sample of the cell suspension was counted and gram stained to confirm a cell concentration of 1×10^9 cells/ml and the presence of gram positive cocci. The suspension was divided into several 250 ml polypropylene centrifuge bottles (Fisher, Chicago, Illinois) and centrifuged at 1000 g and 4°C for 30 minutes in a Sorvall RC-29 centrifuge (Sorvall, Norwalk, Connecticut). The broth was decanted and autoclaved before discarding. The cell sediments were pooled and washed three times with PBS. After the

last wash, the sediment was suspended in 200 ml of PBS containing 0.5% formaldehyde (37%) and incubated at 37°C for three hours. The suspension was centrifuged at 1000 g and 4°C for 30 minutes then washed in PBS four times. The cells were resuspended in PBS to a 10% (v/v) cell concentration and were placed in a boiling water bath for three minutes. The suspension was stored at 4°C in the presence of 0.1% NaN_3 . Cells were washed and resuspended in PBS before each use.

Spleen lymphocytes from male and female (negative control) mice were obtained via a Ficoll-Hypaque separation (described later) and were used as the target cells in the assay.

The assay was performed by adding 50 μl of antiserum to 250 μl of lymphocytes (2×10^6 cells/ml) in 12 x 75 mm disposable culture tubes and incubating for 30 minutes at 4°C. Controls consisted of female lymphocyte targets or normal mouse serum. The culture tubes were centrifuged (International Centrifuge, Boston, Massachusetts) at 125 g and 4°C for 10 minutes, then were washed twice with RPMI 1640. Cell pellets were resuspended in 50 μl of the Staph aureus cell suspension and were incubated at 4°C for 15 minutes. The cells were washed three times with RPMI 1640. After the final wash, the cells were resuspended in 250 μl RPMI 1640. Smears were prepared on microscope slides. After air drying and fixing in methanol, the slides were stained in Giemsa (GIBCO, Grand Island, New York) for 20 minutes. Lymphocytes exhibiting five or more Staph aureus cells were considered positive.

c. Haemagglutination test The haemagglutination test was performed according to the procedure of Shalev et al. (1978) in V-shaped

wells of microtiter plates (Linbro, Flow Labs, McLean, Virginia). Sera were diluted with a 0.5% dextran buffer solution (DB) and 0.1 ml was added to designated wells of the microtiter plate. Red blood cells were collected by bleeding mice from the supraorbital venous sinus and collecting the blood in a 40 times volume of PBS. The cell suspensions were kept on ice, then washed twice in the same volume of PBS. Centrifugations were performed at 4°C. Cell concentrations were adjusted to 10^7 /ml in DB, then 25 μ l were added to designated wells of the microtiter plate. Plates were read when the red blood cells in the negative controls (no serum wells) had completely settled--usually after a two-hour room temperature incubation. The positive control consisted of serial dilutions of rabbit anti C57BL/10Sn sera incubated with C57BL/10Sn red blood cells.

F. Ascites Antibody Production

1. Cell culture and maintenance

One ampule of Ehrlich's-Lettre ascites mouse carcinoma, Strain E, was obtained from the American Type Culture Collection (Rockville, Maryland). The cells were cultured according to the method of Boone et al. (1965). Briefly, 10^6 cells were cultured in 50 ml Falcon flasks (Fisher, Chicago, Illinois) in 10-12 ml of NCTC 135 medium (GIBCO, Grand Island, New York), containing 10% GG-free newborn calf serum and antibiotics. Cells were incubated at 37°C and 7% CO₂. Media changes were made every four to five days. When cultures became confluent, portions of the cells were scraped free with a rubber policeman and

discarded with media changes. Cells were subcultured as needed. Cells were scraped free and about 10^6 cells were inoculated into each freshly prepared flask.

2. Cell passages

Cells were passaged from cell culture to the mouse by dislodging confluent cells with 0.2% trypsin. The cell suspension was washed once with medium, then the cells were counted in a hemacytometer. Between 1.4 and 2.0×10^6 cells in 0.2 ml of culture medium were inoculated, i.p., per mouse. Nontrypsinized cells failed to develop tumors in mice. The ascites were tapped aseptically with a 10 cc syringe and specially prepared 18 gauge needle 14 to 21 days after inoculation. The 18 gauge needle contained six holes perforated along its barrel within 2 cm of the beveled end of the needle. Two-tenths of a ml of ascites (10^8 cells/ml) was injected into each of a second set of mice. The ascites were collected and injected into a third set of mice as described for the second set. Passaged cells were grown in vitro by inoculating 10^6 cells into a Falcon flask containing 10-12 ml of culture medium.

3. Hyperimmune ascites production and testing

Hyperimmune ascites were produced based on the methods of Fathman et al. (1975) and Sachs and Cone (1973). Ascites cells (25×10^6), prepassaged in host strains, were injected concomitantly with 1×10^6 donor spleen cells in a 0.2 ml volume into host mice, previously immunized against the donor spleen cell population. The injection of

tumor and spleen cells resulted in the production of large volumes (up to 10 ml) of ascites fluid containing antibodies to the donor spleen cell population. The hyperimmune ascites was compared to antiserum from the same mouse for reactivity against donor lymphocytes in a lymphocyte cytotoxicity assay. Ascites fluid collected from the third cell passage was used as the negative ascites control and normal host mouse serum served as the negative serum control. The hyperimmune ascites was aliquotted and stored at -70°C .

4. Confirmation of cell lines after in vivo explantation

Chromosomal studies were performed on explanted cells to confirm the cell lines according to Boone et al. (1965). Cultured explanted ascites cells were treated with $1\text{ }\mu\text{g/ml}$ colcemid (GIBCO, Grand Island, New York) during a five-hour incubation period. Cells were detached from the monolayer by treatment with 0.2% trypsin. The cell suspension was centrifuged (Beckman Model TJ-6, Irvine, California) at 125 g and room temperature for 10 minutes. The supernatant was decanted and the cells were resuspended in 5 ml of 0.9% sodium citrate, then placed in a water bath at 37°C for 20 minutes. Cold acetic-alcohol (5 ml) was then slowly added to the cell suspension with gentle agitation. The suspension was set at room temperature for 15 minutes, then centrifuged at 125 g and room temperature for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in 5 ml of cold acetic-alcohol and centrifuged again. The supernatant was decanted and the cell pellet was resuspended in 10 to 20 times its own volume of

acetic-alcohol. Five to ten drops of cell suspension were placed on a glass slide wetted with 50% alcohol. The slide was held horizontally while the fluid was ignited and was then allowed to burn itself out. The slide was gently heated until thoroughly dry. Cells were stained in Giemsa (GIBCO, Grand Island, New York) for about 30 minutes then rinsed in distilled water and dried over a low flame. The slide was placed in xylene for two minutes, then air-dried and examined under oil immersion with a Zeiss microscope (West Germany).

5. Storage of cells

Passaged and nonpassaged cells were periodically frozen and stored in liquid nitrogen. Ascites or cultured cells were counted then centrifuged (Beckman Model TJ-6, Irvine, California) at 125 g and room temperature for 10 minutes. The cells were washed once with culture medium and were then suspended in culture medium containing 10% DMSO to give a final concentration of $1-5 \times 10^6$ cells/ml. The cell suspension was dispensed as 1 ml aliquots into Nunc Intermed storage vials (Denmark). The vials were labeled and were attached to a BF-5 necktube cone adjusted to ring B and the entire freezer apparatus (Union Carbide, Indianapolis, Indiana) was inserted into the neck of a liquid nitrogen storage tank (American Breeders Service, Madison, Wisconsin) for one hour. The vials were then removed from the freezing apparatus and placed in a storage container which was then submerged in liquid nitrogen.

6. Thawing of cells

Cells were thawed rapidly by submerging the storage vial in a 37°C water bath for about one minute. The vial was then submerged in 70% alcohol and transferred to a sterile hood where the cell suspension was aseptically transferred to a 50 ml Falcon flask (Fisher, Chicago, Illinois) containing 10 to 15 ml of culture medium. A 0.1 ml aliquot was removed for a viability count in 0.4% trypan blue dye. Twenty-four hours later, the cells were centrifuged (Beckman Model TJ-6, Irvine, California) at 125 g and room temperature for 10 minutes. The cells were resuspended in 10-15 ml of fresh culture medium and were maintained as described previously.

G. Cytotoxicity Assays

1. Lymphocyte cytotoxicity assay

The rabbit, congenic and NIH antisera and hyperimmune ascites were tested for cytotoxicity against lymphocytes by the trypan blue dye exclusion method (Gorer and O'Gorman, 1956). White blood cells were collected by the Ficoll-Hypaque density gradient centrifugation method (Bøyum, 1968). Spleen suspensions were prepared as described for the production of congenic antisera. About 200 μ l of the spleen suspension was layered over 200 μ l of Ficoll-Hypaque, prepared as shown in Table 2, in 400 μ l microfuge tubes (Beckman, Irvine, California). The tubes were then centrifuged in a Beckman 152 Microfuge (Beckman, Irvine, California) for 2.5 to 3 minutes. The white blood cell layer was removed with a pasteur pipette to clean 400 μ l microfuge tubes. The tubes

were filled with RPMI 1640 and the cells were resuspended in the medium with a pasteur pipette to wash the cells. The tube was then centrifuged in the microfuge (Beckman Model 152, Irvine, California) for 5 to 10 seconds. The supernatant was removed via a pasteur pipette and the cells were resuspended in additional RPMI 1640. After the second wash, the cell pellets were collected into a test tube (12 x 75 mm glass; Fisher, Fair Lawn, New Jersey) and brought to 1 ml with RPMI 1640. A 1:50 dilution was made of the cell suspension which was then counted on a hemacytometer (Brightlight; Fisher, Fair Lawn, New Jersey). The cell suspension was adjusted to 6×10^6 cells/ml with RPMI 1640.

The cytotoxicity assay was performed in 250 μ l microfuge tubes (Beckman, Irvine, California) by adding 10 μ l of 6.0×10^6 cells/ml, 10 μ l of antiserum serially diluted with RPMI 1640, and 10 μ l of guinea pig complement diluted 1:2 with RPMI 1640. The tubes were capped and mixed, then incubated at 37°C. After one hour, 10 μ l of a 0.4% trypan blue solution (GIBCO, Grand Island, New York) were added to each tube and the tubes mixed. After five minutes, the tubes were placed on ice, then counted in a hemocytometer. At least 200 total cells were scored for each tube tested. The live cells were those which had excluded the dye. All serum samples were tested in duplicate with either lymphocytes from the host strain or normal serum serving as the negative control.

The data were reported as: $\% \text{ Killing} = \frac{\text{Number Dead Cells}}{\text{Total Number Cells}} \times 100.$

2. Embryo cytotoxicity assay

The rabbit, congenic and NIH antisera were tested for cytotoxicity against embryos by measuring the inhibition of ^3H -thymidine incorporation into DNA. Figure 2 outlines the procedure. One to five blastocysts or five to ten 8-cells were placed in 25 μl of Whitten and Biggers medium in the well of a 96-well flat bottom microtiter plate, then gassed for 10 to 15 minutes with 7% CO_2 in air. Twenty-five microliters of normal serum or antiserum, diluted with Whitten and Biggers medium, were added to designated wells. The congenic and NIH antisera were added to the microtiter wells at a 1:4 dilution, which seemed to give optimal inhibition of DNA synthesis. After a 0.5 to 5 hour incubation at 37°C and 7% CO_2 in air, 25 μl of guinea pig complement diluted 1:2 with Whitten and Biggers medium were added to all wells and the microtiter plate incubated for an additional hour. Then 75 μl of 8 $\mu\text{Ci/ml}$ of ^3H -thymidine ([methyl- ^3H]-thymidine, 20 Ci/mmole , aqueous solution, New England Nuclear, Boston, Massachusetts) were added to all wells, giving a final concentration of 4 $\mu\text{Ci/ml}$ (0.02 mole thymidine). After a five-hour incubation for blastocysts or a 24-hour incubation for 8-cells at 37°C and 7% CO_2 in air, the microtiter plate was cell harvested and the filters processed.

Due to day-to-day biological variability inherent to DNA synthesis by embryos, similar to that observed with RNA synthesis in embryos (Warner and Tollefson, 1978), each experiment was performed in conjunction with a control. The control was always done in duplicate, and the average cpm in the control was determined after the subtraction of the

Total Volume/Well	<u>Step 1:</u> <u>Short Preincubation of Embryos</u> (10 to 15 minutes at 37°C and 7% CO ₂ in air)
25 µl	25 µl culture medium Embryos
	<u>Step 2:</u> <u>Incubation with Antiserum</u> (1 hour incubation at 37°C and 7% CO ₂ in air)
50 µl	25 µl from Step 1 25 µl normal mouse serum <u>or</u> 25 µl antiserum
	<u>Step 3:</u> <u>Incubation with Complement</u> (1 hour incubation at 37°C and 7% CO ₂ in air)
75 µl	50 µl from Step 2 25 µl guinea pig complement (1:2)
	<u>Step 4:</u> <u>Incubation with ³H-thymidine</u> (5 hour or 24 hour incubation at 37°C and 7% CO ₂ in air)
150 µl	75 µl from Step 3 75 µl ³ H-thymidine (8 µCi/ml)
	<u>Step 5:</u> <u>Termination of Assay</u> (Place plate on ice for 5-10 minutes, then cell harvest and process filter papers)
150 µl	
	<u>Step 6:</u> <u>Counting of Samples</u> (Place filter papers in fluor and count in scintillation counter)
150 µl	

Figure 2. The embryo cytotoxicity assay

background cpm to give "Av. Control cpm." The experimental data (minus background cpm) were then expressed as the ratio:

$$\frac{\text{Experimental cpm}}{\text{Av. Control cpm}}$$

A value of 1.0 reflects no effect, whereas a value of 0.0 reflects complete blocking of ^3H -thymidine incorporation. The ratios were then averaged for a particular set of experiments and the significance of the difference of the mean from a value of 1.0 was determined by Student's t test (Steel and Torrie, 1960). The final results were then reported as:

$$\begin{aligned} \% \text{ Inhibition of } ^3\text{H}\text{-thymidine incorporation} = \\ 1.0 - \frac{\text{Exp. cpm}}{\text{Av. Control cpm}} \times 100. \end{aligned}$$

H. T Cell Growth Factor

1. T cell growth factor production

T cell growth factor (TCGF) was prepared according to the method of Rosenberg et al. (1978a). Spleens from DBA/2 mice were removed aseptically and teased apart in Hank's Balanced Salt Solution (HBSS), then pressed through a 40-gauge wire screen into a 50 ml disposable conical centrifuge tube (Fisher, Chicago, Illinois). The resulting suspension was centrifuged at 500 g and room temperature for five minutes. The supernatant was decanted and the cell pellet was suspended in 10 ml of buffered ammonium chloride solution at room temperature for two to five minutes to lyse the red blood cells. The cell suspension

was diluted to 50 ml with HBSS and was then centrifuged at 500 g and room temperature for five minutes. The supernatant was decanted and the cells were washed two more times. After the final wash, the cell pellet was suspended in supplemented RPMI 1640. A 0.1 ml aliquot was collected, diluted 1:50, and counted in 0.1% trypan blue. The cell suspension was diluted with supplemented RPMI 1640 to give 1×10^7 viable cells/ml then 2 ml of the cell suspension were dispensed per well of a 24-well tissue culture plate (Costar, Cambridge, Massachusetts). Twenty microliters of concanavalin A (Miles-Yeda, Israel) were added to each well to give a final concentration of 10 μ g/ml. The tissue culture plate was then incubated at 37°C and 7% CO₂ in air for 48 hours. The supernatant was removed from each well with a pasteur pipette and collected in a 50 ml conical centrifuge tube which was then centrifuged at 900 g and room temperature for 10 minutes. The supernatant was then passed through a 0.45 μ m filter (Millipore Corp., Bedford, Massachusetts), aliquotted and stored at -70°C until used. TCGF which was to be purified from concanavalin A and serum proteins was not filtered, aliquotted, nor frozen, but was stored at 4°C.

2. Partial purification of T cell growth factor

T cell growth factor was partially purified from concanavalin A and serum proteins according to the method of Rosenberg et al. (1980). Ammonium sulfate precipitation of TCGF supernatants was performed according to the method of Heide and Schwich (1978). Saturated ammonium sulfate was added dropwise to the TCGF supernatant with constant

stirring until 50% ammonium sulfate saturation was reached. The preparation was allowed to stand at room temperature for two hours and was then centrifuged (Beckman Model TJ-6, Irvine, California) at 500 g and room temperature for 30 minutes. The precipitate was resuspended in saline. The supernatant was decanted into the starting beaker and a second ammonium sulfate fractionation was performed by adding sufficient saturated ammonium sulfate to form a 70% saturated solution. The preparation was allowed to stand at room temperature for two hours and was then centrifuged at 500 g and room temperature for 30 minutes. The supernatant was discarded and the precipitate was resuspended in saline and pooled with the precipitate from the 50% saturated ammonium sulfate precipitation. The 10-15 ml of resuspended precipitate was then dialyzed at 4°C against HBSS for one to two days with five to six 0.5 to 1 liter changes of dialysate a day. The TCGF solution was then concentrated (Amicon, Danvers, Massachusetts) at 4°C with a >10,000 MW retension membrane and positive N₂ pressure to a 10 ml volume.

A 2 x 80 cm Sephadex G-100 gel filtration column was prepared and was equilibrated with 0.9% saline. A 10 ml solution containing three standard proteins--bovine serum albumin (MW 69,000), human chorionic gonadotrophin (MW 36,700) and ribonuclease A (MW 13,000) was loaded on the column and eluted in 6 ml fractions with 0.9% saline. A semi-logarithmic plot of elution fraction versus molecular weight yielded a straight line from which the fractions containing TCGF (MW 45,000 and 20,000-30,000 daltons) could be determined. The column was washed with

one to two bed volumes of saline, then the 10 ml TCGF sample was loaded onto the column and eluted in 6 ml fractions with 0.9% saline.

3. Assays for T cell growth factor activity

T cell growth factor activity was assayed by a procedure developed from the methods of Rosenberg et al. (1980) and Gillis et al. (1978) for TCGF activity analysis. B10.CBA_M cytotoxic T cells were harvested from a day 4 mixed lymphocyte reaction (details given later) culture plate, washed in HBSS, and suspended in supplemented RPMI 1640 at a concentration of 5×10^6 cells/ml. One hundred microliters (5×10^5) cytotoxic T cells were added to each well of a 96-well microtiter plate. Serial dilutions of unpurified TCGF were made and 1:4 dilutions of each Sephadex G-100 column fraction from the 15,000 to 60,000 molecular weight range were made. One hundred microliters of a TCGF dilution were added to the designated wells of the microtiter plate. Cytotoxic cells only and cytotoxic cells plus concanavalin A (10 μ g/ml) served as negative and positive controls, respectively. The microtiter plate was incubated at 37°C and 7% CO₂ in air for 24 hours. The 2 μ Ci of ³H-thymidine ([methyl-³H]-thymidine, 6.7 Ci/mmole, aqueous solution, New England Nuclear, Boston, Massachusetts) in 50 μ l of medium were added to each well of the microtiter plate which was then reincubated at 37°C and 7% CO₂ in air for 18 hours. The microtiter plate was processed with a cell harvester and the filters were counted in toluene ~~fluo~~r in a scintillation counter.

4. Protein concentration determination

Protein concentrations in each Sephadex G-100 column fraction were determined by measuring absorbance at 280 nm with a Gilford spectrophotometer (Oberlin, Ohio) and by performing a Lowry test (Lowry et al., 1951). Samples, standards, and blanks were prepared to give a final volume of 1.2 ml of solution, then 3 ml of one part 2.68% Na tartrate plus one part 1% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ plus 100 parts of 2% Na_2CO_3 in 0.1 N NaOH were added to samples, standards, and blanks. The test tubes were vortexed, then were allowed to stand for 10 minutes. Folin reagent (0.3 ml of 1N) was added to all tubes, which were then mixed and were allowed to stand for 30 minutes before absorbance was read at 750 nm. Protein concentrations of samples were then extrapolated from a BSA standard curve.

I. Cell Interaction Assays

1. Mixed lymphocyte reaction (MLR)

a. Generation of cytotoxic T cells to H-Y Cytotoxic T cells were produced, based on the methods of Gordon et al. (1975) and Simpson et al. (1975). C57BL/6 female mice were each injected with 1×10^7 C57BL/6 male spleen cells. Fourteen days later, the female mice were sacrificed and their spleens were aseptically removed and teased apart into HBSS. The spleen suspension was then passed through a 40-gauge stainless steel screen. The suspension was centrifuged (Beckman Model TJ-6, Irvine, California) at 100 g and room temperature for five minutes. The supernatant was decanted and 10 ml of buffered ammonium chloride was

added. The cell suspension was set at room temperature for two to five minutes, then diluted to 50 ml with HBSS and centrifuged. The cells were washed with HBSS two more times, then suspended in 10 ml of supplemented RPMI 1640. An aliquot was collected for a cell count in 0.1% trypan blue. The cell concentration was adjusted to 5×10^6 cells/ml.

C57BL/6 male spleen lymphocytes were collected and isolated in the same way as the female spleen lymphocytes. Stimulator cells were prepared as follows. Half of the male cells and half of the female cells were each treated with mitomycin C at a concentration of 25 $\mu\text{g/ml}$ for 30 minutes at 37°C and 7% CO_2 in air, according to the procedure of Bach and Voynow (1966). After two HBSS washes, the cells were suspended in supplemented RPMI 1640 medium and an aliquot was counted in 0.1% trypan blue. The cell suspensions were adjusted to 5×10^6 cells/ml.

A MLR monitoring 96-well microtiter plate was set up with 0.1 ml of responder cells and 0.1 ml of stimulator cells in designated wells. Responder cells only and stimulator cells only wells were set up as controls. The remaining cell suspensions were added as 1 ml of responder cells and 1 ml of stimulator cells to each well of a 24-well tissue culture plate. Both plates were incubated at 37°C and 7% CO_2 in air. On day 1 of the MLR, 20 μl of a 0.01% PHA-P (Difco, Detroit, Michigan) solution or 20 μl of Concanavalin A (Miles-Yeda, Israel) (1000 $\mu\text{g/ml}$) were added to designated control wells of the MLR monitoring plate. On day 4 of the MLR, 2 μCi of ^3H -thymidine ([methyl- ^3H]-thymidine, 6.7 Ci/mole, New England Nuclear, Boston, Massachusetts) in 50 μl of

media were added to all wells of the MLR monitoring plate. Eighteen to twenty-four hours later, the monitoring plate was cell harvested and the filters were processed. The cytotoxic cells generated in the 24-well tissue culture plate were then collected and tested for activity in a cell mediated lympholysis reaction.

b. Generation of cytotoxic T cells to H-2 Cytotoxic T cells to H-2 were generated according to the method of Rosenberg et al. (1978b). Spleen lymphocyte suspensions were prepared as described for the generation of cytotoxic T cells to H-Y. Stimulator cells were treated with mitomycin C and washed twice. Responder cells were used at 7×10^6 cells/ml and stimulator cells were used at 3×10^6 cells/ml. A MLR monitoring plate was set up with 0.1 ml of stimulator cells (3×10^5) and 0.1 ml of responder cells (7×10^5) in each well. The remaining cell suspensions were added to a 24-well tissue culture plate as 1 ml of responder cells to 1 ml of stimulator cells. The plates were incubated at 37°C and 7% CO₂ in air for four days. The monitoring plate was treated as described previously and harvested on day 5.

A secondary stimulation of cytotoxic cells was performed by collecting the responder cells and adjusting the cell concentration to 4×10^6 responder cells/ml, then adding one ml of 1×10^6 cells/ml of freshly treated mitomycin C stimulator cells and one ml of responder cells to give a final volume of two ml per well. A tertiary stimulation was performed as described for the secondary stimulation.

Cells were grown in the presence of T cell growth factor (TCGF) four days after each stimulation and for six to nine days before the

next stimulation was performed. After the tertiary stimulation, cells were grown in the presence of TCGF and counted every four to five days. Cells were diluted with fresh medium containing TCGF and were plated at 2×10^5 cells per well. The responder cells were tested periodically against lymphocytes in a micro-cell-mediated lympholysis assay or against embryos in a mixed embryo-lymphocyte interaction assay as described in the next sections.

2. Cell-mediated lympholysis (CML)

a. Assaying for H-Y cytotoxic effector cells The detection of H-Y cytotoxic effector cells was performed according to the procedures of Gordon et al. (1975) and Simpson et al. (1975). Sensitized cells were collected from 24-well tissue culture plates and centrifuged at 125 g and room temperature for 10 minutes. The supernatant was decanted and the cells were washed with HBSS, then resuspended in a small volume of culture medium. The cells were counted in a hemacytometer and the concentration was adjusted to 2×10^6 cells/ml. Several serial dilutions were made. Control cell suspensions of unsensitized cells at the same concentration as the sensitized cells were prepared from fresh spleen suspensions treated with the ammonium chloride buffer, then washed three times in HBSS. Medium controls of target cells incubated with medium alone were included in each experiment.

Target cells consisted of mitogen-induced blasts which were prepared by incubating 10 ml of C57BL/6 spleen cells at a concentration of 4×10^6 cells/ml in 30 ml Falcon flasks (Fisher, Chicago, Illinois).

Concanavalin A was added to each flask of cells to yield a final concentration of 10 $\mu\text{g/ml}$. The cells were incubated at 37°C and 7% CO_2 in air for 48 to 72 hours. The cells were then collected and washed once with HBSS. The cells were resuspended in culture medium at a concentration of 3×10^7 cells/ml. The cells were labeled with 250 μCi of ^{51}Cr sodium chromate $\text{Na}[^{51}\text{Cr}]$ (New England Nuclear, 200-500 $\mu\text{Ci/g}$, Boston Massachusetts) per ml of cell suspension. Labeling was performed at 37°C and 7% CO_2 in air for 45 minutes with frequent agitation of the cell suspension. The cell suspension was centrifuged at 125 g and room temperature for 10 minutes. The supernatant was decanted and the cells were washed two times with HBSS. The cells were suspended in culture medium at a concentration of 2×10^6 cells/ml.

The assay was performed in 96-well round bottom tissue culture plates (Linbro: Flow Labs, McLean, Virginia) with three to four replicates. Fifty μl of target cells (1×10^5 cells) were placed in designated wells, then 200 μl of the effector cell suspensions were placed in the appropriate wells. The plate was centrifuged at 40 g and 4°C for five minutes in an International portable refrigerated centrifuge Model PR-2 (Boston, Massachusetts), then was incubated for four hours at 37°C and 7% CO_2 in air. After the incubation period, the plate was centrifuged (International Model PR-2, Boston, Massachusetts) for 20 minutes at 200 g and 4°C. One hundred μl of supernatant was removed from each well and was dispensed in 12 x 75 mm disposable culture tubes containing 200 μl of medium. The tubes were then counted in a Tracor Analytic Automatic γ -counter Model 1197 (Elk Grove Village, Illinois).

Maximum lysis was obtained by adding 200 μ l of 0.1 N HCl to wells containing 50 μ l of ^{51}Cr -labeled target cells.

Results were expressed as:

% Corrected lysis =

$$100 \times \frac{\text{Counts released by experiment} - \text{counts released by control}}{\text{Maximum released counts} - \text{medium background counts}}$$

b. Assaying for H-2 cytotoxic effector cells H-2 cytotoxic

cells were detected by the micromethod of Traill et al. (1981). Sensitized effector cells were pooled, washed once with HBSS, then resuspended in culture medium at a concentration of 8×10^6 cells/ml. A microtest Terasaki plate (Fisher, Chicago, Illinois) was prepared for the assay by using a hot needle to perforate the periphery of the lid with eight holes to allow for maximum gas exchange. The outer wells of the plate were filled with 25 μ l of PBS or culture medium. Various concentrations of sensitized effector cells were dispensed in 20 μ l volumes into designated wells of the Terasaki plate. Target cells were prepared as described for the H-Y CML assay, then 1×10^4 cells were dispensed in a 5 μ l volume into designated wells. The Terasaki plate was covered, inverted, and placed on a damp paper towel and then was incubated at 37°C and 7% CO_2 in air. After four hours, the contents of each well were transferred to 185 μ l of medium in a well of a round-bottom microtiter plate (Linbro: Flow Labs, McLean, Virginia). Each Terasaki well was washed with 50 μ l of medium and then was dispensed into the appropriate well of the round-bottom microtiter plate. The

plate was centrifuged at 125 g and 4°C for 30 minutes, then 100 µl of supernatant was removed and was dispensed into 12 x 75 mm disposable culture tubes, containing 200 µl of culture medium. Chromium release measurements were made in a manual Nuclear Chicago γ-counter (Des Plaines, Illinois). Maximum release was determined by adding 20 µl of 0.1 N HCl to 5 µl of target cells. Results were expressed as

% Specific release =

$$100 \times \frac{\text{cpm of experimental release} - \text{cpm of spontaneous release}}{\text{cpm of maximum release} - \text{cpm of spontaneous release}}$$

3. Mixed embryo lymphocyte interaction assay (MELIA)

The mixed embryo lymphocyte assay was developed utilizing the micro-cell mediated lympholysis procedure of Traill et al. (1981). Embryos of the blastocyst stage of development were collected from superovulated mice and washed three times in Whitten and Biggers medium. The embryos were pronased, then washed three times and incubated at 37°C and 7% CO₂ in air in about 0.5 ml of Whitten and Biggers medium for two to three hours. Cytotoxic T cells were collected from day 5 mixed lymphocyte reaction plates or from long-term T cell growth factor maintained cytotoxic T cells. The cell concentrations were determined with the aid of a hemacytometer. Cell suspensions were centrifuged at 125 g and room temperature for 5-10 minutes. The supernatant was decanted and the cells were washed with HBSS, then brought up to the desired concentration with supplemented RPMI 1640. Various cell concentrations of cytotoxic T cells in 20 µl volumes were dispensed into designated

wells of microtest Terasaki plates (Falcon Plastics, Oxnard, California). One embryo was dispensed into each designated well of the Terasaki plate. All wells were brought to a final volume of 25 μ l by adding 5 μ l of culture medium. The lid of the Terasaki plate was perforated with a hot needle to form several holes around the periphery to allow for gas exchange. The Terasaki plate was then covered, inverted, and placed on a damp paper towel on a small tray. The tray was placed in a 37°C and 7% CO₂ in air incubator for four hours. The plate was then examined under a dissecting microscope and the embryos were removed with a micro-pipette and washed three times in Whitten and Biggers medium. Individual embryos were then assayed for viability in the embryo DNA synthesis assay. Briefly, one embryo was placed in 75 μ l of Whitten and Biggers medium in the well of a 96-well flat-bottom microtiter plate (Linbro: Flow Labs, McLean, Virginia). After a 5-10 minute CO₂ gassing period, 75 μ l of ³H-thymidine (8 μ Ci/ml) were added to all wells and at least two background wells containing no embryos. The plate was incubated for five hours. The embryonic DNA was collected with a cell harvester. The filters were processed and counted in a liquid scintillation counter.

III. RESULTS

A. DNA Synthesis Assay for Embryos

1. Development of the DNA synthesis assay

Initial experiments were designed to optimize the parameters of the DNA synthesis assay. Outbred CF1 embryos were used for these experiments. Figure 3 shows that the optimal concentration of ^3H -thymidine is 2-8 $\mu\text{Ci/ml}$ for blastocysts and 4-125 $\mu\text{Ci/ml}$ for 8-cell embryos when the embryos are incubated for seven hours. The low incorporation of label at high specific activities by blastocysts may be caused by radiation effects (Snow, 1973). Based on these experiments, 4 $\mu\text{Ci/ml}$ was chosen as the concentration of ^3H -thymidine to use in the next experiments.

Figures 4a and 4b show time course studies of the incorporation of ^3H -thymidine into blastocyst (Figure 4a) and 8-cell (Figure 4b) embryos. In both cases, incorporation is linear up to seven hours and then slows down. Therefore, a three-to-five hour ^3H -thymidine labeling time was used for most experiments.

The effect of preincubation at 37°C and 7% CO_2 in air was tested on the subsequent incorporation of ^3H -thymidine. Figure 5 shows that there is little effect of preincubation on 8-cell embryos prior to labeling with ^3H -thymidine. However, blastocysts show a marked increase and then a decrease of the incorporated counts. The decrease may be due to the lack of cell division by late blastocysts in Whitten and Biggers medium. The reason for the initial lag in incorporation is unknown.

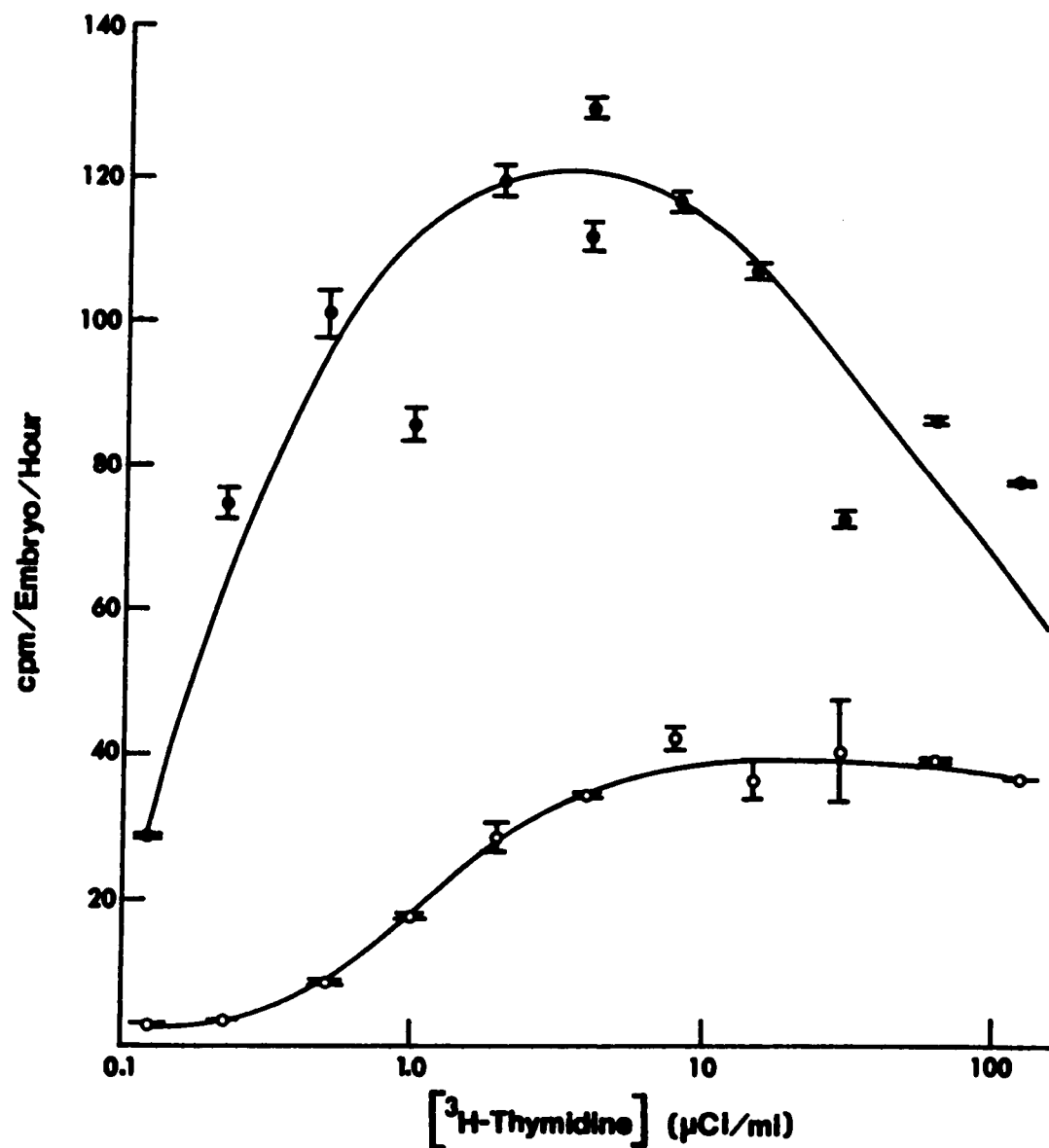


Figure 3. Cpm incorporated into DNA by blastocyst (●) and 8-cell (○) mouse embryos as a function of the concentration of ³H-thymidine. A seven-hour labeling period was used for each experiment

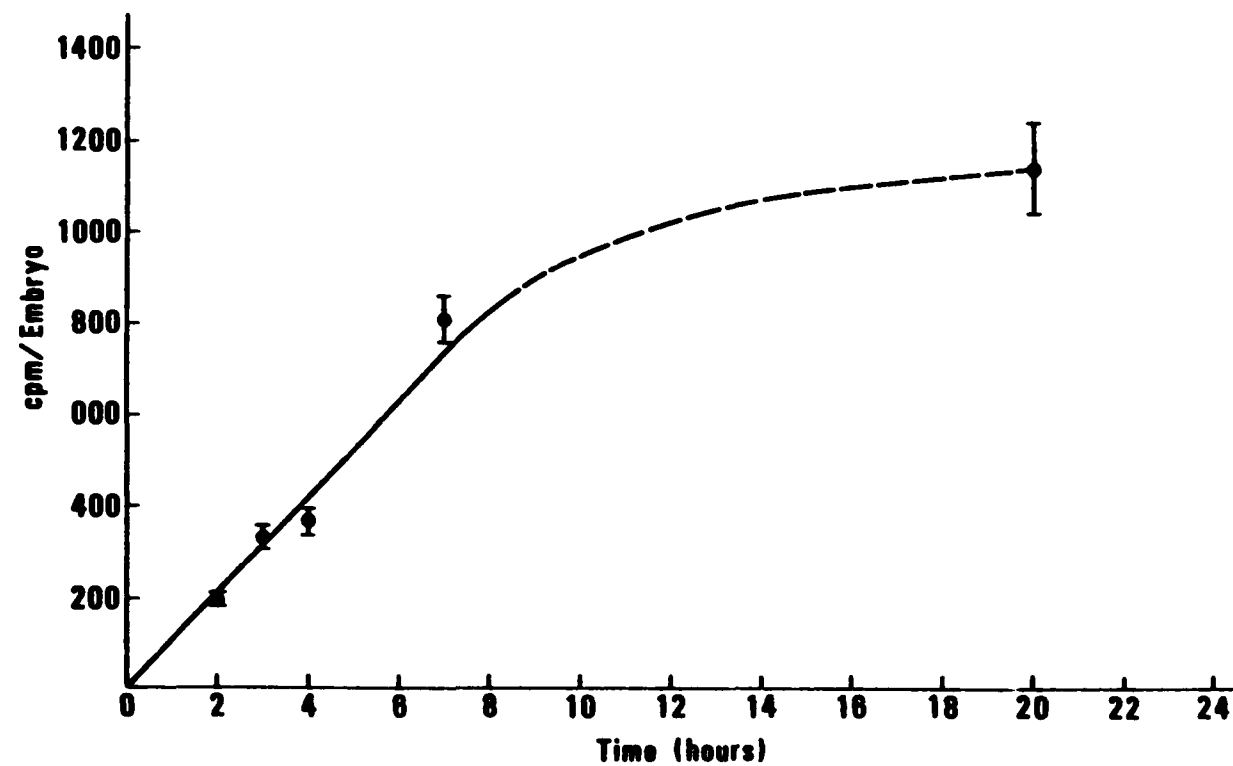


Figure 4a. Cpm incorporated into DNA by blastocyst mouse embryos as a function of time in 4 $\mu\text{Ci/ml}$ ^3H -thymidine

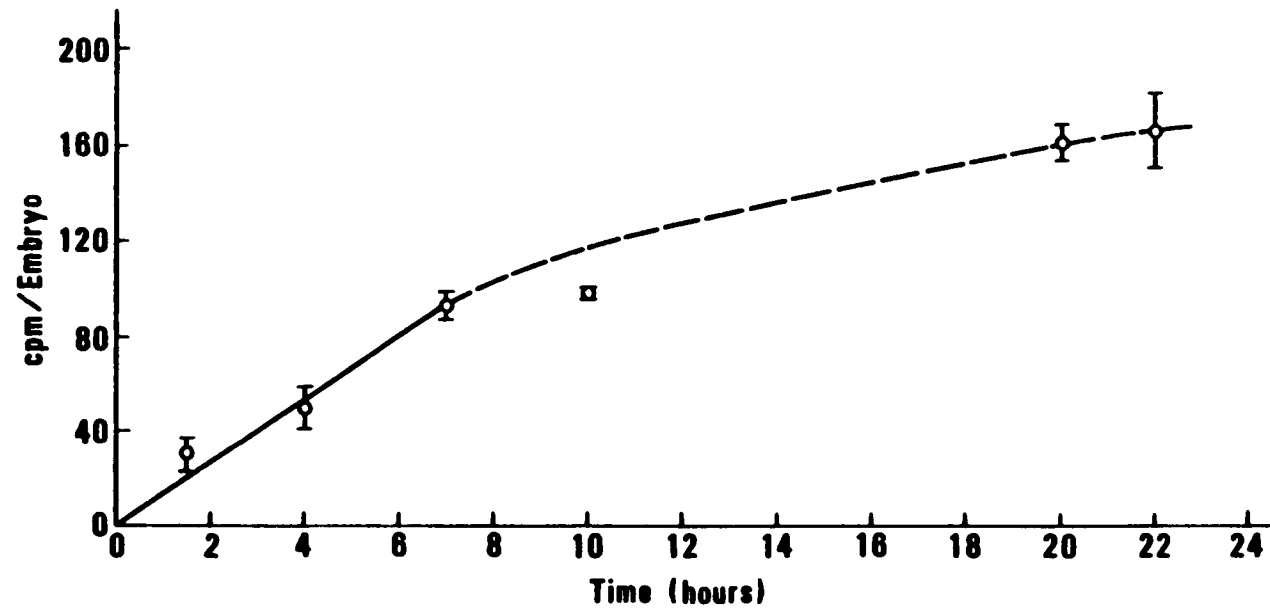


Figure 4b. Cpm incorporated into DNA by 8-cell mouse embryos as a function of time in $4 \mu\text{Ci/ml } ^3\text{H}$ -thymidine

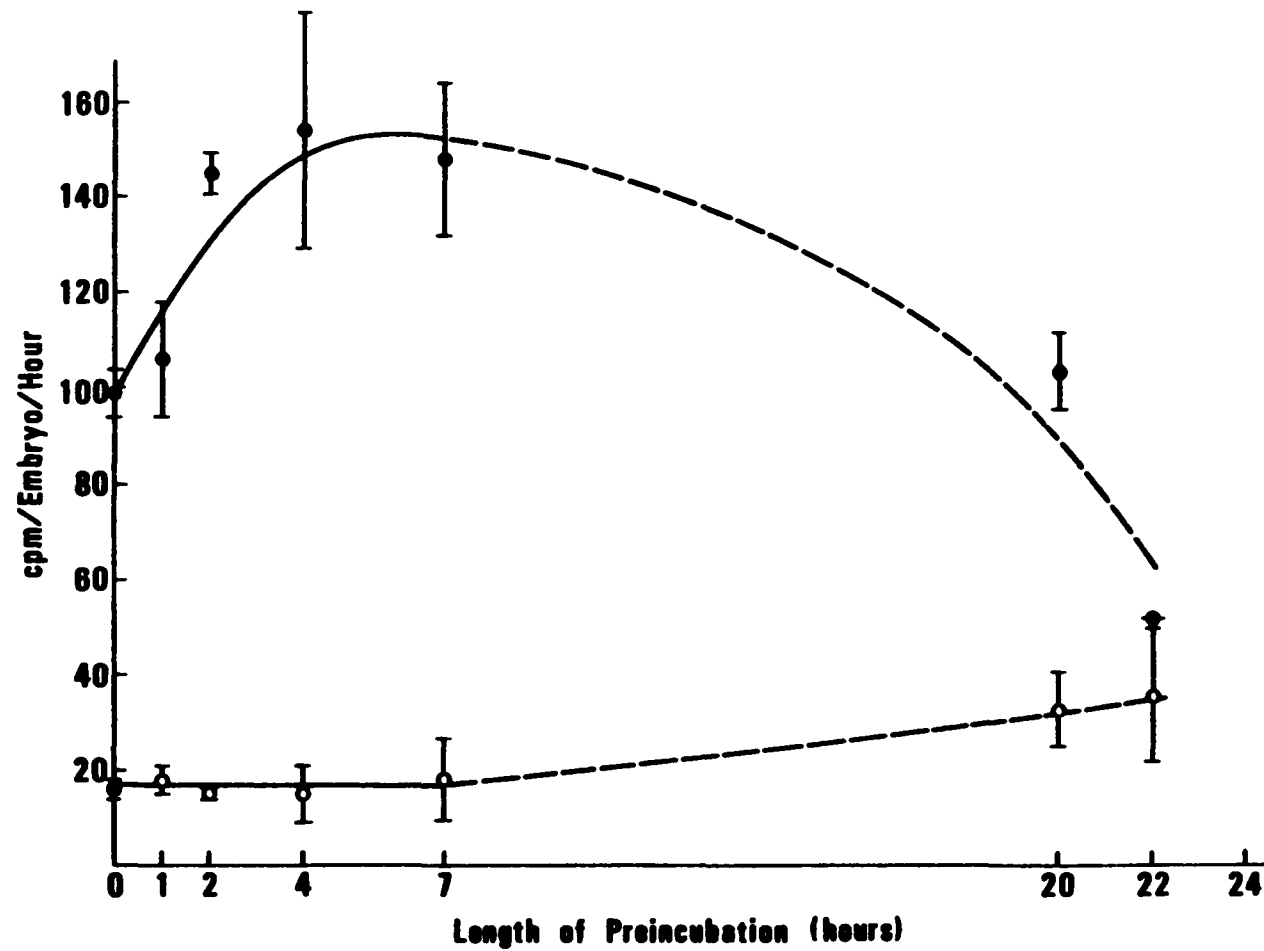


Figure 5. The effect of preincubation on the incorporation of ^3H -thymidine into DNA by blastocyst (●) and 8-cell (○) mouse embryos. Embryos were incubated with $4\text{ }\mu\text{Ci/ml}$ of ^3H -thymidine for three hours after the designated preincubation period

Perhaps the multicellular blastocyst embryo requires 3 to 4 hours to acclimate to in vitro conditions.

To examine whether thymidine pools were saturated in each embryo with 4 $\mu\text{Ci/ml}$ (sp. act. 20 Ci/mole) of ^3H -thymidine, the relationship between label incorporation and the number of embryos present in 50 μl of ^3H -thymidine labeled Whitten and Biggers medium was examined. For both blastocyst (Figure 6a) and 8-cell (Figure 6b) embryos incorporation was linear up to 64 embryos in 50 μl of labeled medium. It should be noted that at the blastocyst stage, individual embryos are easily assayed using this technique. Each CF1 blastocyst incorporates about 200 cpm per embryo per hour with a background of only 20-30 cpm in each well, containing medium and radioactivity, but no embryos.

The presence or absence of the zona pellucida appears to slightly affect ^3H -thymidine incorporation by blastocyst embryos. The optimal concentration of ^3H -thymidine is about the same for blastocyst embryos with and without the zona pellucida (compare Figure 3 and Figure 7a). For 8-cell mouse embryos (Figure 7b) the absence of the zona pellucida appears to shift the ^3H -thymidine concentration for optimal incorporation to the right (see Figure 3). The reason for the shift is not known.

The effect of thymidine incorporation involving two different specific activities of ^3H -thymidine was examined on blastocyst embryos. In Figure 7a, ^3H -thymidine of specific activity 20 Ci/mole was used, while in Figure 8, ^3H -thymidine of specific activity 52 Ci/mole was used. Increasing the specific activity of the ^3H -thymidine increased

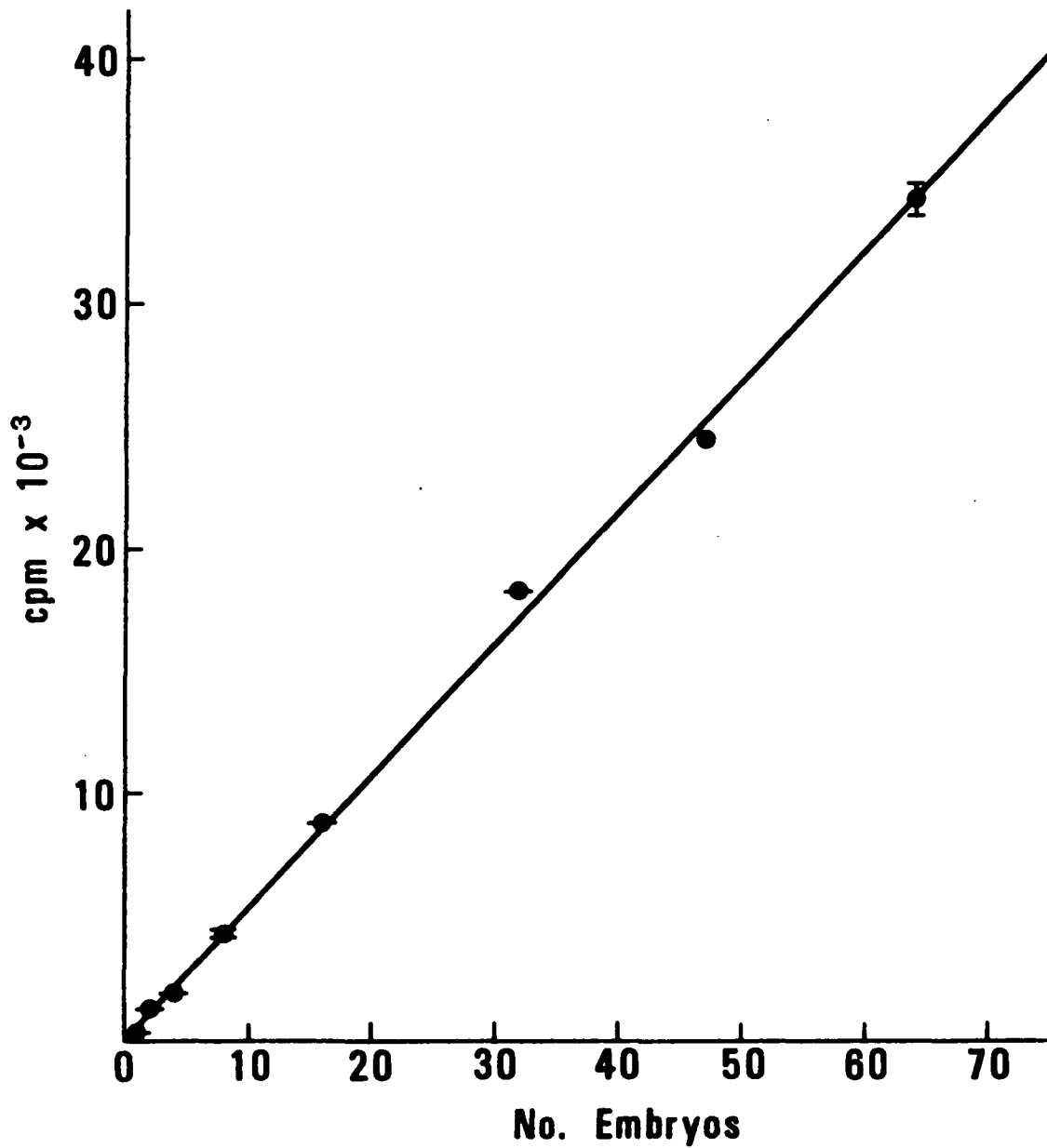


Figure 6a. Cpm incorporated into DNA by varying numbers of blastocyst (•) mouse embryos preincubated for three hours without label and then for three hours in 4 $\mu\text{Ci/ml}$ ^3H -thymidine. The equation for the least squares linear regression line through the points is $y = 536x + 100$; the correlation coefficient, $r = 0.99$

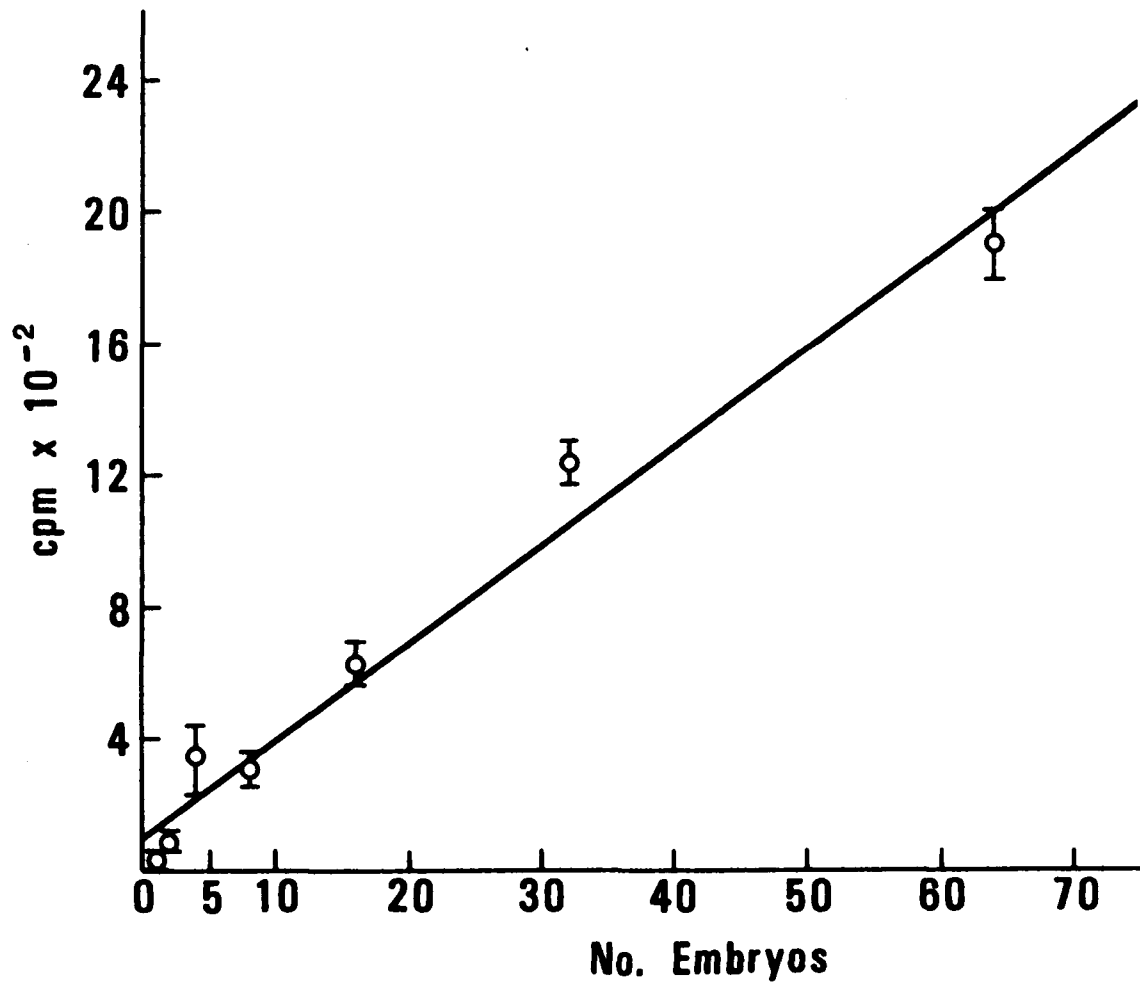


Figure 6b. Cpm incorporated into DNA by varying numbers of 8-cell (●) mouse embryos preincubated for three hours without label and then three hours in 4 μ Ci/ml 3 H-thymidine. The equation for the least squares linear regression line through the points is $y = 65x + 94$; the correlation coefficient, $r = 0.99$

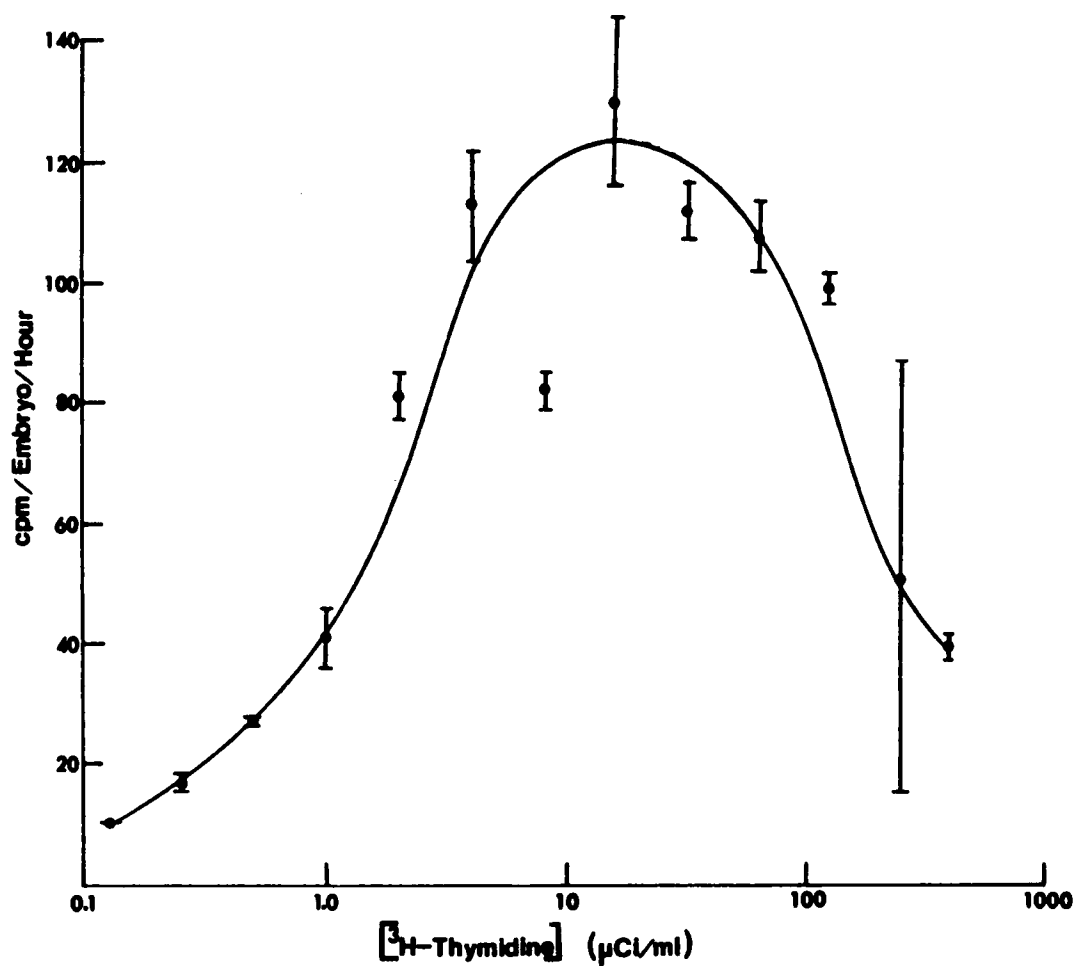


Figure 7a. Cpm incorporated into DNA by blastocyst mouse embryos, which have had their zona pellucida removed by pronase digestion, as a function of concentration of ³H-thymidine. A seven-hour labeling period was used. ³H-thymidine was of specific activity 20 Ci/mmole

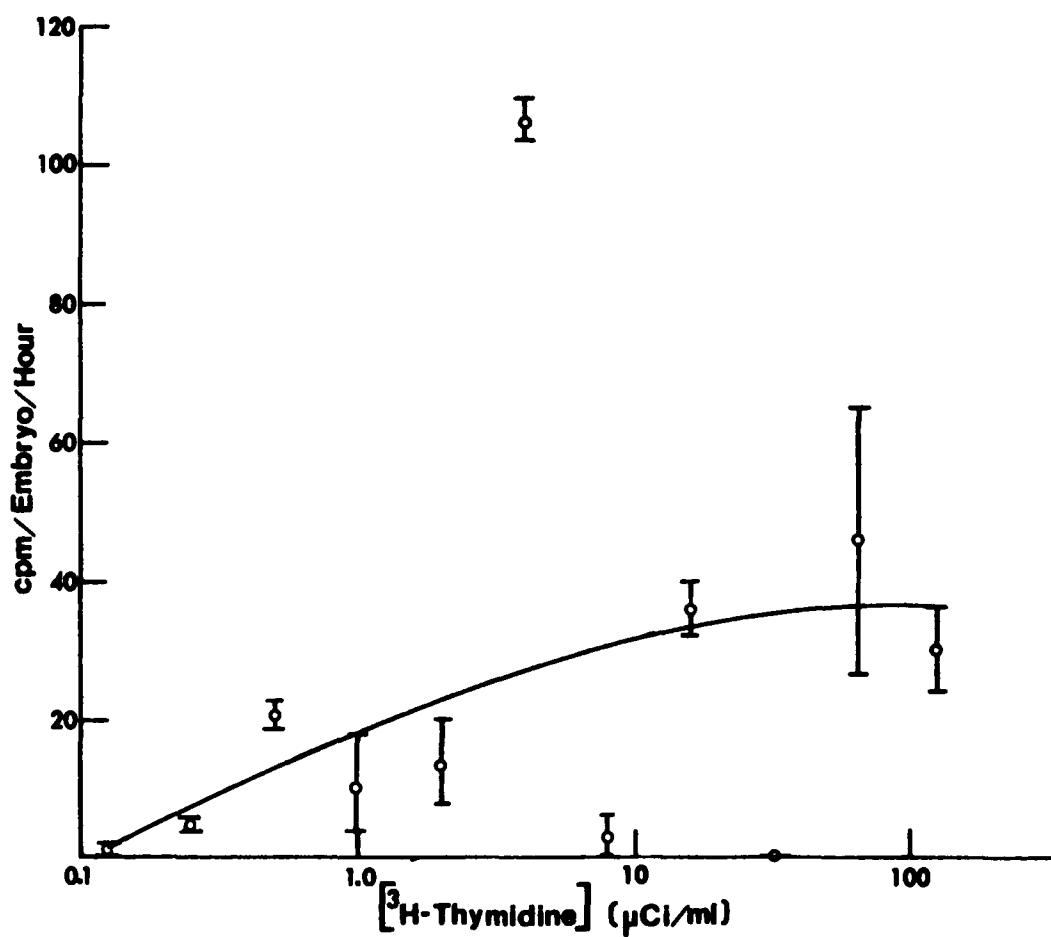


Figure 7b. Cpm incorporated into DNA by 8-cell mouse embryos, which have had their zona pellucida removed by pronase digestion, as a function of concentration of ³H-thymidine. A seven-hour labeling period was used

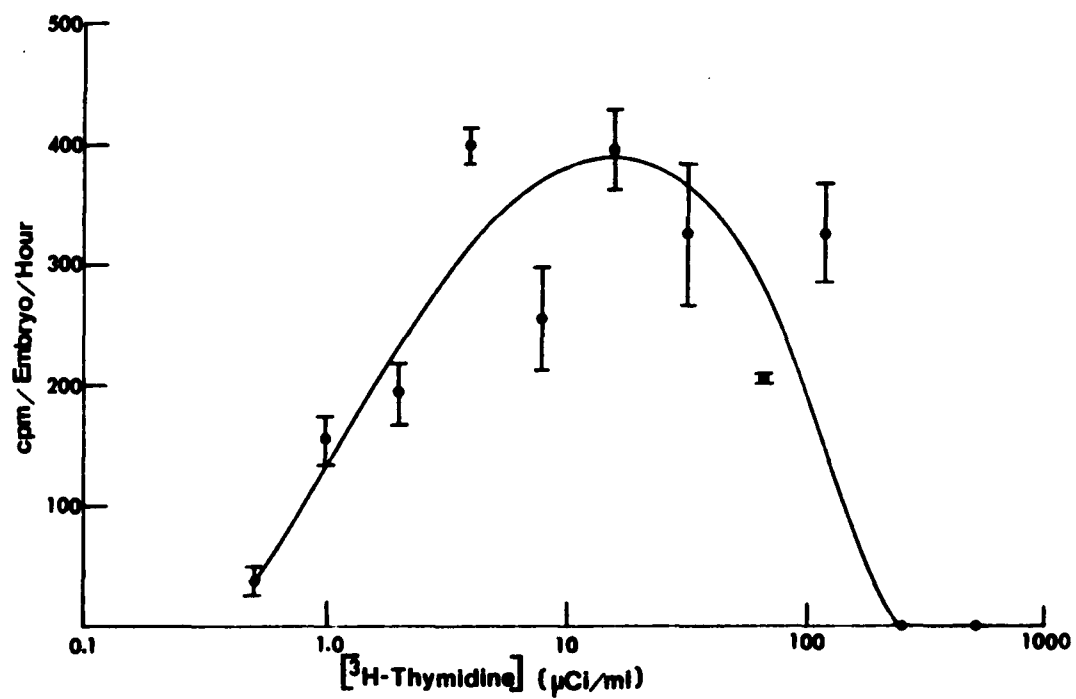


Figure 8. Cpm into DNA by blastocyst embryos, which have had their zona pellucida removed by pronase digestion, as a function of concentration of ³H-thymidine. A seven-hour labeling period was used. The ³H-thymidine was of specific activity 52 Ci/mmole

the total cpm's incorporated per embryo per hour as expected. The incorporation curves follow essentially the same pattern, regardless of specific activity.

In summary, conditions for the DNA synthesis assay were optimized as follows: (1) 4 μ Ci/ml 3 H-thymidine (sp. act. 20 Ci/~~mm~~mole); (2) a labeling period from two to seven hours; (3) a three-hour preincubation period for blastocyst embryos and from zero to seven hours preincubation for 8-cell embryos; (4) 1-64 embryos per assay well; (5) absence of the zona pellucida does not significantly affect incorporation of 3 H-thymidine for blastocyst embryos or 8-cell embryos and, therefore, the zona pellucida was not removed for most experiments; and (6) increasing the specific activity of 3 H-thymidine can be used to increase the total number of cpm's incorporated per blastocyst embryo per hour. However, because of increased background counts with the higher specific activity 3 H-TdR, at least five blastocysts had to be assayed in the same well.

2. Verification of 3 H-thymidine incorporation into the embryonic DNA

Two experiments were performed to prove that 3 H-thymidine was incorporated into the embryonic DNA. First, the effect of mitomycin C, a known inhibitor of DNA synthesis, was examined on CF1 blastocyst embryos. The results, as shown in Table 6, demonstrate almost 100% inhibition of 3 H-thymidine incorporation by a 300 μ g/ml mitomycin C treatment of blastocyst embryos prior to the 3 H-thymidine labeling period.

In the second experiment, the effect of DNase I on embryonic DNA was examined. Figure 9 demonstrates the activity of DNase I in

Table 6. Effect of mitomycin C on CF1 blastocyst embryos. Blastocysts were incubated with mitomycin C for one hour then were incubated with 4 μ Ci/ml of 3 H-thymidine for seven hours

Mitomycin C (μ g/ml)	Number of Embryos Tested	Mean cpm per embryo	Percent Inhibition 3 H-thymidine Incorporation \pm SEM
0	4	898	0
100	4	196	78 \pm 6
200	4	74	92 \pm 3
300	4	26	97 \pm 1

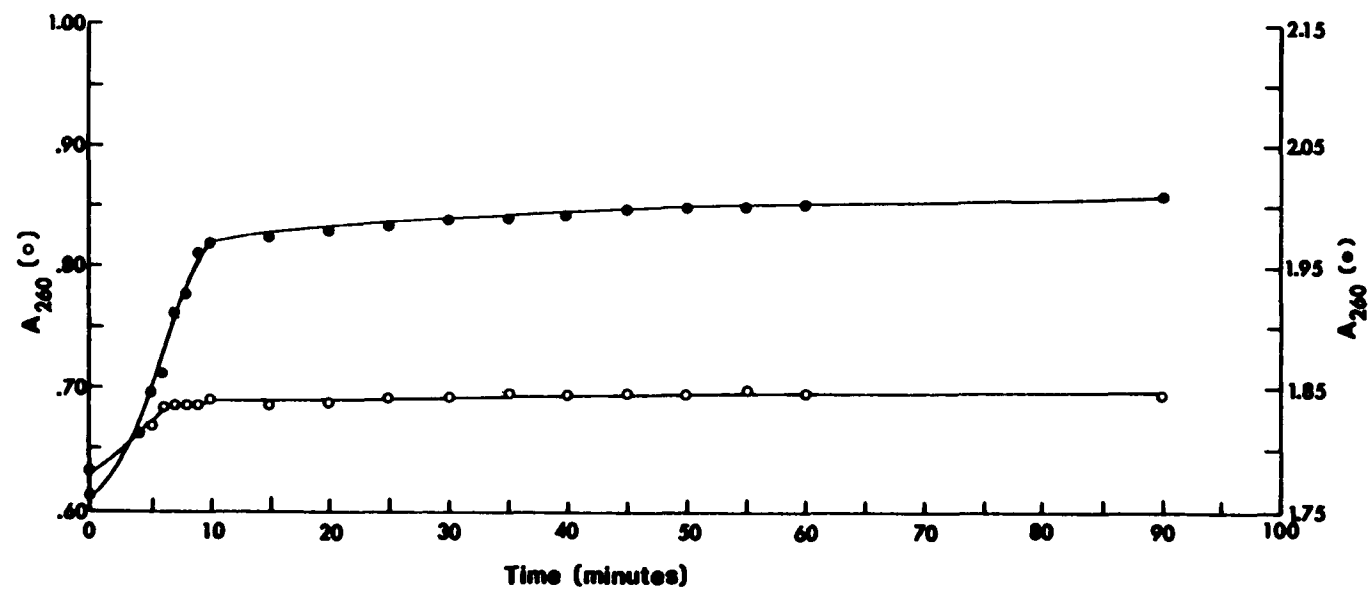


Figure 9. Spectrophotometric measurement of DNase I activity in an acetate buffered substrate solution (●) and in acidified Whitten and Biggers medium (○) containing an equivalent amount of deoxyribonuclease

acidified Whitten and Biggers medium and also in an acetate buffered substrate solution. It was necessary to acidify Whitten and Biggers medium to pH = 5 in order to optimize the activity of DNase I on blastocyst embryos. The specific activity of DNase I in acidified Whitten and Biggers medium was reduced to 4 units/mg as opposed to 12 units/mg for the acetate buffered substrate solution. DNase I was then tested on blastocyst embryos in acidified Whitten and Biggers medium. The results are shown in Table 7. It can be seen that DNase I and Triton X-100, together, greatly decrease incorporated counts. Apparently, Triton X-100 breaks open cell membranes and exposes the embryonic DNA to DNase I which then degrades the labeled DNA into pieces which are too small to be trapped on the glass filter mat during the cell harvesting procedure.

3. ³H-thymidine incorporation by 47, 53, 56, 65, and 89 hour post- hCG embryos

The ability of preimplantation stages of CFl embryos to incorporate 4 μ Ci/ml of ³H-thymidine was examined. Table 8 lists the stages of CFl preimplantation mouse development that were examined. The results are shown in Figure 10. Incorporation of ³H-thymidine per cell per hour appears to increase for the later stages of development (i.e., blastocysts). The most probable explanation is that more cells in any given embryo are labeled at later stages of development. The reason for this is that the cell cycle decreases from 24 to 12 hours during development (McLaren, 1972), but the length of the S phase remains the same (six to seven hours). Thus, during a three-seven hour labeling period, the

Table 7. Verification of ³H-thymidine incorporation into the DNA of blastocyst embryos treated with DNase I and/or Triton X-100

Experiment	DNase I (10 µg/ml)	Triton X-100 (1%)	Number of Embryos Tested	Mean cpm per Embryo	Percent of Control + SEM
No. 1 (Control)	-	-	15	434	100
No. 2	-	+	15	324	75+10
No. 3	+	-	15	284	66+12
No. 4	+	+	15	26	6+2

Table 8. Stage of preimplantation mouse development relative to hours post-hCG injection for CF1 embryos

Hours Post-hCG	Stage of Development	Number of Cells per Embryo \pm SEM ^a	cpm per Embryo \pm SEM	cpm per cell per hour \pm SEM ^b
47	2 cells	2.0 \pm 0.0	3 \pm 1	0.5 \pm 0.1
53	3-4 cells	3.6 \pm 0.1	28 \pm 0	2.6 \pm 0.1
56	4 cells	4.0 \pm 0.1	25 \pm 3	2.1 \pm 0.3
65	6-8 cells	6.8 \pm 1.0	60 \pm 3	2.9 \pm 0.2
89	blastocyst	44.4 \pm 2.2	1159 \pm 56	8.7 \pm 0.5

^aVerbanac and Goldbard, unpublished results.

^bThe result of the embryos' ability to incorporate 4 μ Ci/ml of ³H-thymidine in a DNA synthesis assay.

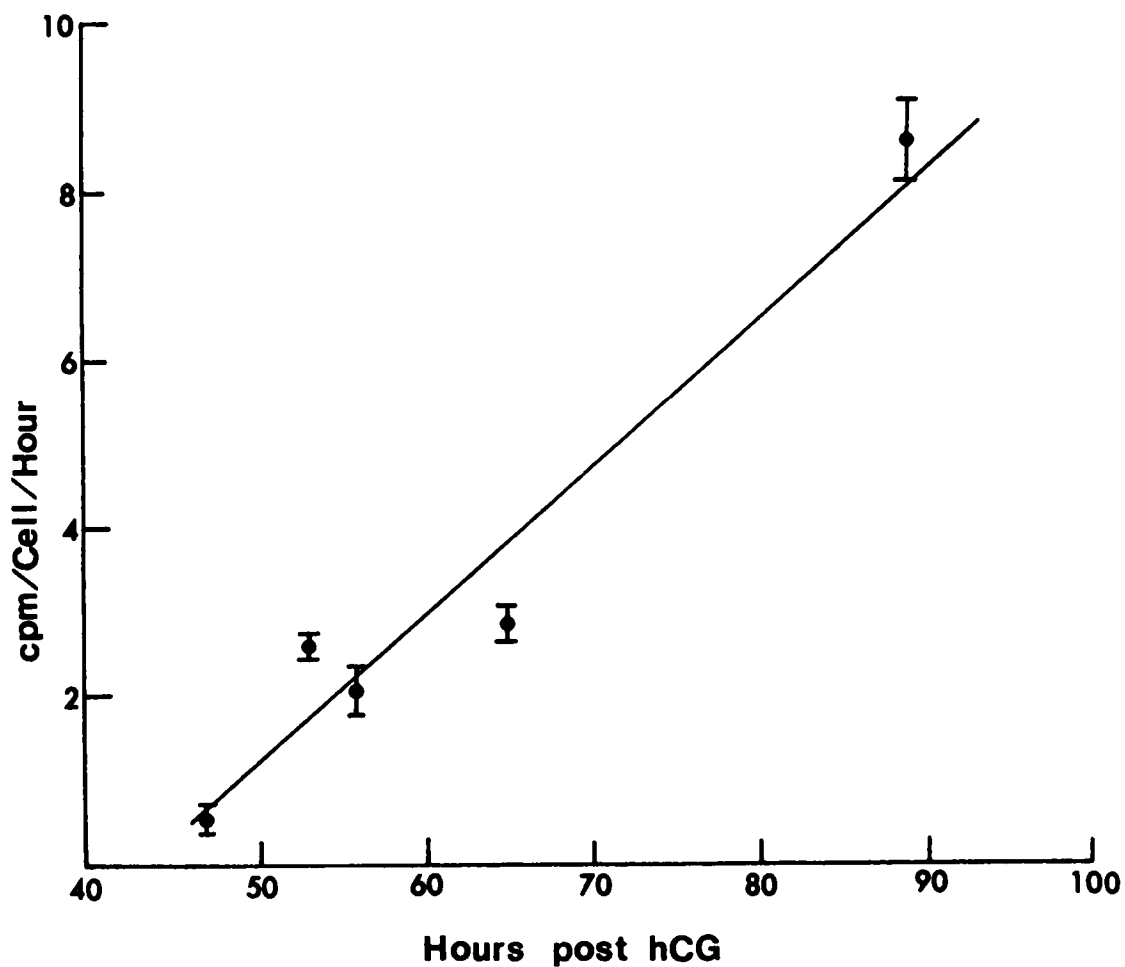


Figure 10. Cpm incorporated into DNA per cell per hour of CF1 embryos at 47, 53, 56, 65, and 89 hours post-hCG injection. Embryos from each stage of development were preincubated three hours prior to a three-hour labeling period with 4 $\mu\text{Ci/ml}$ of ^3H -thymidine. The equation for the least squares linear regression line through the points is $y = 0.2x - 8.0$; the correlation coefficient, $r = 0.97$

shorter the cell cycle, the more likely that the cells will pass through the S phase during the labeling period. Fifty to sixty embryos from the 47, 53 and 56 hours post-hCG stages of development were used per assay in order to obtain ^3H -thymidine incorporation that was above background counts.

4. ^3H -thymidine incorporation by the inner cell mass

The ability of blastocyst inner cell masses (ICM) to incorporate ^3H -thymidine was examined. Immunosurgery was performed on CFl blastocyst embryos according to the method of Solter and Knowles (1975). The results of a 7-hour labeling period and a 24-hour labeling period with ^3H -thymidine are shown in Table 9. It can be seen that ICMs incorporate less than 10% of the total counts incorporated by the intact blastocyst. These results were unexpected in light of studies by Barlow et al. (1972) who demonstrated a higher ^3H -thymidine labeling index for "inside" cells as opposed to "outside" cells of preimplantation mouse embryos based on grain counts. They concluded that the "inside" cells were dividing faster than the "outside" cells. The blastocyst embryo exhibits "inside" cells (i.e., ICM) and "outside" cells (i.e., trophectoderm). It could be that separation of the ICM from the trophectoderm impairs DNA synthesis in the ICM.

5. ^3H -thymidine incorporation as a measure of number of cells per embryo

An experiment was performed to test whether the DNA synthesis assay for embryos could be used as a measure of the number of cells (i.e.,

Table 9. ^3H -thymidine incorporation by CF1 Inner Cell Mass (ICM)

Embryo	Embryo/Well	cpm/Embryo	Mean cpm/Embryo ± SEM	$\frac{\text{ICM}}{\text{ICM} + \text{Trophectoderm}} \times 100$
A. Seven hour incubation with ³ H-thymidine				
ICM + trophectoderm	1	1780	2166±273	
	1	2551		
ICM	1	81	101± 14	5%
	1	120		
B. Twenty-four hour incubation with ³ H-thymidine				
ICM + trophectoderm	1	1460	1357±175	
	1	1774		
	5	1388		
	5	805		
ICM	1	150	110± 32	
	1	165		
	1	30		
	1	0		
	1	42		
	1	11		
	1	163		
	1	1		
	1	245		
	1	418		
	5	92		
	5	59		
	7	52		
				8%

nuclei) per embryo. Our laboratory has observed that different inbred mouse strains have different number of cells per embryo at a given time post-hCG injection. Table 10 shows the data collected for CF1 and five inbred strains of mice (Cozad et al., 1981). One set of embryos was treated by the Tarkowski procedure to determine the number of cells per embryo. The other set of embryos was subjected to the DNA synthesis assay procedure. The correlation of the two sets of data is shown in Figure 11. The DNA synthesis assay can be used as an accurate indicator of number of cells per embryo.

B. The Effect of Compounds on DNA Synthesis in Preimplantation Mouse Embryos

1. The effect of salts

Osmolarity plays an important role in maintaining embryo viability in vitro. Development of one-cell ova to blastocysts occurs best between 0.250 and 0.280 osmol (Whittingham, 1971). The effects of various concentrations of saline and PBS on CF1 blastocyst embryos were examined. The results are shown in Figure 12. Normal saline (0.15 M), when added to an equal amount of double strength Whitten and Biggers medium, inhibited ³H-thymidine incorporation by 40% in blastocyst embryos. The longer preincubation time with NaCl as opposed to PBS probably led to the increased inhibitory effect of NaCl.

2. The effect of various compounds

The effect of mitomycin C on CF1 blastocyst embryos was examined. Mitomycin C inhibits DNA synthesis by covalently cross-linking

Table 10. Determination of the number of cells per mouse embryo

Strain	Hours Post- hCG	Counting Nuclei ^a			DNA Synthesis Assay ^b		
		No. Expts.	No. Embryos	Mean Cell No./Embryo(SE)	No. Expts.	No. Embryos	cpm/Embryo/hour(SE)
CF1	89	5	51	44.4 (2.2)	3	263	178 (26)
CF1	65	4	50	6.8 (1.0)	5	91	16 (1)
C57BL/10Sn	89	8	48	33.1 (2.1)	3	55	112 (31)
CBA	89	7	45	18.9 (1.0)	2	84	91 (7)
SJL	89	5	48	31.9 (2.0)	2	74	107 (26)
BALB/c	89	4	49	22.0 (1.4)	2	59	99 (11)
DBA/1	89	8	47	30.2 (1.5)	2	39	115 (5)

^aNuclei were counted using Tarkowski (1966) procedure.

^bDNA synthesis was measured as described in the text. The assay included a three-hour preincubation, followed by a three-hour labeling period in 4 μ Ci/ml ³H-thymidine.

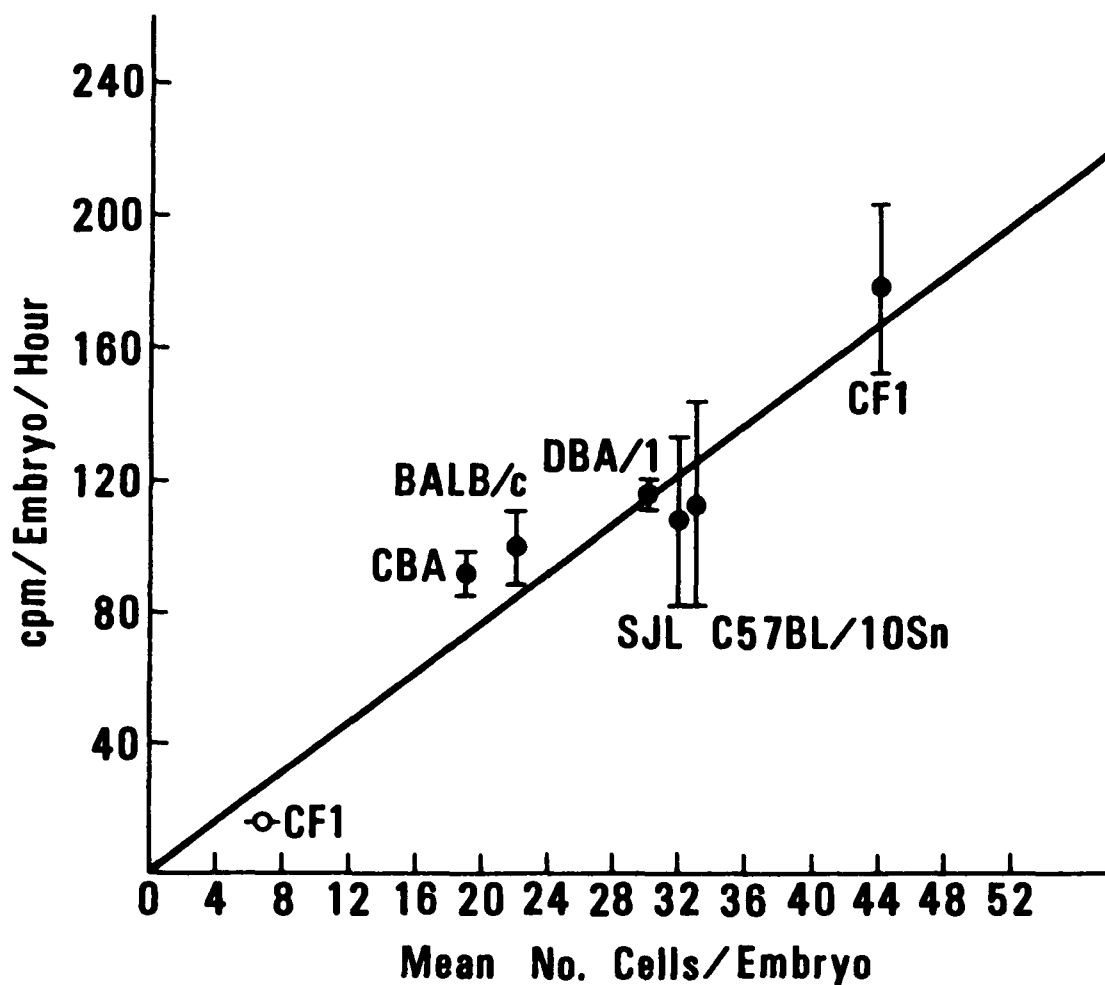


Figure 11. Cpm incorporated into DNA of blastocyst (○) and 8-cell (●) mouse embryos as a function of the number of cells per embryo. The embryos were preincubated for three hours without label and for three hours in $4 \mu\text{Ci/ml}$ ^3H -thymidine. The equation for the least squares linear regression line through the points is $y = 3.8x + 1.6$; the correlation coefficient, $r = 0.96$

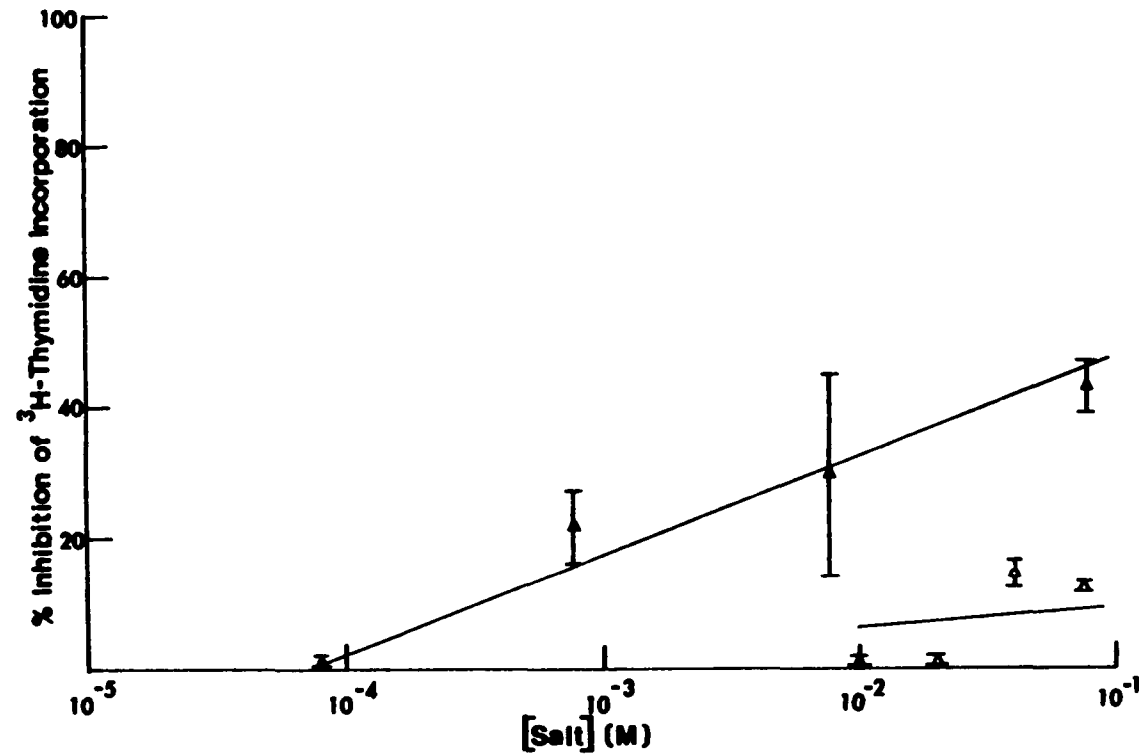


Figure 12. The effect of NaCl (▲) and PBS (△) on ^3H -thymidine incorporation by CF1 blastocyst embryos. Embryos were preincubated with NaCl in Whitten and Biggers medium for seven hours prior to a three-hour labeling period with $4\text{ }\mu\text{Ci/ml}$ of ^3H -thymidine. Embryos were preincubated with PBS in Whitten and Biggers medium for five hours prior to a three-hour labeling period with $4\text{ }\mu\text{Ci/ml}$ of ^3H -thymidine

complementary DNA strands. The effect of mitomycin C on CF1 blastocyst embryos with the zona pellucida present and absent are shown in Figure 13a and 13b, respectively. Similar results were obtained for both graphs, but the effect of mitomycin C is enhanced at lower concentrations (i.e., 50 μ g/ml) when the zona pellucida is absent. In both graphs, a treatment of about 300 μ g/ml of mitomycin C for at least one hour is necessary to give 100% inhibition of ^3H -thymidine incorporation.

α -Amanitin, a known inhibitor of RNA synthesis through binding to Form II and Form III RNA polymerases has been shown to block the cleavage of early mouse embryos (Golbus et al., 1973; Warner and Versteegh, 1974; Levey et al., 1977). It has been suggested that α -amanitin inhibits DNA synthesis in preimplantation mouse embryos. Warner and Hearn (1977) showed that embryos labeled with ^3H -thymidine incorporated about 10% of the label into DNA (presumably by conversion of uridine to deoxycytidine). The incorporation of the label into DNA was inhibited by α -amanitin. The effects of α -amanitin on ^3H -thymidine incorporation by CF1 8-cell and blastocyst embryo is shown in Table 11. Both stages of development are inhibited by α -amanitin, but blastocysts appear to be more susceptible to α -amanitin than 8-cell embryos--especially at the 50 μ g/ml concentration of α -amanitin.

The effect of aphidicolin on CF1 8-cell and blastocyst embryos is shown in Figure 14. Blastocyst and 8-cell embryos are sensitive to aphidicolin at concentrations of 20-50 μ g/ml. Aphidicolin is believed to interact with the catalytic subunit of DNA polymerase α or with an accessory subunit (Huberman, 1981). The inhibition of ^3H -thymidine

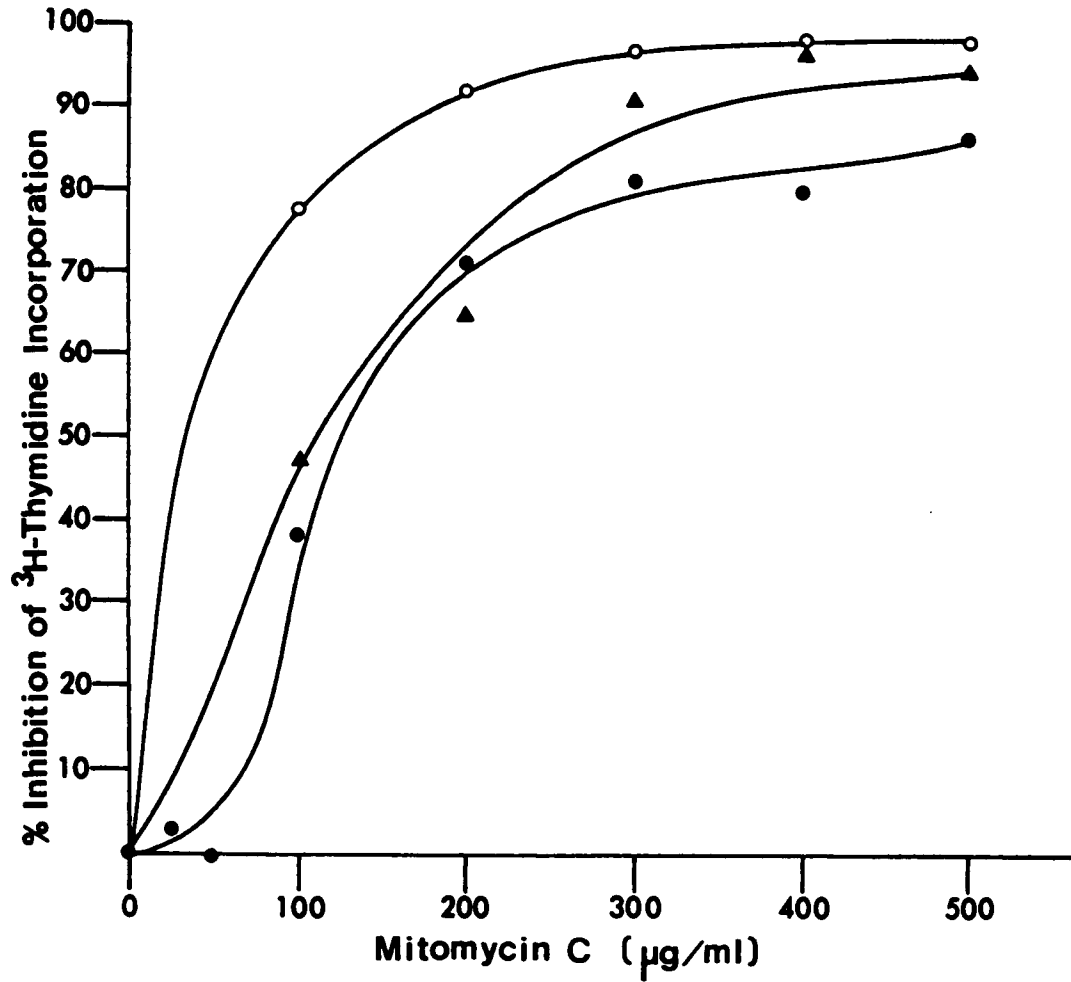


Figure 13a. The % Inhibition of ^3H -thymidine incorporation by CFl blastocyst embryos as a function of mitomycin C treatments for 0.5 (\bullet), 1.0 (\bullet), and 2.0 (\blacktriangle) hours followed by a seven-hour labeling period with ^3H -thymidine

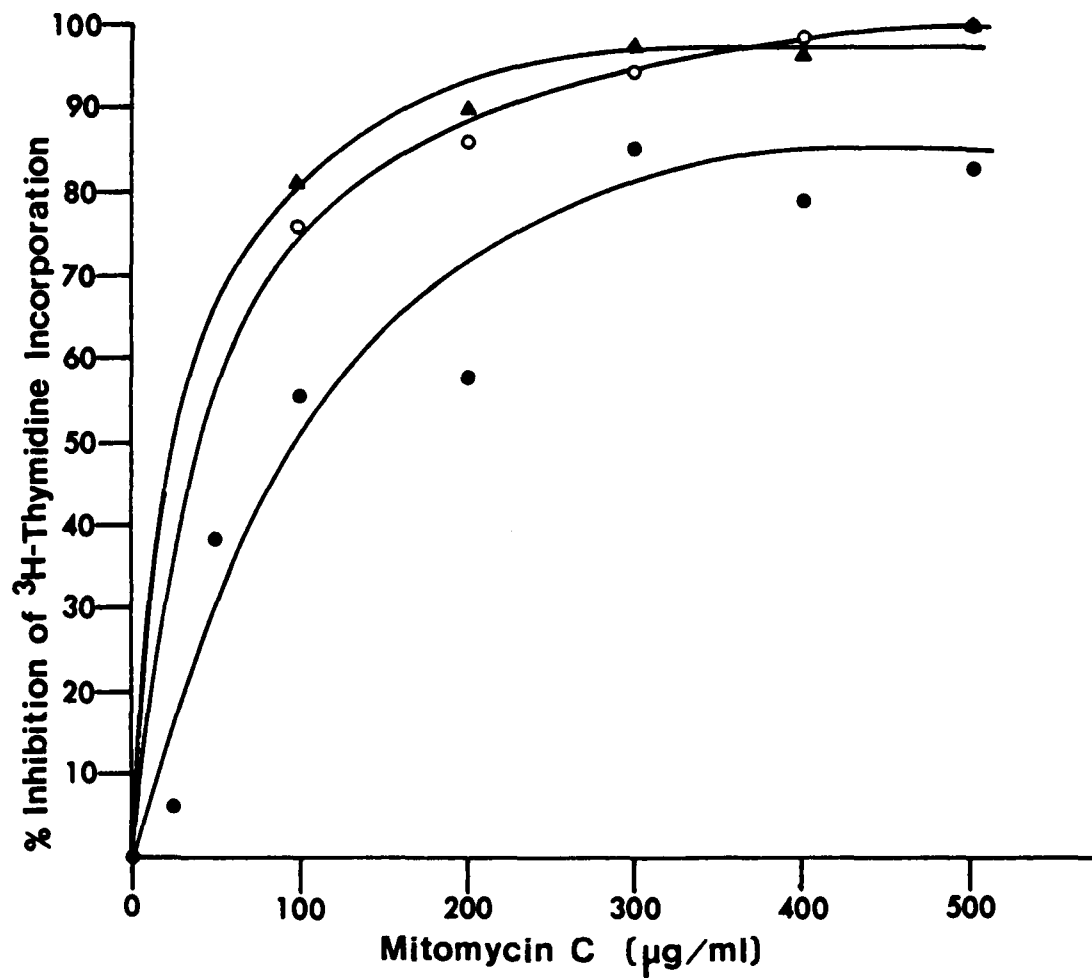


Figure 13b. The % Inhibition of ^3H -thymidine incorporation by CF1 blastocyst embryos without their zona pellucidae as a function of mitomycin C treatments for 0.5 (\bullet), 1.0 (\circ), and 2.0 (\blacktriangle) hours followed by a seven-hour labeling period with ^3H -thymidine

Table 11. The effect of α -amanitin on ^3H -thymidine incorporation by CFl 8-cell and blastocyst embryos. Embryos were incubated with α -amanitin for two hours prior to a three, five or seven hour labeling period with ^3H -thymidine

α -Amanitin ($\mu\text{g/ml}$)	Number of Assays	Number Embryos per Assay	% Inhibition of ^3H -thymidine Incorporation + SEM		
			Time of ^3H -thymidine labeling(hrs.)		
			3	5	7
<u>8-cell</u>					
0	2	9	0	0	0
50	2	9	6 \pm 4	15 \pm 5	0 \pm 0
100	2	9	8 \pm 5	17 \pm 12	8 \pm 2
200	2	9	16 \pm 6	37 \pm 0	25 \pm 11
<u>Blastocyst</u>					
0	2	5	0	0	0
50	2	5	25 \pm 1	34 \pm 6	10 \pm 1
100	2	5	41 \pm 2	24 \pm 2	23 \pm 4
200	2	5	42 \pm 6	28 \pm 5	22 \pm 0

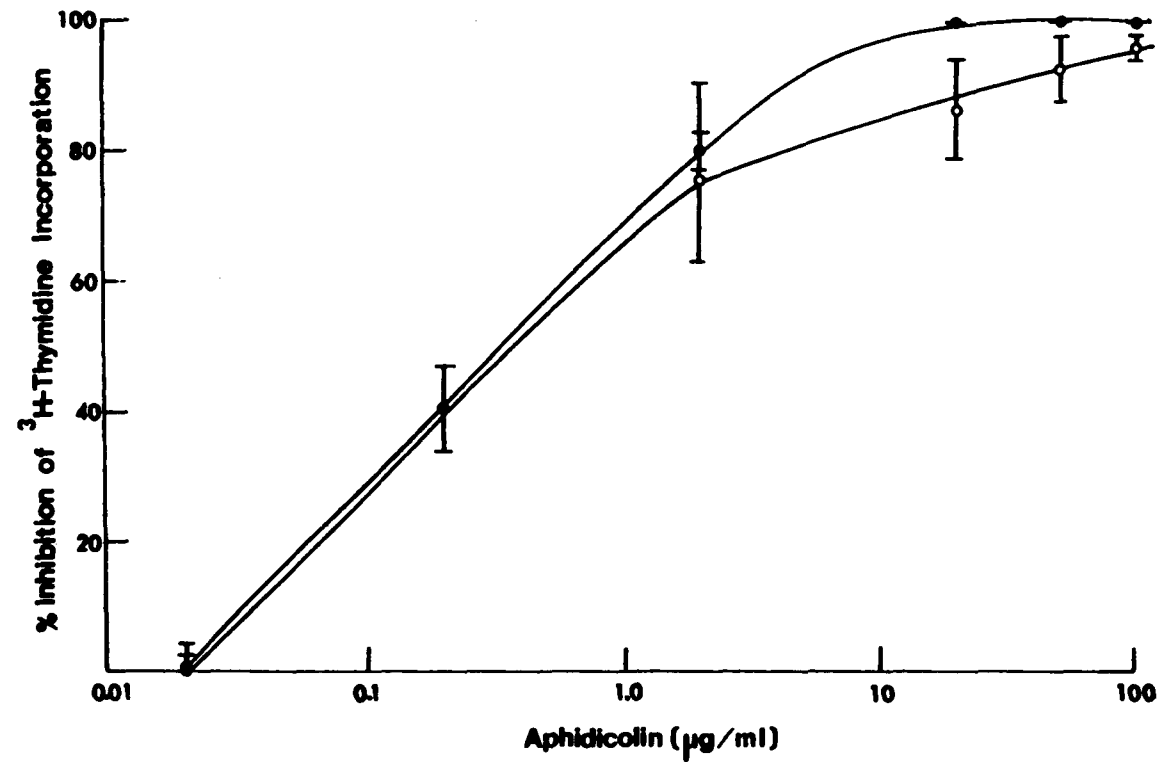


Figure 14. The effect of aphidicolin on CF1 8-cell (○) and blastocyst (●) embryos. Embryos were incubated with aphidicolin for seven hours prior to a three-hour labeling period of ^3H -thymidine

incorporation by aphidicolin suggests that DNA polymerase α or a subunit is active in early embryonic development.

The effects of cycloheximide, puromycin, and theophylline on ^3H -thymidine incorporation by CF1 blastocyst embryos are shown in Figure 15. Theophylline has a negligible effect on blastocyst embryos, whereas cycloheximide and puromycin are inhibitory at 10^{-4} M. Puromycin blocks protein synthesis by interfering with the elongation cycle of translation, while cycloheximide binds to the ribosomes and causes premature termination of peptide synthesis. Therefore, proteins essential for DNA synthesis are probably not produced and subsequently, ^3H -thymidine incorporation is inhibited.

3. The effect of hCG

One of the early indicators of pregnancy in mammals is the production of chorionic gonadotrophins. To examine whether hCG affects embryos, CF1 blastocyst embryos were incubated with hCG or the β -subunit of hCG. The results are shown in Table 12. It can be concluded that hCG and its β -subunit at 1.7 mg/ml and 2.3 mg/ml concentrations, respectively, have no effect on ^3H -thymidine incorporation by blastocyst embryos.

Wiley (1974) used antiserum to hCG in an indirect immunofluorescence assay on mouse embryos. She examined mouse embryos from the unfertilized egg stage to blastocyst outgrowths. Maximal hCG or "hCG-like" concentrations were found at the morula stage with blastocyst exhibiting slight fluorescence. The negative results obtained with 1.7 mg/ml of exogenous hCG could be due to a lack of receptors for hCG on blastocyst

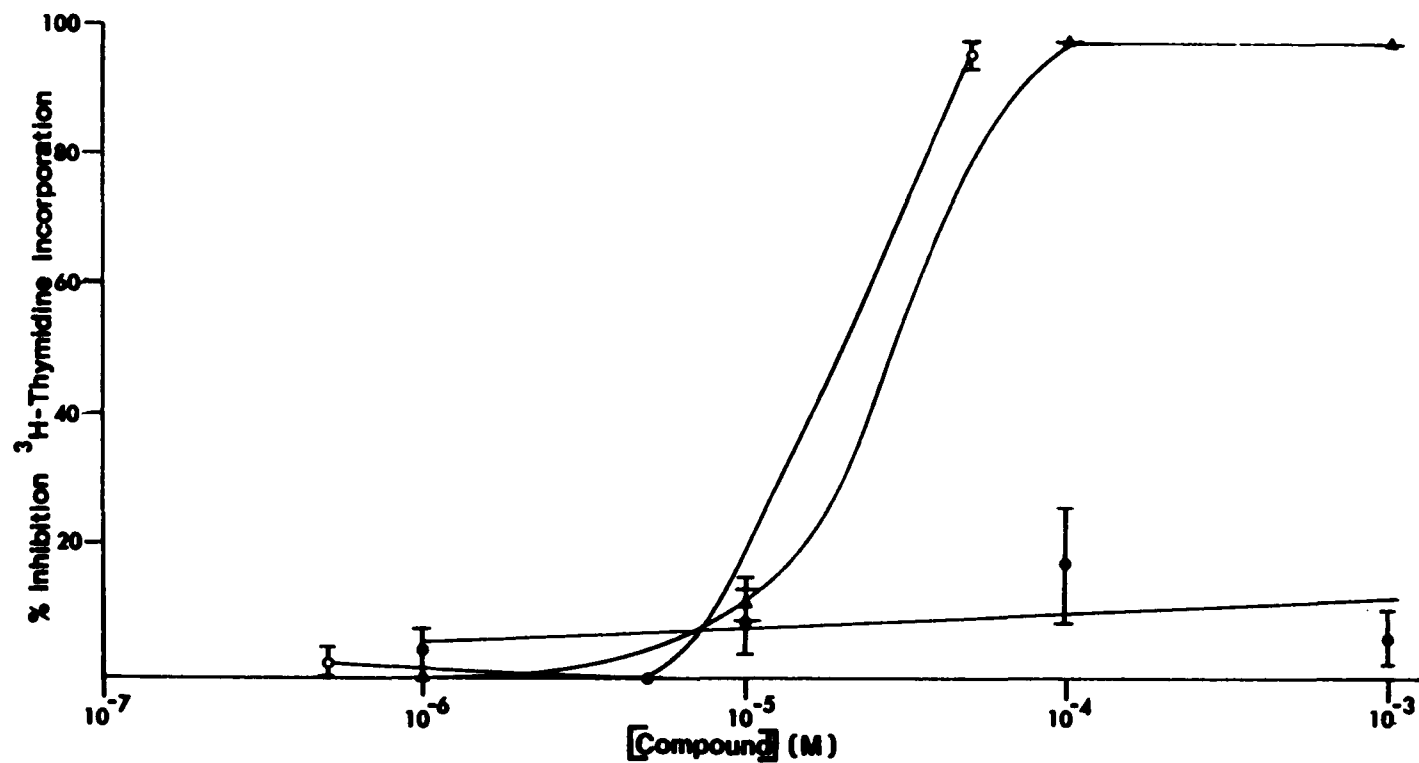


Figure 15. The effects of cycloheximide (\blacktriangle), puromycin (\bullet), and theophylline (\bullet) on CF1 blastocyst embryos. Embryos were incubated with a designated compound for seven hours prior to a three-hour labeling period with ^3H -thymidine

Table 12. The effect of hCG and its β -subunit on the incorporation of ^3H -thymidine by CF1 blastocyst embryos. Embryos were incubated five hours with the designated compound prior to a three-hour labeling period with ^3H -thymidine

Experiment Number	Controls		Experiments		Ratios	% Inhibition of ³ H-thymidine in- corporation <u>±</u> SEM
	Average Number Embryos	Average cpm/embryo	Number of Embryos	cpm/ Embryo	Experiment cpm	
					Average control cpm	
<u>A. hCG (1.7 mg/ml) Treatment</u>						
1	5	898	5	1587	1.77	<u>0</u> +0
			5	1546	1.72	
<u>B. β-Subunit (2.3 mg/ml) Treatment</u>						
1	5	1404	5	1380	0.98	<u>1</u> +1
			5	1777	1.27	
2	5	1737	5	1690	0.97	17 <u>±</u> 10
			5	1219	0.70	

embryos or the receptors could be filled with endogenous hCG and not be affected by exogenous hCG.

C. Development of the Embryo Cytotoxicity Assay

1. Cytotoxic activity of rabbit anti-mouse serum

Rabbit anti-C57BL/10Sn serum was tested against C57BL/10Sn spleen lymphocytes and also against CF1 spleen lymphocytes in a lymphocyte cytotoxicity assay. The results, expressed as % Killing of Lymphocytes, are shown in Table 13. As can be seen, the antiserum gave a titer of 1:128 against both targets. (The titer is the last dilution at which >50% of targets are killed.) The antiserum was then tested against CF1 blastocyst embryos in a cytotoxicity assay. Antiserum was added for one hour, then guinea pig complement for one hour, followed by a five-hour labeling period with ^3H -thymidine. Theoretically, antibody molecules, present in the antiserum, should bind to antigenic determinants on the embryos. The addition of complement would result in complement binding to antibody molecules on the embryonic cells and the initiation of the complement cascade with cell lysis resulting. Bound antibody molecules plus complement have been shown to produce holes as large as 150 Å in diameter (i.e., ribosome size) in the outer cell membrane (Goldberg and Green, 1960). Therefore, enzymes and substrates necessary for DNA replication could easily escape from such cells, thereby diminishing the incorporation of ^3H -thymidine into DNA. Embryos treated with normal serum, lacking antibodies, would not be lysed and would therefore incorporate ^3H -thymidine.

Table 13. Rabbit anti-C57BL/10Sn serum (2°) activity against C57BL/10Sn and CF1 lymphocytes in a lymphocyte cytotoxicity assay

Titer	% Killing of Lymphocytes + SEM	
	C57BL/10Sn Target	CF1 Target
1:2	93 \pm 4	94 \pm 3
1:4	98 \pm 1	95 \pm 0
1:8	95 \pm 0	96 \pm 1
1:16	93 \pm 2	94 \pm 1
1:32	87 \pm 2	87 \pm 5
1:64	85 \pm 1	83 \pm 3
1:128	59 \pm 0	73 \pm 0
1:256	30 \pm 5	43 \pm 8
1:512	9 \pm 1	8 \pm 2
1:1024	8 \pm 2	4 \pm 0
1:2048	6 \pm 0	3 \pm 0

The results for antiserum treatment of CF1 blastocyst embryos and also CF1 lymphocytes are shown in Figure 16. The antiserum effectively (50% killing) inhibits ^3H -thymidine incorporation by embryos to a titer of 1:1024, while the lymphocyte cytotoxicity assay is effective to a titer of 1:256. The embryo cytotoxicity assay is sensitive enough to perform on individual blastocyst embryos.

It is seen that there is a lower inhibition of ^3H -thymidine incorporation at 1:2, 1:4 and 1:8 dilutions of antiserum. The probable explanation is a prozone effect in which the number of antibody molecules binding to antigenic determinants is so great that only one of the two binding sites of the IgG molecule attaches to the embryos. Therefore, neither aggregation nor a conformational change would occur in the IgG molecule, so that the complement cascade with subsequent lysis could not be initiated.

Figure 17 exhibits the morphological changes which can be observed when blastocyst embryos were treated with normal serum or with antiserum, followed by treatment with guinea pig complement. Note that the complement-treated embryos have collapsed blastocoels and the lysed trophoctoderm cells have retracted from the zona pellucida and surround the centrally condensed inner cell mass.

The rabbit anti-C57BL/10Sn serum was titered against CF1 8-cell embryos. The results are shown in Figure 18 and later in Figure 21. As in the case with the blastocyst embryos, the antiserum was cytotoxic to the embryos. The 8-cell embryos appear to be as sensitive to antiserum as the blastocyst embryos. There is 50% inhibition of

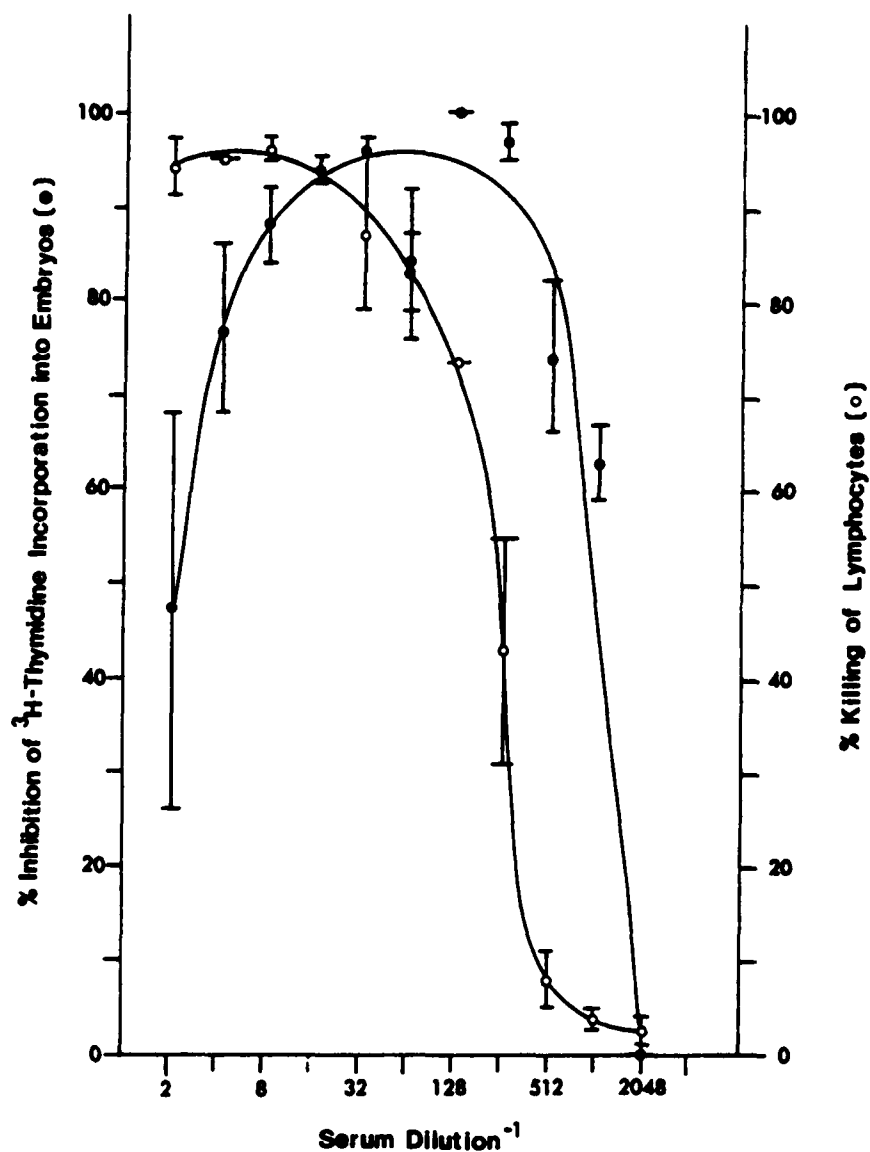


Figure 16. The titer of rabbit anti-mouse serum in cytotoxicity assays of CFl blastocyst embryos (●) and CFl lymphocytes (○). The cytotoxicity on blastocyst embryos was measured by the incorporation of ³H-thymidine (% Inhibition of ³H-thymidine incorporation). The cytotoxicity on lymphocytes was measured by trypan blue dye exclusion (% Killing of lymphocytes)



Figure 17. The effect of a one-hour normal rabbit serum (1:32) or rabbit anti-C57BL/10Sn serum (1:32) treatment of CF1 blastocyst embryos, followed by a one-hour treatment with guinea pig complement. No. 1 designates the group of blastocysts treated with normal serum, while No. 2 designates the group of blastocysts treated with anti-serum. Magnification = 80x

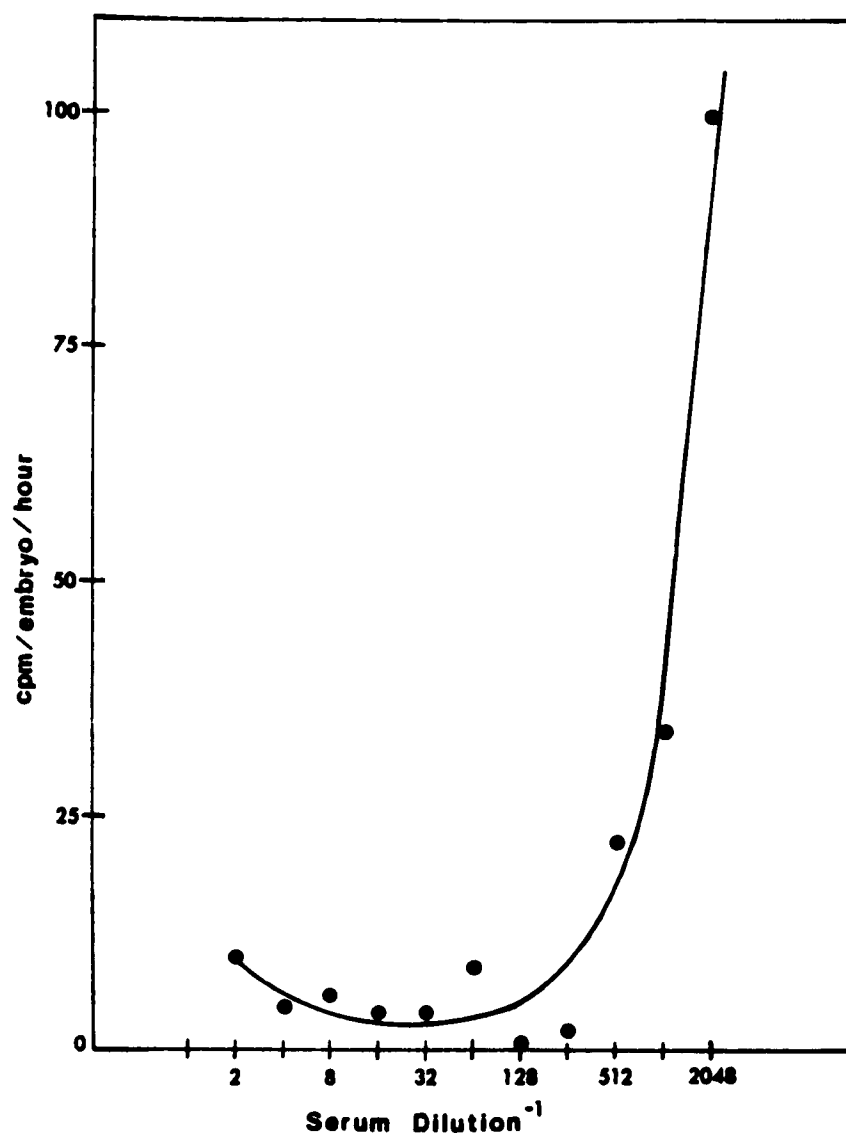


Figure 18. The titer of rabbit anti-mouse serum against CF1 8-cell embryos

^3H -thymidine incorporation at 1:1024 for blastocysts and also at 1:1024 for 8-cell embryos.

2. Necessity of complement in the embryo cytotoxicity assay

To confirm the necessity of complement in the assay system, CF1 blastocyst embryos were treated with rabbit anti-C57BL/10Sn serum then active or heat-inactivated guinea pig complement was added. The results are shown in Figure 19. Active guinea pig complement is necessary for optimal inhibition of ^3H -thymidine incorporation.

3. Optimization of antibody binding to embryos

The length of time that the antiserum needed to be in contact with the embryos before complement addition, was examined concomitantly with the effect of the zona pellucida on antibody diffusion. Sellens and Jenkinson (1975) demonstrated that the zona pellucida impairs the attachment of antibodies to the preimplantation mouse embryo when the antiserum was incubated with embryos for 30 minutes. This limitation was circumvented by allowing a longer incubation time with antiserum. Pronase digestion of the zona pellucida has one major drawback in that there is the potential for altering proteins (and, therefore, antigenic determinants) on the trophectoderm. Figure 20 shows that the absence of the zona pellucida allows optimal binding (and optimal inhibition of ^3H -thymidine incorporation) after 30 minutes, whereas one hour with antiserum prior to the addition of complement was needed to achieve optimal inhibition of ^3H -thymidine incorporation when the zona pellucida was present.

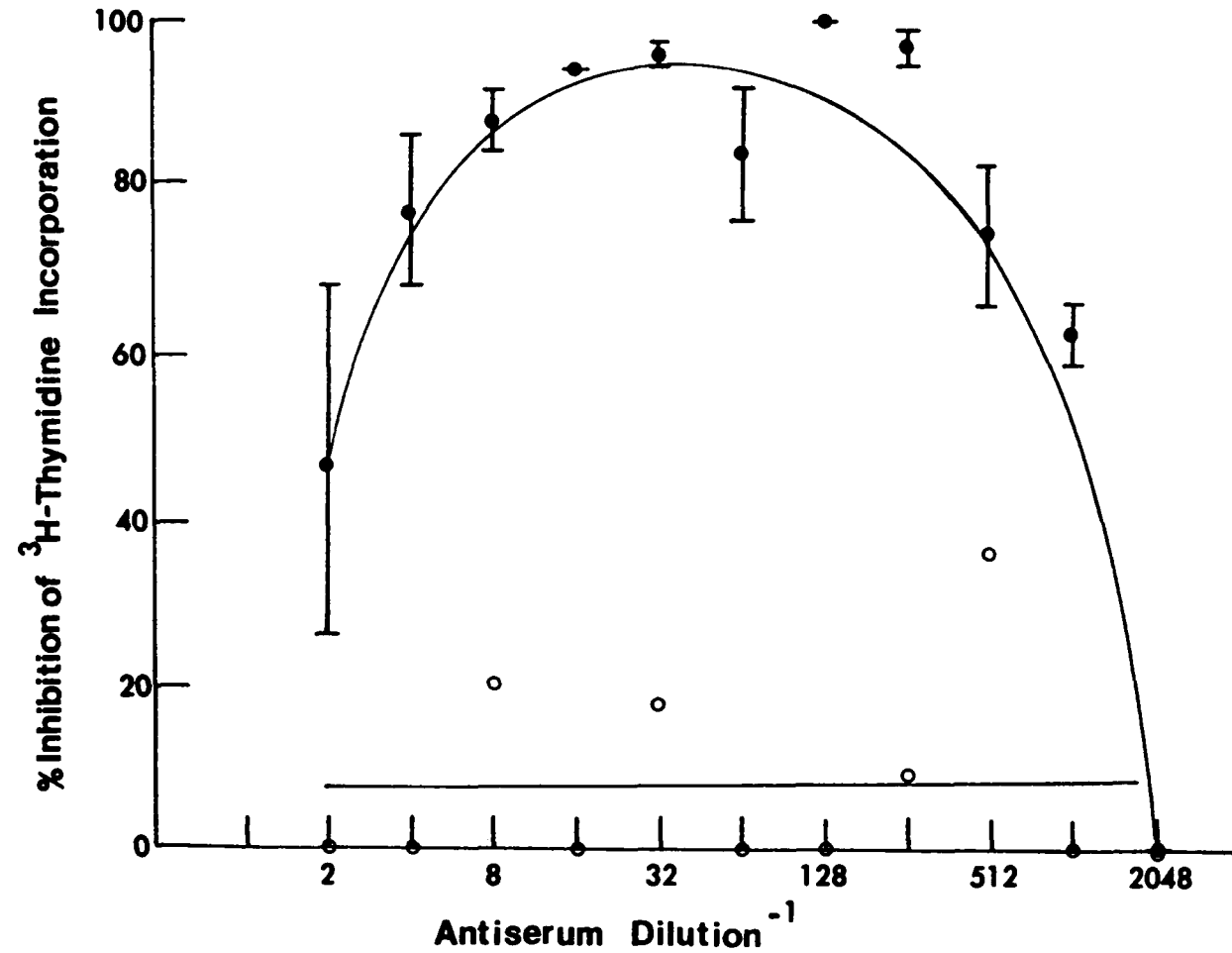


Figure 19. The effect of active (•) and heat-inactivated (◊) guinea pig complement on CF1 blastocysts treated with rabbit anti-C57BL/10Sn serum for one hour. After a one-hour treatment with complement, the embryos were labeled for five hours with ³H-thymidine

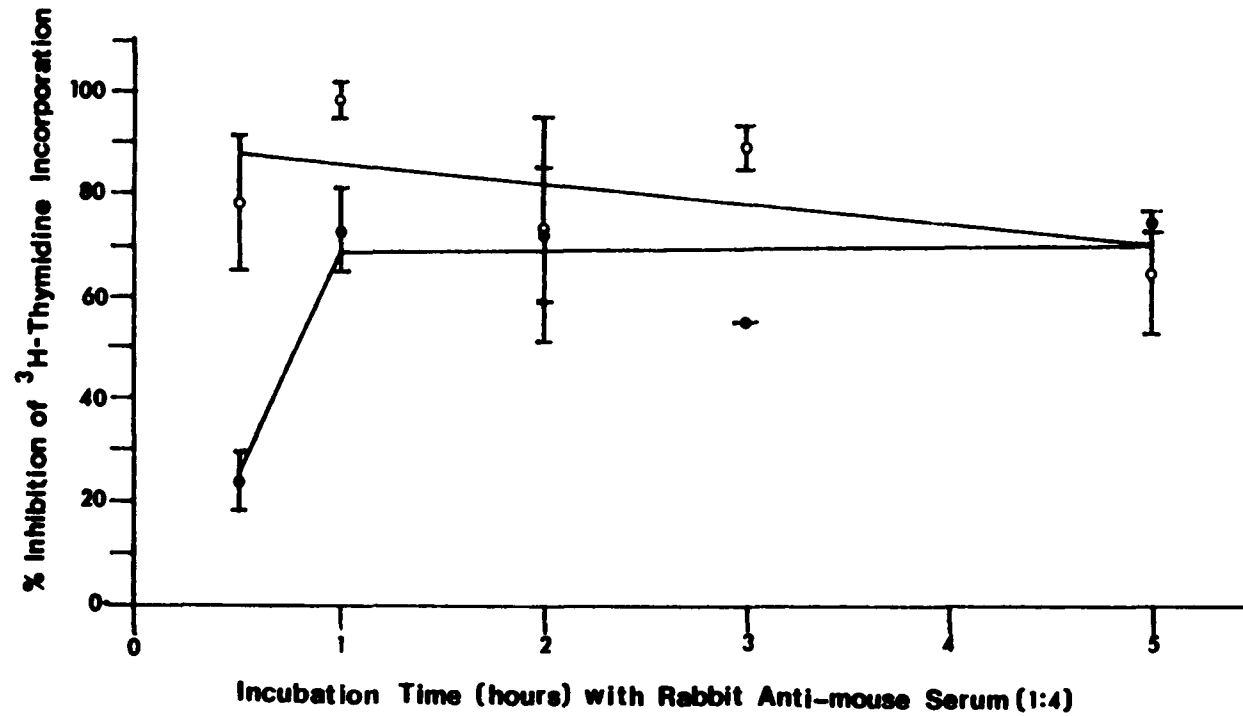


Figure 20. The % Inhibition of ^3H -thymidine incorporation by CF1 blastocyst embryos was examined in relationship to the incubation time with rabbit anti-C57BL/10Sn serum (1:4) prior to complement addition and prior to a five-hour labeling period with ^3H -thymidine. Blastocyst embryos with (●) and without (○) their zona pellucidae were examined

An antiserum time course study was performed on CF1 8-cell embryos and the data are shown in Table 14. Optimal percent inhibition of ^3H -thymidine incorporation occurred after a 30-minute incubation with antiserum, followed by addition of guinea pig complement. When the zona pellucida was removed from 8-cell embryos, both normal serum-treated embryos and antiserum-treated embryos failed to incorporate ^3H -thymidine. Evidently, the serum or the complement contained a "serum factor" that was deleterious to 8-cell embryos lacking the zona pellucida. The reason for this observation is unclear since 8-cell embryos treated with pronase will undergo further cell division in Whitten and Biggers medium.

4. Long-term ^3H -thymidine incorporation by 8-cell embryos

The cytotoxicity assay on blastocyst embryos gave reproducible results for one to five embryos per assay well. The assay, when performed on 8-cell embryos gave repeatable results for nine to ten embryos per assay well. Repeatable results for at least five 8-cell embryos per assay were obtained by utilizing a 24-hour ^3H -thymidine labeling period. The results are shown in Figure 21. It can be seen that the results obtained with a five-hour labeling period of ten 8-cell embryos are essentially identical to those results obtained with a 24-hour labeling period of five 8-cell embryos.

In summary, the embryo cytotoxicity assay can be performed on both CF1 blastocyst embryos and CF1 8-cell embryos. At least a one-hour

Table 14. Rabbit anti-C57BL/10Sn serum (1:8) time course on CF1 8-cell embryos. The embryos were incubated with antiserum for designated time periods prior to a one-hour incubation with guinea pig complement and a five-hour ^3H -thymidine labeling period

Antiserum Incubation (hours)	% Inhibition of ^3H -thymidine Incorporation \pm SEM
0.5	100 \pm 0
1.0	100 \pm 0
2.0	100 \pm 0

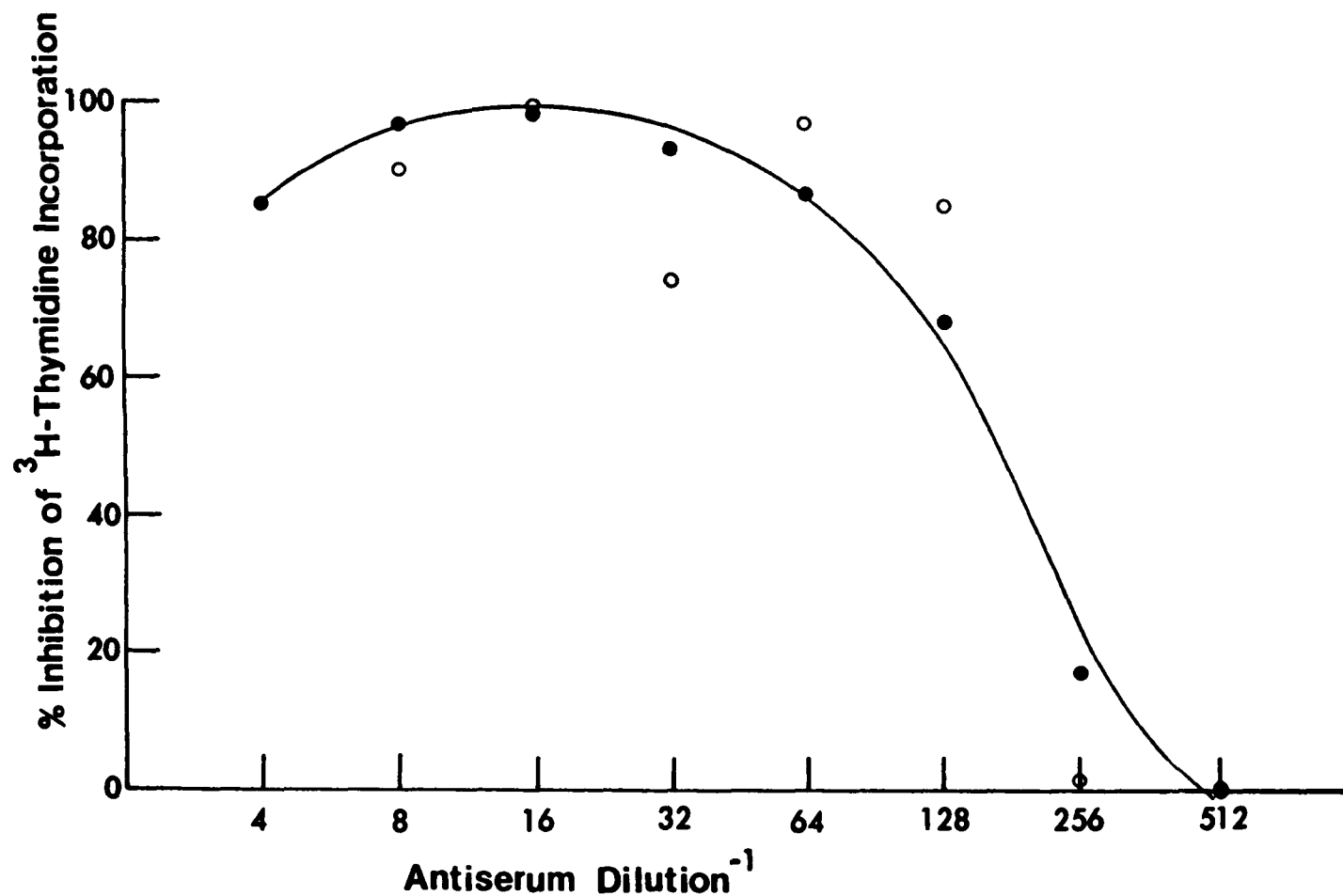


Figure 21. The effects of labeling ten 8-cell embryos with ^3H -thymidine for five hours (●) and of labeling five 8-cell embryos with ^3H -thymidine for twenty-four hours (○). Rabbit anti-C57BL/10Sn serum was titered against the embryos. Embryos were incubated with antiserum for one hour then with guinea pig complement for one hour, prior to the ^3H -thymidine labeling

incubation with antiserum is needed for blastocyst embryos and at least a half-hour incubation with antiserum is needed for 8-cell embryos prior to a one-hour incubation period with guinea pig complement. A five-hour ^3H -thymidine labeling period is sufficient for one to five blastocyst embryos or nine to ten 8-cell embryos. Five 8-cell embryos can be assayed when labeled for 24 hours.

D. Application of the Embryo Cytotoxicity
Assay to the Detection of the Major
Histocompatibility Antigens

1. Production and testing of
H-2 antisera

Congenic antisera were prepared according to a modification of the method of Batchelor (1973). Five bleedings were made and each bleeding was tested against C57BL/10Sn, B10.A, B10.BR, and B10.D2 lymphocytes in a lymphocyte cytotoxicity assay. Antiserum from each bleeding was tested at titers of 1:2, 1:4 and 1:8. The results for the five bleedings of anti-C57BL/10Sn, anti-B10.A, anti-B10.BR, and anti-B10.D2 sera are shown in Tables 15, 16, 17, and 18, respectively. In most cases, the cytotoxicity of the antiserum from the 5^o bleeding at a 1:4 titer exhibited the highest cytotoxicity against its specific target lymphocyte.

H-2 specific antisera were obtained from the National Institutes of Health (Bethesda, Maryland). The NIH antisera are specific to a private specificity and some public specificities at either the K or the D end of the H-2 complex (see Table 5). The cytotoxicity of the NIH antisera against specific target lymphocytes is shown in Table 19. Most of the

Table 15. Summary of C57BL/10Sn antisera (1°, 2°, 3°, 4°, 5° bleedings) made in B10.BR mice. Cytotoxicity against C57BL/10Sn, B10.A, B10.BR, and B10.D2 target lymphocytes was measured in lymphocyte cytotoxicity assays

	Titer	% Killing			
		C57BL/10Sn	B10.A	B10.BR	B10.D2
1° bleeding	1:2	91	72	20	86
	1:4	95	40	11	86
	1:8	90	23	11	41
2° bleeding	1:2	86	50	19	63
	1:4	79	27	9	47
	1:8	44	5	5	28
3° bleeding	1:2	92	23	3	68
	1:4	97	1	2	67
	1:8	90	1	2	20
4° bleeding	1:2	89	15	4	67
	1:4	85	18	3	42
	1:8	92	6	3	12
5° bleeding	1:2	95	65	2	77
	1:4	93	24	4	67
	1:8	85	19	3	44

Table 16. Summary of B10.A antisera (1°, 2°, 3°, 4°, 5° bleedings) made in C57BL/10Sn mice. Cytotoxicity against B10.A, B10.BR, B10.D2, and C57BL/10Sn target lymphocytes was measured in lymphocyte cytotoxicity assays

		% Killing			
		B10.A	B10.BR	B10.D2	C57BL/10Sn
1° bleeding	1:2	70	73	62	2
	1:4	60	45	58	3
	1:8	30	36	34	3
2° bleeding	1:2	70	51	37	5
	1:4	52	45	46	3
	1:8	38	32	29	6
3° bleeding	1:2	81	71	61	7
	1:4	83	67	59	7
	1:8	76	71	55	8
4° bleeding	1:2	88	77	74	2
	1:4	80	67	72	2
	1:8	42	59	35	5
5° bleeding	1:2	89	79	81	5
	1:4	87	81	80	7
	1:8	83	84	65	12

Table 17. Summary of B10.D2 antisera (1°, 2°, 3°, 4°, 5° bleedings) made in C57BL/10Sn mice. Cytotoxicity against B10.D2 C57BL/10Sn, B10.A, and B10.BR target lymphocytes was measured in lymphocyte cytotoxicity assays

	Titer	% Killing			
		B10.D2	C57BL/10Sn	B10.A	B10.BR
1° bleeding	1:2	90	4	87	58
	1:4	82	2	79	42
	1:8	75	4	75	51
2° bleeding	1:2	90	4	86	57
	1:4	79	7	88	58
	1:8	75	7	54	53
3° bleeding	1:2	93	5	91	90
	1:4	95	9	91	75
	1:8	91	6	84	70
4° bleeding	1:2	95	3	97	75
	1:4	94	2	95	61
	1:8	93	2	74	56
5° bleeding	1:2	99	4	97	85
	1:4	96	8	97	95
	1:8	96	9	98	87

Table 18. Summary of B10.BR antisera (1°, 2°, 3°, 4°, 5° bleedings) made in C57BL/10Sn mice. Cytotoxicity against B10.BR, B10.D2, C57BL/10Sn, and B10.A target lymphocytes was measured in lymphocyte cytotoxicity assays

	Titer	% Killing			
		B10.BR	B10.D2	C57BL/10Sn	B10.A
1° bleeding	1:2	72	50	5	53
	1:4	78	57	8	68
	1:8	57	44	7	55
2° bleeding	1:2	86	53	7	75
	1:4	76	46	4	72
	1:8	64	30	6	70
3° bleeding	1:2	94	64	5	87
	1:4	90	76	1	77
	1:8	89	60	2	81
4° bleeding	1:2	95	62	3	89
	1:4	95	57	3	91
	1:8	93	50	1	88
5° bleeding	1:2	89	61	6	92
	1:4	96	64	8	90
	1:8	84	64	6	90

Table 19. Summary of lymphocyte cytotoxicity assays for NIH-23b, NIH-D2, NIH-D31, and NIH-D32 antisera against B10.A, C57BL/10Sn, B10.D2, and B10.BR lymphocytes

Titer	% Killing \pm SEM			
	NIH-D2 (Anti-C57BL/10Sn)	NIH-23b (Anti-B10.A)	NIH-D32 (Anti-B10.BR)	NIH-D31 (Anti-BALB/c) ^a
1:2	86 \pm 6	76 \pm 0	73 \pm 7	60 \pm 4
1:4	78 \pm 1	80 \pm 0	86 \pm 1	73 \pm 3
1:8	68 \pm 12	69 \pm 2	79 \pm 5	62 \pm 6
1:16	57 \pm 3	59 \pm 2	84 \pm 0	62 \pm 1
1:32	64 \pm 10	53 \pm 0	78 \pm 1	52 \pm 2
1:64	41 \pm 6	10 \pm 1	69 \pm 2	25 \pm 1

^aBALB/c mice are haplotype d, as are B10.D2.

NIH antisera exhibit maximum cytotoxic activity at a 1:4 titer and was, therefore, used at a 1:4 dilution when testing embryos.

Ia specific antiserum was obtained from the National Institutes of Health (Bethesda, Maryland). The antiserum is specific to Ia 1,2,3, and 7 specificities. The cytotoxic activity of the antiserum to C57BL/10Sn, B10.A, B10.BR, and B10.D2 lymphocytes is shown in Table 20. The antiserum was used at a 1:4 dilution against C57BL/10Sn, B10.A, B10.BR, and B10.D2 blastocyst embryos.

Anti-B10.D2 ascites and antisera from four C57BL/10Sn mice were tested against B10.D2 lymphocytes in a lymphocyte cytotoxicity assay. The results are shown in Table 21. The cytotoxicity of the ascites parallels the cytotoxicity of the serum. The production of ascites is an excellent way to produce large quantities (i.e., 10-15 ml) of antibody solution that closely parallels the serum's cytotoxicity to an antigen.

Table 22 shows the cytotoxicity of anti-C57BL/10Sn ascites from a B10.D2 mouse. The immune response of this mouse to C57BL/10Sn lymphocyte targets was relatively low.

H-Y antisera were produced and tested by several protocols, as described in the Experiment Procedures. No clearly repeatable results were obtained by using any of the test systems--hemagglutination, sperm cytotoxicity or staph A assays. Only a few labs have successfully and reproducibly produced H-Y antiserum. Many labs have attempted to reproduce these earlier findings with great difficulty. Since H-Y antisera were not successfully produced, an attempt was made to generate

Table 20. Summary of lymphocyte cytotoxicity assays for NIH Ia 1, 2, 3, 7 antiserum against C57BL/10Sn, B10.A, B10.D2, and B10.BR lymphocytes

Titer	% Killing \pm SEM			
	C57BL/10Sn	B10.A	B10.BR	B10.D2
1:2	60 \pm 0	48 \pm 0	60 \pm 4	69 \pm 11
1:4	35 \pm 4	64 \pm 2	68 \pm 11	46 \pm 3
1:8	38 \pm 2	64 \pm 5	81 \pm 5	55 \pm 2
1:16	28 \pm 3	33 \pm 9	66 \pm 2	39 \pm 1
1:32	15 \pm 2	64 \pm 7	40 \pm 4	37 \pm 5
1:64	12 \pm 2	46 \pm 15	35 \pm 1	38 \pm 0

Table 21. Summary of lymphocyte cytotoxicity assays for anti-B10.D2 ascites and serum from four C57BL/10Sn mice against B10.D2 lymphocytes

Titer	% Killing \pm SEM							
	Mouse No. 1		Mouse No. 2		Mouse No. 3		Mouse No. 4	
	Ascites	Serum	Ascites	Serum	Ascites	Serum	Ascites	Serum
1:2	74 \pm 4	87 \pm 2	86 \pm 4	89 \pm 1	85 \pm 2	86 \pm 2	92 \pm 0	84 \pm 1
1:4	83 \pm 2	85 \pm 2	88 \pm 2	83 \pm 3	86 \pm 5	80 \pm 1	90 \pm 3	81 \pm 6
1:8	76 \pm 2	65 \pm 11	87 \pm 0	90 \pm 0	75 \pm 7	85 \pm 3	80 \pm 4	85 \pm 4
1:16	79 \pm 6	75 \pm 2	92 \pm 0	90 \pm 3	72 \pm 1	71 \pm 6	48 \pm 11	79 \pm 2
1:32	79 \pm 2	77 \pm 0	86 \pm 1	87 \pm 2	45 \pm 3	65 \pm 8	15 \pm 2	61 \pm 1
1:64	54 \pm 4	--	--	--	--	--	--	--
1:128	52 \pm 4	--	--	--	--	--	--	--
1:256	18 \pm 1	--	--	--	--	--	--	--
1:512	13 \pm 1	--	--	--	--	--	--	--
1:1024	5 \pm 0	--	--	--	--	--	--	--
1:2048	6 \pm 0	--	--	--	--	--	--	--

Table 22. Summary of lymphocyte cytotoxicity assay for anti-C57BL/10Sn ascites from a B10.D2 mouse against C57BL/10Sn lymphocytes

Titer	% Killing \pm SEM
1:2	46 \pm 4
1:4	44
1:8	12 \pm 0
1:16	5 \pm 4
1:32	2 \pm 1
1:64	4 \pm 3
1:128	3 \pm 2
1:256	1 \pm 1
1:512	4 \pm 3
1:1024	3 \pm 1
1:2048	1 \pm 1

cytotoxic T cells to H-Y. The results of a cell-mediated lympholysis reaction are shown in Table 23. Cytotoxic T cells were successfully generated to H-Y on C57BL/10Sn ♂ target lymphocytes.

2. Determination of optimal antisera dilutions for the embryo cytotoxicity assay

B10.A congenic antiserum was titered at 1:2, 1:4 and 1:8 against B10.A blastocyst embryos in the embryo cytotoxicity assay. The results are shown in Figure 22. The antiserum gave the greatest inhibition when used at a 1:4 dilution.

NIH-23b antiserum (X-B10.A) was titered at 1:2, 1:4, 1:8, and 1:16 against B10.A blastocyst embryos. The results are shown in Figure 23. A 1:4 dilution gave the highest inhibition of ³H-thymidine incorporation. It should be noted that the NIH-23b antiserum is against K^k 11, 23, 25, and 52 (see Table 5), while the B10.A congenic antiserum is against all public and private specificities in both K^k and D^k ends of the H-2 complex. Therefore, the narrower specificity of the NIH antisera could result in less activity with blastocyst embryos and, therefore, less inhibition of ³H-thymidine incorporation. Based on Figures 22 and 23, all congenic and NIH antisera were used at a 1:4 dilution on inbred mouse embryos in the embryo cytotoxicity assay.

3. Effects of NIH and congenic antisera on inbred blastocyst embryos

Tables 24a, 24b, 25, 26, and 27 list the results obtained when NIH and congenic antisera to C57BL/10Sn, B10.A, B10.BR, and B10.D2

Table 23. The effect of C57BL/10Sn ♀ cytotoxic T cells to H-Y against C57BL/10Sn ♂ target lymphocytes in a cell-mediated lympholysis assay. The control consisted of the effect of nonprimed C57BL/10Sn ♀ cells on C57BL/10Sn ♂ target lymphocytes

E:T	Nonprimed C57BL/10Sn ♀ Cells	Primed C57BL/10Sn ♀ Cells	
	\bar{X} cpm \pm SEM	\bar{X} cpm \pm SEM	% Corrected ^a Lysis
20:1	1083 \pm 39	2986 \pm 24	14
10:1	1150 \pm 41	2794 \pm 96	12
5:1	1113 \pm 23	2754 \pm 58	12
2.5:1	1220 \pm 44	3025 \pm 112	13
1.3:1	1090 \pm 23	3180 \pm 207	15
0.6:1	1573 \pm 78	3228 \pm 263	19

^a% Corrected Lysis =

$$\frac{\text{counts released by experiment} - \text{counts released by control}}{\text{maximum released counts} - \text{medium background counts}} \times 100.$$

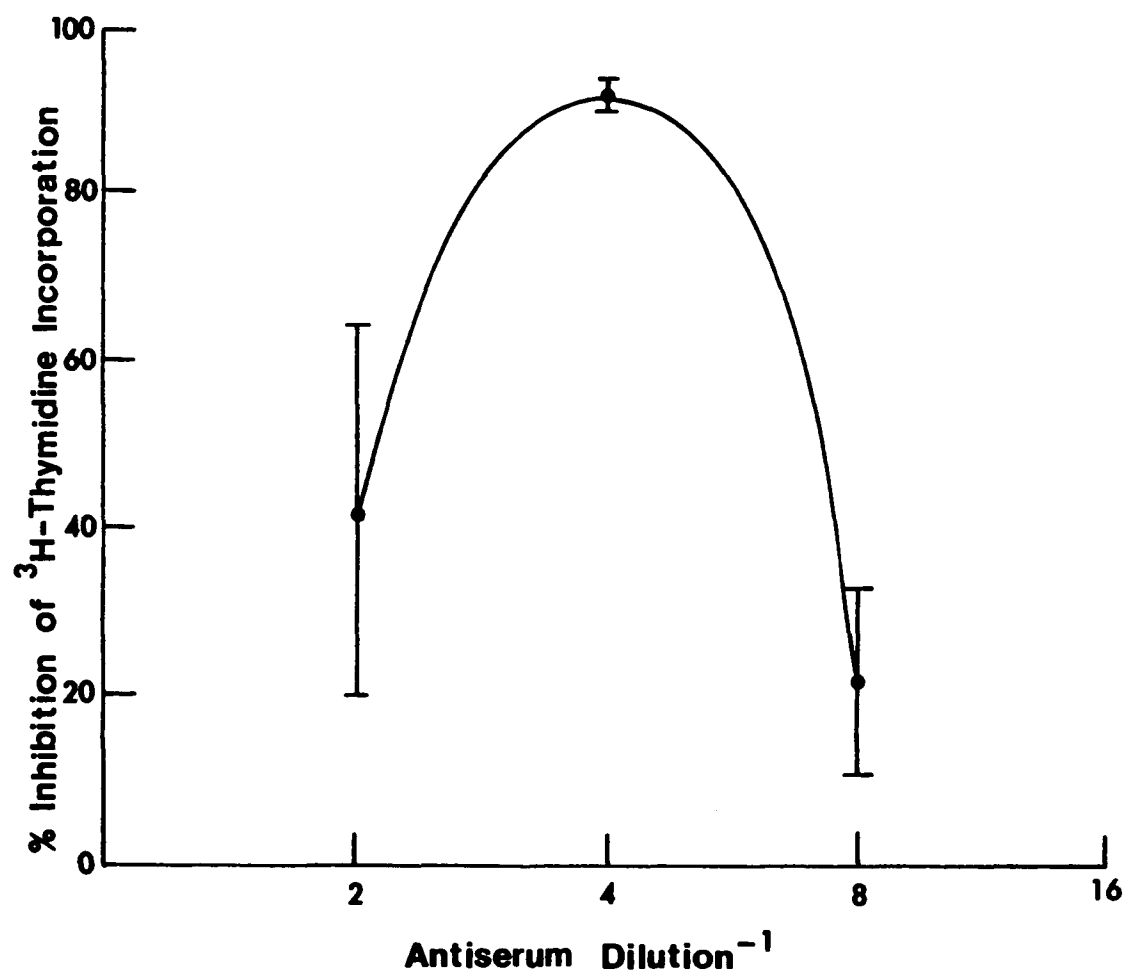


Figure 22. The effect of B10.A congenic antiserum on B10.A blastocyst embryos in the embryo cytotoxicity assay

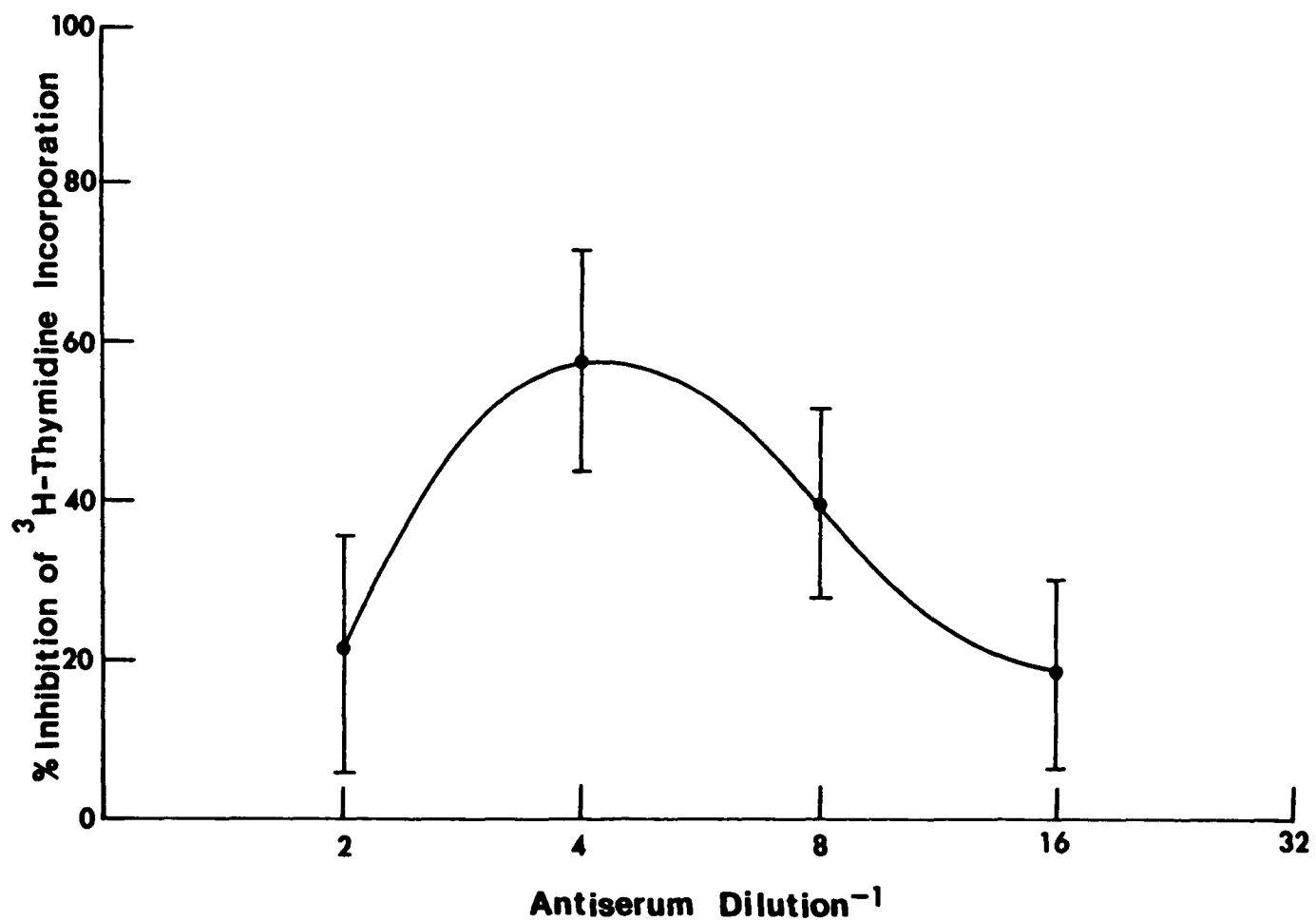


Figure 23. The effect of NIH-23b antiserum (X-B10.A) on B10.A blastocyst embryos in the embryo cytotoxicity assay

Table 24a. Effect of NIH and congenic antisera on C57BL/10Sn blastocyst embryos

Experiment Number	Antiserum	Controls		Experiments		Ratios	
		Average Number	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	Experiment cpm	Average Control cpm
		Embryos	Embryo	Embryos	Embryo		
<u>A. NIH Antiserum Treatment</u>							
1	D-2	4	475	4	313		0.659
				4	167		0.352
				4	413		0.870
2	D-2	3		2	256		1.44
				2	287		1.61
<u>B. Congenic Antiserum Treatment</u>							
1	X-B10	1	210	1	74		0.352
				1	0		0.000
				1	55		0.262
2	X-B10	4	475	4	79		0.166
				4	44		0.0926
3	X-B10.A	3	783	3	898		1.15
				3	945		1.21
4	X-B10.BR	3	783	3	18		0.0230
				2	403		0.515
5	X-B10.BR	3	316	3	23		0.0728
				3	100		0.316
				1	59		0.187
6	X-B10.D2	2	217	2	22		0.101
				2	163		0.751
				2	253		1.17
7	X-B10.D2	1	305	1	118		0.387
				1	198		0.649

Table 24b. Method for the statistical evaluation of data in Table 24a-A
(NIH antiserum treatment on C57BL/10Sn blastocyst embryos)

	Ratios $\left(= \frac{\text{Experiment cpm}}{\text{Average control cpm}} \right)$
Experiment No. 1	0.659 0.352 0.870
Experiment No. 2	1.44 1.61

Mean of control ratio (by definition) (\bar{X}_c) = 1.000
Mean of experimental ratios (\bar{X}_E) = 0.987
Standard deviation (S.D.) = 0.474
Variance (SD^2) = 0.225
Number of experimental observations (n_E) = 5
Degrees of freedom (d.f.) = 5 - 1 = 4

t Test:

$$t = \frac{\bar{X}_c - \bar{X}_E}{\sqrt{\frac{(SD)^2}{n_E}}} = \frac{1.000 - 0.987}{\sqrt{\frac{0.225}{5}}} = 0.06132$$

Compare t value to a "Values of t" table according to degrees of freedoms. Read probably from the top of the column.

$$\therefore p > 0.5$$

Table 25. Effect of NIH and congenic antisera on B10.A blastocyst embryos

Experiment Number	Antiserum	Controls		Experiments		Ratios
		Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	<u>Experiment cpm</u> Average Control cpm
<u>A. NIH Antiserum Treatment</u>						
1	D-23b	1	268	1	151	0.563
				1	30	0.112
2	D-23b	3	214	3	85	0.397
				3	255	1.19
<u>B. Congenic Antiserum Treatment</u>						
1	X-B10.A	4	118	4	73	0.619
				4	30	0.254
2	X-B10.A	1	414	1	57	0.138
				1	106	0.256
				1	348	0.841
				1	386	0.932
				1	151	0.365
3	X-B10.BR	2	462	2	405	0.877
				2	252	0.545
4	X-B10.D2	4	118	4	139	0.180
				4	195	1.65
5	X-B10.D2	2	462	2	516	1.12
				2	397	0.859
6	X-B10	2	462	2	110	0.238
				2	65	0.141

Table 26. Effect of NIH and congenic antisera on B10.BR blastocyst embryos

Experiment Number	Antiserum	Controls		Experiments		Ratios	
		Average Number	Average cpm/ Embryos	Number Embryos	cpm/ Embryo	Experiment cpm	
						Average	Control cpm
<u>A. NIH Antiserum Treatment</u>							
1	D-32	5	559	5	557	0.996	
				5	548	0.980	
2	D-32	3	1653	3	89	0.0538	
				3	532	0.322	
3	D-32	3	592	3	208	0.351	
				3	326	0.551	
4	D-32	3	324	3	213	0.657	
				3	256	0.790	
				3	264	0.815	
<u>B. Congenic Antiserum Treatment</u>							
1	X-B10.BR	5	559	5	344	0.615	
				5	199	0.356	
2	X-B10.D2	3	592	3	882	1.49	
				3	64	0.108	
				3	269	0.454	
3	X-B10.D2	3	186	3	77	0.414	
				3	56	0.301	
4	X-B10.D2	1	187	1	167	0.893	
				1	199	1.06	

5	X-B10	1	695	1	203	0.292
				1	51	0.0734
6	X-B10	3	186	3	61	0.326
				3	107	0.572
7	X-B10.A	3	592	3	468	0.791
				3	438	0.740
				3	287	0.485

Table 27. Effect of NIH and congenic antisera on B10.D2 blastocyst embryos

Experiment Number	Antiserum	Controls		Experiments		Ratios	
		Average	Average	Number	cpm/	Experiment cpm	
		Number	cpm/	Embryos	Embryo	Average	Control cpm
		Embryos	Embryo				
<u>A. NIH Antiserum Treatment</u>							
1	D-31	3	816	3	364	0.446	
				3	281	0.344	
				3	272	0.333	
<u>B. Congenic Antiserum Treatment</u>							
1	X-B10.D2	3	816	3	162	0.199	
				3	185	0.227	
2	X-B10.D2	3	672	3	160	0.238	
				3	167	0.249	
3	X-B10	1	349	1	145	0.416	
				1	325	0.931	
4	X-B10	3	672	3	92	0.137	
				3	178	0.265	
5	X-B10	2	665	2	455	0.684	
				2	197	0.296	
6	X-B10.A	3	672	3	394	0.586	
				3	147	0.219	
7	X-B10.BR	3	672	3	69	0.103	
				3	168	0.250	

blastocyst embryos, respectively, were tested in an embryo cytotoxicity assay. A sample of the method for statistical evaluation of the data is shown in Table 24b. The congenic antisera data on lymphocytes and blastocyst embryos are summarized in Table 28. In all cases, a statistically significant inhibition of ^3H -thymidine incorporation was observed and indicates that the H-2 specific congenic antisera are detecting H-2 specificities on the blastocyst embryos.

A summary of the NIH antisera data on lymphocytes and blastocyst embryos is shown in Table 29. A statistically significant inhibition of ^3H -thymidine incorporation was seen in B10.BR and B10.D2 blastocyst embryos. The data show (consult Table 5) that both K (K^d) and D (D^k) molecules are expressed on blastocyst embryos. Since the anti- D^k serum (D-32) detects only the private specificity D^k (Table 5), it seems likely that this private specificity is expressed on $H-2^k$ blastocyst embryos. The two statistically insignificant results (D^b and K^k) could either reflect a weakness in the antisera (unlikely, since the antisera do kill lymphocytes), or else the absence or low concentration of D^b and K^k molecules on blastocyst embryos.

A summary of the cross-reactions of the congenic antisera on blastocyst embryos of different haplotypes is shown in Table 30. The first line in each experimental group represents reactions with the homologous target. It is seen that H-2 antigens are detected on both lymphocytes and blastocyst embryos. The second line in each experiment group is the negative control; the targets are of the same strain in which the antisera were made. As expected, the lymphocyte controls gave negative results. However, in three cases, the blastocyst embryo controls

Table 28. Effect of congenic antisera on lymphocytes and blastocyst embryos

Congenic Antiserum	Target Strain	% Killing of Lymphocytes \pm SEM ^a	% Inhibition of ³ H-Thymidine Incorporation \pm SEM ^b
X-C57BL/10Sn	C57BL/10Sn	93 \pm 2	83 \pm 6 (P < 0.001)* ^c
X-B10.A	B10.A	87 \pm 1	57 \pm 13 (P < 0.05)*
X-B10.BR	B10.BR	96 \pm 1	51 \pm 9 (P < 0.02)*
X-B10.D2	B10.D2	96 \pm 0	77 \pm 1 (P < 0.001)*

^aCytotoxicity of the antisera on lymphocytes was tested by trypan blue dye exclusion, as described in the Experimental Procedures.

^bCytotoxicity of the antisera on blastocyst embryos was tested by the inhibition of the incorporation of ³H-thymidine into DNA, as described in the Experimental Procedures.

^cP values obtained upon testing of embryo cytotoxicity data by Student's t test. The values reflect the probability that the % Inhibition of ³H-thymidine incorporation is significantly greater than 0%. Statistically significant P values are indicated by an asterisk (*).

Table 29. Effect of NIH antisera on lymphocytes and blastocyst embryos

NIH Antiserum	Target Strain	% Killing of Lymphocytes \pm SEM ^a	% Inhibition of ³ H-Thymidine Incorporation \pm SEM ^b
D-2	C57BL/10Sn	98 \pm 1	1 \pm 21 (P > 0.5) ^c
D-23b	B10.A	80 \pm 1	43 \pm 20 (P > 0.1)
D-32	B10.BR	86 \pm 2	39 \pm 10 (P < 0.01)*
D-31	B10.D2	73 \pm 5	63 \pm 3 (P < 0.01)*

^aCytotoxicity of the antisera on lymphocytes was tested by trypan blue dye exclusion, as described in the Experimental Procedures.

^bCytotoxicity of the antisera on blastocyst embryos was tested by the inhibition of the incorporation of ³H-thymidine into DNA, as described in the Experimental Procedures.

^cP values obtained upon testing of embryo cytotoxicity assay data by Student's t test. The values reflect the probability that the % Inhibition of ³H-thymidine incorporation is significantly greater than 0%. Statistically significant P values are indicated by an asterisk (*).

gave positive results. These numbers are in boxes in Table 30. The third and fourth lines in each experimental group represent targets which should show cross-reactivity based on Table 4. This is generally true for both the lymphocytes and the blastocyst embryos. The most notable exception is the lack of cross-reactivity of B10.A blastocyst embryos treated with anti-B10.D2 serum in experimental group D. Since $H-2^a$ is a recombinant haplotype between $H-2^k$ and $H-2^d$ (see Table 1), this result may suggest the absence of D^d antigens on $H-2^a$ blastocyst embryos.

To further investigate the ability of the antisera to recognize $H-2$ specificities on blastocyst embryos, but not on lymphocytes of the same strain as the embryos, the effect of NIH-D31 was examined on B10.BR lymphocytes and blastocyst embryos. The results are shown in Table 31. NIH-D31 is specific to K^d private specificity 31 and public specificity 34 (see Table 5). B10.BR mice do not express these specificities on adult lymphocytes (see Table 3). Therefore, the data in Table 31 strongly support the hypothesis that $H-2$ antigens exist in a modified form on blastocyst embryos as compared to adult lymphocytes of the same haplotype.

4. Effects of NIH and congenic antisera on inbred 8-cell embryos

The effects of NIH and congenic antisera on 8-cell embryos is shown in Tables 32, 33, 34, and 35 for C57BL/10Sn, B10.A, B10.BR, and B10.D2 8-cell embryos, respectively. The summary of the effect of NIH and congenic antisera on lymphocytes and 8-cell embryos is shown in Table 36. The data show that a statistically significant inhibition of

Table 30. Cross-reactions of congenic antisera with blastocyst embryos of different haplotypes

Experimental Group	Congenic Antiserum	Target Strain
A	X-C57BL/10Sn	C57BL/10Sn B10.BR B10.A B10.D2
B	X-B10.A	B10.A C57BL/10Sn B10.BR B10.D2
C	X-B10.BR	B10.BR C57BL/10Sn B10.A B10.D2
D	X-B10.D2	B10.D2 C57BL/10Sn B10.A B10.BR

^aCytotoxicity of the antisera on lymphocytes was tested by trypan blue dye exclusion, as described in the Experimental Procedures.

^bCytotoxicity of the antisera on blastocyst embryos was tested by the inhibition of the incorporation of ³H-thymidine into DNA, as described in the Experimental Procedures.

^cThe boxes reflect unexpected cross-reactions.

% Killing of Lymphocytes \pm SEM^a % Inhibition of ³H-thymidine Incorporation^b

93 \pm 2	83 \pm 6
4 \pm 2	68 \pm 9 ^c
24 \pm 8	81 \pm 3
67 \pm 4	55 \pm 11
87 \pm 1	57 \pm 13
7 \pm 2	0 \pm 2
81 \pm 3	33 \pm 8
80 \pm 7	60 \pm 13
96 \pm 1	51 \pm 9
8 \pm 5	88 \pm 8
90 \pm 0	29 \pm 12
64 \pm 2	82 \pm 5
96 \pm 0	77 \pm 1
8 \pm 4	49 \pm 16
97 \pm 2	0 \pm 14
95 \pm 3	33 \pm 17

Table 31. Effect of NIH-D31 antisera on B10.BR lymphocytes and B10.BR blastocyst embryos

A. Effect of NIH-D31 titered against B10.BR lymphocytes ^a					
	<u>Titer</u>	<u>\bar{X} % Killing \pm SEM</u>			
	1:2	1+0			
	1:4	2+0			
	1:8	2+0			
	1:16	5+2			
B. Effect of NIH-D31 antisera (1:4) on B10.BR blastocyst embryos ^b					
	<u>Controls</u>		<u>Experiments</u>		<u>Ratios</u>
	<u>Average</u>	<u>Average</u>	<u>Number</u>	<u>cpm/</u>	<u>Experiment cpm</u>
<u>Experiment</u>	<u>Number</u>	<u>cpm/</u>	<u>Embryos</u>	<u>Embryo</u>	<u>Average Control cpm</u>
<u>Number</u>	<u>Embryos</u>	<u>Embryo</u>			
1	2	331	2	177	0.5347
			2	111	0.3353
			2	294	0.8882
			2	194	0.5861
2	2	252	1	140	0.5556
% Inhibition of ³ H-thymidine incorporation \pm SEM = 42 \pm 8 (P < 0.01)* ^c					

^aCytotoxicity of the antisera on lymphocytes was tested by trypan blue dye exclusion, as described in the Experimental Procedures.

^bCytotoxicity of the antisera on blastocyst embryos was tested by the inhibition of the incorporation of ^3H -thymidine into DNA, as described in the Experimental Procedures.

^cP value obtained upon testing of embryo cytotoxicity data by Student's t test. The values reflect the probability that the % inhibition of ^3H -thymidine incorporation is significantly greater than 0%. The asterisk indicates a statistically significant P value.

Table 32. Effect of congenic anti-C57BL/10Sn serum on C57BL/10Sn 8-cell embryos

Experiment Number	Controls		Experiments		Ratios
	Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	$\frac{\text{Experiment cpm}}{\text{Average Control cpm}}$
1	5	121	5	97	0.8017
			5	116	0.9587
			5	183	1.512
2	5	137	2	48	0.3504
3	4	66	4	72	1.090
			4	38	0.5758
			4	4	0.06061
4	4	119	4	60	0.5063
5	5	127	5	87	0.6819
			5	94	0.7402
			5	85	0.6724
			5	75	0.5906
			5	109	0.8551
			5	106	0.8362
			5	75	0.5890

Table 33. Effect of congenic anti-B10.A serum on B10.A 8-cell embryos

Experiment Number	Controls		Experiments		Ratios
	Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	$\frac{\text{Experiment cpm}}{\text{Average Control cpm}}$
1	5	279	5	255	0.9140
			5	204	0.7312
2	4	111	4	119	1.072
			4	60	0.5383
			4	235	2.117
3	4	126	4	109	0.8671
			4	110	0.8690
			4	166	1.319
			4	161	1.280
			4	118	0.9365

Table 34. Effect of NIH and congenic antisera on B10.BR 8-cell embryos

Experiment Number	Controls		Experiments		Ratios
	Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	$\frac{\text{Experiment cpm}}{\text{Average Control cpm}}$
<u>A. NIH-D32 Antiserum Treatment</u>					
1	4	68	4	66	0.9706
			4	123	1.809
2	4	123	4	46	0.3740
<u>B. B10.BR Congenic Antiserum Treatment</u>					
1	5	115	5	92	0.8000
			4	64	0.5565
2	5	173	5	107	0.6185
			5	159	0.9191
			5	120	0.6936
3	4	123	4	89	0.7256
			4	140	1.140

Table 35. Effect of NIH and congenic antisera on B10.D2 8-cell embryos

Experiment Number	Controls		Experiments		Ratios	
	Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	Experiment cpm Average Contol cpm	
A. NIH-D31 Antiserum Treatment						
1	4	152	4	233	1.533	
			4	167	1.099	
2	4	167	4	204	1.222	
			4	0	0.000	
B. B10.D2 Congenic Antiserum Treatment						
1	4	180	4	109	0.6028	
			4	130	0.7222	
			4	72	0.4014	
2	5	116	5	90	0.7724	
			5	0	0.0000	
			4	87	0.7478	
3	4	155	4	157	1.349	
			4	77	0.6659	
			4	91	0.7845	
4	5	118	3	33	0.2797	
5	4	140	4	105	0.7518	
			4	195	1.393	
6	4	123	4	108	0.8780	

Table 36. Effects of NIH and congenic antisera on lymphocytes and 8-cell embryos

Antiserum	Target Strain	% Killing of Lymphocytes ^a + SEM	Total Number Embryos Tested Control	Experiment	³ H-thymidine ^b Incorporation + SEM
A. Effect of NIH Antisera					
D32	B10.BR	86+2	12	12	7+ 7 (P > 0.5) ^c
D31	B10.D2	73+5	16	16	0+ 9 (P > 0.4)
B. Effect of Congenic Antisera					
X-C57BL/10Sn	C57BL/10Sn	93+2	64	68	28+ 8 (P < 0.01)*
X-B10.A	B10.A	87+1	26	42	0+13 (P > 0.5)
X-B10.BR	B10.BR	96+1	33	32	22+ 7 (P < 0.02)*
X-B10.D2	B10.D2	96+0	57	53	28+10 (P < 0.02)*

^aCytotoxicity of the antisera on lymphocytes was tested by trypan blue dye exclusion, as described in the Experimental Procedures.

^bCytotoxicity of the antisera on 8-cell embryos was tested by the inhibition of the incorporation of ³H-thymidine into DNA, as described in the Experimental Procedures.

^cP values obtained upon testing of embryo cytotoxicity data by Student's t test. The values reflect the probability that the % inhibition of ³H-thymidine incorporation is significantly greater than 0%. The asterisk indicates a statistically significant P value.

^3H -thymidine incorporation was obtained from C57BL/10Sn, B10.BR and B10.D2, but not B10.A 8-cell embryos, when treated with the congenic antisera. The NIH antisera failed to detect H-2 specificities on B10.BR and B10.D2 8-cell embryos.

The low percent inhibition of ^3H -thymidine incorporation for statistically significant C57BL/10Sn, B10.BR and B10.D2 8-cell embryos may suggest that the H-2 specificities that are detected may be concentrated on only some of the cells of the embryos at this stage of development. Therefore, ^3H -thymidine incorporation is not greatly inhibited.

The congenic antiserum failed to detect H-2 antigens on B10.A 8-cell embryos. The NIH antiserum had failed to recognize H-2 specificities on B10.A blastocyst embryos, while the congenic antisera had recognized H-2 specificities (see Tables 28 and 29). Evidently, the recombinant event that produced the B10.A haplotype affects either the quantity or the type of H-2 specificities expressed on the B10.A embryo.

5. Effect of NIH Ia 1, 2, 3, and 7 antiserum on inbred blastocyst embryos

The data for the effect of NIH Ia 1, 2, 3, and 7 antiserum on inbred blastocyst embryos are shown in Tables 37, 38, 39, and 40 for C57BL/10Sn, B10.A, B10.BR, and B10.D2 blastocyst embryos, respectively. The results are summarized in Table 41. The antiserum does not significantly affect the ^3H -thymidine incorporation by blastocyst embryos. Therefore, the antiserum does not detect Ia specificities 1, 2, 3, or 7 on C57BL/10Sn, B10.A, B10.BR, or B10.D2 blastocyst embryos.

Table 37. Effect of NIH antiserum on C57BL/10Sn blastocyst embryos

Experiment Number	Controls		Experiments		Ratios
	Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	$\frac{\text{Experiment cpm}}{\text{Average Control cpm}}$
NIH Ia 1, 2, 3, 7 Antiserum Treatment					
1	1	299	1	591	1.976
2	1	350	1	379	1.083
3	2	237	1	344	0.9829
			2	516	2.177
			2	216	0.9114
4	2	262	2	329	1.255
			1	382	1.458

Table 38. Effect of NIH antiserum on B10.A blastocyst embryos

Experiment Number	Controls		Experiments		Ratios
	Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	Experiment cpm Average Control cpm
NIH Ia 1, 2, 3, 7 Antiserum Treatment					
1	1	237	1	263	1.109
			1	0	0.000
			1	346	1.459
			1	460	1.940
2	2	195	2	534	2.738
			2	406	2.082
3	4	26	4	73	2.807
4	2	329	2	124	0.3769
			1	232	0.7052

Table 39. Effect of NIH antiserum on B10.BR blastocyst embryos

Experiment Number	Controls		Experiments		Ratios
	Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	$\frac{\text{Experiment cpm}}{\text{Average Control cpm}}$
<u>NIH Ia 1, 2, 3, 7 Antiserum Treatment</u>					
1	1	220	1	451	2.050
			1	248	1.127
2	2	124	2	245	1.975
			2	0	0.000
3	1	140	1	245	1.750
			1	421	3.007
4	4	149	4	0	0.000
			4	288	1.932
			4	0	0.0000
5	4	164	4	208	1.268
			3	147	0.8963
6	2	346	2	283	0.8179

Table 40. Effect of NIH antiserum on B10.D2 blastocyst embryos

Experiment Number	Controls		Experiments		Ratios
	Average Number Embryos	Average cpm/ Embryo	Number Embryos	cpm/ Embryo	$\frac{\text{Experiment cpm}}{\text{Average Control cpm}}$
<u>NIH Ia 1, 2, 3, 7 Antiserum Treatment</u>					
1	1	312	1	450	1.442
			1	436	1.397
			1	160	0.5128
			1	337	1.080
			1	177	0.5673
			1	0	0.0000
2	1	189	1	61	0.3228
3	1	185	1	395	2.135
4	1	249	1	341	1.369
			1	391	1.570

Table 41. The effect of NIH Ia 1, 2, 3, and 7 antiserum on lymphocytes and blastocyst embryos

Target Strain	% Killing of Lymphocytes + SEM	Total Number of Embryos Tested		% Inhibition of ³ H-Thymidine Incorporation ^b + SEM
		Control	Experimental	
C57BL/10Sn	35 \pm 2	12	10	0 \pm 17 (P \geq 0.1) ^c
B10.A	64 \pm 2	18	15	0 \pm 2 (P \geq 0.2)
B10.BR	55 \pm 4	26	29	0 \pm 26 (P \geq 0.5)
B10.D2	46 \pm 2	8	10	0 \pm 20 (P \geq 0.3)

^aCytotoxicity of the antisera on lymphocytes was tested by trypan blue dye exclusion, as described in the Experimental Procedures.

^bCytotoxicity of the antisera on blastocyst embryos was tested by the inhibition of the incorporation of ³H-thymidine into DNA, as described in the Experimental Procedures.

^cP values obtained upon testing of embryo cytotoxicity data by Student's t test. The values reflect the probability that the % inhibition of ³H-thymidine incorporation is significantly greater than 0%. The asterisk indicates a statistically significant P value.

6. Effect of rabbit anti-mouse
 β_2 -microglobulin on CF1 and
inbred blastocyst embryos

β_2 -microglobulin comprises the 12,000 MW subunit that is noncovalently linked to the 45,000 MW subunit of the H-2 molecule. The xenogenic antiserum to β_2 -microglobulin was tested on CF1 blastocyst embryos and lymphocytes. The results are shown in Table 42. It can be seen that the antiserum is cytotoxic to lymphocytes at 1:2 and 1:4 dilutions, but rapidly loses its cytotoxic effect at higher dilutions. Unexpected results were obtained when the antiserum was incubated with CF1 blastocyst embryos in an embryo cytotoxicity assay. The percent inhibition values indicate that β_2 -microglobulin is not detectable on CF1 blastocyst embryos. Perhaps, a conformational arrangement of the β_2 molecule in relation to the H-2 molecule, prevents the antiserum from detecting its presence. Or else, perhaps the low titer of the antiserum accounts for the negative results.

The embryo cytotoxicity assay was performed with rabbit anti-mouse β_2 -microglobulin on C57BL/10Sn, B10.A, B10.BR, and B10.D2 lymphocytes and blastocyst embryos. The results are shown in Table 43. It can be seen that the lymphocytes are positive for β_2 -microglobulin, while the embryos are negative for β_2 -microglobulin.

Table 42. Summary of lymphocyte cytotoxicity assays of rabbit anti-mouse β_2 -microglobulin against CF1 lymphocytes in a lymphocyte cytotoxicity assay and against CF1 blastocyst embryos in an embryo cytotoxicity assay

Antiserum Titer	Lymphocyte Target % Killing \pm SEM	Blastocyst Target		
		Total Number of Embryos Tested		% Inhibition of ^3H -thymidine Incorporation \pm SEM
		Control	Experiment	
1:2	73	10	10	4 \pm 2
1:4	80 \pm 0	10	10	0 \pm 0
1:8	29 \pm 7	10	10	0 \pm 0
1:16	19 \pm 6	10	10	10 \pm 7
1:32	13 \pm 1	10	10	0
1:64	13 \pm 5	10	10	0 \pm 0
1:128	2 \pm 1	10	10	27 \pm 19
1:256	5 \pm 1	10	10	0 \pm 0
1:512	8 \pm 1	10	10	0 \pm 0

Table 43. The effect of rabbit anti-mouse β_2 -microglobulin (1:4) on inbred lymphocytes in a lymphocyte cytotoxicity assay and on inbred blastocyst embryos in an embryo cytotoxicity assay

Target	Lymphocytes % Killing \pm SEM	Blastocyst Embryos		
		Total Number of Embryos Tested		% Inhibition of ^3H -thymidine Incorporation \pm SEM
		Control	Experiment	
C57BL/10Sn	73 \pm 5	8	4	7 \pm 5
B10.A	83 \pm 5	4	4	16 \pm 0
B10.BR	88 \pm 3	6	6	29 \pm 18
B10.D2	40 \pm 5	4	4	0 \pm 0

E. Development of the Mixed Embryo-Lymphocyte Interaction Assay

1. Blastocyst embryo viability in lymphocyte media

The ability of blastocyst embryos to incorporate ^3H -thymidine was used as a viability measurement of blastocyst embryos in lymphocyte media. Blastocyst embryos were incubated for 4, 7 and 24 hours in different lymphocyte media and in Whitten and Biggers medium. The results are shown in Figure 24. It can be seen that blastocyst embryos in Whitten and Biggers medium alone gradually lose their ability to incorporate ^3H -thymidine. Blastocyst embryos in supplemented RPMI 1640 medium, containing serum, maintain a steady ability to incorporate ^3H -thymidine over time. RPMI 1640 medium, alone, was deleterious to blastocyst embryos, as shown by the decrease in ^3H -thymidine incorporation over time. Supplemented RPMI 1640 medium, containing 10% newborn calf serum was used for experiments in which blastocyst embryos were to be cultured with lymphocytes.

2. Partial purification of T-cell growth factor

T-cell growth factor (TCGF) was purified from concanavalin A according to the methods of Rosenberg et al. (1980) and Heide and Schwich (1978). The $(\text{NH}_4)_2\text{SO}_4$ precipitate was dissolved in saline and layered on a Sephadex G-100 column. The column was eluted with 0.9% saline. Each fraction was examined for protein content by spectrophotometric absorbance at 280 nm and by the Lowry protein test. TCGF exhibits a

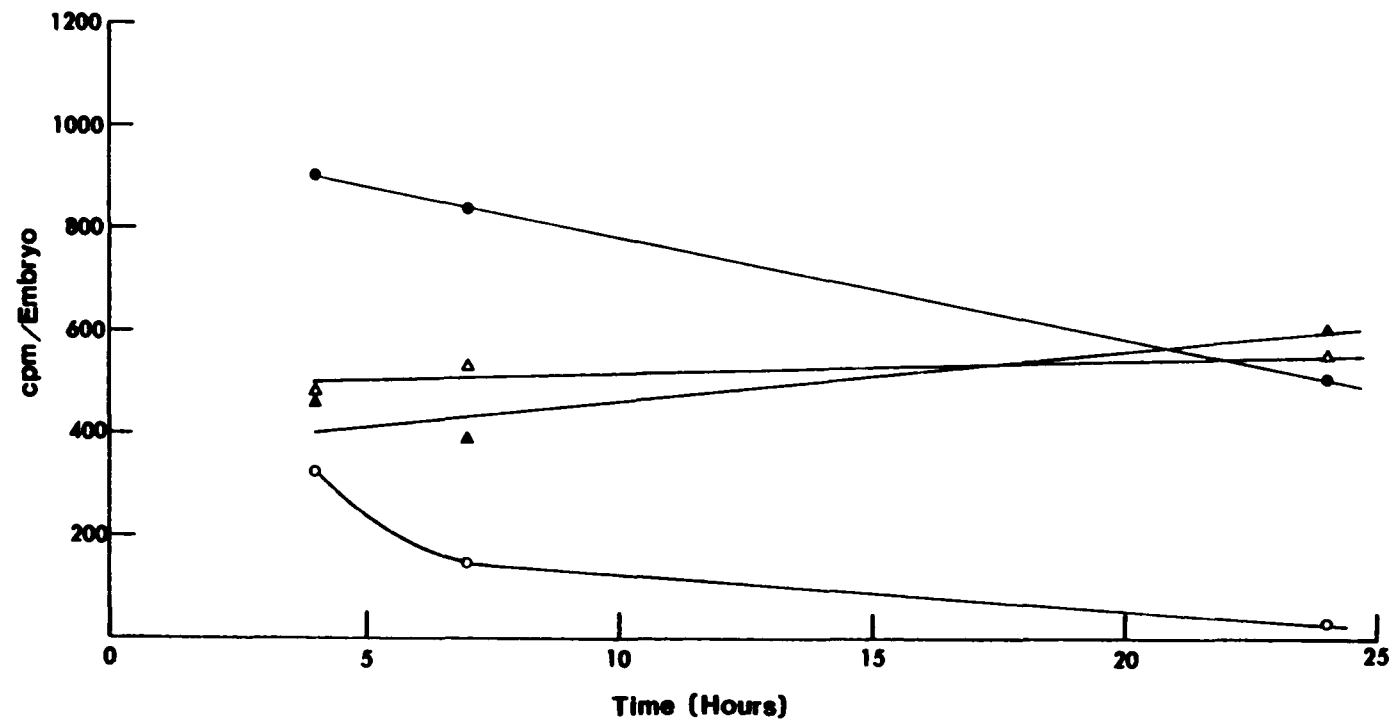


Figure 24. The ability of CF1 blastocyst embryos to incorporate ^3H -thymidine when incubated for 4, 7 and 24 hours in Whitten and Biggers medium (●), RPMI 1640 (◐), supplemented RPMI 1640 with 5% human serum (Δ), and supplemented RPMI 1640 with 10% newborn calf serum (▲)

mitogenic activity when incubated with cytotoxic T cells. Fractions in the appropriate molecular weight ranges were examined for mitogenic activity in a DNA synthesis assay involving cytotoxic cells generated in a mixed lymphocyte reaction.

Figure 25 exhibits the 280 nm absorbance profile for the fractions eluted from the Sephadex G-100 and the mitogenic effect of the fractions on ^3H -thymidine incorporation by cytotoxic T-cells. The TCGF activity is clearly separate from the serum albumin (MW 69,000) and the concanavalin A (MW 90,000) peaks. The mitogenic activity occurred in those molecular weight fractions (MW 50,000-45,000 and MW 30,000-20,000) which characterize TCGF. The fractions containing the three mitogenic activity peaks were pooled and used to maintain long-term cytotoxic T cells.

3. Production and maintenance of cytotoxic T cells in vitro

T-cell growth factor was added to supplemented RPMI 1640, containing 10% NBCS. The medium was then used to culture cytotoxic T cells (B10.CBA_M). Figure 26 demonstrates the growth curve of cytotoxic T cells in TCGF supplemented medium. Sensitizations against CBA_M spleen lymphocytes are indicated as 2°, 3° and 4°. Cytotoxic assays were performed on days marked by T_c. For each data point, the cytotoxic T cells were counted, then centrifuged at 500 g and room temperature. The supernatant was decanted and replaced by fresh medium containing TCGF. For 2°, 3° and 4° sensitizations, CBA_M spleen lymphocytes were present in the TCGF supplemented medium. It can be seen that cytotoxic T cells

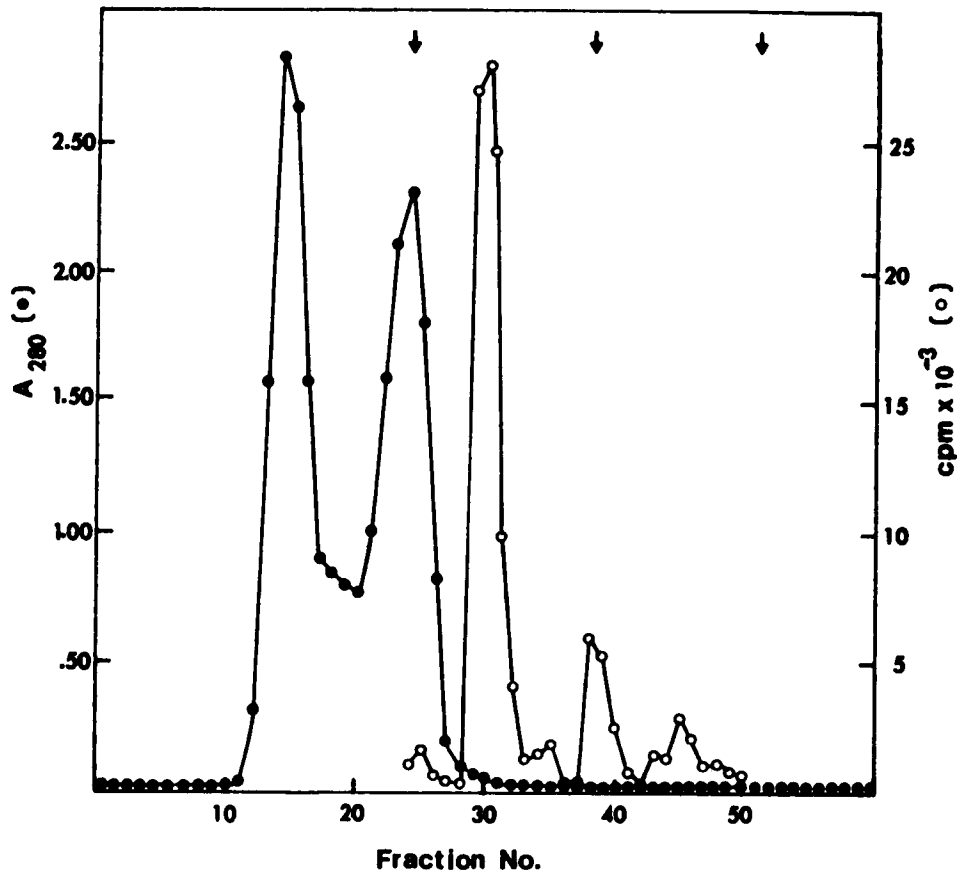


Figure 25. Fraction profile of T-cell growth factor (TCGF) run over a Sephadex G-100 column. The fractions were examined for protein content by spectrophotometric readings at A₂₈₀ (●). TCGF mitogenic activity was measured in a DNA synthesis assay with cytotoxic T cells (○). Protein standard markers were bovine serum albumin (69,000 MW), human chorionic gonadotrophin (26,700 MW) and ribonuclease A (13,000 MW)

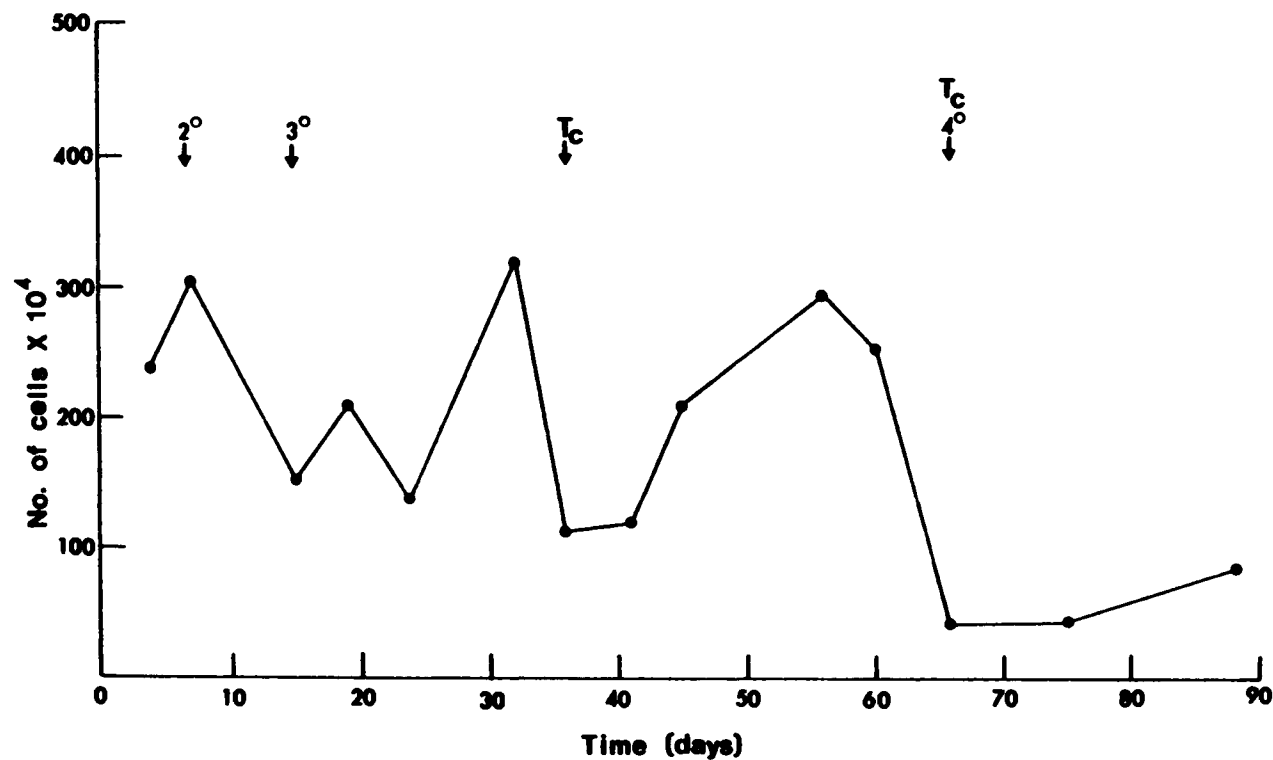


Figure 26. The cell growth of B10.CBA_M cytotoxic T cells in supplemented RPMI 1640 medium, containing 10% newborn calf serum and TCGF (1:10). The initial mixed lymphocyte reaction was set up on day 0 with 3.6×10^7 cells total. 2° , 3° , 4° refer to secondary, tertiary and quaternary sensitizations with CBA_M spleen lymphocytes in medium containing TCGF. T_c refers to the days on which cell-mediated lympholysis and/or mixed embryo-lymphocyte interaction assays were performed

were successfully cultured in the presence of TCGF. Cytotoxic cells were unable to be detected after 60 days of culture.

4. Mixed embryo-lymphocyte interaction assay (MELIA)

Table 44 gives the cytotoxic response of T_c cells to ^{51}Cr -labeled lymphocytes and to B10.BR blastocyst embryos. It can be seen that cytotoxic T cells are capable of affecting the ability of pronased B10.BR blastocyst embryos to incorporate ^3H -thymidine. The ^{51}Cr release by B10 cytotoxic lymphocytes to CBA target lymphocytes confirms the cytotoxicity of the B10 lymphocytes. Since the cytotoxic response of cytotoxic T cells is dependent on K and D antigen recognition, MELIA functionally demonstrates the presence of H-2 K and D antigens on B10.BR blastocyst embryos.

Table 44. The effect of B10.CBA_H cytotoxic T cells against CBA lymphocytes in a ⁵¹Cr^M release microassay and against B10.BR blastocyst embryos in the mixed embryo-lymphocyte interaction assay. Cytotoxic T cells were incubated with lymphocytes or embryos for four hours

Cytotoxic T cell Concentration	CBA Lymphocytes ^a		B10.BR Embryos ^b	
	cpm ± SEM	% Specific Release	cpm ± SEM	% of Control
0	0 ± 0	0	414 ± 45	100
1 x 10 ⁴ cells	313 ± 23	15	14 ± 5	3
4 x 10 ⁴ cells	288 ± 22	4	117 ± 93	28

^a% Specific Release =

$$\frac{\text{cpm of experimental release} - \text{cpm of spontaneous release}}{\text{cpm of maximum release} - \text{cpm of spontaneous release}} \times 100.$$

^b% of Control = $\frac{\text{Average Experimental cpm}}{\text{Average Control cpm}} \times 100.$

IV. DISCUSSION

The first goal of these studies was to develop a cytotoxicity test based on ^3H -thymidine incorporation into the DNA of preimplantation mouse embryos. Parameters for the incorporation of ^3H -thymidine into DNA were optimized and the following is a summary of the final conditions chosen: (1) 4 $\mu\text{Ci/ml}$ ^3H -thymidine (sp. act. 20 Ci/mmol); (2) a ^3H -thymidine labeling period of two to seven hours; (3) a three-hour preincubation period for blastocyst embryos and from zero to seven hours preincubation period for 8-cell embryos; (4) from 1-64 embryos per assay; and (5) presence or absence of the zona pellucida.

The ability of embryos to incorporate ^3H -thymidine was then used as a measurement of embryo viability after treatment with various compounds or antisera and complement. The main advantage of this assay is that it can be performed in a microtiter plate and, therefore, can be processed automatically using a Titertek cell harvester. An assay can be performed and results can be obtained in less than 24 hours, whereas, another assay system (i.e., autoradiography) would require weeks or even months to obtain the final result. Another advantage of this assay is the inherent objectivity in obtaining the final results. The cytotoxicity assay with rabbit anti-mouse sera could be visually scored as to the effect of antisera and complement, since the effect is rather pronounced (see Figure 17), but for antisera with lower cytotoxicity titers (i.e.,

Table 28 and 29) the effect is not as pronounced and is more open to subjective evaluation. Incorporated ^3H -thymidine from experimental tests can be statistically compared to control tests to determine the effectiveness of the antisera and, consequently, the presence of the target antigen.

A few negative aspects of this assay should also be mentioned. For instance, this assay would not be feasible to use for cell cycle studies of the G_1 , S and G_2 phases where autoradiography and cytophotometry have played a predominant role in determining which cells within the embryo were entering the different phases of the cell cycle (reviewed by Sherman, 1979). Also, this assay is not practical to use if embryonic DNA is to be collected and used for another test purpose. The DNA is bound to the GF/C filter papers during the cell harvesting procedure and is not easily eluted from the papers.

As a viability measure, this assay is particularly good because it is (1) sensitive--an individual blastocyst embryo can be successfully assayed; (2) reproducible--as long as control embryos and test embryos are assayed at the same time, so that ratios from experiment to experiment can be compared; and (3) automated--the number of assays that can be performed is limited only by the availability of embryos or test compounds.

The DNA synthesis assay, as described in this dissertation has four potential applications: (1) study of the mechanisms and control of DNA synthesis in preimplantation mouse embryos; (2) evaluation of the number of cells per embryo; (3) study of the effects of various compounds which

might affect cell division, and, therefore, DNA synthesis, in preimplantation mouse embryos; and (4) use of the assay as a measure of embryo viability after treatment with antisera and complement. The first application is not discussed in this dissertation. The second application is shown to be feasible in a given population of embryos, as demonstrated in Figure 11. This is important when measuring enzyme activities in embryos, because the data may then be expressed on a per cell as well as per embryo basis.

The third application was used to study the effects of salt, α -amanitin, aphidicolin, theophylline, cycloheximide, puromycin, and hCG on cell division by preimplantation mouse embryos. Increased salt concentrations impair the ability of blastocyst embryos to incorporate ^3H -thymidine (Figure 12) and corroborates early reports where increased salt concentrations prevented cleavage division (Whittingham, 1971).

α -Amanitin, an RNA inhibitor, was shown to affect ^3H -thymidine incorporation in 8-cell and blastocyst embryos (Table 11). It was interesting to note that 8-cell embryos were more refractory to inhibition than were blastocyst embryos. This result was opposite to that for RNA synthesis studies, in which blastocyst embryos were more refractory to α -amanitin than 8-cell embryos (Warner and Hearn, 1977). This may reflect that 8-cell embryos were more actively synthesizing RNA as compared to DNA and blastocyst embryos were more actively synthesizing DNA as compared to RNA. During cleavage, DNA must be synthesized de novo from each new cell, but pre-existing RNA could be divided among cells because the size of the cells is constantly decreasing during cleavage division

during preimplantation mouse development, though the total size of the embryo is remaining constant. Barlow et al. (1972) demonstrated a higher ^3H -thymidine labeling index for "inside" cells as opposed to "outside" cells of preimplantation mouse embryos. They concluded that the "inside" cells were dividing faster than the "outside" cells. The 8-cell embryo does not exhibit this cellular differentiation into "inside" versus "outside" cells, but the blastocyst embryo exhibits inner cell mass constituents as opposed to outer, trophectodermal cells. The rapidly dividing inner cell mass of the blastocyst could be the main target of α -amanitin action. This idea remains to be tested experimentally.

Weinmann and Roeder (1974) have shown that Form II RNA polymerase is inhibited by 0.03 $\mu\text{g/ml}$ α -amanitin, Form III enzyme by 200 $\mu\text{g/ml}$ and Form I enzyme is refractory to inhibition by α -amanitin. Cleavage division and RNA synthesis are blocked by α -amanitin at concentrations as low as 1 $\mu\text{g/ml}$ (Warner and Versteegh, 1974; Levey et al., 1977; Levey and Brinster, 1978). It was concluded that inhibition of Form II enzyme and, consequently, mRNA synthesis is blocked at 1 $\mu\text{g/ml}$. The high concentration of α -amanitin needed to elicit significant inhibition of DNA synthesis in mouse embryos may suggest that Form III enzyme is directly affected by the inhibitor.

The mechanism of inhibition of DNA synthesis by α -amanitin is unknown. It is possible that the synthesis of an RNA primer, required for the initiation of DNA synthesis, is blocked. It is also possible that crucial mRNA production, such as that for DNA polymerase or thymidine kinase is blocked.

Aphidicolin was shown to affect ^3H -thymidine incorporation in 8-cell and blastocyst embryos (Figure 14). This appears to be the first report of aphidicolin's effect on preimplantation mouse embryos. The effect of aphidicolin on sea urchins (Oguro et al., 1978), rat liver cells (Ohashi et al., 1978) and human embryonic lung cells (Bucknall et al., 1973) have been examined. Aphidicolin doses in the range of 5–15 $\mu\text{g}/\text{ml}$ completely blocked DNA synthesis (i.e., cell cleavage) in these systems. In this study 20 $\mu\text{g}/\text{ml}$ of aphidicolin completely blocked DNA synthesis (i.e., ^3H -thymidine incorporation) in 8-cell and blastocyst embryos. Since aphidicolin is a specific inhibitor of animal DNA polymerase- α , but not β or γ forms (Oguro et al., 1978), it can be concluded that mouse embryos of the 8-cell and blastocyst stages of development express functional DNA polymerase- α . This finding is interesting in light of Kiessling and Weitlauf's (1981) recent report where they detected a DNA polymerase- γ -like enzyme which increased in activity when the morula differentiates into the inner cell mass and the trophectoderm of the blastocyst. They have suggested that DNA polymerase- γ may have a specialized, differentiation function in development. It would be interesting to incubate morula embryos with aphidicolin to see if the differentiation process to the ICM and trophectoderm would still occur. If no inhibition was observed, this would support the hypothesis that a DNA polymerase- γ -like enzyme was responsible for the differentiation process.

Theophylline was seen to have a negligible effect on ^3H -thymidine incorporation by blastocyst embryos (Figure 15). Theophylline inhibits

phosphodiesterase which changes cyclic AMP to AMP. It is possible that phosphodiesterase and cyclic AMP do not play a major role in the biochemistry of the blastocyst embryo. By examining in vitro medium requirements, it was shown that embryos were able to use only certain carbon sources during their development. Growth of 1-cell embryos was supported only by oxaloacetate or pyruvate. Growth of 2-cell embryos was supported by lactate, oxaloacetate, pyruvate, or phosphoenolpyruvate. After the 8-cell stage, embryos could also utilize glucose, malate, and α -ketoglutarate as energy sources (Whittingham, 1971). Therefore, it is quite reasonable to conclude that the embryonic genome is slowly activated during development and that adult enzymes may not be present in early stages of development.

Cycloheximide and puromycin at 10^{-5} M inhibited ^3H -thymidine incorporation by blastocyst embryos (Figure 15) and suggests that blastocyst embryos are producing proteins which are probably essential for DNA replication.

Since embryos are constantly exposed to hormones present in the uterine fluid during early pregnancy, it would seem possible for hormones to affect the molecular machinery of the embryo. Steroid hormones have been shown to have no effect on RNA synthesis in preimplantation mouse embryos (Warner and Tollefson, 1978). Wiley (1980) demonstrated that an hCG-like molecule was present on the surface of inner cell mass cells, but not on the surface of trophectodermal cells. Instead, the hCG-like molecule was detectable in the cytoplasm of trophectodermal cells. The hCG-like molecule was detectable by antiserum to the α -subunit of hCG.

Based on Wiley's findings, and the negative results reported in this thesis for hCG and the β -subunit of hCG (Table 12), it would be interesting to examine the effect that hCG and the α -subunit would have on late 8-cell embryos as they divide in culture to form morulae. Perhaps, the hCG serves the function of indirectly affecting differentiation into not only the ICM and trophoctodermal cells following the morula stage of development, but also affects the differentiation of the ICM and trophoctodermal cells into giant cells and phagocytic-like cells at the time of implantation.

Finally, experiments in which the DNA synthesis assay was used to measure embryo viability, after treatment with antisera and complement, will be discussed. The Cytotoxicity assay for 8-cell and blastocyst embryos consists of incubation with antiserum or normal serum for one hour, then with guinea pig complement for one hour, followed by a five hour incubation period with ^3H -thymidine.

The data (Table 28) demonstrate that mouse blastocyst embryos of the H-2 b, a, k, and d haplotypes express H-2 antigens as demonstrated by the embryo cytotoxicity assay performed with a congenic antiserum directed to cell surface proteins of the H-2 complex. It is possible to obtain complete elimination of DNA synthesis in mouse blastocyst embryos by using a multispecific antiserum directed to mouse cell surface proteins (Figure 16). This implies that both cells of the trophoctoderm and of the inner cell mass are killed by the antisera treatment. However, the use of more restricted antisera (Tables 4 and 5), did not give complete killing of the embryos (Tables 28 and 29).

This may be due to the expression of different cell surface antigens on the inner cell mass as opposed to the trophoctoderm. Therefore, an anti-serum which would bind to one cell type would not necessarily bind to the other cell type. Also, the quantitative expression of H-2 antigens may vary from haplotype to haplotype of mouse embryos. O'Neill and McKenzie (1980) demonstrated that H-2K and H-2D antigen expression on spleen lymphocytes varied quantitatively among the different haplotypes tested.

It is clear from the data that different conclusions about the expression of H-2 antigens on blastocyst embryos are generated by examining different haplotypes. Only when a complete array of antisera are available to detect K and D molecules of each of the haplotypes will definite conclusions be able to be drawn about the presence or absence of these molecules on blastocyst and 8-cell embryos. From the present study, it appears that K^d and D^k molecules are expressed on blastocyst embryos, but D^b , K^k and D^d molecules are not expressed. The development of monoclonal antibodies of defined H-2 specificities should facilitate further studies in this area.

Of special interest in this study are the unpredicted cross-reactivities seen with H-2^k and H-2^b blastocyst embryos (Table 30). Ostrand-Rosenberg et al. (1977) found similar aberrant cross-reactions when studying blastocyst outgrowths of the H-2^q and H-2^s haplotypes. They also noted that blastocyst outgrowths showed different specificities from lymphocytes of the same haplotype. The data shown in Table 30, using the congenic antisera, and the data shown in Table 31, using NIH

antisera specific to K^d on B10.BR blastocyst embryos (H-2 $K^k D^k$) and lymphocytes, suggest that this same conclusion is appropriate when comparing blastocyst embryos and lymphocytes in our system.

There are several possible explanations for the observed aberrant cross-reactivities. One is that the unexpected antigens on embryos may be due to derepression of pre-existing genetic information for H-2 specificities of different haplotypes. Garrido et al. (1976) have shown that foreign H-2 haplotypes could be expressed by certain cell lines after viral infection. Perhaps, embryos, too, possess the genetic information for more than one H-2 haplotype, then upon differentiation, embryos could process their DNA to allow the expression of only one adult H-2 haplotype. Another possibility is that embryos express a general multispecific antigen, which later gives rise to the haplotype specific H-2 antigens found in adult tissues. This hypothesis is especially appealing because it could explain how embryos escape maternal immune surveillance. The multispecific H-2 antigen would present paternal antigens in a form unrecognizable by the maternal system and would, therefore, not elicit an immune response.

Ia antigens do not appear to be present on blastocyst embryos (Table 41) and, therefore, earlier reports (Delovitch et al., 1978; Jenkinson and Searle, 1979) are confirmed.

H-2 antigens were detected on 8-cell embryos of the b, k and d haplotypes in embryo cytotoxicity assays involving congenic antigens (Table 36). The more restricted NIH antisera (D32 and D31) were unable to detect H-2 antigens on 8-cell embryos of the k and d haplotypes.

These results suggest that H-2 antigens are expressed in lower concentrations on 8-cell embryos than on blastocyst embryos, since the restricted NIH antisera (D32 and D31) were able to detect H-2 antigens on blastocyst embryos of the k and d haplotypes (Table 29). Krco and Goldberg (1977) reported the presence of H-2 antigens on 8-cell embryos by visual observation of lysis of one to eight cells of the 8-cell embryo by antiserum and complement. If H-2 antigens are expressed on only some cells of the 8-cell embryo, as suggested by Krco and Goldberg's data, then the sensitivity of the embryo cytotoxicity assay described in this dissertation would have to be enhanced to get more meaningful results. One method of enhancement would be to utilize the "sandwich" technique in which embryos would first be treated with anti-H-2 serum, then incubated with rabbit anti-mouse immunoglobulin, and then finally incubated with guinea pig complement. The rabbit anti-mouse immunoglobulin would bind to two antigenic mouse immunoglobulin molecules which may each be bound to only one H-2 molecule, rather than two. Therefore, complement would bind to the rabbit anti-mouse immunoglobulin molecule and initiate the complement cascade.

The inability to detect β_2 -microglobulin (β_2m) on mouse embryos (Tables 42 and 43) was somewhat puzzling in light of a brief communication by Håkansson and Peterson (1976) in which they used indirect immunofluorescence to detect β_2m on delayed implanting blastocyst embryos and on estradiol-activated blastocyst embryos. There are three possible explanations for this discrepancy. First, there was a difference in antiserum in their study and ours. Håkansson and Peterson used affinity

column purified antibody to human β_2m , whereas rabbit anti-mouse β_2m serum was used in this study. Antibodies raised to human β_2m have been shown to cross-react extensively with mouse β_2m (Rask et al., 1974; Østberg et al., 1975), therefore, this difference may not be critical. Second, the cytotoxic titer, of the rabbit anti-mouse β_2m used in this study was quite low. It decreased greatly at a 1:16 dilution against lymphocyte targets. The low cytotoxicity titer suggests that the β_2m antibody concentration probably was not large enough to be able to detect β_2m on the embryos. Third, this study used early blastocyst embryos (89 hours post-hCG) which had not been induced to enter into a state of delayed implantation nor had been estradiol-activated. It may be that β_2 -microglobulin is not expressed on early mouse blastocysts or that a non- β_2m light chain may be expressed instead. There is some precedence for this in the literature, as discussed below.

F9 antigens are common to primitive teratocarcinoma cells, to preimplantation embryos and to spermatozoa (Artzt et al., 1973). The F9 molecule is composed of two heavy chains (44,000 MW each) crosslinked by a disulfide bridge and two light chains (12,000 MW each) (Vitteta et al., 1975). The light chains were believed to be β_2 -microglobulin chains, but serological studies in conjunction with immunofluorescence have demonstrated that the light chains are not detected by rabbit anti-mouse β_2 -microglobulin (Dubois et al., 1976). Due to the fact that F9 antigens are a product of the T/t locus on chromosome 17 and due to their structural homology with H-2 antigens, it has been postulated that the expression of H-2 and T/t (i.e., F9) antigens may be reciprocal, with

the F9 antigen appearing during early development prior to the appearance of H-2 antigens (Artzt and Bennett, 1975). Therefore, it would seem possible that a transitory form of β_2 -microglobulin may exist, attached to H-2 antigens in early preimplantation development. This transitory " β_2 -microglobulin like" molecule may also provide a conformational form of the H-2 antigen that alters the antigenic determinants so that they differ from those determinants expressed in the adult tissue when β_2 -microglobulin is complexed to the H-2 subunit.

The ability of β_2 -microglobulin to affect conformational change and, subsequently, antigenicity of the H-2 molecule is not clear. Sege et al. (1979) demonstrated that presence or absence of β_2 -microglobulin did not affect the ability of anti-H-2 sera to bind to H-2 antigens. In contrast, in man it has been reported that absence of β_2 -microglobulin from HLA seriously impaired antisera reactivity to the intact HLA molecule (Lancet et al., 1979).

It has been reported that H-2 antigens on mouse lymphocytes are not all expressed with intact β_2m (Sege et al., 1979). If the adult tissue lacks β_2m , it may be that the embryonic H-2 lacks β_2m . An examination of the ability of exogenous β_2m to bind to embryonic H-2 antigens may further elucidate this problem. Schmidt et al. (1981) demonstrated that exogenous β_2m was capable of binding to H-2 on mouse lymphocytes and tumor cells.

One other point of interest (see Table 43) is that the cytotoxicity of rabbit anti-mouse β_2 -microglobulin on lymphocytes is noticeably lower for B10.D2 (H-2^d) lymphocytes than for the other strains tested. Schmidt

et al. (1981) reported a similar low reactivity of anti- β_2m serum for the d haplotype. It seems that the binding affinity of β_2m to $H-2^d$ molecules is lower than that of the other haplotypes.

The last part of this dissertation deals with some preliminary attempts to detect H-2 antigens on mouse embryos by using specific cytotoxic T cells, rather than serum antibody. Ostrand-Rosenberg (1980) demonstrated the feasibility of using cell-mediated immune responses to detect embryonic antigens. She employed two test systems--one, the mixed cell culture system, in which syngeneic lymphocytes were added to syngeneic blastocyst outgrowth cells and blastogenesis by the lymphocytes was measured by 3H -thymidine uptake. The second system involved the growth inhibition assay in which lymphocytes from a five-day mixed cell culture reaction were incubated with target embryonic cell lines for 48 hours, then pulsed with 3H -thymidine for 24 hours. Inhibition of cell growth was recorded as a reduction in cell counts.

The cell-mediated lympholysis reaction involves four stages: (1) cell-cell interaction, (2) programming for lysis, (3) a lethal hit, and (4) the rupture of the target cell's membrane (Henney, 1980). Mg^{2+} is needed for the binding of the effector cell to its target. Ca^{2+} and cAMP appear to have roles in the events following cell-cell interaction, though the exact mechanism of programming for lysis is unclear. The lethal hit has been postulated to involve soluble factors, a membrane associated phospholipase, or even effector cell surface enzymes with trypsin-like activity. The target cells appear to be destroyed by osmotic forces, resulting from the influx of water into the cell (Henney, 1980).

The cell-mediated lympholysis reaction is dependent on H-2K and H-2D antigen recognition by effector cells (cytotoxic T cells).

Cytotoxic T cells were generated and their ability to kill mouse embryos was tested. The preliminary data presented in Table 44 indicate that cytotoxic T lymphocytes, sensitized to H-2^k lymphocytes, are cytotoxic to H-2^k embryos. These results provide functional proof that H-2 antigens are expressed on preimplantation mouse embryos.

The cytotoxic T cell response is very specific. Cytotoxic T cells, sensitized to H-2 antigens, do not kill murine teratoma cells bearing F9, but lacking H-2 antigens, in spite of the structural similarities between H-2 and F9 (Forman and Vitetta, 1975).

The specificity of the H-2 antigens recognized on mouse blastocyst embryos by cytotoxic T cells is uncertain. Vazquez et al. (1980) report that public H-2 specificities are the target determinants for alloreactive cytotoxic T lymphocytes. Therefore, the cytotoxic T cells may be recognizing public H-2 specificities on blastocyst embryos.

The embryo-lymphocyte assay was performed by using cytotoxic T cells, sensitized to adult lymphocytes. It would be interesting to examine the specificity of cytotoxic T cells generated to embryonic cells, especially since the embryo appears to be expressing H-2 antigens which differ from the H-2 antigen of adult tissues. Schirmacher et al. (1980) demonstrated that cytotoxic T lymphocytes sensitized to an H-2^d derived sarcoma were capable of lysing not only the sarcoma cells, but also H-2^k bearing target cells. The H-2^d sarcoma cells were believed to express public specificities common to the H-2^k haplotype.

Cell-mediated cytotoxicity reactions need to be handled carefully in order to obtain meaningful data. Goldstein et al. (1978) found that target cells and sensitizing cells exhibited adsorbed xenogeneic serum constituents on their cell surfaces. Cytotoxic T cells were sensitized to these xenogeneic determinants and, therefore, lysed target cells which had been cultured in the presence of the xenogeneic serum, though the target cells had been washed several times. It therefore appears that the detection of H-2 antigens on preimplantation embryos by using cytotoxic T cells will be possible, but care must be taken in experimental design.

V. CONCLUSIONS

Preimplantation mouse embryos were found capable of incorporating ^3H -thymidine into their DNA. This ability was utilized in three different ways. First, this ability was correlated to the number of cytologically determined cells present in the embryos from different strains of mice. A 0.96 correlation coefficient was obtained, suggesting the feasibility of using DNA synthesis by embryos to determine the average number of cells per embryo in a given population of embryos.

Second, the ability to incorporate ^3H -thymidine was utilized to examine the effect of various compounds on DNA synthesis in preimplantation mouse embryos. Ability to incorporate ^3H -thymidine demonstrated that blastocyst embryos were refractory to theophylline and hCG, while salt, α -amanitin, aphidicolin, cycloheximide, and puromycin impaired ^3H -thymidine incorporation by blastocyst embryos. α -Amanitin is able to directly affect DNA synthesis in blastocyst embryos and to a lesser extent, in 8-cell embryos. Aphidicolin has been used in other animal systems, but not previously on 8-cell and blastocyst embryos.

Third, the ability to incorporate ^3H -thymidine was utilized in two types of cytotoxicity assays as an embryo viability indicator. In the embryo cytotoxicity assay, embryos were incubated with antiserum and complement and cell death was measured by inhibition of ^3H -thymidine incorporation into DNA. The use of congenic and NIH anti-H-2 sera on the embryos, demonstrated that H-2 antigens were detectable on 8-cell

and blastocyst embryos. K^d and D^k molecules were detectable on blastocyst embryos, but D^b , K^k and D^d molecules were not detectable. An interesting aspect of this study was the finding that H-2 antigens on blastocyst embryos exhibit specificities which differ from those specificities present on adult lymphocytes. This general, multispecific H-2 antigen on the embryo was postulated to serve the function of allowing the embryo to escape immune surveillance by the mother during early mammalian embryogenesis. Eight-cell embryos were found to express H-2 antigens, but in lower concentrations than on blastocyst embryos. Ia antigens were not detectable on blastocyst embryos and this finding confirms earlier negative reports. β_2 -microglobulin was not detected on mouse blastocyst embryos which expressed H-2 antigens. The meaning of this result is not clear. A cell-mediated lympholysis reaction, in which cytotoxic T cells were used against blastocyst embryo targets, showed the functional presence of H-2 antigens on blastocyst embryos.

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