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STRAIN DIFFERENCES AND MUTAGEN TREATMENT INFLUENCE
LECTIN BINDING TO MOUSE GERM CELLS

by

Marilyn Schmalz Holm

A Thesis
Submitted to the
Faculty of The Graduate College
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Western Michigan University
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STRAIN DIFFERENCES AND MUTAGEN TREATMENT INFLUENCE
LECTIN BINDING TO MOUSE GERM CELLS

Marilyn Schmalz Holm, M.S.

Western Michigan University, 1986

Carbohydrate moieties on mouse germ cell surfaces were characterized by investigating the binding of FITC-labeled lectins to sperm and testis cells. Differences in lectin binding between different mouse strains, including T/t mice, were found. However, there was no evidence for the involvement of sialic acid or L-fucose in the T/t locus antigens. Antiserum to the t⁰, t⁶ haplotype was equally reactive to both ICR and T/t mouse testis cells in both immunofluorescence and cytotoxicity assays. Trypsinization of testis cells increased binding of soybean agglutinin and decreased binding of wheat germ agglutinin.

A method was developed for flow cytometry of testis cells dual-labeled with a DNA stain plus FITC-lectin. This allowed analysis of haploid (1C), diploid (2C), and dividing (4C) cell populations. Treatment of mice with the mutagen, ethylnitrosourea, resulted in increased binding of soybean agglutinin, wheat germ agglutinin, concanavalin A, and Limax flavus agglutinin in 1C, 2C, and 4C cells.

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Lastly, I would like to dedicate this thesis to my husband, Jim, who has patiently endured countless nights of fast food and midnight oil during my pursuit of this degree.

Marilyn Schmalz Holm

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BINDING TO MOUSE GERM CELLS**

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	ii
LIST OF TABLES	vi
LIST OF FIGURES	vii
INTRODUCTION	1
LITERATURE REVIEW	3
Germ Cells.	3
Testis Cells	3
Sperm	4
Flow Cytometry of Germ Cells	6
<u>T/t</u> Locus	8
Lectins	10
Mechanisms for Altered Lectin Binding by Testis Cells . . .	11
Germ Cells	11
Somatic Cells	13
MATERIALS AND METHODS	14
Materials	14
Diluent	14
Lectins	14
Antisera	16
Mice	16
Sperm Methodology	16
Preparation of Sperm Suspensions	16
Staining of Sperm with Lectins	17

Table of Contents--Continued

Fluorescence Microscopy	18
Testis Cell Methodology	18
Preparation of Testis Cell Suspensions	18
Percoll Density Gradient Centrifugation.	20
Staining of Testis Cells	20
Microscopy	22
Flow Cytometry	22
FITC-Labeled Sperm and Testis Cells	22
Testis Cells Dual-Labeled With FITC and DNA Stain . .	23
<u>T/t</u> Locus Antiserum Methodology	25
Immunofluorescence	25
Cytotoxicity	26
Mutagen Treatment of Mice	27
RESULTS	28
Lectin Binding to Sperm of Different Mouse Strains.	28
Flow Cytometry	28
Fluorescence Microscopy	28
Lectin Binding to Testis Cells of Different Mouse Strains .	32
Effect of Trypsinization on Lectin Binding.	34
Studies with Antiserum to <u>T/t</u> locus Antigen	34
Percoll Density Gradient Centrifugation	36
Effect of Mutagen Treatment on Lectin Binding	36
DISCUSSION	45
Mouse Strain Differences in Lectin Binding	45

Table of Contents--Continued

Sperm Studies	45
Testis Cell Studies	47
Effect of Trypsinization on Lectin Binding	48
Antiserum to <u>T/t</u> Locus Antigen	49
Effect of Mutagen Treatment on Lectin Binding	51
APPENDICES	
A. Lectin Carbohydrate Binding Specificities	55
B. TWOGATEFL Cytofluorograf Program: Figure 1	57
C. FITCPI2 Cytofluorograf Program: Figures 2-4.	60
D. Lectin Binding to Sperm: Figures 5-7.	65
E. Lectin Binding to Testis Cells: Figures 8-14	69
F. Antiserum to <u>T/t</u> Locus: Figure 15	77
G. Mutagen Effects: Figures 16-29	79
BIBLIOGRAPHY	94

LIST OF TABLES

1. Binding of FITC-Lectins to Mouse Sperm	30
2. Mouse Strain Pairwise Comparisons for Lectin Binding to Sperm	31
3. Lectin Binding to ICR and <u>T/t</u> Mouse Sperm and Testis Cells .	33
4. Cytotoxicity of Anti- <u>T/t</u> Locus Serum	35
5. ENU Effects on Body Weight, Testis Weight, and Testis Cell Counts	38
6. Percentages of Cells With 1C, 2C, and 4C DNA After ENU Treatment	39
7. Fluorescence of FITC-Lectins on Testis Cells With 1C DNA . .	42
8. Fluorescence of FITC-Lectins on Testis Cells With 2C DNA . .	43
9. Fluorescence of FITC-Lectins on Testis Cells With 4C DNA . .	44

LIST OF FIGURES

1. TWO GATE FL Cytogram and Histograms	59
2. FITCPI2 Cytogram and Histograms with Linear Scaling	62
3. FITCPI2 Cytogram and Histograms with Logarithmic Scaling	63
4. DNA Fluorescence of Mithramycin and Propidium Iodide Stained Testis Cells	64
5. Binding of BAN and LFA to Mouse Sperm	66
6. Binding of CONA, SBA, and WGA to Mouse Sperm	67
7. Binding of DBA, UEA, and AA to Mouse Sperm	68
8. Lectin Binding to ICR and <u>T/t</u> Mouse Testis Cells	70
9. Binding of FITC-Lectins to ICR Mouse Testis Cells	71
10. Effect of Trypsinization on Binding of LFA and UEA to ICR Mouse Testis Cells	72
11. Effect of Trypsinization on Binding of SBA, WGA, and CONA to ICR Mouse Testis Cells	73
12. Binding of FITC-Lectins to <u>T/t</u> Mouse Testis Cells	74
13. Effect of Trypsinization on Binding of LFA and UEA to <u>T/t</u> Mouse Testis Cells	75
14. Effect of Trypsinization on Binding of SBA, WGA, and CONA to <u>T/t</u> Mouse Testis Cells	76
15. Immunofluorescence of Antiserum to <u>T/t</u> Locus with ICR and <u>T/t</u> Mouse Testis Cells	78
16. Effect of ENU on Percentages of 1C, 2C, and 4C Testis Cells.	80
17. Binding of FITC-Lectins to 1C Testis Cells	81
18. Binding of FITC-Lectins to 2C Testis Cells	82
19. Binding of FITC-Lectins to 4C Testis Cells	83
20. Effect of ENU on Background Fluorescence of Testis Cells	84

List of Figures--Continued

21. Effect of ENU on Binding of FITC-SBA to Testis Cells	85
22. Effect of ENU on Binding of FITC-WGA to Testis Cells	86
23. Effect of ENU on Binding of FITC-BAN to Testis Cells	87
24. Effect of ENU on Binding of FITC-AA to Testis Cells	88
25. Effect of ENU on Binding of FITC-LFA to Testis Cells	89
26. Effect of ENU on Binding of FITC-CONA to Testis Cells . . .	90
27. Effect of ENU on Lectin Binding to Testis Cells Measured by Percent Positive Cells	91
28. Effect of ENU on Lectin Binding to Testis Cells Measured by Mean Fluorescence Intensity	92
29. Effect of DNA Content on Mean Fluorescence Intensity	93

INTRODUCTION

For some time one of the goals of research at Western Michigan University has been the development of a germ cell mutation assay based on a locus or loci which control the binding of lectins to sperm (Ginsberg and Ficsor, 1985; Smith, 1984). Current assays for detection of germ cell mutagens, such as the mouse specific locus test, the dominant lethal, dominant visible and heritable translocation tests, are both expensive and time consuming. If a simpler, cheaper assay could be developed, many more chemicals to which humans are exposed could be tested for their ability to cause germ cell mutations, with a possible decrease in birth defects in humans. Also, a simple test to determine whether a person's germ cells have been damaged after exposure to a suspected mutagen could be extremely valuable, particularly to assess environmental or work place damage to fertility.

Previous studies at Western Michigan University showed that exposure of mouse sperm to the known mutagens, Mitomycin C and ethylnitrosourea, resulted in germ cell damage (Ficsor, Oldford, Loughlin, Panda, Dubien, and Ginsberg, 1984) and increased binding of the isolectin B₄ from Bandeireae simplicifolia by the sperm of ICR mice, which normally did not bind this lectin (Smith, 1984).

The objective of the current research was to further characterize the surface of mouse germ cells with respect to their carbohydrate moieties by studying the binding of FITC-labeled lectins.

The effect of treatment with ENU on lectin binding by mouse testis cells was investigated in the hope of developing a simple test for germ cell damage. Differences in lectin binding by sperm of different mouse strains, including T/t mice, were investigated, since they may be indicative of genetic differences. It was hoped that the nature of the antigenic determinants of the T/t locus antigens, which are thought to involve sugar residues, could be further elucidated.

LITERATURE REVIEW

Germ Cells

Testis Cells

The cell found in mouse testis cell suspensions consist of spermatogonia, primary spermatocytes of the leptotene, zygotene, pachytene and diplotene stages, secondary spermatocytes, spermatids of steps I to XV, and sperm. The majority (66-68%) are spermatids in various stages of development (Meistrich, Bruce, and Clermont, 1973). In addition, there are Sertoli cells, Leydig cells, macrophages and red blood cells (Meistrich et al., 1973). These cells have been separated by velocity sedimentation (Meistrich et al., 1973; Lam, Furrer, and Bruce, 1970), and by centrifugal elutriation (Grabske, Lake, Gledhill, and Meistrich, 1975). Fractions obtained with of these procedures have sometimes been subsequently separated further by Percoll density gradient centrifugation (Stern, Kleene, Gold, and Hecht, 1983; Meistrich, Longtin, Brock, Grimes and Mace, 1981). Identification is easily made by PAS-hematoxylin staining (Meistrich et al, 1973; Rodriguez, Panda, and Ficsor, 1983). Early spermatids are usually found at 23-33% Percoll (Meistrich et al., 1981).

Velocity sedimentation separation after labeling with tritiated thymidine (Lam et al., 1970) has facilitated elucidation of the kinetics of differentiation. It has revealed seven size classes of cells in the differentiation pathway (Goldberg, Geremia, and Bruce, 1977). Briefly, spermatogonia synthesize DNA, divide twice by

mitosis into primary spermatocytes, which mature through the preleptotene, leptotene, zygotene, and pachytene stages. These go through the 1st meiotic division to become secondary spermatocytes and the 2nd meiotic division to become spermatids. In 15-16 steps the round spermatids lose cytoplasm and elongate as they mature into spermatozoa. The whole cycle, from spermatogonia to spermatozoa takes approximately 26 days and spermatogonia differentiate into round spermatids in approximately 18 days (Goldberg et al., 1977; Oakberg, 1956; Leblond and Clermont, 1952; Fawcett, 1975).

Also during spermiogenesis, the typical "somatic-type" of nuclear histones are replaced by histones having higher arginine content (Monesi, 1964). RNA synthesis occurs in primary pachytene spermatocytes while DNA is being synthesized (Monesi, 1965) and in round spermatids (Meistrich et al., 1981).

Sperm

During epididymal transit of sperm, surface changes take place. As ram sperm become fertile they pass from the corpus into the cauda epididymidis and changes in the lipid bilayer take place. The membrane becomes less able to withstand abrupt temperature change. The ability to bind the lectins Ricinus communis agglutinin and Ulex europeas agglutinin Type I (UEA) is lost and the ability to bind concanavalin A (CONA) and wheat germ agglutinin (WGA) is gained (Hammerstedt, Hay, and Amann, 1982). As rabbit sperm become capacitated, they lose the ability to bind CONA (Gordon, Dandekar, and Bartoszewicz, 1975). Hamster sperm lose the ability to bind CONA,

Lens culinaris agglutinin (LCA), peanut agglutinin (PNA) and WGA. They do not bind seven other lectins, including soy bean agglutinin (SBA), Bandieraea simplicifolia lectin (BAN), Dolichose biflorus agglutinin (DBA), and the fucose-binding lectin, UEA, (Ahuja, 1984; Kinsey and Koehler, 1978). Similar findings have been reported for guinea pigs (Schwarz and Koehler, 1979) and monkeys (Fain-Maurel, Dadoune, and Reger, 1984). The reduction in surface carbohydrate over the acrosomal region may be a necessary part of membrane alterations preparing sperm for membrane fusion events prior to the acrosome reaction and fertilization (Schwarz and Koehler, 1979). Changes in membrane galactosyl, galactosaminy, and sialyl glycoproteins to lower molecular weight, highly glycosylated forms also occur. High molecular weight forms of glycoproteins and sialoglycoproteins appear in ram sperm during epididymal maturation (Voglmayr, Fairbanks, and Lewis, 1983). X-bearing sperm have a higher net negative charge on their surface than Y-bearing sperm due to higher sialic acid content (Kaneko, Oshio, Kobayashi, Iizuka, and Mohri, 1984). The binding of capacitated sperm to the zona pellucida is inhibited by mono- and oligosaccharides related to fucose, galactose, and acetylated amino sugars and by glycoproteins with carbohydrate groups rich in or terminated by galactose or N-acetylglucosamine residues (Ahuja, 1982). Mouse sperm surface galactosyltransferases are thought to participate during fertilization by binding N-acetylglucosamine residues in the egg zona pellucida (Shur and Hall, 1982). Thus, it appears that cell surface carbohydrates play an important role in the fertilization ability of spermatozoa.

Flow Cytometry of Germ Cells

Sperm are notoriously difficult to study with a flow cytometer because of their tiny size, assymmetric shape, and long tails. It is usually necessary to use a special orienting flow cytometer so that the sperm are oriented uniformly as they pass through so that the laser beam strikes their flat face.

Several groups have done work with sperm and testis cells mostly determining DNA content (Evenson, Darzynkiewicz, and Melamed, 1980; Meistrich, Lake, Steinmeta, and Gledhill, 1978; Pinkel, Lake, Gledhill, Van Dilla, Stephenson and Watchmaker, 1982; Zante, Schumann, Göhde, and Hacker, 1977). One can quantitate mutagenesis by measuring the increase in coefficient of variation for the DNA of cells in the various stages of the cell cycle (Pinkel et al., 1982; Pinkel, Gledhill, Van Dilla, Lake, and Wyrobek, 1983). It is even possible to determine the relative numbers of X and Y chromosome-bearing sperm by this technique (Pinkel et al., 1982, 1983). The measurement of DNA content in germ cells is particularly difficult because the highly condensed chromatin and arginine-rich histones prevent complete penetration of the fluorescent dye. For this reason most groups have resorted to proteolytic enzymatic treatment with papain to cause nuclear swelling and stoichiometric staining (Zante et al., 1977). Many work with cell nuclei only (Meistrich et al., 1978), making surface fluorescence studies impossible.

Analysis of pepsin treated mouse testis cells by Hacker, Schumann, and Göhde, 1980; Hacker, Schumann, Göhde, and Müller, 1981) resulted

in histograms showing 4 DNA peaks. Two of these peaks were cells with 1C DNA (haploid). Round spermatids from steps 1-8 of spermiogenesis stained proportional to their DNA content and were 40% of the total cells. Elongated spermatids from steps 9-16 and spermatozoa were 24-32% of the cells. They only showed 60-80 percent of their DNA fluorescence because of their highly condensed chromatin, unless treated with papain. The 2C DNA (diploid) peak contained 12-17% of the total cells and consisted of G₁ spermatogonia, G₁ primary spermatocytes and secondary spermatocytes. The 4C DNA (dividing) peak was 10-13% of the total and contained G₂ + M spermatogonia and primary spermatocytes. The S-phase region between 2C and 4C contained spermatogonia and primary spermatocytes synthesizing DNA and was 3-7% of the total. Non-germ cells such as Leydig cells, Sertoli cells and macrophages were in the 2C peak.

The method developed for this work utilizes DNA quantitation by flow cytometry as a means of selecting testis cell populations on the basis of their DNA content for concomitant analysis of surface fluorescence by FITC-labeled lectins. Testis cells were dual labeled: the cell surface with fluoresceinated lectin (green) and the DNA in the nucleus with propidium iodide (red). Use of formalin as a fixative and prolonged incubation with the propidium iodide resulted in near stoichiometric DNA staining, so that the 1C, 2C and 4C cell populations could be selected. This technique was used as an alternative to separation of the testis cell populations by velocity sedimentation at unit gravity (Lam et al., 1970; Meistrich et al., 1973) or centrifugal elutriation (Grabske et al., 1975).

T/t Locus

The murine T/t complex is located on chromosome 17. It is 14 crossover units to the left of the H-2 histocompatibility locus (Cheng and Bennett, 1980). It is defined by a series of recessive lethal t mutants which interact with the T (brachyury) mutation to produce tailless mice (Goodfellow, Levinson, Gable, and McDevitt, 1979; Artzt and Bennett, 1975). Although commonly referred to as the murine T/t locus, increasing evidence of its complexity suggests that it is probably not a true locus with a series of alleles, but rather a complex of loci with a series of haplotypes. The haplotypes are defined as variant regions of genome. They are closely related, structurally variant forms of a portion of mouse chromosome 17 which includes the T/t locus as well as the entire major histocompatibility complex (MHC or H-2) and many other normally functioning genes, such as the phosphoglycerate kinase-2 (PGK-2) gene (Silver, 1985). There is speculation (Artzt and Bennett, 1975) that the T/t complex may be an embryonic analogue of the adult MHC. The T/t complex may mediate cell-cell recognition in the embryo, while the MHC mediates cell-cell recognition in the adult. The T/t complex genes may specify cell surface components important in regulating the cellular interactions necessary for channeling differentiation into specific pathways during embryogenesis (Bennett and Artzt, 1982).

These recessive lethal t mutants display a t-specific segregation distortion or transmission ratio distortion. The t sperm survive longer than normal sperm, so transmit the t haplotype to nearly all their offspring. It is not known whether this is because

the t sperm are superior to normal, the t sperm somehow inactivate the normal sperm, or both (Silver, 1985).

There is also a decrease in meiotic recombination between the H-2 locus and the centromere. Several regions of the T/t locus display chromosomal inversions, in which the order of the genes is reversed from normal. These are thought to account for the strong suppression of recombination between wild-type and t haplotype DNA (Silver, 1985).

The recessive t haplotypes interact with a dominant T-locus mutation to produce a tailless phenotype in double heterozygous T/t mice, short tails in heterozygous T/+ mice, normal tails in +/t heterozygous mice, and death during midgestation in homozygous T/T embryos (Silver, 1985). The t/t homozygous mice also die sometime during development (Bennett and Artzt, 1982).

Six partially complementing groups of t mutants have been defined. Homozygous mice of the same complementation group die because of the lethal gene. Goodfellow et al. (1979) think the defect is the same in all complementation groups, because the homozygous lethality occurs at the same time in all the groups. Bennett and Artzt (1982) think the defects are different because death occurs at different times in the different groups. The mice used for this study belong to the t⁰, t⁶ complementation group.

Several workers (Bennett, Goldberg, Dunn, and Boyse, 1972; Cheng and Bennett, 1980; Goodfellow et al., 1979; Yanagisawa, Bennett, Boyse, Dunn, and Dimeo, 1974) have attempted to define the specificities of the various complementation groups by preparing antisera to them by immunizing mice with testis cells from the mice of the appropriate haplotype, then absorbing the sera with sperm from mice of the

other haplotypes. The anti- t^0 serum used in this study was from such an immunization (Cheng and Bennett, 1980). It has been found that the antigenic specificities of the various t haplotypes are for sugar residues, so it has been hypothesized that the genes of the T/t locus code for glycosyltransferases or regulators of glycosyltransferases which modify the oligosaccharide structures to give specificity to the T/t antigens via their terminal sugar residues (Cheng and Bennett, 1980). The specificities worked out so far are beta-D-galactose for t^{w12} and t^{w32} , N-acetyl-D-galactosamine for t^{w1} , L-fucose for t^0 and t^{w18} , and sialic acid for T (Cheng and Bennett, 1980). These specificities were determined by glycosidase treatment of the target cells prior to complement mediated cytotoxicity assays on sperm. Goodfellow et al. (1979) have also used sperm cytotoxicity assays with these antisera.

The T/t complex antigens are found on the surface of early embryos and embryonal carcinoma cells (Feizi, 1985; Artzt and Bennett, 1985), in addition to sperm and testicular cells. The F9 teratocarcinoma antigen, which may be a wild-type product of T/t complex antigens binds peanut agglutinin, so contains galactose (Cheng and Bennett, 1980). Several proteins specific for various T/t haplotypes have also been identified by two-dimensional gel electrophoresis (Silver, Artzt, and Bennett, 1979; Danska and Silver, 1980).

Lectins

Lectins are homogeneous protein extracts from plants and other sources, such as snails or fish. Most are glycoproteins which agglu-

tinate erythrocytes and malignant cells. They bind sugars specifically (analogous to monoclonal antibodies) and precipitate polysaccharides and glycoproteins specifically. Many, such as concanavalin A (CONA) from the Jack bean (Canavalia ensiformis) and phytohemagglutinin (PHA) from the red kidney bean (Phaseolus vulgaris) are also mitogenic and can stimulate resting lymphocytes to divide.

Lectins are very useful tools for studying specific binding sites on proteins, for use as reagents for isolating polysaccharides and glycoproteins, for studying antigen-antibody reactions, the immune response, and the events initiating cell division and malignant changes. They are also used for investigating the architecture of cell surfaces (Lis and Sharon, 1973). A chart of the lectins used here and their carbohydrate binding specificities appears in Appendix A.

Mechanisms for Altered Lectin Binding by Testis Cells

Germ Cells

Altered lectin binding in germ cells, such as sperm and haploid testicular cells (spermatids) may be caused by damage to the DNA of the spermatogonia, the spermatocytes or the spermatids from which the sperm are descended, resulting from mutations of the genes controlling lectin binding. The earlier such mutations occur in the development of the sperm, the more likely they are to be replicated in the offspring of non-mutant individuals, rather than simply causing sperm damage.

A recent report (Bode, 1984) discusses the use of ethylnitrosourea (ENU) to induce new t-locus mutations in the spermatogonia of mice. From a single dose of 250 or 300 micrograms/g, qk (quaking)

and tf (tufted) mutants were generated at an average frequency of 1/1500, compared to a spontaneous rate of 10^{-5} per locus. After a male is treated with ENU, new rounds of spermatogenesis temporarily cease. The surviving mutagenized spermatogonial stem cells repopulate the testis, spermiogenesis cycles begin again and the male regains fertility after 10-15 weeks. The sperm that were screened for mutations were not those present at the time of injection, but were the progeny of the different clones of mutagenized spermatogonial stem cells. The abundance of a given mutation in the total sperm population is determined by the total number of surviving spermatogonial cells and their subsequent relative transmission capability. Since there are many clones of mutagenized spermatogonia, and one in five mutagenized males has sperm mutant at any of the 5 loci involved, then for a mutation frequency of 1/1500, each male can produce $1500/5 = 300$ different mutants.

Unless a given locus shows haploid expression, effects seen 2 weeks after treatment usually indicate direct damage to sperm development (Ginsberg and Ficsor, 1985). The potential effects on lectin binding of damage to the sperm or spermatids, other than DNA damage, are the same as for somatic cells. Recent studies at Western Michigan University (Ficsor et al., 1984; Rodriguez et al., 1983) have focused on methods for detecting germ cell damage in mice. Sperm enzyme activities (succinic dehydrogenase and acrosin), sperm motility and testis weight all decreased significantly after treatment with ENU or mitomycin C. Testis weight and acrosin activity in spermatogonial cells were the most sensitive indicators of germ cell damage.

Somatic Cells

Altered lectin binding by somatic cells, such as in diploid testicular cells, can be caused by several factors. One cause is physical or chemical alteration of the cell membrane. Enzymatic treatment, heat, fixation, treatment with detergents, even lectins themselves, could all result in different lectin binding. Alteration of the cells' internal biochemistry by drugs could cause altered lectin binding via altered synthesis of cell membrane components. Mutation of the DNA of somatic cells is one such event which could alter lectin binding if expression of genes controlling surface carbohydrates were changed. Mutagenic drugs, carcinogenic drugs, ultraviolet and x-irradiation could all cause such damage.

The mutagenic drug N-ethyl-N-nitrosourea used in this study exerts its effect by alkylation of O⁶ of guanine, and induces sister chromatid exchange and mutagenesis of cultured cells (Swenson, Harbach, and Trzos, 1980). It is possible that sister chromatid exchange is a manifestation of recombinational repair of chemically damaged DNA. O⁶-alkylguanine is removed by repair systems in some cell types, but not in others and can be carried into DNA synthesis (Swenson et al., 1980). Unlike point mutagenesis, in which miscoding O⁶-alkylguanine has been implicated in the past, sister chromatid exchange occurs through more complicated processes involving DNA recombination. It is therefore an indicator of a wider variety of DNA damage from potential mutagenic events. Altered lectin binding to cells, whether somatic cells or germ cells, can indicate an even wider variety of damage, to DNA or to the cell.

MATERIALS AND METHODS

Materials

Diluent

Phosphate buffered saline, pH 7.6, (PBS) consisted of 0.20 grams KCl, 0.20 grams KH_2PO_4 , 8.00 grams NaCl, 2.16 grams $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (1.69 grams anhydrous Na_2HPO_4) in enough H_2O to make 1 liter total volume.

Lectins

The following lectins, labeled with fluorescein isothiocyanate (FITC), were diluted in PBS and used at 25 micrograms/ml final concentration. Their major specificities are shown in Appendix A. Soy bean lectin (SBA) from Glycine max, which binds to N-acetyl-beta-D galactosamine and beta-D-galactose (Lis and Sharon, 1972; Debray, DeCout, Strecker, Spik, and Montreuil, 1981), was purchased FITC-labeled from Miles-Yeda Ltd. (cat. #79-103, lot #FC13). Wheat germ agglutinin (WGA) from Triticum vulgaris, which binds to N-acetyl-D-glucosamine (Debray et al., 1981; Imbar, 1973; Marchesi, 1972), was purchased FITC-labeled from Miles-Yeda Ltd. (cat. #79-102, lot #FW15). Asparagus pea lectin (AA) from Tetragonolobus purpureas (Lotus tetragonolobus), which binds to N-acetyl-D-galactosamine, D-galactose, alpha-L-fucose and agglutinates human type O erythrocytes (Debray et al., 1981; Yariv, Kalb, and Blumberg, 1972), was purchased FITC-labeled from Sigma Chemical Co. (cat. #L-7506, lot #61F-9640).

UEA-I lectin (UEA) from Ulex europeus, which binds alpha-L-fucose (Debray et al., 1981), was purchased FITC-labeled from Polyscience Co. (cat. #15439, lot #35692). Isolectin B₄ (BAN) from Bandeireaea simplicifolia I (BSI-B₄), which binds to alpha-D-galactose and agglutinates human type B erythrocytes (Hayes and Goldstein, 1974; Murphy and Goldstein, 1978; Murphy and Goldstein, 1979; Wood, Kabat, Murphy, and Goldstein, 1979), was purchased FITC-labeled from Sigma Chemical Co. (cat. #L-1134, lot #111F-3926). Horse gram lectin (DBA) from Dolichos biflorus, which binds to N-acetyl-D-galactosamine and agglutinates human type A erythrocytes (Etzler, 1972), was purchased FITC-labeled from Polyscience Co. (cat. #15355, lot #35849). Sea Slug Agglutinin (LFA) from Limax flavus, which binds to sialic acid, was purchased from Calbiochem-Behringer (cat. #122129, lot #405173). It was FITC-labeled by stirring 0.5 mg protein/0.2 mg FITC-celite (hyflo super-cel from Johns-Manville Co.) in 1 ml 50 micromolar pH 8.0-borate buffer for 30 minutes at 0° C, centrifuging for 2 minutes in an Eppendorf microfuge, dialyzing the supernatant for 24 hours at 4° C vs. 1.5 liters 20 mM Tris-HCl pH 7.4 buffer containing 10 mM MgCl₂, with two changes of buffer, and centrifuging again (Rinderknecht, 1960). The 0.45 ml FITC-LFA supernatant recovered contained 145 micrograms/ml protein (29.0% recovery), determined by the Miller modification of the Lowry method (Miller, 1959). Concanavalin A (CONA), Jack Bean Phytohemagglutinin from Canavalia ensiformis, which binds to alpha-mannose and alpha-glucose (Debray et al., 1981), polysaccharides (Imbar, 1973) and glycoproteins such as immunoglobulins and blood group substances (Agrawal and Goldstein, 1972), was

purchased from Sigma and FITC-labeled by J. Monticello as described above. It contained 2.295 micrograms/ml protein (91.8% recovery).

Antisera

Mouse Anti- t^0 antiserum was a gift of Dorothea Bennett at the Sloan-Kettering Institute for Cancer Research, New York. Polyvalent FITC-labeled goat anti-mouse Ig from Southern Biotechnology Associates, Inc. (Cat. #1020-02) was a gift of The Upjohn Co.

Mice

ICR mice were derived from breeding stock purchased from Harlan Sprague Dawley, Inc., Indianapolis, IN. and maintained at Western Michigan University, Kalamazoo, MI. DBA/2J and T/t^6 (TT6/Le T_{tf/t^6} , t-6 lethal group: t^0, t^6) mice were derived from breeding stock purchased from The Jackson Laboratory, Bar Harbor, ME and maintained at Western Michigan University, Kalamazoo, MI. $+/T$ and $+/t^6$ mice were obtained by crossing T/t^6 males with $+/+$ (DBA/2J) females at Western Michigan University, Kalamazoo, MI. C57BL/6, B6D2F1 (C57BL/6 x DBA) and Balb c mice were obtained from Jackson Laboratories, Bar Harbor, ME and housed at The Upjohn Company, Kalamazoo, MI.

Sperm Methodology

Preparation of Sperm Suspensions

Mice were sacrificed by cervical dislocation and the sperm were stripped from the vas deferens into 1.0 ml of PBS. Clumps of sperm

were dispersed by gentle pipetting with a Pasteur pipet. The sperm were centrifuged at room temperature for 5 minutes at 3000 rpm (2500 x g) and resuspended into 1.0 ml PBS.

Staining of Sperm With Lectins

Dry Method

Slides were prepared by spreading 10 microliters of sperm suspension onto a glass slide and allowing it to dry. Slides could be stored dried for several weeks or stained immediately. For staining, 20 microliters of lectin diluted to 25 micrograms/ml in FBS were spread on the slides. The slides were incubated at 37° C in a humidified box for 1 hour, then washed with distilled H₂O and drained until dry. Coverslips were mounted with 10 microliters 50% glycerol in PBS. This is the method of Smith (1984).

Wet Method

Sperm were stained in suspension by mixing 10 microliters of 25 micrograms/ml lectin with the pelleted sperm and incubating 1 hour at 37° C. The sperm were then washed once in 1 ml PBS and resuspended to the original volume (usually 0.5 ml) in PBS. Either slides were prepared by mounting 10 microliters sperm plus 10 microliters 50% glycerol on a slide with a coverslip or the stained cells were fixed in 1% formalin and examined in suspension by flow cytometry.

Staining was occasionally good, but poorly reproducible by this method. Incubation with lysolecithin (lysophosphatidyl choline) from Sigma (cat. #L-4129) at 5-50 micrograms/ml for 0-10 minutes, followed

by washing with 1% bovine serum albumin in PBS (Schroff, Bucana, Klein, Farrell, and Morgan, 1984) failed to improve the staining quality and resulted in cell loss.

Fluorescence Microscopy

Slides of sperm which had been stained with FITC-lectins with either the wet or the dry method were examined under water immersion with a Leitz fluorescence microscope using the #1 fluorescein cube.

Fluorescence was scored according to the following code:

a = acrosome	+ = 1 = faint
h = head	++ = 2 = faint-medium
m = midpiece	+++ = 3 = medium
t = tail	++++ = 4 = medium-bright
w = whole sperm	+++++ = 5 = bright

For statistical analysis of the data by oneway analysis of variance the + symbols were later converted into numbers 1-5 and fluorescence in the whole sperm was recorded as occurring in all of the other regions.

Testis Cell Methodology

Preparation of Testis Cells

Mice were sacrificed by cervical dislocation. The testes were dissected from the mice and placed in several mls. PBS in a petri dish. The tunica albuginea was torn away with forceps and removed to free the seminiferous tubules, which were then diced with scissors

into 3 mm sections. The tubules were transferred to a 50 ml conical nalgene centrifuge tube. The petri dish was washed with PBS and the washings combined with the tubules to make 20 ml total volume. The tubules were aspirated with a Pasteur pipette 20 times, then allowed to stand for 15 minutes, while the tubules settled out. The supernatant was transferred to another tube and centrifuged for 5 minutes at room temperature at 800 rpm in an IEC centrifuge at 170 x g. The pellet was resuspended in 20 ml PBS and centrifuged again, then the cell pellet was resuspended in 0.5 ml PBS and refrigerated until used. This is essentially the method of Lam et al. (1970).

Cells for trypsin treatment were resuspended to 20 ml after the first centrifugation and 100-1000 microliters of 0.5 mg/ml trypsin solution were added (final concentration = 2.5-25 micrograms/ml). The cells were allowed to incubate 20 minutes at room temperature, then they were centrifuged as above, washed once with 1 mg/ml bovine serum albumin, then once with PBS, and resuspended in 0.5-2.5 ml PBS as above. The best results were obtained with 2.5 to 5 micrograms/ml final concentration of trypsin. The higher concentrations resulted in loss of most of the cells.

In some of the early experiments 10 micrograms/ml DNAase was used along with trypsin during the aspiration of testis cells to help free them from the seminiferous tubules (Cheng and Bennett, 1980; Meistrich et al., 1973) and digest the sticky DNA originating from damaged cells which might cause clumping (Ficsor, 1985; Meistrich et al., 1981). This was later found to be unnecessary, so was discontinued to minimize trauma to the cells (Lam et al., 1970).

Percoll Density Gradient Centrifugation

The testis cells in 0.5 ml PBS were layered over a discontinuous gradient of 20 to 45% Percoll, in 5% increments of 0.9 ml each in cellulose nitrate tubes (90 x 15 mm, 12 ml capacity). They were centrifuged at 4° C for 20 minutes in a Sorvall HS-4 swinging bucket rotor in a Sorvall refrigerated centrifuge at 9000 x g. This was essentially the method of Meistrich et al. (1981) and Stern et al. (1983). The bands of cells clearly visible at each interface were manually removed with a plastic bulb Pasteur pipet. Each cell fraction was washed in 3-4 ml PBS, centrifuging 10 minutes at 1000 rpm. in a refrigerated IEC centrifuge.

Staining of Testis Cells

Hematoxylin-Eosin Staining

Slides were made from each of the bands of the Percoll gradient. They were stained with hematoxylin-eosin by a standard procedure (Buthala and Blashfield, 1980). Briefly, the slides were fixed in Bouin's fixative for 1-2 hours, stained in 1:20 alum-hematoxylin for 6 minutes, differentiated in 1% NaHCO₃ for 3-4 minutes, counter-stained in 1% eosin for 1-2 minutes, washing in distilled water between each step. They were then dehydrated by successive rinsing in 95% and 100% ethanol, then cleared in xylene and mounted.

Lectin Staining

The cells were pelleted by centrifugation at 1000 rpm for 10

minutes in a refrigerated IEC centrifuge. The cell pellet was resuspended to about 10^7 cells/ml in PBS, then 10 to 50 microliter aliquots were mixed with an equal volume of FITC-lectin at 50 micrograms/ml in PBS, making a final lectin concentration of 25 micrograms/ml. The cells were incubated with the lectins at 37° C for 1 hour, then washed by adding 1 ml PBS and centrifuging again. The cells were then resuspended in 0.1 ml PBS, fixed by mixing with 0.1 ml 2% formalin to make a final concentration of 1% formalin, and stored at 4° C for 1 to 14 days.

DNA Staining

Cells were stained with lectins and fixed as above. The day before flow cytometry, the cells were pelleted, resuspended in 0.1 ml PBS containing 1 mg/ml ribonuclease A (Sigma Chemical Co., Cat. #R-4875), which had previously been heated at 100° C for 10 minutes to inactivate DNAase. They were incubated at 37° C for 15 minutes, then washed once in PBS. The pelleted cells were resuspended to approximately 10^6 cells/ml in 50 micrograms/ml propidium iodide (Calbiochem-Behring Corp., Cat. #537059) and stored at 4° C until analysis.

In preliminary experiments the cells were similarly fixed and stained with 50 micrograms/ml mithramycin (Mithracin from Miles Laboratories) in 15 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ or with 50 micrograms/ml ethidium bromide in 1.5 mg/ml MgCl_2 , pH 7.4 0.5 M Tris. Ethidium bromide and propidium iodide staining were done both with and without RNAase. RNAase was found to be essential to avoid staining of RNA in the cells along with the DNA. Mithramycin staining was always without

RNAase. In some experiments the cells were fixed by gradual addition of 70% EtOH at 0° C with rapid mixing. In a few experiments the cells were incubated with 5 micrograms/ml Hoescht 33258 at 37° C for 30 min. concurrently with the lectins.

Microscopy

Slides stained with hematoxylin and eosin were examined at 40x magnification with a Leitz microscope and the various cell types were identified by comparing them to photographs by Meistrich et al. (1973). Fluorescence microscopy was performed as above using the #1 fluorescence cube on the cells stained with FITC-lectins in suspension as well as on cells in which the DNA was stained with propidium iodide, ethidium bromide or mithramycin. Propidium iodide stained the nucleus orange, ethidium bromide stained it red and mithramycin stained it yellow. Cells in which the DNA was stained blue with Hoescht were examined using the #4 UV cube.

Flow Cytometry

An Ortho Cytofluorograf model 50-H dual laser (argon and krypton) flow cytometer equipped with a 2150 computer system was used for these studies, courtesy of The Upjohn Company.

FITC-Labeled Sperm and Testis Cells

For quantitation of green fluorescence, the argon laser set for blue-green light at 488 nm wavelength and 400 mw was used for excitation. Green fluorescence was collected by photomultiplier tube (PMT)

1 on a logarithmic scale (to increase sensitivity and range). Forward angle blue light scatter (a measure of cell size) was collected by PMT 2 on a linear scale, and right angle light scatter (a measure of cell reflectivity) was collected by PMT 3 on a log scale. The total light from the cells (integrated area under the curve of the signals) was sent to the computer. The program TWOGATEFL was used to generate one cytogram and three histograms for each sample. Appendix B contains examples (Figure 1) and the program listing. The cytogram was a plot of forward scatter vs. right angle scatter. It was used to select the cells of interest in up to two regions. At the same time dead cells and small debris can be gated out. The three histograms were plots of green fluorescence intensity vs. cell number for the cells in the two regions selected, as well as the ungated total fluorescence. This was used to compare the relative amounts of fluorescence in the selected cell populations of various samples.

To improve the weak and variable signal from the small, asymmetric sperm cells a hooded fiber optic was used on the forward scatter signal. An attempt to improve the signal further by changing to logarithmic scaling on the forward scatter signal and linear scaling on the green fluorescence did not help, so was abandoned.

Testis Cells Dual-Labeled with FITC and DNA Stain

Green fluorescence from FITC was quantitated in each of three populations of cells in each sample. Populations were selected on the basis of their DNA content, which was proportional to red fluorescence from propidium iodide. The argon laser used for excitation

was set for blue-green light at 488 nm wavelength and 400 mw. Green fluorescence was collected by PMT 1 with a 515-530 filter as area on a logarithmic scale to increase both sensitivity and range. Red fluorescence was collected by PMT 3 with a 600 nm long pass filter as both area (integrated area under the curve of the signal = total light from the cells) and peak (peak amplitude of the signal = light from the brightest part of the cell). These were also both scaled logarithmically for the assay, but linearly for assessing stoichiometric DNA staining. The program FITCPI was written by Ann Berger for this purpose. The program FITCPI2 (modified from FITCPI by the author to generate histograms from three regions rather than one) was used to generate one cytogram and five histograms. Appendix C contains the program listing and examples of linear DNA scaling in Figure 2 and logarithmic DNA scaling in Figure 3. The cytogram was a plot of area vs. peak for red fluorescence. This was used to select cell populations on the basis of their DNA content and set regions around each one (1C = haploid, 2C = diploid, 4C = diploid cells in G₂ + M). At the same time 1C doublets which have the same area as 2C cells, but the same peak as single 1C cells were gated out. The green fluorescence from each of the three regions, as well as ungated green fluorescence and ungated red fluorescence, were each plotted vs. cell number in the five histograms.

A similar program DNAMITH was used in preliminary experiments with standard mithramycin staining of testis and spleen cells to identify the cell populations. Excitation was at 457 nm and yellow fluorescence was collected with PMT 1. Mithramycin could not be used

with FITC staining because the wavelengths of yellow and green fluorescence are too close. Figure 4 in Appendix C contains DNA cyto-grams and histograms on a linear scale for the same testis cells stained with either mithramycin (DNAMITH) or propidium iodide (FITCPI2). In the mithramycin stained cells, the upper curve had 4 DNA peaks at channels 113, 166, 245, and 498. These probably correspond to 1C, 1C+, 2C, 4C DNA. The percentages of cells were 63.1% in 1C + 1C+, 24.7% in 2C, and 12.3% in 4C. The lower mithramycin curve also had 4 peaks at channels 126, 174, 276, and 574. They contained 46.7, 17.8, 16.0 and 13.4% of the cells, respectively. The propidium iodide stained cells, in contrast, had only 3 peaks at channels 200, 440, and 780. They contained 69%, 18.1%, and 12.9% of the cells, respectively. If we assume the two lower peaks in the mithramycin curves are both spermatids, these results match those of Hacker et al. (1980 and 1981), who found 24-32% 1C elongated spermatids (which stained 70% less than expected), 40% 1C round spermatids, 12-17% 2C cells, 10-13% 4C cells. It is not clear why our mithramycin stained spermatids stained slightly more than expected, while theirs stained less. With propidium iodide both round and elongated spermatids appear to stain the same.

T/t Locus Antiserum Methodology

Immunofluorescence

Testis cells which had been isolated as described above were washed once in PBS and half the cells of one mouse resuspended to 0.5

ml in PBS. To 0.05 ml aliquots of cells, 0.05 ml of either a 1:12.5 dilution of mouse anti- t^0 antiserum (final concentration 1:25) or PBS were added. The cells were incubated with the antiserum for 60 minutes at 4° C, then washed once with 1 ml PBS. The cell pellets were resuspended in 0.1 ml of a 1:50 dilution of goat anti-mouse Ig and incubated another 60 minutes at 4° C. The cells were washed once in 1 ml PBS, resuspended in 0.1 ml PBS, fixed by mixing with an equal volume of 2% formalin, and refrigerated until examined by flow cytometry using the TWOGATEFL program.

Cytotoxicity

Half the testis cells from one mouse were resuspended in 0.5 ml of Cedarlane Cytotoxicity Medium (Cedarlane Laboratories, Ltd.), which consisted of RPMI-1640 tissue culture medium to which 0.3% bovine serum albumin and 25 mM HEPES buffer had been added. To 0.05 ml aliquots, 0.05 ml of a 1:12.5 dilution of mouse anti- t^0 antiserum (final concentration 1:25) or Cytotoxicity Medium were added. The cells were incubated with the antiserum (Ab) for 60 minutes at 4° C, then washed by adding 1 ml Cytotoxicity Medium and centrifuging. The cells were resuspended in 0.1 ml Cytotoxicity Medium and divided into 4 aliquots of 0.025 ml each. To each aliquot, 0.25 ml of double strength Low-Tox-M Rabbit Complement (Cedarlane Laboratories, Ltd.) were added to make final concentrations of 1:5, 1:10, 1:20 or no complement (C). The cells were incubated with complement at 37°C for 60 minutes, then put on ice. An equal volume of 0.2% Trypan blue was added and the after 3-5 minutes the live (clear) and dead (blue)

cells were enumerated with a hemocytometer.

Mutagen Treatment of Mice

Groups of 3 mice were injected intraperitoneally with either 50 or 100 mg/kg of the mutagen, ethylnitrosourea (ENU), a gift of Dr. David Swenson at The Upjohn Co., or the vehicle, 10% DMSO in saline. They were killed 18 days later to obtain their testis cells. The timing was planned so that the cells which were spermatogonia at the time of treatment would be stage V-IX spermatids at the time of cell testing.

RESULTS

Lectin Binding to Sperm of Different Mouse Strains

Flow Cytometry

Staining of sperm with FITC-labeled SBA, WGA, BAN, DBA, UEA, and AA in suspension increased fluorescence over background, but staining was very weak and variable. Of these, SBA and WGA were much brighter than the rest. Due to the poor quality and poor reproducibility of staining in suspension, use of the flow cytometer for sperm was abandoned.

Fluorescence Microscopy

Slides of sperm from 9 different mouse strains (or genotypes) were prepared and stained with 8 different FITC-labeled lectins by the dry method in a series of experiments. The 9 mouse strains (genotype determined by phenotypes for the T/t x T/t crosses) were: T/t (no tail), +/T (short tail), +/t (normal tail), T/t x DBA (normal tail), DBA/2J, ICR, Balb c, C57BL/6, and B6D2F1 (C57BL/6 x DBA/2J). The 8 FITC-lectins were BAN, LFA, CONA, SBA, WGA, DBA, UEA, AA, plus a no lectin control. Most of the lectins were tested at least twice on each strain. Slides were scored for fluorescence intensity in the acrosome, head, tail, or midpiece. The numerical scores were then subjected to a one way analysis of variance to determine which of the strains were significantly different from each other ($p = 0.05$ level) in the way they bound the various lectins.

The mean scores for lectin binding to each region are given in Table 1. They are shown graphically in Figures 5-7 (Appendix D). All strains showed faint autofluorescence of the midpiece in the no lectin controls. Strains C57BL/6 and B6D2F1 showed faint autofluorescence in the other regions as well. The midpieces of all the T/t locus mice, DBA/2J and ICR mice stained brightly with BAN. LFA stained the midpieces and heads of all the strains brightly. It stained the tails of T/t x DBA and Balb c mice brightly, the other strains moderately. It stained the acrosomes brightly only in the T/t x DBA/2J mice, medium in the +/+ and ICR mice. CONA produced faint to medium staining of the midpieces of all strains and stained the acrosome of DBA mice moderately. SBA produced bright staining of the acrosome and faint to medium staining of the midpiece in all the strains. It stained the heads and tails of C57BL/6 and B6D2F1 mice faint to medium. WGA stained the acrosome brightly in the +/+, +/t, T/t x DBA/2J, ICR and Balb c mice. It stained the midpiece moderately in all the T/t locus mice and faint-medium in the C57BL/6 and B6D2F1 mice. It stained the heads and tails faint-medium in all the strains except T/t x DBA/2J, ICR and Balb c. DBA and UEA produced faint-medium staining of the midpieces of all the mice, medium staining of the heads of Balb c and C57BL/6 mice and faint staining of the acrosomes and tails of C57BL/6 mice. AA stained only the midpieces of all the strains faint to medium.

The results of the pairwise comparisons are shown in Table 2. The most significant differences between the strains were in the binding of BAN. It bound to the midpieces of ICR, DBA/2J, and the

Table 1
Binding of FITC-Lectins to Mouse Sperm

Mouse Strain	Site	Lectin								
		None	BAN	LFA	CONA	SBA	WGA	DBA	UEA	AA
T/t (no tail)	a	0	0	1.00	0	5.00	0	0	0	0
	m	1.00	4.25	4.00	1.00	1.50	3.50	2.00	2.00	1.50
	t	0	0	2.25	0	0	1.00	0	0	0
	h	0	0.50	3.50	0	0	2.00	0	0	0
		n=4	n=4	n=4	n=2	n=4	n=4	n=4	n=4	n=4
+/T (short tail)	a	0	0	2.00	0	5.00	4.00	0	0.50	0
	m	1.00	2.50	4.00	2.00	1.00	2.00	2.00	2.00	1.50
	t	0	0	2.50	0	0	2.00	0	0	0
	h	0	2.00	4.00	0	0	1.50	0.50	0.50	0
		n=2	n=2	n=2	n=1	n=2	n=2	n=2	n=2	n=2
+/t (normal tail)	a	0	0	0	0	5.00	2.50	0	1.00	0
	m	1.00	3.50	4.50	2.00	2.00	3.00	2.00	2.50	2.00
	t	0	0	2.00	0	0	0.50	0	0	0
	h	0	0.50	3.50	0	0	2.50	0	0	0
		n=2	n=2	n=2	n=1	n=2	n=2	n=2	n=2	n=2
T/t x DBA/2J (normal tail)	a	0	0	4.00	0	5.00	5.00	0	0	0
	m	1.00	3.50	4.00	1.00	2.00	3.00	2.00	2.00	2.00
	t	0	0	4.00	0	0	0.50	0	0	0
	h	0	1.00	4.00	0	0	0	0	0	0
		n=2	n=2	n=1	n=1	n=2	n=2	n=2	n=2	n=2
DBA/2J	a	0	0	0	3.00	5.00	1.50	0	0	0
	m	1.00	3.00	4.00	3.00	0.50	1.00	2.00	0.50	1.50
	t	0	0	1.50	0	0	1.00	0	1.50	0
	h	0	0.50	2.50	0	0	1.50	0	0	0
		n=2	n=2	n=2	n=1	n=2	n=2	n=2	n=2	n=2
ICR	a	0	0	2.00	0	5.00	4.00	0	0.50	0
	m	1.00	2.50	3.50	1.00	2.00	0.50	2.00	1.00	1.00
	t	0	0	2.50	0	0	0.50	0	0	0
	h	0	1.00	3.50	0	0	0	0	0	0
		n=1	n=2	n=2	n=1	n=2	n=2	n=2	n=2	n=2
Balb c	a	0	1.00	0	0	4.50	4.33	0	1.33	0
	m	0.67	1.00	5.00	1.00	0.50	0	2.67	1.33	0.75
	t	0.33	1.00	4.00	0	0.25	0.33	0.33	1.67	0.50
	h	0	1.00	4.00	0	0.25	0	2.66	1.33	0
		n=3	n=3	n=1	n=1	n=4	n=3	n=3	n=3	n=4
C57BL/6	a	0.12	0.75	0	2.00	5.00	3.33	1.50	1.25	0
	m	1.00	1.00	4.00	1.00	1.55	1.83	1.00	2.00	1.00
	t	0.75	0.75	2.00	0	1.33	2.00	1.00	1.50	0.60
	h	0.25	1.00	4.00	0	2.67	2.67	1.75	1.50	0.40
		n=8	n=4	n=1	n=1	n=9	n=6	n=4	n=4	n=5
B6D2F1 (C57BL/6 x DBA/2J)	a	0	0.33	0	2.00	5.00	1.80	0.33	0.67	0.25
	m	0.80	0.67	4.00	1.00	0.80	2.40	0.66	0.67	0.75
	t	0.60	0.67	2.00	0	1.20	2.20	0.67	1.00	0.50
	h	0.20	0.67	4.00	0	1.80	2.40	0.67	0.67	0.50
		n=5	n=3	n=1	n=1	n=5	n=5	n=3	n=3	n=4

a = acrosome, m = midpiece, t = tail, h = head

Table 2
Mouse Strain Pairs Which Differ in Lectin Binding

Lectins Bound Differently (p < 0.05)									
Strain	DBA/2J	T/t	+/T	+/t	T/t x DBA	ICR	Balb c	C57BL/6	B6D2F1
DBA/2J	-		BAN ^h LFA ^h				BAN ^{amt} DBA ^h	BAN ^{amt} DBA ^a UEA ^h NONE ^t	BAN ^m
T/t		-	BAN ^{mh}		WGA ^a	BAN ^m	BAN ^{am} UEA ^t DBA ^h WGA ^{am}	BAN ^{amh} UEA ^h DBA ^a NONE ^t	BAN ^m
+/T	BAN ^h LFA ^h	BAN ^{mh}	-	BAN ^h			BAN ^{amt} UEA ^t	BAN ^{amth} DBA ^a NONE ^t	BAN ^{mh}
+/t			BAN ^h	-			BAN ^{amt} UEA ^t DBA ^h	BAN ^{amt} UEA ^h DBA ^a NONE ^t	BAN ^m
T/t x DBA		WGA ^a			-		BAN ^{amt} UEA ^t DBA ^h	BAN ^{amt} UEA ^h DBA ^a NONE ^t	BAN ^m
ICR	BAN	BAN ^m				-	BAN ^{amt} UEA ^t DBA ^h	BAN ^{amt} UEA ^h DBA ^a	BAN ^m
Balb c	BAN ^{am} DBA ^h	BAN ^{amh} DBA ^h UEA ^t WGA ^{am}	BAN ^{am} UEA ^t	BAN ^{am} DBA ^h UEA ^t	BAN ^{am} DBA ^h UEA ^t	BAN ^{am} DBA ^h UEA ^t	-	SBA ^{ah} DBA ^a	BAN ^a
C57BL/6	BAN ^{am} DBA ^a UEA ^h NONE ^t	BAN ^{am} DBA ^a UEA ^h NONE ^t	BAN ^{amh} DBA ^a NONE ^t	BAN ^{am} DBA ^a UEA ^h NONE ^t	BAN ^{am} DBA ^a UEA ^h NONE ^t	BAN ^{am} DBA ^a UEA ^h	SBA ^{ah} DBA ^a	-	
B6D2F1	BAN ^m	BAN ^m	BAN ^{mh}	BAN ^m	BAN ^m	BAN ^m	BAN ^a		-

a = acrosome, m = midpiece, t = tail, h = head

various T/t locus sperm significantly more than to the midpieces of Balb c, C57BL/6 and B6D2F1 sperm. It also bound to the heads in +/T sperm significantly more than to the sperm heads in most of the other strains. The only significant difference in the binding of LFA was that it bound to the heads of DBA/2J sperm less than to those of +/T sperm. It also bound less to the heads and tails of DBA/2J sperm than to the other strains, but the difference was not significant. SBA bound significantly more to the heads and acrosomes of C57BL/6 sperm than to the other strains. WGA bound significantly less to the acrosomes of T/t sperm than to those of T/t x DBA/2J sperm, but significantly more to the midpieces of T/t sperm than to those of Balb c sperm. UEA and DBA bound to the heads and/or tails of Balb c and C57BL/6 sperm significantly more than to the those of T/t locus, DBA, and ICR sperm.

Lectin Binding to Testis Cells of Different Mouse Strains

Flow cytometry was used to quantitate binding of FITC-lectins to testis cells. Figure 9 (Appendix E) shows that in testis cells from ICR mice the lectins SBA, WGA, and CONA increased fluorescence relative to the background fluorescence. CONA had a broader peak, which indicates greater heterogeneity in its binding, i.e., it did not bind to all the cells equally. LFA bound only very slightly and UEA did not bind at all. In another experiment with testis cells from ICR mice, the lectins SBA, WGA, and CONA again bound well, LFA bound very slightly, while BAN and AA did not bind. The histograms showing this are those for control mouse #1 in Figures 17-20 (Appendix G). The

Table 3

Lectin Binding by ICR and T/t Mouse Sperm and Testis Cells

Lectin	Sperm		Testis Cells	
	ICR	<u>T/t</u>	ICR	<u>T/t</u>
SBA	+	+	+	+
WGA	+	+	+	+
CONA	+	+	+	+
BAN	+	+	-	
LFA	+	+	±	±
DBA	±	±		
UEA	±	-	-	-
AA	-	-	-	

percent positive cells are shown graphically in Figure 8 (Appendix E).

Lectin binding by T/t mouse testis cells was virtually identical to that for ICR mouse cells. They also showed excellent binding of SBA, WGA, CONA, and, very slightly, LFA, while UEA did not bind. The percent positive cells are also shown in Figure 8, and the histograms are in Figure 12 (Appendix E).

A comparison of lectin binding by ICR and T/t mouse sperm and testis cells is shown in Table 3. Binding of most of the lectins was similar, except that CONA bound to testis cells better than to sperm, BAN bound to sperm, not to testis cells, and LFA bound much better to sperm than to testis cells.

Effect of Trypsinization on Lectin Binding

The histograms in Figures 9-11 (Appendix E) show that prior treatment of testis cells from an ICR mouse with trypsin at a concentration of 2.5 micrograms/ml increased binding of SBA and decreased binding of WGA. The binding of CONA, LFA, and UEA were unaffected. The histograms in Figures 12-14 show similar results with testis cells from a T/t mouse. Trypsinization with either 2.5 or 5 micrograms/ml increased binding of SBA and decreased binding of WGA. This time CONA binding also was increased. There was a slight increase in binding with UEA and LFA, but this occurred in the control cells to the same extent, so is just an increase in background fluorescence. Both concentrations of trypsin affected the cells similarly.

Studies with Antiserum to T/t Locus Antigen

The results of the immunofluorescence study with anti-t⁰ serum are shown in Figure 15 (Appendix F). Two populations (large and small cells) were observed in the testis cell suspensions. Fluorescence was quantitated for both cell sizes and for the whole preparation as shown in Figure 1 (Appendix B). Region 1 is large cells, region 2 is small cells. The antibody clearly bound to both the T/t⁶ and the ICR cells with the same intensity. It appeared to bind better to the large testis cells (probably spermatocytes and spermatogonia) than to the small cells (spermatids). Similarly absorbed normal mouse control serum was not available to run, so it is possible that some fluorescence is due to non-specific antibody binding.

The cytotoxicity data are shown in Table 4. There was no

Table 4

Cytotoxicity of Anti-T/t Locus Serum

Mouse	Ab	C	% Cyto.	(Ab+C)-(C)
ICR	0	0	17.5	
	1:25	0	10.8	-6.7
	0	1:20	61.0	
	1:25	1:20	58.7	-2.3
	0	1:10	75.5	
	1:25	1:10	58.6	-16.9
	0	1:5	83.7	
	1:25	1:5	88.5	4.8
<u>T/t</u> ⁶	0	0	12.8	
	1:25	0	9.6	-3.2
	0	1:20	55.5	
	1:25	1:20	60.3	4.8
	0	1:10	69.5	
	1:25	1:10	63.5	-6.0
	0	1:5	74.8	
	1:25	1:5	85.7	10.9

greater cytotoxicity to the T/t cells than to the ICR cells, at all three complement levels. In fact, there was very little antibody mediated cytotoxicity at all, however the complement killed over 50% of the cells at the lowest concentration (1:20) used. Sperm are known to be exquisitely sensitive to the heterophile antibody in the normal rabbit serum, which was the complement source (Goodfellow et al., 1979). Apparently testis cells are very sensitive also, because low toxicity absorbed rabbit serum was used. The antiserum caused agglutination, but no cytotoxicity in the absence of complement.

Percoll Density Gradient Centrifugation

Several attempts were made to separate the various populations of mouse testis cells using density gradient centrifugation through Percoll. The purpose was to study lectin binding to the different cell types. Although the smaller, more dense spermatids did tend to move farther toward the bottom of the gradient than the larger, less dense cells, they were spread throughout the 25-35% Percoll layers. Other cell types were also present in large numbers in all the layers. Since good separation was not achieved, the method was abandoned.

Effect of Mutagen Treatment on Lectin Binding

Groups of 3 ICR mice were weighed and treated intraperitoneally with 50 or 100 mg/kg ENU. Control mice received 10% DMSO in saline, which was the diluent for the ENU. Eighteen days later, the mice were again weighed and the testis cells removed and weighed. The

sperm was also collected for enzyme assays, but these are not included in this report. Testis cell suspensions were prepared, incubated with FITC-labeled lectins, fixed, incubated with a DNA stain (propidium iodide), and analyzed by flow cytometry.

The amount of lectin binding to cells with 1C, 2C, or 4C DNA was quantitated. These cell populations were defined by regions 1, 2 and 3, respectively, which are shown in Figure 3 (Appendix C). These regions were set with testis cells from a normal ICR mouse at the beginning of the analysis of the experimental samples and were not changed during the course of the analysis. The peak fluorescence channels on a linear scale are shown in Figure 2 (Appendix C). They were 170, 333, and 703 for regions 1, 2, and 3, respectively. The corresponding mean fluorescence channels were 163.5, 337.3 and 705.6. This indicates near stoichiometric DNA staining in the 1C, 2C and 4C cell populations. The actual sample fluorescence was quantitated on a logarithmic scale, shown in Figure 3 (Appendix C). The peak fluorescence channels on this scale were 320, 475, and 657 for regions 1, 2, and 3, respectively, and the corresponding mean fluorescence channels were 323.1, 482.6, and 643.6. The percent of the total cells in each of these regions were 58.9%, 19.1%, and 16.5% for the 1C, 2C, and 4C populations of these normal mouse testis cells. They do not add up to 100 because doublets and small debris were counted in the cell total, but not included in the regions. 45,000 cells were analyzed for each sample where possible. In some of the ENU treated mice there were not sufficient cells left, so the analysis was done on all the cells available.

Comparisons were made between binding of the lectins vs background fluorescence within each drug treatment group and between drug doses within each lectin for the three cell types. The types of fluorescence data collected were histograms and numerical values for peak channel, mean channel, and % positive cells. The mean fluorescence channel is more meaningful than peak fluorescence channel in the case of skewed peaks. The percent positive cells is the percentage of cells with greater fluorescence than their controls without FITC-lectin. The percentages of cells in each DNA content group were also recorded. The numerical data were subjected to ONEWAY analysis of variance to determine which groups were significantly different from their controls at the 0.05 level.

Table 5 shows the effects of ENU treatment on body weight, testis weight and testis cell number. There was essentially no change in body weight. However, testis weight and total cell numbers decreased in a dose dependent manner after ENU treatment.

Table 5

ENU Effects on Body Weight, Testis Weight, and Testis Cell Counts

Treatment	Initial Body Weight Ave (g) SEM		Final Body Weight Ave (g) SEM		Final Testis Weight Ave (g) SEM		Cells/ Testis Ave
10% DMSO Control	29.3	1.80	30.7	1.88	0.219	0.011	2.1×10^7
50 mg/kg ENU	27.7 ↓5.5%	0.24	29.7 ↓3.3%	0.24	0.132 ↓39.7%	0.014	8.8×10^6 ↓58.1%
100 mg/kg ENU	26.3 ↓10.2%	1.59	29.3 ↓4.6%	1.47	0.109 ↓52.6%	0.013	3.6×10^6 ↓82.9%

Table 6

Percentages of Cells With 1C, 2C and 4C DNA After ENU Treatment

Treatment	Lectin	% 1C Ave	Cells SEM	% 2C Ave	Cells SEM	% 4C Ave	Cells SEM
10 % DMSO Control	None	53.8	3.02	12.5	0.20	14.4	0.73
	SBA	47.2	1.84	13.6	0.71	15.4	0.78
	WGA	46.7	2.01	13.8	0.55	15.6	0.55
	BAN	47.0	2.99	13.5	0.28	16.1	0.87
	AA	*46.0	2.09	13.5	0.26	16.6	0.95
	LFA	48.5	3.20	13.4	0.52	15.4	0.90
	CQNA	*37.1	2.32	*14.2	0.40	*18.5	1.18
	All	46.6	1.30	13.5	0.11	16.0	0.28
50 mg/kg ENU	None	† 45.2	1.93	† 16.3	0.75	† 18.5	1.38
	SBA	†*37.8	1.28	† 16.6	0.52	† 20.7	1.70
	WGA	†*34.7	1.08	† 17.2	1.11	† 21.7	2.14
	BAN	46.1	1.13	15.7	0.90	18.8	1.56
	AA	*40.0	2.26	† 16.8	0.78	20.4	1.60
	LFA	44.7	1.82	† 15.7	0.70	† 20.1	1.42
	CQNA	*36.0	3.60	† 17.9	0.69	19.6	2.29
	All	† 40.5	1.13	† 16.6	0.39	† 20.0	0.24
100 mg/kg ENU	None	53.7	2.40	† 19.8	0.60	† 10.6	0.61
	SBA	*40.5	2.78	†*22.1	0.95	*14.9	1.67
	WGA	†*38.8	2.70	†*24.3	1.15	*14.2	1.31
	BAN	56.3	4.09	† 19.5	0.63	† 9.6	0.71
	AA	*43.1	2.36	†*22.5	0.51	*13.6	0.55
	LFA	51.8	3.83	20.4	0.47	10.2	0.41
	CQNA	*45.6	3.98	† 21.8	0.34	† 12.3	1.03
	All	47.1	1.75	† 21.5	0.37	† 12.2	0.46

* = P < 0.05 for Lectins vs. None, † = P < 0.05 for ENU vs. Control

Significantly decreased percentages of 1C cells and increased percentages of 2C and 4C cells were found after 50 mg/kg ENU treatment. However, after 100 mg/kg ENU, the percentages of 1C cells were unchanged, 2C cells were increased and 4C cells were decreased. This is shown in Table 6 and displayed graphically in Figure 16 (Appendix G).

Histograms for all the lectins plotted vs. their controls for one mouse of each group are shown in Figures 17, 18, and 19 (Appendix G) for cells with 1C, 2C, and 4C DNA, respectively. One can see that the lectins SBA, WGA, and CONA bound to the control cells as expected, while BAN and AA did not. LFA bound very slightly. Interestingly, SBA bound less well to 4C cells. It is also clear that binding of SBA, WGA, CONA, and LFA lectins increased with ENU treatment and that this occurred in all the cells, regardless of DNA content.

The dose related increase in lectin binding after ENU treatment is even more obvious in Figures 20-26 (Appendix G), in which each lectin is plotted individually. Binding increased in 1C, 2C, and 4C cells with SBA, WGA, CONA, and LFA. It increased slightly with AA too at the high dose in 4C cells only. It did not occur with BAN. The CONA curves are skewed, indicating that not all the cells increased in fluorescence. Also, there was more cell loss and greater heterogeneity of binding with CONA than with the other lectins. The apparent cell loss is probably due to agglutination, since doublets and cell aggregates were gated out and not included in any of the three regions. The SBA and WGA curves are definitely biphasic for the 2C cells, again indicating that not all the cells bound lectin. In most cases, however, the whole peak moved, so it can be assumed

that all the cells bound the lectin equally. It also becomes obvious that as DNA content increased, so did fluorescence. This happened to background fluorescence as well as to cells which bound FITC-lectins, so was ascribed to larger surface area of the larger cells.

Data from all three of the mice in each group are plotted in these figures. One can see that nearly identical histograms were obtained with the mice in each group, making even small changes more meaningful. The numerical fluorescence data are shown in Tables 7, 8, and 9 for cells with 1C, 2C, and 4C DNA, respectively. The tightness of the data is also shown by the large number of significant differences among the various groups in the tables. Although LFA binding was very slight in the control cells, it is significant at the 0.05 level in both percent positive cells and mean channel (fluorescence intensity), regardless of DNA content. Looking at percent positive cells, ENU increased binding only in the four lectins which bound it normally, and the LFA increase was not significant. In mean channel, however, ENU increased fluorescence significantly in all the cells, even those without lectin.

It is apparent from Tables 7-9 that mean channel is a more sensitive indicator of small, but significant, differences in fluorescence than either peak channel or percent positive cells. However, the percent positive cells provides the best visualization of increases over background. Comparison of Figures 27 and 28 (Appendix G) show this clearly. These figures show the ENU-induced increase in binding, but not as well as the histograms. Figure 29 (Appendix G) shows the increase in fluorescence with DNA content very well.

Table 7

Fluorescence of FITC-Lectins on Testis Cells With 1C DNA

Mouse Treatment	FITC Lectin	Peak Channel		Mean Channel		% Positive	
		Ave	SEM	Ave	SEM	Ave	SEM
10% DMSO Control	None	187.7	3.76	182.8	4.12	0.9	0.11
	SBA	*288.3	22.45	*306.5	8.75	*61.6	4.88
	WGA	*318.3	9.49	*340.3	6.02	*81.4	4.32
	BAN	192.7	5.36	197.4	2.12	3.9	0.86
	AA	192.0	8.08	193.3	2.92	3.3	0.50
	LFA	207.0	2.08	*216.3	3.29	*10.6	0.51
	CONA	*411.7	14.83	*365.7	9.75	*78.8	2.47
50 mg/kg ENU	None	189.0	7.00	† 196.6	3.87	1.7	0.75
	SBA	†*420.0	9.02	†*403.6	15.03	†*86.9	4.84
	WGA	†*446.7	22.25	†*422.1	12.06	†*92.0	2.39
	BAN	† 211.7	6.64	† 210.0	3.72	4.0	1.83
	AA	204.3	0.88	† 211.2	3.82	5.2	2.91
	LFA	*239.0	10.53	†*250.2	9.36	*19.1	5.79
	CONA	†*530.7	17.45	†*486.4	17.17	†*91.2	0.80
100 mg/kg ENU	None	† 207.3	8.35	† 206.3	3.12	0.9	0.08
	SBA	†*446.0	15.56	†*422.1	5.53	†*87.2	1.23
	WGA	†*476.7	5.24	†*452.2	3.02	†*92.2	0.40
	BAN	† 226.0	2.00	†*223.4	4.16	3.1	0.28
	AA	† 231.3	10.65	†*226.7	5.88	4.6	0.58
	LFA	†*273.3	7.31	†*277.8	6.26	*22.9	2.88
	CONA	†*552.0	13.86	†*510.5	1.39	†*89.6	2.62

* = P < 0.05 for Lectins vs. None, † = P < 0.05 for ENU vs. Control

Table 8

Fluorescence of FITC-Lectins on Testis Cells With 2C DNA

Mouse Treatment	FITC Lectin	Peak Channel		Mean Channel		% Positive	
		Ave	SEM	Ave	SEM	Ave	SEM
10% DMSO Control	None	303.3	2.19	308.2	5.77	5.5	1.90
	SBA	*410.7	10.84	*414.0	5.33	*61.5	5.12
	WGA	*466.0	11.35	*449.2	1.87	*82.5	2.21
	BAN	317.3	5.36	*337.9	0.96	13.7	1.46
	AA	311.7	2.33	*324.4	3.16	9.6	2.08
	LFA	334.0	17.00	*353.0	3.93	*21.5	2.15
	CONA	*510.0	29.67	*474.4	6.55	*80.3	2.48
50 mg/kg ENU	None	317.0	8.14	313.2	7.07	3.6	1.21
	SBA	+*512.0	34.64	+*483.4	11.73	*76.3	7.28
	WGA	+*552.0	19.07	+*501.2	9.68	*83.6	3.56
	BAN	318.7	3.53	*349.5	5.14	14.5	1.99
	AA	328.7	4.37	328.5	2.90	8.0	3.20
	LFA	367.3	11.25	+*380.2	8.16	*28.1	7.55
	CONA	+*615.3	19.93	+*573.3	10.59	+*89.2	2.32
100 mg/kg ENU	None	325.3	13.28	321.9	3.47	2.7	0.98
	SBA	+*559.3	20.69	+*495.4	8.77	*75.7	4.25
	WGA	+*575.7	23.13	+*513.7	4.05	*79.0	2.75
	BAN	342.0	12.34	+*367.6	7.25	*17.2	0.17
	AA	+ 347.3	8.21	+*345.8	9.01	9.2	1.46
	LFA	+*403.7	6.74	+*405.0	6.45	*33.2	5.88
	CONA	+*667.7	9.82	+*604.9	4.31	*87.5	2.77

* = $P < 0.05$ for Lectins vs. None, + = $P < 0.05$ for ENU vs. Control

Table 9

Fluorescence of FITC-Lectins on Testis Cells With 4C DNA

Mouse Treatment	FITC Lectin	Peak Channel		Mean Channel		% Positive	
		Ave	SEM	Ave	SEM	Ave	SEM
10% DMSO Vehicle	None	389.7	4.10	391.4	2.59	2.9	0.64
	SBA	*439.0	4.58	*467.6	3.99	*43.1	6.97
	WGA	*495.7	6.23	*510.4	1.70	*78.5	5.59
	BAN	395.3	2.60	*409.3	0.99	7.8	0.55
	AA	395.0	2.89	*403.1	0.81	6.5	1.11
	LFA	*413.7	6.69	*429.1	0.78	*15.8	2.26
	CONA	*510.7	6.23	*546.1	7.12	*81.9	5.29
50 mg/kg ENU	None	395.7	7.31	397.1	1.55	3.2	0.18
	SBA	*485.3	20.08	†*516.3	10.61	*58.1	6.03
	WGA	*538.7	13.59	†*561.4	8.43	*86.4	3.77
	BAN	402.7	5.70	414.6	1.85	7.5	0.86
	AA	398.0	1.15	410.5	2.63	6.5	1.25
	LFA	434.3	6.33	*449.7	7.44	*22.1	3.79
	CONA	†*679.3	41.95	†*632.0	8.23	*89.0	2.45
100 mg/kg ENU	None	† 426.3	2.91	† 429.4	3.33	3.0	0.49
	SBA	†*623.3	21.75	†*592.5	10.28	†*75.9	3.44
	WGA	†*651.7	32.35	†*614.0	6.62	*81.9	3.14
	BAN	425.0	14.00	†*459.1	6.88	†*15.0	1.07
	AA	† 458.3	15.16	†*452.0	7.59	9.0	1.35
	LFA	†*532.0	17.67	†*516.8	9.10	†*44.3	6.89
	CONA	†*747.3	12.78	†*719.0	4.35	*90.9	3.33

* = P < 0.05 for Lectins vs. None, † = P < 0.05 for ENU vs. Control

DISCUSSION

Mouse Strain Differences in Lectin Binding

Sperm Studies

The finding that LFA bound more to the heads +/T mouse sperm than to the DBA/2J sperm is especially interesting, because this lectin binds sialic acid, thought to be a marker for the T/t locus (Cheng and Bennett, 1980). The fact that all the other strains bound this lectin well at all sites except the acrosome, weakens the evidence for this being a T locus effect.

It was hoped that there would be differences in the binding of AA and UEA by the T/t mice because these lectins bind L-fucose, which is thought to be a marker for the t⁰ and t⁶ haplotypes, (Cheng and Bennett, 1980). Both of these lectins bound slightly better to the midpiece region of the T/t sperm than to the midpiece of the other strains, but the only differences in UEA or AA binding which were statistically significant were greater UEA binding to the heads of C57BL/6 and tails of the Balb c sperm than to the other strains. With DBA and SBA, too, binding was greater and to more regions of the sperm in those two strains. BAN did consistently bind significantly better to T/t, DBA/2J, and ICR sperm midpieces than to the other strains. It is indicative of more galactose on their surfaces. Galactose is supposed to be a marker for the t^{w12} and t^{w32} haplotypes (Cheng and Bennett, 1980). We found no clear evidence of fucose on our t⁶ mice. We found strong evidence for sialic acid, but on all

the mice, not just T mice.

Binding of BAN, LFA, SBA, and WGA indicates the presence of galactose, sialic acid, galactosamine, and glucosamine, respectively on the surface of the dried sperm of all the strains. Lack of significant binding of DBA, UEA, and AA indicates a lack of N-acetyl-D-Galactosamine and L-fucose on all these sperm. These results are consistent with published data for the hamster (Ahuja, 1984), for lack of DBA and UEA binding. However, we found binding of SBA to sperm acrosomes, while they did not. They found binding of CONA to sperm heads, we found binding of this lectin only to acrosomes of DBA mice and midpieces of some of the other mice. We found binding of BAN to midpieces and some heads, they did not. Other workers (Schwarz and Koehler, 1979) have found binding of SBA, WGA and CONA to acrosomes of guinea pig sperm. These results are also in contrast to those of Smith (1984) who reported no binding of BAN to DBA, C57, ICR, BALB c, T/t⁶, or +/t⁶ mouse sperm.

It was disappointing to be unable to use the flow cytometer for analysis of lectin binding to sperm from different mouse strains because of the greater sensitivity and more accurate quantitation of fluorescence it provides. However the poor staining of the sperm in suspension and the poor ability of the flow cytometer to detect fluorescence on them prevented this. One reason the dry slide method resulted in greater staining might be that the drying process results in uncovering of more lectin binding sites on the sperm surface than are normally exposed. This is the reason lysolecithin was tried, since it is reported to uncover intracytoplasmic antigens, which may

then be detected by immunofluorescence (Schroff, et al., 1984).

Testis Cell Studies

Testis cells from both ICR and T/t⁶ mice bound SBA, WGA and CONA well, LFA slightly, but did not bind UEA, AA or BAN. These data indicate the presence of galactosamine, glucosamine, and mannose on these cells. There does not appear to be detectable L-fucose, sialic acid or galactose, even on the T/t⁶ mouse, despite the reported (Cheng and Bennett, 1980) association of L-fucose with the t⁶ haplotype and sialic acid with the T antigen.

The major contrast to the binding data for the sperm is the binding of BAN and LFA by the sperm, but not by the testis cells. This indicates presence of galactose and sialic acid on sperm, but not on testis cells. It is probable that this is a real developmental difference between spermatids and mature sperm in the surface carbohydrates expressed (Voglmayr et al, 1983; Kaneko et al, 1984).

It was not surprising that sialic acid and galactose were found on the sperm cells. Sialic acid is often found on the outer cell membrane and is known to exist on sperm membranes (Voglmayr et al, 1983; Kaneko et al, 1984). It is thought to regulate molecular and cellular recognition. Sialic acid can either mediate recognition as part of a receptor or antigenic determinant or prevent it by masking specific recognition sites (Schauer, 1985). In erythrocytes sialic acid prevents phagocytosis by macrophages, which have a galactose specific lectin on their surface (Schauer, 1985). Often galactose is masked by sialic acid. In juvenile rat ovaries sialic acid masks

gonadotropin receptors and decreases during maturation. Perhaps galactose and sialic acid are added to the surface of sperm to serve a masking function.

Effect of Trypsinization on Lectin Binding

Trypsinization of the testis cells prior to incubation with the lectins was an attempt to increase visualization of lectin binding, particularly the binding of LFA and UEA to the supposed T/t specific carbohydrates. It was hoped that the trypsin would uncover masked sites in the T/t mice, not in the ICR mice. This did not occur. As before, UEA failed to and LFA bound only weakly bind to both strains, indicating that sialic acid and L-fucose are probably not present in increased amounts on the surface of testis cells from our T/t mice.

The observation that trypsinization increased binding of SBA to testis cells while it decreased binding of WGA is further evidence for masked carbohydrate binding sites on the surface of germ cells. Proteolysis can remove some binding sites while uncovering others. The specificity of SBA is for N-acetyl-galactosamine, so these sites may be uncovered. The specificity of WGA is for N-acetyl-glucosamine, so these sites are removed or covered. It does not seem likely that glucosamine is somehow converted into galactosamine by trypsinization. It is more likely that additional cryptic SBA sites are uncovered by trypsin and some WGA sites are removed. It is known that the specificity of lectins changes depending on the fine structure of complex membrane glycoprotein, not just monosaccharide specificity (Debray et al., 1981). Therefore changes in either the

saccharidic sequences on glycoproteins or glycolipids or the conformation of membrane glycoproteins could result in changes to lectin binding sites.

The surface of malignant cells is very different from that of normal cells, and trypsin may mimic the changes which occur during transformation. There is evidence that some lectin binding sites, such as those for CONA (but not those for WGA) on lymphocytes are floating in the fluid cell membrane and can be induced to form caps in normal lymphocytes, but clusters in malignant ones when complexed with the lectin (Imbar, 1973). The induction of movement could result in exposure, concentration or rearrangement of sites. The distribution of CONA binding sites has been shown to change after trypsin treatment, presumably due to increased fluidity of the membrane after trypsinization (Imbar, 1973).

SBA and WGA agglutinate somatic cells which have either been transformed with viral or chemical carcinogens or irradiation or treated with trypsin (Lis and Sharon, 1972 and 1973). The agglutination is specifically inhibited by N-acetyl-D-galactosamine and, to a lesser extent by D-galactose, indicating it is due to the carbohydrate, not just increased stickiness. It is thought that cryptic receptors are exposed (Lis and Sharon, 1973). Apparently testis cells, like all cells, have masked galactose sites on their surfaces in addition to exposed ones.

Antiserum to T/t Locus Antigen

Both the immunofluorescence and the cytotoxicity studies re-

vealed that the anti- t^0 serum probably recognizes antigens on the surface of testis cells from both T/t^0 and ICR mice. They may or may not be the same antigens. The apparent inability of the anti- t^0 serum to detect t^6 antigens is probably not due to a difference between the t^0 and t^6 haplotypes, since they are assumed to be in the same t-6,t-0 lethal group (Jax Mice price list, 1985). The fact that sperm and testis cells of both strains bound the same lectins (while other strains differed) may mean that ICR mice share the T/t antigens. Goodfellow et al. (1979) failed to detect T/t locus-specific antibody in anti- T/t^{W2} mouse sera. It is becoming increasingly clear that the cell surface antigenic determinants thought to be specific for the various t haplotypes (Cheng and Bennett, 1980; Goodfellow et al., 1979; Yanagisawa et al., 1974) are in fact carbohydrate structures found in both wild-type and t -carrying mice, but expressed at different levels determined by the t -haplotypes (Silver, 1985). It is possible also that the genes which specify germ cell carbohydrate structure are not the t haplotypes but are simply located within the same T/t locus. They appear to correlate with the t haplotypes because of decreased recombination. Apparently there was enough of this antigen on the surface of both the ICR and T/t mice to cause similar immunofluorescence. In both cell preparations immunofluorescence was greater in the larger cells, which are primarily spermatogonia and spermatocytes rather, rather than in the smaller spermatids, possibly due to greater surface area.

The actual genotype of our T/t mice is difficult to ascertain. They were derived from $T/t \times T/t$ matings and tail length phenotype

was used to determine the hypothetical genotype. Mice with no tail were assumed to be T/t, mice with short tails were assumed to be +/T, and mice with normal tails were assumed to be either +/t or +/+. There were no T/T or t/t mice because they are lethal genes. It has now been shown that the t gene which controls tail phenotype (tet) is independent of both lethal genes and sperm genes, although closely linked to them (Silver, 1985). Therefore, we cannot be absolutely sure of the genetics of our mice. This may account for our failure to detect T/t locus differences by lectin binding or antiserum.

Effect of Mutagen Treatment on Lectin Binding

The loss in testis weight and testis cell number after ENU treatment indicates germ cell damage and has been observed before with this mutagen (Ficsor et al., 1984). Histopathological studies have ascribed this to loss of spermatogonia within 1 week of treatment, spermatocytes and spermatids 2-4 weeks after treatment, and spermatozoa 4-7 weeks after treatment (Rodriguez et al., 1983). The increase in 2C and 4C cells and concomitant decrease in 1C cells we observed 18 days after treatment with 50 mg/kg ENU confirms the loss of haploid spermatids. These cells would have been spermatogonia at the time of ENU treatment (Goldberg et al., 1977). The decrease in 4C cells and increase in 2C cells seen with 100 mg/kg indicates greater destruction of spermatogonia and less repopulation of testis by survivors. These changes in the percentages of the three cell types are similar to those reported by Hacker et al. (1980) after x-irradiation, in that the spermatids were less sensi-

tive to irradiation than the 2C and 4C cells. They saw initial decreases in 2C, 4C, and S-phase cells during the first week, followed by increases in these cell types and decreases in 1C cells 21 days after irradiation.

The percentages of the 1C, 2C, and 4C cells we found compare favorably to those reported for flow cytometry of testis cells (Hacker et al., 1980 and 1981). We did not see the second 1C peak of elongated spermatids, either because the formalin fixation and longer time of DNA staining allowed proportional uptake of the stain or because the timing of the experiment was such that few were present when the cells were harvested. The experiment was planned so that the descendants of mutagen treated spermatogonia would be round spermatids.

As early as one week after ENU treatment, new rounds of spermiogenesis cease, and if the dose is high greater than 250-300 mg/kg the mouse remains sterile. If the dose is lower, surviving mutagenized spermatogonial stem cells repopulate the testis and spermatogenesis begins again. A single dose of 250 mg/kg ENU induces mutants at an average frequency of 1/1100 per locus. This efficient procedure has been used to produce new mouse T locus mutants by Bode (1984). The loss of haploid cells in our mice is evidence that the ENU dose was high enough to cause mutagenesis in addition to more generalized toxic effects on germ cells.

ENU consistently and significantly increased binding of SBA, WGA and CONA, lectins which normally bind to mouse testis cells. It also caused binding by LFA and AA, which do not normally bind well. The

binding of AA was increased only in 4C cells and only at the high ENU dose. It seems likely that it is due to damaged spermatogonia that have survived, but not divided. The increased binding of LFA was greatest in 4C cells, but occurred in the other cell types as well. The increased binding of the other lectins seemed to occur in all the cells equally, regardless of their DNA content. In no case was the increase greater in 1C cells than in 2C or 4C cells. This does not rule out a mutation, since a large effect on 1C cells would only be expected if the gene was expressed in haploid cells (Ginsberg and Ficsor, 1985). Increased heterogeneity of binding was typically seen in the cells from treated mice, evidenced by broader, lower peaks. This was especially noticed with CONA. Increased coefficient of variation such as this often occurs after irradiation or mutagen treatment and may reflect abnormal meiotic segregation (Hacker et al., 1981).

Given the toxicity of ENU for germ cells, a great deal of generalized cell damage would be expected. It is likely that this accounts for the majority of the effects seen this early after treatment. Increased lectin binding could be due to increased synthesis of new sites as the damaged cells repair their membranes. New sites could also be more available for binding because they are not yet masked as in the normal cell.

It is also possible that mutation toward greater lectin binding has occurred in some of the cells. Considering that the mutation frequency of ENU for the T locus genes is approximately 1/1500 per locus (Bode, 1984), it is highly unlikely that we would be able to

detect the few mutant cells that might be present with this method. In addition, one must normally observe many generations of progeny to provide evidence of heritability.

Future experiments directed toward detecting mutations might utilize the sorting capability of the cytofluorograf to enrich for mutant cells in testis cell populations long enough after ENU treatment for the repair process to be completed. Then changes in binding would be more likely to be due to mutation. If the mutant cells could be cultured, induced to divide, or hybridized with myeloma cells, the progeny could be examined for the mutation.

Appendix A

Lectin Carbohydrate Binding Specificities

LECTIN CARBOHYDRATE BINDING SPECIFICITIES

Lectin	Source	Major Specificity
Soy Bean Agglutinin (SBA)	<u>Glycin max</u>	N-acetyl- β -galactosamine β -galactose
Wheat Germ Agglutinin (WGA)	<u>Triticum vulgare</u>	N-acetyl- β -glucosamine
Horse Gram Lectin (DBA)	<u>Dolichos biflorus</u>	N-acetyl-D-galactosamine Type A erythrocytes
BSI-B ₄ Isolectin (BAN)	<u>Bandeireae simplicifolia</u>	galactose Type B erythrocytes
Asparagus Pea Lectin (AA)	<u>Lotus tetragonolobus</u>	α -L-fucose, D-galactose N-acetyl-D-galactosamine Type O erythrocytes
UEA-I Lectin (UEA)	<u>Ulex europeus</u>	α -L-fucose
Sea Slug Agglutinin (LFA)	<u>Limax flavus</u>	sialic acid
Concanavalin A (CONA)	<u>Canavalia ensiformis</u> Jack bean	α -mannose α -glucose

Appendix B

TWOGATEFL Cytofluorograf Program: Figure 1

TWO GATE FL

```

00 RPL/2150 SYSTEM ...ORTHO DIAGNOSTIC SYSTEMS.....
05 REM HIST/LIST PROTOCOL THREEPTDIF: 3 PT DIFF W/ GATE FOR IMMUNOFLUORESCENCE
07 REM
10 REM X1=PMT2 (90' SCATTER)=H1
20 REM Y1=PMT3 (FORWARD SCATTER)=H2
30 REM X2=PMT1 (GREEN FLUORESCENCE)=H3
40 REM
100 GET H1 FROM PORT1    \GET 90' SCATTER RAW PARAMETER
110 GET H2 FROM PORT2    \GET FRW-SC RAW PARAMETER
120 GET H3 FROM PORT3    \GET GRN-FL RAW PARAMETER
200 LIST H1,H2,H3        \COLLECT ALL 3 RAW PARAMETERS IN LIST MODE
250 LET F1=H3
251 LET F2=H3
260 LET P1=PAIR(H2,H1)    \CYTOGRAM OF FRW-SC(Y-AXIS) VS 90'-SC(X-AXIS)
267 REM
268 REM LINE 270 UNCONDITIONALLY CREATES HISTOGRAMS OF RAW PARAMETERS
269 REM   AS WELL AS THE CYTOGRAM
270 HIST H1,H2,H3,P1
274 REM
275 REM LINE 280 AND 281 CREATES HISTOGRAM F1(GRN-FL) FROM
276 REM REGION 1 OF P1 AND F2 (GRN-FL) FROM REGION 2 OF P1
280 IF REG 1 OF P1 THEN HIST F1
281 IF REG 2 OF P1 THEN HIST F2
290 LABEL H1 WITH "90' SCATTER"
300 LABEL H2 WITH "FORWARD SCATTER"
310 LABEL H3 WITH "GREEN FLUORESCENCE"
320 LABEL F1 WITH "GRN-FL, REG1 OF P1"
321 LABEL F2 WITH "GRN-FL, REG2 OF P1"
330 LABEL P1 WITH "FRW-SC/Y VS 90'-SC/X"
9999 END

```



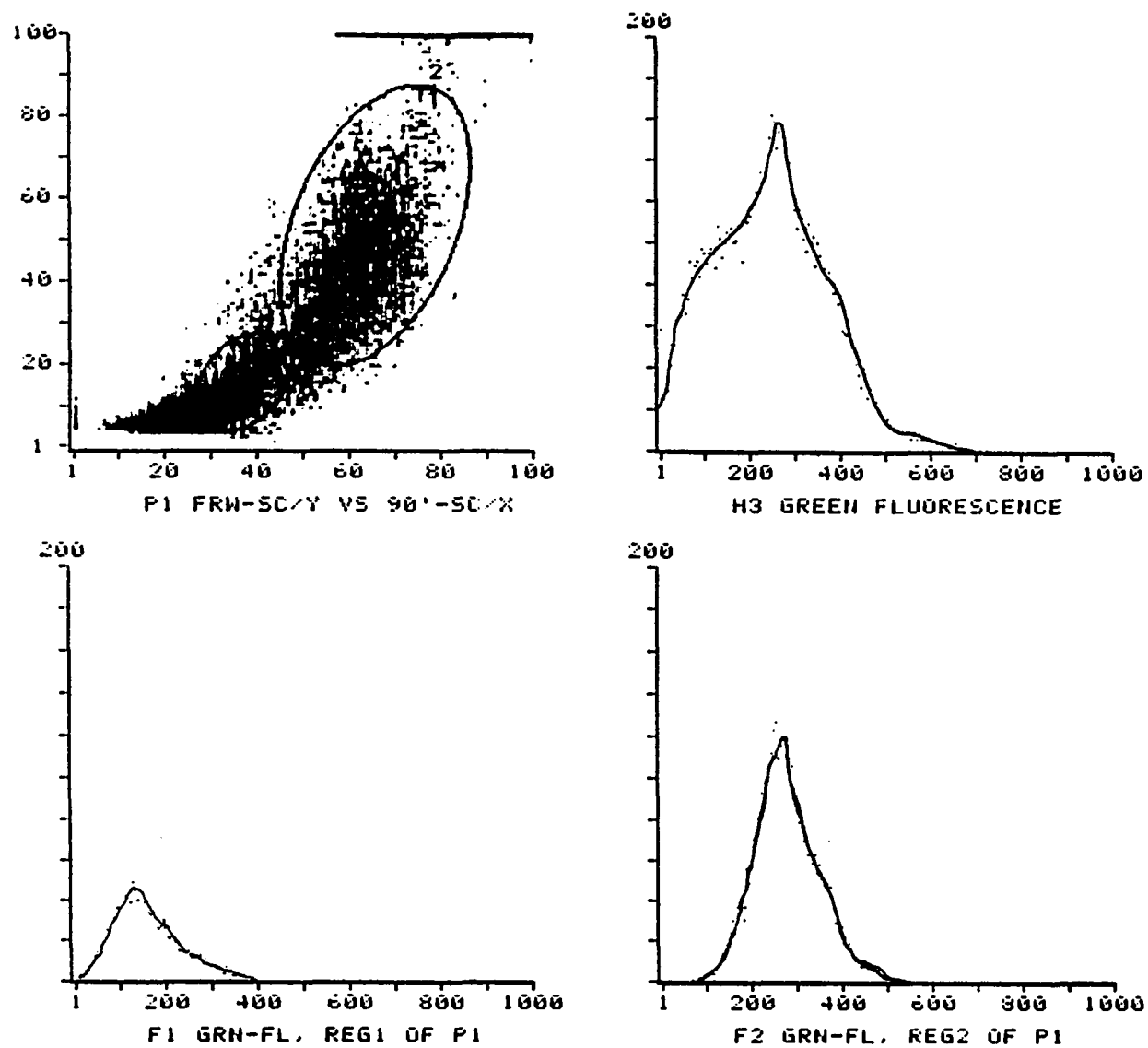


Figure 1. TWOGATEFL Cytogram and Histograms.

Appendix C

FITCPI2 Cytofluorograf Program: Figures 2-4

```
1 REM CELLS SHOULD BE LABELLED WITH FITC-LECTIN, FIXED WITH 1% FORMALIN,
2 REM TREATED WITH 1 MG/ML RNAse FOR 15 MIN AT 37 DEG C,
3 REM STAINED WITH 50 UG/ML PROPIDIUM IODIDE OVERNIGHT IN THE COLD
4 REM ARGON LASER SHOULD BE AT 488 NM
5 REM
6 REM
7 REM
8 REM
9 REM PARAMETERS SHOULD BE SET UP AS FOLLOWS*
10 REM
11 REM SELECT PMT3 (RED FL) ON X1 AREA
12 REM SELECT PMT3 (RED FL) ON Y1 PEAK
13 REM SELECT PMT1 (GRN FL) ON X2 AREA
14 REM
15 REM
100 GET A1 FROM PORT1\RED AREA
110 GET Y1 FROM PORT2\RED PEAK
120 GET S1 FROM PORT3\GRN FL
130 LET P1=PAIR(Y1,A1)
140 HIST P1
141 HIST A1
142 HIST S1
150 REM
160 REM
170 LET M1=S1
171 LET M2=S1
172 LET M3=S1
180 IF REG 1 OF P1 THEN HIST M1\HIST GRN FL OF P1R1
181 IF REG 2 OF P1 THEN HIST M2
182 IF REG 3 OF P1 THEN HIST M3
230 LABEL P1 WITH "DNA PEAK (Y) VS AREA (X)"
240 LABEL M1 WITH "FITC FLUOR FROM P1R1"
241 LABEL M2 WITH "FITC FLUOR FROM P1R2"
242 LABEL M3 WITH "FITC FLUOR FROM P1R3"
250 LABEL S1 WITH "UNGATED FITC FLUOR"
1000 END
```

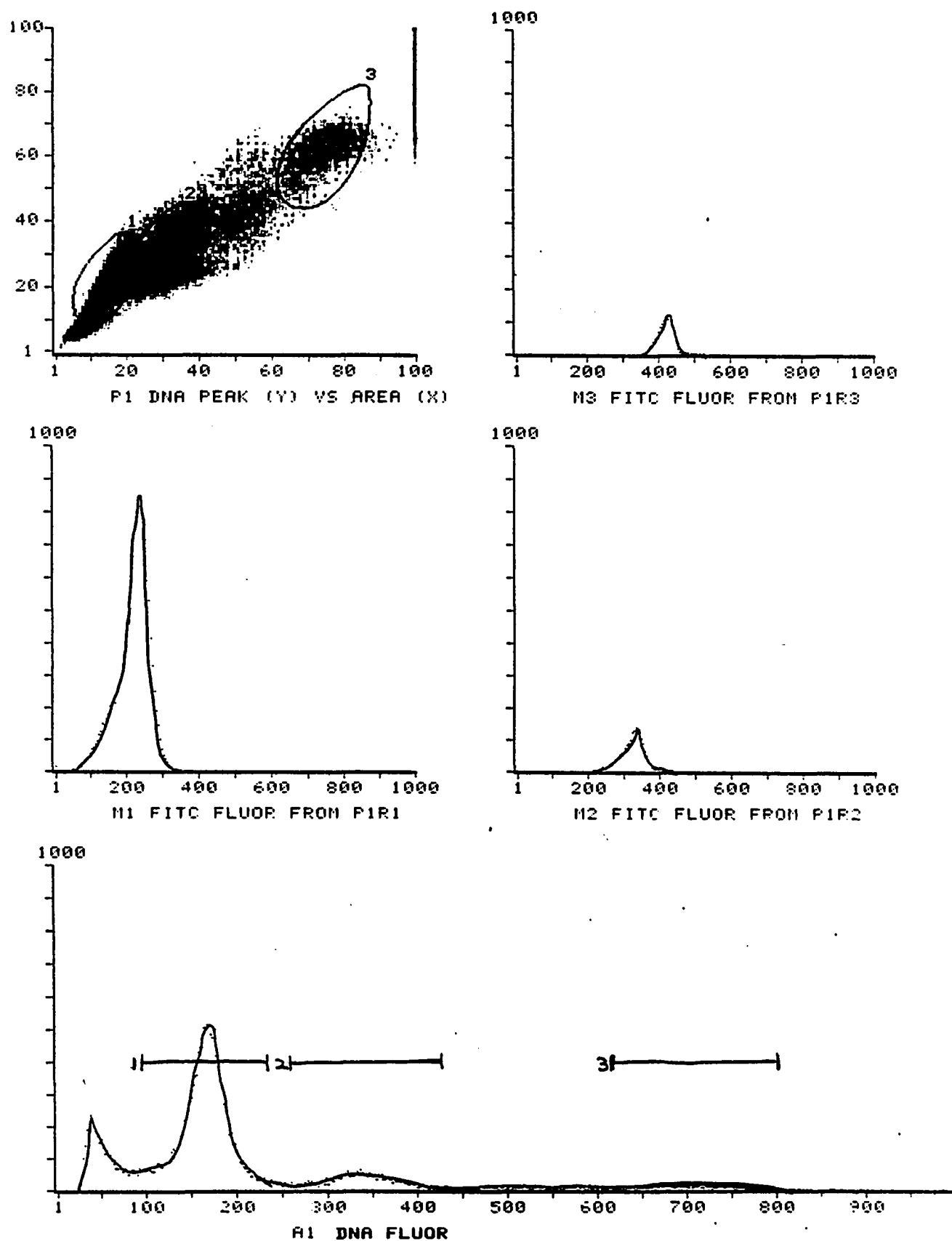


Figure 2. FITCPI2 Cytogram and Histograms With Linear Scaling.

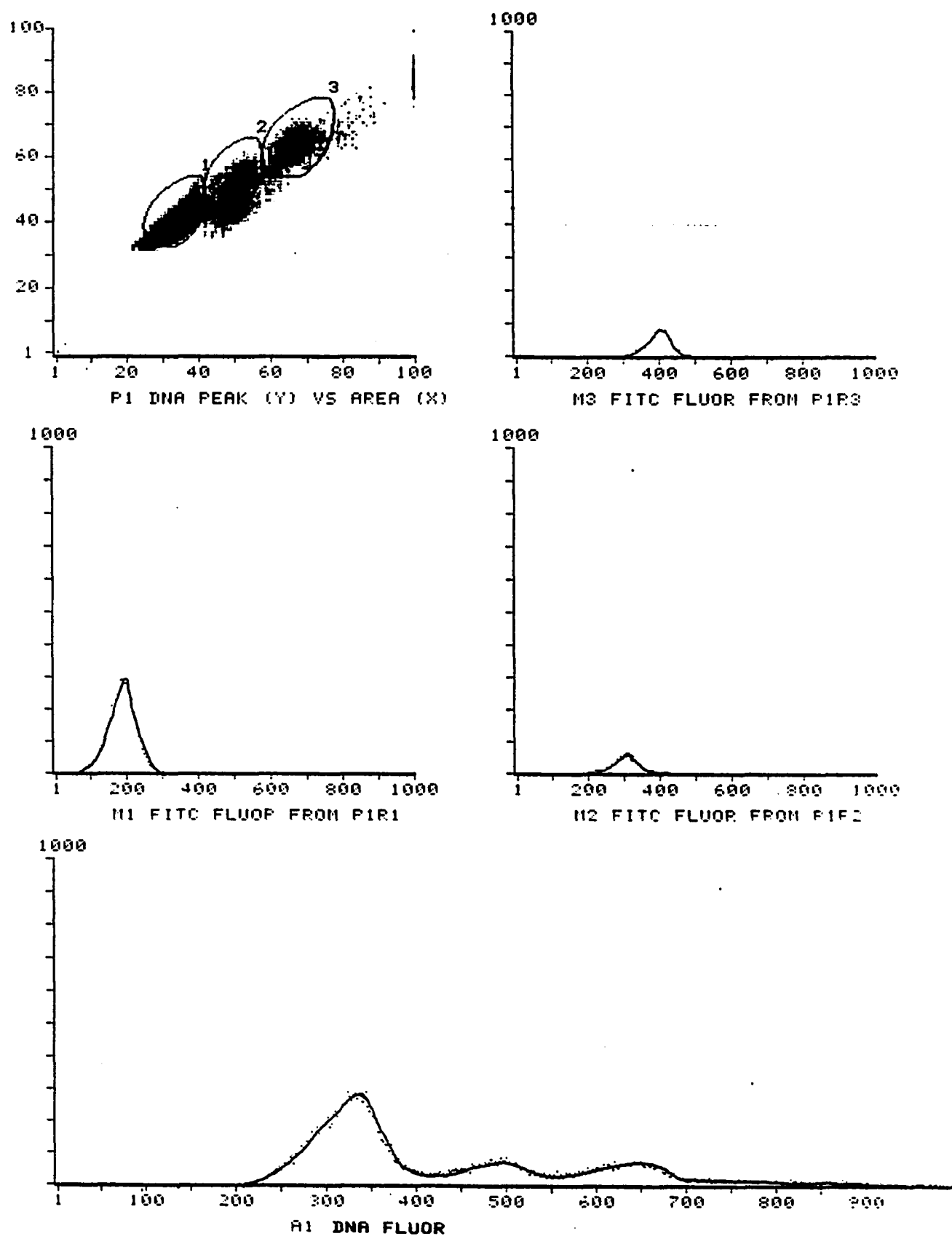


Figure 3. FITCPI2 Cytogram and Histograms With Logarithmic Scaling.

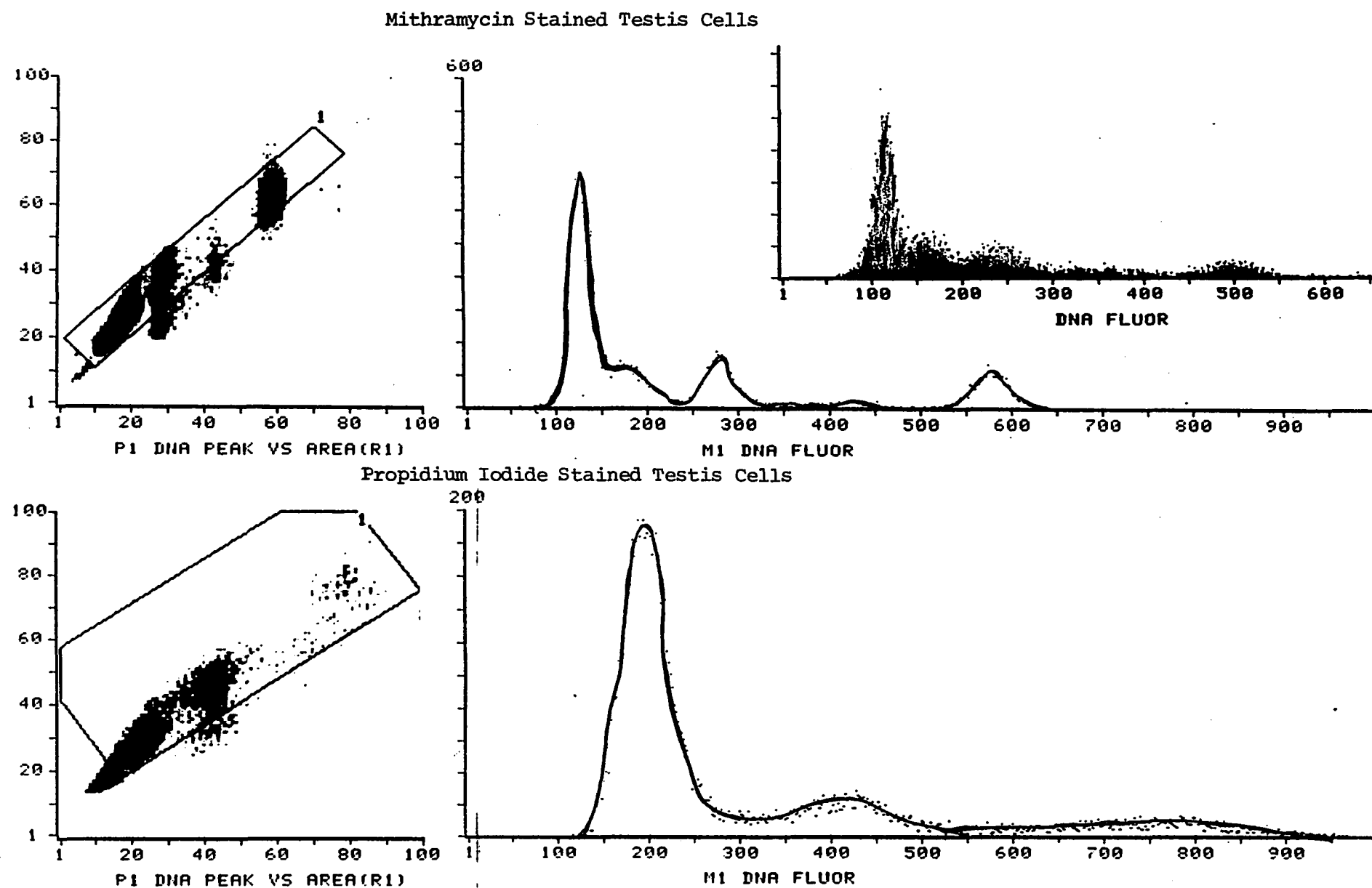
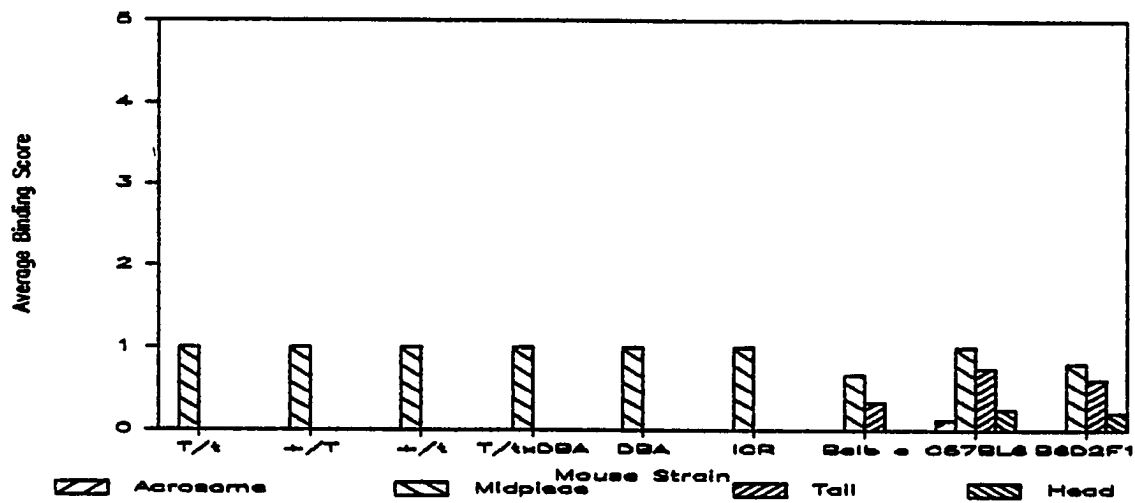


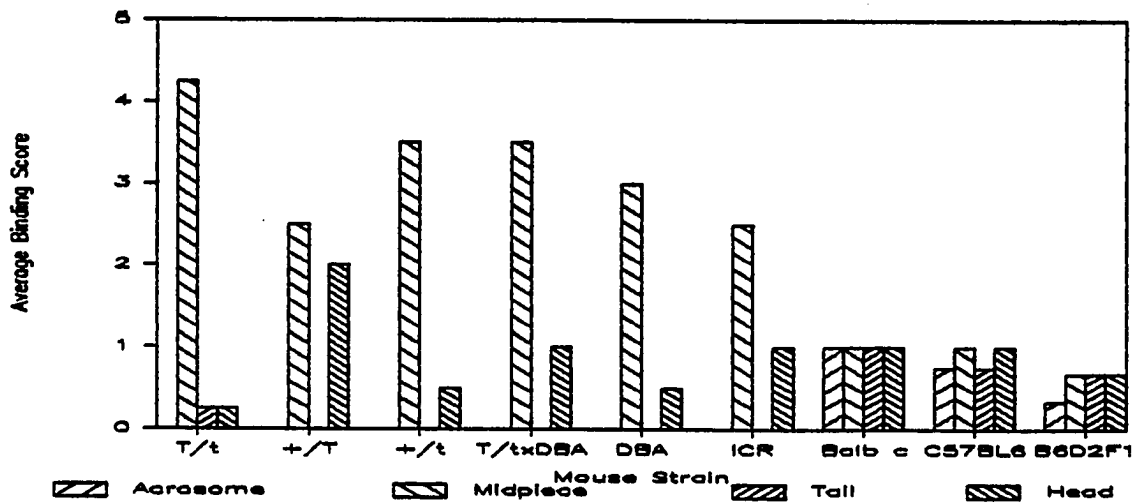
Figure 4. DNA Fluorescence of Mithramycin and Propidium Iodide Stained Testis Cells.

Appendix D

Lectin Binding to Sperm: Figures 5-7



BAN



LFA

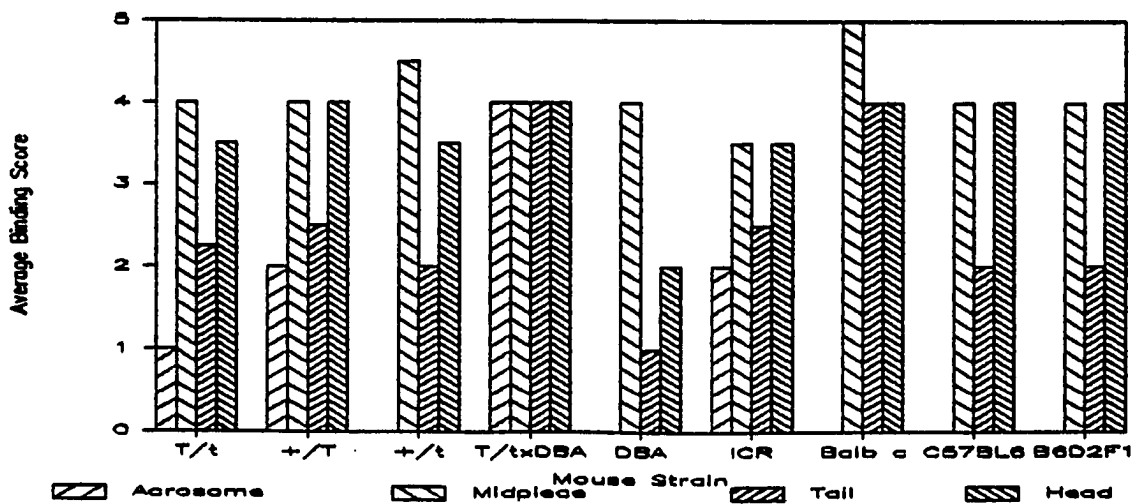
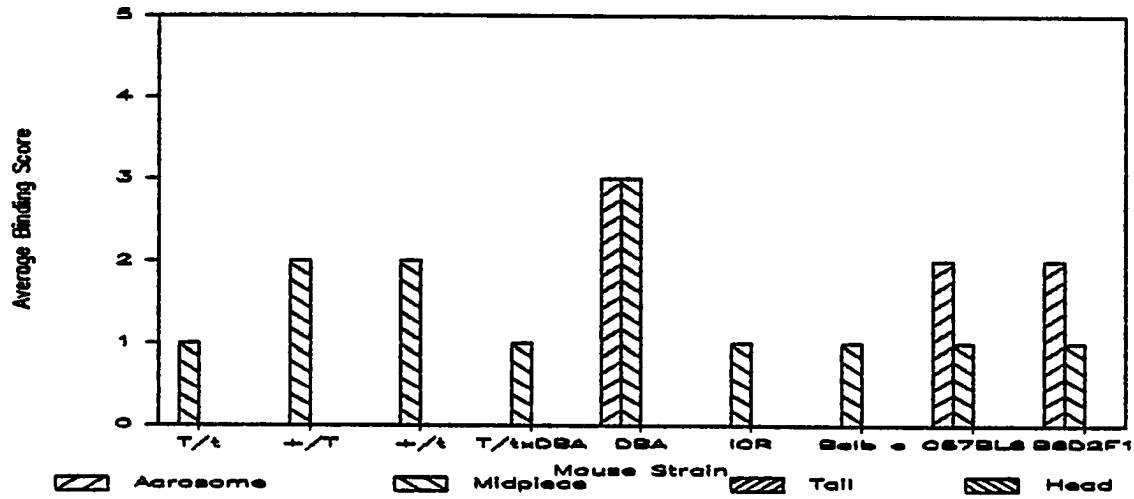
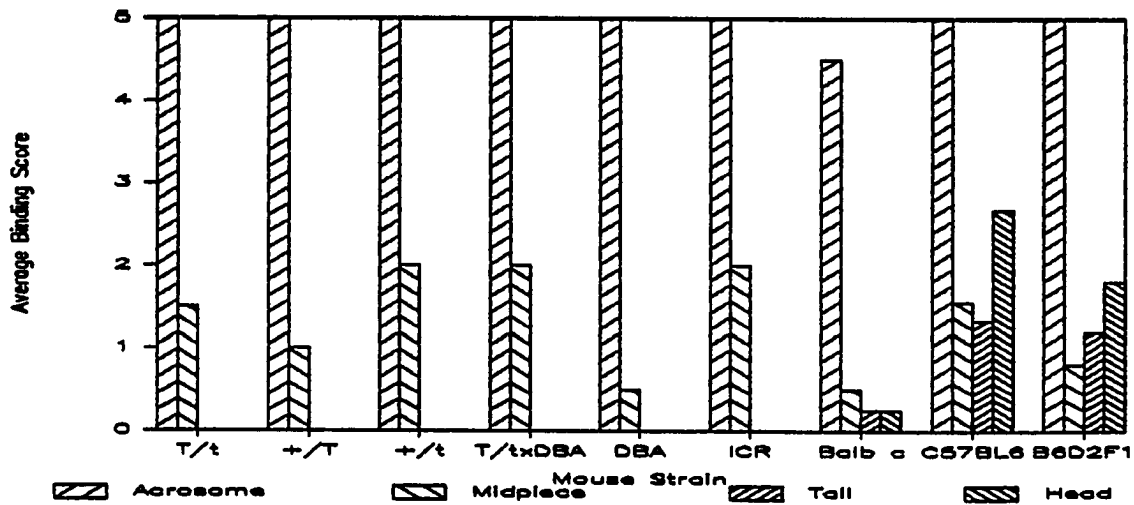


Figure 5. Binding of BAN and LFA to Mouse Sperm.



SBA



WGA

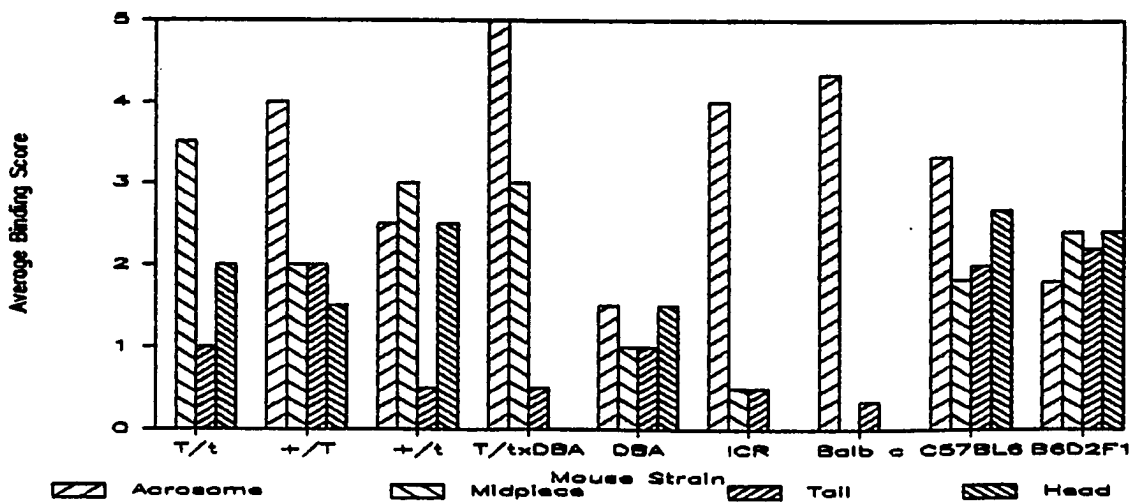
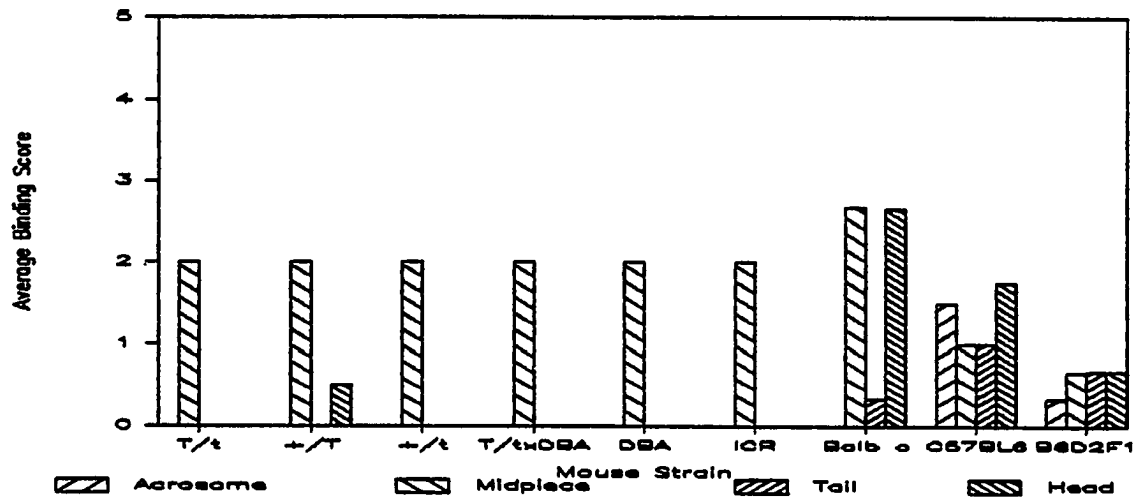
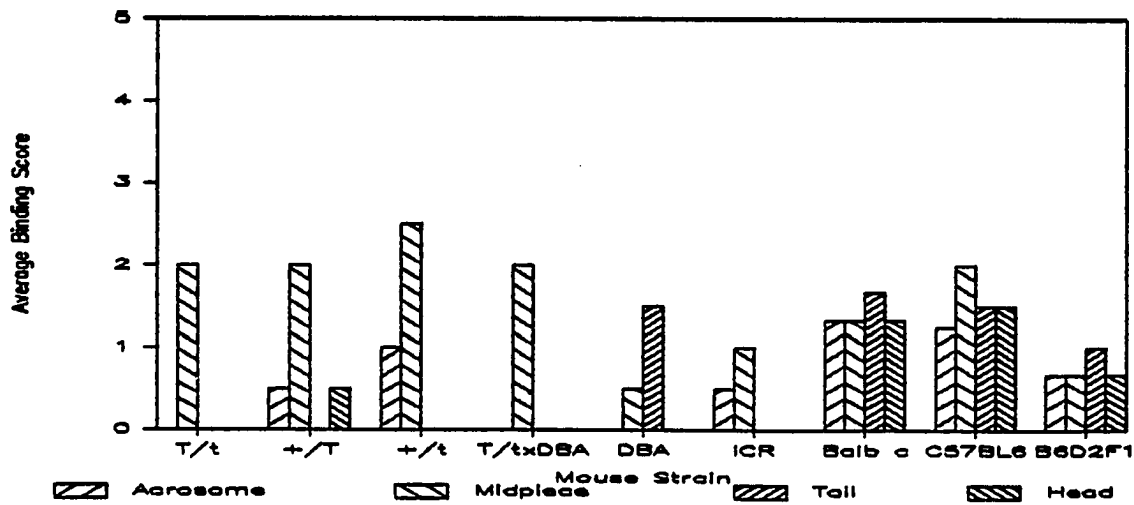


Figure 6. Binding of CONA, SBA, and WGA to Mouse Sperm.



UEA



AA

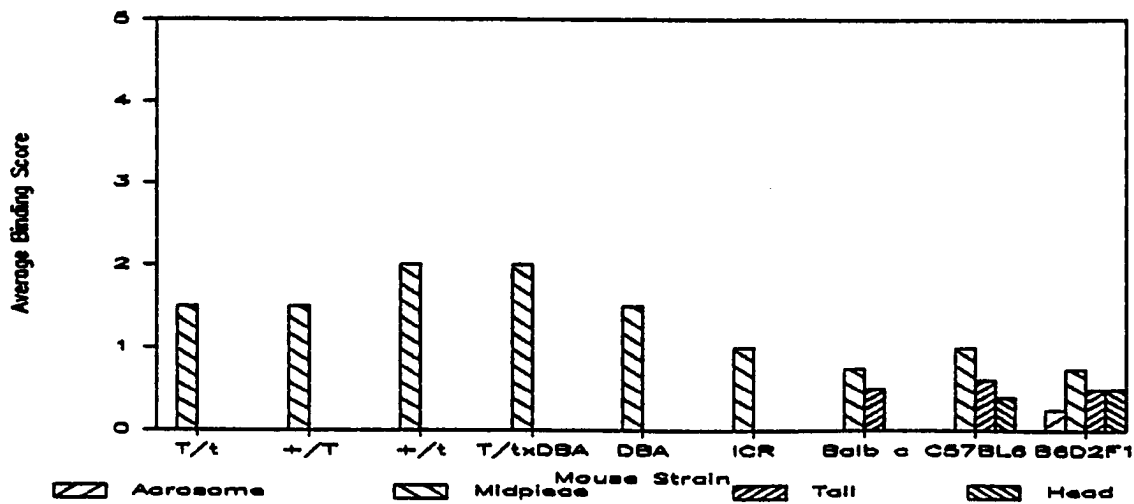


Figure 7. Binding of DBA, UEA, and AA to Mouse Sperm.

Appendix E

Lectin Binding to Testis Cells: Figures 8-14

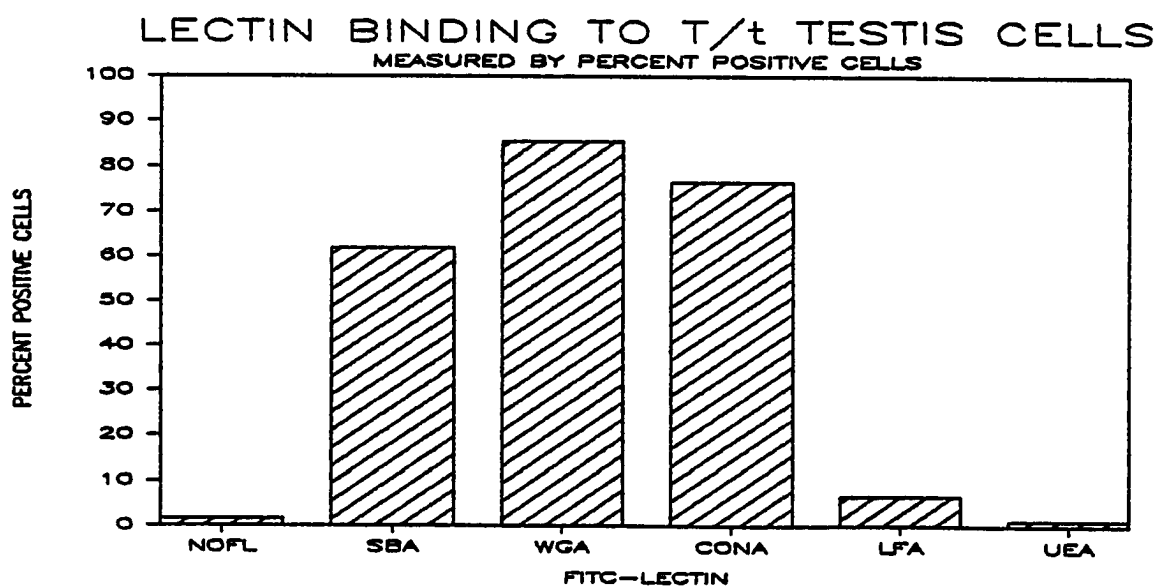
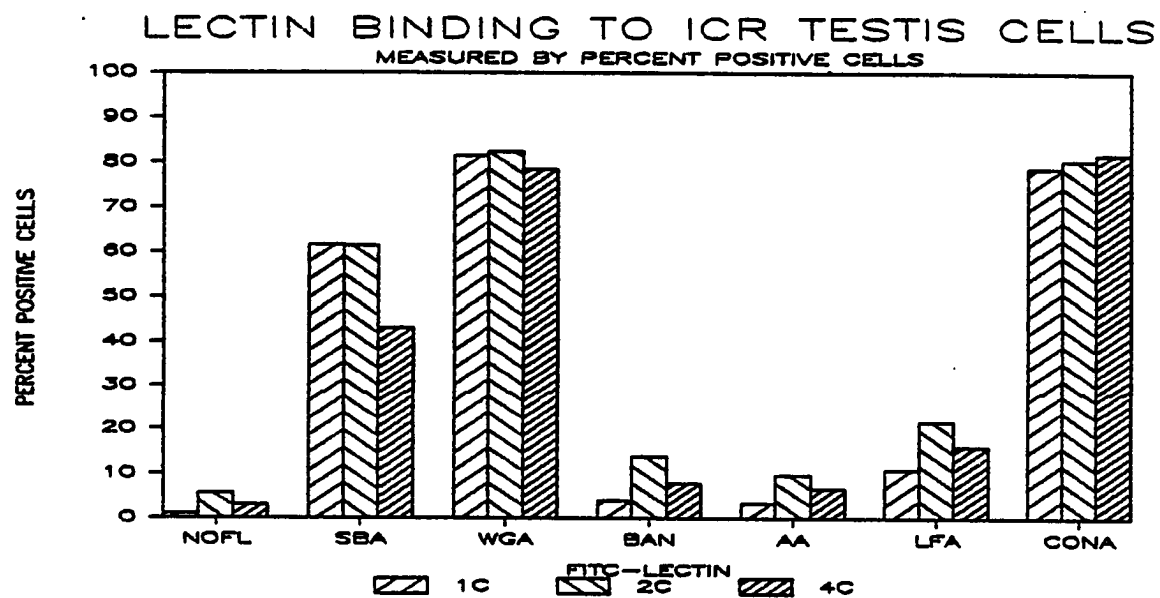


Figure 8. Lectin Binding to ICR and T/t Mouse Testis Cells.

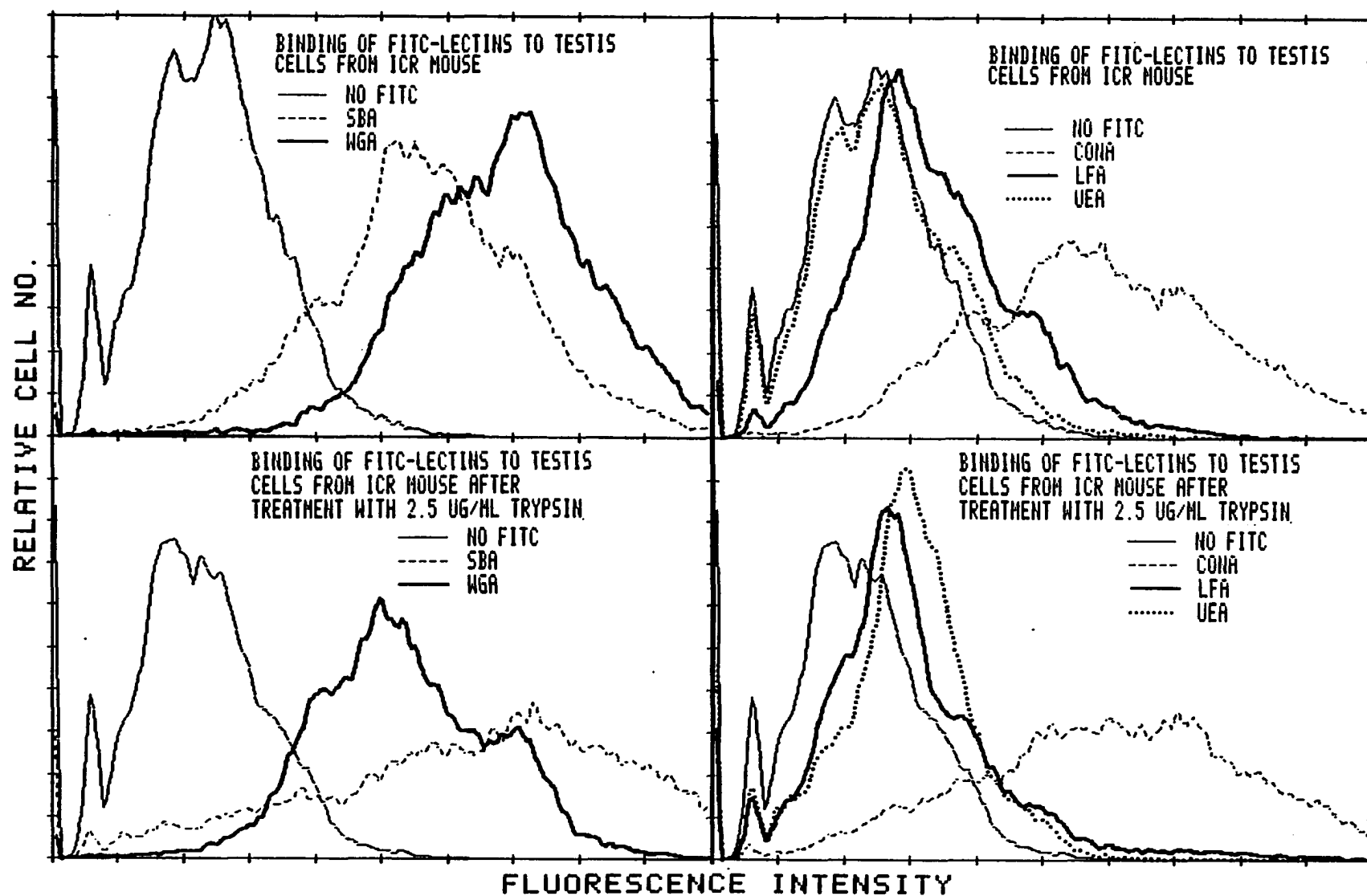


Figure 9. Binding of FITC-Lectins to ICR Mouse Testis Cells.

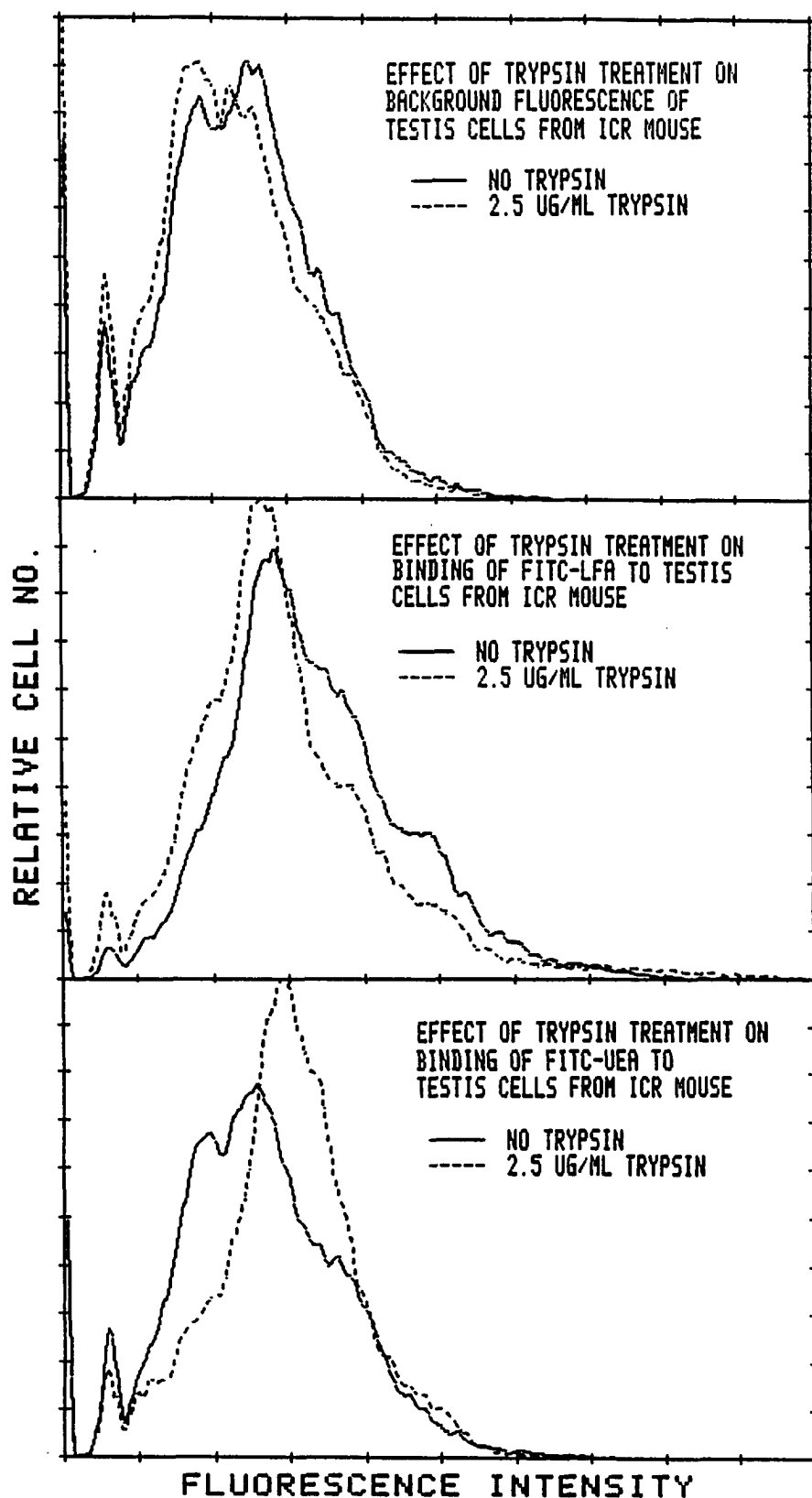


Figure 10. Effect of Trypsinization on Binding of LFA and UEA to ICR Mouse Testis Cells.

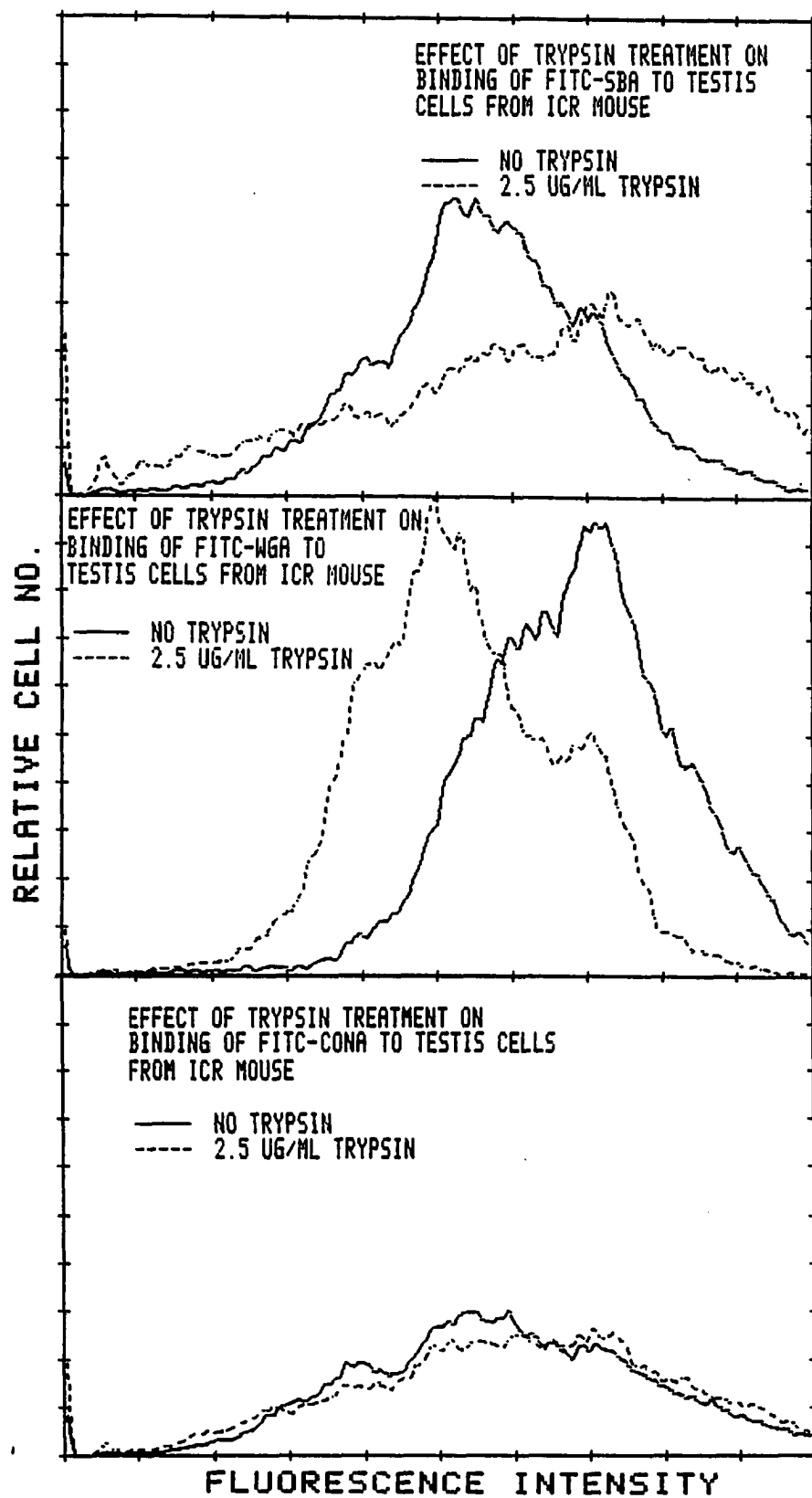


Figure 11. Effect of Trypsinization on Binding of SBA, WGA, and CONA to ICR Mouse Testis Cells.

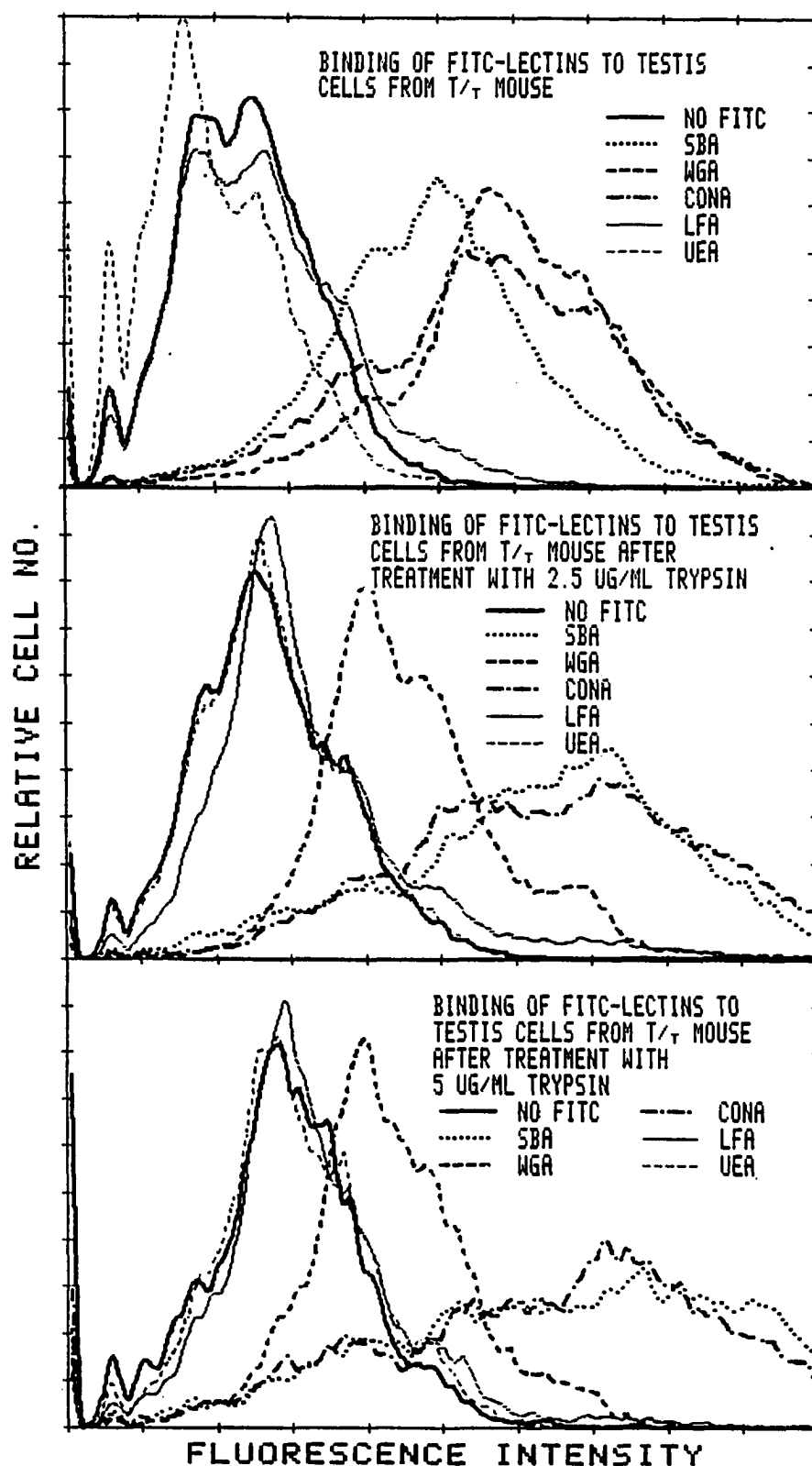


Figure 12. Binding of FITC-Lectins to T/t Mouse Testis Cells.

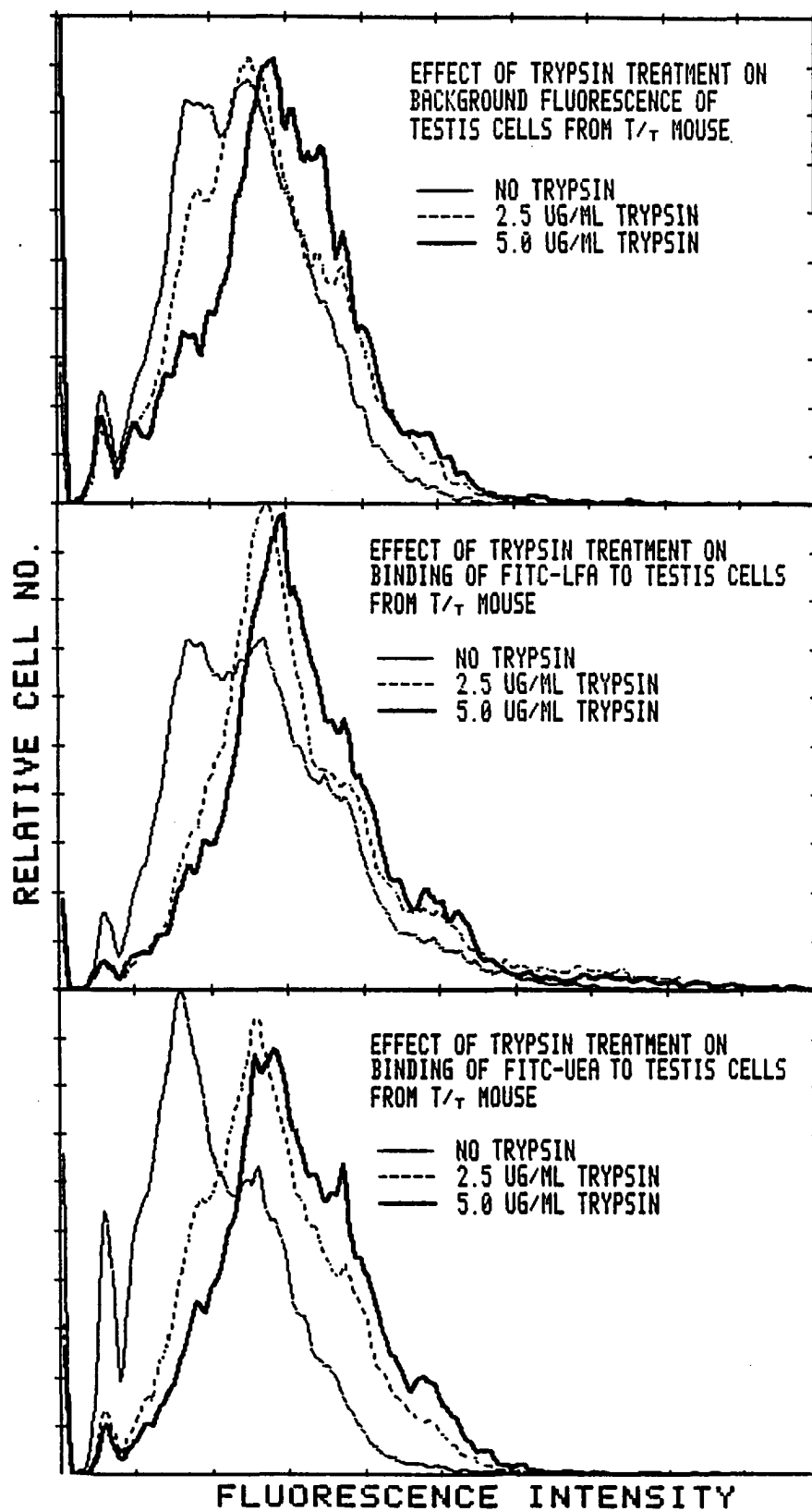


Figure 13. Effect of Trypsinization on Binding of LFA and UEA to T/t Mouse Testis Cells.

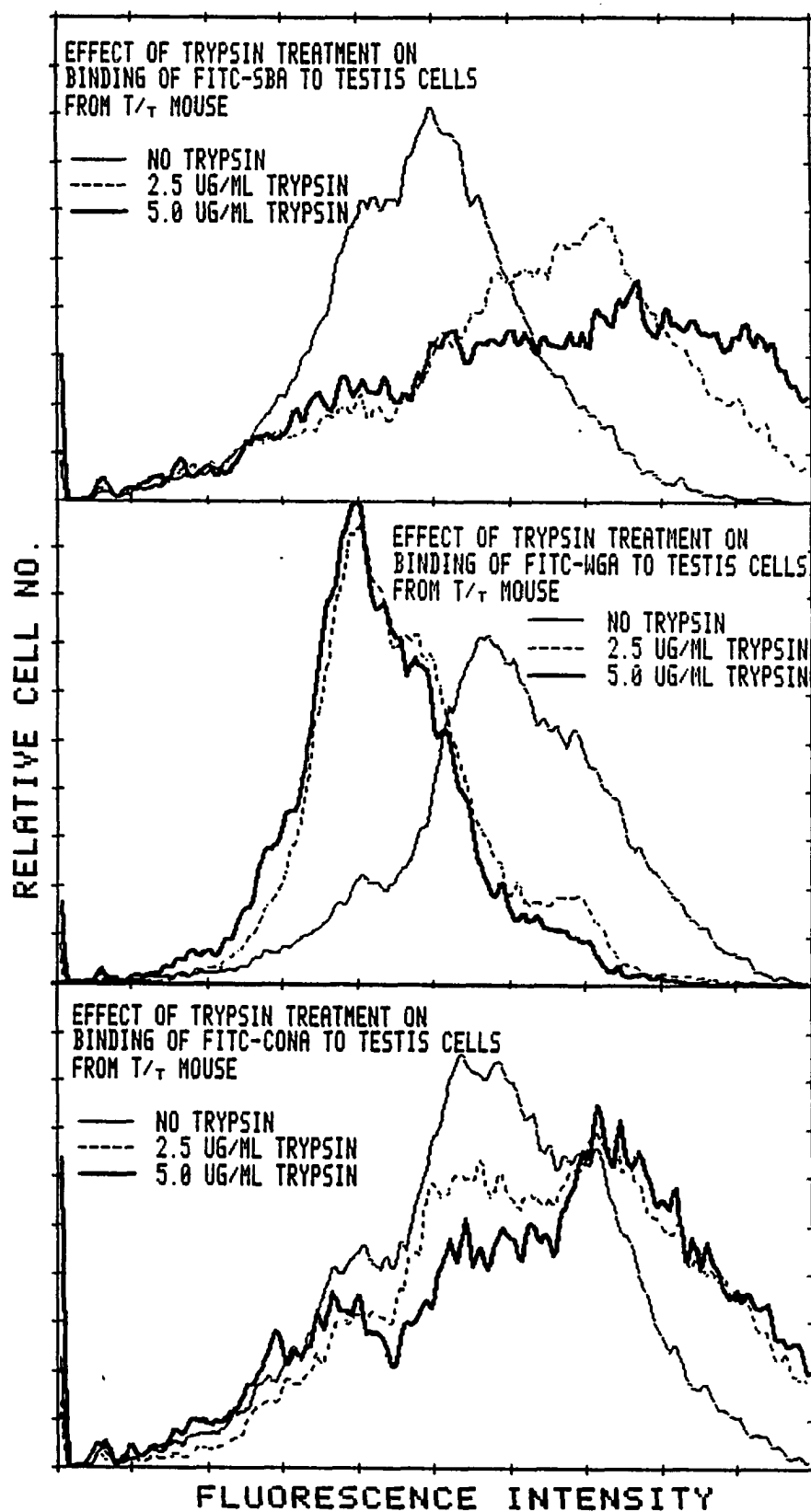


Figure 14. Effect of Trypsinization on Binding of SBA, WGA, and CONA to T/t Mouse Testis Cells.

Appendix F

Antiserum to T/t Locus: Figure 15

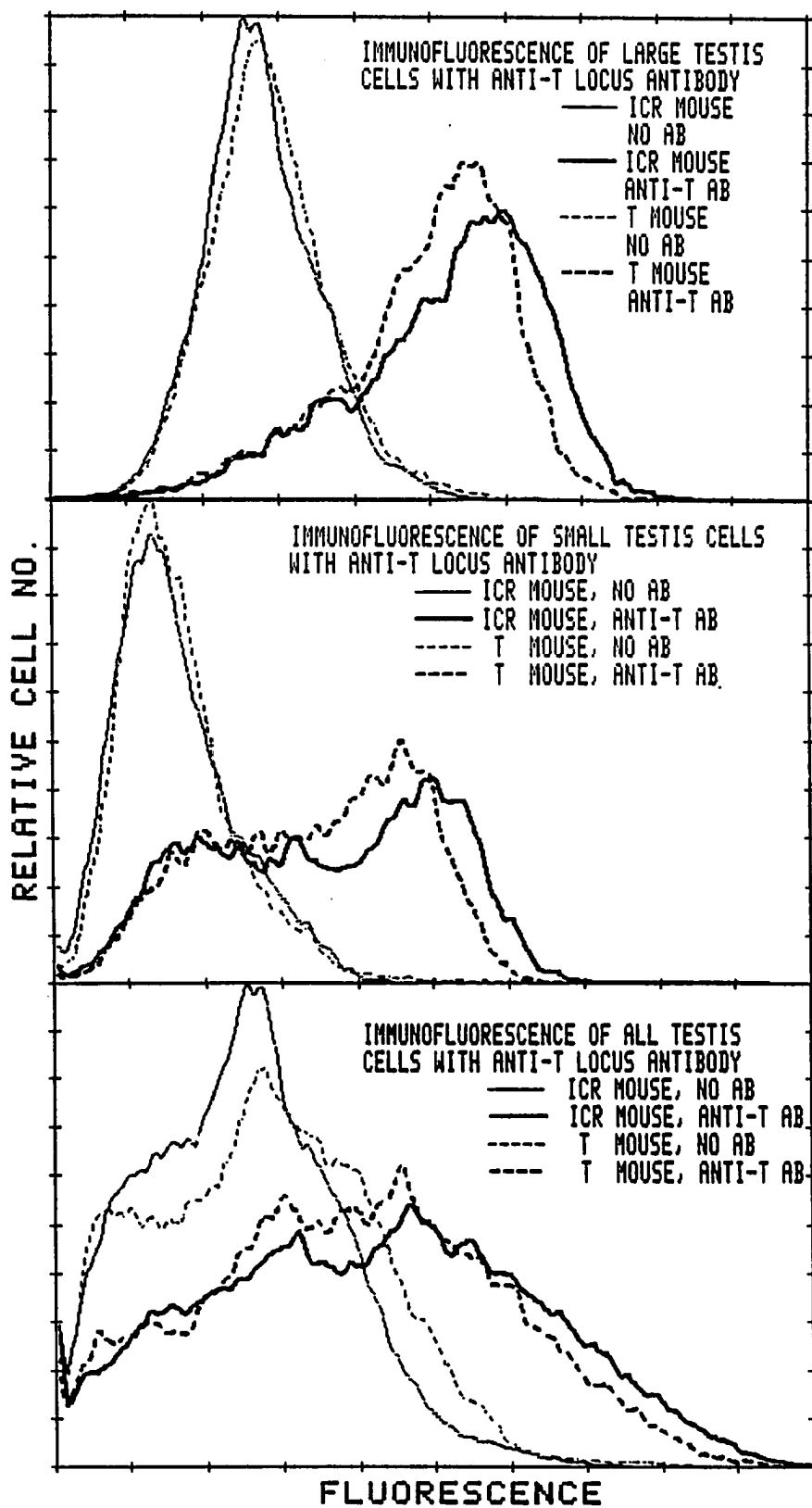


Figure 15. Immunofluorescence of Antiserum to T/t Locus With ICR and T/t Mouse Testis Cells.

Appendix G

Mutagen Effects: Figures 16-29

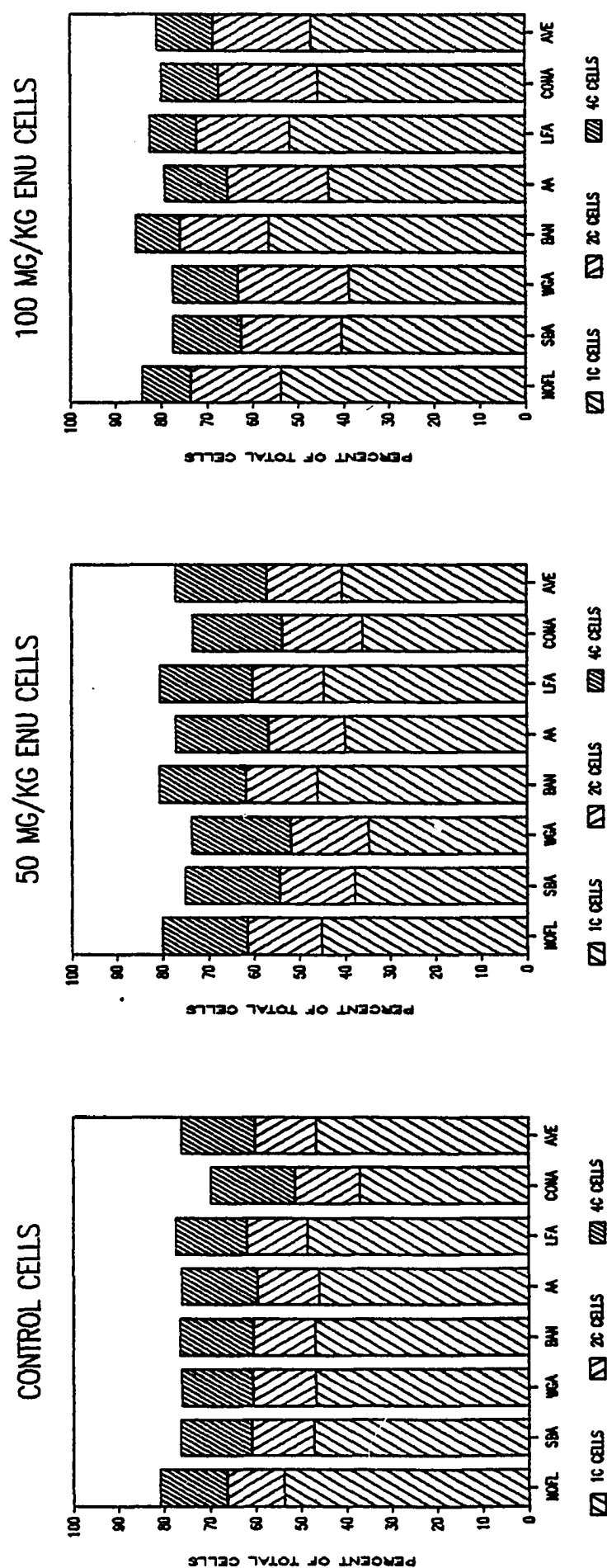


Figure 16. Effect of ENU on Percentages of 1C, 2C, and 4C Testis Cells.

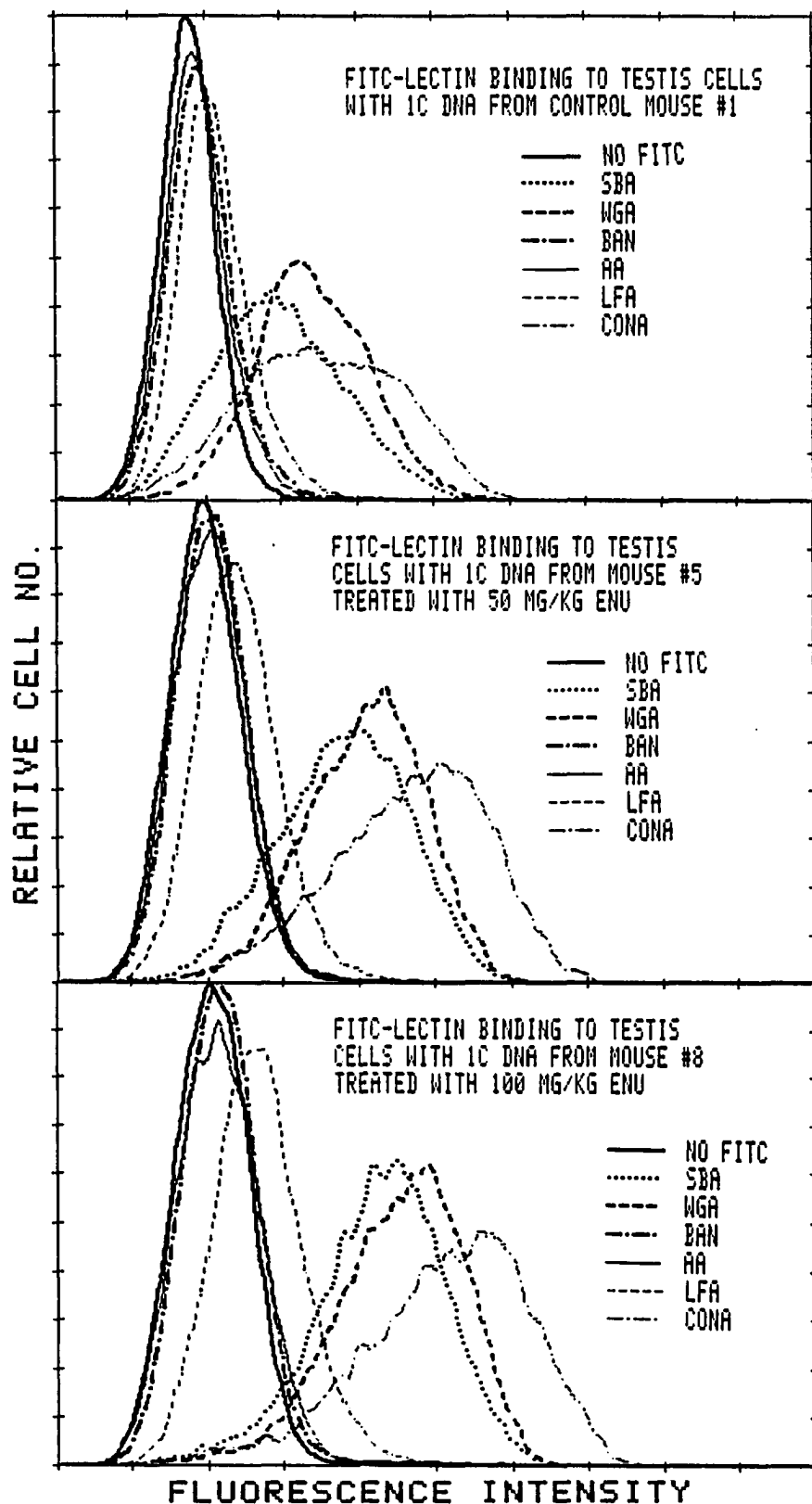


Figure 17. Binding of FITC-Lectins to 1C Testis Cells.

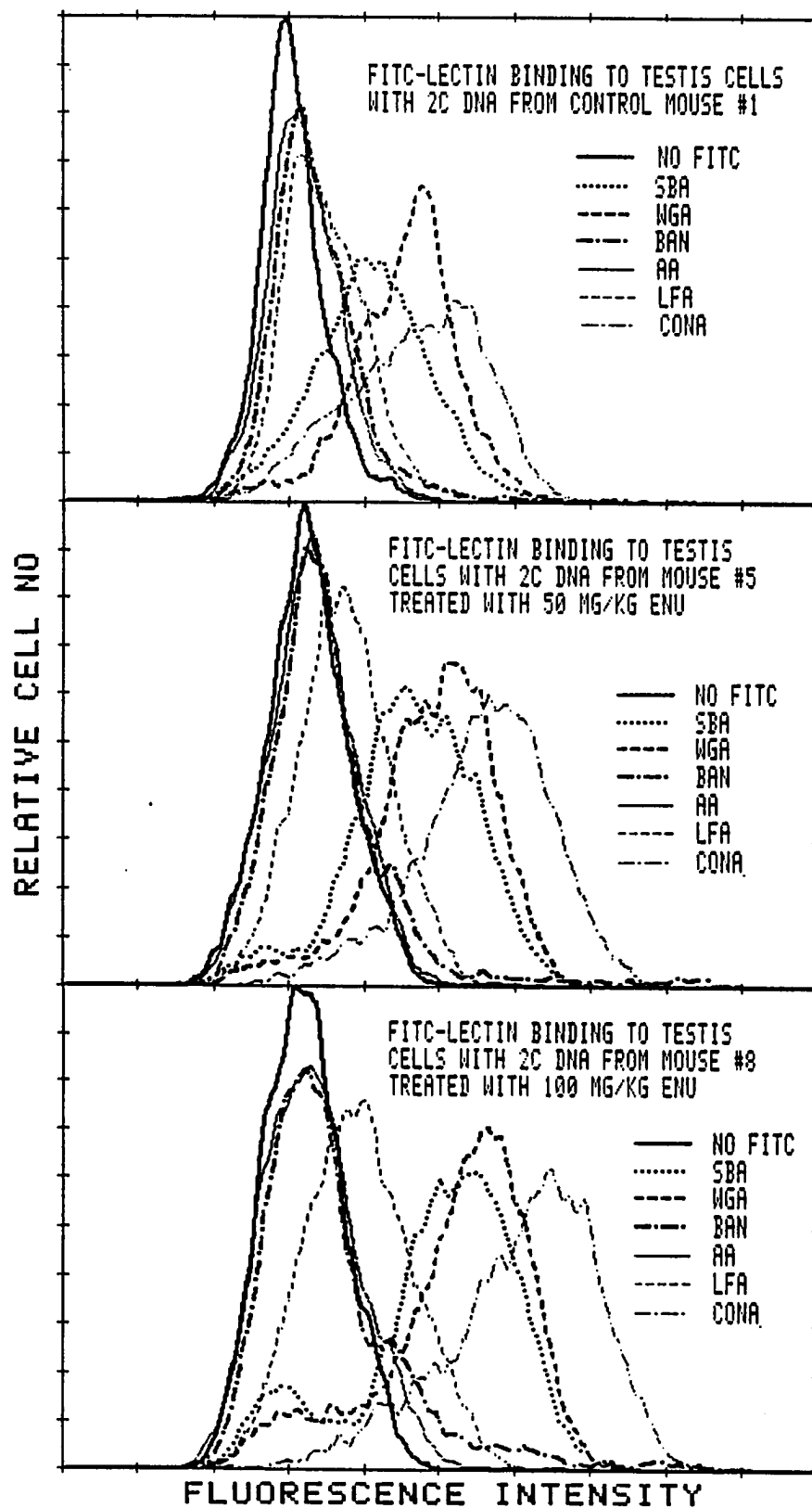


Figure 18. Binding of FITC-Lectins to 2C Testis Cells.

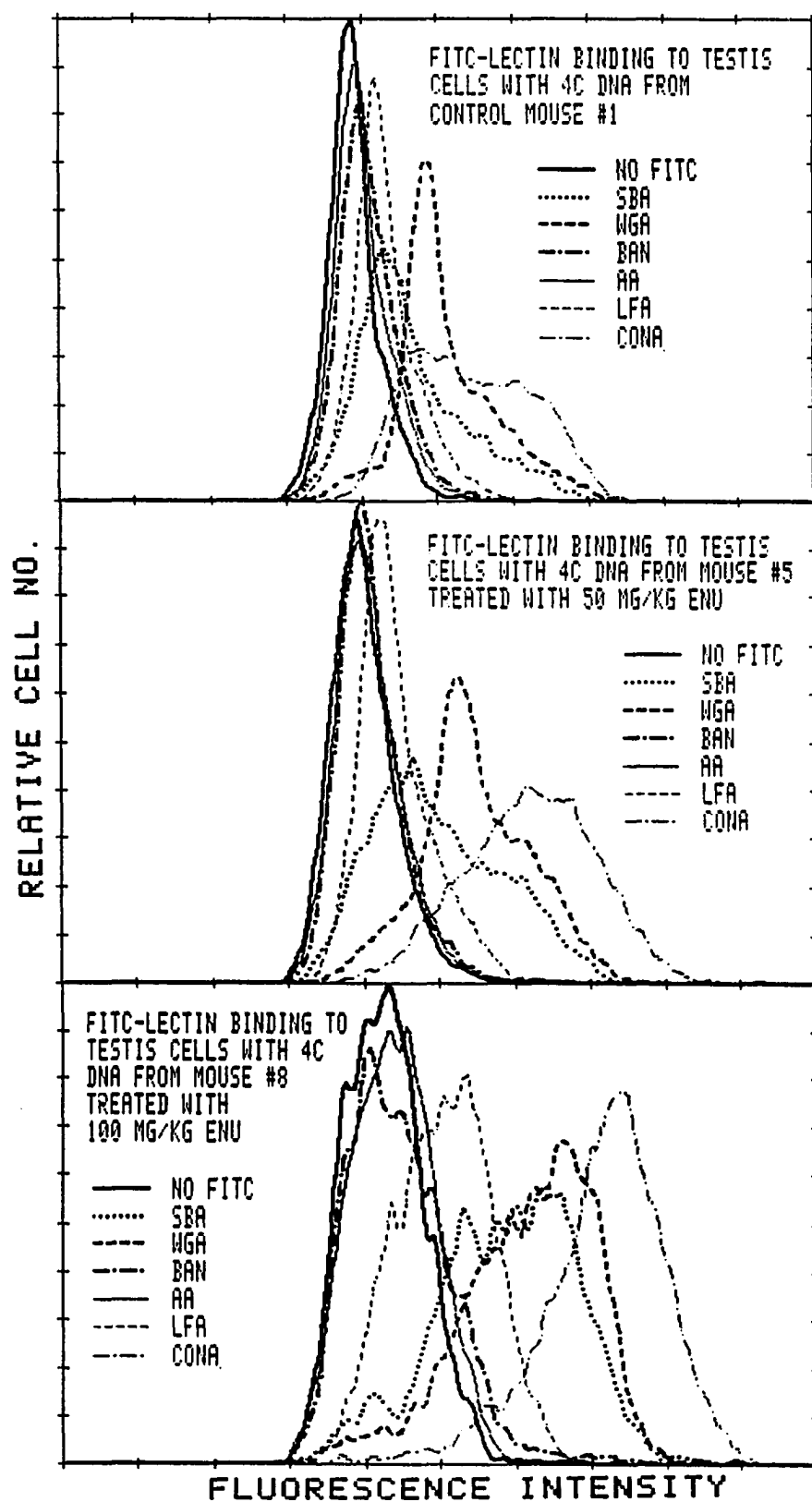


Figure 19. Binding of FITC-Lectins to 4C Testis Cells.

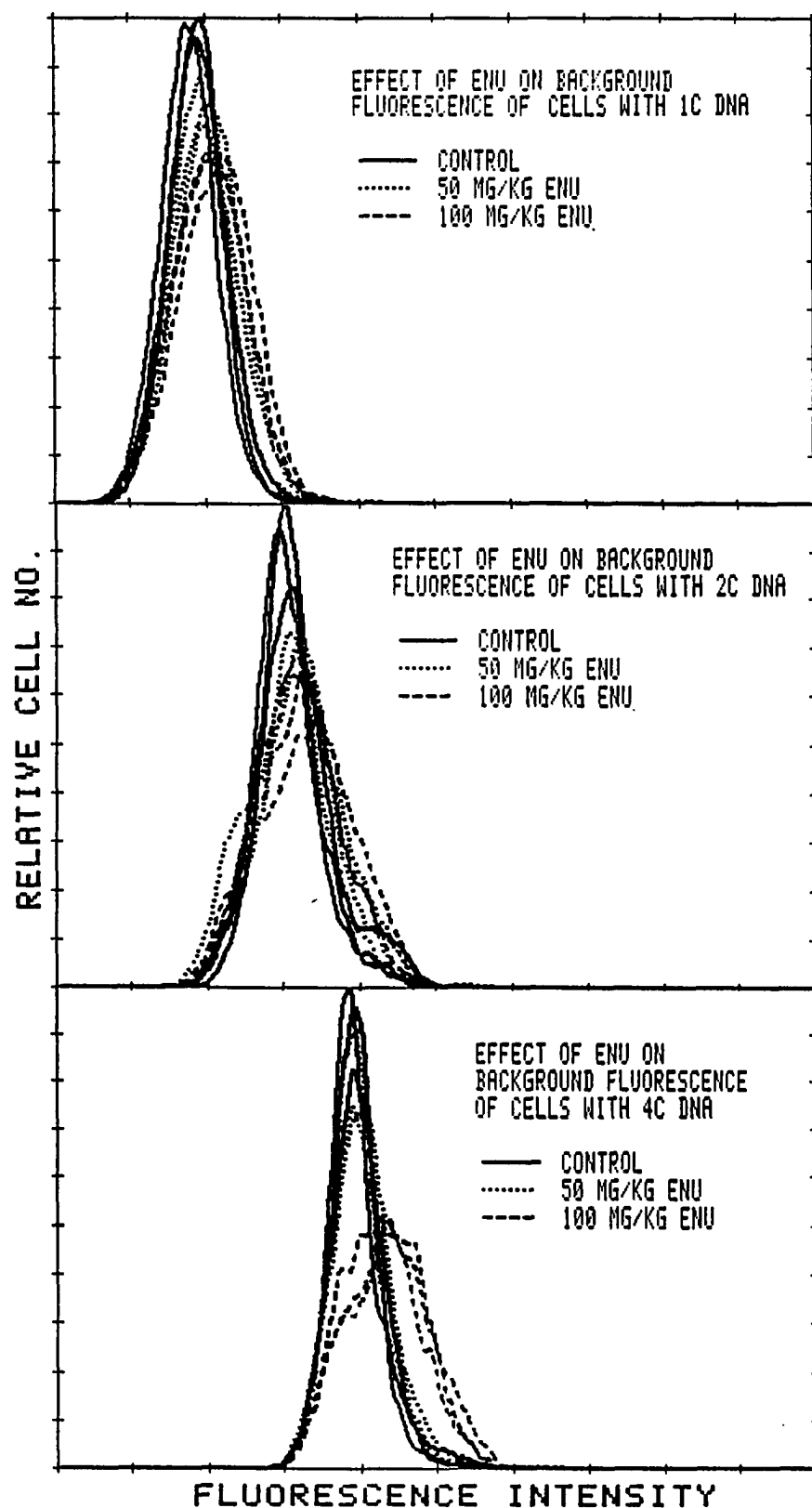


Figure 20. Effect of ENU on Background Fluorescence of Testis Cells.

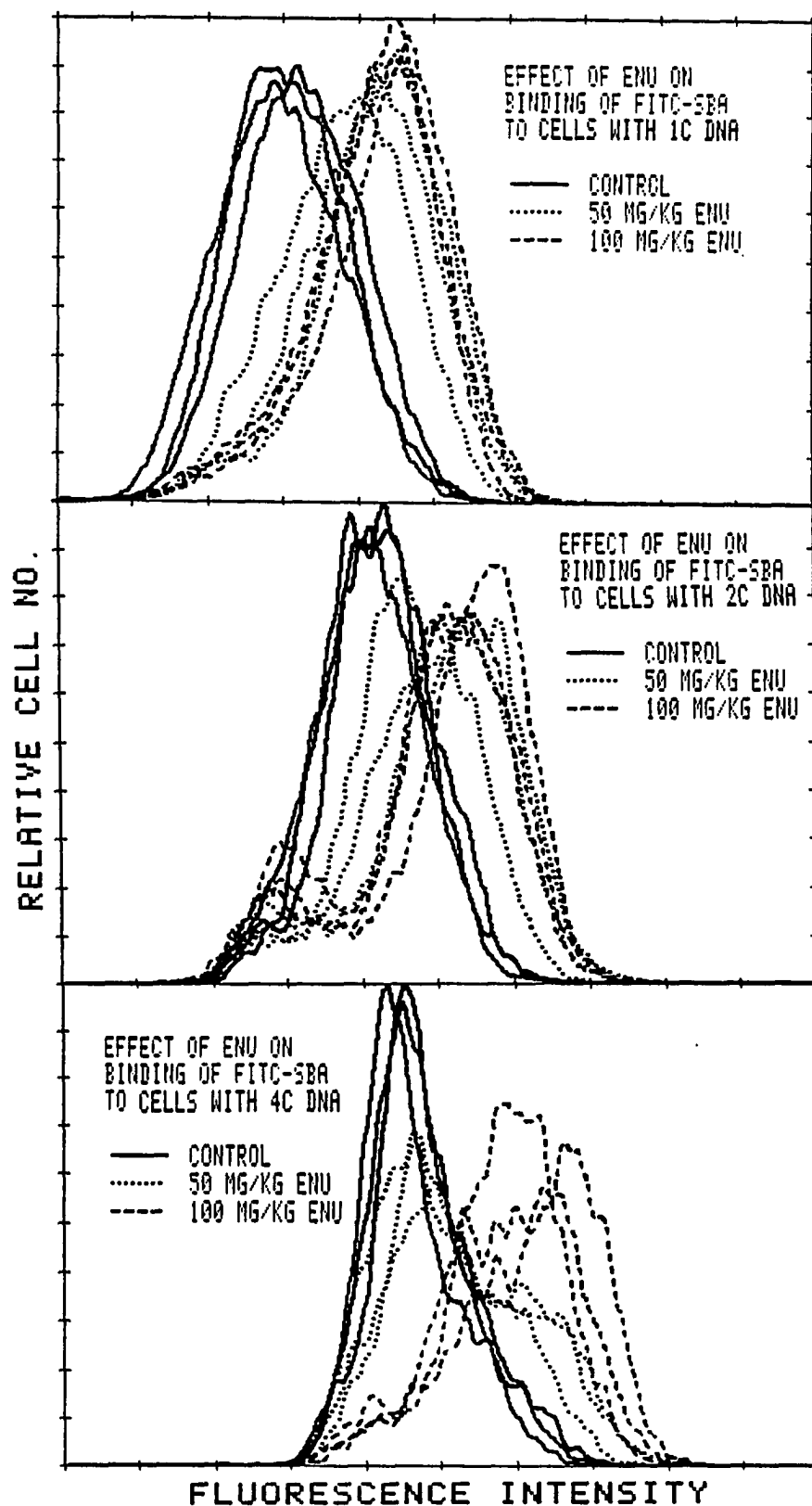


Figure 21. Effect of ENU on Binding of FITC-SBA to Testis Cells.

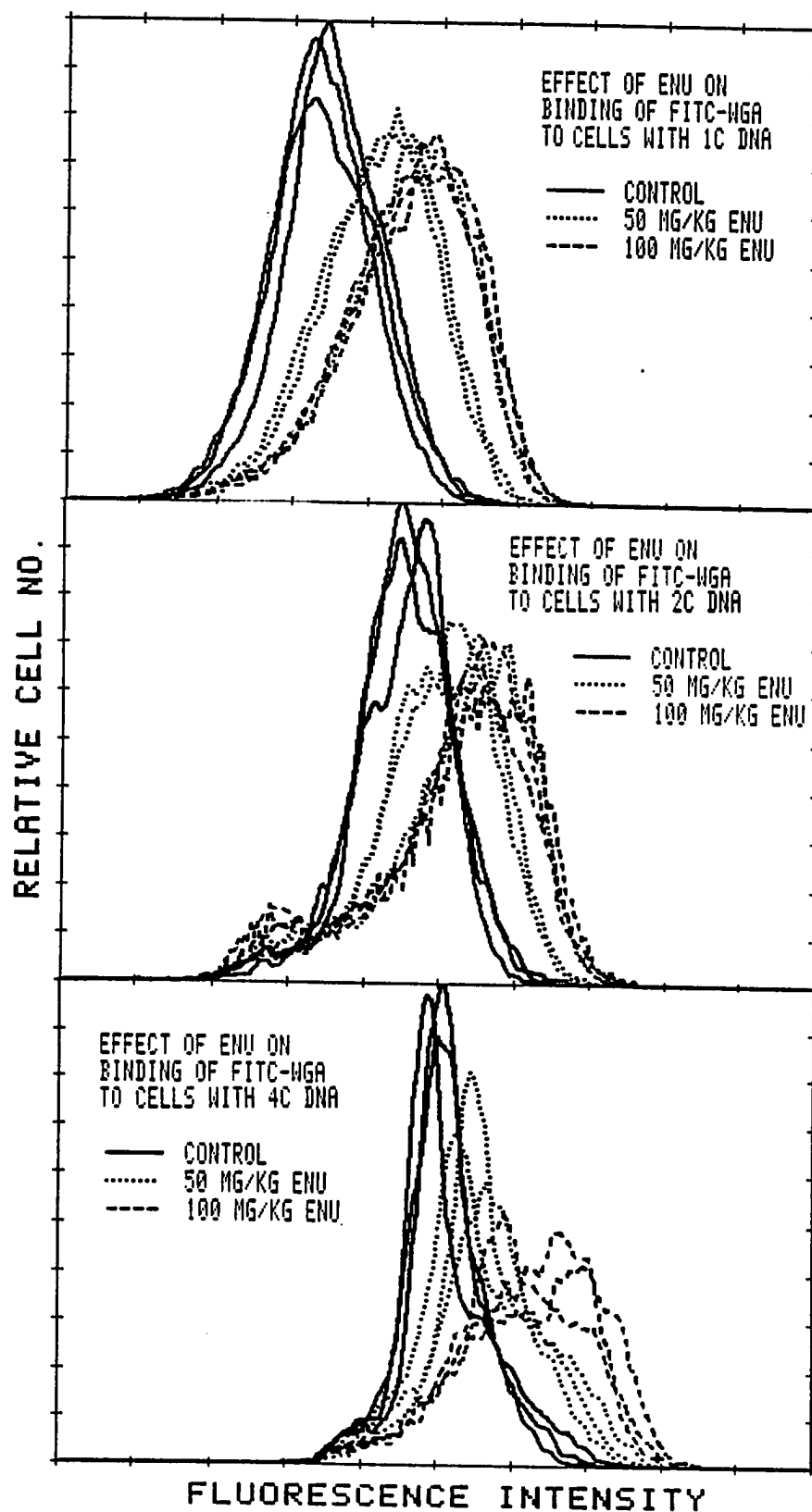


Figure 22. Effect of ENU on Binding of FITC-WGA to Testis Cells.

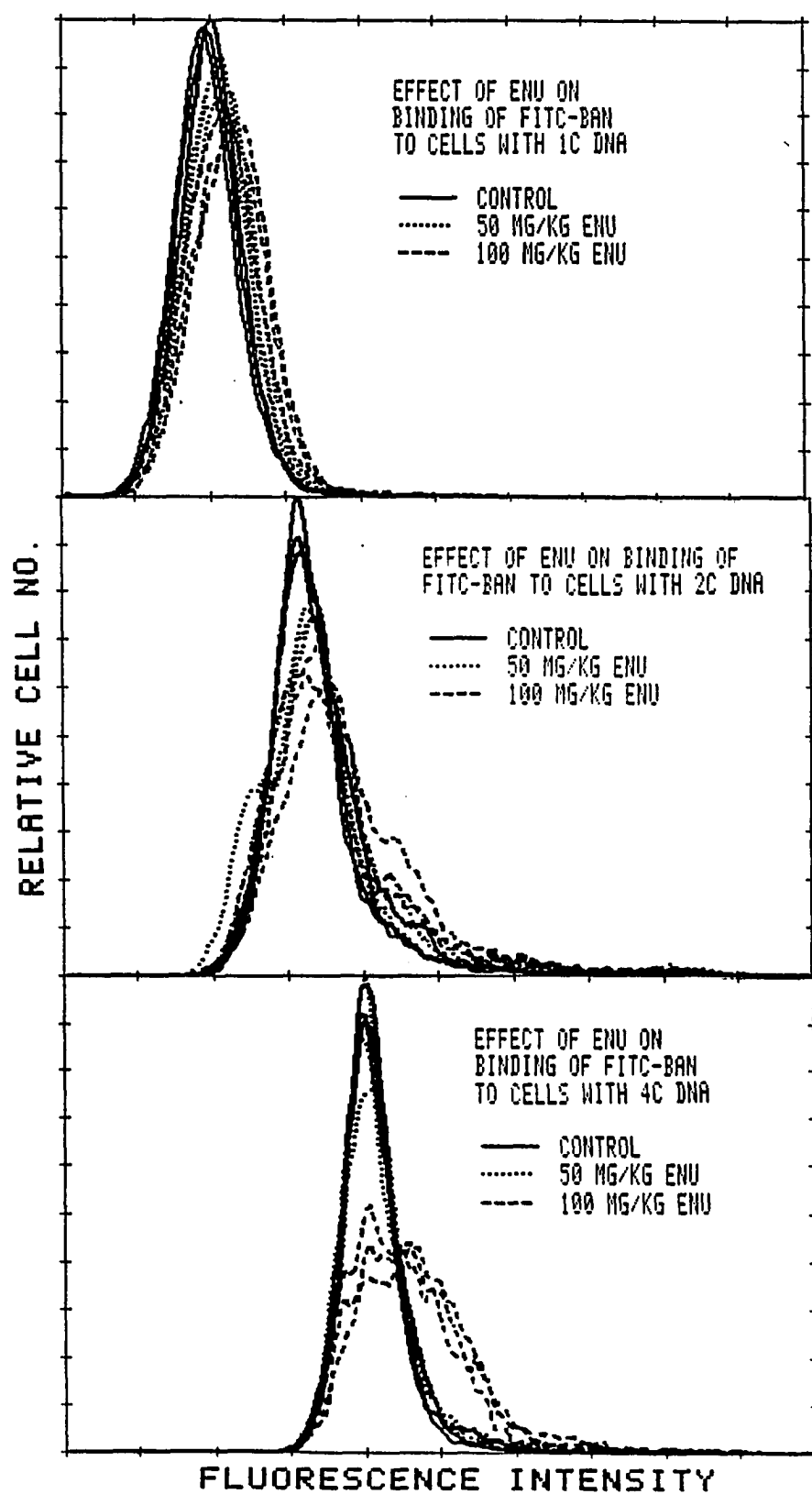


Figure 23. Effect of ENU on Binding of FITC-BAN to Testis Cells.

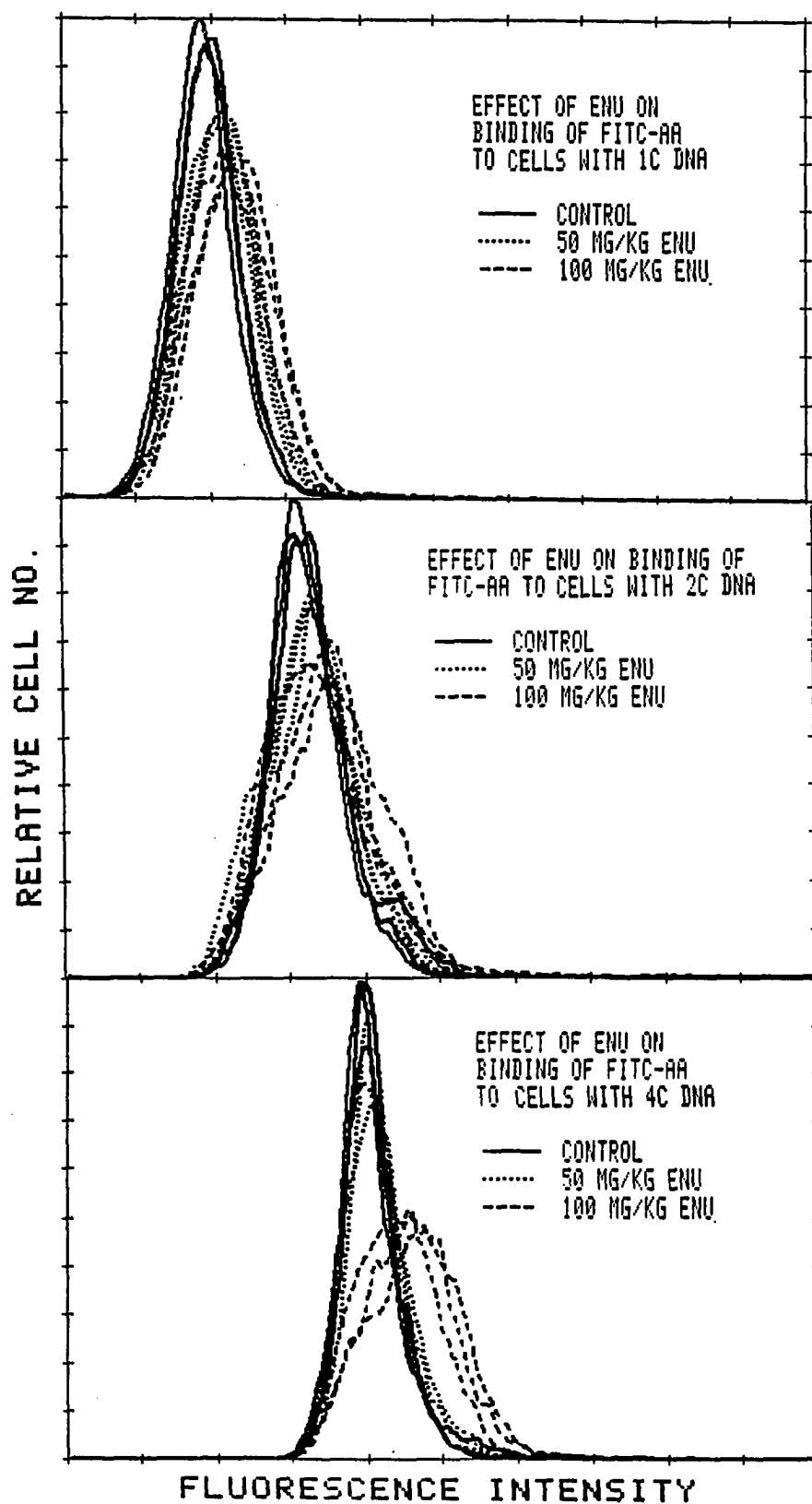


Figure 24. Effect of ENU on Binding of FITC-AA to Testis Cells.

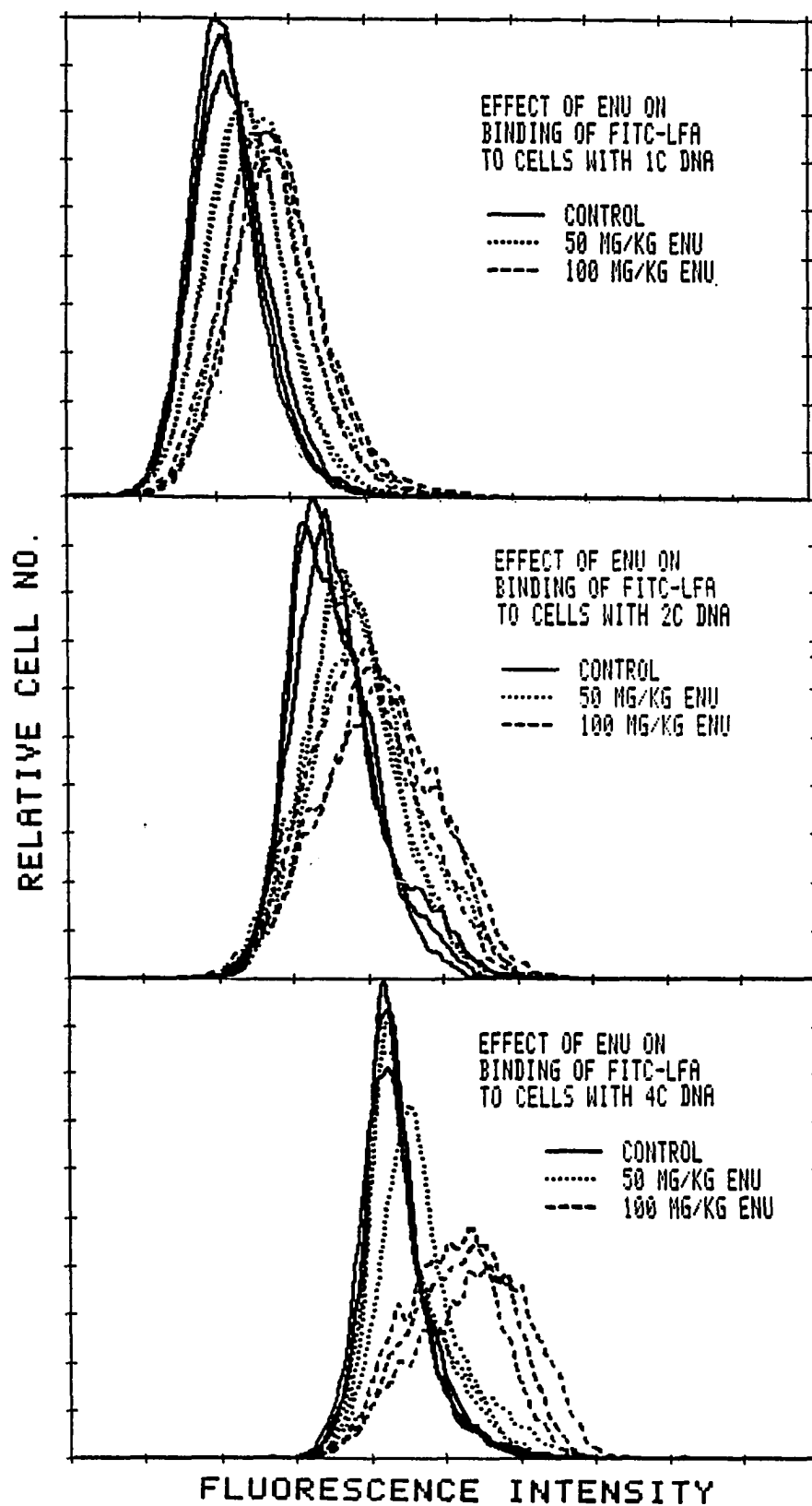


Figure 25. Effect of ENU on Binding of FITC-LFA to Testis Cells.

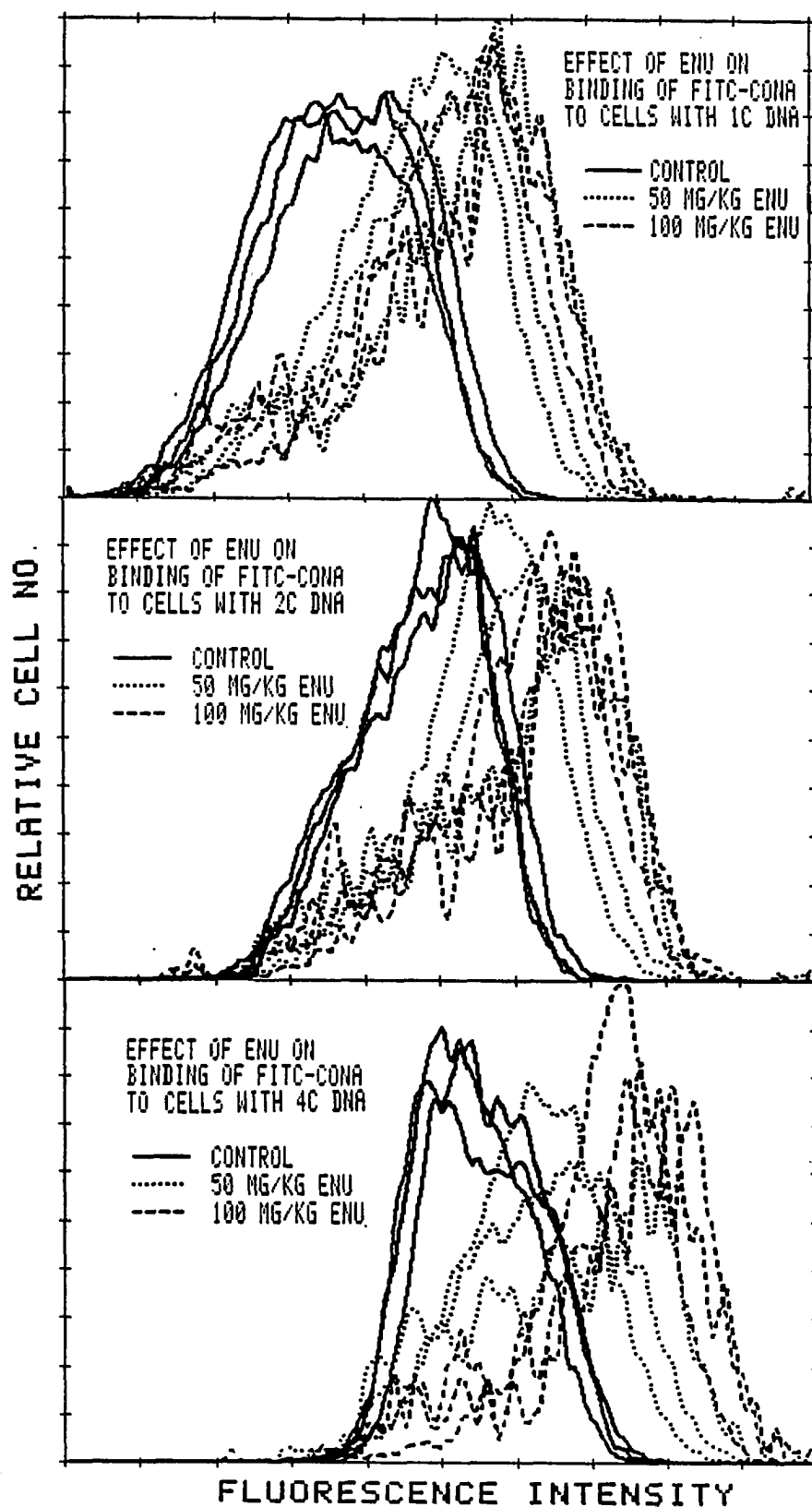


Figure 26. Effect of ENU on Binding of FITC-CONA to Testis Cells.

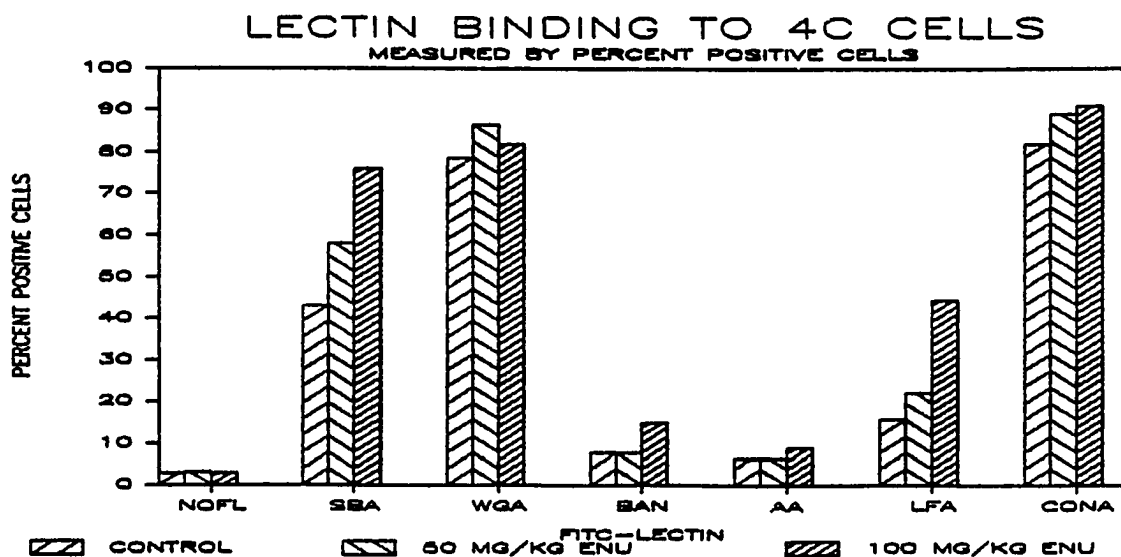
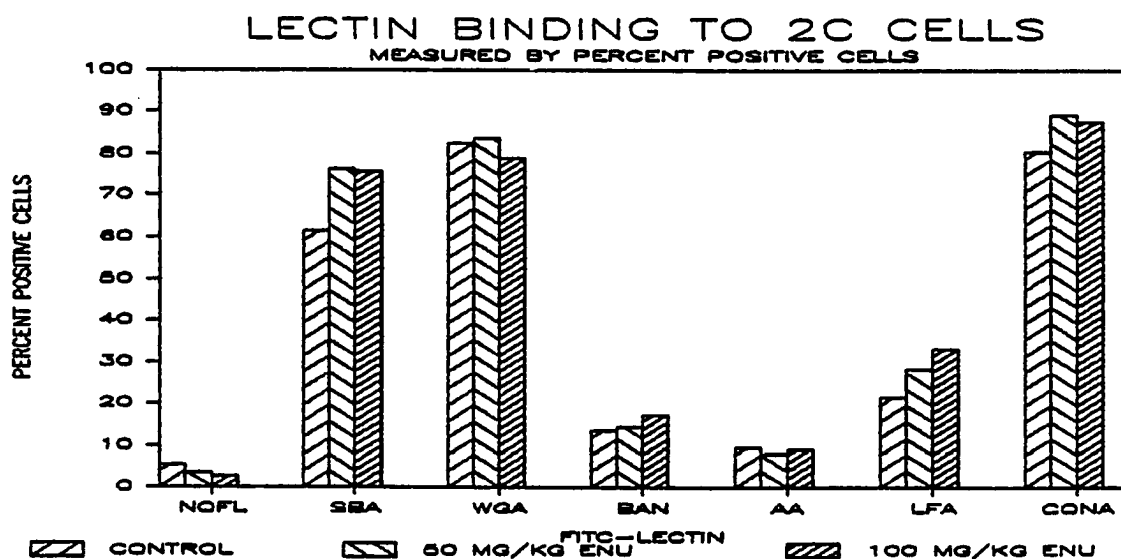
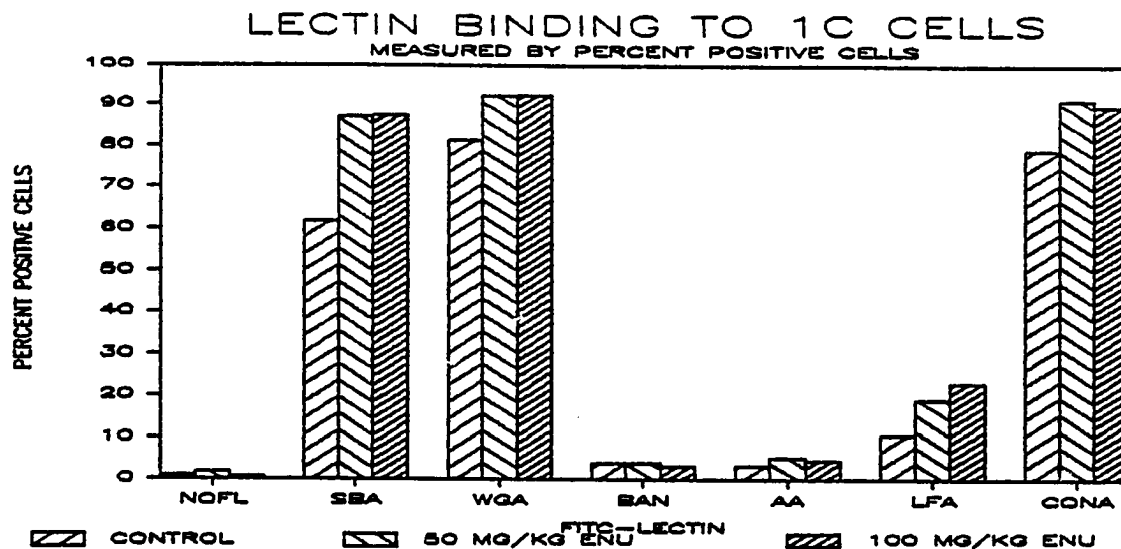
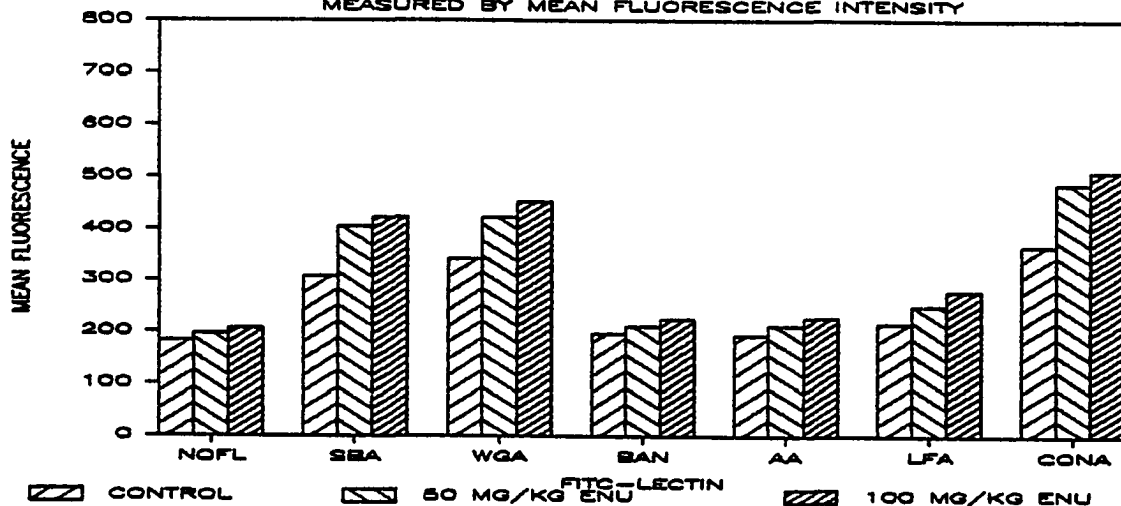


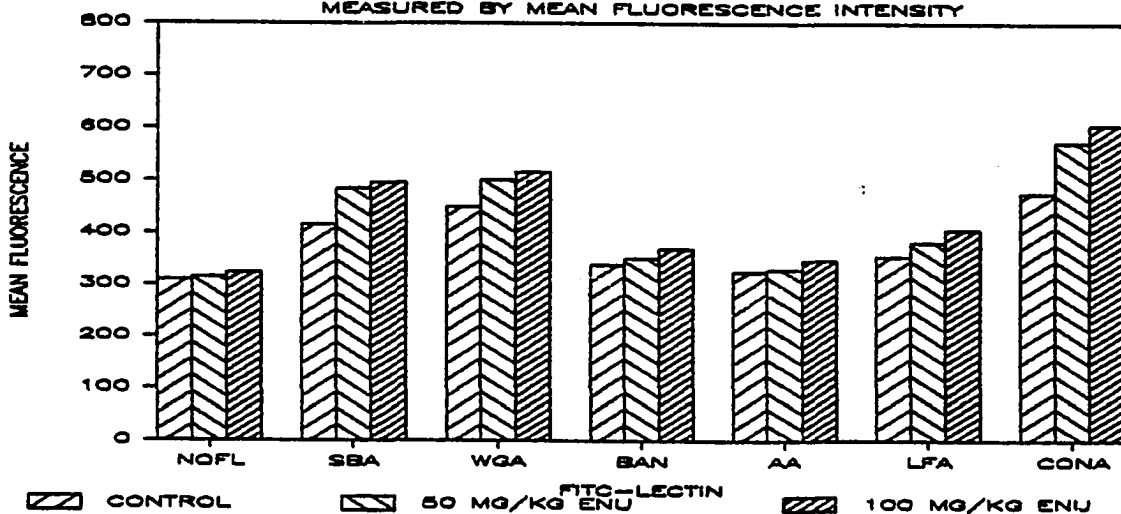
Figure 27. Effect of ENU on Lectin Binding to Testis Cells Measured by Percent Positive Cells.

LECTIN BINDING TO 1C CELLS MEASURED BY MEAN FLUORESCENCE INTENSITY

92



LECTIN BINDING TO 2C CELLS MEASURED BY MEAN FLUORESCENCE INTENSITY



LECTIN BINDING TO 4C CELLS MEASURED BY MEAN FLUORESCENCE INTENSITY

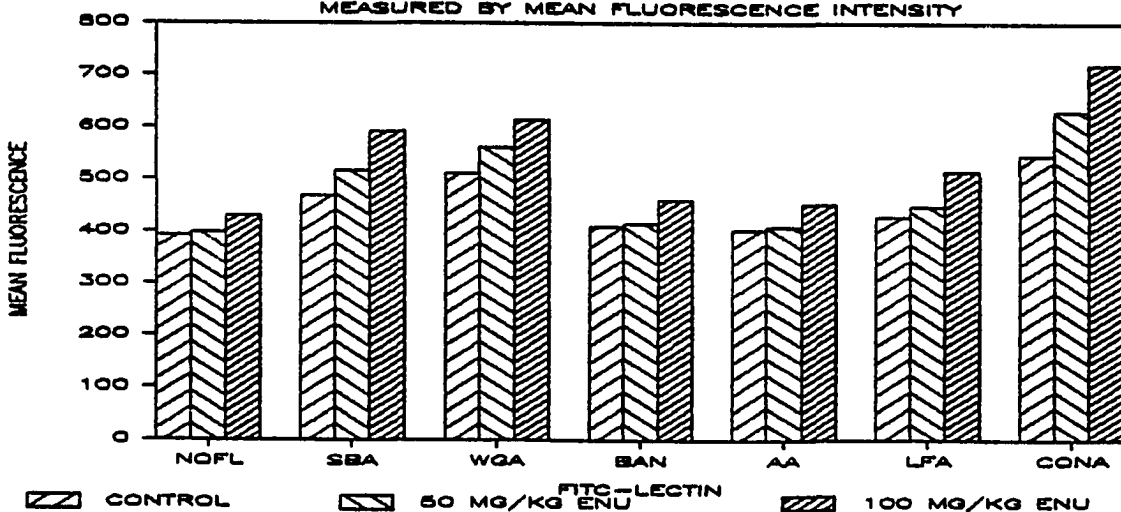


Figure 28. Effect of ENU on Lectin Binding to Testis Cells Measured by Mean Fluorescence Intensity.

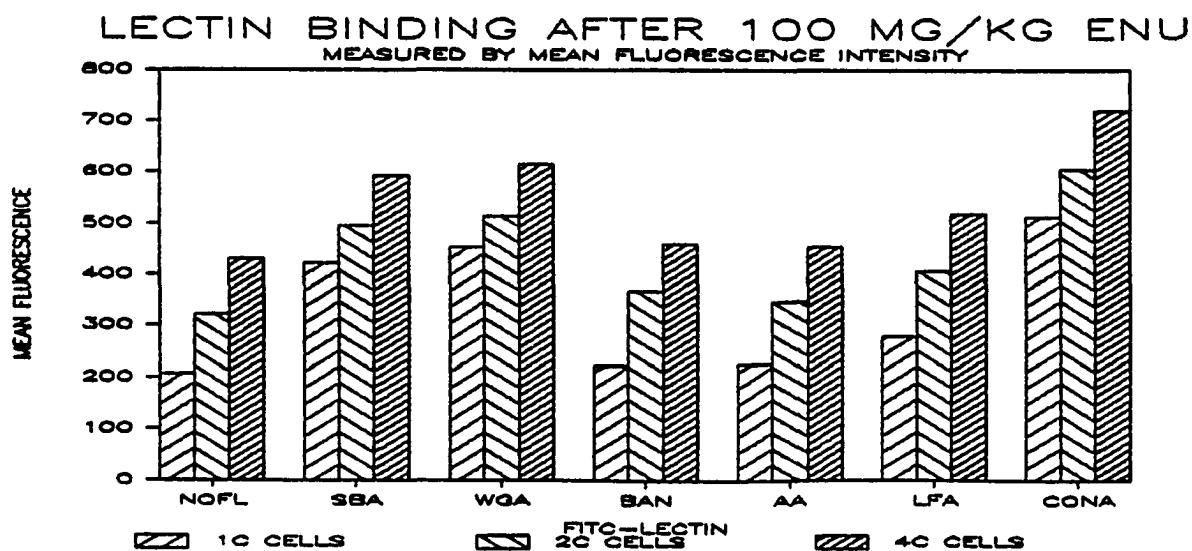
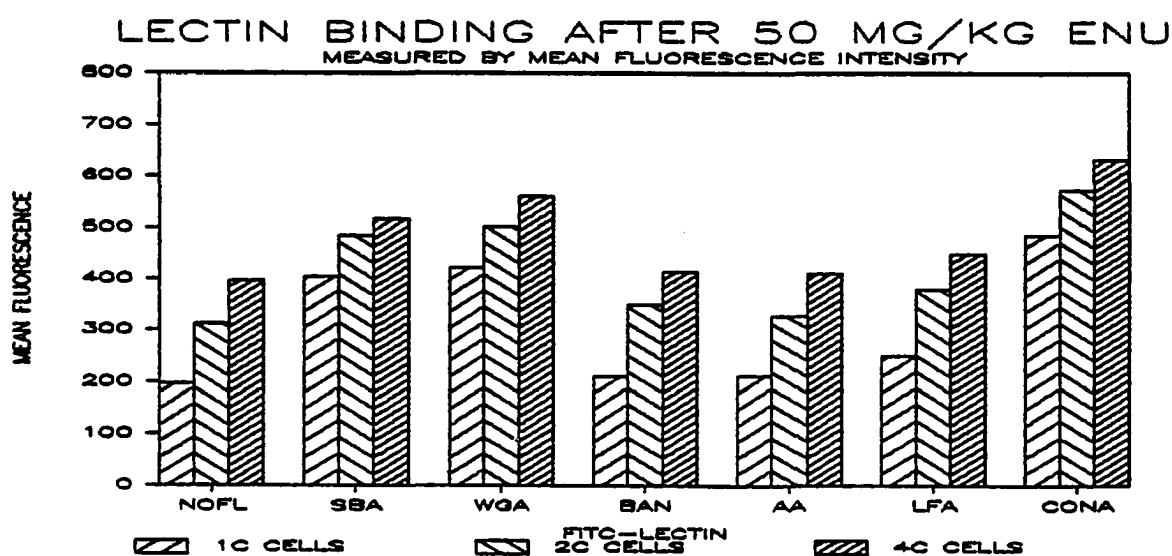
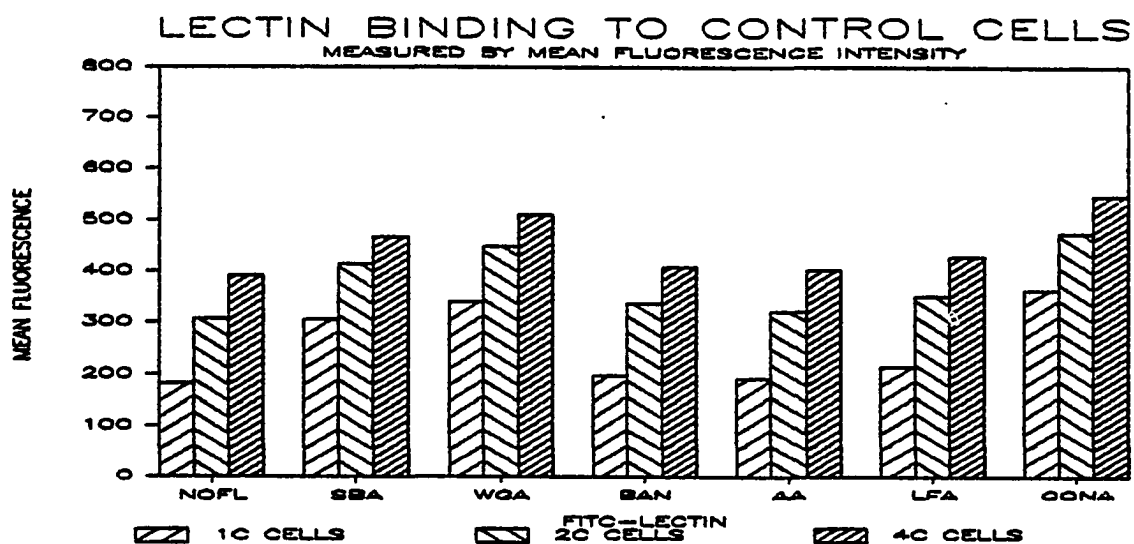


Figure 29. Effect of DNA Content on Mean Fluorescence Intensity.

BIBLIOGRAPHY

- Agrawal, B. B. L., and Goldstain, I. J., (1972), Concanavalin A, the Jack Bean (Canavalia ensiformis) phytohemagglutinin, Methods in Enzymology, **28**, Part B, 313-318.
- Ahuja, K. K. (1982). Fertilization studies in the hamster: The role of cell-surface carbohydrates, Experimental Cell Research, **140**, 353-362.
- Ahuja, K. K. (1984). Lectin-coated agarose beads in the investigation of sperm capacitation in the hamster, Developmental Biology, **104**, 131-142.
- Artzt, K. and Bennett, D. (1975). Analogies between embryonic (T/t) antigens and adult major histocompatibility (H-2) antigens, Nature, **256**, 545-547.
- Barondes, S.H. (1984). Soluble lectins: A new class of extracellular proteins, Science, **223**, 1259-1264.
- Bechtol, K. B., Brown, S. C., and Kennett, R. H. (1979). Recognition of differentiation antigens of spermatogenesis in the mouse by using antibodies from spleen cell-myeloma hybrids after syngeneic immunization, Proceedings of the National Academy of Sciences of the United States of America, **76**, 363-367.
- Bennett, D., and Artzt, K. (1982). Genetic and developmental analysis of T/t-complex mutations in the mouse, Human Genetics, Part A: The Unfolding Genome, New York: Alan R. Liss, 309-315.
- Bennett, D., Goldberg, E., Dunn, L. C., and Boyse, E. A. (1972). Serological detection of a cell-surface antigen specified by the T (brachyury) mutant gene in the house mouse, Proceedings of the National Academy of Sciences of the United States of America, **69**, 2076-2080.
- Beranek, D. T., Weis, C. C., and Swenson, D. H. (1980). A comprehensive quantitative analysis of methylated and ethylated DNA using high pressure liquid chromatography, Carcinogenesis, **1**, 595-606.
- Bode, V. C. (1984). Ethylnitrosourea mutagenesis and the isolation of mutant alleles for specific genes located in the T region of mouse chromosome 17, Genetics, **108**, 457-470.
- Buthala, D. A., and Blashfield, K. (1980). Tissue and organ culture laboratory manual, Kalamazoo: Western Michigan University, 85.
- Cheng, C. C., and Bennett, D. (1980). Nature of the antigenic

- determinants of T locus antigens, Cell, 19, 537-544.
- Clausen, O. P. F., and Abyholm, T. (1980). Deoxyribonucleic acid flow cytometry of germ cells in the investigation of male infertility, Fertility and Sterility, 34, 369-374.
- Danska, J. S., and Silver, L. M. (1980). Cell-free translation of a T/t complex cell surface-associated gene product, Cell, 22, 901-904.
- Debray, H., DeCout, D., Strecker, G., Spik, G., and Montreuil, J. (1981). Specificity of twelve lectins towards oligosaccharides and glycopeptides related to N-glycosylproteins, European Journal of Biochemistry, 117, 41-55.
- Doyle, R. J. (1982). Lectins in diagnostic microbiology, Biotech, 1, 1-8.
- Etzler, M. (1972). Horse gram (Dolichos biflorus) lectin, Methods in Enzymology, 28, Part B, 340-344.
- Evenson, D. P., Darzynkiewicz, Z., and Melamed, M. R. (1980). Flow cytometric analysis of sperm chromatin structure, Acta Pathologica Microbiologica Scandinavica Supplement (Flow Cytometry IV), 274, 191-195.
- Fain-Maurel, M. A., Dadoune, J. P., and Reger, J. F. (1984). A cytochemical study on surface charges and lectin-binding sites in epididymal and ejaculated spermatozoa of Macaca fascicularis, The Anatomical Record, 208, 375-382.
- Fawcett, D. W. (1975). The mammalian spermatozoon, Developmental Biology, 44, 395-435.
- Feizi, T. (1985). Demonstration by monoclonal antibodies that carbohydrate structures of glycoproteins and glycolipids are onco-developmental antigens, Nature, 314, 53-57.
- Ficsor, G., Oldford, G. M., Loughlin, K. R., Panda, B. B., Dubien, J. L., and Ginsberg, L. C. (1984). Comparison of methods for detecting mitomycin C- and ethyl nitrosourea- induced germ cell damage in mice: Sperm enzyme activities, sperm motility, and testis weight, Environmental Mutagenesis, 6, 287-298.
- Ficsor, G. (1985). Personal communication via letter from friend in Switzerland.
- Ginsberg, L. C., and Ficsor, G. (1985). Germ cell mutagen assay, Research Grant Proposal #200-38-7892, Western Michigan University.
- Goldberg, R. B., Geremia, R., and Bruce, W. R. (1977). Histone synthesis and replacement during spermatogenesis in the mouse,

Differentiation, 7, 167-180.

Goodfellow, P. N., Levinson, J. R., Gable, R. J., and McDevitt, H. O. (1979). Analysis of anti-sperm sera for T/t locus-specific antibody, Journal of Reproductive Immunology, 1, 11-21.

Gordon, M., Dandekar, P. V., and Bartoszewicz, W. (1975). The surface coat of epididymal, ejaculated and capacitated sperm, Journal of Ultrastructure Research, 50, 199-207.

Grabske, R. J., Lake, S., Gledhill, B. L., and Meistrich, M. L. (1975). Centrifugal elutriation: Separation of spermatogenic cells on the basis of sedimentation velocity, Journal of Cell Physiology, 86, 177-190.

Hacker, U., Schumann, J., and Göhde, W. (1980). Effects of acute gamma-irradiation of spermatogenesis as revealed by flow cytometry, Acta Radiologica Oncology, 19, 361-368.

Hacker, U., Schumann, J., Göhde, W. and Müller, K. (1981). Mammalian spermatogenesis as a biologic dosimeter for radiation, Acta Radiologica Oncology, 20, 279-282.

Hammerstedt, R. H., Hay, S. R. and Amann, R. P. (1982). Modification of ram sperm membranes during epididymal transit, Biology of Reproduction, 27, 745-754.

Harris, G. (1983). DNA damage and repair in immunologically active cells, Immunology Today, 4, 80-83.

Hayes, C. E. and Goldstein, I. J. (1974). An alpha-D-galactosyl-binding lectin from Bandeiraea simplicifolia seeds, Journal of Biological Chemistry, 249, 1904-1913.

Imbar, M. (1973). Difference in the mobility of lectin sites on the surface membrane of normal lymphocytes and malignant lymphoma cells, International Journal of Cancer, 12, 93-99.

Kaneko, S., Oshio, S., Kobayashi, T., Iizuka, R. and Mohri, H. (1984). Human X- and Y- bearing sperm differ in cell surface sialic acid content, Biochemical and Biophysical Research Communications, 124, 950-955.

Kinsey, W. H. and Koehler, J. K. (1978). Cell surface changes associated with in vitro capacitation of hamster sperm, Journal of Ultrastructure Research, 64, 1-13.

Lam, D. M. K., Furrer, R., and Bruce, W. R. (1970). The separation, physical characterization, and differentiation kinetics of spermatogonial cells of the mouse, Proceedings of the National Academy of Sciences of the United States of America, 65, 192-199.

- Leblond, C. P., and Clermont, Y. (1952). Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulfuric acid technique, American Journal of Anatomy, 90, 167-215.
- Lis, H., and Sharon, N. (1972). Soy bean (Glycine max) agglutinin, Methods in Enzymology, 50, Part B, 360-365.
- Lis, H., and Sharon, N. (1973). The biochemistry of plant lectins (phytohemagglutinins), Annual Reviews in Biochemistry, 42, 541-574.
- Marchesi, V. (1972). Wheat germ (Triticum vulgaris) agglutinin, Methods in Enzymology, 28, Part B, 354-356.
- Meistrich, M. L., Bruce, W. R., and Clermont, Y. (1973). Cellular composition of fractions of mouse testis cells following velocity sedimentation separation, Experimental Cell Research, 79, 213-227.
- Meistrich, M. L., Lake, S., Steinmeta, L. L., and Gledhill, B. L. (1978). Flow cytometry of DNA in mouse sperm and testis nuclei, Mutation Research, 49, 383-396.
- Meistrich, M. L., Longtin, J., Brock, W. A., Grimes, S. R., and Mace, M. L. (1981). Purification of rat spermatogenic cells and preliminary biochemical analysis of these cells, Biology of Reproduction, 25, 1065-1077.
- Miller, G. L. (1959). Protein determination for large numbers of samples, Analytical Chemistry, 31, 964.
- Monesi, V. (1964). Autoradiographic evidence of a nuclear histone synthesis during mouse spermiogenesis in the absence of detectable quantities of nuclear ribonucleic acid, Experimental Cell Research, 36, 683-688.
- Monesi, V. (1965). Synthetic activities during spermatogenesis in the mouse, Experimental Cell Research, 39, 197-224.
- Murphy, L. A., and Goldstein, I. J. (1978). Bandeiraea simplicifolia I isolectins, Methods in Enzymology, 50, Part C, 345-350.
- Murphy, L. A., and Goldstein, I. J. (1979). Physical-chemical characterization and carbohydrate-binding activity of the A and B subunits of the Bandeiraea simplicifolia I isolectins, Biochemistry, 18, 4999-5005.
- Oakberg, E. F. (1956). Duration of spermiogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium, American Journal of Anatomy, 99, 507-515.
- Pinkel, D., Lake, S., Gledhill, B. L., Van Dilla, M. A., Stephenson,

- D., and Watchmaker, G. (1982). High resolution DNA content measurements of mammalian sperm, Cytometry, 3, 1-9.
- Pinkel, D., Gledhill, G. L., Van Dilla, M. A., Lake, S., and Wyrobek, A. J. (1983). Radiation-induced DNA content variability in mouse sperm, Radiation Research, 95, 550-565.
- Reisner, U., and Sharon, N. (1980). Cell fractionation by lectins, Trends in Biochemical Sciences, 5.
- Rinderknecht, H. (1960). A new technique for the fluorescent labelling of proteins, Experientia, XVI, 430-431.
- Rodriguez, M., Panda, B. B., and Ficsor, G. (1983). Testes weight reflect ethylnitrosourea induced histopathology in mice, Toxicology Letters, 17, 77-80.
- Schauer, R. (1985). Sialic acids and their role as biological masks, Trends in Biochemical Sciences, 10, 357-360.
- Schroff, R. W., Bucana, C. D., Klein, R. A., Farrell, M. M., and Morgan, A. C. (1984). Detection of intracytoplasmic antigens by flow cytometry, Journal of Immunological Methods, 70, 167-177.
- Schwartz, M. A., and Koehler, J. K. (1979). Alterations in lectin binding to guinea pig spermatozoa accompanying in vitro capacitation and the acrosome reaction, Biology of Reproduction, 21, 1295-1307.
- Sharon, N. (1984). Surface carbohydrates and surface lectins are recognition determinants in phagocytosis, Immunology Today, 5, 143-147.
- Shur, B. D., and Hall, N. G. (1982). A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida, Journal of Cell Biology, 95, 574-579.
- Silver, L. M. (1985). Mouse t haplotypes, Annual Review of Genetics, 19, 179-208.
- Silver, L. M., Artzt, K., and Bennett, D. (1979). A major testicular cell protein specified by a mouse T/t complex gene, Cell, 17, 275-284.
- Smith, S. (1984). Unpublished data, Western Michigan University.
- Stern, L., Kleene, K. C., Gold, B., and Hecht, N. B. (1983). Gene expression during mammalian spermatogenesis III. Changes in populations of mRNA during spermiogenesis, Experimental Cell Research, 143, 247-255.
- Swenson, D. H., Harbach, P. R., and Trzos, R. J. (1980). The rela-

- tionship between alkylation of specific DNA bases and induction of sister chromatid exchange, Carcinogenesis, 1, 931-936.
- Toda, N., Doi, A., Jimbo, A., Matsumoto, I., and Seno, N. (1981). Interaction of sulfated glycosaminoglycans with lectins, Journal of Biological Chemistry, 256, 5345-5349.
- Van Dilla, M. A., Pinkel, D., Gledhill, B. L., Lake, S., Watchmaker, G. and Wyrobek, A. J. (1980). Flow cytometry of mammalian sperm: progress report, Flow Cytometry, IV, 279-283.
- Vanhe-Perttula, T., and Arya, M. (1985). Lectin staining of rat testis and epididymis after ligation of excurrent ducts at different levels, Biology of Reproduction, 33, 477-485.
- Voglmayr, J. K., Fairbanks, G. and Lewis, R. G. (1983). Surface glycoprotein changes in ram spermatozoa during epididymal maturation, Biology of Reproduction, 29, 767-775.
- Wood, C., Kabat, E. A., Murphy, L. A., and Goldstein, I. J. (1979). Immunochemical studies of the combining sites of the two isolectins, A₄ and B₄, isolated from Bandeiraea simplicifolia, Archives of Biochemistry and Biophysics, 198, 1-11.
- Yanagisawa, K., Bennett, D., Boyse, E. A., Dunn, L. C., and Dimeo, A. (1974). Serological identification of sperm antigens specified by lethal t-alleles in the mouse, Immunogenetics, 1, 57-67.
- Yariv, J., Kalb, A. J., and Blumberg, S. (1972). Lotus tetragonolobus L-fucose-binding proteins, Methods in Enzymology, 28, 356-360.
- Zante, J., Schumann, J., Göhde, W., and Hacker, U. (1977). DNA-fluorometry of mammalian sperm, Histochemistry, 54, 1-7.