

Serological Detection of a Cell-Surface Antigen Specified by the T (Brachyury) Mutant Gene in the House Mouse

(autoantigen/embryology/sperm)

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ABSTRACT A cell-surface component specified by a mutant gene at the T-locus in the mouse has been detected on sperm by serological methods. The gene product thus recognized is not present on other adult cells.

At present, there is in mammals no single morphogenetic event of which the mechanisms and controls are really understood. One recurrent theme common to the thinking of most embryologists has been that during the sorting out of cells, which comprises a large part of embryonic development, the surfaces of the cells must come to bear differential displays that can serve as recognition devices.

Among many others, two of us (D.B., L.C.D.) have been interested for a long time in using developmental genetics as an approach to the problem of the control of differentiation. Our approach has been to use as analytical tools lethal mutants with marked morphogenetic effects on specific populations of cells in the early embryo, whose effects are therefore readily approachable descriptively. Such mutants should provide delicate ways of detecting factors or events essential to embryogenesis, since their effects may initially be restricted to the abnormality or absence of a single gene product. We have concentrated on genetic and embryological studies of mutant alleles at the complex T-locus in linkage group IX of the mouse. Our efforts and those of others (for review see refs. 1 and 2) have revealed in the region of the T-locus a well defined system of morphological mutants. The abnormalities produced by these mutants in both heterozygous and homozygous condition have led us to suspect that aberrations of the cell surface may be responsible (3).

Genetics

The T-locus is marked by a dominant mutant, *T*, which in heterozygotes leads to a shortened tail and in homozygotes to lethality midway through embryogenesis. Recessive alleles at the locus are identified by their interaction with *T* to produce taillessness. Several recessive embryonic lethal alleles of independent origin have been detected and maintained in balanced lethal tailless lines as *T/tⁿ*. The lethal alleles that have so far been well studied are found by genetic test to fall into six different complementation groups designated *T, t⁰, t⁹, t¹², t¹⁰¹, and t¹⁰⁵*. Alleles of any one complementation group have similar homozygous effects on embryonic development and these are different from those of any other group in time and in detail.

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Embryology

The earliest effect of each of the six lethal T-locus alleles can be characterized, in general terms, as an interference with apparent switch points, which occur as ectodermal and primitive streak derivatives diverge along separate pathways. The embryology of the lethal homozygotes suggests an inability of particular groups of cells either to reach their normal location or to maintain their morphological positions or their viability once they have attained their destinations. An extensive discussion of the detailed morphogenetic defects seen in all the homozygous lethal T-locus mutants is not appropriate here. It will suffice to say that each of these mutants appears to affect very early and basic processes in the organizational and morphogenetic events occurring during embryogenesis. The T-locus itself must therefore be considered a major region controlling such early events. Our panel of mutant alleles thus represents valuable material for testing the contention that the cell surface is the mediator of cellular interrelationships during embryogenesis.

Effects on spermatozoa

Another class of abnormalities shown by T-locus mutants is the alteration of the production or function, probably both, of sperm. This effect, which is unique amongst mutant genes known in mammalian systems, results in characteristic aberrations in the ratio observed at birth when *t*-alleles are transmitted from male, but not from female, heterozygotes. Progeny from such heterozygotes preponderantly receive the *t*-allele. This distortion of transmission ratio is seen to the same degree regardless of whether male heterozygotes are *T/t* or *+/t*, indicating that the aberration can be attributed to the recessive allele. In this respect at least, *T* and *+* are equivalent in sperm. Alteration of the transmission ratio of *t*-alleles may rest either on increased production of *t*-bearing sperm or on some fertilization advantage of *t*-bearing sperm relative to *+* or *T* (for review see ref. 4). Since there is no evidence of disturbed spermatogenesis in *t* heterozygotes, the implication is that *t*-bearing sperm may possess some property that confers a selective advantage during fertilization, which must further imply that this property is controlled by the haploid genotype of the sperm itself.

Taking into account the nature of the T-locus and its effects on both embryonic differentiation and sperm function, a case can be made for the hypothesis that one of the

functions of these genes is to specify cell surface components essential to both embryonic and sperm differentiation.

Others of us (E.A.B., E.G.) have approached the study of differentiation from a somewhat different angle, by concentrating on the immunogenetics of the surfaces of nucleated cells primarily of the lymphocyte series, and more recently of sperm. We know: (i) that the products associated with single genes are identifiable as antigens on the cell surface; (ii) that these gene-specified components of the cell surface are organized in detectable topographical patterns; and (iii) that different populations of differentiated cells within the same individual may be identified and categorized by quantitative or qualitative differences in the constellation of genetically specified components of the cell surface they possess (for review see ref. 5). However, with the single exception of the immunoglobulins that are incorporated in the cell surfaces of lymphocytes, in no case has the function of the genetically controlled alloantigens thus identified been shown to have any definable function.

Thus, the interests of developmental geneticists who suspected that gene-specified components of the cell surface were important in differentiation have converged with the interests of immunologists searching for functions of the gene-specified surface components identified as antigens.

We report here the serological detection of a cell surface antigen specified by an allele at the T-locus.

MATERIALS AND METHODS

Mouse Stocks. Closed-colony but not strictly inbred stocks: T/t^{w1} , T/t^{w2} , $T/+$, and $+/+$. t^{w1} and t^{w2} are, respectively, a lethal and a semilethal allele obtained from a wild population in New York (6) and maintained in balanced stocks as above since 1952 by L.C.D. The T mutant in all these stocks is the one originally found and described by Dobrovolskaia-Zavadskaia in 1927 (7) and maintained by L.C.D. since 1930. $T/+$ and $+/+$ stocks are similar in genetic background to each other and to T/t^{w1} and T/t^{w2} . Inbred stocks: BALB/cHu - $T^J/+$ (abbreviation, BALB/ T^J) and BALB/cHu (abbreviation, BALB) were kindly given us in 1970 by Dr. Katherine Hummel of the Jackson Laboratory. BALB/ T^J carries an independent mutation to T (T^J), which appeared in Dr. Hummel's BALB stock.

Antisera. (1) $+/+$ anti- T/t^{w2} sperm; (2) BALB σ^7 anti-BALB/ T^J sperm; (3) BALB/ T^J σ^7 anti-BALB/ T^J sperm.

Mice aged 2-4 months received 1×10^7 sperm subcutaneously, followed by 5×10^7 sperm intraperitoneally every 2 weeks. Recipients were individually tested for cytotoxic antibody against donor sperm.

Absorption of Antisera to Remove Sperm Autoantibody.[¶] The following applies to antisera 1 and 2: Sera of adequate titer were absorbed with recipient-type sperm to remove sperm autoantibody and tested for residual activity against the particular type of sperm used for immunization. For this

[¶] It will be shown in *Results* that BALB/ T^J males produce antibody against an antigen specified by the T-locus when immunized with BALB/ T^J sperm. Antigen T is truly an autoantigen in this case, and the anti-T antibody a true autoantibody. Nevertheless, we shall restrict the term "sperm autoantibody" in this paper to its more common connotation: namely to antibody reactive not only with sperm of the antiserum-donor's type but also with sperm of all mice.

absorption, four parts of undiluted antiserum were mixed with one part (by volume) of testicular cells and incubated for 30 min at 0° with occasional shaking. Testicular cells for absorption were prepared by mincing and gentle homogenization of the testis in phosphate-buffered saline (Dulbecco's PBS) containing 5% heat-inactivated fetal-calf serum ("IPT" = immunoprecipitin tested, free of IgG, Grand Island Biological Co.) and 0.5% fructose. Sometimes the absorption had to be repeated in order to remove all activity against recipient-type sperm. Absorbed sera with adequate titer against the specific immunizing sperm, were analyzed further as described in *Results*. The following applies to antiserum 3: this antiserum (BALB/ T^J anti-BALB/ T^J sperm) is produced by immunization with syngeneic sperm; therefore, absorption of sperm autoantibody[¶] was in this case done with BALB sperm, which should remove all activity except that directed specifically to antigen T.

Harvesting of Antisera. Selected mice yielding good antiserum were bled from the tail 7, 10, and 14 days after each inoculation of sperm. Successive sera were stored at -70° and pooled as necessary to provide sufficient volume for a group of tests. Because a few mice would commonly produce better antisera than others, pooling of sera from

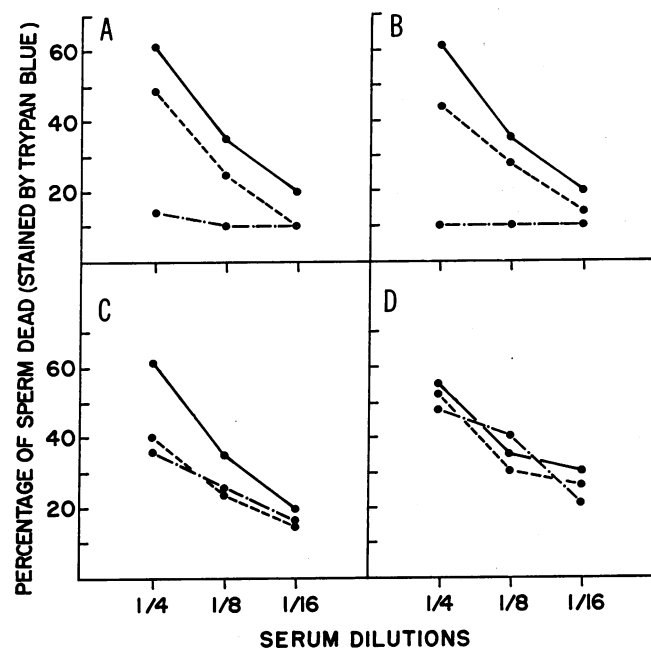


FIG. 1. Tissue representation of antigen T determined by absorption of $+/+$ anti- T/t^{w2} serum with various cell types and subsequent testing on BALB/ T^J sperm. Control (—): no absorption except for routine pre-absorption with $+/+$ testicular cells to remove sperm autoantibody. (A) absorbed with BALB sperm (---); absorbed with BALB/ T^J sperm (-·-·). (B) absorbed with BALB testicular cells (---); absorbed with BALB/ T^J testicular cells (-·-·). (C) absorbed with BALB brain cells (---); absorbed with BALB/ T^J brain cells (-·-·). (D) absorbed with T/t^{w2} lymphocytes and thymocytes (---); absorbed with $+/+$ lymphocytes and thymocytes.

Result: Positive cells: sperm and testicular cells from BALB/ T^J males. Negative cells: sperm and testicular cells from BALB males; lymphocytes and thymocytes from T/t^{w2} or $+/+$ males, brain cells from BALB and BALB/ T^J . The fall in titer seen in cells designated as negative is no more than to be expected from the absorption procedure itself.

two or more mice was seldom resorted to, nor was it usually necessary in view of the substantial volumes of serum that can be accumulated from a single mouse according to the bleeding schedule described. Immunized mice received as many as 10 inoculations, but activity was usually detectable after five inoculations.

Preparation of Sperm. Sperm were obtained from the vas deferens and epididymis of freshly killed males. For use in immunizations, sperm were suspended at a concentration of 5×10^7 /ml in Earle's balanced salt solution containing penicillin and streptomycin. For cytotoxicity tests, only epididymal sperm were used; they were suspended in phosphate-buffered saline containing 0.5% fructose and 5% fetal-calf serum (IPT).

Cytotoxicity Test. The cytotoxicity test for use with sperm is described elsewhere (8). Briefly, antiserum is set up in 2-fold dilutions (0.05-ml volumes) in phosphate-buffered saline with 5% fetal-calf serum (IPT) and 0.5% fructose. Equal volumes of sperm suspension (see above) and horse serum (diluted 1/16; complement source) are then added, and the tubes are incubated for 45 min in a 37° water-bath. Then 0.1 ml of trypan blue prepared as described (8) is added to each tube, and the proportion of dead (stained) sperm counted in a counting-chamber. Controls consisted of tubes in which phosphate-buffered saline was substituted for either antiserum or horse serum. The proportion of dead sperm in these controls did not exceed 20% (otherwise the results were discarded); for brevity, these controls are omitted in *Results*. Selected horse serum was chosen as the source of complement because it has far less natural cytotoxicity for sperm than rabbit serum (otherwise the best source of complement in this system), so that prior absorption with mouse cells for removal of natural heteroantibody could be dispensed with.

RESULTS

Detection of antigen-T on sperm

Antiserum $+/+$ ♂ anti- T/t^{w2} sperm (Table 1). Antiserum $+/+$ anti- T/t^{w2} was cytotoxic for sperm from males of genotype T/t^{w2} , $T/+$, and BALB/ T^J , but not for sperm from $+/+$ segregants of the same stocks. The specificity of the cytotoxic reaction for antigen-T is virtually guaranteed by negative reactions for BALB ($+/+$) sperm contrasted with the positive results for the coisogenic BALB/ T^J ($T^J/+$) sperm and the fact that antiserum produced against sperm

carrying the original T mutant was equally cytotoxic for sperm carrying the independent mutant T^J . In addition, many other $+/+$ genotypes from other stocks were tested; all these tests, not shown in Table 1, were uniformly negative. The fact that a higher percentage of lysis was obtained with sperm from T/t^{w2} genotypes suggests that the antiserum includes antibody against antigen specified by t^{w2} as well as by T .

Antiserum BALB ♂ anti-BALB/ T^J sperm (Table 2). This antiserum, produced by immunizing BALB males with sperm of the coisogenic mutant BALB/ T^J stock, also was cytotoxic for sperm from both BALB/ T^J and $T/+$ mice, but not for sperm from $+/+$ genotypes. This is illustrated in Table 2, which shows results of blind tests on sperm from nine mice of various $T/+$ or $+/+$ genotypes.

Identification of T as an autoantigen

Because the effects of the T-locus mutants appear probably to be restricted to embryos and sperm, it seemed likely that the distribution of antigen-T would be similarly restricted. Anti-T sera were not cytotoxic for thymocytes, lymphocytes, or epidermal cells (prepared as described in ref. 9), nor was antigen T detectable on lymphocytes and thymocytes, or brain cells by the more sensitive criterion of absorption (Fig. 1). Testicular cells and sperm, on the other hand, absorbed anti-T activity specifically (Fig. 1).

Our inability to detect antigen-T on adult cells other than sperm and testicular cells raised the question whether antigen-T might function as an autoantigen. Table 3 shows that this is indeed the case; immunization of BALB/ T^J males with sperm from syngeneic BALB/ T^J animals produced antisera which (after absorption with BALB sperm) was cytotoxic for sperm of all T -containing genotypes tested.

DISCUSSION

The purpose of this initial serological study was to look for evidence that the T locus does in fact code for components of the cell surface. This is crucial to the argument that morphogenetic events depend on properties of the cell surface; and that hypothesis, though now much in favor and supported by considerable circumstantial evidence, lacks direct proof. The observation that the allele T specifies an antigen on the surface of sperm is thus a needed vindication of the prediction that genes with a morphogenetic role will be found to code for components of the cell surface. It also, incidentally, supports the premise that the altered transmission ratios

TABLE 1. Cytotoxicity tests on mouse epididymal sperm with antisera* $+/+$ anti- T/t^{w2}

Sperm tested	Result of cytotoxicity test (% sperm dead)											
	Test no. †											
	1	2	3	4	5	6	7	8	9	10	11	12
T/t^{w2}						71	53	55	74	75	77	76
$T/+$	41	51				42						
BALB/ T^J	72	40	53	40	46							
$+/+$	<20	<20				<20	<20	<20	<20	<20	<20	<20
BALB	<20	<20	<20	<20	<20							

* Serum dilution: 1/2.

† Each test number in all tables identifies a group of samples run concurrently with the same reagents; tests in separate columns were always done on different batches of cells, and frequently with different sera.

TABLE 2. Cytotoxicity tests with serum* of BALB male mice immunized with BALB/T sperm: T-specificity confirmed by blind tests on epididymal sperm of mice of selected genotypes

Sperm tested	Result of cytotoxicity test (% sperm dead)
BALB/T ^J	60
BALB/T ^J	41
BALB/T ^J	50
T/+	75
T/+	45
BALB	<20
BALB	<20
+/+	<20
+/+	<20

* Serum dilution: 1/4.

produced by *t*-alleles of the T locus are due to properties of the sperm surface coded by these alleles, for it is highly probable that if *T* codes for a cell surface component, as reported here, so will all other alleles, *t* and +, at this locus. And this implies that the constitution of the sperm surface must reflect, at least in part, the haploid genome of the sperm; for otherwise, i.e., if the final constitution of the sperm surface were determined at a stage when its precursors were still diploid, it would not be likely for a gene coding for a sperm surface component to cause preferential transmission of one allele.

Haploid gene effects in sperm of animals have not in general been detected (10); the only two impressive examples of putative haploid effects on gamete function are in fact the aberrant ratios produced by *t*-alleles, and the preponderance of males over females conceived in many mammalian species; the latter presumably being explained by a fertilizing advantage of Y-bearing sperm. The scarcity of haploid effects observed in mammalian sperm may stem from several facts: (a) As in all other cell types, only a minority of genes may be expected to be active in sperm or their precursors, (b) RNA synthesis is minimal in secondary spermatocytes and spermatids, the only stages of differentiation at which RNA synthesis could be directed by the haploid genome (11), and (c) in heterozygotes, any odd number of crossovers between a given gene and its centromere would result in secondary spermatocytes retaining the heterozygous condition until the spermatid stage, when levels of RNA synthesis are extremely low. These factors would tend to prevent or obscure expression of a haploid phenotype in sperm. It may be significant, therefore, that the two putative examples of haploid effects mentioned above occur in situations where crossing-over is reduced or absent. Thus: (a) The recessive lethal *t*-alleles that produce altered transmission ratios are known to prevent recombination at least between *T* and a marker locus *tf*, which is about 8 units distal to *T* in relation to the centromere, (13) and they may well suppress crossingover in the opposite direction; in any case the T-locus is so close to the centromere, perhaps even adjacent to the centromeric heterochromatin according to Klein (14), that even the allele *T*, which is not known to suppress recombination, might be expected to produce a detectable haploid effect; (b) X and Y chromosomes are thought not to undergo crossingover (12),

TABLE 3. Cytotoxicity tests on mouse epididymal sperm with serum* of BALB/T^J males immunized with BALB/T^J sperm

Sperm tested	Result of cytotoxicity test (% sperm dead)		
	Test no.		
	1	2	3
BALB/T ^J	60	30	54
BALB	<20	<20	<20
T/+	66	30	
+/+	<20	<20	
T/ <i>t</i> ^{w2}		30	

* Serum dilution: 1/4.

and, therefore, secondary spermatocytes are truly hemizygous for these chromosomes.

Our results with anti-T serum are compatible with some degree of haploid influence because in cytotoxic tests considerably less than 100% of sperm from T/+ heterozygotes were susceptible to anti-T antibody, suggesting that some sperm had less or no antigen T. This interpretation is unwarranted however, as the serological system is a relatively weak one, with low antibody titers; thus there is no justification for concluding that sperm not lysed in the cytotoxic test are devoid of antigen. Furthermore, the considerations concerning the effect of crossingover on the gene content of immature sperm suggest that even where a haploid effect might be demonstrable it would be partial rather than complete. Again, information regarding synthetic inactivity in spermatogenic cells suggests that the mature sperm would have at least some of the membrane specified by its original diploid genotype. It seems possible that the sperm produced by a heterozygote where haploid effects occur comprise subpopulations that differ quantitatively rather than qualitatively with respect to gene products appearing in haploid as well as diploid phases of sperm differentiation.

Our choice of *T* as the first allele for serological study was governed mainly by the availability of the mutant coisogenic BALB/T^J stock, which was invaluable in proving the specificity of cytotoxic anti-T antisera for a product of the *T* allele of the T-locus. The fact that T can be an auto-antigen, i.e., that anti-T antibody is produced by inbred T^J/+ males immunized with T^J/+ sperm of males of the same inbred strain, supports the serological indication that the gene *T* is not expressed in adult cells other than sperm. If *T* were expressed more generally on adult somatic cells, the expectation is that self-tolerance of antigen T would normally be maintained throughout life, in which case autoimmunization would normally be impossible. These facts are consistent with our original premise that the T-locus may control cell surface constituents important only in embryogenesis and spermatogenesis.

Thus, in addition to being an allo-antigen and a "differentiation antigen" (5), antigen T might be classed as a sperm autoantigen, the first indeed whose coding has been traced to a particular gene.

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