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Primitive Teratocarcinoma Cells Express a Differentiation Antigen Specified by a Gene at the T-Locus in the Mouse

(quantitative absorption/cytotoxicity test/embryonic antigen/morula/t^{12})

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ABSTRACT **A cell-surface antigen common to mouse primitive teratocarcinoma cells, morulae, and sperm has been shown by serological methods to be specified by a wild-type T-locus gene whose mutant form is lethal in homozygotes at the morula stage.**

Embryonic cell-surface components are commonly thought to mediate morphogenetic events via differential surface displays providing recognition devices by which embryonic cells may identify others as similar or different (1). While various differentiated cell populations of the adult individual can be discriminated specifically by genetically determined cell surface markers (2), the experimental analysis of embryonic morphogenesis in terms of cell-surface components has been frustrated because embryonic antigens specified by known genetic systems have not as yet been identified. We report here that an early embryonic cell-surface antigen previously demonstrated on primitive teratocarcinoma (PTC) cells is specified by a wild-type allele at the T-locus in the mouse, where a morphogenetically important series of mutations has already been defined.

PTC cells in culture elicit, after hyperimmunization of syngeneic male mice, the formation of specific antibodies cytotoxic to various PTC cell lines but not to a variety of differentiated cells which take origin from the same tumor. These anti-PTC sera are also negative on all other mouse cells tested with only two exceptions: (*i*) mouse morula cells, the reaction increasing from very little, if any, with 1-cell eggs to a maximum at the 8-cell stage, a result which suggests the progressive expression of a surface antigen(s) during the earliest stages of development and (*ii*) male germ cells, notably sperm cells (3).

The finding that male germ cells are the only adult cells tested so far which share an antigen in common with PTC and normal morula cells suggests a way of analyzing the genetic origin of this antigen. Several surface antigens have been detected on sperm cells; these include H-2 (4), H-Y (5), and so-called "sperm auto-antigens" (6). Furthermore, a series of surface antigens genetically determined by alleles of the T-locus has been detected on sperm cells (6, 7).

A gene at the T-locus appears to be a good candidate for specifying the antigen common to PTC, male germ cells, and morulae because those mutations at the T-locus which determine surface antigens on the sperm are known to compromise embryonic development in specific ways (8) that are interpretable as due to cell-surface defects (1). Since the surface antigen revealed on morula cells by means of anti-PTC sera is likely to play an important role in cleavage stages, the best candidate appears to be that gene whose mutation (t^{12}) is known, in the homozygous condition, to act earliest and block development at the morula stage. This hypothesis can be tested because mouse sperm cells have been shown to express antigens determined by both wild-type and mutant *t*-alleles (7). Specific anti-PTC antiserum can, therefore, be absorbed quantitatively with sperm from mice of various *T* genotypes (+/+ or +/t^{12}) and tested for residual cytotoxic activity on PTC cells. The results show that the major antibody component of the anti-PTC serum recognizes specifically the product of the wild allele $+^{t^{12}}$.

MATERIALS AND METHODS

Mice. -129/Sv-CP (129) mice were maintained at The Pasteur Institute. The following T-locus stocks were maintained at Cornell University Medical College:

(*1*) BT BRTF/Nev, the T-locus background. This stock segregates for the dominant marker Brachy and carries the outside recessive marker tufted; $\frac{T\ tf}{+\ tf}$, short tailed tufted and $\frac{+\ tf}{+\ tf}$, normal tailed tufted.

(*2*) The tailless stock $\frac{T\ \ tf}{t^{w32}\ +}$, t^{w32} is an allele lethal at morula stage (9) in the same complementation group as t^{12} and is, therefore, operationally identical to it (10). We shall refer to t^{w32} as t^{12} throughout. These mice are maintained in the balanced lethal cross $\frac{T\ tf}{t^{12}\ +} \times \frac{T\ tf}{t^{12}\ +}$ forcibly heterozygous for t^{12} (t^{12} prevents recombination between *T* and *tf*).

(*3*) The selected normal tailed F_1 of the cross of the above two stocks $\left(\frac{T\ tf}{t^{12}\ +} \times \frac{+\ tf}{+\ tf}\right)$ which is $\frac{+\ tf}{t^{12}\ +}$.

(*4*) The tailless stock $\frac{T\ \ tf}{t^{w1}\ +}$, t^{w1} is a late acting (after 9 days) lethal *t*-allele (11, 12) controlling an antigen detectable on sperm which does not cross-react with the antigen specified by t^{12} (7).

Abbreviations: PTC, primitive teratocarcinoma; C′, complement; NA_{50}, number of cells necessary to absorb 50% cytotoxic acitivity.

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Antiserum. 129 Male anti-PTC-F9 (anti-F9) serum was prepared as previously described (3) and heat inactivated (56° for 30 min). The experiments described here were done with serum obtained from a single bleeding of an individual mouse taken 7 days post 7th immunization; the cytotoxic titer was 1:3200 on F9. This serum was diluted to 1:800 in test medium (Hanks' containing 4% heat inactivated, immunoprecipitin tested, fetal-calf serum—GIBCO) and aliquots of it were kept frozen at −70° until needed for quantitative absorption.

Cells. PTC-F9-41 (F9), a pleuropneumonia-like-organism-free derivative of the original F9 cell line was cultivated in complete medium and harvested with EDTA as previously described (3). GiL4 (13), a C57Bl/6 virally induced leukemia was harvested from mice growing ascites. Sperm for absorptions were prepared at room temperature by stripping the vas deferens and mincing the whole epididymis. Sperm obtained from one mouse were washed twice in 10 ml of Hanks' balanced salt solution +0.5% fructose by centrifuging at 3000 × *g* for 15 min. They were resuspended in a small volume and counted. Appropriate different numbers of sperm cells (different volumes) from the concentrated suspension were delivered to disposable plastic centrifuge tubes. The tubes were filled to capacity (0.4 ml) with test medium and spun for 10 min in a Beckman microfuge at maximum speed.

Quantitative Absorptions were performed in the same tubes by the method of Boyse *et al.* (14) using 0.06 ml of anti-F9 serum at a dilution of 1:800, absorbing 20 min at room temperature and 70 min at 4° with increasing numbers of sperm of each genotype. The serum was then separated from the cells at maximum speed on the Beckman microfuge and tested for residual cytotoxic activity on F9. Two control absorptions were done: (*i*) with F9 to obtain maximum absorption and (*ii*) with GiL4 to indicate the level of nonspecific absorption. Conditions for these control absorptions were slightly different; the entire absorption (90 min) was performed at 4°, because at room temperature with the cell concentrations used, F9 cells spontaneously aggregate producing considerable variation in the total surface area available from a given number of cells. The serum dilution was 1:400 instead of 1:800 because so few F9 cells (they are extremely large) removed all activity.

The standard point of comparison of the absorption capacity of one cell type to another was taken as the number of absorbing cells necessary to reduce the cytotoxic activity of anti-F9 to 50% lysis when tested on F9 cells (NA_{50}).

Cytotoxicity Test has been previously described (3). Briefly, equal volumes (0.04 ml) of: F9 cells (1.4×10^5 cells per ml) suspended in test medium; anti-F9 absorbed with different numbers of sperm cells and absorbed rabbit complement (C′) were incubated for 45 min at 37° with occasional vigorous shaking. The % dead cells was determined after addition of trypan blue. For each test done, two internal controls were included; a tube of unabsorbed anti-F9 + C′ to indicate preabsorption cytotoxicity, and a tube in which antiserum was replaced by test medium to control for C′ toxicity.

RESULTS

Effect of Anti-sperm Serum on PTC Cells. Anti-sperm serum was prepared by hyperimmunizing a C57Bl/6J female with C57Bl/6J.H-2^k sperm. This serum was cytotoxic for sperm of all genotypes tested because of sperm-specific autoantibody (6). It was also cytotoxic for F9 cells, killing 50% above controls up to a dilution of 1:256. The effect on F9 could not be ascribed to the presence of H-2^k (because F9 is derived from 129 mice which are H-2^b); nor of H-Y (because absorption with male or female lymphocytes does not differentially alter activity on sperm). It was, therefore, due to the presence of either sperm autoantigens or, more likely, of a wild-type t-antigen on these cells.

To remove autoantibody, the anti-sperm serum was massively absorbed (1 volume:1 volume repeated twice) with testicular cells pooled from mice of different genotypes (T/t^{w1}, T/t^{w5}, and T/t^{12}). The absorbed serum remained cytotoxic for +/+, but not for any T/t^z sperm while sperm containing one + allele such as $T/+$ or $+/t^z$ showed intermediate sensitivity (7). It seemed, therefore, that the absorbed serum was specific for the product of a + allele at the T-locus and it was designated "anti-+" serum.

When assayed on F9 cells by cytotoxicity test, however, this anti-+ serum was negative (for summary of results of anti-sperm serum, see Table 1). The most obvious conclusion would then be to ascribe the effect of anti-sperm serum on F9 to sperm autoantibody, a rather puzzling conclusion indeed. Another interpretation can be given, however, provided the genetic structure of the T-locus is reconsidered. In fact, for some time, there have been reasons for supposing that this region consists of more than one gene.

Without going into details, all recessive mutations at the T-locus express one character in common, namely interaction with *T* to produce taillessness. Each different recessive allele, however, also expresses effects unique to itself, which involve embryonic lethality and sperm antigen. The nonparallelism of these effects and the complementation pattern of these mutations can best be accounted for by assuming (*i*) that recessive *t*-alleles have at least two separable components: t^T (allelic to *T* and responsible for interaction leading to taillessness) and t^z (closely linked and responsible for sperm and viability effects) and (*ii*) that the t^z components comprise a series of different closely linked genes controlling a series of products, presumably surface antigens, present both on sperm and on specific cells at certain stages of embryonic development. By modifying a model originally proposed by Lyon and Meredith (15), the chromosome bearing a wild-type T-locus haplotype could then (without implying prejudice to actual order or number of genes) be represented by $+^T +^{t^z} +^{t^v} \ldots +^{t^i}$; the Brachy mutation by $\underline{T +^{t^z} +^{t^v} \ldots +^{t^i}}$; and a recessive lethal *t* mutation (for instance t^v) would need two mutant

TABLE 1. *Summary of cytotoxicity of serum made against sperm cells*

	+/+ sperm	T/t^z sperm	PTC-F9
C57Bl/6 anti-C57Bl/6.H-2^k sperm; "anti-sperm"	Positive	Positive	Positive
Anti-sperm absorbed with T/t^{w1}, T/t^{w5}, T/t^{12} sperm; "anti-$+^T$"	Positive	Negative	Negative

TABLE 2. *Putative T-locus genotypes and NA_{50} values obtained by quantitative absorption of anti-F9 with sperm*

T-locus stock	+/+	T/t^{12}	T/t^{w1}	$T/+$	$+/t^{12}$
Phenotype	normal tail	no tail	no tail	short tail	normal tail
Genotype* with respect to t^{12}	$\frac{+^T\ \boxed{+^{t12}}\ +^{tw1}\ \ldots}{+^T\ \boxed{+^{t12}}\ +^{tw1}\ \ldots}$	$\frac{T\ \boxed{+^{t12}}\ +^{tw1}\ \ldots}{t^T\ t^{12}\ \ +^{tw1}\ \ldots}$	$\frac{T\ \boxed{+^{t12}}\ +^{tw1}\ \ldots}{t^T\ \boxed{+^{t12}}\ t^{w1}\ \ldots}$	$\frac{T\ \boxed{+^{t12}}\ +^{tw1}\ \ldots}{+^T\ \boxed{+^{t12}}\ +^{tw1}\ \ldots}$	$\frac{+^T\ \boxed{+^{t12}}\ +\ ^{tw1}\ \ldots}{t^T\ \ t^{12}\ \ +\ ^{tw1}\ \ldots}$

Exp.						Ratio $\left[\frac{NA_{50}\ +^{t12}/t^{12}}{NA_{50}\ +^{t12}/+^{t12}}\right]$
107	6.4†	13.7				2.1
108	4.4	11.4				2.6
111		15.1	6.4			2.4
112	4.4				10.5	2.4
113	5.3				12.8	2.4
114			5.7	4.8		—
115	3.2			3.4		—

* The order of the genes is arbitrary, the $+^{t12}$ allele is boxed for greater clarity.
† $NA_{50} = 10^{-6} \times$ the number of cells necessary to reduce anti-F9 activity to 50% lysis of F9.

components: $t^T\ +^{tx}\ t^y\ \ldots\ +^{tz}$. In this scheme, a heterozygote of the type T/t^{12} has the structure

$$\frac{T\ +^{tw1}\ +^{t12}\ +^{tw5}\ \ldots}{t^T\ +^{tw1}\ t^{12}\ \ +^{tw5}\ \ldots}.$$

With this model, which accounts much more satisfactorily for the properties of the mutations at the T-locus, the results of the anti-sperm serum on F9 cells take on new significance. The serum was prepared against

$$\frac{+^T\ +^{tx}\ +^{ty}\ \ldots\ +^{tz}}{+^T\ +^{tx}\ +^{ty}\ \ldots\ +^{tz}}$$

(wild-type) sperm. It was absorbed with a mixture of

$$\frac{T\ +^{tx}\ +^{ty}\ \ldots\ +^{tz}}{t^T\ t^x\ \ +^{ty}\ \ldots\ +^{tz}},\quad \frac{T\ +^{tx}\ +^{ty}\ \ldots\ +^{tz}}{t^T\ +^{tx}\ t^y\ \ \ldots\ +^{tz}},$$

etc. sperm. This would leave activity against $+^T$ antigen but remove activity against the wild-type products of all t genes. This absorbed serum is in fact anti-$+^T$. If should, therefore, be active on normal sperm since all T-locus antigens appear to be expressed on sperm cells, but inactive on T/t^x sperm since they do not contain $+^T$. It should also not be active on F9 cells which, being in some way the analogue of morula cells, should express only the T-locus antigen relevant to that stage of development, namely $+^{t12}$ but not $+^T$. The latter should appear on embryos only several days later, at a stage when the development of T/T homozygous embryos is blocked.

In order to detect the presence of $+^{t12}$ antigen on the surface of F9 cells, quantitative absorption of anti-F9 serum was made comparatively with sperm from homozygous $+^{t12}/+^{t12}$ versus heterozygous $+^{t12}/t^{12}$ mice. Since each allele appears to be expressed on sperm cells, it should require twice as many sperm from the heterozygous as from the homozygous $+^{t12}/+^{t12}$ animal to reduce the cytotoxicity against F9 to 50% of its initial activity.

Quantitative Absorption of Anti-F9 Serum. Control absorptions were first performed to determine background levels of the absorption capacity (number of cells necessary to absorb 50% cytotoxic activity—NA_{50}) for known positive and negative cells. F9 cells were used as the positive control: a small number of cells was sufficient to remove all activity, the NA_{50} being 9×10^4 cells. GiL4, a C57Bl/6 leukemia that had previously been shown to lack any surface antigen cross-reacting with F9 (3), was used as a negative control. Even the use of relatively enormous amounts of GiL4 cells (1×10^8) lowered anti-F9 activity only slightly, from a pre-absorption level of 82% lysed, to 70%.

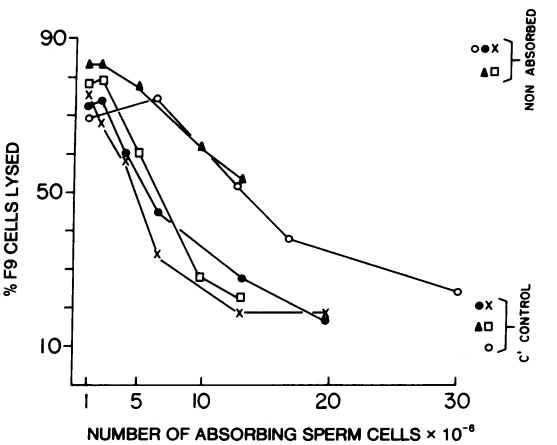

FIG. 1. Residual cytotoxic activity of anti-F9 tested on F9, after quantitative absorption with sperm from T/t^{12} (▲), $+/t^{12}$ (○), +/+ (□), T/t^{w1} (●), and $T/+$ (×) mice. Points for non-absorbed serum and C′ control are included for each genotype. (Data are taken from three experiments.)

Quantitative absorptions of anti-F9 serum were then performed with sperm from mice of five different T-locus genotypes. In each experiment, sperm of two different genotypes were compared under the same test conditions (Table 2). One test for each genotype is graphed in Fig. 1. Although from one experiment to another, the NA_{50} for the same genotype exhibits some variation, in the same experiment the results appear to depend exclusively on the number of $+^{t12}$ alleles. When sperm from homozygous $+^{t12}/+^{t12}$ are compared, their NA_{50}'s are alike. When sperm from heterozygous $t^{12}/+^{t12}$ are compared, they also have similar NA_{50}'s. But, when homozygous and heterozygous $+^{t12}$ are compared, the NA_{50} of the heterozygotes turns out inevitably to be twice higher (averaging 2.38 in five independent experiments) than that of the homozygotes. In contrast, changing the number of wild-type alleles at other genes of the T-locus, such as T or t^{w1}, does not significantly alter the NA_{50}. We conclude that the product of the $+^{t12}$ allele is a major antigen detected on F9.

It is also evident from these data that the NA_{50} of sperm from the normal tailed $(T/t^{12} \times +/+)F_1$ is the same as the parental heterozygotes T/t^{12}. This result taken together with the NA_{50}'s obtained with sperm from the other T-locus stocks tested represents controls for differences in genetic background.

DISCUSSION

Syngeneic immunization with PTC cells had shown that such cells share a common surface antigen with morula and sperm cells (3). The observations reported in this communication show that this antigen is determined by a gene $(+^{t^{12}})$ of the T-locus. The results obtained with anti-sperm and anti-PTC sera have permitted us to consolidate our concept of the genetic organization of this complex chromosomal region and form a testable working model.

On the one hand, with anti-sperm sera, we can now directly detect on sperm two alleles of the gene Brachy: T, and $+^T$ (6, 7). (The presence of a third allele, t^T is implied, because by definition, there is a factor in every recessive t haplotype which interacts with T to cause taillessness.) On the other hand, with quantitative absorption of anti-F9, we have shown that $+^T$ is not detectable on F9 cells, but another wild-type allele at the T-locus, $+^{t^{12}}$ is; therefore, we conclude that there must be a minimum of two different closely linked genes at the T-locus. Thus, in the T/t^x genotype, the chromosome bearing the recessive t determinant contains a minimum of two mutated factors: t^T, allelic to T and responsible for the phenotypic interaction causing taillessness; and at least one other component, t^x, responsible for lethality and effects on sperm.

Since embryos homozygous for any of five different lethal genes of the T-locus fail to perform a step in differentiation specific for that gene, the products determined by the wild-type alleles of these genes must serve several indispensable functions in normal embryonic development. We have presented evidence that a cell-surface component present on both PTC and morula cells is determined by one of these genes; more precisely by that gene whose mutation is known, in the homozygous condition, to block embryonic development at the morula stage. This strongly supports the idea that genetically controlled differentiation antigens have essential functional roles during embryogenesis. Antigens specified by wild-type alleles of another, later acting, lethal t-mutation are not detectable on PTC. If PTC and morula cells are developmentally analogous, as we believe they are, we may thus suppose that the various stages of embryonic development are marked by the sequential appearance of specific cell surface components.

Further analysis of the genetic determination and stage specificity of morphogenetically important antigens should now be possible, with the use of embryonic tumors of normal genotypes like F9 as well as tumors of abnormal genotypes such as those which can be derived from at least one t-mutant (t^{w18}/t^{w18}) (16).

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1. Bennett, D., Boyse, E. A. & Old, L. J. (1971) "Cell surface immunogenetics in the study of morphogenesis," in *Proc. III Lepetit Colloquium*, London, Nov. 1971 (North Holland Publ. Co., Amsterdam), pp. 247–263.
2. Boyse, E. A. & Old, L. J. (1969) "Some aspects of normal and abnormal cell surface genetics," *Annu. Rev. Genet.* **3**, 269–290.
3. Artzt, K., Dubois, P., Bennett, D., Condamine, H., Babinet, C. & Jacob, F. (1973) "Surface antigens common to mouse cleavage embryos and primitive teratocarcinoma cells in culture," *Proc. Nat. Acad. Sci. USA* **70**, 2988–2992. See also Correction, *Proc. Nat. Acad. Sci. USA* **70**, 3932.
4. Goldberg, E., Aoki, T., Boyse, E. & Bennett, D. (1970) "Detection of H-2 antigens on mouse spermatozoa by the cytotoxicity test," *Nature* **228**, 570–572.
5. Goldberg, E., Boyse, E. A., Bennett, D. Scheid, M. & Carswell, E. A. (1971) "Serological demonstration of H-Y (male) antigen on mouse sperm," *Nature* **232**, 478–480.
6. Bennett, D., Goldberg, E., Dunn, L. C. & Boyse, E. A. (1972) "Serological detection of a cell-surface antigen specified by the T (Brachyury) mutant gene in the house mouse," *Proc. Nat. Acad. Sci. USA* **69**, 2076–2080.
7. Yanagisawa, K., Bennett, D., Boyse, E. A., Dunn, L. C. & Dimeo, A. (1974) "Serological identification of sperm antigens specified by lethal t-alleles in the mouse," *Immunogenetics* **1**, in press.
8. Bennett, D. (1964) "Abnormalities associated with a chromosome region in the mouse. II. The embryological effects of lethal alleles at the t-region," *Science* **144**, 263–267.
9. Smith, L. J. (1956) "A morphological and histochemical investigation of a preimplantation lethal (t^{12}) in the house mouse," *J. Exp. Zool.* **132**, 51–79.
10. Bennett, D. & Dunn, L. C. (1964) "Repeated occurrences in the mouse of lethal alleles of the same complementation group," *Genetics* **49**, 949–958.
11. Bennett, D., Dunn, L. C. & Badenhausen, S. (1959) "A second group of similar lethals in populations of wild house mice," *Genetics* **44**, 795–802.
12. Bennett, D., Badenhausen, S. & Dunn, L. C. (1959) "The embryological effects of four late-lethal t-alleles in the mouse which affect the neural tube and skeleton," *J. Morphol.* **105**, 105–144.
13. Levy, J. P., LeClerc, J. C., Varet, B. & Oppenheim, E. (1968) "Study of antigenic specificity of Graffi leukemic cells," *J. Nat. Cancer Inst.* **41**, 743–750.
14. Boyse, E. A., Miyazawa, M., Aoki, T. & Old, L. J. (1968) "Ly-A and Ly-B: two systems of lymphocyte isoantigens in the mouse," *Proc. Roy. Soc. London Ser. B.* **170**, 175–193.
15. Lyon, M. F. & Meredith, R. (1964) "Investigations of the nature of t-alleles in the mouse. II. Genetic analysis of an unusual mutant allele and its derivatives," *Heredity* **19**, 313–330.
16. Artzt, K. & Bennett, D. (1972) "A genetically caused embryonal ectodermal tumor in the mouse," *J. Nat. Cancer Inst.* **48**, 141–158.