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Normal adenylate ribonucleotide content in mouse embryos homozygous for the t^{12} mutation

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SUMMARY

The recently improved firefly luciferase assay was used to determine ATP, ADP or AMP in single preimplantation mouse embryos from crosses yielding lethal t^{12}/t^{12} embryos. Normal values of the three adenylate ribonucleotides were found in freshly collected 2-cell and 4-cell embryos and during *in vitro* culture to the blastocyst stage. A decrease in adenylate ribonucleotide content was seen in putative t^{12}/t^{12} embryos only when they were degenerating.

INTRODUCTION

The mechanism of embryonic lethality for mutations which map in the *t*-region of chromosome 17 of the mouse has been a matter of considerable controversy (reviewed in Erickson, Hammerberg & Sanchez, 1980). A major concern has been whether alterations in cell surface molecules, detectable as antigens, cause embryonic arrest with subsequent lethality or whether primary alterations in essential cell functions are the probable causes of embryonic death. Artzt & Jacob (1974) and Bennett (1975) have argued for the former view while Wudl, Sherman & Hillmann (1977) have argued for the latter view. For instance, in the case of t^6/t^6, the loss of inner cell masses (Erickson & Pederson, 1975) and the premature cessation of DNA synthesis in trophoblastic nuclei (Wudl & Sherman, 1978) are compatible with the occurrence of a metabolic lesion with an early effect on inner cell mass cells and a slower effect on trophoblast cells. The evidence, that male transmission ratio distortion determined by the *t* allele may be mediated by altered sperm intermediary metabolism, has been reviewed (Erickson, Lewis & Butley, 1981).

Ginsberg & Hillmann (1975) studied ATP and ADP levels in groups of embryos from experimental and control matings involving the t^{12} mutation. t^{12}/t^{12} embryos were expected to compose about 40 % of the embryos in the experimental groups because of the transmission ratio distortion for this *t* mutation in

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males. They found that the "t^{12}" litters had excessive rates of ATP synthesis with decreased ratios of ATP/ADP compared to embryos from control crosses. This was also found for the t^{w32} mutation, a mutation from the same complementation group with a similar time of lethality. Erickson, Betlach & Epstein (1974) did not find a statistically significant difference in ATP levels between such experimental and control matings from crosses involving t^{12}. Using newly developed methods for measuring ATP, ADP or AMP in single embryos, we have found normal values of these three adenylate ribonucleotides in single embryos from crosses yielding t^{12}/t^{12} embryos, both at the 2-cell and 4-cell stage and during *in vitro* culture. A decrease in adenylate ribonucleotide content was found in putative t^{12}/t^{12} embryos only when they were degenerating.

MATERIALS AND METHODS

Mice

T/t^{12} tailless mice were originally obtained from Dr. Salome Glücksohn-Waelsch who also provided the animals for Hillman's mouse colony (Hillmann, Hillmann & Wileman, 1970). The inbred background of these T/t^{12} mice is their own inbred background. They have been maintained for 100 generations of brother and sister matings and are not related to any standard inbred strain. Males were mated with NMRI females (randombred, wild type for T-complex genes, Inst. für Tierzucht, Hannover, West Germany) and the offspring were grouped for normal and short tails. It was found, that the male transmission frequency for the t^{12} allele was approximately 80 %. Normal-tailed ($+/t^{12}$) and short-tailed ($+/T$) mice were used to produce the experimental embryos. From $+/t^{12} \times +/t^{12}$ matings embryos of three genotypes are obtained ($+/+$, $+/t^{12}$ and t^{12}/t^{12}).

Determinations of the adenine ribonucleotide content were not performed on whole litters of such matings as in the earlier study (Ginsberg & Hillman, 1975) but on single embryos. Embryos from $+/T \times +/t^{12}$ matings served as controls, since they are producing viable embryos carrying t-alleles. In the earlier study, however, wild-type Swiss albino mouse embryos had been used as controls (Ginsberg & Hillmann, 1975). Males and females were caged overnight and the day of the observation of the vaginal plug is considered day 0 of pregnancy.

Collection and cultures of embryos

Since homozygous lethal t^{12} embryos are degenerating *in utero* at the morula stage, they may be lost. Ginsberg & Hillman (1975) have, therefore, performed their studies with embryos that were placed into culture at the 2-cell stage until they reached the desired cleavage stage. In a similar manner we have cultured 4-cell and 8-cell embryos from $+/T \times +/t^{12}$ and from $+/t^{12} \times +/t^{12}$ matings in plastic culture dishes (NUNC, Nunclon, Denmark) containing Whitten's medium (Whitten, 1971) in a humidified 5 % CO_2 in air atmosphere for 48 h to

the blastocyst stage. 2-cell embryos were flushed from the oviducts on day 1 of pregnancy and 4-cell and 8-cell embryos on day 2 at 10 a.m. using phosphate-buffered saline (Whittingham & Wales, 1969) as described previously (Schiffner & Spielmann, 1976).

Determination of ATP, ADP and AMP in Single Preimplantation Embryos

Immediately after flushing from the genital tract or removal from the incubator, the embryos were taken up singly in 10 µl of Tris-EDTA buffer, transferred to an Eppendorf micro test tube (volume 1·5 ml, Eppendorf GmbH, Germany), and kept frozen in liquid nitrogen. Our assay conditions for ATP, ADP and AMP in single preimplantation mouse embryos, using purified firefly luciferin luciferase, have been described in detail elsewhere (Spielmann, Jacob-Müller & Schulz, 1981). Only one of the three adenine ribonucleotides can be determined in a single embryo which is destroyed during the assay procedure. In these measurements, only ATP or ATP + ADP or ATP + ADP + AMP are determined per embryo and both ADP and AMP have to be calculated from these data by subtraction (ADP = (ATP + ADP) − ATP; AMP = (ATP + ADP + AMP) − (ATP + ADP)). ADP and AMP values are given without any standard deviation since they are low compared to ATP and since they are not obtained for single embryos but for groups of embryos by subtracting the average values.

RESULTS

In a preliminary experiment the *in vitro* development of 4-cell and 8-cell embryos was studied. As seen in Table 1, embryos from both experimental and control cultures show no differences in the number of degenerated embryos after 24 h in culture. However, after 48 h of culture, among the embryos from crosses

Table 1. In vitro *development from the 4-cell and 8-cell to the blastocyst stage of preimplantation mouse embryos carrying the early lethal mutation* t^{12}

	Development Stage		
Genotype	Morula	Blastocyst	Degenerated
	After 24 h in culture		
$+/T \times +/t^{12}$	142 (100 %)	0	0
$+/t^{12} \times +/t^{12}$	113 (100 %)	0	0
	After 48 h in culture		
$+/T \times +/t^{12}$	8 (6 %)	134 (94 %)	0
$+/t^{12} \times +/t^{12}$	21 (19 %)	67 (59 %)	25 (22 %)

Embryos were cultured in groups of five–ten in Whitten's Medium.

where about 40 % of the embryos would be expected to be t^{12} homozygotes, 19 % were delayed morulae and 22 % were degenerated, contrasting to no embryos in these classes in the control cross. We presume that the 22 % degenerated embryos were t^{12} homozygotes as were the delayed morulae. The combined 41 % abnormal embryos is very close to the expected number because of the transmission ratio distortion for t^{12} in males.

When 2-cell embryos were flushed from the plugged females and assayed as single embryos, no differences were found for ATP, ADP or AMP between the

Table 2. *Adenylate ribonucleotide content of preimplantation mouse embryos carrying the* t^{12}*-mutation during development* in vivo *and* in vitro *(pmol/embryo)*

2-cell stage, development *in vivo*					
Genotype	ATP	ATP + ADP	ADP	ATP + ADP + AMP	AMP
$+/T \times +/t^{12}$	0·54 ± 0·07 (34)	0·65 ± 0·09 (27)	0·11	0·78 ± 0·08 (23)	0·13
$+/t^{12} \times +/t^{12}$	0·51 ± 0·06 (51)	0·64 ± 0·07 (44)	0·13	0·75 ± 0·09 (41)	0·11

Table 3. *Adenylate ribonucleotide content of preimplantation mouse embryos carrying the* t^{12} *mutation during development* in vivo *and* in vitro *(pmol/embryo)*

4-cell and 8-cell stage, development *in vivo*					
Genotype	ATP	ATP + ADP	ADP	ATP + ADP + AMP	AMP
$+/T \times +/t^{12}$	0·53 ± 0·06 (25)	0·58 ± 0·05 (24)	0·05	0·66 ± 0·08 (27)	0·08
$+/t^{12} \times +/t^{12}$	0·52 ± 0·09 (32)	0·57 ± 0·07 (35)	0·05	0·67 ± 0·09 (34)	0·10

Table 4. *Adenylate ribonucleotide content of preimplantation mouse embryos carrying the* t^{12} *mutation during development* in vivo *and* in vitro *(pmol/embryo)*

Blastocysts and morulae, development *in vitro* from the 4-cell and 8-cell stage					
Genotype	ATP	ATP + ADP	ADP	ATP + ADP + AMP	AMP
$+/T \times +/t^{12}$ blastocysts	0·39 ± 0·04 (46)	0·51 ± 0·06 (23)	0·12	0·62 ± 0·05 (28)	0·09
$+/t^{12} \times +/t^{12}$ blastocysts	0·40 ± 0·06 (29)	0·49 ± 0·06 (25)	0·09	0·58 ± 0·06 (23)	0·09
morulae	0·42 ± 0·07 (21)	0·51 ± 0·05 (19)	0·09	0·59 ± 0·04 (17)	0·08
$+/t^{12} \times +/t^{12}$ degenerated	0·11 ± 0·07 (27)	0	0	0	0

single embryos from the control cross and the experimental cross (expected to produce about 40 % t^{12}/t^{12}; Table 2). As seen in Fig. 1, the values for the single embryos in both experimental and control crosses showed a unimodal distribution. Similar results were found with 4-cell embryos flushed from the females and assayed for adenylate ribonucleotide levels (Table 3). Again, there was no indication of any bimodality of values in the embryos from the $+/t^{12} \times +/t^{12}$ cross (Fig. 1). In order to compare the levels of adenylate ribonucleotides

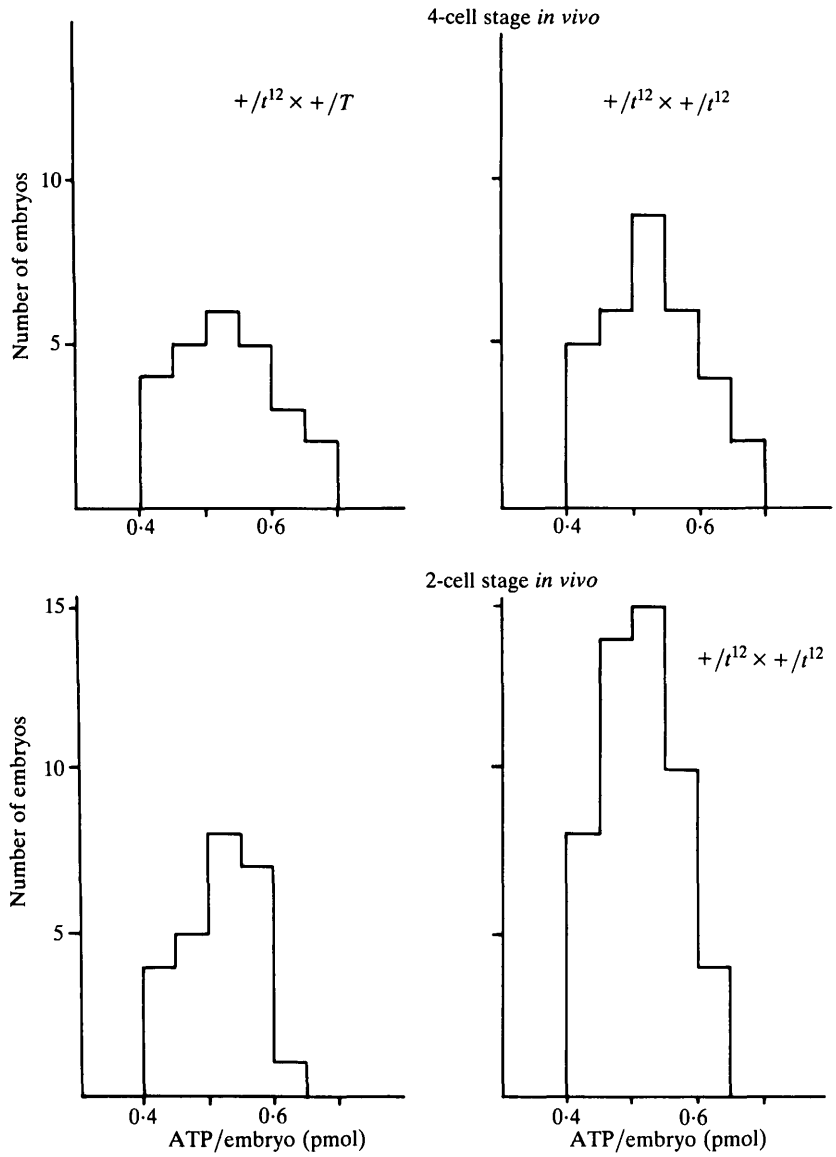

Fig. 1. Concentration of ATP in single embryos from experimental or control crosses at two stages of development by 0·05 pmol intervals.

in the normal blastocysts with levels in the blastocysts, delayed morulae and degenerated embryos in the cross of $+/t^{12} \times +/t^{12}$, the embryos flushed at the 4-cell stage were cultured *in vitro* for 48 h and the various types of embryos assayed as seen in Table 4. It is apparent that there was no difference in the blastocysts or morulae of the experimental or control crosses, although the degenerated embryos, presumptive t^{12}/t^{12}, showed greatly decreased ATP levels and no detectable ADP or AMP.

DISCUSSION

Morphological methods revealed that t^{12} homozygous embryos effectively stopped development as 30-cell morulae about 80 h after fertilization (Smith, 1956). Ultrastructural studies by Hillmann and co-workers showed that both t^{12}/t^{12}, and the other member of this complementation group (t^{w32}/t^{w32}), embryos could be distinguished from their littermates by the presence of nuclear lipid droplets and excessive cytoplasmic lipid as early as the 2-cell stage (Hillmann, *et al.* 1970; Hillmann & Hillmann, 1975). In studies using ^{32}phosphate to measure rates of synthesis and levels of ATP and ADP, Ginsberg & Hillmann found higher levels of total ATP and ADP with lower ATP/ADP ratios for litters of embryos including t^{12}/t^{12} and t^{w32}/t^{w32} than for control litters (1975). In their studies, all the embryos were flushed at 2-cell stages and cultured to later stages. They found that these increased levels of adenylate ribonucleotides continued until the 8-cell stage in t^{w32} litters and until the early morulae stage in t^{12} litters. At that time (the time corresponding to the lethality of the homozygous t^{12} and t^{w32} embryos), the levels of ATP in mutant embryos fell below control levels.

Our results differ from theirs in two major points. One, no difference was found between experimental and control animals and two, the levels of adenine ribonucleotides that they detected were considerably higher than those that we found at all stages. This is in particular demonstrated by their unusually high ADP values in experimental litters, which were five to ten times higher at the 2-cell and 4-cell stage than in wild-type embryos. The actual ADP data for the homozygous lethal t^{12} embryos are at least twice as high in their study since only 40 % of the embryos were carrying this genotype. The same calculation also holds for ATP values of homozygous t^{12} embryos in experimental litters at the 2-cell and 4-cell stage. Taking into account the methodological approach of the study, the actual concentrations in homozygous t^{12} embryos are e.g. at the 2-cell stage 3 pmoles ATP and 3 pmoles ADP compared to 1·5 pmoles ATP and 0·15 pmoles ADP per embryo in wild-type litters.

At the same time there are several arguments in favour of our results. We have been using an assay system that is more sensitive by a factor of 10 since we have been using purified enzymes, which are not contaminated with enzymes metabolizing ATP and ADP. Such chemicals could not be obtained for laboratory use before 1980. Our data for ATP and ADP are very similar to results

reported by other groups using the crude firefly system (Quinn & Wales, 1973) or other methods (Clegg & Piko, 1977). In addition we have used more appropriate control embryos and we have also performed a considerably higher amount of determinations for each data point compared to at least three measurements that were done in the study on whole litters. Finally the increased sensitivity of the assay system has allowed us to look for the distribution pattern of the ATP values (Fig. 1). There was no indication for a bimodality of the distribution of ATP that one would expect from the investigation on whole litters. The extremely high ATP and ADP values of the homozygous t^{12} embryos would not escape our highly sensitive methodology (Moyer & Henderson, 1983).

The only data on ATP synthesis in homozygous t^{12} embryos have again been obtained on litters containing about 40 % of such embryos (Ginsberg & Hillman, 1975). Appropriate conclusions on adenine ribonucleotide metabolism in single embryos in relation to the actual concentrations of the present study are, therefore, impossible and do require further investigations.

The question of cell autonomous lethality was raised by Boris Ephrussi as early as 1936 in regards to brachyury homozygotes (Ephrussi, 1936). He studied the ability of the T/T embryonic tissue to survive in tissue culture and found that the cells survived much longer in tissue culture than they would have survived *in vivo*. Salome Gluecksohn-Schoenheimer confirmed this (1944) when she explanted such embryos to the chick allantois and also saw a longer survival. Thus, it is clear that the *in utero* death of T/T homozygous embryos was not due to an immediate cell lethality but was due to an inability to survive in the female uterus. In this case, the death *in utero* can be easily explained by the lack of connections to the placenta, and hence, a lack of oxygenation and nutrition. Beatrice Mintz (1964) explored the possibility with regard to t^{12}-homozygous lethality by making chimaeras of embryos, some of which would be t^{12}/t^{12}, with control embryos. She was able to find blastocysts in which some of the cells showed characteristic t^{12}-homozygous abnormalities amongst normal-appearing cells, i.e., the t^{12} lethality seemed to be autonomous but the effect of t^{12}/t^{12} cells in these chimaeras beyond the blastocyst stage is unknown, as the embryos were not transferred back to pseudopregnant mothers.

It would seem fairly obvious that the lack of any 'housekeeping' function would be a cell autonomous lethal and that the alterations in ATP levels reported by Ginsberg & Hillmann (1975) appeared compatible with such a deficiency. With our inability to reproduce such a finding, the alternative possibility of blocked developmental patterns with subsequent death of the embryo might seem to be a possibility. However, claims that the t^{12} locus determined the F9 antigen have been largely refuted. The evidence for a determination of the F9 teratocarcinoma antigen by the t^{12} allele was of two sorts: 1) Absorption studies with sperm showed that it took twice as many sperm from a t^{12}-heterozygous animal ($t^{12}/+$ or t^{12}/T) to absorb anti-F9 reactivity compared with spermatozoa from a variety of other mice, including other *t*-alleles (Artzt & Jacob, 1974;

Marticorena, Artzt & Bennett, 1978). 2) The apparent segregation of the F9 antigen on embryos in crosses of $+/t^{12} \times +/t^{12}$ and $+/t^{w5}$ (Kemler, *et al.* 1976). Since there is a locus in the *T* region which has effects on the expression of H-Y antigen (Kralová & Lengerová, 1979), it seems reasonable that such a locus could have an effect on expression of other antigens, such as the F9 antigen. because of the crossover suppression of t^{12}, allelic variation in a locus controlling the amount of antigens will also be carried with a particular *t*-allele lethal effect. Thus, the data on absorption of antisera by t^{12} sperm, in comparison to other kinds of sperm, are not definitive. The argument that the expected number of embryos (about 1/3 because of transmission ratio distortion) in crosses of $+/t^{12} \times +/t^{12}$ did not show F9 antigen has been obviated because the authors who collaborated in this report have since changed their position and state that 'regardless of *t* genotype, all four-cell to morula (sic) stage embryos display at least some F9 antigen' (Marticorena *et al.* 1978). Erickson & Lewis (1980) have studied the expression of F9 antigen on a very large number of embryos from variety of crosses involving early developmental lethals. In this work, no evidence for segregation of t^{12} antigen in crosses of $+/t^{12} \times +/t^{12}$ was found. Thus, neither adenylate ribonucleotide charge nor embryonic antigens seem to be the answer to the question of t^{12} homozygous lethality.

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