Variation among inbred strains of mice in adenosine 3':5' cyclic monophosphate levels of spermatozoa

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SUMMARY

Spermatozoa from inbred strains of mice were found to vary significantly for levels of cyclic AMP when extractions were performed in a reproducible manner. The F_1 hybrid between high and low spermatozoal cAMP strains showed spermatozoal cAMP levels typical of the low strain. An analysis of spermatozoal cAMP in individual mice from the backcross of the F_1 to the high strain suggested that alleles at more than one locus determine strain differences in spermatozoal cAMP. The major histocompatibility locus of mice, *H-2*, which had been found to have an effect on liver cAMP levels did not seem to affect spermatozoal cAMP levels. *t*-Alleles, which appear to alter fertilization rates by effects on motility, had no apparent affects on spermatozoal cAMP.

1. INTRODUCTION

Adenosine 3':5' cyclic monophosphate (cAMP) is believed to play a role in the induction or maintenance of sperm motility since there is a high correlation between the measured levels of cAMP and sperm motility under a variety of conditions (Hoskins & Casillas, 1975). These cAMP augmenting conditions include the addition of cyclic nucleotide phosphodiesterase inhibitors (Garbers, First & Lardy, 1973), dilution (Cascieri, Amann & Hammerstedt, 1976), and sea-urchin-egg factors (Garbers & Hardman, 1976). Furthermore, most of the components of cAMP-modulated regulatory systems are found in spermatozoa (Gray *et al.* 1976): adenyl cyclase (Casillas & Hoskins, 1970), cyclic nucleotide phosphodiesterase (Christiansen & Desautel, 1973), cAMP-dependent protein kinase (Hoskins, Casillas & Stephens, 1972), and a phosphoprotein phosphatase which dephosphorylates proteins phosphorylated by this protein kinase (Tang & Hoskins, 1975). However, the natural stimulus for mammalian sperm adenyl cyclase (Garbers & Hardman, 1975) and the motility-coupled intermediate phosphorylated by the cAMP-dependent protein kinase (Tamblyn & First, 1977) have not been identified. As part of our studies on the genetic control of sperm function, we have studied

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variations in endogenous cAMP levels among inbred strains of mice. These strains include both standard laboratory stocks and lines carrying t-alleles which allow the spermatozoa bearing them to fertilize eggs more successfully than spermatozoa without t-alleles (Gluecksohn-Waelsch & Erickson, 1970). The strain variations found should provide genetic material for elucidating the role of cAMP in sperm function.

2. MATERIALS AND METHODS

(i) *Mice*

Standard inbred strains were obtained from the Jackson Laboratory, Bar Harbor, Maine. T/t^{12} and T/t^0 were obtained from Dr Salome Gluecksohn-Waelsch as balanced lethal lines; the former has been maintained by brother–sister matings for 46 generations (Gluecksohn-Waelsch & Erickson, 1970), the latter is F4 since an outcross to A/HeJ . T^{Or1}, maintained on a C57Bl/6 background, was obtained from Dr Jean-Louis Guenét – these three stocks are maintained in our laboratory. All mice were kept in a room with controlled lighting (14 h light, 10 h dark). Cedar shavings and pine shavings 1 : 2 were used as bedding. The female parent is written first in the symbolization of hybrids.

(ii) *Sperm preparation and extraction*

We developed a reproducible method of handling spermatozoa from individual mice such that initial aliquots of sperm suspension would be extracted 25 min after the animal was killed, since we were primarily interested in genetic variations in spermatozoal cAMP levels and not in *in vivo* levels of cAMP. To accomplish this, mature mice (10 weeks to 6 months of age but within a 2-week range for any 1 set of comparisons) were killed by cervical dislocation and the epididymides and vasa deferentia rapidly dissected out. The spermatozoa in the vasa were stripped into 0·5 ml of buffer (0·139 M-NaCl, 0·0374 M fructose, 0·0005 M-Na$_2$HPO$_4$, 0·0013 M-NaH$_2$PO$_4$, 0·007 M KCl, 0·0014 M-MgSO$_4$) while the epididymides (caput, corpus and cauda) were sliced to about 1 mm pieces in 0·5 ml. After 10 min, vasa spermatozoa were suspended with a Pasteur pipette, mixed with the sliced epididymides, and tissue fragments were removed from the mixture with a no. 16 screen. The 1 ml of sperm suspension was mixed and allowed to settle for 10 min. Then two 200 μl aliquots of the supernatant were pipetted into small glass centrifuge tubes immersed in a boiling water bath. This method of deproteinizing tissue has been found to be superior to perchloric or trichloracetic acid precipitation (Cooper, McPherson & Schofield, 1972) and in addition avoids difficulties involved in removing those organic acids before performing the cAMP assay. The sperm samples were boiled for 10 min and immediately removed to an ice bucket. After chilling, the tubes were centrifuged for 12 min at 1300 g at 4 °C; 100 μl of each supernatant was stored for the cAMP assay, 50 μl from each supernatant was pooled to be treated with phosphodiesterase and then assayed, and the pellet was saved for protein determinations. The samples were preserved at -70 °C for up to a week before use.

(iii) *cAMP assay*

cAMP was assayed by the competitive-binding protein assay of Gilman (1970) as modified by Brown *et al.* (1971) using a commercially available kit (Amersham/Searle).

(iv) *Demonstration of specificity of cAMP assay*

Phosphodiesterase treatment was used to demonstrate that cAMP was being measured. It was performed by diluting the sperm extract 1:10 with 0·5 M Tris, pH 7·5, adding 10 μg 3′:5′-cyclic nucleotide phosphodiesterase (0·21 units/mg, Sigma Chemical Co. no. PO134) per 90 μl sample, incubating 30 min at 30 °C, and extracting by boiling as with the sperm samples. These more dilute, phosphodiesterase-treated samples were then assayed by the more sensitive radioimmunoassay method of Steiner, Parker & Kipnis (1972) using a commercially available kit (New England Nuclear). The phosphodiesterase treatment reduced the concentration of cAMP to nearly undetectable levels: the reduction ranged from 89 to 94 %. Proteins were determined by the method of Lowry and results are expressed in pm cAMP/mg of spermatozoal protein.

(v) *Statistical analyses*

A one-way analysis of variance was performed with the F test of the equality of different means. Schaffe's multiple comparison procedure was used to contrast specific linear combinations of strain means.

3. RESULTS

(i) *Strain variation in cAMP*

Spermatozoal cAMP levels were found to differ markedly among several inbred strains of mice, Table 1. Three inbred strains were found to have low levels that were indistinguishable from each other while two other strains of mice had high levels (not statistically different from each other). The values for the two high strains were very significantly different from the low spermatozoal cAMP strains. The values, of course, do not necessarily reflect *in vivo* levels but measure concentrations of cAMP in a mixture of epididymal and vas deferen spermatozoa maintained at high concentration ($10-30 \times 10^6$/ml) for 20 min in a fructose-containing medium.

(ii) *Effects of* H-2 *on sperm cAMP*

We wished to determine whether or not allelic variation at the *H-2* locus, the major histocompatibility locus of mice, would affect spermatozoal cAMP levels since variation at this locus affects liver cAMP concentration (Meruelo & Edidin, 1975). The low spermatozoal cAMP strain, A/J, is *H-2*[a] while the high spermatozoal cAMP strain, C57Bl/10J, is *H-2*[b]. Congenic lines were studied for this purpose. These are inbred lines of mice in which the *H-2*[b] haplotype is introduced on to the A/J genetic background (A.BY/SNJ) and in which

the $H\text{-}2^a$ haplotype of A/J is introduced on to the genetic background of C57Bl/10J (B10.A/J). This reversal of $H\text{-}2$ alleles between the two inbred lines did not significantly alter the original spermatozoal cAMP levels of the strains (Table 2) although an insignificant shift of each strain's value towards that source of the $H\text{-}2$ allele is evident.

Table 1. *cAMP in sperm: strain variation*

Strain		pmol cAMP/mg protein	Probability of this difference by chance	
SEC/1ReJ	(8)	$4\cdot61 \pm 0\cdot75$*		
DBA/2J	(5)	$4\cdot03 \pm 1\cdot5$	$0\cdot488$	
A/J	(5)	$4\cdot70 \pm 1\cdot9$		$<0\cdot001$
CBA/J	(3)	$10\cdot44 \pm 1\cdot3$	$0\cdot310$	
C57Bl/10J	(5)	$13\cdot30 \pm 4\cdot2$		

* (Number of mice) mean ± standard deviation.

Table 2. *cAMP in sperm: H-2 independence*

Strain	Allele	pmol cAMP/mg protein	Probability of this difference by chance	
A/J	a	(5) $4\cdot70 \pm 1\cdot9$*	$0\cdot574$	
A.BY/SNJ	b	(5) $5\cdot65 \pm 0\cdot4$		$<0\cdot001$
C57Bl/10J	b	(5) $13\cdot30 \pm 4\cdot2$	$0\cdot169$	
B10.A/J	a	(5) $10\cdot91 \pm 2\cdot4$		

(Number of mice) mean ± standard deviation.

(iii) *Genetics of spermatozoal cAMP*

Crosses were made between inbred strains of mice with high and low levels of spermatozoal cAMP in order to study the genetics of this difference. As seen in Table 3, the F_1 mice from the cross between the A/J and C57Bl/6J strains showed levels of spermatozoal cAMP statistically indistinguishable from those of the A/J strain, i.e. low spermatozoal cAMP is dominant. (The numbers and values for the A/J strain differ in this table because a new set of strain-specific data was generated to go with the crosses since a long time interval occurred between these and the other experiments. Note that the two sets of A/J data are highly concordant.) The dominant effect was also noted in backcrosses where the mean value was still not distinguishably different from that of the A/J strain. If alleles at a single gene determined the difference in spermatozoal cAMP noted between the two strains, one half of the backcross males should have been like the C57Bl/6J parent. However, no bimodality in spermatozoal cAMP was found in individual backcross males (data not shown).

(iv) *Effects of* t-*alleles on spermatozoal cAMP*

Since *t*-alleles may alter sperm motility (Gluecksohn-Waelsch & Erickson, 1970), we also studied cAMP in the spermatozoa of males with various combinations of *t*-alleles. Since each *t*-allele is maintained in its own inbred line, outcrosses of the *t*-allele stocks to the inbred lines utilized in this study were also used

Table 3. *cAMP in sperm: F1 and F2 crosses*

Strain	pmol cAMP/mg Protein	Probability of this difference by chance
C57Bl/6J	(7) $12 \cdot 66 \pm 5 \cdot 1$*	$< 0 \cdot 001$
A/J	(7) $3 \cdot 64 \pm 0 \cdot 7$	$0 \cdot 434$
A/J × C57Bl/6J†	(3) $5 \cdot 07 \pm 1 \cdot 9$	$0 \cdot 573$
C57Bl/6J × (A/J × C57Bl/6J)	(15) $6 \cdot 01 \pm 1 \cdot 2$	

* (Number of mice) mean ± standard deviation.
† Female indicated first in crosses.

Table 4. *cAMP in sperm:* t-*alleles*

Strain	pm cAMP/mg protein
T/t^{12}	(10) $11 \cdot 44 \pm 1 \cdot 67$*
Outcrossed to A/J:	
$+/t^{12}$	(6) $8 \cdot 49 \pm 1 \cdot 56$
$T/+$	(5) $8 \cdot 21 \pm 1 \cdot 35$
Outcrossed to C57Bl/10J:	
$+/t^{12}$	(4) $15 \cdot 44 \pm 6 \cdot 47$
$T/+$	(7) $11 \cdot 89 \pm 4 \cdot 36$
T/t^{0}	(8) $9 \cdot 07 \pm 2 \cdot 47$
Outcrossed to A/J:	
$+/t^{0}$	(2) $4 \cdot 8 \pm 0 \cdot 68$
Outcrossed to C57Bl/10J:	
$+/t^{0}$	(7) $6 \cdot 64 \pm 0 \cdot 99$
$T/+$	(2) $7 \cdot 03 \pm 0 \cdot 84$
$T^{Or1}/+$	(4) $7 \cdot 59 \pm 2 \cdot 43$
$+/+$ (T^{Or1} background)	(3) $5 \cdot 07 \pm 0 \cdot 41$
Outcrossed to T/t^{0}:	
T^{Or1}/t^{0}	(3) $9 \cdot 02 \pm 2 \cdot 68$

* (Number of mice) mean ± standard deviation.

(Table 4). For instance, the T/t^{12} inbred line had mean spermatozoal cAMP levels of 11·4 pmol/mg protein; when outcrossed to the A/J strain, the dominant effect of A/J seen in its crosses with C57Bl/6J was not seen. Instead A/J × T/t^{12} males showed intermediate values and no difference between $+/t^{12}$ (males which would show segregation distortion) and $T/+$ males was found. Similarly, $T/+$ and $+/t^{12}$ males from the outcross to C57Bl/10J did not differ from each other and small numbers of mice from crosses of the T/t^{0} line suggested a similar pattern of events. T^{Or1}, a deletion in the *t*-region with mild segregation distortion (Erickson, Lewis & Slusser, 1978), also did not have apparent effects on sperm cAMP.

4. DISCUSSION

Our results show that inbred strains of mice vary in their levels of spermatozoal cAMP (as measured after 20 min of *in vitro* incubation in the presence of fructose) Differences between inbred strains of mice maintained under constant laboratory conditions are usually due to genetic differences between the strains. The low spermatozoal cAMP found in F_1 males from the cross of strains with high and low levels of spermatozoal cAMP is best explained by a dominant effect of the gene(s) involved in determining the measured differences. The continuous variation and low cAMP values found in males from the backcross of F_1 mice to a strain with high spermatozoal cAMP levels suggests that several genes may be involved in this strain difference in spermatozoal cAMP.

There have been several reports of differences between inbred strains of mice for cAMP levels in various tissues: liver (Meruelo & Edidin, 1975), whole brain (Orenberg *et al*. 1975) and cerebral cortex (Sattin, 1975). These studies are difficult to compare because different methods of measuring cAMP and expressing the results are used. Nonetheless, it is interesting to compare the relative ordering of strains, from high to low levels, found in the various studies. The relative strain activities are different in different tissues. Thus, the A/J strain shows high liver cAMP levels while A/J sperm and brain cAMP levels are about the lowest found. It is clear that variation in *H-2* alleles can only play a small part in strain variation in spermatozoal cAMP. The effect of *H-2* has not yet been studied in brain or cerebral cortex.

Although these strains vary in epididymal and vas deferens concentrations of prostaglandins E and F (Badr, 1975) and although most prostaglandins function as activators of adenyl cyclase, it is unlikely that variations in levels of prostaglandins are related to strain variations in spermatozoal cAMP. First, prostaglandins have not been found to have an effect on spermatozoal cAMP *in vitro* (Hoskins & Casillas, 1975). Secondly, the A/J strain was shown to have high levels of testicular and epididymal prostaglandins (compared to C57Bl/6J, for instance) and, thus, would be expected to have high, rather than low, levels of spermatozoal cAMP. On the other hand, strain variation in levels of cAMP in brain could be related to those in spermatozoa. Cyclic AMP levels in brain (Orenberg *et al*. 1975) have been measured in three strains of mice in which we have determined spermatozoal cAMP levels and the rank orders are the same. Many antigens are shared by brain and sperm and the spermatozoal membrane displays several pharmacological properties characteristic of the neuromuscular post-synaptic junction (reviewed in Erickson, 1977). cAMP has been implicated as a mediator for several neurotransmitters of the central nervous system (Nathenson, 1977) but not acetylcholine, which is the transmitter at the neuromuscular junction. Since the *in vivo* stimulus for spermatozoal adenylate cyclase has not yet been found, it is possible that the physiological stimulus is one of these neurotransmitters. Thus, a parallel between sperm and brain cAMP levels among inbred strains of mice may not be entirely fortuitous.

A number of variations in parameters related to fertilization have been described for inbred strains of mice. No clear correlation to the strain variation in spermatozoal cAMP is apparent. For instance, Braden (1957) found 1·1, 1·0 and 0·7 % polyspermy in C57Bl/Fa, A/Fa and CBA/Ca strains, respectively. The rank order of these strains is the same for the number of supplementary sperm in the perivitelline space (Braden, 1958) and these rank orders do not reflect that found for spermatozoal cAMP levels. One study of strain variation in the success of *in vitro* fertilization allows comparison to our work. Kaleta (1977) found a 95 % success of *in vitro* fertilization with the CBA/Kw strain and only 8·6 % success with sperm and ova of the C57Bl/Kw strain. Thus, variation in levels of sperm cAMP are not necessarily related to the success of *in vitro* fertilization. High-speed cinemicrographic analyses of sperm flagellar beat-frequency did not disclose strain differences comparable to the differences in cAMP, but the statistical variation of this variable (between mice of one strain) was very large (Katz, Erickson & Nathanson, unpublished observations).

It is interesting to note that motility studies of spermatozoa from T/t^n segregation-distorting males were interpreted as showing two populations of sperm (Yanagisawa, 1965). We have not found related changes in cAMP levels when sons of T/t^0 males with and without segregation-distortion elements were compared. Of course, the two kinds of sperm that are hypothesized (assuming postmeiotic gene expression) would only make up half of the population of sperm and this dilution would obscure differences.

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