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Effects of Supplemental Methionine on Antiserum-Induced Dysmorphology in Rat Embryos Cultured In Vitro

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ABSTRACT

Background: Heterologous antiserum to the visceral yolk sac (AVYS) is teratogenic, inducing a spectrum of malformations in vivo and producing similar effects in vitro. Numerous studies support the concept that AVYS-induced malformations result from embryonic nutritional deficiency, without affecting the maternal nutritional status. This has provided a useful model with which to investigate the nutritional requirements of the early embryo, as well as the role of various nutrients in the etiology of congenital defects.

Methods: In the current investigation, we examined the effects of methionine and other nutrients on AVYS-induced embryotoxicity in vitro. For these experiments, we cultured rat embryos (9.5 p.c) for 48 hr with AVYS and/or methionine at several concentration levels.

Results: The addition of L-methionine to AVYS-exposed cultures reduced dysmorphology and open neural tube; this effect was concentration dependent. AVYS-induced dysmorphology was completely prevented at a concentration of L-methionine corresponding to 50-fold the basal serum concentration. Utilization of D-methionine, L-leucine, or folic acid (5-methyltetrahydrofolate, MTHF) instead of L-methionine had no protective effects.

Conclusions: These results suggest that, although AVYS limits the supply of all amino acids to the embryo, embryopathy largely results from a deficiency of methionine. Furthermore, although endocytosis and degradation of proteins by the VYS supplies most amino acids to the embryo, free amino acids may be compensatory when this source is reduced. These results support those of previous investigations that suggest methionine is required for normal NT closure and that methionine is a limiting nutrient for embryonic development.

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INTRODUCTION

Although the etiology of many human birth defects remains unknown (Brent, '86; Beckman et al., '97a), it has become increasingly evident that a significant proportion may result from effects relating to maternal and/or embryonic nutritional status. It is now well known that periconceptional supplementation with folate, or vitamin supplements containing folate, reduces both the overall incidence and the recurrence of neural tube defects (NTDs) (MRC, '91; Czeizel and Dudas, '92). Unfortunately not all NTDs are prevented by folate supplementation, and the mechanism by which folate prevents NTDs has not been determined. While reduced levels of folate have been reported in some studies for women with at least one prior NTD birth (Bunduki et al., '95), this is not a universal observation (Scott et al., '90; Economides et al., '92), and attention has turned to the hypothesis that folate supplementation prevents NTDs by correcting subtle alterations in folate metabolism, rather than overt deficiency (Kirke et al., '96). This hypothesis is supported by observations that alterations in key components of folate metabolism, such as homocysteine (Steegers-Theunissen et al., '91, '94) and vitamin B_{12} (Kirke et al., '96), are also associated with an increased risk of NTD.

There is growing evidence that supplementation with methionine may also be able to prevent some NTDs, and that a methionine deficiency may increase risk of NTD. In vitro culture of rat embryos using serum-deficient methionine results in abnormalities including open NT (Flynne et al., '87; Coelho et al., '89), while supplementation of the culture serum with free methionine promoted normal development and NT closure (Flynne et al., '87; Coelho et al., '89). It has further been shown that methionine supplementation of whole rat embryo cultures reduces the dysmorphology that results from culture of embryos with anti-laminin antibodies (Chambers et al., '95b), valproic acid (Nosel and Klein, '92), and nitrous oxide (Fujinaga and Baden, '94). Methionine supplementation in vivo reduced the spontaneous incidence of spina bifida in *Axd* mice by 41–47% (Essien, '92; Essien and Wannberg, '93) and reduced the incidence of NTDs resulting from administration of valproic acid to pregnant dams (Ehlers et al., '96).

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The majority of amino acids used during organogenesis by rat embryos are derived from digestion of maternal proteins within the visceral yolk sac (VYS) (Beckman et al., '90, '97b; Lloyd et al., '96). Greater than 90% of methionine, leucine, and serine used for protein synthesis by the embryo have been shown to be derived from this source, rather than from free amino acids (Rowe and Kalaizis, '85; Lloyd et al., '96; Beckman et al., '97b). This is perhaps not surprising, as free amino acids account for only 1% of the total amino acids present in rat serum (Koszalka et al., '94). Despite the requirement for methionine for both protein synthesis and for synthesis of the methyl group donor S-adenosylmethionine (SAM), methionine constitutes only 1% of all serum amino acids, both free and incorporated into protein (Milakofsky et al., '84; Koszalka et al., '94). Thus, it is of great interest that rat embryos grown in cultures deficient in free methionine develop abnormally, and supplementation of these cultures with free methionine prevents the dysmorphology (Flynne et al., '87; Coelho et al., '89). This implies that free methionine, although apparently present in insignificant amounts in the serum as compared with methionine derived from maternal proteins, may assume greater importance under circumstances in which the primary methionine supply is limited, both in vitro and in vivo.

Investigations from our laboratory have long focused on the mechanisms by which the early embryo obtains nutrients and on the deleterious effects of embryonic malnutrition (Brent and Fawcett, '98). Much of our current understanding of early embryonic nutrition is derived from studies that use various teratogens to inhibit the transfer of nutrients to the embryo. In particular, the discovery of teratogenic antibodies raised to certain rat tissues, especially antiserum to the VYS (Brent et al., '71), has provided a model that has greatly facilitated both the study of the nutritional requirements of the organogenesis stage rat embryo and the mechanisms by which the embryo derives these nutrients (for review, see Brent and Fawcett, '98). Anti-VYS serum (AVYS) inhibits VYS endocytosis (Freeman et al., '82; Lerman et al., '86). Because most amino acids required by the rat embryo during organogenesis are derived from endocytosed protein, exposure to AVYS significantly decreases the availability of amino acids to the embryo, inducing a nutritional deficiency in the embryo without altering the nutritional status of the mother. Exposure of yolk sacs to AVYS during organogenesis results in aberrant in vitro development and in congenital malformations in vivo, including a high incidence of exencephaly. Direct exposure of the embryo the AVYS does not induce embryopathy (New and Brent, '72). Although AVYS inhibits the uptake of macromolecules such as protein, evidence to date suggests that the transport processes of small molecules, such as free amino acids, are not significantly affected by AVYS (Lerman et al., '86; Beckman et al., '90).

For the present investigation, we have addressed the possibility that AVYS-mediated teratogenesis may result in part from a methionine deficiency. This hypothesis was based on the current understanding of amino acid supply to the embryo, the relatively low concentration of methionine in serum as compared with other amino acids, and the previous reports that methionine deficiency resulted in NTDs in vitro. Because most amino acids are supplied to the embryo by endocytosis and degradation of protein in the VYS endoderm, inhibition of VYS endocytosis would theoretically decrease availability of all amino acids. However, because of its low concentration and potential developmental importance, methionine may become limiting before other more abundant amino acids in AVYS-treated embryos. For these experiments we first measured the effects of various doses of AVYS on embryonic development in vitro and compared these results with development in vivo after a teratogenic in vivo dose of AVYS was administered. We then supplemented 9.5-day rat embryo culture with various concentrations of methionine to determine if methionine could ameliorate the embryopathic effects of AVYS in vitro. Finally we conducted experiments to determine if supplemental folic acid reduced AVYS-induced embryopathy.

MATERIALS AND METHODS

Animals and timed pregnancies

Wistar rats weighing 200–250 g (Charles River Laboratories, Wilmington, DE) were used for all experiments. The rats were mated overnight, and the presence of sperm in the vaginal lavage the next morning was used to indicate 0.5 days postconception (p.c.). Food and water were available ad libitum.

Whole embryo culture

Mid-head-fold stage embryos from pregnant rats were obtained at 9.5 days p.c. Embryos were cultured according to the method of New ('78). Pregnant females were euthanized and gravid uteri were removed. Embryos were isolated for culture by dissecting uterine, decidual, and Reichart's membrane tissues away from the embryo in phosphate buffered saline containing 0.1% glucose at 37°C. Embryos with attached yolk sacs and ectoplacental cones were cultured at 37°C under constant rotation (35–40 rpm) in whole rat serum that was prepared by immediate centrifugation, heat-inactivated at 56°C for 30 min, and stored at −70°C before use. Culture serum was supplemented with 50 μg/ml streptomycin and 50 U/ml penicillin (Sigma Chemical Co., St. Louis, MO). At 3 hr after culture initiation, embryos were evaluated for normal VYS expansion and absence of damage resulting from the isolation procedure. Defective embryos were excluded from further analysis. Up to four embryos were cultured together, using 1 ml of rat serum per embryo. Cultures were gassed with $5\%O_2{:}5\%CO_2{:}90\%N_2$ on the first day of culture, with $20\%O_2{:}5\%CO_2{:}75\%N_2$ on the second day of culture, and with $40\%O_2{:}5\%CO_2{:}55\%N_2$ for the final

6 hr. Embryos were harvested from culture 48 hr after initiation of the treatments and evaluated.

Evaluation of embryos

At the termination of culture, embryos were transferred to sterile phosphate-buffered saline (PBS) containing 1% glucose at 37°C, and immediately examined using a stereomicroscope. The presence of a heartbeat was used to indicate viability, and only viable embryos were evaluated. The maximum diameter of the visceral yolk sac was measured using an ocular micrometer, and the relative degree of axial rotation was assessed. Embryos were then dissected free of the VYS, ectoplacental cone, and amnion; growth parameters and morphology were assessed. Crown-rump length, defined as the maximum diameter of the embryo in its natural position (Brown and Fabro, '81), was measured using an ocular micrometer before removal of the amnion. After evaluation, embryos and visceral yolk sacs were frozen at −70°C. Total protein was determined by dissolving yolk sacs and embryos in 0.5 M NaOH for 1 hr at 37°C. The protein content was determined using the method of Lowry et al. ('51).

Teratogenic antiserum and culture supplements

Cultures were supplemented with L- or D-methionine (Sigma) dissolved in sterile deionized water in a volume no greater than 50 ml to achieve a final concentration of 100 or 500 mg methionine/ml culture medium, which corresponded to 10-fold or 50-fold basal serum concentrations respectively. Alternatively, culture medium was supplemented with L-leucine to achieve a final concentration of 328 or 656 μg/ml (corresponding to 10-fold or 20-fold basal concentrations respectively), or with 5MTHF (Sigma) to achieve a final concentration of 25 μg/ml or 500-fold the basal serum concentration. Control cultures received an equal volume of sterile deionized water. After the initial 3 hr of culture, one-half of the cultures were treated with AVYS. The AVYS used for this study was a sheep anti-whole visceral yolk sac that has been stored lyophilized at −20°C since its initial preparation (Brent et al., '71), and has been used in previous investigations (Brent et al., '71; New and Brent, '72; Fawcett et al., '95; Beckman et al., '97b). Antiserum was reconstituted with sterile deionized water and was added to embryo cultures in a volume no greater than 15 ml, to achieve a final concentration of either 50, 75, or 100 mg antiserum protein per ml culture serum. An equal volume of sterile deionized water or normal (pre-immune) sheep serum (NSS) was added to control cultures.

Embryopathy of AVYS in vivo

Pregnant rats (9.5 p.c.) were administered a single intraperitoneal injection of AVYS (70 mg protein/ml) at a dose of 100 mg/kg body weight (LD_{50}). On day 11.5 days p.c., 48 hr postinjection, dams were euthanized, using CO_2. Gravid uteri were removed, the total sites counted, and then 5 sites were randomly chosen and removed for analysis. Embryos were dissected free of the uterine wall and decidua; the diameter of the VYS and vitelline circulation was assessed. Embryos were then removed from the VYS and assessed for morphology and size, using the same criteria used for the evaluation of cultured embryos.

Statistical analysis

All statistical analysis was performed with the aid of Sigma Stat statistical software (SPSS). Embryo and VYS size and protein measurements were compared using one-way analysis of variance (ANOVA). Pairwise comparisons were achieved using Student-Newman-Keuls method. Developmental parameters and incidence of malformations were analyzed using the chi-square test with the Yates correction, or the Fisher's exact test where appropriate. The level of significance were set at $P \leq 0.05$ for all tests.

RESULTS

Comparison of AVYS in vitro dose response with in vivo teratogenic effects

In the first series of experiments, we ascertained the effect of several doses of AVYS on embryonic development in vitro using 48-hr embryo culture. We then compared the outcome of embryos with embryos exposed for 48 hr in vivo by injecting pregnant dams with a teratogenic dose of AVYS on 9.5 days p.c. The results from these experiments are presented for comparison in Table 1.

When pregnant dams were administered a dose of AVYS on day 9.5 p.c. and embryos were examined on day 11.5 p.c., most embryos were found to be dysmorphic. In particular, these embryos had open NTs and absent or abnormal development of optic structures. Although approximately one-fourth of embryos appeared morphologically normal, all were severely growth retarded as evidenced by the protein content of embryos and VYSs and the number of somites, both of which were significantly reduced. Administration of an equal dose of preimmune sheep serum (NSS), rather than AVYS, did not significantly affect embryonic development as previously reported (Brent et al., '71; New and Brent, '72); however, two embryos from one litter had at least one dysmorphic feature involving the NT and/or eye which resulted in an incidence of 8% dysmorphology overall.

Embryos cultured for 48 hr in vitro in whole rat serum were smaller than same stage embryos but normal in morphology and consistent with previously published data with regard to size and protein content. (Table 1). Embryos had an average of 26 somite pairs, three brachial arches, full dorsal flexion, and the vast majority had completed NT closure. Embryos and VYSs averaged approximately 170–180 μg and 100 μg of protein respectively. The addition of NSS to cultures did not adversely affect development; these data have been included with the data for embryos cultured without additional supplements. The addition of AVYS to

TABLE 1. Comparison of rat embryos from dams treated with a teratogenic dose of AVYS (LD_{50}) in vivo and rat embryos grown with several concentrations of AVYS in vitro 48 hr postexposure (11.5 days p.c)[†]

	In vivo		In vitro			
AVYS	0 mg/kg	100 mg/kg	0 μg/ml	50 μg/ml	75 μg/ml	100 μg/ml
No. of embryos	25	43	62	39	20	33
Crown-rump (mm)	4.2 ± 0.3	2.4 ± 0.3*	3.2 ± 0.4	3.1 ± 0.4	3.0 ± 0.3	1.8 ± 0.4*
VYS (mm)	4.8 ± 0.3	3.1 ± 0.3*	3.7 ± 0.5	3.6 ± 0.6	3.7 ± 0.3	2.8 ± 0.4*
Embryo protein (μg)	368.5 ± 38.2	92.9 ± 15.8*	176.7 ± 49.0	172.9 ± 48.1	172.2 ± 29.4	50.6 ± 23.8*
VYS protein (μg)	151.4 ± 14.5	62.6 ± 11.1*	100.7 ± 27.4	105.2 ± 27.0	106.3 ± 20.6	48.9 ± 17.7*
No. of somites	30.0 ± 1.9	24.2 ± 6.8*	26.5 ± 4.1	25.5 ± 4.9	24.3 ± 6.1	5.8 ± 8*
No. of arches	3.1 ± 0.3	2.7 ± 0.5	3.0 ± 0.4	2.8 ± 0.6	3.0 ± 0.5	1.3 ± 1.4*
Rotation (%)	100	97.7	95.2	71.8*	45.0*	3*
VYS circulation (%)	100	100	98.4	92.3	100	52*
Forelimb bud (%)	100	95.3	100	100	100	36*
Hindlimb bud (%)	100	88.4	56	25.6*	10*	0*
Otic (% normal)	100	97.7	100	97.4	95	58*
Optic (% normal)	92	25.6*	93.5	33.3*	5*	0*
Neural tube (% closed)	96	30.2*	93.5	41.0*	5*	0*
Dysmorphic (%)	8	74.4*	6.4	66.7*	95*	100*

*Significantly different from control values $P < 0.05$.
[†]Values are mean ± SD, or percentage of embryos where indicated.

rat embryo cultures significantly inhibited embryonic growth and development and significantly increased morphologic abnormalities, especially with regard to optic structures and NT development. Consistent with the in vivo effects of AVYS, the effects observed with AVYS in vitro were dose dependent. A concentration of 50 μg AVYS/ml culture medium significantly inhibited axial rotation, somite number, and hindlimb bud development of the embryos. In addition, only 33% of embryos had normal eye development, and only 41% had completed NT closure. These abnormalities usually manifested in the same embryos. Open NTs in this group were restricted to the anterior regions of the neural groove. Surprisingly, exposure of embryos to AVYS did not measurably affect the protein content of either the VYS or embryo at this dose. Increasing the concentration of AVYS to 75 μg/ml resulted in a decreased proportion of embryos completing axial flexion and an increased proportion of embryos with abnormal or absent eye development and open NTs (Table 1). Most embryos treated with 75 μg/ml AVYS had open NTs (95%). The severity of the open NT was also increased; 45% of embryos treated with 75 μg/ml AVYS had an open NT encompassing the entire neural groove. Finally, addition of AVYS to cultures at 100 μg/ml resulted in the most severe effects on cultured embryos. Protein accretion was only one-third of control embryos, and embryos had significantly reduced somite number, reduced or absent limb bud development and lacked axial flexion. Additionally, the vast majority of these embryos had open NTs encompassing the entire neural groove. Because the embryos failed to achieve appropriate rotation, the cranial and caudal aspects of the neural tubes that were in opposition became fused together in a large proportion of these embryos. A significant number of embryos treated with 100 μg/ml AVYS were also absent or had dysmorphic otic vesicles and the majority (52%) of yolk sacs lacked a vitelline circulation. Despite these severe effects on growth and development, most embryos (>98%) treated with this dose of AVYS were viable at examination. It should be noted that although the embryos were significantly retarded with regard to growth and development, the failure of the embryos to initiate NT closure cannot be explained entirely by developmental delay, because the morphology of the NT at all treatment doses of AVYS was frankly abnormal. Open neural tubes were consistently flared ventrally. Furthermore, in the more severely affected embryos, the anterior and posterior NT were fused, a malformation which has been previously described as dorsifusion by other investigators (Chambers et al., '95a).

Effect of supplemental free L-methionine on AVYS-induced dysmorphology

Results of morphologic assessment of embryos cultured for 48 hr in the presence of AVYS serum at either 50 or 100 μg/ml were entirely consistent with those described for the dose response analysis presented in Table 1; these results are presented in Table 2. Addition of 100 μg/ml L-methionine (100 μg or 500 μg/ml) alone had no effect on the growth or development of embryos. The addition of D-methionine alone at 100 μg/ml significantly ($P < 0.05$) affected embryonic development with regard to optic morphology as compared with embryos cultured without supplements, or embryos cultured with L-methionine or NSS. Eye vesicles were present but abnormal in 35% of these embryos. Some embryos cultured with 100 μg/ml D-methionine also had an open anterior NT; however, this value was not significantly different from control values, perhaps because of the small sample size.

The addition of free L-methionine to cultures significantly improved the outcome of embryos treated with AVYS, particularly at a dose of 50 μg/ml AVYS. When cultures were supplemented with free L-methionine at 10 times the reported basal serum concentration, (100 μg/ml; Milakofsky et al., '84), the percentage of em-

TABLE 2. Effect of supplemental L-Methionine on AVYS-induced dysmorphology in rat embryos cultured in vitro from 9.5 to 11.5 days[†]

	0 μg AVYS/ml				50 μg AVYS/ml			100 μg AVYS/ml			
Dose of AVYS supplement	None	D-Methionine 100 μg/ml	L-Methionine 100 μg/ml	L-Methionine 500 μg/ml	None	L-Methionine 100 μg/ml	L-Methionine 500 μg/ml	None	D-Methionine 100 μg/ml	L-Methionine 100 μg/ml	L-Methionine 500 μg/ml
No. of embryos	88	17	26	22	55	60	32	48	22	44	32
Crown-rump (mm)	3.2 ± 0.4	3.1 ± 0.3	3.2 ± 0.4	3.3 ± 0.3	3.2 ± 0.4	3.1 ± 0.5	3.3 ± 0.3	2.0 ± 0.6*	1.9 ± 0.5*	2.2 ± 0.4**	2.4 ± 0.4**
VYS (mm)	3.9 ± 0.6	3.7 ± 0.4	3.7 ± 0.3	4.5 ± 0.5	3.7 ± 0.6	3.6 ± 0.6	4.0 ± 0.2	3.0 ± 0.4*	2.8 ± 0.3*	2.9 ± 0.4*	3.1 ± 0.3*
Embryo protein (μg)	187.1 ± 47.5	151.6 ± 42.1	180.0 ± 34.5	186.9 ± 33.1	180.0 ± 55.3	175.2 ± 53.2	176.8 ± 25.4	63.7 ± 36.3*	55.3 ± 17.8*	66.4 ± 11.9*	70.5 ± 26.9*
VYS protein (μg)	110.7 ± 30.1	83.3 ± 14.3	91.5 ± 14.8	133.8 ± 25.2	104.9 ± 27.9	105.9 ± 23.4	117.8 ± 11.1	58.4 ± 22.6*	58.1 ± 9.8*	54.2 ± 10.1*	64.7 ± 13.5*
No. of somites	27.2 ± 3.9	26.5 ± 5.0	24.1 ± 7.7	29.0 ± 3.4	24.0 ± 6.1*	21.4 ± 5.3*	29.6 ± 2.5	7.4 ± 7.8*	8.4 ± 6.4*	12.8 ± 10.0**	13.3 ± 9.1**
No. of arches	3.0 ± 0.4	2.9 ± 0.3	2.9 ± 0.5	3.0 ± 0.6	2.8 ± 0.6*	2.8 ± 0.5*	3.3 ± 0.5	1.6 ± 1.4*	1.7 ± 1.2*	2.5 ± 1.1**	2.1 ± 1.2**
Rotation (%)	96.6	82.4	96.1	90.1	70.9*	80*	100	2.1*	0*	2.3*	12.5
VYS circulation (%)	98.9	94.1	100	95.4	92.7	100	100	66.7*	54.5*	87.5*	93.8
Forelimb bud (%)	100	94.1	96.1	100	100	100	100	50.0*	50.0*	77.3**	81.2**
Hindlimb bud (%)	58.0	52.9	46.1	68.2	21.8*	48.3	53.1	0*	0*	0*	6.2*
Otic (% normal)	100	94.1	96.1	100	96.4	98.3	100	56.2*	59.1*	97.7	87.5
Optic (% normal)	98.9	64.7*	88	90.9	36.4*	56.7*	96.9	0*	0*	2.3*	3.1*
Neural tube (% closed)	96.6	82.4	100	100	45.4*	75.0**	100	0*	0*	0*	6.2*
Dysmorphic (%)	2.4	35.3*	11.5	9.1	65.4*	46.7*	3.1	100*	100*	100*	96.9*

*Significantly different from control values $P < 0.05$.
**Significantly different from control values and AVYS alone $P < 0.05$.
[†]Values are mean ± SD or percentage of embryos where indicated.

bryos treated with 50 μg/ml AVYS completing normal NT closure increased significantly from 45% to 75% (Table 2). This dose also restored hindlimb bud development to control values, and modestly increased the percentage of embryos with normal optic development and axial rotation. However, a significant number of embryos in this group were still dysmorphic (47%; Table 2).

Supplementation of cultures with L-methionine at 50 times the basal serum concentration significantly improved embryonic development such that embryos treated with 50 μg/ml AVYS in the presence of 50-fold L-methionine were indistinguishable from control embryos. Specifically, all (100%) of these embryos completed NT closure and appropriate axial rotation. In addition, only one embryo from this group had an optic abnormality, an incidence that does not differ from control values.

Results obtained with L-methionine supplementation of embryo cultures at higher doses of AVYS were not as dramatic as those obtained with 50 μg/ml AVYS. Supplementation of embryo cultures treated with 100 μg/ml AVYS with L-methionine at 10-fold the normal rat serum concentration (770 μM) led to significant improvement for some developmental parameters as compared with embryos treated with AVYS alone. In particular, supplementation with L-methionine significantly improved the number of somite pairs as well as crown-rump length. Embryos cultured with AVYS and 10-fold L-methionine also had significantly fewer otic malformations compared with embryos cultured with AVYS alone. A significant improvement was also noted in the development of the vitelline circulation. Supplementation of cultures with 50 times the basal concentration of free L-methionine in the rat serum, followed by treatment of the cultures with 100 μg/ml AVYS, achieved essentially the same results obtained with the 10-fold concentration of L-methionine. The percentage of embryos achieving partial closure of the NT was significantly increased in AVYS-treated cultures supplemented with L-methionine as compared with cultures treated with 100 μg/ml AVYS alone. This resulted in a concurrent decrease in the percentage of embryos treated with antiserum that had open NTs encompassing the entire neural groove.

Despite these differences, all embryos exposed to the higher dose of AVYS (100 μg/ml) and supplemented with L-methionine were still abnormal, and protein accretion in either the VYS or embryo was not affected. Addition of D-methionine instead of L-methionine in these experiments yielded results virtually identical to those obtained with AVYS alone, indicating that the effects of L-methionine are stereo specific. Furthermore, although D-methionine did not increase the toxicity of AVYS in culture, in experiments in which D-methionine was added to cultures alone the number of embryos with abnormal optic development was significantly increased, indicating that D-methionine itself may have adverse effects on embryonic development.

Effects of 5MTHF and L-leucine on AVYS-induced dysmorphology in vitro

Embryos cultured for 48 hr in whole rat serum with supplemental folic acid (5MTHF) or L-leucine grew normally and did not differ from embryos grown without supplements. The addition of 50 μg/ml AVYS to these cultures resulted in similar morphology as that reported for the previous experiments described above with the exception that a significant decrease in total protein accretion by both the embryo and VYS were noted. The addition of either 5MTHF or L-leucine to AVYS-treated cultures for 48 hr did not affect embryonic growth or morphology, compared to embryos grown with AVYS alone. Culture of embryos with 50 μg/ml AVYS and either leucine or 5MTHF produced dysmorphology in more than two-thirds of embryos and did not differ significantly from embryos cultured with AVYS alone in which 50% had NTD and almost 60% were dysmorphic (Table 3).

DISCUSSION

The degradation of endocytosed proteins within lysosomes of the VYS endoderm provides most (85–97%) of the amino acids used by the organogenesis stage rat embryo (Rowe and Kalaizis, '85; Lloyd et al., '96). It has been well documented that the primary effect of AVYS is an inhibition of VYS endocytosis that produces an embryonic nutritional deficiency (Freeman et al., '82; Lerman et al., '86; Brent et al., '90; Beckman et al., '91). Although AVYS disrupts the supply of all amino acids to the embryo, amino acids such as methionine are in such short supply that the embryo may become deficient in these before other, more abundant, amino acids. In the present study, we have addressed the possibility that the embryopathy induced by AVYS results, at least in part, from a deficiency in methionine, rather than a generalized reduction of amino acids. Experiments performed to assess the ability of methionine to overcome AVYS-induced teratogenicity in vitro support the hypothesis that AVYS-induced embryopathy results in large part from reduced availability of methionine.

The effects of AVYS, like other teratogens, are dose dependent. Thus, for our initial experiments, we needed to determine the relationship between the in vivo versus the in vitro effects of AVYS. The predominant malformations observed with a teratogenic (LD_{50}; 100-mg/kg) dose of AVYS administered during organogenesis in vivo are micro/anophthalmia and exencephaly (Brent et al., '71, '90; Fawcett et al., '95; Brent and Fawcett, '98). NTD encompassing other regions of the NT, such as spina bifida, are infrequent. The present study confirmed that 48 hr after administration of a teratogenic dose of AVYS to 9.5-day pregnant rats in vivo, the most common defects observed in the embryos are anophthalmia and a failure of anterior NT closure. On the basis of these results, we were able to determine that the concentration of AVYS that resulted in a similar morphology in vitro was 50 μg/ml

TABLE 3. Effect of leucine and folate on incidence of dysmorphology in cultured rat embryos resulting from in vitro exposure to AVYS from 9.5 to 11.5 days

	0 μg/ml culture medium			50 μg/ml culture medium			
AVYS supplement	None	L-Leucine	MTHF[†]	None	L-Leucine 10×	L-Leucine 20×	MTHF (μg/ml)
No. of embryos	51	11	12	26	28	21	32
Crown-rump (mm)	3.5 ± 0.4	3.3 ± 0.3	3.5 ± 0.5	2.99 ± 0.7*	2.7 ± 0.5*	2.94 ± 0.4*	3.0 ± 0.5*
VYS (mm)	4.4 ± 0.5	4.2 ± 0.56	4.0 ± 0.4	3.57 ± 0.8*	3.46 ± 0.42*	3.8 ± 0.4*	3.6 ± 0.5*
Embryo protein (μg)	206.6 ± 38.1	195.8 ± 34.5	209.4 ± 48.03	147.0 ± 52.2*	135.9 ± 36.4*	143.2 ± 39.1*	153.2 ± 38.6*
VYS protein (μg)	118.7 ± 15.4	114.4 ± 18.8	104.7 ± 30.9	82.1 ± 26.3*	75.8 ± 24.0*	106.4 ± 24.6	85.7 ± 20.6*
No. of somites	28.84 ± 2.9	28.45 ± 3.9	28.6 ± 1.5	24.7 ± 7.7	22.65 ± 7.9*	22.4 ± 7.4*	22.6 ± 6.3*
No. of arches	2.9 ± 0.3	3.0 ± 0	3.0 ± 0	2.60 ± 0.85	2.7 ± 0.5	2.6 ± 0.6	2.6 ± 0.8
Rotation (%)	96.1	91	91.7	73.1*	64.3*	76.2*	62.5*
VYS circulation (%)	98	100	100	80.8*	89.3	95.2	93.8
Forelimb bud (%)	100	100	100	92.3	100	100	96.9
Hindlimb bud (%)	62.7	72.7	91.7	23.1*	28.6*	23.8*	15.6*
Otic vesicles (%)	100	91	100	92.3	96.4	100	90.6
Optic vesicles (%)	98	91	100	50*	42.9*	19*	28.1*
Neural tube (% closed)	98	91	100	50*	50*	19*	34.4*
Dysmorphic (%)	3.9	9.1	0	57.7*	57.1*	80.9*	71.9*

*Significantly different from control value ($P < 0.05$).
[†]MTHF; 5-methyltetrahydrofolate.

AVYS. At this dose, a significant majority of embryos failed to close the anterior NT and had a high incidence of optic dysmorphology, but were of normal size and had completed axial flexion. Higher doses of AVYS in vitro led to a failure to close the entire NT, lack of axial flexion, and significant inhibition of other growth parameters. This morphology is more consistent with that expected for a dose that would not permit survival if administered in vivo; thus, none of these malformations would be observed at term.

It is noteworthy that the plasma concentration of AVYS resulting from an in vivo teratogenic dose is approximately 50-fold higher than the concentration of AVYS found to exert a similar morphologic effect on the embryos in vitro. This difference is best explained by the decreased accessibility of the VYS to the maternal circulation in vivo, and the length of time the VYS is exposed. Previous investigations have demonstrated that the VYS-reactive IgG is rapidly removed from the maternal circulation (Fawcett et al., '95). This is due, at least in part, to the presence of cross-reactive proteins in other rat tissues. In culture the VYS is directly exposed to the entire dose administered over a more prolonged period of time. Thus while maternal plasma levels of AVYS may be higher with an in vivo administration, the amount of AVYS that the conceptus is exposed to is likely to be only a fraction of the maternal plasma levels. Based on the morphological criteria used for the present study, it is likely that the overall exposure achieved in vivo is similar to that obtained by the lower in vitro dose.

Once we identified an in vitro concentration of AVYS that produced a similar embryopathy as that resulting from a teratogenic in vivo dose, and that would permit survival to term, we were able to assess the effects of methionine on AVYS induced embryopathy in vitro using both a "teratogenic" (50 μg/ml) and an "embryolethal" (100 μg/ml) dose of AVYS. At the higher of the two concentrations of AVYS (100 μg/ml), supplemental L-methionine significantly improved the degree of NT closure, although the defect was still present. Supplemental L-methionine also reduced the incidence of other defects observed, especially with regard to otic development, and somite number. When L-methionine was supplied at 50-fold the basal concentration and the lower "teratogenic" dose of AVYS was used (50 μg/ml), the embryopathy was completely prevented and the AVYS-exposed embryos were indistinguishable from littermate control embryos. No effects were observed when D-methionine was used instead of L-methionine at the same concentration. Furthermore, supplementation of cultures with L-leucine or 5MTHF did not alter the outcome of embryos treated with AVYS. These data strongly support the concept that the effects of L-methionine are specific to this amino acid, although the efficacy of other compounds for amelioration of AVYS-induced embryopathy cannot be ruled out at this time.

Previous reports have established that adequate methionine is essential for proper NT development in the rat. Culture of embryos in serum deficient in methionine produces NTDs in vitro (Flynne et al., '87; Coelho et al., '89). Furthermore, supplemental methionine ameliorates the embryopathy induced by a variety of substances both in vivo (Essien, '92; Essien and Wannberg, '93; Ehlers et al., '96) and in vitro (Flynne et al., '87; Coelho and Klein, '90; Van Aerts et al., '93, '94; Chambers et al., '95b). Of particular interest to the present investigation are studies demonstrating that supplemental free methionine ameliorates embryopathy induced by anti-laminin antibodies in vitro (Chambers et al., '95b). As in the present study, D-methionine proved ineffective, as were other amino acids or vitamins (Chambers et al., '95). The in vitro mechanism by which anti-laminin induces embryopathy may be similar to AVYS in that it may involve inhibition of nutrient transport to the embryo. Anti-laminin has been reported to inhibit both endocytosis, as measured by uptake of sucrose, and the transport of free amino acids. (Chambers et al., '95a), and could therefore lead to nutritional deprivation of the embryo. However, al-

though anti-laminin may induce NTD by a similar mechanism as AVYS in vitro, it is likely that the in vivo mechanism of anti-laminin embryotoxicity differs from AVYS. While AVYS induces malformations in vivo including NTD, anti-laminin antibodies have not been associated with increased incidence of NTD or other malformations in vivo, but rather are embryolethal (Foidart et al., '83) and have been associated with reproductive failure and/or spontaneous abortion (Szarfman et al., '82; Foidart et al., '86; Weeks et al., '89; Chambers et al., '95a,b).

Previous studies have demonstrated that AVYS inhibits endocytosis by the VYS but does not affect the transport of small molecules, such as leucine, α-amino isobuteric acid, and deoxyglucose (Lerman et al., '86; Beckman et al., '90). Transport of free methionine would not be expected to be affected by exposure to AVYS, as confirmed by recent data from our laboratory (Fawcett et al., '99). Thus, it is likely that transport of supplemental free methionine can compensate for the loss of methionine normally provided by endocytosis and protein degradation, even though greater than 90% of the methionine used by the embryo is normally derived from endocytosis (Lloyd et al, '96). This may explain why free methionine was able to overcome AVYS-induced embryopathy in vitro in the present investigation. It should be noted that although transport of free amino acids is not affected, even at the higher of the two concentrations used in the present study (100 μg/ml; Lerman et al., '86; Beckman et al., '90), the effects of this concentration on the VYS may be so severe that deficiencies in other amino acids/nutrients may also occur, resulting in growth retardation and reduced protein accretion. Under these circumstances, additional methionine cannot completely prevent the embryopathic effects of AVYS.

Attempts to purify the antigenic target of AVYS have shown that the antigen responsible for teratogenesis may be a high-molecular-weight glycoprotein on the apical membrane of the visceral endoderm (Leung, '82; Leung et al., '85). Combined evidence from adsorption using several rat tissues, immunodiffusion, electrophoresis, and Western blot has demonstrated that the antigen has immunologic homology with a protein on the brush border of kidney proximal tubules. Although adsorption of AVYS with kidney proximal tubules decreases teratogenicity, adsorption with basement membrane material, including collagen, laminin, or basement membrane tissues, did not reduce AVYS teratogenicity, indicating that the antigen has no homology to these proteins (Jensen et al., '75; Brent et al., '90). Previously isolated proposed targets had molecular weights of 30 and 60 kD, respectively (Leung et al., '85). Recently, Moestrup et al. ('98) reported that the target of teratogenic monoclonals to the VYS used in their studies is a 420-kD intrinsic factor-vitamin B_{12} receptor also found on the brush border of kidney proximal tubules. This finding is of great interest in light of the fact that vitamin B_{12}, methionine, and folate pathways are intimately linked. The putative antigens purified thus far in our laboratory are not of high molecular weight; however, the possibility remains that these proteins are subunits of a larger protein (Brent and Fawcett, '98). The similarity of the target of our antiserum to other potential teratogenic targets can only be fully addressed with final purification and sequencing of the proteins currently under study.

The mechanism by which methionine prevents NTDs has not been definitively established. However, evidence from several studies, including the present investigation, indicate that methionine supplementation overcomes a direct deficiency of methionine or SAM, rather than metabolic alterations of other components of one carbon metabolism (Coelho et al., '89; Coelho and Klein, '90; Moephuli et al., '97). In the present study, up to 500 times the basal concentration of folic acid, provided as 5MTHF, failed to improve the outcome of embryos exposed to AVYS. Furthermore, because supplemental methionine did not appear to affect protein content in either normal embryos, or in those exposed to AVYS, it is doubtful that the mechanism involves increased protein synthesis. Klein and colleagues have proposed that insufficient methionine, and consequently reduced SAM, results in reduced methylation of key proteins involved in NT closure (Coelho et al., '89; Coelho and Klein, '90; Moephuli et al., '97). This hypothesis is supported by the demonstration that actin, myosin, and several other proteins in the NT are hypomethylated when embryos are cultured in serum that is deficient in methionine (bovine serum) in vitro (Coelho and Klein, '90; Moephuli et al., '97). In a more recent study, NTDs in rodent embryos grown in methionine-deficient serum could be prevented using homocysteine, as well as methionine, but not folinic acid (Van Aerts et al., '94). At high concentrations, L-homocysteine is embryotoxic in vitro but does not induce NTD. The embryotoxicity resulting from elevated homocysteine was attenuated using serine (a methyl group donor), 5MTHF, or vitamin B_{12} (Van Aerts et al., '94). These results suggest that methionine was provided to the embryo by the methylation of homocysteine and the demethylation of 5MTHF via methionine synthase, and further demonstrates the importance of methylation for normal embryonic development.

The importance of methionine in human pregnancy outcome remains unknown; few studies have addressed this question. In one early report, dietary supplements containing methionine were found to increase favorable pregnancy outcome for women with a history of recurrent spontaneous abortion (Farrari et al., '94). A recent epidemiological study reported an association between dietary intake of methionine and reduced risk of NTD independent of folate status, although the data could easily support an association with other nutrients (Shaw et al., '97). Unfortunately, it is difficult to study the in vivo effects of methionine deficiency using animals models. Several early investigations (Sims, '51; Tagliamonte et al., '76) demonstrated that although pregnant rats could be made methionine deficient, litters were normal with respect to morphology

and levels of methionine. This was largely due to the efficient transfer of methionine from maternal stores to the fetus. Deficiency of methionine resulted in major liver changes, failure to lactate and low serum and protein methionine levels in the dams. Thus, defects that may result as a result of inefficient transport of methionine (from fetal membranes) to the embryo, whether genetic or teratogen-induced, cannot be accurately modeled by maternal dietary deficiency alone. The model used in this laboratory may circumvent these problems since AVYS induces nutritional deprivation of the embryo in vivo as well as in vitro with similar outcome (i.e., NTDs) without affecting maternal nutrition or general health. It will be of considerable interest to determine whether methionine can overcome the malformations induced by AVYS in vivo.

In conclusion, these studies confirm those of previous investigations demonstrating the importance of methionine in embryonic development, especially with regard to NT closure. Supplementation of embryo cultures with methionine significantly improved the outcome of AVYS-treated embryos and NT closure. This effect occurred without significantly altering protein accretion by the embryo, indicating that the action of methionine may occur through other metabolic roles. Further studies are necessary to determine the effect of methionine on AVYS-induced defects in vivo, the levels of methionine required for maximum beneficial effects on development, and whether these levels are attainable, and can be maintained, in utero.

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