Labeling index of AH7974F cells with ^{3}H-TdR

	Time after injection (h)	Labeling index of tumor cells			
		Brain		Liver	
		Mean	SD (n)	Mean	SD (n)
Flash labeling	1*	47.5	3.9 (2)	64.5	3.2 (2)
	6	58.0	1.8 (3)	61.0	1.4 (3)
	12*	30.5	17.5 (2)	69.5	1.8 (2)
	24*	44.5	0.3 (3)	68.7	2.9 (3)
	48*	52.0	2.1 (2)	75.0	1.4 (3)
Continuous labeling	6*	60.6	2.4 (3)	75.3	2.1 (3)
	12*	48.0	0.4 (3)	91.6	1.4 (3)
	24*	47.3	6.0 (2)	89.5	1.8 (2)

* There was a statistical significance in the labeling indexes between tumor cells in the brain and liver ($p < 0.01$).

constituted by 2-3 tumor cells which compressed the hepatic cell strands.

The scarcity of tumor formation in the brain after the injection of AH7974F cells has been considered to be due to the low number of tumor cell emboli in the brain[11]. In the present experiments we have revealed the characteristics of the proliferation-kinetics of the tumor cells arrested in the brain. The results of flash-labeling indicated that the AH7974F cells arrested in the brain remained viable, and the results of continuous labeling and silver-grain dilution tests suggested that many of tumor cells in the brain did not divide, although they remained viable. On the other hand, it is apparent from the table and the figure, that almost all of the AH7974F cells arrested in the liver divided. Such differences in proliferation-kinetics among the tumor cells arrested at these sites was also reflected in the final formation of tumors. They rarely formed in the brain but frequently formed in the liver. These results appear to support the 'seed and soil' hypothesis of the mechanism of organ specificity of cancer metastasis.

1 We thank Professor Garth L. Nicolson of The University of Texas System Cancer Center, M.D. Anderson Hospital and Tumor Institute, Houston, for revision of this manuscript. This work was supported by Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

2 R.A. Willis, in: The spread of tumors in the human body. Butterworth, London 1973.
3 S. Paget, Lancet *1*, 571 (1889).
4 B. Luke, C. Breedis, Z.P. Woo, L. Berwick and P. Nowell, Cancer Res. *12*, 734 (1952).
5 D.L. Kinsey, Cancer *13*, 674 (1960).
6 H.I. Pilgrim, Cancer Res. *29*, 1200 (1969).
7 R.C. Parks, J. natl Cancer Inst. *52*, 971 (1974).
8 J.W. Proker, Br. J. Cancer *34*, 651 (1976).
9 G.L. Nicolson, BioScience *28*, 441 (1978).
10 T. Kawaguchi, M. Endo, S. Yokoya and K. Nakamura, Experientia *37*, 414 (1981).
11 T. Kawaguchi and K. Nakamura, Gann *68*, 65 (1977).
12 S. Asahina, Fukushima Med. J. *27*, 65 (1967).

Abnormal development of cultured rat embryos in rat and human sera prepared after vitamin A ingestion[1]

C.E. Steele[2], J.D. Plenefisch and N.W. Klein

Physiological Laboratory, Cambridge CB2 3EG (England), and Department of Animal Genetics, and Genetics and Cell Biology Section, University of Connecticut, Storrs (Connecticut 06268, USA), 2 March 1982

Summary. Human and rat sera were assayed for teratogenic activity using a whole rat embryo culture technique. Sera prepared from blood withdrawn 1-5 h after the ingestion of vitamin A capsules caused developmental retardation and craniofacial abnormalities. Control sera permitted normal growth and differentiation.

It has been suggested that relatively small increases in plasma vitamin A concentration - of exogenous and/or pathological origin - could adversely affect embryonic development without any noticeable effect on the mother[3]. This is based on indirect evidence from a study of the teratogenic effects of low doses of retinol (vitamin A alcohol) added to the medium in which rat embryos were cultured[4] and on the few cases of human malformations following maternal ingestion of large amounts of vitamin A[5].

The whole embryo culture technique[6] used to demonstrate the direct effect of vitamin A on embryogenesis has now been successfully applied to the assay of teratogenic activity of human[7] and primate[8] sera, using these sera supplemented with glucose as the culture medium for rat embryos. We have adopted this approach to assay human sera for teratogenic activity following the ingestion of commercially available vitamin A capsules. Having first established the time course of vitamin A uptake using rats we were able to obtain typical vitamin A-induced abnormalities in serum from human subjects who had taken an overdose of vitamin A.

Materials and methods. Vitamin A. 'Natural Vitamin A' capsules were purchased from a drugstore. Each capsule contained 25,000 USP units of vitamin A from fish liver oil, 625% the minimum daily adult requirement of vitamin A. The form of vitamin A was not specified.

Dosing and bleeding. The contents of 4 vitamin capsules (100,000 USP units in 1 ml fluid) were administered by gavage to each of 3 non-pregnant female CD rats (Charles River Breeding Laboratories, Inc., Wilmington, MA, USA). 3 control rats received a similar volume of pure safflower oil. All rats were allowed food and water ad libitum. 1 control and 1 experimental rat were bled for serum

Table 1. Development of rat embryos in serum from rats dosed with safflower oil (control) or vitamin A

Serum	No. of embryos	Heartbeat at end of culture	Yolk sac circulation at end of culture	Turning complete	Head closed	Somites (x ± SE)	Protein (μg; x ± SE)
1-h control	3	3	3	3	3	23 ± 1	200 ± 6
2-h control	3	3	1	3	3	22 ± 1	209 ± 12
5-h control	3	3	3	3	3	22 ± 0	198 ± 3
1-h vit A	3	3	2	2	3	20 ± 1	178 ± 14
2-h vit A	3	3	0	0	2	15 ± 0	130 ± 17
5-h vit A	3	3	0	0	0	11 ± 1	99 ± 12

Table 2. Development of rat embryos in human serum from 2 human volunteers before and after vitamin A ingestion

Serum		No. of embryos	Heartbeat at end of culture	Yolk sac circulation at end of culture	Turning complete	Head closed	Somites (x ± SE)	Protein (μg; x ± SE)
Subject 1	pre-vit A	3	3	3	3	3	21 ± 1	138 ± 6
	5-h vit A	3	3	3	2	0	19 ± 1	105 ± 9
Subject 2	pre-vit A	3	3	3	3	3	21 ± 0	141 ± 11
	5-h vit A	3	3	3	3	0	18 ± 0	122 ± 16

preparation under ether anesthesia 1, 2 and 5 h after dosing.

2 healthy male human volunteers who had fasted overnight and were not receiving any medication were bled prior to taking 4 vitamin capsules each, equivalent to 4 times the manufacturer's suggested daily dose. They were bled again 5 h after ingestion of the vitamin A, the time at which the maximum effect was seen in dosed rats (see results). For the sake of brevity the sera will be referred to as pre-vit A serum and 5-h vit A serum.

Serum preparation. Blood was centrifuged immediately after withdrawal[9], the serum decanted and stored at −20 °C. All sera were heat-inactivated at 56 °C for 30 min and antibiotics added prior to use. Human sera were analyzed for glucose concentration by the glucose oxidase method[10] and were adjusted to a final concentration of 3 mg/ml.

Embryo culture. CD rat embryos of the early-headfold stage were explanted in Tyrode saline during the morning of the 10th day of gestation (1st day = day of the +ve vaginal smear). 3 embryos were cultured for 48 h in 2 ml of each serum sample contained in a bottle rotated at 30 rev./min[11]. For the first 20 h of culture the serum was equilibrated with a gas mixture containing 5% O_2. This was changed to 15%, 30% and 40% after 20, 27 and 44 h, respectively[8]. All gas mixtures contained 5% CO_2, the remainder being nitrogen.

At the end of the culture period the presence or absence of heartbeat and yolk sac circulation was noted. The embryos were then dissected from the yolk sac and amnion and the following recorded: the degree of 'turning' (whether the embryo had adopted the characteristic fetal position), whether or not the neural tube had closed, and somite number. Finally, the protein content of the embryos was determined by the microspectrophotometric method of Lowry et al.[12].

Results. Embryonic development in serum from rats dosed with vitamin A. Both differentiation (somite number) and growth (protein content) of embryos cultured in serum from rats receiving vitamin A were retarded compared with that in serum from control rats (table 1). The effect was least pronounced in 1-h vit A serum and most severe in 5-h vit A serum; 3-h vit A serum gave an intermediate result. There was no effect on closure of the head in 1-h vit A serum but in 5-h vit A serum all embryos had open heads as well as the posterior part of the embryo protruding from the yolk sac. The most severely affected embryos had no otocysts, pharyngeal arches or limb buds.

Embryonic development in serum from human subjects receiving vitamin A. The effects of vitamin A ingestion on differentiation and growth were less pronounced in human sera compared with rat serum (table 2). However, there was a diminution in somite number and protein content in 5-h vit A human serum compared with pre-vit A human serum. The effect of vitamin A on head closure was similar to that in rat serum. All embryos cultured in 5-h vit A human serum had open heads and all those in pre-vit A serum had closed heads. 1 embryo cultured in 5-h vit A human serum failed to develop optic vesicles.

Discussion. The ingestion of 100,000 USP units (30,000 μg) of vitamin A by rats and human subjects rendered their serum teratogenic to rat embryos within 5 h of dosing. The abnormalities seen in embryos cultured in these sera were similar to those previously observed following direct addition of 0.5–3.0 μg/ml retinol to rat serum[4]. They were less severe than those following the direct addition of 5–20 μg/ml retinol when the embryo consisted of only a thickened area on the yolk sac with little or no differentiation[4]. These results seem to confirm the original suggestion that in vivo only a small proportion of the administered vitamin A reaches the embryo in active form[3].

Experiments are in progress to quantify the factors affecting the teratogenic activity of vitamin A in human serum including the minimum oral dose, minimum exposure time of the embryo and the variation between human subjects. In the meantime, if it can be assumed that the response of explanted rat embryos to serum as a culture medium is an indication of the teratological risk to the individual donating the serum, then the existing data have important clinical implications. We conclude that overdoses of vitamin A may result in human congenital malformation.

1 Financial support for this project was provided by Department of Energy contract EVO 3139 (Office of Health and Environmental Research), USA (Scientific contribution 940, Storrs Agricultural Experiment Station), and by the Medical Research Council, U.K.

2 To whom reprint requests should be addressed.

3 Morriss, G.M., and Thomson, A.D., Lancet *2* (1974) 899.

4 Morriss, G.M., and Steele, C.E., J. Embryol. exp. Morph. *32* (1974) 505.
5 Pilotti, G., and Scorta A., Minerva ginec. *17* (1965) 1103.
6 New, D.A.T., Biol. Rev. *53* (1978) 81.
7 Chatot, C.L., Klein, N.W., Piatek, J., and Pierro, L.J., Science *207* (1980) 1471.
8 Klein, N.W., Plenefisch, J.D., Carey, S.W., Timm Fredrickson, W., Sackett, G.P., Burbacher, T.M., and Parker, R.M., Science, in press (1982).
9 Steele, C.E., and New, D.A.T., J. Embryol. exp. Morph. *31* (1974) 707.
10 Bergmeyer, H.U., and Bernt, E., in: Methods of enzymatic analysis. p. 1205. Ed. H.U. Bergmeyer. Academic Press, New York 1974.
11 New, D.A.T., Coppola, P.T., and Terry, S., J. Reprod. Fert. *35* (1973) 135.
12 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.

The germ cell – oncogenic and embryogenic correlates

B. Iyengar[1]

Department of Pathology, Maulana Azad Medical College, New Delhi (India), 30 October 1981

Summary. This study is based on 100 consecutive germ cell tumors of the gonads, received during a 5-year period. Benign teratomas, with totipotent differentiation are the commonest ovarian germ cell tumors, whereas the nullipotent germinomas from the bulk of the testicular tumors. Differentiation in the rapidly proliferating testicular teratomas, occurs in the form of embryoid bodies and organoid structures. From an analysis of the germ cell tumors it is evident that the ovum confers differentiating functions on the zygote, while the spermatozoon confers functions of organization and proliferation. This difference in the behavior of the 2 germ cells is due to local feedbacks within the cortical and medullary zones of the gonads.

Embryogenesis and oncogenesis have 2 important features in common. Both processes involve rapid proliferation and changing levels of differentiation, although one is well regulated and the other is erratic and uncontrolled.

The role of the gonadal germ cells in embryogenesis has been outlined in the present work by observing their behavior during oncogenesis.

Materials and methods. The study is based on the morphology of 100 gonadal germ cell tumors, received in the Department of Pathology, Maulana Azad Azad Medical College, New Delhi, during a 5-year period. Multiple sections have been taken from each tumor, to ensure a proper diagnosis. The tumor distribution in the 2 sexes have been compared.

Results. Germ cell tumors: Of the 100 tumors in both sexes, the largest number are benign teratomas (50). Germinomas form the second largest group, there being 27 in all. There are 15 malignant teratomas, while germinomas mixed with other tumor types are 5 in number. 2 are undifferentiated malignant tumors and there is 1 gonadoblastoma (table).

A comparison of the ovarian and testicular germ cell tumors: Ovarian germ cell tumors form 63% and the testicular tumors form 37% of the total.

The variant potential of the germ cells is evident from the relative frequency of the different tumor types (table). The nullipotent germinomas form the bulk of the testicular tumors (22), in contrast to the high incidence of totipotent, well differentiated benign teratomas of the ovaries (49). The testicular germinomas form 81.5% and the ovarian germinomas form 18.5%. The ovarian teratomas show differentiation into the 3 germ layers with the formation of mature but haphazard tissues. The malignant teratomas (5) arise from unipotent, committed cell lines within the ovary. Only one benign teratoma is seen in the testis, while malignant teratomas have a high incidence (10). These show an organoid differentiation, with the formation of embryoid bodies which mimic the early, 2- to 3-layered blastoderm, with little further differentiation. The anaplastic tumors have an equal distribution in the 2 gonads.

Discussion. The germ cells arise from the yolk sac to migrate into the cortex and medulla of the genital ridge[2]. In the ovary, the cortical germ cells remain to become the oocytes. As only 1 ovarian follicle matures at each cycle[3,4], the oocytes are very stable cells.

In the testis, it is the medullary germ cells which persist and come to line the seminiferous tubules. The spermatogonium undergoes constant mitosis and is a highly labile cell.

In both the gonads the germ cells are supported by cells from the cortex (the granulosa and Sertoli cells)[5–8], and the interstitial cells which arise from the medulla (the theca and Leydig cells)[9]. Thus the difference in the germ cell activity is due to their location within the stroma of the cortex or the medulla. The reversal of the gonadal sex by extirpation of one of these zones supports this fact[10].

It is evident that there is a distinct difference in the behavior of the germ cell present in the 2 gonads, determining the pattern of incidence and the morphogenesis of the germ cell tumors.

The occurrence of a large number of benign and well differentiated tumors reflects the totipotential nature of the ovum. Even the rarer malignant teratomas arise chiefly from fully differentiated cells. This has been a consistent observation made by many other workers also[11,13].

The pattern of testicular germ cell tumors is another well-defined feature[12,14–16]. The frequency of germinomas indicates that this germ cell maintains its nullipotency. The differentiating tumors are all malignant teratomas with organoid differentiation and the formation of embryoid bodies indicating organizational properties. As noted by other workers[17], inspite of rapid proliferation in most testicular germ cell tumors, the ovarian tumors are twice as frequent.

Comparison of ovarian and testicular germ cell tumors

Sample No.	Tumors	Ovarian No.	Ovarian %	Testicular No.	Testicular %	Total No.
1	Benign teratoma	49	77.8	1	2.7	50
2	Malignant teratoma	5	8.0	10	15.0	15
3	Mixed tumor	2	3.1	3	5.0	5
4	Germinoma	5	8.0	22	59.5	27
5	Anaplastic tumor	1	1.6	1	2.7	2
6	Gonadoblastoma	1	1.6	0	0	1
	Total	63	63.0	37	37.0	100