Inhibition of phorbol myristate acetate and phytohemagglutinin stimulation of human lymphocytes by 13-cis-retinoic acid and ethyl etrinoate[1]

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Summary. The retinol analogues 13-cis-retinoic acid and ethyl etrinoate inhibit the mitogenic activity of phorbol myristate acetate and phytohemagglutinin on human lymphocytes. This inhibitory effect is greater against the stimulation with phorbol myristate acetate than with phytohemagglutinin.

Experiments have shown that retinol and retinoic acid can prevent experimentally-induced benign and malignant tumors in animals, particularly those induced with the tumor promoter phorbol myristate acetate (PMA)[2-7], but they have limited usefulness as agents of chemoprevention because of poor tissue distribution and excessive toxicity (hyper-vitaminosis A)[7,8]. Synthetic analogues have been shown to be more effective in the prevention of some experimentally-induced cancers and less toxic for the animals and cultured organs[9-11]. Retinol is also known to inhibit the mitogenic response of human lymphocytes exposed to PMA[12]. In this study it is shown that the retinol analogues 13-cis-retinoic acid and ethyl etrinoate which are known to prevent tumor induction also inhibit lymphocyte mitogenesis from PMA and phytohemagglutinin (PHA).

Materials and methods. Human lymphocytes from heparinized blood were obtained by separation over a Ficoll-Paque gradient. The cells were suspended in growth medium (RPMI 1640 and 10% fetal calf serum, Grand Island Biological Company, GM) at a concentration of 2×10^6 lymphocytes per ml of GM. Retinol (Sigma Chemical Company), ethyl etrinoate (RO 10-9359, Hoffmann-La-Roche Ltd) were dissolved in dimethylsulfoxide (DMSO) and then further diluted with GM to make stock solutions at 300 µg/ml. Further dilutions of these stock solutions were made with GM. The final concentration of DMSO in the retinoids at 20 µg/ml was 1%. The mitogenic response of the lymphocytes to PHA (Bacto-Phytohemagglutinin P, Difco) and to PMA (Sigma Chemicals) was assessed by the [^{3}H] thymidine uptake using a micro-method[13] with Linbro plates. The optimum dose of 0.4 µl/ml for PHA and 50 ng/ml for PMA had previously been established in our laboratory[12]. The effect of the retinol, 13-cis-retinoic acid, and ethyl etrinoate on the mitogenic response of the lymphocytes to the PHA and PMA was assessed by adding 0.1 ml of these materials at final concentrations of 5, 10 and 20 µg/ml to the wells with the lymphocytes and mitogens. 10 µg/ml of retinol is equivalent to a 3.5 mM solution, 10 µg/ml of 13-cis-retinoic acid equals a 3.3 mM solution and 10 µg/ml of ethyl etrinoate is equivalent to a 2.8 mM solution. Control cultures with lymphocytes and DMSO 1%, and lymphocytes alone with retinol and its analogues were also carried out. The viability of lymphocytes exposed to retinol and the retinol analogues for 72 h at the maximum concentrations used was assessed by the trypan blue exclusion method.

Results. Retinol and its analogues significantly inhibit the mitogenic response to the PHA and PMA. The percent inhibition (taking the response of the lymphocytes to PHA or PMA alone as 100%) by the various concentrations is shown in figures 1 and 2. Retinol had the greatest inhibitory effect with PHA stimulation and ethyl etrinoate least (fig. 1) but with PMA as the stimulant the inhibitory effect of retinol and its analogues was about the same (fig. 2). There was no significant effect of the DMSO 1% on the mitogenic response to either PHA or PMA. Viability of the lymphocytes exposed to retinol and its analogues for 72 h was 98%.

Discussion. The effect of the retinol on the lymphocyte mitogenic response is the same as has been found in our previous study[12] and the inhibitory effect is the same on stimulation with either PHA or PMA. The 13-cis-retinoic acid and ethyl etrinoate, however, show some difference in

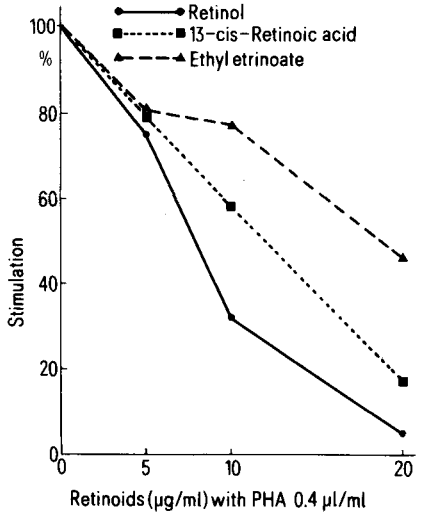

Figure 1. The effect of varying concentrations of retinol, 13-cis-retinoic acid and ethyl etrinoate on PHA stimulated lymphocytes expressed as a percentage of a 100% stimulation with PHA alone. 100% represents 15,000 cpm. Lymphocyte controls had counts of 225-300 cpm.

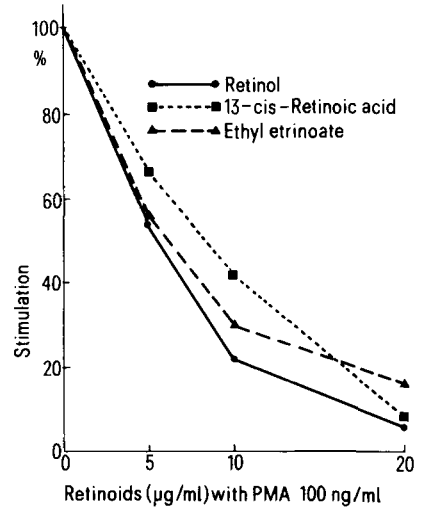

Figure 2. The effect of varying concentrations of retinol, 13-cis-retinoic acid on PMA stimulated lymphocytes expressed as a percentage of a 100% stimulation with PMA alone. 100% represents 42,000 cpm. Lymphocyte controls had counts of 225-300 cpm.

their effect on the response to PHA (fig. 1) compared with that to PMA (fig. 2). The PHA-stimulated lymphocytes have still an 80% and 60% mitogenic response with ethyl etrinoate and 13-cis-retinoic acid respectively even at a concentration of 10 ng/ml of these substances. This concentration (10 µg/ml) is one that could only be achieved in vivo by high pharmacological doses (the physiological level of retinol in the blood is 0.2–0.4 µg/ml). The reason for the difference in their effect on PHA stimulation compared with PMA stimulation is not clear nor can any definite significance be attributed to it, although one would hope that the retinol analogues maintain an anti-tumor promoter activity against PMA stimulation with less effect on the mitogenic response of lymphocytes to PHA than might be expected in an immune reaction. It is generally accepted that the retinol analogues such as 13-cis-retinoic acid are less toxic than retinol in animals while maintaining full anti tumor promoting activity[9-11]. This difference in their inhibitory effect on lymphocyte stimulation with PHA is another factor to be considered in chemoprevention of cancer[14].

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Development of the hypothalamic-hypophysial-gonadotrophic activities in fetal rats

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Summary. Pituitary responsiveness to LHRH and anti-LHRH serum was investigated in fetal rats aged 18.5–22.5 days. Synthetic LHRH injection in utero into fetuses brought about a remarkable depletion of pituitary-LH with a corresponding increase of serum-LH on day 18.5. On the contrary, anti-LHRH serum administration to day-20.5 fetuses caused a significant augmentation of pituitary-LH 1 day later. These data indicate that LH-gonadotrophs respond to LHRH even on day 18.5, and that endogenous LHRH begins to affect LH-gonadotrophs on day 20.5.

Information on the appearance of hypothalamic gonadotropic hormone releasing hormone (LHRH)[3-7] and of gonadotrophs[8-10] is available in rat fetuses. However, the time of the first occurrence of their functional relations has still not been determined, although the existence of the hypothalamic-hypophysial-gonadal axis is well established in fetal rats[11]. Synthetic LHRH induces LH discharge in newborn rats in vivo[12] and fetal rats[13] and fetal mice[14] in vitro. Our previous immunohistochemical study has demonstrated in fetal rats that synthetic LHRH depletes immunoreactive LH from the pituitary, and the storage of pituitary LH follows an administration of anti-LHRH serum[15]. In the present study, we attempted to determine by radioimmunoassay when the fetal pituitary responds to exogenous LHRH and when endogenous LHRH begins to stimulate LH release.

Materials and methods. Female Sprague-Dawley rats were mated overnight, and the vaginal smear was examined the next morning. If spermatozoa were found in the smear, the animals were determined as day 0.5 of gestation. For examination of the response of gonadotrophs to LHRH, day-18.5 and -19.5 fetuses were used. The pregnant rats were anesthesized with ether between 10.00 and 11.00 h, and the uterine horns were exposed by laparotomy. 2 µg of synthetic LHRH (Peptide Institute, Osaka, Japan) dissolved in 10 µl saline was injected i.p. into the fetuses in one uterine horn through the uterine wall. The fetuses in another uterine horn were injected with 10 µl saline and served as controls. After replacement of the uterine horns, the abdominal wall was closed. 2 h later, the rat thus treated was killed by cervical dislocation and the fetuses were removed. The heads of the fetuses were cut and the trunk blood spurting out from the cervix was collected in an ice-cold 1-ml glass tube. The pituitaries removed were dropped into ice-cold 1-ml homogenizers containing 0.1 ml of phosphate buffered saline (0.01 M PO_4–0.15 M NaCl, pH 7.4) and homogenized. The homogenate was transferred into a 0.4-ml plastic tube and stored at −80 °C until the assay procedure was carried out. The blood was collected from 3–11 animals. The serum of each experimental group consisted of 2 blood samples. The serum samples were stored at −80 °C.

Serum LH and FSH concentrations in fetal rats receiving LHRH or saline

Age (day)	Treatments	Gonadotrophins	Concentrations (ng/ml) Sample 1	Sample 2
18.5	Saline	LH	0.13 (11)*	0.32 (8)
18.5	LHRH	LH	0.39 (8)	0.55 (9)
19.5	Saline	LH	0.17 (5)	0.31 (7)
19.5	LHRH	LH	0.90 (8)	1.40 (11)
19.5	Saline	FSH	30.0 67)	37.4 (5)
19.5	LHRH	FSH	41.3 (8)	63.4 (11)

* Number of animals in parentheses.