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EFFECTS OF INSULIN, DEXAMETHASONE AND PHORBOL ESTER ON VIRUS PRODUCTION IN CULTIVATED DBA/2 MOUSE LEUKEMIA (MLA) CELLS

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Summary. — The in vitro production of intracytoplasmic oncovirus A-particles and of atypical mammary tumor virus (MLAV) from a line of DBA/2 mouse leukemia (MLA) cells were compared in the presence of insulin, dexamethasone and 12-0-tetradecanoyl--phorbol-13-acetate (TPA), which are known to stimulate the synthesis of mouse mammary tumor virus. High amounts of A-particles were recovered from cells cultivated in the presence of dexamethasone (1 μg/ml) as compared to untreated controls; in contrast, the production of MLAV was suppressed. Insulin and TPA did not show any significant effects.

Key words: intracytoplasmic A-particles; atypical mammary tumor virus; DBA/2 mouse leukemia cells; dexamethasone; 12-0-tetra--decanoyl-phorbol-13 acetate; insulin

Introduction

Hormonal effect on tumor virus, especially on murine mammary tumor virus (MMTV) production, has been recognized by several authors. McGrath (1971, 1975) reported that the production of MMTV and the formation of three-dimensional "dome" structures by primary mouse mammary tumor cells in culture were mediated by the actions of insulin and hydrocortisone. Further studies (Fine *et al.*, 1974; Parks *et al.*, 1974; Ringold *et al.*, 1975) revealed that the glycocorticoid hormone dexamethasone could elicit a 10- to 20-fold increase in both, the levels of viral RNA and amount of complete virions, while no such effect was detected on the amount of gs-antigen of C-type viruses. In addition, a recent study (Arya, 1980) has demonstrated that TPA, a potent tumor promoter, stimulates the synthesis of MMTV--production in cultured C3H mouse mammary tumor cells.

We established a cell line in vitro from spontaneous leukemia of DBA/2 mouse (designated as MLA-cells) which produced intracytoplasmic A-particles and released a peculiar type of extracellular mature virus particles (MLAV) into the culture fluid (Tamura and Tanaka, 1973). Antigenic and biochemical studies demonstrated that intracytoplasmic A-particles were the precursor of the type-B particles of MMTV (Tanaka *et al.*, 1972; Tanaka, 1977). MLAV was produced by the budding of A-particles, and had

a nucleoid morphologically indistinguishable from that of B-particles of MMTV, as well as an apparently smooth envelope akin to that of C-particles. Further study (Vaidya *et al.*, 1980) showed clearly that MLAV contained MMTV-related antigens, MMTV-specific RNA and, moreover, inoculation of the MLAV into BALB/c mice resulted in the development of mammary tumors in the females.

The present study was designed to ascertain whether insulin, dexamethasone and TPA could enhance the virus production of MLA-cells as seen in the case of other mammary tumor cell lines.

Materials and Methods

Cells. MLA-cells were cultivated in RPMI 1640 medium (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal calf serum, in which the cells were grown in floculating state even at stational incubation.

Drug-treatment of cell culture. A drug was added to eight 800 ml-culture bottles containing 50 ml each of MLA-cell culture at the cell concentration of 4×10^6 per ml, and on the 2nd and 4th days, half of the culture medium in each bottle was replaced with a new medium containing the drug. On the 7th day after treatment, all cultures were harvested and centrifuged at low speed. The MLAV and A-particles were purified from the supernatant and cell pellets obtained. Drugs used were prepared as follows: insulin (Sigma no. 1-5500) and dexamethasone (Sigma no. D-1756) were solubilized in RPMI 1640 medium at the concentration of 1 mg/ml; TPA (P-L Biochemicals, Inc., Milwaukee, Wis.) was solubilized in ethanol at the concentration of 0.1 mg/ml. The drugs were added to make the final concentration of 10 μg/ml for insulin, 1 μg/ml for dexamethasone and 0.1 μg/ml for TPA, respectively.

Purification of A-particles. A-particles were purified by the method described in our previous report (Tanaka *et al.*, 1972). Afther the second sucrose density gradient centrifugation in a 5 ml nitrocellulose tube of Hitachi RSP-50 rotor at 115,000 × g for 120 min, 5 drops of each fraction from the top of the tube obtained by an Autodensi Flow II C (Buchler Instrument Co.) were diluted with 0.5 ml SET (0.25 mol/l sucrose, 0.001 mol/l EDTA and 0.01 mol/l Tris-HCl buffer, pH 7.4) and their optical density was measured at 280 nm.

Purification of MLAV. Culture fluid of MLA-cells was centrifuged at 2,500 × g for 10 min and the supernatant was further centrifuged at 70,000 × g for 70 min. The pellet obtained was resuspended in SET, clarified by centrifugation at 700 × g for 10 min, and layered on 11 ml of sucrose density gradient column of the density 1.10 to 1.20 in ET (0.001 mol/l EDTA and 0.01 mol/l Tris-HCl buffer, pH 7.4) formed in 13 ml nitrocellulose centrifuge tube, and centrifuged at 104,500 × g for 70 min in Hitachi RSP 40T rotor. The band formed at the densities of 1.14—1.16 was harvested by a capillary pipette, diluted 3-fold with ET and centrifuged at 117,000 × g for 60 min in the same rotor. The pellet obtained was resuspended in SET, centrifuged at 300 × g for 10 min, layered again on 11 ml of sucrose gradient column of the density 1.01 to 1.13 and centrifuged at 35,700 × g for 60 min. A band formation of MLAV was observed at the portion of density 1.06 to 1.08. Each of 20 drops was fractionated from the top of the tube by Autodensi Flow IIC and 0.5 ml SET was added to each fraction. The optical density of the fractions was measured at 280 nm.

Electron microscopy. Samples of centrifugal pellets were fixed, without destroying the block, in 2% glutaraldehyde in 0.1 mol/l cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer, stained with 2% uranyl acetate and dehydrated in a graded alcohol series. Then the blocks were embedded in Epon 812 (Luft, 1961), sectioned on an LKB-IV ultratome, and stained on grids with lead citrate (Sato, 1968). The ultrathin sections were examined with a JEM-100CX electron microscope.

Results and Discussion

MLA-cell cultures were divided into 4 groups. No hormones or drugs were added to the first (control) group. Insulin was added to the 2nd, dexa-

methasone to 3rd, and both insulin and dexamethasone to the 4th groups. After one week incubation, A-particles and MLAV were purified from each culture and the patterns of the second sucrose density gradient centrifugation were compared. As shown in Fig. 1, dexamethasone-containing cultures (the 3rd and 4th groups) produced an extremely large amount of A-particles

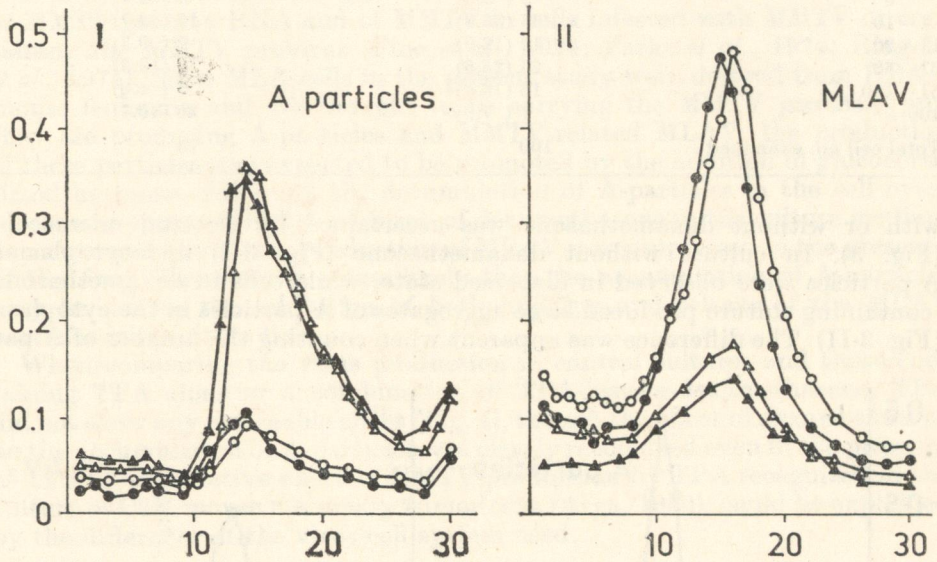

Fig. 1.

Sedimentation patterns of A-particles and MLAV in sucrose density gradient centrifugation Obtained from control cultures (——○——); cultures containing insulin alone (——●——); dexamethasone alone (——△——) and both insulin and dexamethasone (——▲——). I — — A-particles; II — MLAV.

Abscissae: fraction number from top to bottom; ordinates: optical density at 280 nm.

as compared with those of the control and insulin-containing cultures (the 1st and 2nd groups). In contrast, the production of MLAV was markedly enhanced in the control and insulin-treated cultures as compared to that in the dexamethasone-treated ones.

The fact that the peak in the left graph of Fig. 1 is composed of A-particles was confirmed by electron microscopic examination as shown in our previous report (Tanaka *et al.*, 1972). The peak of the right graph of Fig. 1 was pooled, recovered in the pellet by centrifugation, and examined in electron microscope. As seen in Fig. 2, the preparation is composed mainly of MLAV, which are nearly spherical in shape, approximately 110 nm in diameter, and have an eccentric nucleoid surrounded by an intermediate shell. These findings indicate that the peak of the right graphs in Fig. 1 consists of MLAV. The difference in the production of A-particles in cultures

Table 1. Distribution of cell population according to the number of A-particles in the cell cytoplasm

No. of A-particles in the cytoplasm	Culture without drugs	Culture containing dexam. and insulin
	Number (and %) of cells	
0	5 (5.0)	3 (4.0)
1—10	35 (35.0)	17 (22.7)
11—20	12 (12.0)	7 (9.3)
21—50	25 (25.0)	10 (13.3)
51—100	16 (16.0)	3 (4.0)
100—	7 (7.0)	35 (46.7)
Total cell no. examined	100	75

with or without dexamethasone was ascertained by electron microscopy (Fig. 3). In culture without dexamethasone (Fig. 3-I) intracytoplasmic A-particles were observed in dispersed state, while cells in dexamethasone--containing culture produced large aggregates of A-particles in the cytoplasm (Fig. 3-II) The difference was apparent when counting the number of A-par-

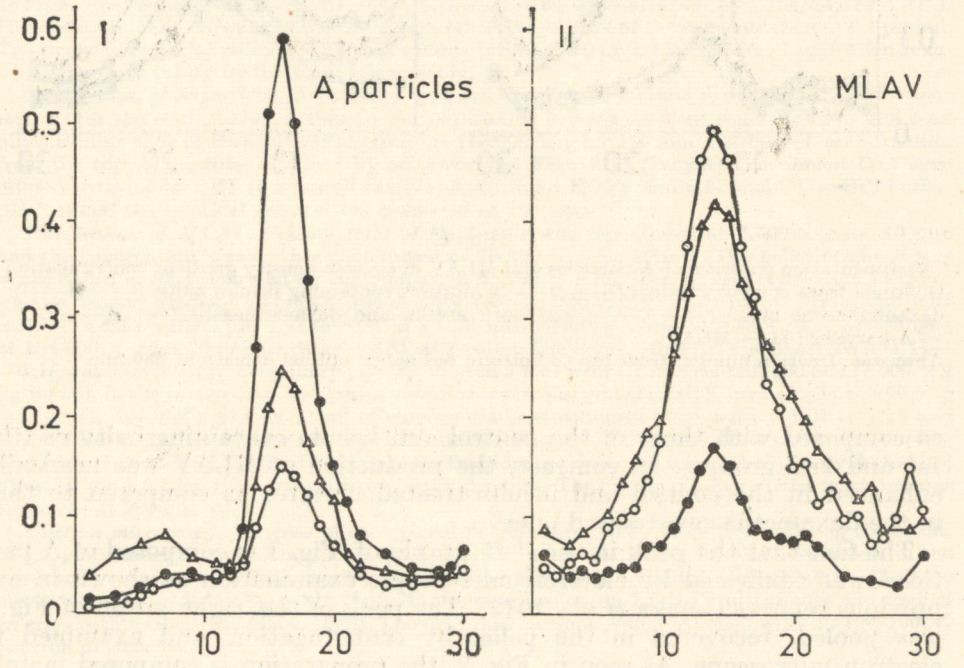

Fig. 4.

Sedimentation patterns of A-particles and MLAV in sucrose density gradient centrifugation Obtained from control cultures (——○——); cultures containing TPA alone (——△——); TPA, insulin and dexamethasone (——●——), I — A-particles; II — MLAV. Abscissae: fraction number from top to bottom; ordinates: optical density at 280 nm.

ticles in the cells sectioned equatorially through Golgi bodies and nucleus (Table 1). In control cultures lacking dexamethasone, 35% of cells contained less than 10 A-particles and more than 70% of cells contained less than 50 A-particles. In contrast, large numbers of A-particles (more than 100) were observed in 46.7% of cells of the dexamethasone-treated culture.

It is well known that the glucocorticoid hormone stimulates the induction of MMTV genome RNA and of MMTV in cells infected with MMTV or containing the MMTV provirus (Fine *et al.*, 1974; Parks *et al.*, 1974; Ringold *et al.*, 1975). Since MLA-cells in the present study were derived from DBA/2 mouse leukemia and are thought to be carrying the MMTV provirus, and they are producing A-particles and MMTV-related MLAV, the production of these particles was expected to be promoted by the addition of glucocorticoid hormone. Although the accumulation of A-particles in the cell cytoplasm was observed by the addition of dexamethasone in the culture medium as mentioned above, the production of MLAV was suppressed in the presence of the drug. Therefore it seems likely that the accumulation of A-particles may be due to the inhibition of both budding and release of the MLAV particles into culture fluid.

When comparing the virus production in control cultures and those containing TPA alone or a combination of TPA-insulin-dexamethasone, TPA did not show any noticeable effect (Fig. 4), though the effect of dexamethasone on the accumulation of A-particles was clearly recognized even in the presence of TPA. A stimulative effect of MMTV production by TPA recognized in the culture of C3H mouse mammary tumor cells (Arya, 1980), could be explained by the difference of the virus-cell system used.

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Explanation of Electron Micrographs (Plates XLVII—XLVIII):

Fig. 2. Ultrathin section of the centrifugal pellet recovered from MLAV peak in Fig. 1 (fractions no. 14—18 of control culture).

Fig. 3. Ultrathin section of MLA-cells cultivated with or without dexamethasone. I — in the absence of drug; II — with dexamethasone. Arrowheads indicate A-particles and an arrow shows MLAV.