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EFFECTS OF ANCHORAGE-MODULATING DOSES OF CONCANAVALIN A, MICROTUBULE-DISRUPTING DRUGS AND MICROFILAMENT PERTURBANTS, CYTOCHALASINS, ON THE PHOSPHATIDYLINOSITOL RESPONSE OF RAT LYMPH-NODE CELLS

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In lymphocytes isolated from rat lymph nodes, concanavalin A stimulated the $^{32}PO_4$ incorporation into phosphatidylinositol and phosphatidic acid in a dose-dependent manner up to 200 μg of the lectin per ml of the lymphocyte culture. [^{3}H]Thymidine incorporation was found to be optimal at 2 μg concanavalin A per ml of the culture when the incorporation was examined at the same cell density as was used in the determination of the $^{32}PO_4$ incorporation. As previously described (Wang, J.L. and Edelman, G.M. (1978) J. Biol. Chem. 253, 3000–3007), the [^{3}H]thymidine incorporation was inhibited at doses higher than 5 μg/ml in a dose-dependent manner. These results indicate that concanavalin A produced the phosphatidylinositol PI response of rat lymph-node cells in the dose range in which the mobility and distribution of lymphocyte surface receptors were modulated by the lectin (Yahara, I. and Edelman, G.M. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 608–612). Colchicine and vinblastine at a concentration of 10^{-4} M did not inhibit the concanavalin A-induced PI response of rat lymph-node cells. Cytochalasins B and D at a concentration of 10^{-5} M enhanced the concanavalin A-induced PI response to some degree. All the results obtained suggest that submembranous assemblies of microtubules and microfilaments do not play an indispensable role in the sequence of events involved in the PI response of rat lymph-node cells.

Introduction

Binding of concanavalin A to the lymphocyte surface produces a small but rapid loss of phosphatidylinositol accompanied with accumulations of phosphatidic acid and diacylglycerol. Selective acceleration of the $^{32}PO_4$ incorporation into the lymphocyte phosphatidylinositol [1] occurs following this initial breakdown of preexisting phosphatidylinositol. This stimulation of the phosphatidylinositol metabolism is usually known as 'the phosphatidylinostiol (PI) response' in lymphocytes.

Concanavalin A in concentrations greater than 5–10 μg/ml restricts the mobility of a variety of lymphocyte surface receptors and antigens at 20 and 37°C, but not at 4°C [2,3]. This restriction by concanavalin A can be reversed by colchicine and related drugs [4]. It has been proposed that cross-linkage of certain cell surface receptors can modulate the movement of other receptors via alterations in a cytoplasmic assembly consisting of microtubules and microfilaments [5,6].

The binding of concanavalin A and other mitogenic lectins to the lymphocyte surface triggers the stimulation of DNA synthesis and blast transformation. The mitogenic stimulation by concanavalin A is optimal at 0.5–2 μg lectin/ml [6–8]. Concanavalin A is inhibitory for the DNA synthesis in the dose range in which the cell surface modulation by the tetravalent lectin occurs. At high doses of concanavalin A, the mitogenic signal is present and lymphocytes are committed to blast transformation, but are also

simultaneously blocked from entering the S phase of cell cycle by a dominant negative growth signal probably generated by the anchorage modulation [9]. Moreover, divalent succinyl-concanavalin A, which does not modulate receptor mobility, is mitogenic even at a high dose range [6,10].

In this paper, we report that the PI response induced by concanavalin A in rat lymph-node cells is not inhibited in the high dose range of the lectin in which the lectin was inhibitory for the DNA synthesis. In addition, we describe the effects of microtubule-disrupting drugs, colchicine and vinblastine, and cytochalasins B and D, which inhibit microfilament-dependent cell functions [11,12], on the lymphocyte PI response.

Materials and Methods

Materials. Colchicine, vinblastine sulfate and cytochalasins B and D were purchased from Sigma Chemical Company (St. Louis). [^{32}P]Phosphate (carrier-free) was obtained from Japan Atomic Energy Research Institute (Tokyo). [*methyl*-^{3}H]-Thymidine (6.7 Ci/mmol) was purchased from New England Nuclear (Boston). Concanavalin A was obtained from Pharmacia Fine Chemicals (Uppsala). Fetal calf serum was obtained from Flow Laboratories (Stanmore).

Isolation of rat lymphocytes. Lymph-node cells were isolated from cervical and mesenteric lymph nodes of rats of Wistar strain. To avoid the mixed lymphocyte reaction, lymphocytes obtained from each rat were separately handled in our experiments. The isolated lymphocytes were washed three times with phosphate-free Tris-buffered saline (137 mM NaCl/5 mM KCl/25 mM Tris-HCl/0.5 mM $MgCl_2$/0.9 mM $CaCl_2$, pH 7.4) containing glucose (1 mg/ml). The cells were finally suspended at a cell density of $(2–4) \cdot 10^7$/ml in the phosphate- and calcium-free Eagle's minimal essential medium containing 10 mM Hepes, 4 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin.

Lipid analysis. Incubations were terminated by addition of 1 ml 10% (w/v) trichloroacetic acid to each of the cell suspensions (0.5 ml). Lipids in the acid precipitate were extracted with 2 ml chloroform/methanol/concentrated HCl (200 : 100 . 1, v/v) and the extract was back-washed [13]. Carrier phosphatidic acid, phosphatidylserine, phosphatidylinositol, and CDP-diacylglycerol (a kind gift from Dr. Akamatsu) were added to each extract. Phospholipids were separated by two-dimensional TLC on a silica gel H plate impregnated with magnesium acetate [14]. Chloroform/methanol/28% ammonia/water (130 70 : 10 : 2, v/v) and chloroform/methanol/acetic acid/water (80 : 40 · 7.4 : 1.2, v/v) were used as the developing solvents in the first and second dimensions, respectively. Lipids separated on TLC plates were located by iodine vapor. Each located area was scraped into a vial, the sample was mixed with 0.3 ml water and 5 ml ACS II (Amersham), and radioactivity was determined with a Beckman LS-9000 liquid scintillation spectrometer.

[^{3}H]Thymidine incorporation into lymphocyte DNA. Cultures of $6.5 \cdot 10^6$ rat lymph-node cells were made in 0.5 ml Eagle's minimum essential medium (for suspension culture) containing 10% fetal calf serum and various concentrations (0–200 μg/ml) of concanavalin A. After 24 h of culture, the media were changed similar to those used in the start of the cultures. Tubes were pulsed with 2 μCi [*methyl*-^{3}H]-thymidine between 45 and 49 h. Incorporation of the radioactivity into trichloroacetic acid-insoluble materials was determined.

Results

The $^{32}PO_4$ incorporations into phosphatidylinositol and phosphatidic acid of rat lymphocytes were markedly stimulated by concanavalin A (Table I). The stimulation was dose dependent at least up to 200 μg lectin/ml culture. The amounts of the $^{32}PO_4$ incorporations at 15 and 30 min after the lectin addition indicate that no time lag was present in the stimulation. At the concanavalin A concentration of 20 μg/ml, the enhancement of the incorporations was about 10-fold into phosphatidylinostiol and about 8-fold into phosphatidic acid. The effect of concanavalin A on the lipid metabolism of lymphocytes had a specificity; the $^{32}PO_4$ incorporations into phosphatidylcholine and phosphatidylethanolamine were not affected by the lectin. A small stimulation was found in the incorporation into phosphatidylserine. The marked increase in the $^{32}PO_4$ incorporation into phospholipids in concanavalin A-stimulated lymphocytes does not accompany a gross increase in

TABLE I

EFFECT OF VARIOUS AMOUNTS OF CONCANAVALIN A ON THE $^{32}PO_4$ INCORPORATION INTO PHOSPHOLIPIDS OF RAT LYMPH-NODE CELLS

The incubation was started by addition of [^{32}P]phosphate to a lymphocyte suspension (2 10^7 cells/ml) at 50 μCi/ml. After 30 min of incubation at 37°C in an atmosphere of 5% CO_2/95% air, 0.5 ml-portions of the cell suspension were transferred to test tubes containing various amounts (0–100 μg) of concanavalin A in 20 μl 0.9% NaCl. The incubation was continued for an additional 15 or 30 min. The $^{32}PO_4$ incorporations into individual classes of phospholipids in 10^7 cells are shown by a relative ratio. The ratio was calculated by dividing the radioactivity (cpm) in each phospholipid with the radioactivity in phosphatidylcholine at 30 min of incubation (141 ± 2 cpm). Results are presented as the average of an experiment performed in duplicate and are representative of three trials

Phospholipids	Incubation time (min)	Incorporated radioactivity (relative ratio) in the presence of the indicated amount of concanavalin A (μg/ml)				
		0	0.2	2	20	200
Phosphatidylcholine	30	1.00				
	45	2.54	2.64	2.62	2.86	2.87
	60	4.34		4.45	4.74	5.24
Phosphatidylserine	30	0.12				
	45	0.33	0.18	0 52	1 06	2.27
	60	0.39		0.49	1.28	1.41
Phosphatidic acid	30	3.42				
	45	4.90	4.88	7.28	16.70	19.23
	60	5 86		10.24	21 99	22.58
Phosphatidylinositol	30	3 68				
	45	5.31	6.12	9.24	21 19	50.23
	60	8 03		18.65	48.82	89 60
Phosphatidylethanolamine	30	0 73				
	45	1 65	1.95	1.60	1.80	1.64
	60	1.91		1.91	1.99	2.20

the amount of lymphocyte phospholipid because a concanavalin A-dose dependent increase was also observed in the specific radioactivity of the total phospholipid; the increases at 15 min after the lectin addition were 2.3-fold at 2 μg/ml, 4.3-fold at 20 μg/ml, and 8.6-fold at 200 μg/ml.

The dose responses of concanavalin A for blast transformation as judged by [^{3}H]thymidine incorporation into DNA were examined by the use of the same concanavalin A preparation as was used in the experiments shown in Table I. The thymidine incorporation and the $^{32}PO_4$ incorporation shown in Table I were examined at a comparable cell density. As previously described [6–8], the [^{3}H]thymidine incorporation was optimal at 2 μg concanavalin A/ml culture. At doses higher than 5 μg/ml, the [^{3}H]thymidine incorporation was inhibited in a dose-dependent manner; at 200 μg concanavalin A/ml, the incorporation was 1/46th of that observed at 2 μg/ml. Therefore, the lymphocyte PI response was induced by concanavalin A at doses of the lectin in which the blast transformation as measured by increases in DNA synthesis is profoundly inhibited by the lectin.

Microtubule-disrupting drugs, colchicine and vinblastine, at a concentration of 10^{-4} M did not have an obvious effect on the lymphocyte PI response induced by concanavalin A (Table II). Vinblastine sulfate had several noticeable effects on the basal, unstimulated, phospholipid metabolism of rat lymph-node cells as revealed by the $^{32}PO_4$ incorporation, the drug increased the $^{32}PO_4$ incorporation into the total phospholipids by 2.7-fold; the increased incorporation reflect the 4–9-fold increases in the incorporations into acidic phospholipids, whereas the incorporation into phosphatidylcholine was 1/4th of the control value.

TABLE II

EFFECTS OF MICROTUBULE-DISRUPTING DRUGS AND CYTOCHALASINS B AND D ON THE $^{32}PO_4$ INCORPORATION INTO PHOSPHOLIPIDS OF CONCANAVALIN A-STIMULATED AND UNSTIMULATED RAT LYMPH-NODE CELLS

The incubation was started by addition of [^{32}P]phosphate to a lymphocyte suspension ($4 \cdot 10^7$ cells/ml) at 50 μCi/ml. After 30 min of incubation at 37°C in an atmosphere of 5% CO_2/95% air, 0.5 ml-portions of the cell suspension were transferred to test tubes containing either 10 μl 0 9% NaCl (marked by –) or 50 μg concanavalin A in 10 μl 0.9% NaCl (marked by +), in addition, the tubes contained either 50 nmol colchicine in 10 μl water, or 50 nmol vinblastine sulfate in 10 μl water, or 5 nmol cytochalasin B in 10 μl dimethyl sulfoxide/water (1 9, v/v), or 5 nmol cytochalasin D in 10 μl dimethyl sulfoxide/water (1 9, v/v) as indicated in the table. The incubation was continued for an additional 30 min. The $^{32}PO_4$ incorporations into individual classes of phospholipids in $2 \cdot 10^7$ cells during the last 30 min of the incubation are shown by a relative ratio. The ratio was calculated by dividing the radioactivity (cpm) incorporated into each phospholipid during the second half of the incubation with the radioactivity incorporated into phosphatidylcholine during the second half of the incubation in the absence of both concanavalin A and the drugs (680 cpm) Increases in the specific radioactivity of total phospholipids during the second half of the incubation are shown as cpm/nmol of phospholipid phosphorus. Results of one experiment are shown and are representative of three trials Con A, concanavalin A, S.A , specific radioactivity.

Reagents added	Con A addition	$^{32}PO_4$ incorporation (relative ratio)					Increase in S A of total phospholipids (cpm/nmol)
		Phosphatidylcholine	Phosphatidylinositol	Phosphatidic acid	Phosphatidylserine	CDPdiacylglycerol	
None	–	1 00	0.65	0.66	0.19	0.51	38
None	+	1 23	12 4	4.71	1.53	1.22	240
Colchicine	–	1.38	1 15	1.44	–0.52	–0 80	61
Colchicine	+	1 32	12 3	4.48	–0.42	–0.21	226
Vinblastine	–	0 24	2.88	2.53	1.70	2 13	104
Vinblastine	+	0.27	15.6	8.38	0.75	0.57	234
Cytochalasin D	–	1 06	2.56	2 66	0 05	0.37	76
Cytochalasin D	+	1 18	18 6	3.78	0 23	0.40	232
Cytochalasin B	–	0.74	0 92	0.21	0 63	2.03	17
Cytochalasin B	+	0.80	18 2	2.43	1.41	0.61	228

Microfilaments perturbants, cytochalasins B and D, at a concentration of 10^{-5} M increased the lymphocyte PI response by 1.5-fold (Table II). Cytochalasin D affected the basal phospholipid metabolism of lymphocytes; the drug increased the $^{32}PO_4$ incorporation into the total phospholipids by 2-fold; the increased incorporation reflect about 4-fold increase in the incorporations into phosphatidylinositol and phosphatidic acid. Addition of dimethyl sulfoxide at the same concentration as in the cytochalasin D experiment, 0.2% in the final concentration, did not affect the concanavalin A-stimulated and unstimulated $^{32}PO_4$ incorporations into phospholipids. Cytochalasin B decreased the $^{32}PO_4$ incorporation into the total phospholipids of unstimulated lymphocytes to about half of the control value; this observation can probably be explained by the effect of the drug as an inhibitor of hexose transport [15–17]; the decrease was evident in the incorporations into phosphatidic acid and phosphatidylcholine.

Discussion

Binding of concanavalin A to the lymphocyte surface induces a number of biochemical processes at the membrane level the PI response [1], methylation of phosphatidylethanolamine [8], activation of lysolecithin acyltransferase [18], increase in the membrane microviscosity [19], modulation of mobility and distribution of cell surface receptors [2,3], and activation of the transport of amino acids [20], sugars [21], and small ions [22]. The relationship between these early events and the processes leading to blast transformation is still obscure. It was reported that the PI response was induced by mitogenic lectins but not by nonmitogenic lectins

[23]. However, bacterial lipopolysaccharide and Pa-1 pokeweed mitogen, both B cell mitogens, did not induce the PI response in mouse B lymphocytes [19,24].

We found that the PI response induced by concanavalin A in rat lymph-node cells was dose dependent up to 200 μg lectin/ml culture (Table I), whereas the same lectin preparation inhibited the [^{3}H]thymidine incorporation in a dose-dependent manner at doses higher than 5 μg/ml as previously described [6–8]. Therefore, concanavalin A produced the PI response in the dose range in which the mobility and distribution of cell surface receptors were modulated by the lectin. Our result suggests that the magnitude of the lymphocyte PI response is dependent on the amount of concanavalin A receptors occupied by the lectin. It was reported that binding of ^{125}I-labeled concanavalin A to mouse spleen cells ($2 \cdot 10^7$ cells/ml) was saturated at 50 μg lectin/ml [2].

Concanavalin A and other mitogenic lectins rapidly stimulate methylation of phosphatidylethanolamine in mouse spleen T-lymphocytes [8]. A parallelism between dose-response curves of concanavalin A for the phospholipid methylation and thymidine incorporation was reported [8]; high doses of the lectin were inhibitory for the methylation just as those were for the DNA synthesis.

From the analysis of the effect of anchorage-modulating doses of concanavalin A on lymphocyte mitogenesis, McClain and Edelman [9] proposed that "any measured biochemical result of lectin binding that shows a concanavalin A dose-response curve with a falling limb at high lectin concentrations is probably a later event not in the initial stimulatory pathway". When we consider the two early membrane events induced by concanavalin A binding to the lymphocyte surface, the phospholipid methylation [8] and the PI response, the PI response can be an earlier event because the concanavalin A dose-response curve for the PI response does not have the falling limb at high lectin concentrations whereas that for the methylation has the falling limb [8]. The initial event in the PI response of rat lymph-node cells, a rapid loss of the pre-existing phosphatidylinositol accompanied by accumulations of diacylglycerol and phosphatidic acid, was found at 1–5 min after the concanavalin A addition to the cells (Hasegawa-Sasaki, H. and Sasaki, T., unpublished results). The activation of the phospholipid methylation had been reported to have a peak at 10 min after the concanavalin A addition [8].

Colchicine, vinblastine and related drugs inhibit the mitogenic stimulation of lymphocytes from various species by lectins such as concanavalin A [5,25,26]. Based on the analyses of the colchicine blockade, Edelman et al. [5,6,26–28] advanced the hypothesis that cytoplasmic microtubular proteins are somehow implicated in the regulation of early biochemical event that commits resting lymphocytes to undergo blast transformation and DNA synthesis. Colchicine and vinblastine at a concentration of 10^{-4} M, a concentration in which a marked inhibition of the concanavalin A-induced mitogenic response had been observed [26], did not inhibit the concanavalin A-induced PI response of rat lymph-node cells (Table II). Schellenberg and Gillespie [29] reported that in human peripheral blood lymphocytes, colchicine and vinblastine inhibited the enhanced incorporations of *myo*-[^{3}H]inositol and [^{32}P]phosphate into phosphatidylinositol and phosphatidic acid caused by concanavalin A. According to these authors' results, these drugs did not inhibit completely; a marked, although diminished, stimulation of the $^{32}PO_4$ incorporation into phosphatidylinositol was reported to occur in the presence of 10^{-4} M colchicine or vinblastine. Schellenberg and Gillespie [29,30] mainly used *myo*-[^{3}H]inositol to assess the PI response, it should be noted that the use of labeled inositol to measure phosphatidylinositol synthesis is unreliable because it appears to reflect the activity of the freely reversible CDP-diacylglycerol inositol phosphatidyltransferase [31,32]. The discrepancy between the results reported by Schellenberg and Gillespie and the results obtained by us may possibly originate from the difference in the effects of colchicine and vinblastine on the PI responses of human peripheral blood lymphocytes and rat lymph-node cells.

Cytochalasins affect the mitogenic stimulation of lymphocytes [33–36]. Relatively low concentrations (10^{-8}–10^{-7} M) of cytochalasin B enhances, whereas higher concentrations (above 10^{-6} M) of cytochalasin B inhibits the mitogenic response to concanavalin A and to other mitogens. Cytochalasin D was about 10-times as potent as cytochalasin B as an inhibitor of concanavalin A-induced lymphocyte proliferation

[36]. From the analysis of cytochalasin-binding sites of lymphocytes, Mookerjee et al. [36] proposed that cytoskeletal actin plays an important role in the modulation of lymphocyte mitogenesis. Cytochalasins B and D at a concentration of 10^{-5} M, a concentration in which 100% inhibition of the concanavalin A-induced mitogenic response had been observed [36], enhanced to some degree the PI response of rat lymph-node cells induced by concanavalin A (Table II).

Stimulation of the PI response of rat lymph-node cells by anchorage-modulating doses of concanavalin A and lack of inhibitory effects of both microtubule-disrupting drugs and microfilament perturbants, cytochalasins, on the PI response all suggest that submembranous assemblies of microtubules and microfilaments do not play an indispensable role in the sequence of events involved in the PI response of rat lymph-node cells. Although the results suggest that the PI response is the earliest event induced by concanavalin A binding to lymphocytes, it remains to be determined whether the PI response is an essential early event involved in the blast transformation of T lymphocytes.

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