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## Gamma-hexachlorocyclohexane inhibits the initiation of lymphocyte growth by phytohemagglutinin*

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THE INITIATION of growth in cultured human lymphocytes by phytohemagglutinin (PHA) is characterized by a rapid acceleration of phosphatidylinositol turnover.[1] Hours are required for the stimulation of lymphocyte RNA synthesis,[2] more than a day for the initiation of DNA synthesis,[3] and the growth which the increased nucleic acid synthesis reflects.[4,5] The acceleration of phosphatidylinositol turnover, however, occurs within minutes after exposure of lymphocytes to PHA. To determine the relationship between this early alteration of phosphatidylinositol metabolism and the induction of lymphocyte growth by PHA, we examined the effect of preventing the phosphatidylinositol response on the ensuing growth response. Gamma-hexachlorocyclohexane (gammexane, the hexachlorocyclohexane isomer with the same configuration as *muco*-inositol[6]) has been shown by Hokin and Brown[7] to inhibit selectively the increase in $^{32}P_i$ labeling of phosphatidylinositol produced by acetylcholine treatment of brain slices. The present report demonstrates that gammexane blocks the initial acceleration of phosphatidylinositol turnover by PHA. Gammexane also prevents the stimulation of [$^3$H]-cytidine and [$^3$H]thymidine incorporation, which reflects the induction of RNA and DNA synthesis. These findings support the view that the stimulation of phosphatidylinositol turnover is related to the induction of growth by PHA.

Human peripheral lymphocytes were prepared as described previously,[1] except that heparin was added to the blood to give a final concentration of 8 units/ml. The lymphocytes (1 to 2 × $10^6$/ml) were suspended in a modified Eagle's HeLa medium (EHM) which contained 7% bovine serum. The suspension was incubated 1–2 days at 37° under an atmosphere of 95% air : 5% $CO_2$ prior to beginning

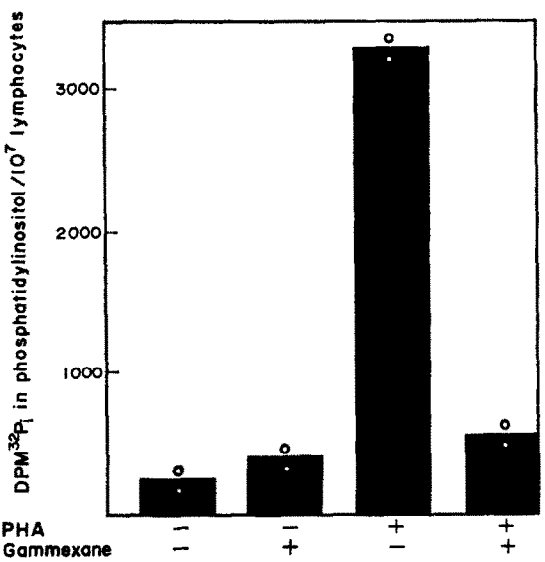


FIG. 1. Effect of gammexane on $^{32}P_i$ incorporation into phosphatidylinositol in control and PHA-treated lymphocytes. Cultures containing 6 × $10^6$ lymphocytes in 3 ml of phosphate-free Eagle's HeLa medium were incubated 1 hr. Then 200 $\mu$c $^{32}P_i$ was added to each culture and, after 50 min of additional incubation, 10 $\mu$l of 0·3 M gammexane in dimethylsulfoxide (DMSO) or 10 $\mu$l DMSO alone was added to cultures. After 10 min of further incubation, PHA was added to appropriate cultures.

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experiments. During this incubation period, polymorphonuclear leukocytes die. After 1 day, the nucleated cells in the suspension were greater than 99 per cent lymphocytes. There still remained, however, 2–3 red blood cells per lymphocyte. For $^{32}P_i$ (carrier-free, E. R. Squibb & Sons, New York) incorporation studies, cells were washed with 0·9% NaCl and resuspended in 2- to 3-ml aliquots of phosphate-free EHM (3–5 × $10^6$/ml). Cell suspensions were incubated 30 min at 37°, $^{32}P_i$ (50–100 μc/ml) was added, and 1 hr later PHA (70 μg/ml, Phytohemagglutinin M, Difco, Detroit, Mich.) was added. The individual phospholipids were isolated and their radioactivity was determined as described previously,[1] except that the cells were washed with 5 mM $MgCl_2$ instead of 0·09% NaCl.

For a study of glucose utilization, the lymphocytes (20 × $10^6$/ml) were resuspended in 1-ml aliquots of glucose-free EHM in Warburg flasks. [$^{14}$C]D-glucose, uniformly labeled (1·5 μc/flask, 3·0 mc/mmole, New England Nuclear Corp., Boston), gammexane, and PHA were tipped in from the side arm to begin the incubation. After 1 hr, the cell suspension was acidified with 0·15 ml of 0·5 N $HClO_4$ and the radioactivity of the various fractions was measured.

The effect of gammexane on $^{32}P_i$ incorporation into phosphatidylinositol in the presence and absence of PHA was determined. As shown in a representative experiment (Fig. 1), 1 mM gammexane abolishes the 30-fold stimulation of $^{32}P_i$ incorporation into phosphatidylinositol which is characteristic of the early action of PHA. In three experiments gammexane inhibited this phospholipid response by 85 per cent (±7·5%, S.E.M.).

To assess the specificity of gammexane inhibition, the beta-isomer of hexachlorocyclohexane was tested. This isomer at 1 mM had no effect on the control or PHA-stimulated rate of $^{32}P_i$ incorporation into phosphatidylinositol.

To examine the possibility that gammexane inhibited the phospholipid response by interfering with energy production, its effect on glucose utilization was assessed. A 1-hr treatment of lymphocytes with PHA stimulated the conversion of [$^{14}$C]glucose into $CO_2$ (20%), into acids in the medium (130%, presumably lactic and pyruvic acid),[8] and into total lipids (50%, Table 1). Since it did not substantially inhibit these processes, it appears unlikely that gammexane interferes significantly with energy production in the PHA-stimulated lymphocytes.

The effect of gammexane on the ability of PHA to stimulate RNA synthesis and initiate DNA synthesis was tested using the incorporation of [$^3$H]cytidine and [$^3$H]thymidine respectively. [$^3$H]-cytidine incorporation actually measures increased cytidine kinase as well as RNA synthesis.[9] Gammexane (0·2 mM) completely prevented the PHA stimulation of cytidine incorporation while

TABLE 1. EFFECT OF GAMMEXANE ON [$^{14}$C]GLUCOSE UTILIZATION BY PHA-TREATED LYMPHOCYTES*

| | dis./min/$10^7$ Lymphocytes | | |
|---|---|---|---|
| Sample | $^{14}CO_2$ | [$^{14}$C]lipid | [$^{14}$C]acids in medium |
| No PHA | 6075† | 97 | 1515 |
| PHA | 7325 | 147 | 3445 |
| PHA + 1 mM gammexane | 6675 | 197 | 4470 |

* Stoppered Warburg flasks containing 20 × $10^6$ lymphocytes in 1·5 ml of glucose-free Eagle's HeLa medium were incubated 1 hr with 1·5 μc [$^{14}$C]D-glucose (uniformly labeled) ± PHA ± gammexane (10 μl of 0·15 M gammexane in DMSO or 10 μl DMSO alone). Reaction was terminated by addition of $HClO_4$ (final concentration, 0·5 N). $^{14}CO_2$ was collected on Hyamine hydroxide-containing filter paper placed in center well. The radioactivity was determined by liquid scintillation spectrometry. [$^{14}$C]lipid was obtained by extracting the acid-insoluble fraction with 1:1 ethanol–diethyl ether. $^{14}$C-labeled acids were removed from the media with Dowex-1-Cl. Radioactivity of the latter two fractions was measured by a gas-flow low background counter.

† Values are means of duplicate determinations.

TABLE 2. EFFECT OF GAMMEXANE ON [$^3$H]CYTIDINE AND [$^3$H]THYMIDINE INCORPORATION

| Sample | [$^3$H]cytidine incorporation* (counts/min ± S.E.M.) |
|---|---|
| No PHA | 5250 ± 300 |
| No PHA + 0·2 mM gammexane | 4770 ± 385 |
| PHA | 7550 ± 248 |
| PHA + 0·2 mM gammexane | 4780 ± 112 |

| Sample | [$^3$H]thymidine incorporation† (counts/min) |
|---|---|
| No PHA | 55 |
| No PHA + 0·04 mM gammexane (0 hr) | 56 |
| PHA | 4325 |
| PHA + 0·04 mM gammexane (0 hr) | 2584 |
| PHA + 0·04 mM gammexane (24 hr) | 5518 |

* Gammexane was added to triplicate 1-ml cultures ($4 \times 10^6$ lymphocytes) 10 min before PHA, and 2 $\mu$c [$^3$H]5-cytidine (6 c/m-mole, Schwarz, Orangeburg, N.Y.) was added at the time of PHA addition. Four hr later, cells were precipitated and washed with 5% trichloroacetic acid, then with 1:1 ethanol–ether. The residue was dissolved in formic acid and radioactivity was determined by liquid scintillation spectrometry.

† Two $\mu$c [$^3$H]Me-thymidine (110 mc/m-mole, Schwarz) was added to duplicate 3-ml cultures ($3 \times 10^6$ lymphocytes) 24 hr after PHA. Gammexane was added either 10 min before (0 hr) or 24 hr after PHA (24 hr). At 36 hr, radioactivity was determined in same manner as that described above.

affecting control cultures (Table 2) only minimally. Gammexane (0·04 mM) markedly inhibited [$^3$H]thymidine incorporation (40 per cent) when added just prior to PHA, but had little effect on control cultures. Gammexane actually stimulated DNA synthesis by 30 per cent in PHA-treated cultures when added 24 hr after the mitogen.

In contrast to the activity of the gamma-isomer, beta-hexachlorocyclohexane (1 mM) did not affect PHA-stimulated [$^3$H]thymidine incorporation. Thus, the isomer specificity displayed in the inhibition of the growth and the phospholipid responses to PHA is identical. These results support the conclusion that the acceleration of phosphatidylinositol turnover is involved in the initiation of growth in cultured human lymphocytes by PHA.

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## Fatty acids of liver mitochondrial and microsomal lipids in the rat exposed to phenothiazine derivatives

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DERIVATIVES of phenothiazine, e.g. chlorpromazine (2-chloro-10 [3-dimethylaminopropyl] phenothiazine) (CPZ) and prochlorperazine (2-chloro-10-[3-(-methyl-4-piperazinyl) -propyl] phenothiazine) (PCP) are major tranquilizers and can produce the symptoms of central nervous system depression in the rat. Although the specific site(s) of action of these drugs is not known, evidence by several workers suggests that they may directly affect the components of membranes.[1–3] Morphological changes have been described in liver and brain mitochondria of rats and monkeys following the administration of prochlorperazine.[4] The results indicated that a biochemical lesion may have been produced in the metabolism of the lipid components of these organelles. To examine the possibility that phospholipids are involved, gas–liquid chromatography was used to determine the effect of chlorpromazine and prochlorperazine *in vivo* on the proportions of fatty acids in the lipids of rat liver mitochondrial and microsomal fractions and on the fatty acids of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of the mitochondrial fraction.

The procedures employed were as follows: Male albino rats (Wistar strain, Woodlyn Farms, Guelph, Ontario, weighing 170–190 g), pre-adapted for 14 days to a purified diet containing 20% casein, 20% corn oil, 50% sucrose, 4% salt mixture, U.S.P. XIV, 1% vitamin mixture, and 5% alphacel, were injected intraperitoneally with a saline solution providing 5 mg/100 g body weight of chlorpromazine hydrochloride (Poulenc Limited, Montreal) or prochlorperazine ("Stemetil," Poulenc Limited, Montreal). At 4 and 24 hr after drug injection, groups of three animals were killed by decapitation and the livers rapidly removed. With a Dounce homogenizer, a 10% homogenate of liver tissue was prepared in 0·25 M sucrose (containing $10^{-3}$M EDTA) from which the mitochondrial and microsomal fractions were separated by differential centrifugation.[5] The lipid was removed from each pelleted sub-cellular fraction by double extraction with water–methanol–chloroform (0·8 : 2 : 1, by vol.) and separation of the chloroform phase according to the method of Bligh and Dyer.[6] Fatty acid analyses of the total mitochondrial and microsomal lipid extract and of lecithin and phosphatidylethanolamine of the mitochondrial fraction were performed by gas–liquid chromatography.[7] Phospholipids were separated by thin-layer chromatography on silica gel H in a solvent system consisting of chloroform–methanol–glacial acetic acid–water (99 : 60 : 15 : 5, by vol.). One ml of butylated hydroxytoluene (BHT) in chloroform (9 mg/ml) was added as an antioxidant to the solvent to give a final concentration of 0·005%.[8] The phospholipid distribution in the liver mitochondrial fractions was estimated from the amount of lipid phosphorus in each phospholipid spot measured according to Bartlett[9] as modified by Parker and Peterson.[10]

The relative proportions (weight per cent) of major fatty acids of the liver mitochondrial and microsomal fractions from control and CPZ or PCP-treated rats were compared at 4 and 24 hr after drug injection (Table 1). In animals exposed to CPZ or PCP, the proportion of linoleic acid (18 : 2) was found to be significantly higher ($P = 0·05$) at 4 hr in the mitochondrial and microsomal fractions. Concurrently, the proportion of arachidonic acid in animals exposed to the drugs tended to decrease. These effects were no longer evident 24 hr after drug treatment. The differences in proportions of fatty