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γ-HEXACHLOROCYCLOHEXANE STIMULATION OF MACROPHAGE PHOSPHOLIPID HYDROLYSIS AND LEUKOTRIENE PRODUCTION

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Abstract—γ-Hexachlorocyclohexane stimulates arachidonic acid release from macrophage phospholipids. It is also a powerful stimulator of leukotriene C_4 production, yet (by comparison with zymosan) produces only a small effect on prostaglandin production. This suggests that synthesis of leukotrienes and prostaglandins can be independently regulated. The most likely mechanism of action of γ-hexachlorocyclohexane is through its effect on phosphatidylinositol metabolism.

Activation of macrophages by various stimuli is associated with increased turnover of phosphatidylinositol, increased release of arachidonic acid from its esters, and increased production of arachidonic acid metabolites such as leukotrienes and prostaglandins [1–3]. Turnover of phosphatidylinositol can occur via a cycle in which the intermediate compounds are diacylglycerol, phosphatidic acid and CDP-diacylglyceride. The final step in this cycle, combination of CDP-diacylglyceride with inositol to reform phosphatidylinositol, can be inhibited in brain tissue by γ-hexachlorocyclohexane [4]. In agreement with a similar action in macrophages, we have found this compound to inhibit [^{3}H]inositol incorporation into phosphatidylinositol and to cause accumulation of [^{14}C]glycerol-labelled phosphatidic acid. We have therefore investigated γ-hexachlorocyclohexane as a novel modulator both of arachidonic acid release from macrophage phospholipids, and of production of arachidonic acid metabolites.

MATERIALS AND METHODS

Materials. γ-Hexachlorocyclohexane was purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). β-Hexachlorocyclohexane was purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.). For addition to cultures they were dissolved at 200 mM in dimethylformamide and an appropriate quantity of this dimethylformamide solution was added to the culture medium with vigorous mixing. Defatted serum albumin and zymosan A were also purchased from Sigma. The zymosan, from *Saccharomyces cerevisiae*, was boiled 30 min in distilled water, then washed once prior to use. The following were purchased from Amersham International (Amersham, Bucks, U.K.): [1-^{14}C]arachidonic acid, sp. act. 55 Ci/mole (item CFA 504); [2-^{3}H]inositol, sp. act. 3900 Ci/mole (item TRK 317); [2-^{14}C]glycerol, sp. act. 30 Ci/mole (item CFA 302). Phosphatidic acid, β-[1-^{14}C]arachidonyl-α-stearoyl was prepared by digestion of L-α-phosphatidylcholine-β-[1-^{14}C]arachidonyl-α-stearoyl (Amersham, item CFA 655) with phospholipase D [5]. The specific activity of the original labelled phosphatidylcholine was 59 Ci/mole. Eagles MEM was purchased from Flow Laboratories. It was supplemented with 292 mg/l glutamine, 100,000 units/l penicillin and 2.2 g/l sodium bicarbonate prior to use. Foetal calf serum was purchased from Sera Lab. It was inactivated by heating 30 min at 56° prior to use. Lactalbumin hydrolysate came from Koch Light Laboratories. Phospholipid separations were made by high-pressure liquid chromatography (HPLC) using Spherisorb S5W column packing from Phase Separations (Queensferry, Clwyd, Wales, U.K.). The Techsphere 5 C18 packing, used for leukotriene separation, was purchased from HPLC Technology (Wilmslow, Cheshire, U.K.). Silica gel-60 plates for thin-layer chromatography (TLC), thickness 0.25 mm, were from Merck (item 5721). For HPLC, all solvents (except water) were HPLC grade (Fisons). They were degassed and filtered through 0.5 μm solvent-resistant filters (Millipore, catalogue No. FHLP 04700). Deionized water for HPLC was filtered through Millipore CSWP 04700 filters. For work other than HPLC, 'Analar' grade solvents were employed. Most phospholipid standards were purchased from Lipid Products (South Nutfield, Surrey, U.K.). Lysophosphatidic acid was purchased from Serdary Research Laboratories (London, Ontario, Canada). HETE standards were a kind gift from Miss Hazel Pickett. Prostaglandin E_2 and $F_{2\alpha}$ standards were purchased from Sigma; prostaglandin 6oxo$F_{1\alpha}$ and thromboxane B_2 came from Upjohn Co. (Kalamazoo, MI). Lactate dehydrogenase was assayed using the 'Statzyme' kit produced by Worthington Diagnostics (Freehold, NJ). Spray-dried *Micrococcus luteus* for lysozyme assay was purchased from Miles Laboratories (Elkhart, IN).

Animals. Adult female MF1 outbred mice were purchased from Olac Ltd. (Bicester, Oxon, U.K.).

1. *Assay for arachidonic acid release following γ-hexachlorocyclohexane stimulation.* Resident cells

were lavaged from the peritoneum of 50–100 mice, washed and cultured at 37° in a humidified atmosphere of 5% CO_2. Aliquots (2.4 ml) were cultured in 50 mm diameter petri dishes at 2×10^6 cells/ml in Eagles MEM supplemented with 15% heat-inactivated foetal calf serum. After 18 hr non-adherent cells were poured off and the remaining adherent cells were incubated for 1 hr with 2.4 ml Eagles MEM + 0.2% lactalbumin hydrolysate containing 1 μCi [^{14}C]arachidonic acid added as 5 μl of an ethanolic solution. At the end of this period, plates were washed once with MEM + 1% defatted bovine serum albumin, then cultured for 4 hr at 37° in the same medium containing appropriate concentrations of γ-hexachlorocyclohexane added in dimethylformamide (final concentration 1.25 μl/ml). By binding released [^{14}C]arachidonic acid, defatted albumin prevents re-incorporation into cell lipids. Activity in all washings was measured, from which activity in the cells at the beginning of the 4 hr culture period was calculated. Activity released over 4 hr was measured in supernatants spun for 5 min at 400 *g* to remove cell debris. Control experiments showed that dimethylformamide alone had no significant effect on arachidonic acid release at 1.25 μl/ml.

Experiments with β-hexachlorocyclohexane followed a similar protocol.

2. *Assay for [2-^{14}C]glycerol incorporation into phosphatidic acid.* Resident murine peritoneal cells were obtained and set up in culture as above, but a different technique for prelabelling the cells was employed. Cell monolayers were incubated with Eagles MEM + 0.2% lactalbumin hydrolysate for 5 hr (rather than 1 hr) and 1.2 μCi/dish [2-^{14}C]glycerol was present in place of arachidonic acid. The labelled glycerol was washed away before addition of γ-hexachlorocyclohexane. The period of contact with γ-hexachlorocyclohexane was reduced to 20 min, at the end of which cells adhering to the dish were extracted with 2 × 2.5 ml aliquots $CHCl_3$–MeOH, 2:1 (v/v). Phosphatidic acid (0.1 mg) was added as carrier. The extracts were washed twice with 3 ml 0.5 M $MgCl_2$, blown down under nitrogen, and taken up successively in chloroform and methanol for spotting onto oxalate-coated TLC plates. These plates were prepared by briefly soaking standard TLC plates in 0.5 M oxalic acid and heating them for 60 min at 110° immediately prior to use. Using the solvent system $CHCl_3$–MeOH–10 N HCl (87:13:0.5, v/v), approximate R_f values were: phosphatidic acid, 0.55; lysophosphatidic acid, 0.40; other phospholipids, 0.05–0.15. Plates were exposed to iodine vapour to reveal location of the phosphatidic acid spot, and radioactivity was determined using a quantitative thin-layer plate scanner (Berthold). Efficiency of extraction of phosphatidic acid was checked by 'spiking' unlabelled cells with known amounts of ^{14}C-labelled phosphatidic acid.

3. *Assay for [2-^{3}H]inositol incorporation.* Cells were cultured as in (1) above, except that in place of [^{14}C]arachidonic acid, the cells were incubated with 0.4 μCi [2-^{3}H]inositol during the 4 hr incubation in the presence of defatted serum albumin. After 4 hr, the cells were washed, and phosphatidylinositol was extracted as described elsewhere [1]. Radioactivity was determined by scintillation counting. HPLC on a small number of extracts was used to confirm that label incorporated into cells was indeed in phosphatidylinositol. In some experiments another control was included. Uptake of label from the medium was monitored to determine whether γ-hexachlorocyclohexane had any effect on [^{3}H]inositol uptake by cells, in addition to any effect on incorporation into phosphatidylinositol.

4. *Identification of phospholipid classes from which labelled arachidonic acid is released.* After culture, exudate cell monolayers were extracted with $CHCl_3$–MeOH (2:1, v/v). Extracts were washed twice with 0.5 M $MgCl_2$, and blown down under N_2. The major classes of phospholipids were separated by HPLC using a modification of the method described by Hax and Guerts van Kessel [6]. The stationary phase was Spherisorb S5W, the moving phase hexane–isopropyl alcohol–H_2O in ratios varying between 6:8:0.9 and 6:8:1.0, v/v. Column dimensions were 4.9 mm diameter ×25 cm length. The flow rate was 2 ml/min. Position of peaks was identified by absorption at 207 nm.

5. *Identification of arachidonic acid metabolites.* Cells were cultured as described in (1) above. After the final 4 hr culture period, the spun supernatant medium was acidified to pH 3 with 0.2 M citric acid, extracted with ethyl acetate and then run on silica gel-60 in the system toluene–dioxan–glacial acetic acid (70:30:1.5, v/v). Appropriate R_f values were: prostaglandin $F_{2\alpha}$/prostaglandin 6oxo$F_{1\alpha}$, 0.11; prostaglandin E_2, 0.17; thromboxane B_2, 0.23; 5,12-diHETE, 0.40; 5-HETE, 0.57; other HETEs, 0.63; arachidonic acid, 0.78. Leukotrienes were not extracted by ethyl acetate and were therefore assayed by a separate method.

6. *Production and isolation of leukotrienes.* Leukotrienes were produced as described elsewhere [7]. They were separated and fractionated as described by Osborne *et al.* [8]. The final stage of the fractionation was by HPLC. The best HPLC separations were obtained with a Techsphere OD5 C18 reverse-phase column (4.9 mm × 12.5 cm) with eluting solvent MeOH–H_2O–glacial acetic acid (65:35:0.06, v/v, pH 5.3). Flow rate was 1 ml/min. Peaks were detected by absorption at 280 and 237 nm. Fractions were assayed for biological activity by contraction of guinea-pig ileum. The ileum was suspended in oxygenated Tyrode's solution supplemented with 10^{-6} M methysergide and 10^{-6} M mepyramine and held at 37°.

7. *Assays for cell viability.* In some experiments, the macrophage monolayers used for [^{14}C]arachidonic acid release measurements were disrupted with 0.05% Triton X-100 at the end of the experiment and, in addition to arachidonic acid release, lactate dehydrogenase (LDH) and lysozyme were measured in both cell disruptates and supernatants. LDH was assayed photometrically by reduction of NAD [9]. Lysozyme was assayed by lysis of a suspension of *Micrococcus luteus* [10].

RESULTS

γ-Hexachlorocyclohexane was a powerful stimulus for arachidonic acid release from macrophage membrane phospholipids. It was active at concentrations

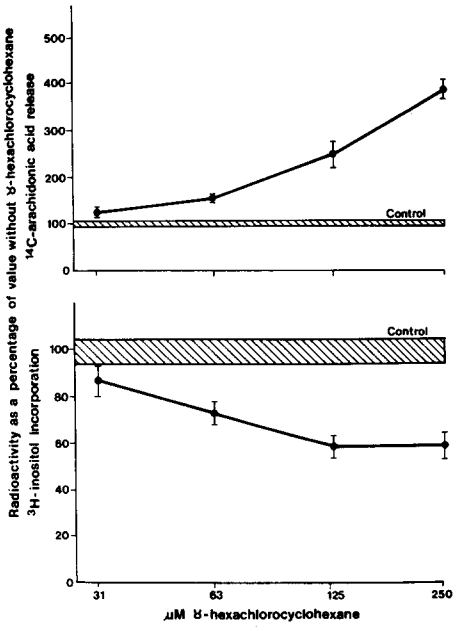

Fig. 1. Effects of exposure to γ-hexachlorocyclohexane upon (a) release of ^{14}C-label from macrophage lipids prelabelled with [^{14}C]arachidonic acid and (b) incorporation of ^{3}H-label into phosphatidylinositol by macrophages exposed to [^{3}H]inositol. All values are expressed as a percentage of a control value for cells without γ-hexachlorocyclohexane. The shaded area shows this 100% control value ± one standard error. Vertical bars show ± one standard error of other points. The rate of uptake of [^{3}H]inositol by the cells was measured in another experiment, and exceeded the rate of incorporation into phosphatidylinositol by *ca* 50-fold. At concentrations at which γ-hexachlorocyclohexane significantly inhibited [^{3}H]inositol incorporation into phosphatidylinositol, the effect on total [^{3}H]inositol uptake from the medium was small (<15%) and not statistically significant.

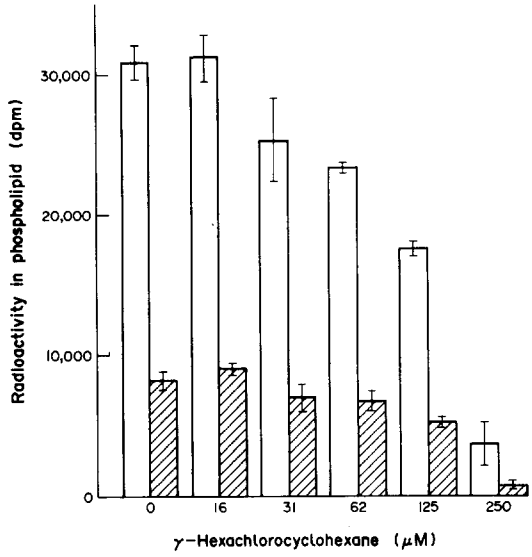

Fig. 2. ^{14}C-Label remaining in phosphatidylcholine or phosphatidylinositol after 4 hr incubation of [^{14}C]arachidonic acid prelabelled cells with different concentrations of γ-hexachlorocyclohexane. Open bars = radioactivity in phosphatidylcholine. Shaded bars = radioactivity in phosphatidylinositol. Vertical lines show ± one standard deviation (n = 4).

below those which had an effect on LDH release or lysozyme secretion. In dose–response studies, there was a relationship between the effect of the compound upon phosphatidylinositol turnover and the effect on arachidonic acid release. Figure 1 shows the relationship between the concentration of γ-hexachlorocyclohexane to which murine macrophages were exposed and either incorporation of [^{3}H]inositol into phosphatidylinositol or release of [^{14}C]arachidonic acid from prelabelled lipids. The minimum concentrations of γ-hexachlorocyclohexane (63 μM) required to produce a significant change in either inositol uptake or arachidonic acid release were similar.

63 μM was also the minimum concentration of γ-hexachlorocyclohexane required to increase significantly the amount of ^{14}C-labelled phosphatidic acid formed 20 min after stimulation of [^{14}C]glycerol prelabelled cells. We performed three experiments each with triplicate determinations of phosphatidic acid. 3.4% of label was present in phosphatidic acid in control cells. This was not increased by 16 or 32 μM γ-hexachlorocyclohexane, whereas 64, 125 and 250 μM γ-hexachlorocyclohexane all increased the amount of label in phosphatidic acid to between 4.6 and 5.3%. Mean recovery of phosphatidic acid by our extraction procedure was 92%. A two-way analysis of variance showed the effect of γ-hexachlorocyclohexane upon incorporation of [^{14}C]glycerol into phosphatidic acid was significant at the 5% level.

β-Hexachlorocyclohexane was without effect on arachidonic acid release at 63, 125 or 250 μM.

In some experiments, we looked at the effect of γ-hexachlorocyclohexane on the distribution of [^{14}C]arachidonic acid between cell lipids. At all concentrations at which it produced an effect, γ-hexachlorocyclohexane stimulated arachidonic acid release not only from phosphatidylinositol but also from phosphatidylcholine (Fig. 2).

In other experiments, we measured the different arachidonic acid metabolites formed as a result of γ-hexachlorocyclohexane treatment. We first tested γ-hexachlorocyclohexane (and zymosan) in the presence of defatted albumin. In these conditions, there was only a small increase in production of PGE_2 and other cyclooxygenase products after γ-hexachlorocyclohexane. In contrast, zymosan as well as stimulating arachidonic acid release also markedly stimulated prostaglandin production (Fig. 3). However, γ-hexachlorocyclohexane, particularly at high concentrations, did increase production of lipoxygenase products. The failure of γ-hexachlorocyclohexane to stimulate prostaglandin production cannot be explained by an inhibition of cyclooxygenase because when γ-hexachlorocyclohexane and zymosan stimuli were combined there was no reduction in the ability of zymosan to stimulate prostaglandin synthesis.

One of the more important lipoxygenase products produced by macrophages is leukotriene C_4, which we assayed by contraction of guinea-pig ileum. The

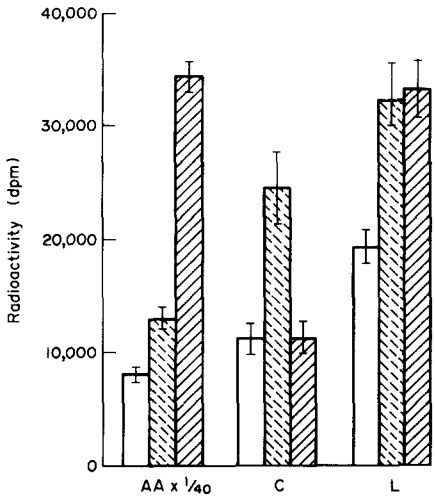

Fig. 3. Radioactive arachidonic acid metabolites released following 4 hr incubation with either 200 μM γ-hexachlorocyclohexane or 100 μg/ml zymosan. Cell culture conditions were as in the experiments shown in Figs 1 and 3; supernatants were extracted with ethyl acetate. AA = arachidonic acid, L = principal extracted lipoxygenase products (5-HETE, other monoHETEs and 5,12-diHETE), C = principal cyclooxygenase products (PGE_2 and PG6oxo-$F_{1\alpha}$ or $PGF_{1\alpha}$). Dpm in arachidonic acid have been divided by 40 to bring them on scale. □, Unstimulated cells; ▨, cells stimulated with 100 μg/ml zymosan; ▩, cells stimulated with 200 μM γ-hexachlorocyclohexane. Vertical bars show ± one standard deviation ($n = 5$). In cultures such as shown here, over 98% of the cells are macrophages as indicated by morphology, uptake of zymosan particles and presence of non-specific esterases.

biological activity of leukotriene C_4 is antagonized by FPL 55712. γ-Hexachlorocyclohexane at concentrations down to 63 μM caused macrophages to release substantial amounts of an ileum-contracting activity which was appreciably (if not completely) inhibited by FPL 55712. Fractionation of our macrophage culture supernatants by HPLC gave almost all the ileum-contracting activity in a fraction which co-eluted with a leukotriene C_4 standard. Biological activity of this fraction was totally inhibited by FPL 55712. The UV absorption spectrum was identical to that of the leuokotriene C_4 standard (λ_{max} = 271, 281, 291 nm). Small quantities of leukotriene D_4, leukotriene B_4, 5*S*,12*R*-diHETE, 5*S*,12*S*-diHETE and various monoHETEs were also identified but these only made a small contribution to the total ileum-contracting activity, as did the prostaglandin fraction. Using the same concentrations of γ-hexachlorocyclohexane and zymosan as specified in Fig. 3, we found in five experiments that the mean quantity of biologically active leukotriene C_4 extracted per ml supernatant was 47 ng (range 7–130 ng) using 200 μM γ-hexachlorocyclohexane as the stimulus, but only 3 ng (range 2–6 ng) using 100 μg/ml zymosan as the stimulus.

The β-isomer of hexachlorocyclohexane was unable to stimulate leukotriene production at 50, 100 or 200 μM (LTC_4 production with 200 μM β-hexachlorocyclohexane was less than 2% of production with the same concentration of γ-hexachlorocyclohexane).

DISCUSSION

γ-Hexachlorocyclohexane causes considerable release of arachidonic acid from macrophage phospholipids. It is also one of the most potent stimulators of macrophage leukotriene C_4 production that we have yet observed. It is likely that these two phenomena are linked. There are three reasons for suggesting this. First, in other systems, availability of free arachidonic acid has been shown to be a factor regulating production of lipoxygenase enzyme metabolites [11]. Second, in percentage terms, stimulation of cells with γ-hexachlorocyclohexane causes an even greater increase in free arachidonic acid than it causes an increase in lipoxygenase product formation (Fig. 3). Third, the minimum concentrations of γ-hexachlorocyclohexane required to stimulate leukotriene production and arachidonic acid release are similar.

By comparison with zymosan, when tested under the conditions specified in this paper, γ-hexachlorocyclohexane is a far better stimulus for formation of lipoxygenase products than cyclooxygenase products. This is of considerable interest. There is evidence from various sytems that production of prostaglandins is regulated by the availability of free arachidonic acid [12, 13]. Our results suggest that there are at least two different pools of free arachidonic acid, one more available to the lipoxygenase, the other to the cyclooxygenase enzyme. The existence of at least two independent arachidonic acid pools in macrophages has previously been suggested by Hsueh and co-workers [14]. They proposed that one of the pools was located in the lysosomes and one elsewhere. Two different phospholipase A_2 enzymes can be isolated from murine macrophages [15]. A recent paper describes the converse of our observations, namely stimulation of prostaglandin production without stimulation of leukotriene production [16]. In this paper, the effect is again ascribed to the existence of different arachidonic acid pools within the macrophage.

We have investigated the mechanism by which γ-hexachlorocyclohexane stimulates arachidonic acid release. γ-Hexachlorocyclohexane has been used by several workers as a regulator of phosphatidylinositol turnover, but as with any inhibitor, there is the possibility of effects on other systems. In support of γ-hexachlorocyclohexane acting through its effect on phosphatidylinositol turnover is the similarity in the concentrations of γ-hexachlorocyclohexane required either to stimulate arachidonic acid release or to inhibit inositol incorporation into phosphatidylinositol. Our measurements of [^{3}H]inositol uptake into cells suggest that the effect of γ-hexachlorocyclohexane upon incorporation of labelled inositol into phosphatidylinositol is a genuine effect on phosphatidylinositol synthesis rather than an indirect effect on inositol transport into the cell. In further support of the involvement of effects on phosphatidylinositol turnover, the β-isomer of hexachlorocyclohexane, which is reported to be inactive at inhibiting phosphatidylinositol synthesis [4], is also inactive at stimulating either arachidonic acid release or leukotriene production.

Work on platelets and polymorphs has suggested a number of different pathways by which phospha-

tidylinositol breakdown might be coupled to arachidonic acid release. One suggestion is hydrolysis of diacylglycerol formed by action of phospholipase C upon phosphatidylinositol [17]. Macrophages contain appreciable amounts of phosphatidylinositol-specific phospholipase C [18]. Another suggestion is that the diacylglycerol is phosphorylated to phosphatidic acid and it is phosphatidic acid which plays the central role in the control of arachidonic acid release [19]. Phosphatidic acid is a potent calcium ionophore [20]. One view of the role of phosphatidic acid is that it causes activation of calcium-dependent phospholipase A_2 enzymes. At least one of the phospholipase A_2 enzymes in murine macrophages is known to be calcium-dependent [15]. Another role that has been suggested for phosphatidic acid is as a substrate for a phosphatidate-specific phospholipase A_2. Lysophosphatidic acid could then be reacylated by the direct transfer of arachidonate from phosphatidylcholine [19]. Either mechanism of phosphatidic acid's action would explain how an agent acting on phosphatidylinositol turnover might (as we observed with γ-hexachlorocyclohexane) also stimulate the release of arachidonic acid from phosphatidylcholine (Fig. 2).

We attempted to measure directly the effect of γ-hexachlorocyclohexane upon cellular phosphatidate, but the quantities of phosphatidic acid were too small to assay by our available assay methods. We therefore labelled macrophages with [^{14}C]glycerol. Whilst accumulation of [^{14}C]glycerol-labelled phosphatidic acid is not direct evidence that the total amount of phosphatidic acid in the cell is increased by γ-hexachlorocyclohexane, it at least suggests that γ-hexachlorocyclohexane is having some effect on phosphatidic acid metabolism.

To summarize, the most likely mechanism of action of γ-hexachlorocyclohexane is through its effect on phosphatidylinositol metabolism. We are, however, cautious before extrapolating these results to support the concept that phosphatidylinositol turnover has a role in arachidonic acid release in macrophages. Although this is an attractive concept, any study such as this one is limited by the specificity of the inhibitor employed. There may well be other effects of γ-hexachlorocyclohexane, as yet unexplored, apart from effects on the metabolism of phosphatidylinositol.

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