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EFFECT OF LINDANE ON HUMAN LYMPHOCYTE RESPONSES TO PHYTOHEMAGGLUTININ

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Abstract—Human peripheral blood mononuclear cells, stimulated by phytohemagglutinin, were exposed to various concentrations of the organochlorine insecticide, lindane. At various intervals of time, viability and cellular growth, as well as accumulation and incorporation of macromolecular synthesis precursors were measured in the cell culture. Concentrations of lindane in the range of 10^{-4}M exerted an inhibition on the macromolecular biosynthesis in the three cellular models: unstimulated lymphocytes, PHA-activated lymphocytes and dividing blast cells. In the mitogen-stimulated cells, the inhibitory effect of lindane seemed to be restricted to a delaying one, caused by the alteration of an event occurring early after the PHA activation. It might be that lindane, shrinking the cellular soluble pool of labelled precursors and blocking the aggregation of the stimulated lymphocytes, acts on the surface membrane of the cells. The resulting disorganization of membrane architecture could therefore be related to the hydrophobic nature of the molecule.

Despite the countless advantages to man conferred by the use of lindane and other organochlorine insecticides, the increasing environmental contamination by these substances has resulted in great amounts of research studying various aspects of the physiological systems. However, little information exists on the effects of lindane on the immune response: the finding of Dési demonstrated a decrease in the antigen–antibody agglutination titer of rabbit serum after chronic treatment with lindane [1]. Rosival *et al.* observed an inhibition of the specific production of antibodies against human serum albumin in rats acutely intoxicated by lindane [2].

These studies only concerned the suppression of humoral immune response; thus it would be interesting to enlarge such research to analyse lindane interferences with cellular immune responses. Fisher and Mueller have described in phytohemagglutinin (PHA)-stimulated human lymphocytes an inhibition of [^{3}H]thymidine incorporation by 0.4×10^{-4}M of lindane, as well as the prevention of [^{3}H]-5-cytidine incorporation increase induced by PHA with 2×10^{-4}M of lindane [3]. The alteration of growth response to PHA reflected by these two parameters was related to the inhibition of phosphatidylinositol (PI) turnover acceleration, which occurs within minutes after exposure of lymphocytes to PHA. It was suggested that lindane, the gamma-isomer of hexachlorocyclohexane with the same configuration as muco-inositol, would prevent PHA-activation of lymphocytes by interfering with the stimulation of PI turnover. Like Hokin and Brown, who studied the inhibition by hexachlorocyclohexane of acetylcholine-stimulated PI systhesis in cerebral cortex slices [4], Fisher and Mueller used the lindane concentration of 1 mM, much higher than that which allows a decrease in lymphocytes growth [3].

Recent findings concerning drugs on concanavalin A (Con A) stimulated [^{3}H]inositol incorporation into PI have shown that a correlation between the prevention of increased PI turnover and the inhibition of other events which follow lymphocyte activation is still debatable: Greenberg and Mellors [5] have observed in mouse lymphocytes that Con A stimulated [^{14}C]acetate incorporation into PI is suppressed by 10^{-4}M of delta-9-tetrahydrocannabinol, whereas Nahas and Desoize [6] have noted in PHA-activated human lymphocytes an inhibition of thymidine uptake in the concentration range of 10^{-4}M. In human lymphocytes, 10^{-6}M of colchicine has been shown to prevent the Con A-stimulated [^{3}H]inositol incorporation into PI [7], while the same concentration will not affect the early calcium uptake response to lectin [8] and the early increased protein synthesis occurring after PHA activation [9].

In the present investigation, a number of experiments were carried out to analyze the kinetics of the inhibitory activity of lindane. The effects of the insecticide on cell viability and cellular growth, as well as accumulation and incorporation of macromolecular synthesis precursors were studied in cultures of human lymphocytes which had been stimulated by PHA. We observed that the same concentrations of lindane will inhibit both the blastogenic response and the clustering of the lymphocytes by PHA. Analysis of the kinetic data may indicate that lindane has a delaying effect on the increase in protein and RNA synthesis, likewise on the onset of DNA synthesis, because it blocks an event occurring early after PHA activation, at a stage prior to the entrance of the cells into the S-phase. Comparison of our results with data concerning the effects of colchicine and chlorpromazine, a local anesthetic, leads us to suggest a disorganizing effect of γ-hexachlorocyclohexane on plasma membrane architecture.

Lindane concentrations required to induce any effects on human lymphocytes must be about 10^{-4}M: such doses bring about changes in other kinds of cells [1] and this general toxic effect of lindane could explain

the particular effect at the level of the cellular immune response and at the level of the production of antibodies.

MATERIALS AND METHODS

Reagents. Medium RPMI 1640 (Grand Island Biological Co., Glasgow, Scotland), containing penicillin (100 u/ml) and streptomycin (100 μg/ml) was supplemented with 5% autologous serum, unless otherwise mentioned. E-PHA (purified erythroagglutining PHA, Wellcome Reagents Limited, Beckenham, England) was reconstituted with culture medium and a final culture concentration of 4 μg/ml of PHA was utilized for the standard experiment. Lindane or γ-hexachlorocyclohexane (Société Pepro, Lyon, France) was dissolved (72.75 mg/ml) in dimethyl sulfoxide (DMSO) (Merck, Darmstadt, Germany) and stored at 4°. The final concentration of DMSO in the culture medium with and without lindane, did not exceed 0.2 per cent. Further dilutions in RPMI medium were made immediately before use.

Mononuclear cells. Heparinized peripheral venous human blood was obtained from healthy male and female volunteers aged 25–35. Human mononuclear cells were obtained by centrifugation on Ficoll-hypaque followed by two low speed centrifugations to remove platelets as previously described [10].

Culture conditions. When the micromethod for lymphocyte culture was used, 2×10^5 mononuclear cells were cultured in triplicate or quadruplicate in microtiter plates (Greiner, Bishwiller, France) to a final volume of 0.260 ml. Cultures were maintained in an atmosphere of 5% CO_2–95% air. Mitogen and lindane were added at the initiation of culture, except where otherwise noted in some experiments. 1 μCi of [^{3}H]uridine (CEA, Gif-sur-Yvette, France) (15–25 Ci/mmol) or 1 μCi of [^{3}H]leucine (15–30 Ci/m-moles) was added to separate wells at the onset of the culture. 1 μCi of [^{3}H]thymidine (0.5–2 Ci/m-moles) was added to the other wells 6 hr before harvest.

Harvesting and counting techniques. Cultures were harvested with a mechanical 24-place harvester (MASH, Microbiological Associates, Bethesda, M.D, U.S.A.) by precipitation into glass fibre filter paper. The filter was pre-wetted and the cells were repeatedly washed with 0.9% NaCl, precipitated with 5% (w/v) trichloroacetic acid and then dried by two additional washes with 95% ethanol. Retained radioactivity was measured by transferring the filters to counting vials, the addition of 10 ml of counting solution (PPO 0.4% w/v, POPOP dimethyl 0.01% w/v, in toluene; Merck, Darmstadt, Germany) and the assay in an automatic liquid scintillation counter.

Uridine or thymidine incorporation into acid-soluble and acid-insoluble material. The incorporation of labelled substances was measured during the final 15 min of 18 and 42 hr incubations at 37° by a modification of the technique of Peters and Hausen [11]. All experimental conditions were examined in triplicate or in quadruplicate. 1×10^6 cells suspended in medium RPMI 1640 with 5% serum, PHA (4 μg/ml) and lindane (2×10^{-4}M) were added to 13 × 100 mm plastic test tubes to a total volume of 0.6 ml. After the desired incubation period, the cell suspensions were centrifuged for 5 min at 300 *g* and the cells were washed with 3 ml of medium RPMI to remove free stimulant and lindane. The cell pellet was resuspended in 0.25 ml of medium containing: for the determination of uridine uptake, 2.5 μCi (PHA-stimulated lymphocytes) or 10 μCi (unstimulated lymphocytes) of [^{3}H]uridine; for the determination of thymidine uptake, 0.5 μCi (PHA-stimulated lymphocytes) of [^{3}H]thymidine. The cells were then incubated for 15 min. The incubation was terminated by the addition of 3 ml ice-cold phosphate-buffered saline (PBS) containing unlabelled uridine or thymidine (0.2 mM). The cells were centrifuged at 1500 *g* for 3 min and washed 3 times with 3 ml of uridine or thymidine–PBS. The cell pellets were resuspended in 0.5 ml of distilled water and disrupted by sonication. 0.5 ml or 10% trichloracetic acid (TCA) was added to the cell homogenate. The mixture was allowed to stand in the cold for at least 1 hr before being centrifuged. 0.7 ml of the supernatant was then mixed with 10 ml of Instagel scintillator fluid (Packard, Groningen, The Netherlands) for radioactivity measurement. The sediment was washed 3 times with 5% trichloroacetic acid and dissolved in 0.05 N NaOH at 56°. Radioactivity was determined using toluene scintillator, as previously described.

Protein levels of lymphocytes, homogenates and acid insoluble material were determined by the method of Lowry [12], using bovine serum albumin as a standard. The DNA of the acid-insoluble pellet was analysed by Giles and Myers' modification of the diphenylamine reaction, using calf thymus DNA as a standard [13].

Reversibility of lindane effect. Human lymphocytes were incubated with PHA and 2×10^{-4}M lindane for 20 hr. The test tubes were then centrifuged at 1200 rev/min for 3 min and the medium was removed with a fine needle; cells were then washed with 1 ml of medium, the supernatant was removed and the cells were resuspended in fresh medium containing 5% human serum and mitogen, and without lindane. [^{3}H]Thymidine was added on day 3 and the uptake was determined 6 hr later. Parallel cultures of PHA-stimulated lymphocytes were treated by 2×10^{-4}M of lindane during the whole period of 72 hr, and the results were compared.

Statistical analysis. The means of experimental triplicates or quadruplicates were compared by using Student's *t* test for unpaired data. The error bars appearing in the accompanying figures represent the standard deviation from triplicate or quadruplicate determinations.

RESULTS

The conditions of lindane treatment of human lymphocytes stimulated with PHA. Lindane was dissolved in dimethylsulfoxide (DMSO) and 0.2% of the solution was added to the culture. All experiments were made with control cultures containing 0.2% DMSO; preliminary assays had demonstrated that the presence of DMSO did not bring any change in the studied parameters. The rate of [^{3}H]thymidine incorporation was measured on 72 hr-stimulated lymphocytes by the micromethod: the PHA blastogenesis of cultures without DMSO (28,887 + 1901 c.p.m.) and with 0.2% DMSO (29,354 ± 2413) were not significantly different. The incorporation of [^{3}H]uridine into the acid-soluble pool and into the acid-insoluble material was measured separately: the differences of the accumulation rates in the

Table 1. Effects of lindane on viability and protein concentration of human lymphocytes stimulated with PHA for 72 hr.

Lindane concentration (M)	Viable cells Cell number per ml medium ($\times 10^4$)	Dead cells	Protein concentration μg per ml medium
	± S.E.	± S.E.	±S.E.
0	50.8 ± 9.5	4.1 ± 0.2	120.9 ± 3.9
2×10^{-4}	50.3 ± 7.0	3.5 ± 1.9	121.8 ± 1.6
3×10^{-4}	37.9 ± 5.3	7.2 ± 1.9	100.0 ± 4.8

All values represent means of 3 microtiter wells ± standard deviations.

soluble pool between the cultures without DMSO (6160 ± 342 c.p.m./100 μg protein) and with DMSO (6140 ± 368) were not significant. The rates of [^{3}H]uridine incorporation into the acid-insoluble material of non-DMSO-treated cells (572 ± 43 c.p.m./100 μg protein) and of DMSO-treated cells (503 ± 30) were little different ($P < 0.05$).

The use of labelled lindane, which was added in the culture medium in a similar manner, had allowed us to ascertain the solubility of the total product in the medium, when the concentrations were up to 2×10^{-4}M. With the lindane concentration of 3×10^{-4}M however, a fraction was precipitated: the radioactivity in the supernatant, after a 4 hr incubation at 37°, was 55,468 c.p.m. instead of 64,153 c.p.m. at the beginning of the incubation; 2.58×10^{-4}M of the product would be effectively dissolved.

The effect of lindane on viability of human lymphocytes stimulated with PHA for 72 hr. PHA (4 μg/ml) was added to microtiter wells containing human lymphocytes (2×10^5). Immediately thereafter, lindane, dissolved in DMSO, was added to the wells.

Drug toxicity was tested by trypan blue exclusion after 72 hr of incubation. The results, shown in Table 1, demonstrate that viability was unaffected by 2×10^{-4}M lindane. With 3×10^{-4}M lindane, total cell number was lower and this change was accompanied by a decrease in protein content: this could be due to the higher ratio of dead cells, although it was not significant, as well as to the inhibition of blast transformation and cell division.

The present study has essentially been carried out with both lindane concentrations, 2×10^{-4} and 3×10^{-4}M.

The effect of lindane on the blastogenic response of human lymphocytes to PHA. [^{3}H]Thymidine was added on day 3 and the uptake was determined 6 hr later. The activity of lindane on [^{3}H]thymidine incorporation depended on the serum concentration in the culture medium (Fig. 1): with the lowest studied dose of lindane (1×10^{-4}M), the inhibition reached 11.8% at the highest concentration of serum (20%) and 41.4% at the lowest concentration (2%). Serum present in culture medium showed an antagonistic influence in opposition to lindane toxicity.

Lymphocytes cultured with PHA alone demonstrated the formation of a few very large clusters. In the presence of 2×10^{-4}M or 3×10^{-4}M of lindane, no aggregate formation was evident on the monolayer of cells, which thus corresponds to an inhibition of the PHA response.

Figures 2 and 3 show the effects of lindane on the

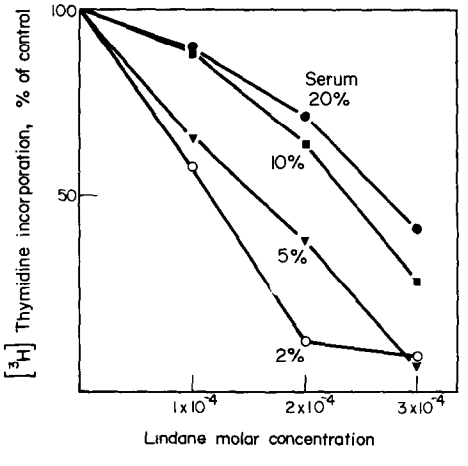

Fig. 1. Effect of lindane on the mitogenic response to PHA (4 μg/ml) of human lymphocytes. The values shown represent c.p.m. of tritiated thymidine uptake by cell cultures harvested on day 3. The determinations were done for different serum concentrations in culture medium. Lindane was added to cultures immediately before mitogen addition. The values are expressed as percentages of control values.

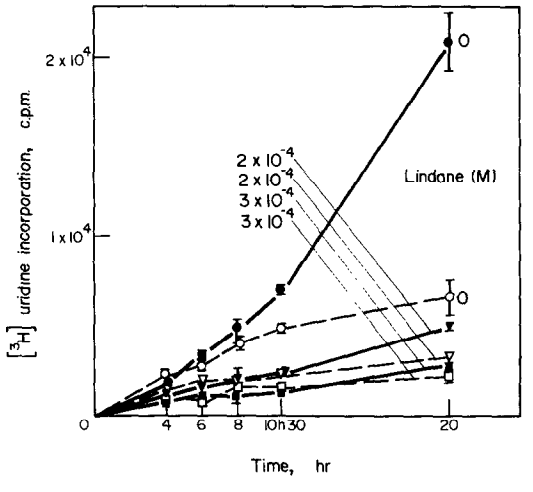

Fig. 2. Effect of various doses of lindane on the kinetics of uridine incorporation in PHA-stimulated (——●) and unstimulated (- - - - ●) lymphocytes within the first hours of culture. The values shown represent c.p.m. of tritiated uridine uptake by cell cultures harvested at the hour indicated and are given as means of 3 determinations ± standard deviations. Lindane was added to culture immediately before mitogen addition and [^{3}H]uridine at time 0.

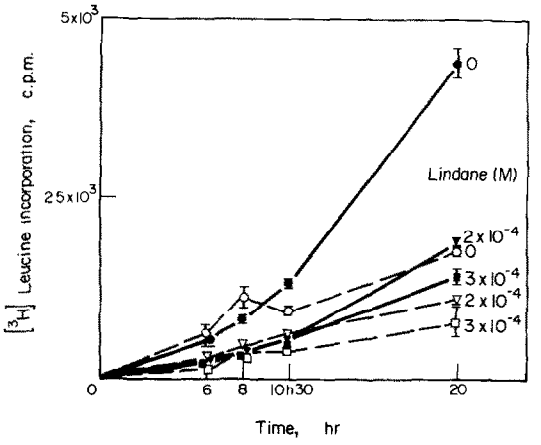

Fig. 3. Effect of various doses of lindane on the kinetics of leucine incorporation in PHA-stimulated (——●) and unstimulated (- - - - ●) lymphocytes, within the first hours. Lindane was added to culture immediately before mitogen addition and [³H]leucine, at time 0.

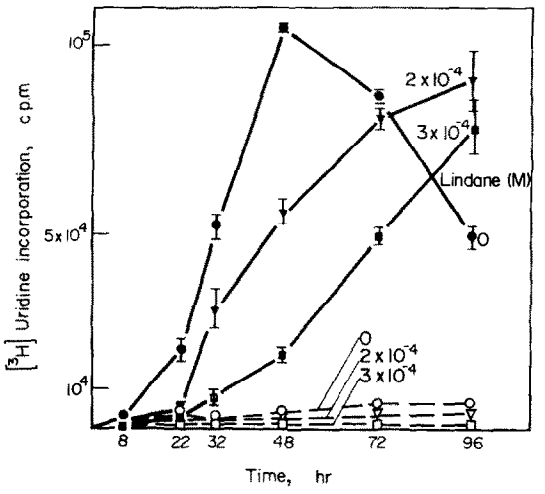

Fig. 5. Effect of various doses of lindane on the kinetics of uridine incorporation in PHA-stimulated (——●) and unstimulated (- - - - ●) lymphocytes, between 8 hr and 96 hr of culture. Lindane was added to the culture immediately before mitogen addition and [³H]uridine, at time 0.

PHA-stimulation of uridine and leucine incorporation within the first hours following the initiation of cultures. [³H]uridine and [³H]leucine were added at the beginning of incubation. Three conclusions can be drawn: (1) the time allowed for the lindane effect was less than 6 hr. (2) During the first 8 hr of incubation, PHA does not induce any change in the incorporation rate of the two precursors: lindane brought about the same inhibition in cultures with or without PHA. The presence of the mitogenic agent did not interfere with the binding or cell uptake of lindane, nor with its inhibitory action. (3). In this experiment, in incubation times longer than 8 hr, PHA activation of lymphocytes appeared through an increase in uridine and leucine incorporation. Lindane (2×10^{-4}M and 3×10^{-4}M) completely prevented the stimulation of this incorporation in 10 hr and 20 hr incubations, while it affected unstimulated cultures only minimally.

Figures 4–6 show the time course of the effect of various concentrations of lindane on PHA-induced blastogenesis, between 24 hr and 96 hr cultures. The IC_{50} for [³H]thymidine, [³H]uridine and [³]leucine incorporation (the lindane concentration which induces 50% inhibition of incorporation) was about 2×10^{-4}M, after 48 hr of culture, and about 3×10^{-4}M, after 72 hr. The entry into the first S-phase measured by [³H]thymidine incorporation into DNA

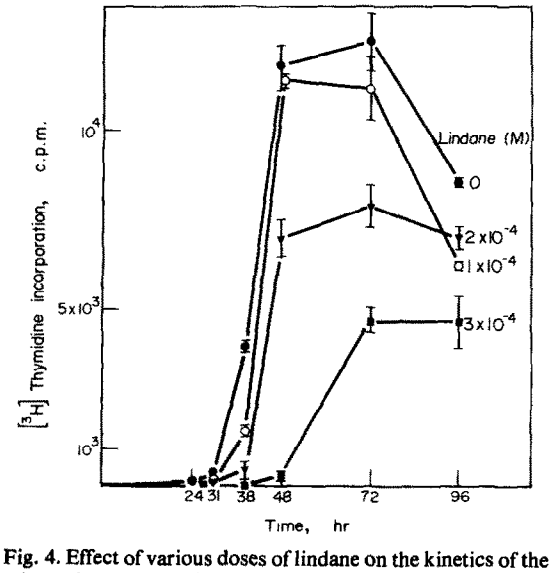

Fig. 4. Effect of various doses of lindane on the kinetics of the mitogenic response of human lymphocytes to PHA, between 24 hr and 96 hr of cultures. Lindane was added to culture immediately before mitogen addition and [³H]thymidine was present during the final 6 hr of incubation.

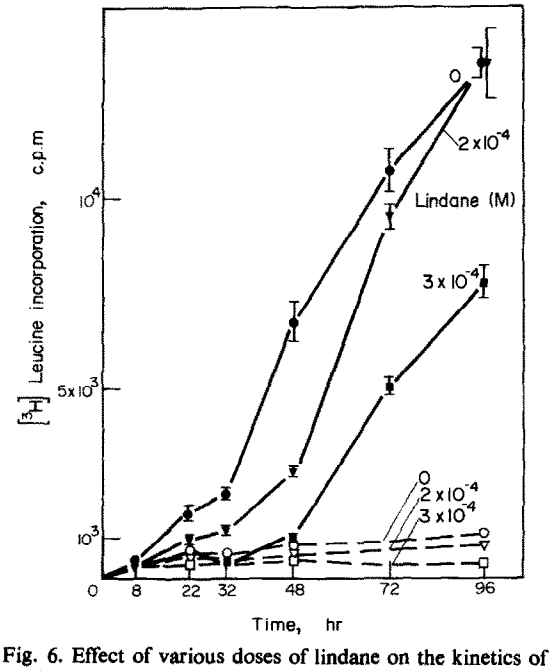

Fig. 6. Effect of various doses of lindane on the kinetics of leucine incorporation in PHA-stimulated (——●) and unstimulated (- - - - ●) lymphocytes, between 8 hr and 96 hr of culture. Lindane was added to culture immediately before mitogen addition and [³H]leucine, at time 0.

Table 2. Effects of lindane on protein content and DNA concentration of human lymphocytes stimulated by PHA

Treatment time (hr)	Lindane molar concentration	Protein concentration $\mu g/10^6$ cells (at the initiation of the culture)	DNA concentration $\mu g/10^6$ cells
0	—	44.0 ± 2.4	Not done
18	0	63.4 ± 2.4	5.40 ± 0.10
	3×10^{-4}	57.2 ± 1.1	5.35 ± 0.09
42	0	62.9 ± 8.4	5.65 ± 0.09
	3×10^{-4}	62.5 ± 3.0	5.50 ± 0.00
66	0	93.2 ± 4.1	7.17 ± 0.66
	3×10^{-4}	56.3 ± 2.3	5.01 ± 0.40
90	0	127.4 ± 8.3	12.23 ± 0.77
	3×10^{-4}	80.6 ± 0.8	6.17 ± 0.50

All values represent means of 3 plastic test tubes ± standard deviations.

occured 24 hr after the initiation and the PHA activation of the culture (Fig. 4). On the other hand, when the cells were cultured with PHA and lindane, the start of [^{3}H]thymidine incorporation was delayed by 7 hr with 2×10^{-4}M and by 14 hr with 3×10^{-4}M of lindane. After 72 hr, the [^{3}H]thymidine incorporation remained significantly inhibited by the same concentrations, but after 96 hr, this inhibition was no longer observed because of a sharp decline of [^{3}H]thymidine incorporation in non-treated PHA-activated lymphocytes.

This delayed and reduced response to PHA in the presence of lindane was also observed by studying the mitogen stimulation of [^{3}H]uridine or [^{3}H]leucine incorporation. With 3×10^{-4}M of lindane, the prevention of the PHA-induced increase in uridine and leucine uptake, shown in Figs. 2 and 3, was prolonged to 22 hr and 32 hr respectively. After 48 hr, uridine uptake decreased in control PHA-activated lymphocytes. In cells exposed to PHA and lindane, the increase with time in [^{3}H]uridine incorporation was maintained beyond 48 hr, reaching the highest value measured at 48 hr in the control PHA-activated lymphocytes (Fig. 5). On the other hand, the leucine uptake by the PHA-activated lymphocytes treated with 2×10^{-4}M lindane had increased at 96 hr to the same level as the uptake by the non-treated PHA-activated cells (Fig. 6). Table 2 shows that an increase in protein synthesis accompanying blastogenesis precedes an increase in DNA synthesis with lymphocytes exposed to PHA alone after 66 hr and with lymphocytes treated equally by lindane after 90 hr. Again, lindane seems to delay the blastogenic response to PHA.

The effect of lindane on the incorporation of [^{3}H]*uridine and* [^{3}H]*thymidine into acid-soluble and acid-insoluble material of human lymphocytes in culture.* In Table 3, the rates of [^{3}H]uridine and [^{3}H]thymidine incorporation are expressed per 100 μg protein. Taking notice of the changes in culture protein concentration after PHA stimulation and lindane exposure enables consideration of the differences in cell number between treated and control cultures. The cells were pulse-labelled with [^{3}H]uridine or [^{3}H]thymidine

Table 3. Influence of lindane on the incorporation of uridine and thymidine into acid-soluble and -insoluble material of human lymphocytes in culture

Lindane molar concentration	[^{3}H]Uridine incorporation c.p.m. $\times 10^{-3}$/100 μg protein (% of control)				[^{3}H]Thymidine incorporation c.p.m. $\times 10^{-3}$/100 μg protein (% of control)
	−PHA		+PHA		+PHA
Treatment time (hr)	18	42	18	42	42
Into acid-soluble material					
0	21.99 ± 1.26	8.28 ± 1.02	91.13 ± 7.06	238.45 ± 12.09	3.61 ± 0.41
2×10^{-4}	—	—	—	—	—
	7.56 ± 1.73 (34.4%)	2.49 ± 0.99 (30.1%)	32.59 ± 3.34 (35.8%)	69.84 ± 16.63 (29.3%)	2.75 ± 0.20 (76.05%) ($P < 0.05$)
Into acid-insoluble material					
0	5.26 ± 1.15	2.28 ± 0.21	45.03 ± 2.94	80.03 ± 0.79	41.96 ± 3.46
2×10^{-4}	—	—	—	—	—
	1.86 ± 0.04 (33.1%)	0.59 ± 0.01 (25.9%)	15.42 ± 0.93 (34.2%)	39.38 ± 2.22 (49.2%)	16.87 ± 1.97 (40.2%)

All values represent means of at least 3 plastic test tubes ± standard deviations.
Figures in parentheses represent the percentage of the mean given by non-treated cultures.
The PHA-stimulated lymphocytes were pulse-labelled at the time indicated with [^{3}H]uridine or [^{3}H]thymidine for 15 min.
Radioactivity of acid-soluble and acid-insoluble material was determined as described under Materials and Methods.

Table 4. Effects of lindane removal 20 hr after PHA initiation of human lymphocytes incorporation

Lindane concentration (M)		Thymidine incorporation (72 hr) as	
		C.p.m. ($\times 10^{-3}$)	Per cent of control without lindane
Lindane present in culture		± S.E.	
0–20 hr *	0	5.82 ± 0.34	—
	2×10^{-4}	3.44 ± 0.17	59.0
0–72 hr	0	5.23 ± 0.33	—
	2×10^{-4}	0.37 ± 0.03	7.0

All values represent means of at least 4 plastic test tubes ± standard deviations.
* After 20 hr of culture, the test tubes were centrifuged at 1200 rev./min for 3 min and the media was removed with a fine needle. Cells were then washed with 1 ml of media, the wash was removed and cells resuspended in mitogen containing drug-free media. [^{3}H]thymidine was added on day 3 and the uptake was determined 6 hr later. Parallel cultures of PHA-stimulated lymphocytes were treated by 2×10^{-4}M of lindane during 72 hr.

for 15 min and the incorporation of labelled compounds into the acid-soluble pool and into the acid-insoluble material were measured separately.

In the unstimulated lymphocytes, the reduction of [^{3}H]uridine accumulation into the acid-soluble pool was comparable with the inhibition of [^{3}H]uridine incorporation into the acid-insoluble material. The decrease of these two parameters was more pronounced after 42 hr of treatment than after 18 hr. In the PHA-stimulated lymphocytes, the same parallel between the changes in the accumulation and in the incorporation of [^{3}H]uridine could be demonstrated after 18 hr. The incorporation rate was decreased less after 42 hr than after 18 hr: as demonstrated in Fig. 5, the rate of inhibition induced by lindane on [^{3}H]uridine incorporation in PHA-activated lymphocytes diminished over time, whereas it increased in unstimulated lymphocytes.

In the PHA-stimulated lymphocytes, no correlation could be established between the important decrease of [^{3}H]thymidine incorporation into the acid-insoluble material and the minor reduction of [^{3}H]thymidine accumulation into the acid-soluble pool.

The effect of a short exposure to lindane during the first hours of PHA-stimulation of lymphocytes. Table 4 shows that the inhibitory effect of lindane was partially reversible when lindane was present during the initial period of 20 hr. Lindane can also further alter the cellular metabolism of the 72 hr-treated lymphocytes during the period between 20 and 72 hr: the partial reversibility of lindane effect, or the maintenance of lindane action beyond the first hours of PHA-stimulation, may explain the reduced inhibition in the 20 hr-treated cultures, compared with the 72 hr-treated cells.

The effect of lindane addition at intervals before or after the stimulation of lymphocytes with PHA. Since PHA binds rapidly to cell surfaces at 37°, experiments were performed in which PHA and lindane were added at various times in respect to one another. Table 5 shows that a 30 min preincubation of lymphocytes with lindane greatly increased the inhibition of PHA response.

Figure 7 demonstrates the effect of adding lindane at various times after the addition of PHA to the human lymphocytes culture. Delaying the addition of lindane caused a marked loss in the inhibitory effect on macromolecular synthesis, which was proportional to the delay between the addition of PHA and lindane. Exposing 48 hr-stimulated lymphocytes to lindane induced decreases in the incorporation of the three precursors whose level remained below 50 per cent.

DISCUSSION

To induce lymphocyte transformation, a mitogen must bind to the surface membrane of lymphocytes and trigger intracellular events leading to blast transformation, DNA synthesis and division. These intracellular changes are caused by post-binding events which take place within the cell membrane: for instance, the increased turnover of phosphatidylinositol and lipids [14] and the increased permeability of the membrane to ions [15], sugars [16], aminoacids [17] and nucleosides [18, 19]. This involves an increased incorporation of substrates into cellular components. The present studies demonstrate that lindane inhibits macromolecular biosynthesis in the three cellular models: unstimulated small lymphocytes, PHA-activated lymphocytes and dividing blast cells. The delay in the PHA-induced onset of thymidine uptake results in a decrease of the blast cells division during the optimum culture conditions. It can be suggested that lindane, which shrinks the intracellular soluble pool of labelled precursors and blocks the aggregation of stimulated lymphocytes, will alter membrane-mediated events linked to the mitogen-activation process. Such effects can only be obtained with concentrations of insecticide in the range of 10^{-4}M, in culture medium containing 5% serum.

Fisher and Mueller [3] have described the prevention of [^{3}H]cytidine incorporation in human lymphocytes by 2×10^{-4}M lindane after 4 hr and the partial inhibition of DNA synthesis by 0.4×10^{-4}M lindane after 24 hr. In this study, 2×10^{-4}M lindane completely prevented the mitogen stimulation of [^{3}H]uridine and [^{3}H]leucine incorporation, as well as the onset of DNA synthesis.

This complete inhibition of the mitogen stimulation disappeared after several hours, so that the inhibitory effect of lindane seemed restricted to a delaying effect. Such an escape of lymphocytes from a drug-induced

Table 5. Effects of addition of lindane before PHA initiation of human lymphocyte stimulation

	Thymidine incorporation (72 hr) as Lindane concentration (M)	C.p.m. ($\times 10^{-3}$)	Per cent of control without lindane
		± S.E.	
Addition of lindane and PHA at the same time	0	3.07 ± 0.53	—
	1×10^{-4}	1.74 ± 0.31	62.6
	2×10^{-4}	2.28 ± 0.27	74.5
	3×10^{-4}	1.23 ± 0.11	44.4
Addition of lindane 1 hr before PHA	0	3.66 ± 0.25	—
	1×10^{-4}	1.45 ± 0.25	39.6
	2×10^{-4}	1.66 ± 0.25	45.3
	3×10^{-4}	0.75 ± 0.05	20.4

All values represent means of at least 4 plastic test tubes ± standard deviations.

inhibition had already been observed with 1 μM of colchicine on Con A-stimulated human lymphocytes by Wang *et al.* [20, 21]. Other similarities between the effects of colchicine and lindane may be reported: both reagents, added at various times after mitogen activation of human lymphocytes, showed a decline in their inhibitory effects, in relation to the time of addition. This suggests that the longer the exposure to mitogen, the more cells that become refractory to inhibition. The results agree with the hypothesis that lindane, like colchicine, could inhibit mitogenic stimulation of lymphocytes by blocking an event occurring near the time at which the cell is stimulated to undergo blast transformation.

According to Peters and Hausen [11] for uridine and Fridlender *et al.* [22] for thymidine, the increased incorporation of these precursors, occurring in mitogen-activated lymphocytes, appears to be primarily due to an enhanced cell permeation after PHA binding, which might be responsible for an expansion of the intracellular nucleotide pool. Since our study demonstrates a decrease in [^{3}H]uridine and [^{3}H]thymidine accumulation into the acid-soluble pool, it could be that lindane interferes with the increase of membrane permeation, perhaps by altering processes involved in the stimulation of transport sites.

On the other hand, an inhibition of incorporation, as well as a decrease in the size of the intracellular pool of uridine could even be shown in unstimulated small lymphocytes, although it was less marked than in PHA-activated cells. In such conditions, an alteration of membrane transport sites by lindane could be suggested to occur in the non-stimulated cells. Thus, the prevention of increased precursor uptake by lindane in PHA-stimulated lymphocytes might be due to a modification of both the basal and the activated transport sites. Likewise, the partial inhibition of precursors incorporation induced by lindane in 18 and 48 hr-stimulated cultures might be caused by an alteration of these different transport sites. Similar inhibiting effects on the transport process of lymphocytes, with and without mitogen, were reported by Greene *et al.* [8]; they demonstrated the early inhibition of the [^{14}C]aminoisobutyric acid uptake, by microtubule-disrupting concentrations of colchicine and vinblastine.

The percentage of inhibition caused by lindane on precursor incorporation in PHA-activated lymphocytes diminished over time, whereas in unstimulated

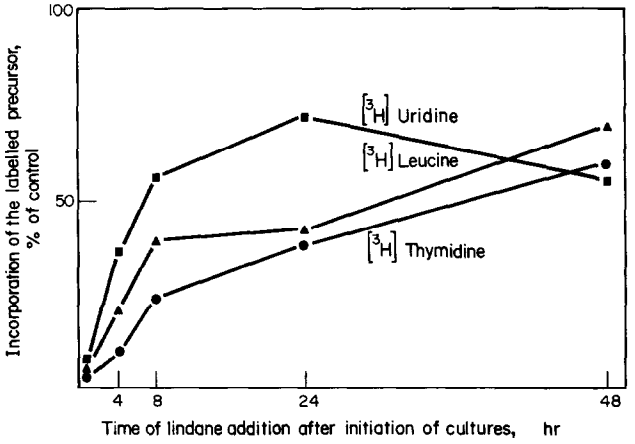

Fig. 7. Effect of 3×10^{-4}M of lindane on the incorporation rate of [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]leucine on day 3, when added at various intervals after stimulation with PHA. The labelled precursors were present during the final 2 hr of incubation. Values are expressed as precentages of control values.

lymphocytes, it increased during the 96 hr incubation. Two changes may occur in normal PHA-activated cultures after 3 or 4 days, which explain the slowing down observed in macromolecular synthesis: according to Desoize [23] the culture medium runs out of essential nutrients like glucose; and according to Bernard and Waksman [24], the 'cell–cell contact' or topoinhibition caused by lectin-induced agglutination has inhibiting effects on cellular proliferation in response to mitogenic stimuli. However, the PHA-activated cultures treated by lindane, which exhibit a delaying effect in their development, did not undergo these regulatory processes after 3 or 4 days and could have not exhausted some components of the medium; they would be allowed to overtake the optimum activity of the PHA-activated non-treated cultures. In control unstimulated lymphocytes as well, these regulatory processes did not occur, and the inhibitions induced by the addition of lindane increased in relation to the incubation time.

At the intercellular level, lindane was found to prevent the cluster formation caused by lymphocyte aggregation, which according to Ferguson *et al.* [25] might represent an active energy and temperature-dependent event, as well as a necessary prerequisite for the transformation process. Among the drugs described which block the active agglutination of mitogen-stimulated lymphocytes, chlorpromazine and lidocaine [25, 26], two local anesthetics, might prevent the development of cell-aggregating properties which facilitate cell-cell interaction. Furthermore, as was observed with lindane, if these drugs were added to lymphocyte cultures after mitogen exposure, [^{3}H]thymidine uptake was proportional to the delay between the time of mitogen stimulation and anesthetic addition.

It can be seen from the present study that the effect of lindane on human lymphocytes shows some similarities with the changes induced by the two local anesthetics, chlorpromazine and lidocaine, and by microtubule-disrupting agents, like colchicine. In the former case, according to Ferguson *et al.* [25], the known membrane active actions of chlorpromazine and lidocaine strongly suggest that the inhibition of mitogen-induced lymphocyte transformation by these drugs takes place on the cell surface membrane.

In the latter, according to Greene *et al.* [8], the alteration induced by colchicine in the microtubule assembly may affect membrane architecture, interfering in stimulated lymphocytes with the propagation of the stimulus from the membrane to the cell interior. These two studies lead us to support the hypothesis that the alteration of the membrane structural integrity could be one early cause of lindane effects on human PHA-stimulated lymphocytes. The inhibition of phosphatidylinositol turnover acceleration by lindane, described by Fisher and Mueller [3], may only be the result of these membrane disorganizing effects.

Since the observed effects were obtained with doses in the range of 10^{-4}M, the action of lindane on human lymphocytes is not related to the insecticide effects which involve stereo-chemical specificity and give a nervous response at 10^{-4}M or less. According to Uchida *et al.* [27], the effects of lindane concentrations superior to 10^{-4}M are only caused by the hydrophobicity of the molecule, resulting in a depressant effect common to the other hexachlorocyclohexane isomers, DDT and aliphatic alcohols. This hypothesis could be supported with further studies at the cellular or intracellular level, like the inhibition of eucaryotic microorganism multiplication [28], the decrease in uridine transport and the pinocytosis rate of peritoneal macrophages [29] or the increase in the permeability of liver lysosomes *in vitro* [30].

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