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Effects of DDT on the Calcium Transport and Thymidine Uptake of Bovine Lymphocytes

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It is known that organochlorine compounds in the environment present risks which may be related to an interference with calcium metabolism. Additionally there is strong evidence that DDT and perhaps chemically related compounds act in insects by virtue of their inhibition at the micromolar level, of a number of metal activated ATP'ases, including calcium ATP'ase (Elder et al 1979). Furthermore there is evidence that several of the chlorinated hydrocarbon insecticides (dieldrin, benzene hexachloride, heptachlor and DDT) are carcinogenic (Epstein 1977).

A number of the organochlorine insecticides (arochlor 1254, DDT, chlordane) are known to have a profound effect on immune responses. This effect has been investigated by Street and Sharma (1975) and Lee et al. (1979 a and b) who have shown that several compounds (carbaryl, dieldrin, lindane, chlordane, carbofuran, arochlor 1254, methylparathion and DDT) will inhibit lymphocyte mitogenesis often at the micromolar level.

The evidence in favour of a role for intracellular calcium levels as signals for the initiation of cell division is summarised by Metcalfe et al. (1981), and forms the basis for a calcium hypothesis for the control of cell growth. Lymphocytes undergoing transformation provide evidence for an involvement of calcium in the steps preceeding mitogenic activation which occur within 20 hours of exposure to a mitogen (Hovi et al 1979). Additionally increased rates of calcium uptake have been shown in lymphocytes exposed to mitogens (Freedman 1979). It has been suggested that this early increase in calcium flux is an initiating factor for lymphocyte mitogenesis, although Hesketh et al. (1983) claim that it is not a large short term pulse of calcium that is the pre-requisite for commitment to mitogenesis, but a prolonged small increase in free cytoplasmic Ca^{2+}concentration within a defined and narrow range that is needed for such a commitment.

It has been suspected for some time that the incidence of lymphomas (Cantor 1982) and leukemias (Blair & White 1981; Adamson & Seiber 1981; and McCabe et al 1984), may be higher in farming populations with significant past exposure to several organochlorine insecticides including chlorinated phenoxy acids,

dieldrin, benzene hexachloride, heptachlor and DDT. It is also known that immune suppression can provoke an increase in the incidence of certain cancers (Sheil 1977), although whether this is an example of (lack of) immune surveillance for potential cancer cells as envisaged by Burnet (1956) or of some less direct mechanism such as a reduced resistance to a viral infection, is still unclear. For these reasons it is important to know whether DDT and other chemically related organochlorine compounds modulate calcium levels within lymphocytes, and if so whether this modulation relates to the observed effects of these compounds on lymphocyte mitogenesis. Such studies may also be of some value in providing a clue as to the relationship between chemically induced leukemias and DDT exposure.

MATERIALS AND METHODS

Blood was collected from healthy cattle into EDTA (10mg/ml blood) and centrifuged at 800g for 30 mins to obtain a "buffy coat" which was removed and diluted with phosphate buffered saline (P.B.S.) modified to contain low Ca^{2+} (Krebs & DeGasquet 1964). The diluted buffy coat suspension was layered onto Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centrifuged at 800g for 40mins. The lymphocyte band was removed with a Pasteur pipette, diluted and washed 4 times in the modified PBS. The lymphocytes were counted on a Neubauer haemocytometer using trypan blue exclusion to assess viability, and adjusted to a final concentration of 10^8 cells/ml in the low calcium PBS, prior to their use for Ca uptake measurements.

For transformation assays the lymphocyte separation was similar except that calcium free PBS was used for washing prior to a final suspension of the cells (5 x 10^6 cells/ml) in Eagles Minimal Essential Medium (MEM) (Gibco Laboratories, Grand Island, New York) and supplemented with 10% autologous serum and Kanamycin (Kantrex, Bristol Laboratories Pty, Brookvale, NSW Australia) to a final concentation of 100ug/ml.

The range of concentrations of DDT found within human populations is reportedly from; 10^{-4}M in body fat; approx 10^{-5}M in breast milk; and of the order 10^{-7}M in plasma and urine (Sunshine 1969). All of these reported concentrations are in excess of the solubility of DDT in water (approx 10^{-8}M at 25°C). Presumably this is largely due to hydrophobic associations. To encompass this range of levels of exposure by the lymphocytes (and to permit accumulation of DDT into the cells at levels related to their in vivo situations), quantities of DDT were added to the cell suspensions such that the final nomimal concentration (assuming no selective partitioning into cellular or subcellular compartments), fell within the range 10^{-7} to 10^{-4}M. The DDT was kindly donated by Montrose Chemical Corp, California. A stock solution in ethanol was appropriately diluted in the low Ca PBS for the calcium uptake experiments and in Eagles MEM for the lymphocyte transformation assays.

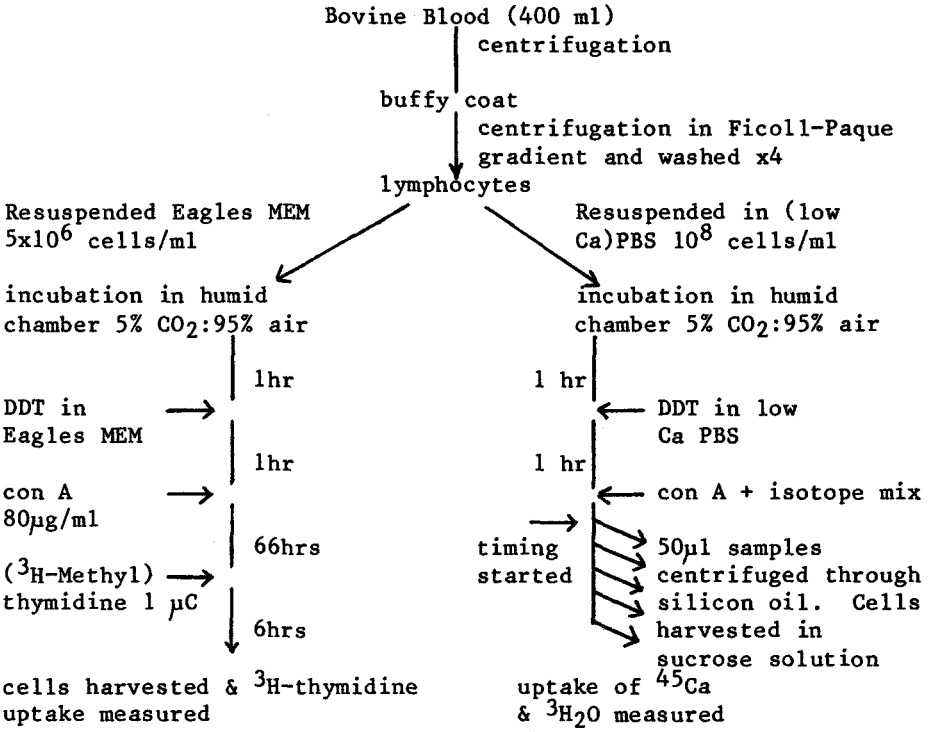

Figure 1. Summary of experimental conditions and sequences for the isolation of lymphocytes and lymphocyte (con A induced) transformation and ^{45}Ca uptake in the presence of DDT. See text for full details.

For the transformation assays, samples (100µl) of the lymphocyte suspension were plated into Linbrook (96 well) plates (Flow Laboratories, Connecticut). DDT (50µl) in the PBS was added to give final well concentrations between 10^{-7} and 10^{-4}M and incubated at room temperature for 1 hour. Eagles MEM or concanavalin A (con A) (80µg/ml) was added to 4 replicates of each DDT concentration. The cultures were maintained at 37° in a humid chamber containing 5% CO_2, 95% air for 72 hours. Prior to the final 6 hours incubation, samples of test and control wells were taken for viability by trypan blue exclusion. (^{3}H-methyl)-thymidine (1µC) was added to each well. Following the final 6 hours incubation the cells were harvested into glass fibre filter paper (grade 934 AH, Microbiological Associates, Bethesda, Ma). The filters were dried overnight prior to counting using a standard scintillation cocktail. Mean and standard deviations were calculated as was the response for each nominal concentration of DDT used as a % of the (con A) induced response in the absence of the DDT.

For calcium uptake assay, triplicate samples of cells were incubated at 37° for an hour prior to addition of DDT. The cells were then incubated for a further hour prior to addition of con A

and isotope mixture. The con A concentration was determined from dose response curves showing maximal ^{3}H-thymidine uptake after 72 hours. Timing was started on the addition of 20µl PBS or con A and 20µl of isotope mixture containing $^{45}CaCl_2$ and $^{3}H_2O$, so that the final incubation medium contained 1µC/ml of ^{45}Ca and 5µC/ml of $^{3}H_2O$. Cells to be incubated for periods longer than 10 mins were also supplemented with 0.1% glucose. Since $^{3}H_2O$ will equilibrate rapidly across cell membranes, it was used as an indicator of cell numbers (Freedman & Khan 1979).

At specified time intervals after the commencement, 50µl samples were removed and centrifuged at 12,900g for 20 sec through a barrier of 200µl silicon oil (Versilube F-50, General Electric, Waterford, N.Y.) which was layered over 20µl sucrose solution (1.45M) contained within a 400 µl microfuge tube. Centrifugation was complete within a few secs and was continued for 20 secs. The tubes were frozen by immersion into liquid N_2 and the bottom sucrose compartment was amputated into plastic minivials prior to counting for both ^{3}H and ^{45}Ca. Cell digestion proved to be unnecessary. The ratio $^{45}Ca/^{3}H_2O$ was calculated and was used as a measure of calcium uptake per lymphocyte.

RESULTS AND DISCUSSION

The effects of DDT over a range of nominal concentrations up to 10^{-4}M, on the incorporation of ^{3}H-thymidine by the lymphocyte population subsequent to its stimulation by con A is shown in table 1.

Table 1. Influence of DDT on con A induced bovine lymphocyte mitogenesis, measured by ^{3}H-thymidine uptake.

DDT (molar)	Unstimulated lymphocytes +DDT	Stimulated with 80µg con A/ml +DDT	% suppression
0	315 ± 68	55937 ± 4871	
10^{-7}	488 ± 29	37291 ± 3943	33
10^{-6}	302 ± 23	35564 ± 5289	40
10^{-5}	369 ± 57	36358 ± 4918	35
10^{-4}	422 ± 16	27375 ± 3127	51

The mean of 4 replicates ± standard deviation are tabulated. % suppression of con A stimulation was calculated as

$$100-100\ \left[\frac{\text{net con A stimulation with DDT}}{\text{net con A stimulation without DDT}}\right]$$

It can be seen that even the lowest concentration of DDT tested (10^{-7}M) provoked a 33% inhibition which could not be increased beyond approx. 50% even at a 10^{3} fold higher concentration. Following the 66 hours of incubation in the presence of the DDT there was no observed difference in viability of the cells between test and control as measured by trypan blue exclusion,both sets of samples showing approx 90% viability.

Table 2. Short term ^{45} Calcium Uptake (expressed as $^{45}Ca:^{3}H$) in Bovine Lymphocytes in the Presence and absence of 10^{-4} M DDT

Time (mins)	0 µg Con A/ml	120 µg Con A/ml
1	.1871 ± .04	.1923 ± .006
3	.2727 ± .05	.2369 ± .003
5	.2794 ± .04	.2140 ± .021
7	.3157 ± .06	.2465 ± .001
9	.3335 ± .06	.2408 ± .030

Mean of 3 replicates. Calcium uptake measured as the ratio $^{45}Ca/^{3}H$. Other details as in text.

Table 2 and 3 show the effects of con A and DDT either together or alone on the accumulation of ^{45}Ca by the lymphocytes. No immediate stimulus of calcium flux into the cells could be detected following stimulation with con A, however commencing at about 15 hours and in the absence of DDT there was a sustained increase in calcium accumulation. This time period coincides with the late G_1 stage of the cell cycle which is known to be calcium dependent in human lymphocytes (Bard et al 1978). By contrast prolonged incubation of lymphocytes with mitogen and DDT did not show this late (G_1 phase) accumulation of ^{45}Ca. Instead they showed an initial increase in ^{45}Ca uptake which was essentially complete within the first few minutes.

It is possible that the upset in calcium signals is responsible for the low proliferative response of the cells which we have observed here. To recover from the large initial calcium flux the cells may respond by sequestering and/or pumping out excess Ca, eventually lowering its intracellular level down to the level of the G_o(resting) phase.

It is interesting to note that total inhibition of cell division did not occur in bovine lymphocytes even when exposed to final nominal DDT concentrations up to 10^{-4}M and when the uptake of calcium by the cells was already considerably altered. Those cells which nevertheless proceed to S-phase may have become tolerant to the altered intracellular calcium levels. Some cancer cells have been shown to have altered calcium and calmodulin levels and their ability to evade growth regulatory signals may be a consequence of this (Hickie 1982). In this context DDT is tumorigenic in mice (Tomatis et al 1972) and in rats (Ortega et al 1956) on daily doses from 0.2mg/Kg.

The results obtained here suggest that while intracellular calcium levels may play an important role in cellular regulation and in the timing and magnitude of cell division, the fluctuations of intracellular calcium levels are not the onlysignals for cell division. The inability of DDT to abolish completely the mitogenesis of lymphocytes even though it has seriously perturbed their intracellular calcium levels, suggests that the biochemical

Table 3. 20 hour ^{45}Calcium uptake in stimulated bovine lymphocytes in presence and absence of DDT

Time (mins)	0 Con A 0 DDT	100 µg Con A per ml 0 DDT	0 Con A 10^{-5} M DDT	100 µg Con A per ml 10^{-5} M DDT	0 Con A 10^{-4} M DDT	100 µg Con A per ml 10^{-4} M DDT
10	.1702 ± .02	.1838 ± .02	.1682 ± .02	.1682 ± .02	.2473 ± .01	.2180 ± .03
20	.1936 ± .007	.1900 ± .02	.1873 ± .03	.1709 ± .01	.2878 ± .03	.2542 ± .02
30	.2498 ± .07	.1946 ± .03	.1599 ± .02	.2051 ± .01	.3075 ± .02	.2825 ± .02
60	.2503 ± .09	.2087 ± .03	.2511 ± .02	.2228 ± .02	.3990 ± .03	.4141 ± .04
120	.2336 ± .001	.2692 ± .01	.2392 ± .03	.2950 ± .007	.4743 ± .08	.4006 ± .15
240	.2459 ± .03	.2694 ± .06	.2375 ± .01	.3040 ± .11	.4825 ± .04	.4994 ± .05
360	.2649 ± .03	.2411 ± .02	.2630 ± .03	.2512 ± .02	.4551 ± .07	.5312 ± .08
480	.2809 ± .07	.2962 ± .01	.2545 ± .06	.2828 ± .09	.4789 ± .14	.4335 ± .12
600	.3503 ± .05	.3438 ± .04	.3206 ± .04	.3429 ± .003	.4858 ± .05	.6128 ± .13
960	.3147 ± .05	.5177 ± .07	.3502 ± .04	.3782 ± .15	.3751 ± .18	.5141 ± .2
1080	.3118 ± .04	.6244 ± .08	.3482 ± .05	.5223 ± .13	.4515 ± .04	.5329 ± .15
1200	.3117 ± .05	.7182 ± .11	.3153 ± .05			.5978 ± .07

Calcium Uptake measured as the ratio ^{45}Ca:^{3}H. Other details as in text.

lesion(s) produced by DDT can have quite different effects on different cells, or even on the same cell types under different conditions. Such an observation is in accord with a view of the cell as a dynamic system of interrelated biochemical oscillations (Gilbert 1974), in which oscillations of intracellular calcium are only a part (and not crucial under all circumstances) of the proliferation cycle.

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