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ENHANCEMENT OF CONCANAVALIN A-INDUCED VACUOLATION IN MACROPHAGES BY LOCAL ANAESTHETICS (CHLORPROMAZINE)

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Summary

The extensive vacuolation induced in mouse peritoneal macrophages in response to interaction with concanavalin A is markedly enhanced in the presence of chlorpromazine (10^{-5} M). At a low concentration of concanavalin A (5 μg/ml) chlorpromazine induces more than double the total number of vacuoles (> 2 μm). At higher concentrations of concanavalin A (10—40 μg/ml) though the total number of concanavalin A induced vacuoles is not affected, the size distribution of the vacuoles is changed by chlorpromazine; the number of huge vacuoles (> 5 μm) is doubled. Neither [^{3}H]concanavalin A binding nor its interiorization are affected by the simultaneous presence of chlorpromazine with concanavalin A in the incubation medium. A two-fold increase in chlorpromazine concentration ($2 \cdot 10^{-5}$ M) results in macrophage contraction and inhibition of concanavalin A-induced vacuolation. The data suggest that chlorpromazine affects vacuole formation at the stage of intracellular fusion of concanavalin A-bearing pinosomes.

Mouse peritoneal macrophages undergo extensive vacuolation in response to interaction with concanavalin A. The vacuolation process consists of surface binding of concanavalin A, redistribution of concanavalin A-receptor conjugates to form clusters that are subsequently pinocytosed and an extensive coalescence of the pinocytic vesicles to form numerous small (< 2μm) medium (2—5 μm) and huge (> 5μm) vacuoles [1—3]. The molecular basis for the frequent intracellular fusion processes, involving both mutual fusion of concanavalin A-bearing pinosomes and lysosomal fusion with the developing vacuoles exhibited in the system, is as yet unknown. This is essentially true of membrane fusion in general. Current notions on membrane fusion based on studies concerning phospholipid membranes and biological membranes are concentrated mainly on two parameters as probable determinants in the process; (a) membrane fluidity and (b) Ca^{2+} displacement

from anionic groups of acidic membrane phospholipids [4]. The molecular structure of local anaesthetics endows them with the ability to affect both parameters [4,5]. In the following we report on the ability of a phenothiazine-type tranquillizer, chlorpromazine, to enhance concanavalin A-induced macrophage vacuolation.

Peritoneal macrophages were collected from BALB/C mice and cultivated for 48 h either on cover glasses or directly on Falcon plastic tissue culture dishes in Dulbecco's modified Eagle's medium (medium) containing 20% of heat-inactivated new born calf serum [2]. Macrophages were exposed to the specified concentrations of concanavalin A (Miles-Yeda, Israel) in medium for 90 min at 37°C, with or without 10^{-5} M chlorpromazine-HCl (Taro, Israel). The extent of concanavalin A-induced macrophage vacuolation depends on the concentration of concanavalin A in the incubation medium (Figs. 1 and 2). The simultaneous exposure to chlorpromazine and concanavalin A enhances vacuolation while chlorpromazine in itself fails to induce vacuole formation. A qualitative representation of enhanced vacuolation due to the presence of chlorpromazine is given in Fig. 1, whereas the quantitative aspect of the vacuolating activity of both concanavalin A and chlorpromazine is given in Fig. 2. The number of concanavalin A-induced vacuoles increases markedly with increasing concanavalin A concentration from 5 μg/ml to 10 μg/ml, and only moderately upon further increase of concanavalin A concentration (up to 40 μg/ml). The simultaneous presence of chlorpromazine and concanavalin A enhances macrophage response to the low concanavalin A concentration; i.e. at 5 μg/ml of concanavalin A + chlorpromazine vacuolation is approaching the high vacuolation exhibited from a concentration of 10 μg/ml or more (Fig. 2A). Differential enumeration of vacuoles according to their diameter, 2—5 μm and > 5 μm, reveals a linear increase in the number of the huge vacuoles. The ($> 5\mu$m) with concanavalin A concentration (Fig. 2B). Chlorpromazine has an enhancing effect on the development of the huge vacuoles; at concanavalin A concentrations of 5, 10 and 20 μg/ml the presence of chlorpromazine results in more than a 2-fold increase in the number of the hugh vacuoles. The concanavalin A concentration dependence of huge vacuole development in the presence of chlorpromazine implies an approach to a plateau value in the number of vacuoles, a phenomenon easily understood on the basis of available cytoplasmic space. Macrophages are heterogeneous in their response to concanavalin A. At 5 μg concanavalin A/ml, 40% of the cells are devoid of vacuoles ($> 2\mu$m). At concanavalin A concentrations of 10 μg/ml and upto 40 μg/ml the number of nonresponding cells is decreased to a constant value of 20%. Chlorpromazine does not induce nonresponding cells to undergo vacuolation, the same number of cells devoid of vacuoles ($> 2\mu$m) have been enumerated in its presence as well as in its absence. Chlorpromazine could affect vacuole formation by affecting either the binding or interiorization of concanavalin A (or both) or the processes that follow lectin interiorization. In order to assess the stage at which the drug exerts its effect on vacuole formation a quantitative analysis of [^{3}H]concanavalin A binding and internalization has been carried out (Table I). The results imply that neither binding nor internalization of concanavalin A are significantly affected by

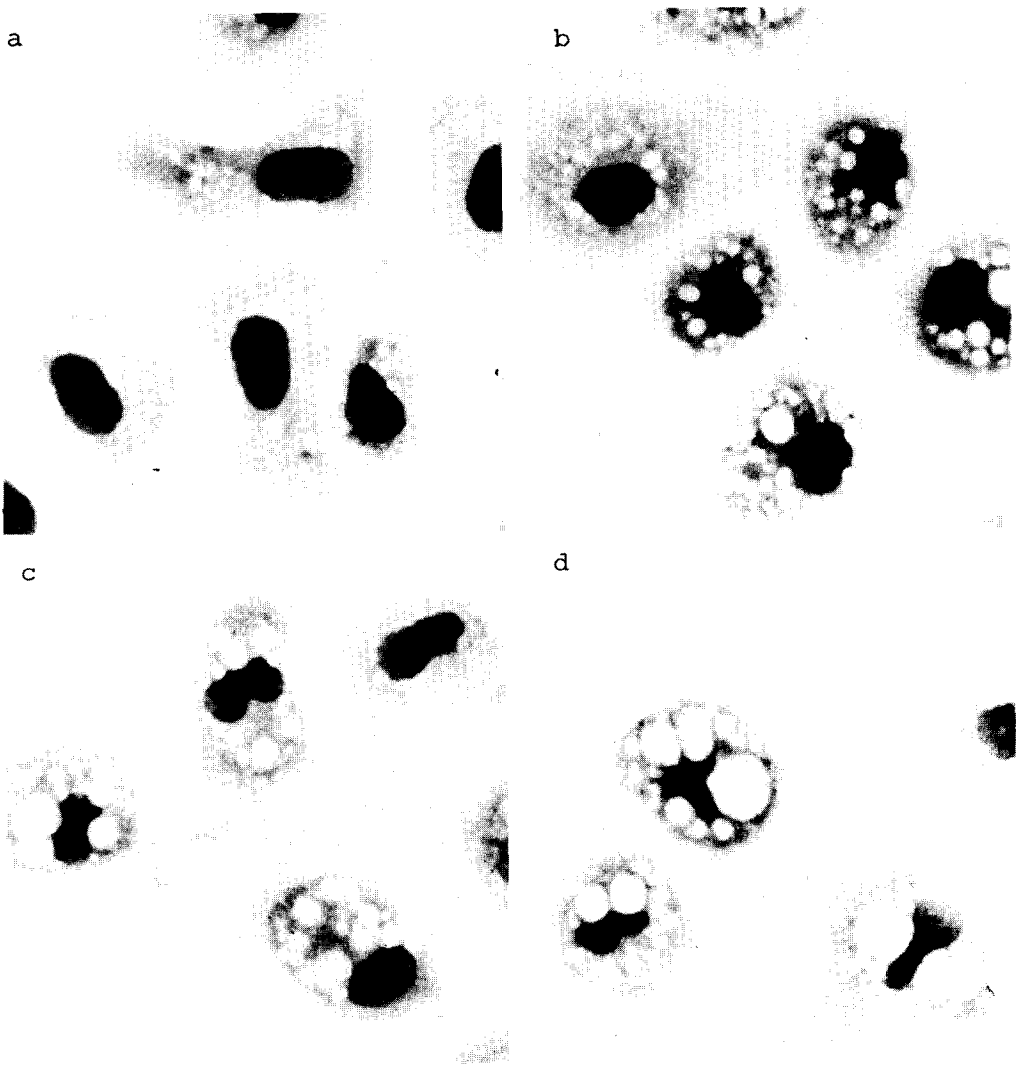

Fig.1. Effect of chlorpromazine on concanavalin A vacuolation. Macrophages were incubated in medium (37°, 90 min) with 5 μg concanavalin A/ml (a,b) or with 40 μg concanavalin/ml (c,d) in the presence (b,d) or absence (a,c) of 10^{-5} M chlorpromazine. Subsequent to incubation, cultures were fixed (2% glutaraldehyde in phosphate-buffered saline, pH 7.4, 30 min, 4°) and stained (May-Grunwald-Giemsa). × 900.

the presence of chlorpromazine and thus the enhanced vacuolation cannot be attributed to an increase in the overall number of concanavalin A-receptor conjugates interiorized.

Internalization of concanavalin A in the absence of chlorpromazine (40 μg/ml; 15 min, 37°C) and a subsequent stripping off of surface-bound concanavalin A by use of the concanavalin A-inhibitor α-methyl mannoside (0.1 M, 30 min at 22°C) yields upon reincubation in medium for 75 min (37°C) moderate cell vacuolation (about a 1/3 of the extent observed when concanavalin A is present during the 90 min period of incubation at 37°C). The presence of chlorpromazine (10^{-5} M) in the last incubation step markedly

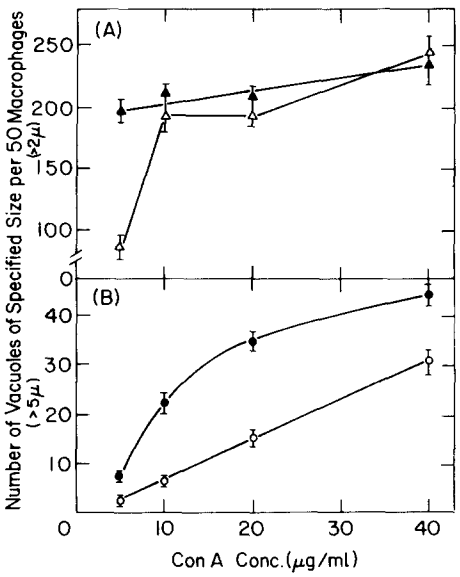

Fig.2. Effect of chlorpromazine on concacanavalin A-induced vacuolation-dependence on concanavalin A concentration. Full and empty symbols denote the presence or absence of chlorpromazine in the incubation media. Experimental conditions are those given in the legend to Fig. 1. Mean values ± S.E.M. are given for 9 groups of 50 cells enumerated in triplicate cultures.

TABLE I

THE EFFECT OF CHLORPROMAZINE ON BINDING AND INTERNALIZATION OF [^{3}H]CONCANAVALIN A

Macrophage cultures were incubated (60 min, 37°C) with [^{3}H]concanavalin A (spec. activity $6.5 \cdot 10^6$ cpm/mg) in the presence or absence of chlorpromazine. Surface bound concanavalin A and internalized concanavalin A denote the amount of [^{3}H]concanavalin A that is removable and the amount that is irremovable from macrophages by exposure to αMM (0.1 M in phosphate-buffered saline, 30 min, 22°C). The results are averages of four plates ± S.E. (standard error).

[^{3}H]concanavalin A (μg/ml)	Chlorpromazine (M)	Surface-bound [^{3}H]concanavalin A (cpm/plate) ± S.E.	Internalized [^{3}H]concanavalin A (cpm/plate) ± S.E.	Total macrophage-associated [^{3}H]concanavalin A (cpm/plate)
5	—	736 ± 68	1027 ± 44	1763
5	10^{-5}	658 ± 72	862 ± 132	1520
40	—	1390 ± 126	1684 ± 285	3074
40	10^{-5}	1297 ± 98	1712 ± 262	3009

enhances macrophage vacuolation; the total number of vacuoles (> 2 μm) is doubled, whereas the number of large size vacuoles (>5μm) increases by 5 fold. This observation implies that chlorpromazine affects the intracellular fusion of preformed concanavalin A-bearing pinosomes.

The morphologic appearance of the vacuoles as seen in the electron microscope, the location of products of acid phosphatase activity within the vacuoles (cytochemical observations) and the intracellular distribution of fluorescein-conjugated concanavalin A are essentially identical in macrophages undergoing concanavalin A-induced vacuolation in the presence (10^{-5} M) or absence of chlorpromazine (not shown).

Local anaesthetics interact with membranes affecting different membrane parameters in a concentration dependent pattern that is extremely subtle,

i.e. exhibiting differential effects (biphasic effects) at low and high drug concentrations [5]. The response of macrophages to chlorpromazine also follows a biphasic pattern. While at 10^{-5} M chlorpromazine enhances concanavalin A-induced vacuolation, a 2-fold increase in the concentration of chlorpromazine to which macrophages are exposed results in their contraction with a concomitant inhibition of concanavalin A-induced vacuolation. It is noteworthy that incubation in medium in the presence of $2 \cdot 10^{-5}$ M chlorpromazine (30 min, 37°C) reduces the ATP content of macrophages to 50% the value of control cells or cells exposed to 10^{-5} M chlorpromazine (to be published).

The manifold observations described in the literature concerning the effect of local anaesthetics on physical parameters of phospholipid membrane model systems as well as on physiological phenomena in cultured cells do not enable a formulation of a unified theory on the molecular basis of their action. Local anaesthetics have been shown to increase phospholipid fluidity both in model and natural membranes (measured by ESR and NMR [6—8]) to increase the susceptibility of untransformed cells to agglutination by concanavalin A [9] and to inhibit concanavalin A-induced lymphocyte mitogenesis [10], surface capping of concanavalin A-receptor conjugates [11] and virus-induced cell fusion [12].

The evidence presented above strongly suggests that chlorpromazine affects concanavalin A-induced vacuolation at the stage of intracellular fusion. A fluidizing effect of chlorpromazine on membrane lipids could stem from either the insertion of the lipid soluble portion of the molecule into the phospholipid domain of the membrane or from its ability to displace membrane bound Ca^{2+} [12]. An additional aspect to be considered is a possible action of chlorpromazine on the cytoskeleton. Xylocaine has been reported to cause a reversible disappearance of microtubules in rabbit vagus nerve [13]. An enhancement of concanavalin A-induced vacuolation upon interaction of macrophages with colchicine has been recently recorded [14]. Currently, efforts are being made to outline the molecular parameters that determine the capacity of a compound to induce intracellular membrane fusion, and to dissociate between effects of the lipid soluble portion and the positive charge common to many local anaesthetics. It is of interest that hydrocortisone commonly used as an antiinflammatory drug [16] has an inhibitory effect on concanavalin A-induced vacuolation [17]. Inhibition by hydrocortisone of concanavalin A-induced vacuolation is in accord with the implication that the therapeutic effect of hydrocortisone is a result of its ability to stabilize membranes against fusion.

In view of the above, we propose that studies concerning parameters affecting the frequent fusion events that precede vacuolation may be of relevance to other fusion events such as those encountered in secretion of neurotransmitters, and other secretory granules.

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