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EFFECT OF AY 9944 AND CHLORPROMAZINE ON CONCANAVALIN A-INDUCED STIMULATION OF HUMAN LYMPHOCYTES

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Abstract—Amphiphilic molecules AY 9944 and chlorpromazine (CPZ) inhibited DNA synthesis in Concanavalin A-stimulated lymphocytes in a dose-dependent manner. While AY 9944 strongly decreased 7-dehydrocholesterol conversion to cholesterol, CPZ did not significantly affect this reaction. Moreover, the inhibitory effect of AY 9944 and CPZ on DNA synthesis took place in the presence of cholesterol in the culture medium. These findings suggest that the mechanism of inhibition of DNA synthesis by AY 9944 or CPZ is not related to endogenous cholesterol synthesis or exogenous cholesterol supply. Results are discussed in relation to the amphiphilic properties of AY 9944 and CPZ and to the interaction of these drugs with membranes or other intracellular targets such as calmodulin.

Numerous studies have shown a relationship between the activity of the enzyme 3-hydroxy 3-methylglutaryl Coenzyme A (HMG CoA) reductase and DNA synthesis [1–3]. DNA synthesis is strongly reduced in cells treated by various inhibitors of HMG CoA reductase such as oxysterols [4], compactin [5, 6], and mevinolin [7]. Recently Kay and Wilce [8] found that AY 9944,* an inhibitor of 7-dehydrocholesterol reductase [9], strongly reduced phytohemagglutinin (PHA)-induced stimulation of human lymphocytes cultured in a delipidated medium. This effect appeared to be partially reversed when the medium was supplemented with low density lipoproteins [8], the main plasma cholesterol carrier [10]. Thus, these workers concluded that AY 9944 inhibited PHA-induced lymphocyte stimulation by means of a decrease in cholesterol synthesis. These observations support the hypothesis that some endogenous cholesterol synthesis is required for the primary step(s) of cell proliferation. However, there was no direct evidence for a relationship between cholesterol synthesis and lymphocyte activation. It must be also considered that AY 9944 is an amphiphilic compound which can interact with membranes [11] and with various intracellular components. In previous studies [12], we demonstrated that this drug is a powerful antagonist of calmodulin, binding to this calcium-regulatory protein with a greater affinity than well-known calmodulin inhibitors trifluoperazine (TFP) or chlorpromazine (CPZ). Moreover, Salisbury *et al.* [13] demonstrated that calmodulin antagonists CPZ or TFP inhibit lectin endocytosis and block lymphocyte activation. Thus, the inhibition of lymphocyte stimulation by AY 9944 could also be interpretated as the result of its ability to block calmodulin-dependent processes. In the present work, we comparatively studied the effects of AY 9944 and of the calmodulin antagonist CPZ [14], on lipid (especially sterol) synthesis and thymidine incorporation in Concanavalin A (Con A) stimulated human lymphocytes. We found that AY 9944 inhibited lymphocyte activation even in the presence of extracellular cholesterol supply. CPZ also inhibited lymphocyte stimulation although this phenothiazine did not alter the conversion of 7-dehydrocholesterol to cholesterol.

* Abbreviations used: AY 9944, [*trans*-1,4 bis (2-chlorobenzylaminomethyl) cyclohexane dihydrochloride]; CPZ, chlorpromazine; TFP, trifluoperazine; Con A, Concanavalin A; PHA, phytohemagglutinin; LDL, low density lipoprotein.

MATERIALS AND METHODS

Chemicals and radiolabelled compounds. [^{3}H-methyl]-thymidine (5Ci/mmol) was purchased from Amersham, U.K. [1-^{14}C] sodium acetate (50 mCi/mmol) was ordered from C.E.A., France. AY 9944 was a generous gift of Ayerst Laboratories, U.S.A. Concanavalin A (Con A) was acquired from Miles Laboratories, U.S.A. and chlorpromazine (CPZ) from Serva.

Cell culture. Human peripheral blood lymphocytes were isolated on a Ficoll-Metrizoate gradient (Lymphoprep, Nyegaard, Oslo). The cells were cultured in 10 ml tubes (Nunc) at 10^6 cells/ml of the culture medium (RPMI 1640, L-glutamine, 25 mM Hepes, penicillin 250 IU/ml, streptomycin 250 μg/ml) supplemented with 10% autologous plasma inactivated by heating 30 min at 56°. Cultures have been incubated at 37° in a 5% CO_2 incubator for 18 hr before starting all experiments. Lymphocytes were stimulated by the lectin Convanavalin A (Con A) (5 μg/ml). Con A-stimulated lymphocytes were harvested after 72 hr of culture. When added, AY 9944 and CPZ were brought to the culture medium at the same time that Con A. Cell viability was assayed by the Trypan blue test and was found to be above 90%.

DNA synthesis determination. [^{3}H-methyl]-thymidine (1.25 μCi/ml) was added to the culture medium 4 hr before harvesting the cells. Cell suspensions were layered on Whatman glass microfiber filters GF/C and washed 4 times with cold phosphate buffer saline (PBS) pH 7.4. The radioactivity of the dried filters was measured in a liquid scintillation counter.

Lipid synthesis determination. [1-^{14}C] sodium acetate (12.5 μCi/ml) was added to the culture medium 4 hr before the harvesting. Cells were harvested by centrifugation in a cold PBS pH 7.4. The cell suspension was then extracted with chloroform/methanol (2/1, v/v) according to the method of Folch *et al.* [15]. The recovery of sterols was monitored by incorporation of [^{3}H]-cholesterol before extraction. Lipid analysis was performed by thin layer chromatography (TLC) on silicagel plates (Silicagel F 1500 Schleicher and Schull), according to the method of Ditullio *et al.* [16] modified by Barbu *et al.* [17]. The upper half of TLC plate was impregnated with silver nitrate by immersion in a 10% silver nitrate solution. The chromatogram was initially developed in a solvent system consisting of chloroform/methanol/water (65/25/4, v/v). As the solvent front reached approximately 50% of the TLC plate, the solvent system was switched to chloroform/diisobutylketone (90:10, v/v). The different sterols, isoprenoids and other lipids were identified after autoradiography by comparison with purified unlabelled standards. Cholesterol and lanosterol were ordered from Sigma, 7-dehydrocholesterol and lathosterol were purchased from Steraloids, U.S.A. Radioactive spots were cut out from the TLC plates and their radioactivity measured by liquid scintillation.

RESULTS

It can be observed in Fig. 1 that a marked decrease in thymidine incorporation took place for AY 9944 at 10^{-5} M (about 70–80% inhibition) or chlorpromazine (CPZ) over 10^{-5} M (90% inhibition at 5×10^{-5} M). In fact AY 9944 appears to be more effective than CPZ for inhibiting [^{3}H]-thymidine incorporation by Con A-stimulated cells.

Figure 2 displays the results of the comparative study of the effects of AY 9944 and CPZ on cholesterol and 7-dehydrocholesterol biosynthesis from [^{14}C]-acetate during Con A-induced stimulation. In view of the Kay's hypothesis of a relationship between the 7-dehydrocholesterol to cholesterol conversion and stimulation of cell proliferation, we especially focused our attention on the [^{14}C]-7-dehydrocholesterol/[^{14}C]-cholesterol ratio. It clearly appears from Fig. 2 that where, as expected, the ratio was dramatically increased in cells treated with AY 9944 above 5×10^{-6} M, there was no significant alteration of this ratio under CPZ treatment even at 5×10^{-5} M, a concentration which severely impaired [^{3}H]-thymidine incorporation into DNA. This is illustrated by Fig. 3, which shows the increase in [^{14}C]-acetate incorporation into 7-dehydrocholesterol and the correlated decrease of the radioactivity incorporated into cholesterol in cells treated with AY 9944 10^{-5} M. On the same plate, it can be seen that for CPZ at 5×10^{-5} M, a concentration

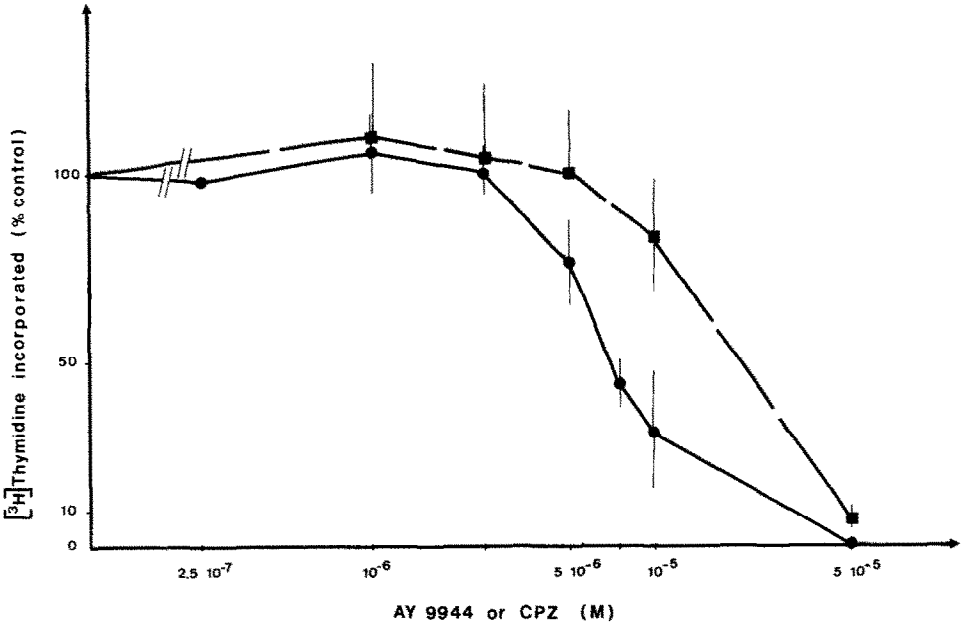

Fig. 1. Effect of AY 9944 and CPZ on [^{3}H]-thymidine incorporation. 2×10^6 peripheral blood lymphocytes were cultured 72 hr in 2 ml of complete medium supplemented with 5 μg/ml Con A and increasing concentrations of AY 9944 (●) or of CPZ (■). Cells were labelled with 2.5 μCi of [^{3}H]-thymidine 4 hr before the harvest. 100% = 406908 ± 45857 CPM. Data represent means of three experiments.

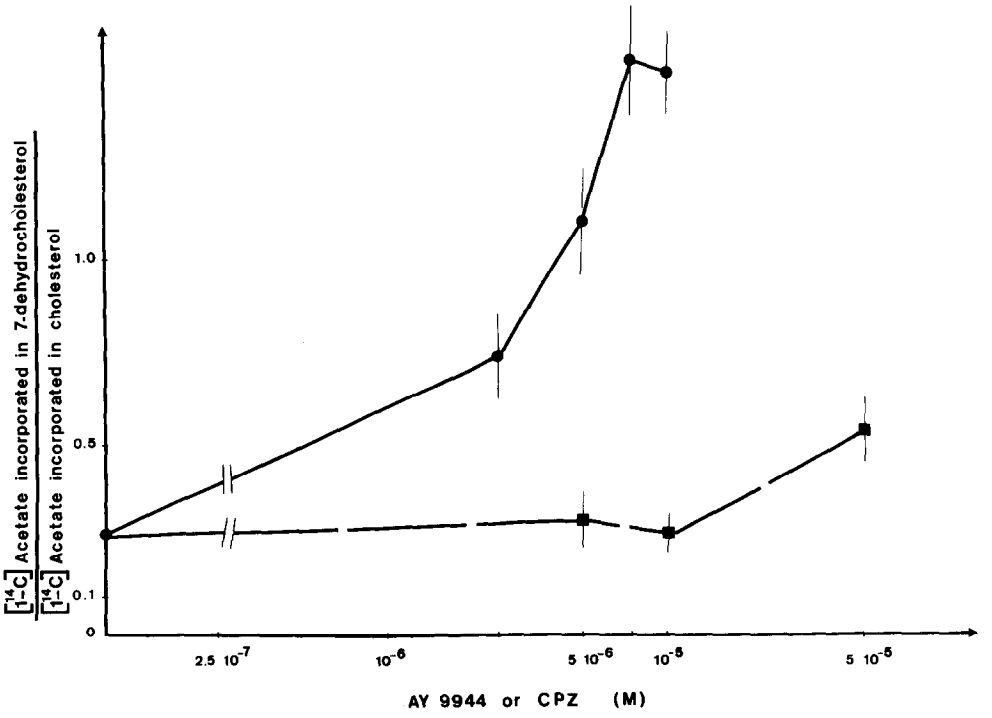

Fig. 2. Effect of AY 9944 and CPZ on sterol synthesis. 2×10^6 peripheral blood lymphocytes were cultured 72 hr in 2 ml of complete medium supplemented with 5 μg/ml Con A and increasing concentrations of AY 9944 (●) or of CPZ (■). Cells were labelled with 25 μCi of [1-^{14}C] acetate 4 hr before the harvest. Lipids were extracted and separated by TLC as described in Materials and Methods. The following values were found in Con A-stimulated lymphocytes in the absence of drugs: [^{14}C]-acetate incorporated into cholesterol: 3555 ± 311 cpm/10^6 cells. [^{14}C]-acetate incorporated into 7-dehydrocholesterol: 960 ± 30 cpm/10^6 cells. Data represent means of three experiments.

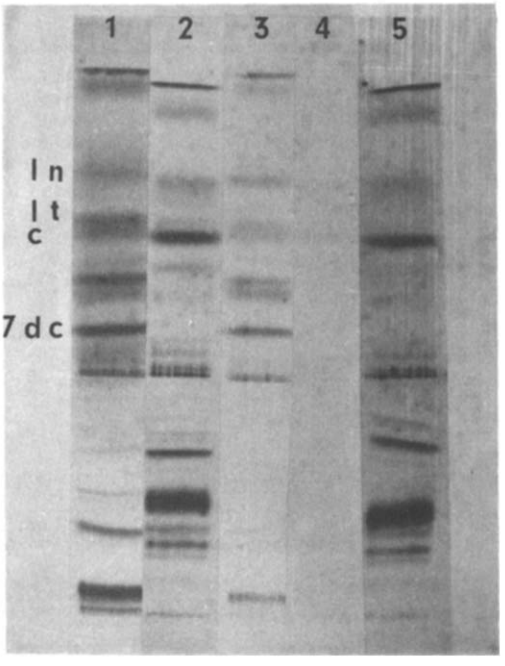

Fig. 3. Autoradiography of a TLC plate showing the incorporation of [1-^{14}C] acetate in sterols by human lymphocytes: c, cholesterol; 7dc, 7-dehydrocholesterol; lt, lathosterol; ln, lanosterol; 1, AY 9944 7.5 μM; 2, CPZ 10 μM; 3, AY 9944 10 μM; 4, CPZ 50 μM; 5, control.

which also strongly inhibits lymphocyte stimulation, there is clearly no decrease of the conversion of 7-dehydrocholesterol to cholesterol.

DISCUSSION

From these results, several conclusions can be drawn.

(1) In our experimental conditions, AY 9944 or CPZ is able to inhibit the effect of Con-A on human lymphocyte proliferation, even in a medium containing exogenous cholesterol (lipoproteins). This is a first difference with the results obtained by Kay *et al.* which found that addition of LDL to their culture medium (which was essentially devoid of cholesterol) partially restored the lectin-induced stimulation. In our case, the inhibitory effect of either AY 9944 or CPZ took place even in the presence of cholesterol in the culture medium.

(2) The inhibition of Con A-induced stimulation by AY 9944 and CPZ seems to be unrelated to the rate of the 7-dehydrocholesterol to cholesterol conversion, as CPZ, at doses which strongly reduced thymidine incorporation into DNA, did not significantly affect the ratio of [^{14}C]-acetate incorporated into 7-dehydrocholesterol versus [^{14}C]-acetate incorporated into cholesterol.

Actually, our results are closely similar to the inhibition of Con A-induced lymphocyte stimulation by trifluoperazine (TFP) reported by Salisbury *et*

al. [13]. TFP, as well as CPZ, is an amphiphilic compound which strongly interacts with membranes and alters their physico-chemical properties [18, 19]. Moreover, these drugs, as well as many other amphiphilic compounds, are known as non-specific calmodulin antagonists [20]. AY 9944 shares many properties with these drugs: it is an amphiphilic cation, which also induces non-specific lipidosis in animals [21] and inhibits various membrane-bound enzymes such as Na^+, K^+-ATPase or sphingomyelinase [22]. Finally, it is a powerful ligand of calmodulin in the range of concentration used in our experiments, with greater affinity than CPZ, as we previously described [12]. In fact, cultured fibroblasts treated with AY 9944 rapidly exhibit a rounded shape with thin bipolar extensions, which strongly suggests an effect of the drug on the cytoskeleton, as it was also reported for TFP [23].

One of the observed effects of AY 9944 and perhaps several of them, could account for the inhibition of the lectine-induced lymphocyte proliferation. The alteration of the membrane physico-chemical properties, the inhibition of the sodium/potassium pump, the blockade of the calmodulin-dependent endocytosis of the lectin itself are probably concomitantly involved in the observed phenomenon.

The last question which must be discussed is the partial reversion of the inhibitory effect of AY 9944 observed by Kay and Wilce [8] in their experimental conditions. It must be considered that LDL has been reported by several authors to antagonize the lectin-induced stimulation of lymphocytes [24, 25]. Thus, the partial reversion by LDL of the inhibitory effect of AY 9944 on Con A-induced stimulation of lymphocytes observed by Kay and Wilce [8] is rather surprising. If we consider the amphiphilic properties of AY 9944, an interaction between the drug and the LDL phospholipids may be hypothesized. Thus, the actual concentration of the drug in the cell membrane or at the level of its intracellular targets such as calmodulin could be reduced by the presence of LDL in the culture medium. This could explain the partial reversion of the inhibitory effect of AY 9944 by LDL observed by Kay and Wilce [8].

In conclusion, from our experiments, we postulate that the inhibition of the mitogenic effect of lectins on human lymphocytes by AY 9944 is related to the effect of the drug on membrane and/or intracellular targets such as calmodulin rather than to the inhibition of the 7-dehydrocholesterol to cholesterol conversion. Experiments are now performed to determine if AY 9944 inhibition of DNA synthesis could be reversed by mevalonate like with other hypocholesterolemic drugs such as compactin or mevinolin [6, 7] and if its effect on other intracellular targets than calmodulin could be involved in the observed phenomenon.

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