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Combined effects of AY9944 and plasma LDL (or whole plasma) on lymphocyte blastic transformation

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During blastic transformation of lymphocytes stimulated by lectins, a peak in cholesterol synthesis occurs in phase G1 of the cell cycle [1, 2]. Such cholesterol synthesis is indispensible for cells to enter into phase S. Various inhibitors of cholesterol synthesis, 20, α-hydroxycholesterol, 25, α-hydroxycholesterol, 7-cetocholesterol, acting at the step of HMGCoA reduction, are capable of inhibiting the incorporation of [^{3}H]thymidine into cell DNA and the occurrence of blastic forms in stimulated lymphocytes [1–3]. Kay and Wilce [4] have further observed that AY9944 (*trans* 1,4bis(2-chlorobenzylaminomethyl)cyclohexane), an inhibitor of the ultimate stage of cholesterol synthesis,

acting at the step of 7-dehydrocholesterol reduction [5], can also, in a cholesterol-free medium, inhibit the incorporation of [^{3}H]thymidine, an inhibition which is partially reversible by the simultaneous addition of low density lipoproteins (LDL) to the medium (that is to say, by an addition of exogenous cholesterol) [4].

Nevertheless, LDL, or at least a fraction thereof, and VLDL (very low density lipoproteins) are themselves inhibitors of blastic transformation [6–8]. The inhibitory effect of LDL is apparent on cell plasmic membrane [9], where LDL block the initial stages of cell stimulation [10–14], irrespective of cholesterol synthesis. On the other hand, in a 10% serum medium, despite contact with exogenous cholesterol, an increase in the dose of AY9944 inhibits the incorporation of [^{3}H]thymidine in stimulated lymphocytes [15]. This suggests the idea that AY9944, in addition to its effect on cholesterol synthesis, also has a different action, possibly related to its amphiphilic properties and to its strong membrane polarity [16].

In this work, we observed that the plasma in the medium was able to neutralize the inhibitory effect of AY9944 on blastic transformation, and we hypothesized that plasma LDL were responsible for this phenomenon. Accordingly, we demonstrated that if AY9944 and LDL are added to the medium simultaneously, these two agents neutralize each other's inhibitory effect on blastic transformation. On the contrary, if the cells are pre-incubated with AY9944 and subsequently separated and placed in contact with LDL during stimulation, the inhibitory effects of these two agents are cumulative.

Materials and methods

Human peripheral lymphocytes were prepared by Böyum's method [17], under precise conditions previously described [18].

LDL were prepared by the method of Havel *et al.* [19]. The serum of AB(+) blood (CNTS, Paris) was collected, combined with 0.4% EDTA and centrifuged at slow speed. The density of the serum was then brought up from 1.006 to 1.024 by the addition of a suitable volume of saline solution with a density of 1.357, containing 153 g of NaCl, 354 g of KBr and 0.4 g of EDTA per liter. This was centrifuged at 155,000 *g*, at 10°, for 18 hr. The upper half of the supernatant, which contains VLDL, was eliminated. The rest of the supernatant was collected and its density raised to 1.050 with the preceding saline solution. This was centrifuged again at 155,000 *g*, for 22 hr. The upper part of the supernatant, containing LDL and free of high density lipoprotein (HDL), was collected. LDL were purified by another identical centrifugation. They were finally dialysed at 4° against a solution containing 175 g of NaCl, 3.5 g of EDTA and 15.8 g of Tris buffer per liter. Proteins of the LDL fraction prepared in this manner were titrated by the method of Lowry *et al.* [20].

Lymphocyte blastic transformation was evaluated by using either [^{3}H]thymidine incorporation into cell DNA or the blast counting method.

In some experiments, cell stimulation was effected at various plasma concentrations in the medium. When we used very low plasma concentrations, down to 1.5%, the viability of cells was only acceptable for incubation times not exceeding 48 hr, that is too short for blast counting. Therefore, in these experiments, we evaluated blastic transformation by [^{3}H]thymidine incorporation. Moreover, plasma contains glycoproteins capable of blocking concanavalin A [21]. A precise quantitative study of this phenomenon* led us to use doses of concanavalin A which were proportional to the different concentrations of plasma, so that blastic transformation would always be of the same order (in any event, for each dose of concanavalin A, we referred the inhibitory effect of AY9944 on blastic transformation to the value obtained in the absence of AY9944). Lymphocytes were incubated in RPMI 1640 medium, supplemented with 0.3 g of L-glutamine, 25 mM of Hepes buffer, 10^5 I.U. of penicillin and 0.3 g of streptomycin per liter, to which 1.5, 7.5 or 15% autologous plasma were added. Four hundred thousand cells were incubated in 150 μl of medium (final volume), under 5% CO_2, at 37°. When present, AY9944 (Ayerst Laboratories, U.S.A.) was used at a concentration of 5 or 10 μM. For blastic transformation, 2, 10 or 20 μg of concanavalin A (Sigma Chemical Co., U.K.) were added per tube, these doses being proportional to the different concentrations of plasma. After 48 hr of incubation, each tube receives 2 μCi—that is 10 μl, or 80 pM—of [^{3}H-methyl]thymidine (CEA, Saclay). Two hours later, incorporation was stopped by the addition of 1 ml of a 25% solution of trichloracetic acid (TCA). After 15 min, the TCA-insoluble material was centrifuged, washed four times with TCA and directly transferred into scintillation phials, where it was dried and its radioactivity counted.

In other experiments, cell stimulation was effected in a normal medium of 10% plasma. In this case, the viability of cells was acceptable even for 96-hour incubation, and we preferred to use blast counting, which gives the total number of stimulated cells (including those lymphoblasts which have the slowest cell cycle and which are seen in G1, before thymidine incorporation). The incubation medium was the same as above, but it contains 10% autologous plasma. This time, 1.5 million cells were incubated in 1.5 ml of medium, in closed tubes. AY9944 was used at a concentration of 5 μM and LDL (from the preceding preparation) were used at a concentration of 360 μg of protein per tube (in addition to the LDL in the 10% plasma). For blastic transformation, 30 μg of concanavalin A were added per tube, so that the percentage of lymphoblasts which was obtained was about 75%. Incubation was carried out for 72 or 96 hr. At the end of the incubation, the cell suspension was centrifuged, the cells taken out, spread out on a slide, fixed by methanol, colored according to May–Grumwald–Giemsa and observed in immersion. Cells with expanded and basophilic cytoplasm were considered blastic. The percentage of lymphoblasts was determined from the observation of 500 cells.

Results and discussion

We describe, in Table 1, the effect of AY9944 on blastic transformation at various plasma concentrations in the medium. It can be seen that AY9944 is capable of inhibiting blastic transformation, as has already been reported [4, 15]. In addition, the inhibitory effect observed, which increases with the dose of AY9944, diminishes when plasma concentration is higher. Cell viability is not responsible for these results. At plasma concentrations of 7.5 and 15%, the percentage of dead cells, evaluated by the method of trypan blue exclusion, was less than 5%. At a plasma concentration of 1.5%, cell death, although higher, never exceeded 20%, irrespective of the dose of AY9944. It is thus possible to hypothesize the existence of a plasma factor capable of neutralizing AY9944.

This factor may be plasma LDL. We can see, on Table 2, the effect of the simultaneous addition of AY9944 and LDL to the medium during cell stimulation. AY9944 by itself, at a concentration of 5 μM, lowers blastic transformation to half the reference value. LDL alone lower blastic transformation to two-thirds of the reference value. But if AY9944 and LDL are added together to the medium, their inhibitory effects totally cancel out. We thus conclude that AY9944 and LDL neutralize each other, either in the medium (which is the most probable explanation) or on the cellular level.

Table 3 describes the effect of LDL on blastic transformation of lymphocytes pre-incubated with AY9944. Cells were first incubated with AY9944, then later sep-

* Rampini C, unpublished results.

Table 1. Effect of AY9944 on blastic transformation of lymphocytes at various plasma concentrations in the medium

Plasma concentration (%)	Concentration of AY9944 0	5 μM	10 μM
1.5 (N = 6)	100	20.6 (±6.5)	7.2 (±3.3)
7.5 (N = 12)	100	64.8 (±5.4)	28.0 (±5.8)
15 (N = 6)	100	83.9 (±7.6)	64.0 (±11.0)

Lymphocyte blastic transformation was evaluated by [^{3}H]thymidine incorporation, using 2-hr pulses, 48 hrs after the onset of stimulation with concanavalin A and expressed in percentage of observed values in the absence of AY9944. These values were, for plasma concentrations of 1.5, 7.5 and 15% respectively, 29700 (±9500), 63000 (±7600) and 50200 (±13300) cpm.

The doses of concanavalin A, which were proportional to the different concentrations of plasma, were respectively 2, 10 and 20 μg per tube.

Table 2. Effect of the simultaneous addition of AY9944 and LDL to the medium during stimulation of lymphocytes

	Without	With AY9944 alone	With LDL alone	With AY9944 and LDL
Percentage of lymphoblasts	74 (±5)	41 (±7)	52 (±9)	75 (±8)
Percentage of the first column	100	55	70	101

Lymphocyte blastic transformation was evaluated by the percentage of lymphoblasts obtained 96 hr after the onset of stimulation with concanavalin A.

The plasma concentration of the medium was 10%. The concentration of AY9944 was 5 μM. The quantity of LDL used was 360 μg of protein per tube (1.5 ml of medium). The dose of concanavalin A was 30 μg per tube.

Values are the average of six experiments.

Table 3. Effect of LDL on the blastic transformation of lymphocytes pre-incubated with AY9944

	Without	With AY9944 alone (pre-incubation)	With LDL alone	With AY9944 (pre-incubation) and LDL
Percentage of lymphoblasts	81 (±8)	47 (±7)	57 (±7)	23 (±9)
Percentage of the first column	100	58	70	28

The cells were pre-incubated 24 hr with or without AY9944. They were then separated and incubated with concanavalin A with or without LDL. Blastic transformation was evaluated by the percentage of lymphoblasts obtained 72 hr after the onset of stimulation with concanavalin A.

The plasma concentration of the medium was 10%. The concentration of AY9944 was 5 μM. The quantity of LDL used was 360 μg of protein per tube (1.5 ml of medium). The dose of concanavalin A was 30 μg per tube.

Values are the average of six experiments.

arated and stimulated by concanavalin A, with the addition of LDL. The added LDL were thus never in direct contact with AY9944 in the medium, in order to avoid any mutual neutralization of these two agents out of the cells. In these conditions, i.e. on the cellular level, the inhibitory effects of AY9944 and LDL on blastic transformation combined in a synergic manner.

Our results indicate the existence of a direct interaction between AY9944 and LDL in the medium, so that these two agents neutralize each other's inhibitory effect on blastic transformation of lymphocytes. This fact could explain the apparently contradictory results obtained in different studies [4, 15], during which AY9944 and LDL were always in contact with each other in the culture medium. One of these studies thus concluded that, in a cholesterol-free medium, AY9944 inhibited blastic transformation by

means of a blockade of cholesterol synthesis, since addition of LDL, which provide exogenous cholesterol, reversed the inhibition of cell stimulation [4]: nevertheless, the added LDL might simply neutralize AY9944 in the medium. Conversely, another study concluded that AY9944 inhibited the initial stages of blastic transformation, irrespective of cholesterol synthesis, since the inhibition was observed in a normal medium of 10% serum, that is to say, in the presence of exogenous cholesterol [15]: but in this case, AY9944 might neutralize LDL in the medium, so that it also blocked the supply of exogenous cholesterol to cells.

The neutralization of LDL by AY9944 could correspond to a blockade of the protein moiety of LDL, which is responsible [9, 13] for their inhibitory effect on blastic transformation. It must be noted that LDL with selectively modified lysine residues lost their ability to suppress phosphatidylinositol turnover in stimulated lymphocytes, but that nevertheless, they bind to cells [13]. Similarly, the neutralization of LDL by AY9944 could not signify that the receptor recognition site of LDL is blocked.

Thus, the combined effects of AY9944 and plasma LDL on the cellular level can only be studied if the action of these two agents is dissociated in time. We then observed a synergic inhibitory effect of AY9944 and LDL on blastic transformation. It has been established that LDL inhibit lymphocyte stimulation on the level of the plasmic membrane [9], at the very first stages of the mitogen's action [10–14]. However, the mechanism of the inhibitory effect of AY9944 on blastic transformation has not yet been well established (see above). AY9944 may inhibit the early stages of cell stimulation—because of its membrane polarity [15, 16]—but it may also block later events during G1: cholesterol synthesis [4, 5], IL-2 formation or IL-2 effect (IL-2 receptors are detected only about 10 hr after the onset of stimulation with lectin [22]). We observed that AY9944 inhibited the early "phosphatidylinositol response" in concanavalin A-stimulated lymphocytes [23]. Thus, it is most likely that AY9944 affects the very initial stages of cell stimulation.

Direct extracellular interaction between AY9944 and LDL could be, *in vivo*, an essential factor in the teratological effects of AY9944 on the brain [24, 25]. It is possible that the neutralization of plasma LDL by AY9944 brings about a reduction in the exogenous contribution of cholesterol in the nerve cells, at a stage of ontogenesis at which the endogenous synthesis of cholesterol is a limiting factor (which is itself lowered by AY9944). Moreover, the neutralization of AY9944 by plasma LDL can partially explain the influence of hyper- or hypocholesterolemia on this drug's teratological effects [26, 27].

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Further characterization of neurotensin binding in the rat brain: levocabastine-displaceable neurotensin binding sites are not histamine-H_1 receptors

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Neurotensin was discovered in 1973 by Carraway and Leeman in extracts from bovine hypothalamus [1], and was found to be involved in numerous physiological processes both in the central nervous system and the periphery [2].

In radioligand binding studies, several lines of evidence suggested the existence of a high and a low affinity neurotensin binding site in rat brain membranes (NT_H- and NT_L-sites) [3, 4]. Levocabastine [5], a histamine-H_1 antagonist, structurally unrelated to neurotensin, was shown to inhibit selectively neurotensin binding to the NT_L-sites in rat, mouse and hamster brain, but not in other species including human. Levocabastine became a useful tool which enabled separate measurement of the labelling of high and low affinity neurotensin binding sites (K_D = 0.7 and 7.1 nM, respectively) [6].

NT_H-sites in rat brain displayed the characteristics of a neurotransmitter receptor, whereas the NT_L-sites have an as yet undefined role and are likely to be considered as chemical recognition sites for neurotensin or acceptor sites [6–8].

In the present report we will define additional biochemical properties of both neurotensin binding sites, in order to further scrutinize the distinction between NT_H and NT_L-sites in the rat brain and to analyse whether any relationship would exist between the low affinity neurotensin binding site and the histamine-H_1 receptor.

Material and methods

[^{3}H]*Neurotensin binding.* [^{3}H]Neurotensin binding (2 nM) was performed in 0.5 ml of Tris–HCl buffer (50 mM, pH 7.4) containing total particulate fraction corresponding to 5 mg original rat forebrain, 0.1% bovine serum albumin, 1 mM EDTA and 0.2 mM bacitracin. The incubation of 20 min at 25° was terminated by rapid filtration through Whatman GF/B glass fibre filters under reduced pressure. The filters were rinsed twice with ice-cold Tris–HCl buffer (50 mM, pH 7.4) and the radioactivity retained on the filters was measured by liquid scintillation spectrometry.

Filters were soaked in 0.1% polyethylenimine for 2 hr prior to use, in order to reduce adsorption of the radioligand to the filters.

Selective binding to the NT_H-sites was measured in the presence of 1 μM levocabastine, which occludes the NT_L-sites. Nonspecific binding was determined by addition of 1 μM unlabelled neurotensin to the incubation medium.

Binding to NT_L-sites was calculated as the difference between total binding and binding in the presence of 1 μM levocabastine.

[^{3}H]*Levocabastine binding.* Binding experiments were performed in 1 ml of Tris–HCl buffer (50 mM, pH 7.4) containing total particulate fractions from rat cortex corresponding to 10 mg of original tissue per assay, 2 nM [^{3}H]levocabastine, 0.1% bovine serum albumin, 1 mM EDTA and 0.2 mM bacitracin. Incubation was run for 20 min at 25° and ended by rapid filtration through Whatman GF/B glass fibre filters under reduced pressure. Filters were rinsed twice with ice-cold Tris–HCl buffer (50 mM, pH 7.4) and retained radioactivity was counted by liquid scintillation spectrometry.

Filters were soaked in 0.1% polyethylenimine for 2 hr prior to use, in order to reduce adsorption of the radioligand to the filters.

Nonspecific binding was measured in the presence of 1 μM unlabelled levocabastine.

[^{3}H]*Pyrilamine binding.* [^{3}H]Pyrilamine binding (2 nM) was performed in total particulate fractions from guinea-pig cerebellum (10 mg original tissue/assay) diluted in 1.1 ml phosphate buffer (10 mN NaH_2PO_4, 40 mM K_2HPO_4, pH 7.5). Incubation was run for 30 min at 25° and terminated by rapid filtration under reduced pressure. Filters were rinsed twice with ice-cold Tris–HCl buffer (50 mM, pH 7.4) and radioactivity retained on the filters was counted by liquid scintillation spectrometry. Astemizole (1 μm) was used for the determination of the nonspecific binding.

Materials. [3,11-Tyrosyl-3,5-^{3}H(N)]neurotensin (40.9–74.5 Ci/mmol or 1.5–2.7 TBq/mmol) and [^{3}H]pyrilamine (27.9 Ci/mmol or 1.0 TBq/mmol) were purchased from New England Nuclear (Dreieich, F.R.G.). [^{3}H]Levocabastine (16.0 Ci/mmol or 0.6 TBq/mmol) was synthesized by C. Janssen (Janssen Research Foundation, Beerse, Belgium). Unlabelled neurotensin was obtained from U.C.B. (Braine-l'Alleud, Belgium) and unlabelled levocabastine was from Janssen Pharmaceutica (Beerse, Belgium). Instagel II scintillation fluid was purchased from Packard (Downers Grove, IL).

Results and discussion

Association and dissociation of [^{3}H]*neurotensin binding.* Binding of [^{3}H]neurotensin to NT_H- and NT_L-sites was measured after different incubation times, between 1 and