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CYTOTOXICITY OF CHLORAMBUCIL AND CHLORAMBUCIL–FATTY ACID CONJUGATES AGAINST HUMAN LYMPHOMAS AND NORMAL HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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Abstract—The cytotoxic activity of chlorambucil (Chl) and of chlorambucil–fatty acid conjugates of different degree of unsaturation have been assayed *in vitro* upon two human lymphoma cell lines and comparatively, upon quiescent and mitogen-activated lymphocytes from healthy blood donors. The cell toxicity observed with Chl–arachidonic acid and Chl–docosahexaenoic acid against lymphoma cells was, at any experimental condition used, equal or higher than the individual toxic potential of either chlorambucil or fatty acids. The two conjugates, like chlorambucil alone, were toxic against mitogen-activated lymphocytes. Contrary to chlorambucil, Chl–arachidonic at any concentration tested, lacked of toxicity towards normal non-activated lymphocytes. Chl–oleic acid conjugate was, whatever the cell species tested, much less toxic than Chl alone. In conclusion, the coupling of chlorambucil with polyunsaturated fatty acids increases: (a) the selectivity against neoplastic *versus* quiescent lymphocytes and (b) the toxicity for B-lymphoma cells. The selective effect of Chl–fatty acid conjugates is discussed in relation with the expression of an AFP/AFP-receptor autocrine system in malignant lymphoblastoid cells and in mitogen-activated lymphocytes.

Chemotherapeutic agents currently used for antitumor therapy are selected for their toxicity upon rapidly proliferating cells. A limitation of their use lies on the deleterious effects that they exert upon normal tissues and especially on inmunocompetent cells [1–3]. Many attempts have been made to increase the selectivity of anticancer drugs towards malignant cells and/or to decrease their adverse effects on normal cells. The coupling of drugs with enzymes, radioisotopes, DNA, toxins and mitogens has been tested with this purpose [4, 5]. A great deal of work has also been made by conjugating drugs with antibodies directed against specific tumor antigens [6–11]. An alternate approach, though limited to the chemotherapy of hepatomas and other alpha-fetoprotein (AFP) secreting tumors, was followed by Deutsch and coworkers [12] using daunomycin–fatty acid derivatives. The potent antitumor activity of these conjugates was supposed to depend upon the high affinity of AFP for fatty acids and the fact that these tumors synthesize AFP.

Work from our laboratory has provided evidence that, contrary to normal resting cells, many tumor cells of varied origin express, *in vitro* and *in vivo*, specific cell surface receptors to AFP [13, 14] and that these receptors, through their interaction with AFP, can be involved in the transfer of fatty acids into the cells [15]. We have explored the possibility of improving the selectivity of antitumor drugs by the use of their fatty acid conjugates on neoplastic cells expressing AFP-receptors. Covalent conjugates of chlorambucil and fatty acids of different degree of unsaturation have been prepared and assayed to check their cytotoxic activities *in vitro* upon two human lymphoma cell lines (RAJI, a B-cell-derived lymphoma and CEM, a T-cell-derived one) and, comparatively, upon quiescent and mitogen activated lymphocytes from normal donors. The choice of this model was determined by previous studies [14] showing that these human T and B lymphoblastoid cell lines expressed both AFP and AFP-receptors.

MATERIALS AND METHODS

General chemical methods. All reactions were conducted under nitrogen with exclusion of the light. Solutions were concentrated to dryness under reduced pressure below 30°. Thin layer chromatography was performed on Silica Gel 60 F_{254} (Merck, Darmstadt, F.R.G.) and Silica Gel 60 (230–400 mesh) was used for flash chromatography [16]. Chlorambucil derivatives were detected by examination under UV light and unsaturated fatty acids were located by exposure to iodine vapours. R_f values are given for hexane-ethylacetate (7/3, v/v) as the eluent. ^{1}H-NMR spectra were recorded for solutions in $CDCl_3$ with tetramethylsilane as the internal standard, using a Bruker MSL 300 spectrometer.

Preparation of chlorambucil hydroxyethylester (ChEtOH). To a stirred solution of chlorambucil (304 mg, 1 mmol) in dry dichloromethane were added dimethylaminopyridine (6.1 mg, 0.05 mmol),

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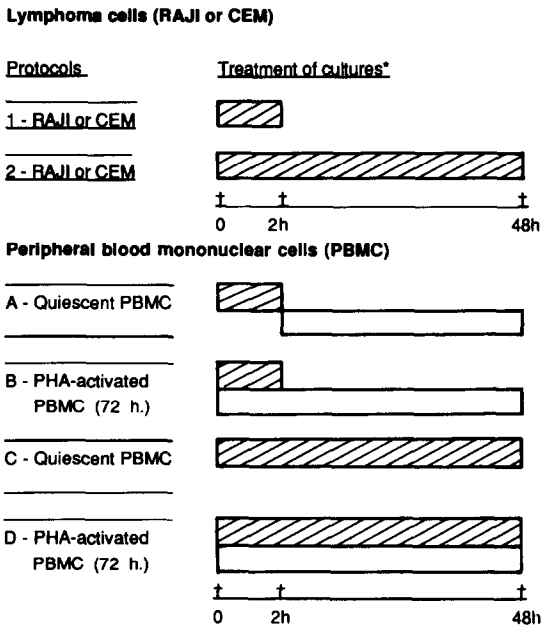
dicyclohexylcarbodiimide (227 mg, 1.1 mmol) and triphenylmethoxyethanol (334 mg, 1.1 mmol) obtained by selective monotritylation of ethyleneglycol. The mixture was kept for 1.5 hr at room temperature and then filtered. The filtrate was washed with dilute acetic acid and water, dried (Na_2SO_4) and concentrated. Flash chromatography (continuous gradient from petroleum ether to petroleum ether–ethyl acetate (8/2, v/v)) of the residue gave the triphenylmethoxyethyl ester of chlorambucil (ChEtOTr) (531 mg, 90%), R_f 0.78, which was detritylated as follows: a solution of the product in ethyl acetate (2 mL) and aqueous 90% formic acid (4 mL) was stirred at room temperature for 25 min, then diluted with ethyl acetate (60 mL), washed with half-saturated aqueous NaCl, saturated $NaHCO_3$ and water. The organic layer was dried and concentrated. Flash chromatography (continuous gradient from hexane to hexane–ethyl acetate (1/1, v/v) of the residue gave 257 mg (82%) of chlorambucil hydroxyethylester, R_f 0.13, which could be stored at −18° under nitrogen for a few months.

Coupling of fatty acids with chlorambucil hydroxyethylester. The esterification was performed as described above using 0.25 mmol of fatty acid, 87 mg (0.25 mmol) of ChEtOH, 750 μl of dry dichloromethane, 2 mg of dimethylaminopyridine and 57 mg (0.275 mmol) of dicyclohexylcarbodiimide. Chromatographic purification (continuous gradient from petroleum ether to petroleum ether–ethyl acetate (8/2, v/v)) gave the fatty acid–chlorambucil conjugate in a yield of 78–88%, R_f 0.64–0.71 depending on the fatty acid used. It was immediately dissolved in petroleum ether and aliquots containing 2 mg of the product were distributed in small tubes. The content of the tubes was concentrated in a vacuum desiccator then filled with nitrogen and stored at −18°.

Lymphoma cells. The human lymphomas RAJI (B-cell derived) and CEM (T-cell derived) were used for this study. Cells were cultured in RPMI 1640 medium (Flow, Irvine, U.K.) supplemented with 10% fetal calf serum, 2 mM glutamine and antibiotics (complete RPMI). The seeding density in these experiments was 2.5×10^5 cells/mL for RAJI cells and 4×10^5 for CEM cells and the doubling time was approximately 24 hr in control cultures.

Peripheral blood mononuclear cells. Peripheral blood mononuclear cells (PBMC) were isolated from blood of normal donors by gradient centrifugation in Ficoll-Paque (Pharmacia, Uppsala, Sweden), as described previously [17]. After the isolation, cells were cultured in complete RPMI at a cell density of 1×10^6 cells/mL. Viability was controlled by Trypan Blue exclusion and in all cases it was greater than 95% at the beginning of the cultures. Non-activated PBMC were incubated with the cytotoxics as soon as they were isolated. Activated PBMC were incubated with the cytotoxics after 72 hr in culture in contact with 2 μg/mL phytohemagglutinin (PHA-M, Sigma, Poole, U.K.) in the cell culture medium. In these experimental conditions, 2×10^5 72 hr-activated PBMC incubated with 1 μCi of [^{3}H]thymidine (Amersham, Bucks, U.K.) during the last 7 hr of culture, incorporated 110,000 cpm, whereas the same number of non-activated PBMC incubated in the same conditions, incorporated only 1200 cpm of [^{3}H]thymidine.



* Incubation time in the presence of Clh or Clh-FA (cross-hatched rectangles) and in the presence of phytohemagglutinin (PHA) (white rectangles).

Fig. 1. Schematic representation of the protocols used to test the toxicity *in vitro* of chlorambucil (Chl) or chlorambucil–fatty acid conjugates (Chl–FA) against human lymphoma cells (RAJI or CEM) and normal human quiescent or PHA-activated PBMCs.

Toxicity assays. Just before use, chlorambucil (Chl) or chlorambucil–fatty acid conjugates (chlorambucil-oleic acid conjugate (Chl-18:1), chlorambucil–arachidonic acid conjugate (Chl-20:4) and chlorambucil–docosahexaenoic acid conjugate (Chl-22:6)) were dissolved in 96% ethanol and aliquots of these solutions were added to complete RPMI medium. In all cases, final ethanol concentration in the medium was 0.4% (v/v). As illustrated in Fig. 1, cells were treated with the drugs for 2 hr (protocols 1, A and B) or 48 hr (protocols 2, C and D).

RAJI or CEM cells were treated according to protocols 1 and 2 (Fig. 1) with concentrations ranging in both cases between 5 and 150 μM of drug or fatty acid conjugates. In the protocol 1, the medium was discarded after the 2 hr of incubation, and the cells were washed three times with PBS and resuspended in the same volume of complete RPMI. At time 0 and after 24 and 48 hr of culture, the cells were subjected to the colorimetric proliferation test of Mosmann [18]. This test relates the number of viable cells present in the culture with the amount of formazan crystals produced by the cells from a solution of MTT (dimethylthiazolytetrazolium bromide, Sigma). Cells incubated for 2 hr in complete RPMI with 0.4% (v/v) of ethanol and after, cultured for 48 hr in the same medium without ethanol, were used as controls. In the long-term-incubation treatment

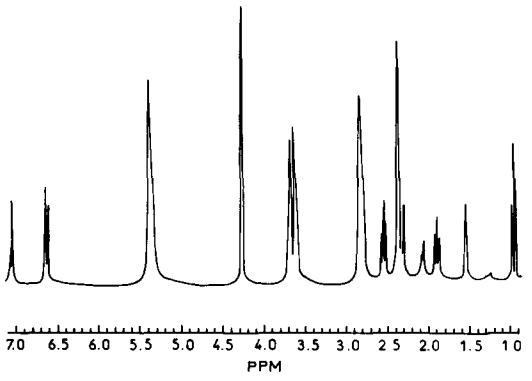


Fig. 2. ^{1}H-NMR spectrum (300 MHz, $CDCl_3$) of chlorambucil–docosahexaenoic acid conjugate.

(protocol 2), the drug-containing medium was present throughout the time of culture and the cells were subjected to the same proliferation test at time 0, and after 24 and 48 hr in culture. Control cells were cultured during the same periods of time in complete RPMI with 0.4% (v/v) of ethanol. See also Fig. 1.

Quiescent (non-activated) and PHA-activated PBMC were treated as illustrated in Fig. 1 (Protocols A, B, C and D). Important to note is that: (a) protocols A and C, delineated for the treatment of quiescent, non activated PBMC, differ not only in the incubation period with the drugs tested (2 and 48 hr, respectively) but also in the addition of PHA to the cells subjected to the 2 hr treatment with the drugs (protocol A). In fact, this protocol evaluates the capability of drug-treated quiescent lymphocytes to undergo subsequent PHA activation; and (b) whatever the protocol used, the viability of treated cells was determined at time 0 and after 24 and 48 hr of culture.

Results are expressed in terms of cellular growth (% of the control) calculated as number of viable cells from the 492 nm absorbance values obtained by application of the colorimetric proliferation test of Mosmann [18]:

Growth inhibition (% of the control) (G.I.)
= (Control cells − Drug incubated cells/Control cells) × 100

Cellular growth (% of the control) (C.G.)
= 100 − G.I.

RESULTS

Chemistry

In order to preserve the pharmacologically active moiety of the molecule, chlorambucil was linked with fatty acids through its carboxyl group by the two-carbon chain bridge of ethylene glycol. Treatment of chlorambucil with 2-triphenylmethoxyethanol in the presence of dicyclohexylcarbodiimide and dimethylaminopyridine [19] gave the triphenylmethoxyethylester in good yield, the structure of which was confirmed by ^{1}H-NMR spectroscopy. The spectrum contained *inter alia* a 15-proton multiplet centered at ∂ 7.34 for the triphenylmethoxy group, a 4-proton multiplet centered at ∂ 6.82 for the aromatic protons of chlorambucil, and an 8-proton multiplet at ∂ 3.63 for the *N*-chloroethyl groups of chlorambucil.

Removal of the trityl group with 90% aqueous formic acid [20] afforded a more polar compound (ChEtOH). The ^{1}H-NMR spectrum of ChEtOH was essentially the same as that of ChEtOTr except for the absence of the aromatic protons of the trityl group.

Esterification of chlorambucilhydroxyethyl ester with various fatty acids gave hydrophobic compounds (ChEtOFat) which were characterized by ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectrum of the chlorambucil–docosahexaenoic acid conjugate is

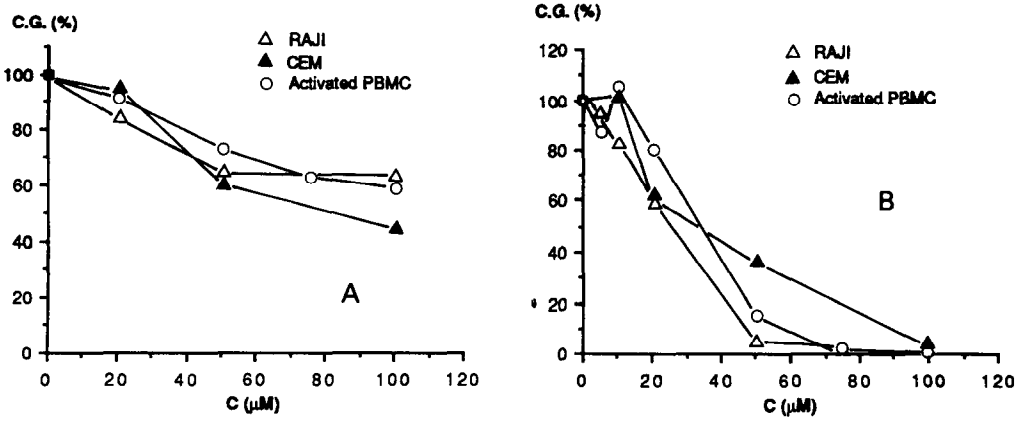


Fig. 3. Cytotoxic effects of arachidonic (A) or docosahexaenoic acids (B) on 72 hr PHA-activated PBMCs, RAJI and CEM cells. The cells were cultured during 48 hr in the presence of different μM concentrations (C) of fatty acids. The cells were then subjected to the proliferation test of Mosmann. The cellular growth (C.G., % of the control) was calculated from these data. Data are the mean of two independent experiments and the determinations were made in quadruplicate. SD was always less than 10% of the mean.

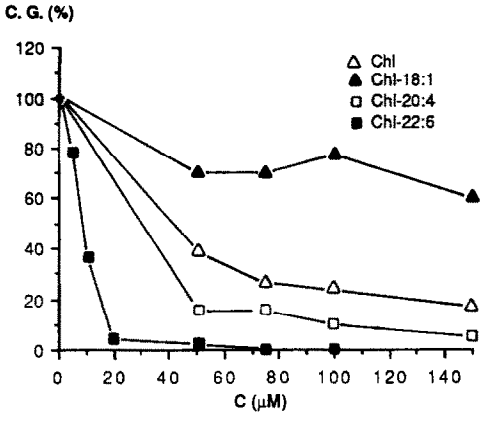


Fig. 4. Cytotoxic effect of several concentrations (C) of chlorambucil (Chl) and of conjugates of chlorambucil with oleic acid (Chl-18:1), arachidonic acid (Chl-20:4) or docosahexaenoic acid (Chl-22:6) on RAJI cells after 2 hr of incubation (protocol 1). The cells were subjected to the proliferation test of Mosmann after 48 hr of culture in drug-free, complete RPMI. The cellular growth (C.G., % of the control) was calculated from these data. Data are the mean of three (chlorambucil alone) or two (Chl-conjugates) independent experiments and the determinations were made in quadruplicate. SD was always less than 20% of the mean.

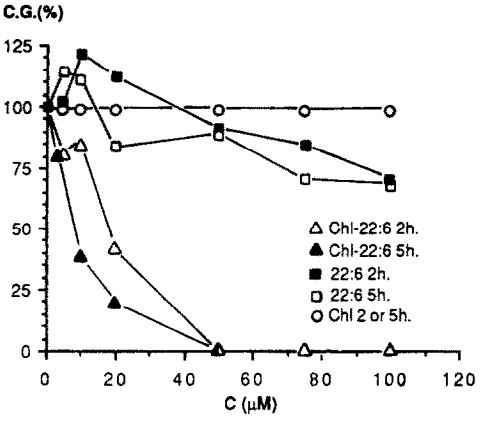


Fig. 5. Comparison of the toxic effects of several concentrations (C) of chlorambucil–docosahexaneoic (Chl-22:6), chlorambucil (Chl) and docosahexaneoic acid (22:6) on RAJI cells after short-time incubations (2 or 5 hr). The cells were subjected to the proliferation test of Mosmann immediately after the incubations. The cellular growth (C.G., % of the control) was calculated from these data. Data are the mean of two independent experiments and the determinations were made in quadruplicate. SD was always less than 10% of the mean.

shown in Fig. 2. It contains, *inter alia*, the characteristic signals of chlorambucil: a 4-proton multiplet at ∂ 6.85 (aromatic protons) and an 8-proton multiplet at ∂ 3.66 (*N*-chloroethyl groups), and, in addition, signals for the 12 olefinic protons of the fatty acid centered at ∂ 5.38, as well as signals for 10 allylic protons centered at ∂ 2.83, confirming the structure of the conjugate.

Toxicity of the fatty acids per se

Preliminary experiments were carried out to explore the toxic activity of fatty acids *per se* in the experimental conditions used in this work. Using protocol 1 (short-term drug treatment) on RAJI cells, only high concentrations (more than 75 μM) of docosahexaenoic acid were toxic (data not shown). After long-term incubations (24 or 48 hr) arachidonic and docosahexaenoic acids showed significant toxic activity upon RAJI and CEM cells and also against activated PBMCs. The toxic effect of arachidonic acid (20:4n-6, Fig. 3A) was moderate and always lower than that of docosahexaenoic acid (22:6n-3) at similar concentrations (Fig. 3B). In the same conditions, oleic acid lacked of toxicity at any concentration (data not shown).

Toxicity of Chl and Chl–FA towards human lymphoma cell lines

Figure 4 shows the results obtained with RAJI (B-cell origin) cells using the 2 hr incubation protocol (see Fig. 1). Relative to the effect of chlorambucil alone, the toxicity of chlorambucil–fatty acid conjugates depends on the fatty acid coupled to the drug. The greatest inhibition of growth was observed with the docosahexaenoic acid conjugate and the lowest with that of oleic acid, at all concentrations tested. In contrast with the effect of oleic acid, docosahexaenoic– and arachidonic–chlorambucil conjugates were more toxic than chlorambucil alone. This indicates that the coupling reaction between fatty acids and chlorambucil modifies the toxic capacity of the drug, either enhancing (22:6n-3 and 20:4n-6) or diminishing it (18:1n-9).

The high toxicity of Chl-22:6 towards RAJI cells was further investigated, making determinations of viability immediately after short-time (2 and 5 hr) incubation periods with the conjugate and, comparatively, with the drug or the fatty acid separately (Fig. 5). It seems clear from these data that the greater toxicity of Chl-22:6 is due to the conjugate itself and does not result from the summation of the individual toxic effects of Chl and 22:6.

Using the long-term-incubation protocol, the toxicities of the fatty acid conjugates or of chloambucil alone were very similar to those reported using the 2 hr treatment. The only difference was observed with the oleic acid conjugate, which proved to be slightly more toxic when present throughout the time of the culture instead of during a 2 hr period (Data not shown).

When using the 2 hr treatment, Chl and Chl–FA conjugates were less toxic against CEM cells (Fig. 6A) than against RAJI ones (See Fig. 4). However, when the drugs were present in the medium throughout the time of the culture, the growth of CEM cells was greatly inhibited by Chl, Chl-20:4 and Chl-22:6 after either 24 or 48 hr (Fig. 6B) of culture.

Toxicity of Chl and Chl–FA towards quiescent and PHA-activated normal human PBMC

Following protocols A and B, the oleic acid conjugate lacked of toxicity against either quiescent or PHA-activated PBMC, at any concentration tested

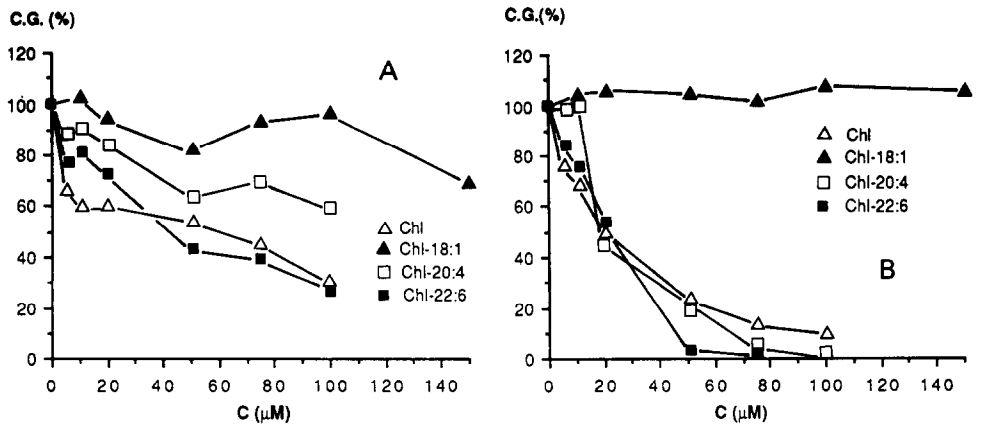


Fig. 6. Cytotoxic effect of chlorambucil (Chl) and of the chlorambucil–oleic acid (Chl-18:1), chlorambucil–arachidonic acid (Chl-20:4) and chlorambucil–docosahexaenoic acid (Chl-22:6) conjugates on CEM cells. (A) Cells were treated as indicated for RAJI ones in Fig. 4. (B) cells were incubated with different concentrations of the drugs (C) during 48 hr (Protocol 2). After incubation, cells were subjected to the proliferation test of Mosmann. The cellular growth (C.G., % of the control) was calculated from these data. Data are the means of two independent experiments and the determinations were made in quadruplicate. SD was always less than 10% of the mean.

(Fig. 7A). Chl-18:1 was not toxic when it was present throughout the culture period (Protocols C and D, data not shown). By contrast, chlorambucil alone was equally toxic against quiescent or PHA-activated PBMC whatever the protocol used.

Figure 7B shows the results obtained with the arachidonic acid-chlorambucil conjugate after a treatment similar to that of Fig. 7A. This conjugate proved to be more toxic on activated PBMC than chlorambucil alone but, contrary to the latter, lacked of toxicity against quiescent PBMC. When the cells were incubated with Chl-20:4 during 48 hr (Protocols C and D), the drug conjugate was less toxic than Chl alone on non-activated PBMC and more toxic on activated ones (data not shown). Figure 7C compares the toxicity of the Chl-22:6 conjugate with that of Chl alone using protocols A and B. At concentrations greater than 20 μM, the conjugate and the drug alone show similar toxicities on either quiescent or activated PBMC. However, in the long-term incubation protocols, although it was more toxic than Chl alone on activated PBMC, it was much less toxic than Chl on quiescent cells (data not shown).

The interest of Chl-22:6 conjugate lies on its high efficiency at low concentrations (5, 10 and 20 μM) against malignant cells. At these low concentrations, Chl-22:6 is not toxic against quiescent PBMC but inhibits the growth of the malignant cell lines tested with much higher efficiency than all the other compounds assayed (Chl, Chl-18:1 or Chl-20:4). The effects observed with these concentrations of the Chl-22:6 conjugate on the growth of the different kinds of cells studied are summarized in Tables 1 and 2. The results obtained at concentrations up to 20 μM and for both groups of protocols provide evidence that Chl-22:6: (a) lacks of toxicity against quiescent PBMC; (b) exhibits similar toxicities upon the T-lymphoma cells and normal activated PBMC and (c) exerts its highest toxicity upon the B-lymphoma cells.

As a summary of the results obtained with the different experimental protocols and cell types used and to allow appropriate comparisons to be made, IC_{50} values are given for Chl, Chl-20:4 and Chl-22:6 in Table 3. IC_{50} is defined as the concentration necessary to cause a 50% inhibition of the cellular growth of control cells.

DISCUSSION

From the results presented here it can be concluded that the coupling of chlorambucil with polyunsaturated fatty acids may improve the toxic activity of the drug, as well as its selectivity against neoplastic cells when the latter are compared with quiescent cells. The *in vitro* system used in the present work allowed to make a good comparison of the effect of Chl and Chl–fatty acid conjugates upon tumor cells (lymphomas, RAJI and CEM) and their normal counterparts (quiescent and PHA-activated lymphocytes).

The first point which deserves attention concerns the differences observed in the toxic potential of the drug conjugates according to the fatty acid moiety. There is no clear explanation for this but the differences may result, in part, from the metabolic status of the cell (quiescent *versus* activated). Studies from Resch and co-workers [21, 22] have shown that the enhancement of fatty acid uptake by rabbit thymocytes after activation with concanavalin A varies with the fatty acid involved: i.e. arachidonic acid was much more internalized than oleic acid. No data are available in the literature concerning the uptake of 22:6n-3 by lymphoid cells. As reported above, Chl–oleic acid conjugates are less toxic than Chl alone, while the reverse occurs with both Chl–arachidonic acid and Chl–docosahexaenoic acid conjugates.

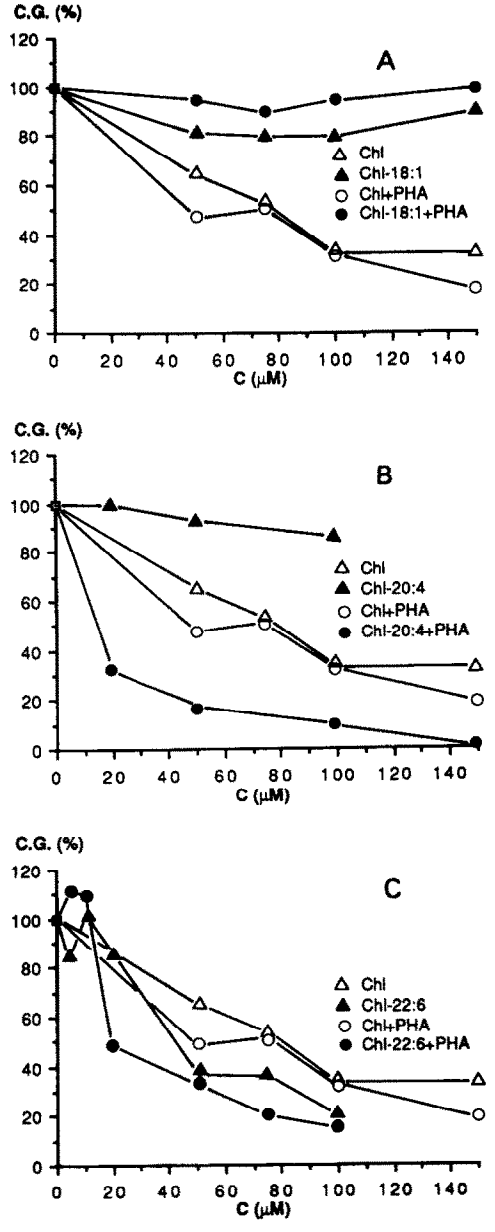


Fig. 7. Cytotoxic effect of several concentrations (C) of chlorambucil and conjugates of chlorambucil with oleic acid (Chl-18:1, A), arachidonic acid (Chl-20:4, B) and docosahexaenoic acid (Chl-22:6, C) on quiescent or PHA-activated (+ PHA) normal human PMBC after 2 hr of incubation followed by 48 hr of culture in drug-free, complete RPMI, supplemented with PHA (2 μg/mL) (Protocols A and B). The cells were then subjected to the proliferation test of Mosmann. The cellular growth (C.G. % of the control) was calculated from this data. Data are the mean of three (Chl) or two (conjugates) independent experiments and the determinations were made in quadruplicate. SD was always less than 15% of the mean.

More important is the considerable gain of cell toxicity of chlorambucil on B-lymphoma cells when the drug is conjugated with arachidonic or docosahexaenoic acid (Fig. 4). The toxic activity observed always went beyond the summation of individual effects of chlorambucil and fatty acids. The killing effectiveness of Chl-22:6 on RAJI cells after short-term treatment with the conjugate and at concentrations as low as 20 μM is remarkable.

As yet known [3] chlorambucil proved not to be selective between lymphoma cells and normal PBMC, as it was equally harmful upon quiescent and activated lymphocytes (Figs. 4 and 7). By contrast Chl-18:1 and Chl-20:4, at any concentration tested (Fig. 7A and B), and Chl-22:6 at concentrations up to 20 μM (Tables 1 and 2) lacked of toxicity toward quiescent lymphocytes. This was evidenced by the fact that they were able to undergo normal PHA-activation after a 2 hr treatment with the conjugates and subsequent 48 hr of culture in drug-free medium (protocol A). Both Chl-20:4 and Chl-22:6 were toxic against mitogen-activated lymphocytes. Interesting to note, however, is that at low concentrations, Chl-22:6 was much more toxic upon B lymphoma cells than toward normal activated PBMC (Tables 1 and 2). Since the toxic effect of Chl–fatty acid conjugates varies according to the type of cells (i.e. malignant cells, quiescent and activated normal lymphocytes), it would be possible to find adequate conditions of drug concentration and protocol of treatment to assure good killing efficiency upon lymphomas while, totally or partially, preserving the pool of normal resting immunocompetent cells.

Chlorambucil is a well-known alkylating agent, frequently used in chemotherapy against human lymphomas and leukemias [23–25]. It is a highly hydrophobic molecule which can enter into the cells by a passive diffusion process [26, 27], apparently only affected by physicochemical changes (e.g. the intra or extracellular pH) [28]. By contrast, fatty acid uptake by cells seems to be carrier-mediated [15, 29]. In recent years, biochemical studies have been trying to elucidate the mechanism of entry into cells of these molecules. Evidence has grown on the involvement of specific cell proteins, the AFP receptor and the membrane fatty acid binding proteins (FABP) [29], which regulate the influx of fatty acids into the cells. FABP have been characterized and isolated from several tissues and types of cells (cardiac myocytes, liver, adipose tissue, gut) [29].

On the other hand, alpha-fetoprotein internalization through specific cell-surface AFP-receptors has been demonstrated in a variety of tumors including the lymphomas used in the present work [14, 30], as well as in PHA-activated normal human PBMC [31]. Moreover, the time course expression of AFP-receptors by these cells is accompanied by the local synthesis of AFP and thus makes possible the existence of an AFP/AFP-receptor autocrine pathway [31, 32]. The latter is important because, in adult individuals, liver synthesis and serum concentration of AFP are too low to turn operational cell surface AFP-receptors [32]. The high affinity of AFP for polyunsaturated fatty acids [33, 34] and especially for docosahexaenoic acid suggested that the physiological role of AFP and its receptors might be the transfer of these fatty acids to growing cells. Experimental support of AFP-mediated facilitation of fatty acid transfer to cells expressing AFP-receptors was obtained with a cloned cell line derived

Table 1. Cellular growth of the different kind of cells used in the study after the 2 hr treatments with low concentrations of Chl-22:6 conjugate and 48 hr of culture in drug free, complete RPMI

	C.G. (% of the control)			
Chl-22:6 (μM)	RAJI	CEM	Activated PBMC	Non-activated PBMC
5	80	80	100	100
10	40	80	100	90
20	5	70	50	100
50	0	55	35	50

Table 2. Cellular growth of the different kind of cells used in the study after 48 hr of incubation with low concentrations of Chl-22:6 conjugate

	C.G. (% of the control)			
Chl-22:6 (μM)	RAJI	CEM	Activated PBMC	Non-activated PBMC
5	40	85	65	100
10	0	80	45	100
20	0	55	10	100
50	0	5	0	37

Table 3. Concentrations (μM) necessary to cause a 50% inhibition of the cellular growth of control cells (IC_{50}) in the different cell types used, at the times indicated and using the different experimental protocols (1, 2, A, B, C and D)

Treatment*	Chl				Chl-20:4				Chl-22:6			
	2 hr		Long-term		2 hr		Long-term		2 hr		Long-term	
Time of culture†	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Non-activated PBMC	∞	75	150	35	∞	∞	70	50	∞	48	∞	70
Activated PBMC	135	75	100	15	35	15	20	10	62	20	19	9
RAJI	150	45	87	35	50	30	75	18	9	8	6	4
CEM	160	70	75	25	∞	130	60	20	120	45	30	22

* Treatment = time of incubation in the presence of the drug: 2 hr (protocols 1, A or B); long-term (protocols 2, C or D).

† Time at what aliquots of cultured cells were subjects to the proliferation test of Mosmann.

∞ = no effect.

from a rat rhabdomyosarcoma [15, 35]. The results presented here fit well with such a mechanism since the toxicity of conjugates of chlorambucil with polyunsaturated fatty acids is high towards cells expressing AFP-receptors (B- and T-lymphoma cells and activated PBMC) and low against quiescent lymphocytes which do not express this receptor. At the time when Deutsch and his co-workers used for the first time antitumor drugs conjugated to fatty acids [12], the existence of AFP-receptors as well as the synthesis of AFP by malignant and normal growing cells of non hepatic origin were not yet established. At present, then, the use for therapeutic purposes of fatty acids as drug-carriers into malignant cells offers a much larger field of applications.

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