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Publication n°4

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British Journal of Nutrition (2006) vol. 73, pages 258-268

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Anne De La Torre[1], Eric Debiton[2], Pierre Juanéda[3], Denys Durand[1], Jean-Michel Chardigny[3], Chantal Barthomeuf[2], Dominique Bauchart[1] and Dominique Gruffat[1 *]

[1] INRA, Unité de Recherche sur les Herbivores, Equipe Nutriments et Métabolismes, 63122, Saint Genès-Champanelle, France. [2] Laboratoire de Pharmacognosie/Biotechnologies; UMR 484 INSERM, 63005 Clermont-Ferrand, France. [3] INRA, Unité de Nutrition Lipidique, 21065 Dijon, France.

Abstract : Although many data are available concerning anticarcinogenic effects of industrial CLA, few studies have reported the antitumor properties of CLA mixtures originated from ruminant products. The aim of this study is to investigate the *in vitro* antiproliferative effects of beef CLA mixtures on breast, lung, colon, melanoma and ovarian human cancer cell lines. For this purpose, four fatty acid (FA) extracts prepared from beef lipid and varying in their CLA composition, their corresponding purified CLA-enriched fractions, and mixtures of pure synthetic CLA, the composition of which reproduced that of the four selected beef samples, were tested on cancer cell lines. Cancer cells were exposed for 48 h to medium containing 100 µM of FA and their proliferation was determined by quantifying cellular DNA content (Hoechst 33342 dye). Compared with cells incubated without FA, the number of cancer cells was reduced from 25 to 67% (P<0·0001) following FA treatment. Antiproliferative effects of CLA mixtures varied in magnitude according to the source of FA, the CLA composition and the cell lines. CLA mixtures naturally present in beef inhibited the proliferation of human cancer cell lines, a high content in *cis*-*trans* isomers allowing the most important antiproliferative effect. Beef total FA exhibited a greater growth-inhibitory activity than their corresponding CLA-enriched fractions. These results suggested that either beef FA other than beef CLA could possess antiproliferative properties and/or the existence of complementary effects of non conjugated FA and CLA, which could favour the antiproliferative properties of beef total FA.

Beef: Fatty acids: Conjugated linoleic acid: Human cancer cell: Growth inhibition.

Introduction

Conjugated linoleic acid (CLA) is a collective name for a group of positional and geometric isomers of linoleic acid (*cis*-9,*cis*-12-18:2) in which the double bonds are separated by a single carbon-carbon bond. It is a substance naturally provided by fat from ruminant products (milk and meat) which constitute the major source of dietary CLA for human (Pariza *et al.* 2001) as at least 24 distinct CLA isomers (Cruz-Hernandez *et al.* 2004); the *cis*-9,*trans*-11-CLA isomer (rumenic acid) representing more than 80% of total CLA (Griinari & Bauman, 1999). In addition, synthetic CLA mixtures can be generated industrially by catalytic hydrogenation of vegetable oils (Kritchevsky, 2000). The *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA isomers predominate in these preparations (85-90%), these two isomers being usually represented in equal amounts with the presence of other minor CLA isomers (10 - 15%) (Gnädig *et al.* 2001).

Over the past two decades, extensive research indicates that CLA mixtures could possess numerous beneficial properties for the human health including anticarcinogenic, antiadipogenic, antiatherogenic and antidiabetogenic properties (Belury, 2002). Their anticarcinogenic properties have been widely studied using synthetic CLA i.e. *cis*-9,*trans*-11- and *trans*-10,*cis*-12-CLA isomers tested either individually or in a 50/50 mixture. *In vivo* studies of experimental carcinogenesis using rodents as the animal model for human have shown that synthetic CLA prevent tumor development in mammary, colon, forestomach and skin tumors (Belury & Vanden Hauvel, 1997). In the same way, *in vitro* studies have demonstrated that synthetic CLA inhibit, in a dose- and time-dependent manner, the proliferation of several human tumor cell lines from breast, lung, prostate, skin and colon (Kelly, 2001; Belury, 2002). Although many *in vivo* and *in vitro* experiments have investigated biological properties of synthetic CLA isomers, few have reported antitumor effects of complex CLA mixtures naturally present in lipids of ruminant meat and milk. O'Shea *et al.* (2000) have showed that CLA-enriched milk fat is as effective as synthetic CLA mixture in decreasing breast tumor cell (MCF-7) proliferation. These antiproliferative effects are independent of the composition of FA other than CLA in milk fat samples, suggesting that CLA isomers could be the active compounds (O'Shea *et al*, 2000). Properties of natural CLA mixtures could differ from those of synthetic CLA because of specific properties of each CLA isomer. Trans-10,cis-12- isomer, virtually absent in ruminant products, is actually the most potent isomer to inhibit proliferation of colon cancer cells (Kim *et al.* 2002; Miller *et al.* 2002). *Cis*-9,*trans*-11-isomer exhibited greater antiproliferative effects than its *cis*-9,*cis*-11-conterpart in both colo-rectal and prostate cancer cells (Palombo *et al*, 2002). These different effects of the various isomers could be explained, at least in part, by different mechanisms and/or targets of action of isomers (Pariza *et al.* 2000). For example, *trans*-10,*cis*-12-isomer may act on prostate cancer cells through the modulation of apoptosis and of cell control whilst *cis*-9,*trans*-11-isomer might alter preferentially arachidonic acid metabolism (Ochoa *et al.* 2004).

In this context, the aim of the present study was to investigate the antiproliferative properties of CLA mixtures that occur naturally in beef. For this purpose, four samples of beef fatty acids differing by their CLA composition (different proportions in *cis,trans*-, *cis,cis*- and *trans,trans*-isomers) were selected. Antiproliferative properties of 1) total fatty acids present in the selected beef, 2) CLA-enriched fractions purified from the four fatty acids examples and 3) synthetic CLA mixture composed of 9,11-CLA isomers reproducing the composition of CLA present in selected beef were compared in breast, lung, melanoma, colon, and ovarian human cancer cell lines by the measure changes in cellular DNA content using Hoechst 33342 dye.

Materials and Methods

Fatty acid extraction and analysis

Eight crossbred Charolais x Salers steers (412 ± 33 d-old; live weight: 536 ± 33 kg) were selected on the basis of live weight and daily gain. Animals were assigned at random to two groups (n=4 for each diet) for a 70-d feeding study. Animals were given the basal diet (45% natural hay and 55% concentrate) alone or with extruded linseed-providing lipids at the level of 4% of diet dry matter. All steers were slaughtered conventionally at the abattoir of the Research Center (INRA-Theix) and the carcasses chilled at 4°C for 24 h before sampling. Muscle samples were taken up for chemical analysis from *Longissimus thoracis*, *Rectus abdominis*, *Semitendinosus* and *Pectoralis transversus*. Total lipids were extracted from muscle samples (150 g) according to the method of Folch *et al.* (1957). Extraction and transmethylation of fatty acids (FA) into methyl esters (FAME) was realized by

Table 1 : FA composition of the four beef samples (Mix A to D)* selected for their specific composition out of isomers of CLA

	Mix A	Mix B	Mix C	Mix D
	% total FA			
∑ Saturated FA	50·27	39·84	49·96	39·75
of which 16:0	25·20	21·34	25·55	21·29
of which 18:0	20·36	14·56	19·81	14·47
∑ Monounsaturated FA	39·36	45·74	35·90	43·08
of which *trans*-11 18:1	2·65	2·72	1·95	2·86
of which *cis*-9 18:2	29·31	35·40	28·09	33·84
∑ Polyunsaturated FA	4·40	6·46	6·43	8·04
of which 18:2 *n*-6	3·76	4·44	5·18	6·65
of which 18:3 *n*-3	0·89	1·08	0·77	0·80
Total CLA	0·540	0·932	0·479	0·593
of which ∑ *cis,trans*	0·446	0·526	0·437	0·567
of which ∑ *cis,cis*	0·052	0·066	0·020	0·019
of which ∑ *trans,trans*	0·042	0·039	0·023	0·008
∑ unknown FA	5·43	7·03	7·23	8·54
	% total CLA			
∑ *cis,trans*	82·6	88·7	91·0	95·5
∑ *cis,cis*	9·6	7·1	4·2	3·2
∑ *trans,trans*	7·8	4·2	4·8	1·3

* Mix A: FA of *Longissimus thoracis* muscle (LT) of Charolais x Salers steers fed linseed supplemented diet, Mix B: FA of *Pectoralis transversus* muscle (PT) of Charolais x Salers steers fed linseed supplemented diet, Mix C: FA of LT muscle of Charolais x Salers steers fed control diet, Mix D: FA of PT muscle of Charolais x Salers steers fed control diet

using sodium methanolate solution (0·5 M) according to the method of Christie (2001). FAME composition was determined by gas liquid chromatography (GLC, DI 200 chromatograph, Perichrom, Saulx les Chartreux, France) using a glass capillary column (100 m length x 0·25 mm i.d.) coated with CP-Sil 88 (oven temperature program: 70-215°C). Hydrogen was used as the carrier gas (at a flow rate of 1·1 ml/min). Chromatographic signals were analyzed by Winilab II Chromatography Data System software (Perichrom). The FA composition was calculated using an internal standard method (C19:0). A reference standard (Mix C4-C24 methyl esters, Supelco, PA) and CLA standard mix (Sigma-Aldrich, Isle d'Abeau Chesnes, France) were used to determine recoveries and correction factors for the determination of individual FA composition of beef fat.

Total FA of selected beef samples

Among 32 beef samples analysed, four samples were selected for their specific CLA composition (*Longissimus thoracis* and *Pectoralis transversus* of steers given the control and linseed supplemented diets) and their FA composition was presented in Table 1. Briefly, all beef samples were dominated by *cis,trans* CLA isomer (namely, *cis*-9,*trans*-11-isomer) but additionally, sample A was characterized by a high content of *cis,cis*- and *trans,trans*-CLA isomers (Mix A), sample B possessed a high content of *cis,cis*- and a low content of *trans,trans*-isomers (Mix B), sample C had an equivalent medium content of *cis,cis*- and *trans,trans*-CLA isomers (Mix C) and finally, sample D showed a low *cis,cis*- and *trans,trans*-isomer content. In order to determine the ability of these FA mixtures to inhibit human tumor cell proliferation, approximatively 20 mg of total lipids of each sample were extracted and their FA were saponified by a 10% KOH ethanolic solution over night at room temperature (Bauchart & Aurousseau, 1981). Free FA were solubilized in absolute ethanol at a concentration of 100 mM and kept at -20°C until use (in the two weeks following their preparation).

Preparation of beef CLA-enriched fatty acid fractions

Beef CLA-enriched fractions were prepared as FAME from total FA of the four selected samples. Beef FAME were successively fractionated by preparative and semi-preparative high performance liquid chromatography (HPLC) followed by silver nitrate thin-layer chromatography ($AgNO_3$-TLC). Briefly, the preparative HPLC (a WaterPrepLC/System 500 coupled with axial modul preparative column) was carried out on a reverse phase column (20 cm lengh, 7 cm i.d.) using a Lichroprep. RP 18 (Merck, KGat, 6427 Darmstadt, Germany) as previously described by Sébédio *et al*. (1987). FAME (up to 6 g) were dissolved in acetone and chromatographied with pure methanol as the solvent system (flow rate: 150 ml/min). The fraction containing C18:2 FAME including CLA was collected, dried under nitrogen and dissolved in hexane (up to 40 mg) to be fractionated on a reverse phase column (Nucleosil C18, 5µm, 25 cm x 10 mm i.d.) by semi-preparative HPLC (Spectraphysics SP8810 pump coupled with RID 10A detector) using pure acetonitrile as solvent system (flow rate: 4 ml/min). The FAME fraction containing CLA was thereafter refined by $AgNO_3$-TLC (Merck KGat, 6427 Darmstadt, Germany, ref.5721, 0·25 mm thickness) using pure toluene as eluant according to the method of Morris (1966). FAME were viewed under UV after spraying 2'7' dichlorofluorescein (0·1% in ethanol). The band containing CLA was recovered and FA composition was analyzed by GLC as described earlier. FA composition of these four CLA semi-purified fractions from beef was given in Table 2. Mixes E, F, G and H were the CLA-enriched FAME fractions purified from total FA of Mixes A, B, C and D, respectively (Table 1). FAME mixes were converted into free FA counterparts, solubilized in absolute ethanol at the concentration of 175 mM and kept at -20°C until use (in the two weeks following their preparation).

Table 2 : FA composition of CLA-enriched mixes (mix E to H)* prepared from beef samples selected their specific composition out of isomers of CLA

	Mix E	Mix F	Mix G	Mix H
	% total FA			
non conjugated FA	42·6	29·2	34·4	37·4
14:0	1·3	2·0	3·2	4·4
∑ *trans* 16:1	32·3	21·8	25·3	22·3
∑ *cis* 16:1	3·5	2·3	2·3	4·1
17:0	2·4	1·6	2·1	1·8
∑ 18:2	3·2	1·6	1·5	4·9
∑ unknown FA	3·2	2·1	3·0	4·3
Total CLA	54·2	68·6	62·6	58·3
of which ∑ *cis,trans*	43·7	62·1	56·7	54·9
of which ∑ *cis,cis*	5·6	4·5	2·9	1·9
of which ∑ *trans,trans*	4·9	2·0	3·0	1·5
	% total CLA			
∑ *cis,trans*	80·6	90·5	90·6	94·2
∑ *cis,cis*	10·4	6·6	4·6	3·2
∑ *trans,trans*	9·0	2·9	4·8	2·6

* Mix E: FA of *Longissimus thoracis* muscle (LT) of Charolais x Salers steers fed linseed supplemented diet, Mix F: FA of *Pectoralis transversus* muscle (PT) of Charolais x Salers steers fed linseed supplemented diet, Mix G: FA of LT muscle of Charolais x Salers steers fed control diet, Mix H: FA of PT muscle of Charolais x Salers steers fed control diet.

Table 3 : CLA composition of synthetic CLA mixtures reproducing the composition of CLA present in lipids of selected beef (Mix 1 for Mix A, Mix 2 for Mix B and Mix 3 for Mix C).

	Mix 1	Mix 2	Mix 3
	% total FA		
cis -9,*trans* -11 CLA	82·0	87·0	91·0
cis -9,*cis* -11 CLA	10·0	8·0	4·0
trans -9,*trans* -11 CLA	8·0	5·0	5·0

Preparation of synthetic CLA mixtures

Synthetic CLA mixtures containing *cis*-9,*trans*-11-CLA, *cis*-9,*cis*-11-CLA and *trans*-9,*trans*-11-CLA (Matreya Inc., USA) were prepared to mimic CLA composition of selected beef samples (Table 3). Concentrations of *cis*-9,*trans*-11-, *cis*-9,*cis*-11- and *trans*-9,*trans*-11-CLA isomers corresponded respectively to the concentrations of all *cis,trans*, *cis,cis*- and *trans,trans*-isomers. Thus, compositions of mixes 1, 2 and 3 corresponded to the composition of mixes A, B and C, respectively. A stock solution in absolute ethanol (Sigma-Aldrich, France) was prepared for each synthetic CLA mixture (100 mM) and kept at -20°C until use (in the two weeks following their preparation).

Cell lines and culture conditions

M4Beu, a human melanoma cell line, was established in the laboratory of Dr. J.F. Doré (INSERM, Unit 128, Lyon, France) from metastatic biopsy specimens and has been maintained in culture cell for almost 15 years (Jacubovich *et al.* 1985). Breast adenocarcinoma (MCF7), colon adenocarcinoma (DLD-1), ovary teratocarcinoma (PA-1) and lung non-small-cell carcinoma (A-549) human cell lines were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK). Stock cell cultures were maintained as monolayers in 75-cm^2 culture flasks in a complete medium. This medium contained Glutamax Eagle's Minimum Essential Medium (MEM) with Earle's salts (Gibco-BRL, Paisley, UK, ref. 41090-28) supplemented with 10% of fetal calf serum naturally poor in CLA (Biochrom, batch 431 B, France), 1% of vitamin solution (Gibco-BRL, Paisley, UK, ref. 11102-037), 1% of sodium pyruvate solution (ref.11360-039, Gibco-BRL, UK), 1% of a mixture of non-essential amino-acids solution (ref. 11140-035, Gibco-BRL, UK) and 2 mg gentamicin base (ref. 15710-049, Gibco-BRL, UK). All cell culture solutions were certified endotoxin-tested and sterile-filtered. Cells were grown at 37°C in a humidified incubator and under an atmosphere containing 5% CO_2 during a two week-period of adaptation before the proliferation assay. The same batch of fetal bovine serum was systematically used for all experiments to minimise the effects of inter-batch variability.

Total beef FA and synthetic CLA mixtures were tested on cell lines at a concentration of 100 µM. CLA-enriched fractions were tested at 175 µM because of the presence of additional FA in these mixtures (Table 2), this concentration corresponding to 100 µM of CLA. The non-detergent effect for cellular viability of such concentrations of FA was verified in a primarily study (data not shown).

Proliferation assay

Cells were plated at the density of $5x10^3$ per 150 µl culture medium in 96-well microplates (Nunclon, Nunc, Roskild, Denmark) and allowed to adhere for 16 h before FA treatment. Thereafter, medium was replaced by a fresh complete culture medium supplemented with a given FA preparation (6 wells by treatment) at the final FA concentration of 100 µM for synthetic CLA preparations or for total beef FA and of 175 µM for CLA-enriched fractions (final volume: 200 µl). In these conditions, the final concentration of ethanol in culture medium was 0·25% for all experiments. In parallel, control treatment consisted in cells incubated in ethanol (0·25% vol/vol) without FA. Three independent experiments were performed, each in hexaplicate. After 48 h of continuous FA exposure, the antiproliferative effect of FA was assessed by measurement of DNA content with Hoechst dye 33342, as previously described by Debiton *et al.* (2003). Briefly, on the day of the assay, plates were thawed at room temperature, 100 µL of a SDS solution (0.01%, m/v) in sterile distilled water were added into each well, and plates were incubated for 1 h at room temperature and then frozen at -80°C for 1 h. After thawing, 100 µL of Hoechst dye 33342 solution at 30 µg/mL in a hypersaline buffer (10 mM Tris HCl, pH 7.4, 1 mM EDTA and 2 M NaCl) were added to each well. The plates were then

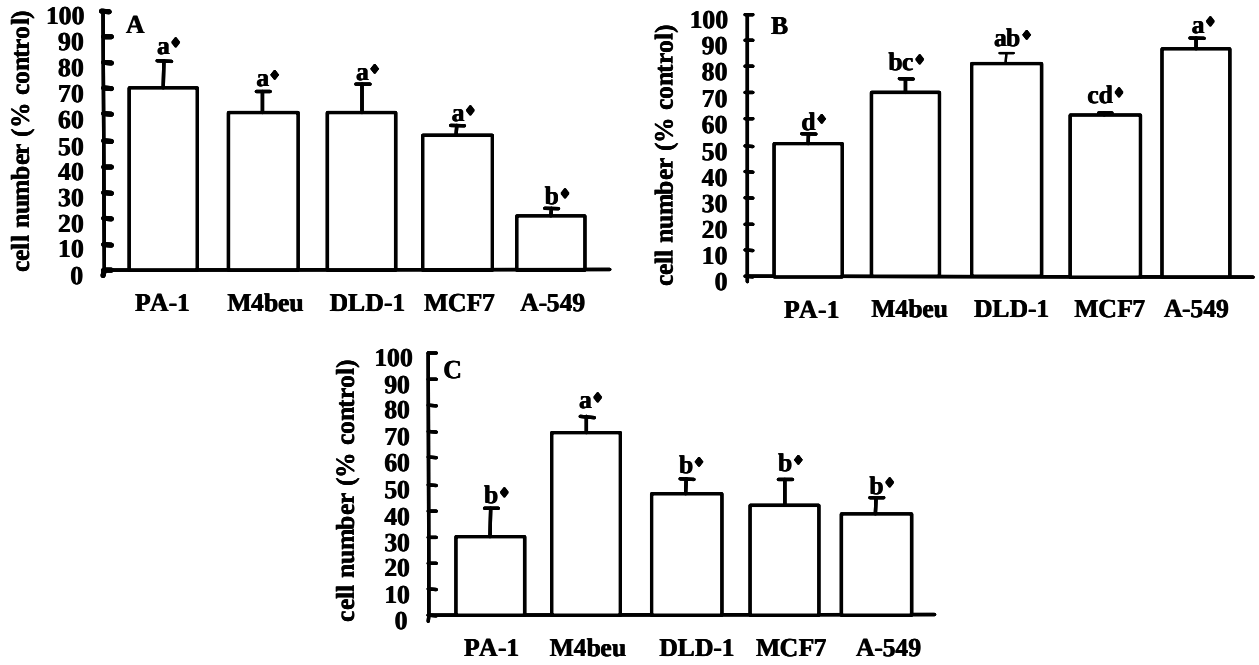
Table 4 : Effects of CLA mixtures composed with synthetic CLA isomers (Mixes 1 to 3), of beef CLA-enriched mixture (mixes E to H)* and beef total FA (mixes A to D)* on human tumor cell lines proliferation (number of cells in percent of control).

		MCF7	M4Beu	PA1	A549	DLD1
				% control		
Synthetic CLA	Mix 1	40	47	55	18	56
	Mix 2	46	67	85	21	78
	Mix 3	57	68	71	25	48
CLA-enriched fraction	Mix E	64	78	55	93	87
	Mix F	59	76	57	92	86
	Mix G	63	70	47	84	79
	Mix H	62	56	45	80	72
Total FA of bovine muscle	Mix A	61	65	48	52	54
	Mix B	37	83	39	33	36
	Mix C	48	59	27	41	48
	Mix D	21	71	5	29	37

Statistical effects (*P*=)						
residual SEM	cell	FA	origin	cell x origin	FA x origin	cell x FA
11.31	0·034	0·0069	0·0001	0·0001	0·6233	0·9812

* Mix A and E: FA of *Longissimus thoracis* muscle (LT) of Charolais x Salers steers fed linseed supplemented diet, Mix B and F: FA of *Pectoralis transversus* muscle (PT) of Charolais x Salers steers fed linseed supplemented diet Mix C and G: FA of LT muscle of Charolais x Salers steers fed control diet, Mix D and H: FA of PT muscle of Charolais x Salers steers fed control diet

Figure 1 : Growth-inhibitory activities on each human tumor cell lines to synthetic 9,11-CLA mixes (A), to CLA-enriched mixtures from beef (B) and to total FA from beef (C).



Cells were cultured in complete medium supplemented with either 100 µM of synthetic CLA isomer mixtures, 175 µM of CLA-enriched mixtures or 100 µM of total FA extracted from selected beef. Control wells were treated with an equivalent volume of ethanol compared to treated cells (0·25% vol/vol) but without FA. Sensitivity of tumour cells against any CLA mixtures (synthetic or natural) was determined by the measure of cellular DNA content by Hoechst 33342 dye. Values are expressed as percent of control (means ± SEM of 3 independent experiments).

♦ Black rhombus indicated a significant difference compared with control, $P < 0{\cdot}05$.

[a, b, c, d] Means without a common letter differ, $P < 0{\cdot}05$

incubated under soft agitation for 1 h protected from light at room temperature. Fluorescence was measured at 360/460 nm. Under these conditions, fluorescence was proportional to the amount of cellular biomass.

Statistical Analysis

Values are expressed as the mean ± SE of three independent experiments. Global effects of cell type and of CLA preparation were tested by analysis of variance (ANOVA) using the GLM procedure in SAS (Statistical Analysis Systems Inc., Cary, NC, USA). Effects tested in the model included the type of cell line (presented as cell), the nature of FA mixtures tested (presented as FA), the origin (synthetic or extracted from beef) of CLA mixtures (presented as origin), the interaction between the type of cells and the origin of FA mixtures (cell x origin), the interaction between the nature of FA tested and their origin (FA x origin) and the interaction between the type of cells and the nature of FA (cell x FA). Significance was set at $P < 0{\cdot}05$. From this statistical analysis, no significant interaction was observed between the nature of FA and their origin and between the nature of FA tested and the type of tumor cells (Table 4). Consequently, the effects of FA on tumor cell growth were independent of the type of cancer cell lines and *vice versa*. On this basis, results for each factor will be presented as follows: i) the magnitude of response of each cancer cell lines to FA, all FA having the same origin taken together, ii) the effects of each FA having the same origin on tumor cell growth, all cell lines taken together.

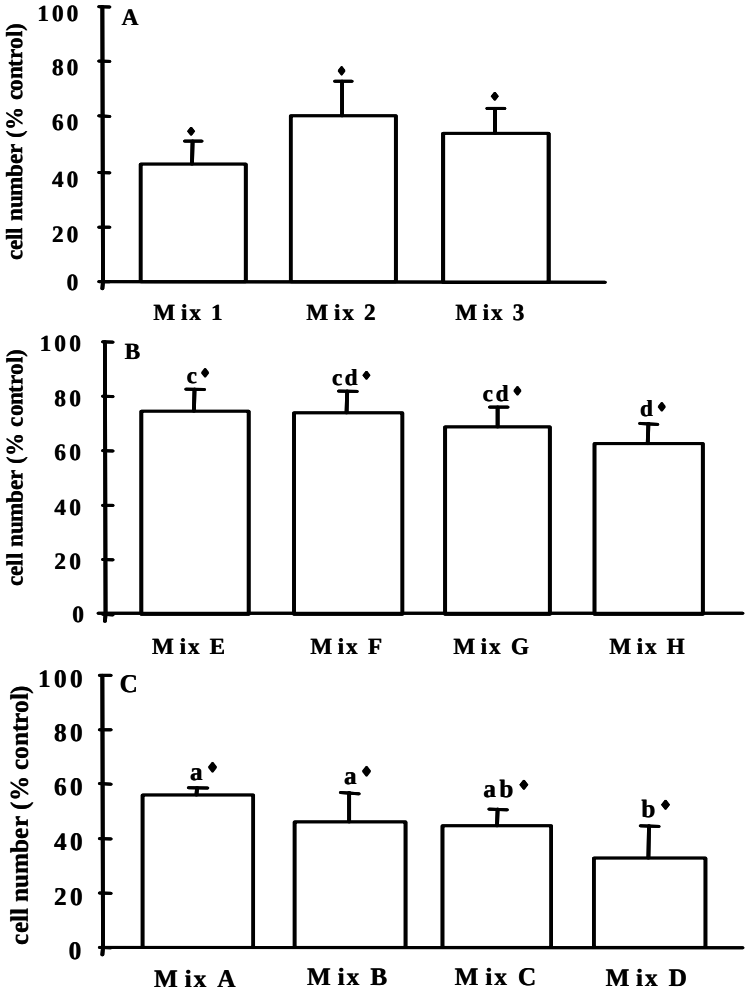
Results

Mean values for all experimental treatments were compared with that of the control treatment and given in Table 4. These global results showed a cell-growth inhibitory activity ($P = 0{\cdot}0069$) of all FA sources and significant differences between responses of cancer cell lines ($P = 0{\cdot}034$). The cell-growth inhibitory activity of CLA mixtures was different ($P = 0{\cdot}0001$) according to their origin, i.e. synthetic CLA (mixes 1 - 3) compared with purified beef CLA (mixes A - H). Since there was no significant interaction between cell lines and FA tested, and in order to clarify the presentation of results, the magnitude of the response of each cell line to the FA source is presented with as those from a common origin taken together and the effects of each FA of similar origin on cell growth presented similarly.

Each tumor cell line responds differently to fatty acids

Relative sensitivity of tumor cell lines to FA mixtures of synthetic 9,11 CLA isomers (Fig. 1A), beef CLA-enriched mixtures (Fig. 1B) and beef total fatty acids (Fig. 1C) were significantly different ($P < 0{\cdot}05$). Indeed, the tumor cells treated with any synthetic CLA mixtures (Fig. 1A) achieved on 70 to 21% of the growth observed in the control. The lung cell line (A-549) was the most sensitive tumor cell line (only 21% of control numbers) among the five cell lines tested ($P < 0{\cdot}05$). As illustrated in Fig. 1B, there is a 13 to 49% reduction in cell growth in treated cells by any CLA-enriched fraction purified from beef (87 to 51% of control numbers), the most resistant tumor cell line being the lung cell line (A-549, >87% of control numbers, $P < 0{\cdot}05$) whereas ovarian tumor cell line (PA-1) was the most sensitive (51% of control numbers, $P < 0{\cdot}05$). The sensitivity of each tumor cell lines exposed to any total fatty acids resulting from beef was ranged from 70 to 30% of control numbers (Fig. 1C). In these conditions, melanoma cell line (M4beu) was the most resistant to FA treatment whereas colon

Figure 2 : Growth inhibitory activity of synthetic 9,11-CLA mixes (A), beef CLA-enriched mixtures (B) and beef total FA (C) on any human tumour cells.



Cells were cultured in a complete medium supplemented with either 100 µM of synthetic CLA isomer mixtures, or 175 µM of CLA-enriched fractions or 100 µM of total FA extracted from selected beef. Control wells were treated with an equivalent volume of ethanol compared to treated cells (0·25% vol/vol) but without FA. Sensitivity of any tumour cells against each CLA mixture (synthetic or natural) was determined by the measure of cellular DNA content by Hoechst dye. Values are expressed as percent of control (means ± SEM of 3 independent experiments).

♦ Black rhombus indicated a significant difference compared with control (P<0·0001)

[a, b, c, d] Means without a common letter differ ([a, b] P < 0·05 and [c, d] P< 0·07, respectively).

(DLD-1), breast (MCF7), lung (A-549) and ovarian (PA1) cell lines were more sensitive (from 30 to 46% of control cells).

Each FA mixture reduces differently human tumor cell growth

The three synthetic CLA mixtures reduced the growth of cancer cells from 40 to 57% (60 to 43 % of control numbers, $P < 0{\cdot}0001$, Fig. 2A). However, variations in CLA composition of these mixtures did not significantly modify their antiproliferative effect.

The four CLA-enriched mixtures (mixes E, F, G and H) purified from beef contained some non conjugated FA but their composition was similar between fractions (Table 2). The tumor cells treated with these CLA-enriched mixtures (Fig. 2B) achieved on 75 to 63% of the growth observed in the control cells ($P < 0{\cdot}0001$). Among these CLA-enriched mixtures, Mix H, characterized by a high content in *cis,trans*-isomers and a low content in *cis,cis*- and *trans,trans*-isomers, possessed the greatest cell-growth inhibitory activity (63% of control cells).

Compared with control, the four total fatty acid mixtures selected from beef (Table 1) decreased significantly human cancer cell growth (from 56 to 33% of control numbers, $P < 0{\cdot}0001$) (Fig. 2C). As with CLA-enriched mixtures, the beef FA mixture containing a high proportion of *cis,trans*-CLA isomers and a low content in *cis,cis*- and *trans,trans*-isomers (Mix D) exerted the greatest cell-growth inhibitory activity (33% of control cells) and reversely, beef FA characterized by a low content in *cis,trans*-isomers with a high content in *cis,cis*- and *trans,trans*-CLA isomers (Mix A) possessed the lowest cell-growth inhibitory activity (56 % of control numbers).

Discussion

Although many data are available concerning anticarcinogenic effects of synthetic CLA (Kritchevsky, 2000; Kelly, 2001; Belury, 2002), only few studies have reported the antitumor properties of CLA mixtures originated from ruminant products (Ip, 1999; O'Shea, 2000). In this context, the aim of this study was to determine on several types of human tumor cells the specific antiproliferative effects of CLA-enriched FA fractions and of total FA extracted from beef differing by their CLA isomers composition.

In these experimental conditions, sensitivity of the five tumor cell lines to FA added to the medium differed as reported earlier (Shultz *et al.* 1992; Mcmillan *et al.* 1995; Palombo *et al.* 2002). Such differences in cell sensitivity could be related to the nature or to the origin of FA supplements (synthetic CLA isomers / beef CLA). Differences in cell sensitivity to each FA could be explained by intrinsic differences in cellular model such as the histogenic cell origin, the variable rates of cell proliferation and the specific uptake of FA by cells and their metabolic utilization.

CLA purification from beef needed a succession of chromatographic procedures which did not allow to obtain pure CLA mixtures. Consequently, we first investigated antiproliferative effects of synthetic CLA mixtures using common 9,11-CLA isomers with *cis,trans, cis,cis* and *trans,trans* configurations to mimic beef CLA composition. Surprisingly, no significant differences in antiproliferative properties were noted between the three synthetic CLA mixtures whereas several studies have shown that the effectiveness of individual CLA isomer to inhibit cell proliferation could be different (Pariza *et al.* 2001; Belury, 2002). A recent study has reported that the inhibitory activity of CLA isomers on cancer cell-growth is linked to the geometrical configuration of their double bonds (Palombo *et al.* 2002). Indeed, *cis*-9,*trans*-11-CLA isomer exhibits a greater antiproliferative effect on both colo-rectal and prostate cells than does *cis*-9,*cis*-11-isomer (Palombo *et al.* 2002). Discrepancies

between experiments (using individual isomers) and our study (using synthetic CLA mixtures) could be explained either by differences in antiproliferative properties between CLA mixtures and their individual constituent isomers, or by a too low concentration of specific isomers such as *trans,trans* isomers in CLA mixtures to exhibit their antiproliferative properties. Consequently, the antiproliferative effect of these CLA mixtures could be likely linked to the ability of the most abundant CLA isomer (*cis*9,*trans*11-isomer) to inhibit growth of cancer cell, differences in this isomer concentrations (from 82 to 91 % of total CLA) being probably not sufficiently contrasted to involve differences between CLA mixtures.

This paper reported for the first time the antiproliferative properties of four CLA-enriched fractions extracted from beef, mainly differing by the proportion of CLA isomers and not by the composition of the other FA which was relatively constant. The mixture that possessed the greater amount of non conjugated FA was the less potent inhibitor of cancer cell-growth indicating that these FA were not implicated in the antiproliferative effects of this mixture. These results suggested, as proposed by O'Shea (2000) who have studied anticancer properties of milk fat, that CLA could be the active ingredient responsible for the antiproliferative effect of mixtures on human cancer cells. In our experimental conditions, the most active CLA-enriched mixture was characterized by the highest proportion in *cis,trans*-isomers and the lowest proportion in *cis,cis*- and *trans,trans*-isomers. Although *cis,trans*-isomers present in ruminant products are dominated by *cis*-9,*trans*-11-isomer (Griinari & Bauman, 1999), other minor isomers could also possess significant antitumor properties as recently reported for *cis*-11,*trans*-13-CLA isomer which inhibits of cancer-cell growth (Palombo *et al.* 2002). Interestingly, total FA mixtures extracted from the beef selected strongly altered the proliferation of human cancer cells in spite of their low content in CLA (< 1% of total FA). These antiproliferative effects of total FA were higher than their corresponding CLA-enriched mixtures (55 vs 30 % of inhibition of cell growth). This suggests the ability of total FA mixtures to inhibit cancer cell-growth could be due to the presence of FA other than CLA, which could potentialize the action of CLA-enriched fractions. Among these FA, stearic, palmitic and oleic acids have no specific antiproliferative properties towards colon (Caco-2) and pancreatic cancer cells (MIA PaCa-2, PANC-1 and CFPAC) (Nano *et al.* 2003) and may even enhance cell growth at low concentration (5 µM) (Falconer *et al.* 1994; Awad *et al.* 2000). Conversely, much evidence has indicated that vaccenic and α-linolenic acids inhibit the growth of cancer cells (Begin *et al.* 1988; Awad *et al.* 1995). Among hypothesis to explain these antitumor properties, Awad *et al.* (1995) have demonstrated that vaccenic acid can be taken up by cancer cells where it could be converted into *cis*-9,*trans*-11-CLA-isomer, a potent inhibitor of tumor growth (Corl *et al.* 2003; Miller *et al.* 2003). In addition, several studies have demonstrated that polyunsaturated FA (PUFA), especially omega 3 FA, exhibit a great potency to inhibit growth of tumor cells (Booyens *et al*. 1984; Begin *et al.* 1988). Indeed, PUFA can undergo peroxidation, which generates free radicals and lipid peroxides leading to DNA damage and thus to inhibition of cell proliferation (Kumar & Das, 1995; Das, 1999). However, interaction between FA, and in particular synergic effects, cannot be excluded.

This study is the first to demonstrate that CLA mixtures naturally present in beef inhibit proliferation of human cancer cell lines. Moreover, at similar concentrations, inhibition was dependant upon the specific composition in CLA isomers present; a high content in *cis,trans*-isomers associated with a low content in *cis,cis*- and *trans,trans*-isomers being the most potent. In addition, total FA mixtures from beef exhibited a greater inhibitory activity on cell growth than their corresponding CLA-enriched mixtures suggesting that FA others than CLA present in bovine tissues possess antiproliferative properties against cancer cells and, on the other hand, that relationships between FA and CLA could influence properties of such natural mixtures.

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