Br. J. Cancer (1975) **32**, 693

CYTOTOXIC EFFECTS OF SPLENIC ULTRAFILTRATES UPON LEUKAEMIC LYMPHOCYTES

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Received 28 July 1975. Accepted 11 September 1975

Summary.—Ultrafiltrates from spleen inhibited both DNA synthesis and the proliferation of normal lymphocytes stimulated in culture from both mouse and man without apparent cytotoxicity. However, the same doses of this spleen ultrafiltrate will kill up to two-thirds of the leukaemic lymphoblasts from both mouse and man after 24 h incubation. This unique lymphocytotoxic effect could also be demonstrated on fresh primary cultures of leukaemic lymphocytes and was highly effective on slowly growing established cell lines under crowd culture conditions. Furthermore, ultrafiltrated thymus extract did not affect the DNA synthesis rates or the viability of NC-37 lymphoblasts, which have B cell characteristic. Thymus extract was cytotoxic to Molt cells, which have T cell characteristics.

IT HAS LONG been known that the rate of cell division in adult tissues is well controlled and is at a level characteristic of the cell type concerned. Theories have at various times been put forward (Bullough, 1967) to account for this phenomenon; during the last decade experimental attention has been attracted to the " chalone " concept, that is, that cells produce inhibitors which specifically repress their own proliferation. This " chalone " concept was originally developed by Bullough and Laurence (1960) and Iversen (1960) to explain the local growth regulation system of the epidermis. " Chalones " have been reported for a number of cell systems (Houck and Hennings, 1973).

It has been shown that extracts of lymphoid tissues from several species were effective in decreasing both DNA synthesis and mitosis by normal human lymphocytes (Moorhead *et al.*, 1969; Garcia-Giralt *et al.*, 1970; Houck, Irausquin and Leiken, 1971; Lord *et al.*, 1974; Attallah *et al.*, 1975); in contrast, the mitosis of leukaemic cells was not inhibited by spleen extracts (Garcia-Giralt and Marcieira-Coelho, 1974) despite the inhibition of ^{3}H-TdR incorporation into the acid insoluble DNA of these cells. It is the purpose of this communication to give a qualitative as well as quantitative description of a unique cytotoxic effect of splenic ultrafiltrates toward leukaemic but not toward normal lymphocytes *in vitro*. However, lymphocyte " chalone " activity was demonstrated in the same extracts against normal lymphocyte proliferation.

MATERIALS AND METHODS

Four established lymphoblast cell lines were used in this study: L-1210 and EL-4 mouse lymphocytes; Molt, established from the peripheral blood of a patient during relapses of acute lymphoblastic leukaemia with T cell characteristics (Minowada, Ohnuma and Moore, 1973) and NC-37, a permanent lymphoblastic line with B cell characteristics (Pattengale, Smith and Gerber, 1973) which originated from the peripheral leucocytes of a patient with pneumonia. The leukaemic lymphocyte cell lines were grown in RPMI 1640 medium containing 20% foetal calf serum, glutamine, and 100 i.u./ml each of penicillin and streptomycin.

The NC-37 human lymphoblastic cells were cultivated in McCoy's medium with 20% foetal calf serum and 100 i.u./ml each of penicillin and streptomycin.

Primary cultures of EL-4 lymphoblasts were also studied. The EL-4 lymphoid tumour, originally induced in C57 mice by the carcinogen 9,10-dimethyl-1,2-banzanthracine (Gorer, 1960) has been carried in several laboratories as a transplantable ascites tumour (provided by Dr John Wunderlich of the National Institutes of Health). These animals were killed and the EL-4 cells were removed from the peritoneum and counted in a haemacytometer. On the basis of the exclusion of vital dye, they were more than 95% viable. These primary cultures were incubated in triplicate.

Desiccated, defatted (by 1,2-dichloroethanol extraction) powders of pooled calf spleen (Viobin Corp., Monticello, Illinois 61856) were extracted overnight in distilled water (20 ml/g) with stirring at 4°C. After centrifugation, the extracts were fractionated between 30,000 and 50,000 daltons and then dialysed by Amicon ultrafiltration (Attallah *et al.*, 1975). The resulting 30,000–50,000 dalton salt-free ultrafiltrate was then lyophilized.

The spleen ultrafiltrates were reconstituted in the appropriate medium before being added to the cultures. DNA synthesis was measured by the amount of ^{3}H-TdR incorporated into the acid insoluble DNA fraction (Houck *et al.*, 1971). Triplicate cultures were incubated at 37°C for 18 h with the spleen extracts, followed by a 6-h pulse of ^{3}H-TdR. The number of viable cells remaining in similar cultures was determined in triplicate by Trypan blue dye exclusion, using a haemacytometer (Moorhead *et al.*, 1969; Attallah *et al.*, 1975). The standard deviation of the mean for this procedure was about 5%; therefore, 2 means differing by more than 10% were always found to be significantly different ($P<0{\cdot}05$). These same spleen extracts were also incubated with human lymphocytes from peripheral blood of normal volunteers and normal mouse spleen cells, the latter purified by Ficoll–Hypaque gradients (Boyum, 1968). Triplicate cultures of 5×10^5 mouse lymphocytes each per ml with and without phytohaemagglutinin (PHA) were incubated for 72 and 48 h respectively with and without 200 μg/ml of ultrafiltered spleen extract which had been added just after PHA, as described previously (Houck *et al.*, 1971; Attallah *et al.*, 1975). Both the viability and the incorporation rates of ^{3}H-TdR into these cells were determined.

RESULTS

Cytotoxic effects of ultrafiltrates upon leukaemic lymphocytes

We have found that relatively large concentrations of ultrafiltered spleen extract containing lymphocyte "chalone activity" can prevent the proliferation of leukaemic lymphocytes *in vitro*. The results, shown in Table I, show that (a) the spleen extracts inhibited the incorporation of ^{3}H-TdR into the acid insoluble DNA of all 4 cell lines and into PHA stimulated normal cells and (b) many of the human and mouse established cell line lymphocytes in the cultures were apparently killed during incubation, whereas the viability of none of the normal cells in culture was affected by incubation with spleen ultrafiltrates.

Essentially similar cytotoxicity to leukaemic lymphocyte (Molt, L-1210) *in vitro* was also demonstrated by ultrafiltered preparations from other desiccated, defatted calf, cow and pig spleen powders (Viobin Corp.). Similar ultrafiltered extracts were prepared from fresh calf spleen and 200 μg/ml of this ultrafiltered material could also kill 45% of the Molt cells *in vitro* after 24 h incubation.

Ultrafiltered extracts of cow and pig kidney were also prepared as described above. Neither of these extracts in concentrations similar to that of the spleen preparations inhibited the incorporation of ^{3}H-TdR into, nor were they cytotoxic to, L-1210 or Molt cells after 48 h incubation. Further, similar ultrafiltered extracts of calf thymus were prepared and 200 μg/ml of this ultrafiltrate reduced the viability of Molt cells by 48% but did not reduce the viability of normal human lymphocytes from that of the incubation control. Finally, thymus extract (200 μg/ml) neither killed nor

TABLE I.—*The Effects of 24-h Incubation at 37°C of 0, 125 and 250 μg/ml of Ultrafiltered Spleen Extracts (in Triplicate) upon the Mean Number of Viable Cells (Capable of Excluding Trypan Blue Vital Dye) Remaining and the Incorporation of ^{3}H-TdR (6-h pulse) into the Acid Insoluble DNA of 4 Established Lymphoblastic Cell Lines*

Cell type	Conc. (μg/ml)	No. of viable cells	% dead cells	Ct/min/10^6 viable cells	% inhibition of DNA
Mouse					
1. L-1210	0	$8{\cdot}1 \times 10^5$/ml	< 10	37500	—
($5{\cdot}5 \times 10^5$/ml)*	125	$4{\cdot}5 \times 10^5$/ml	44	15500	59
	250	$2{\cdot}8 \times 10^5$/ml	65	14200	63
2. EL-4	0	$8{\cdot}0 \times 10^5$/ml	< 10	13800	—
($10{\cdot}2 \times 10^5$/ml)*	125	$3{\cdot}3 \times 10^5$/ml	59	14400	0
	250	$1{\cdot}4 \times 10^5$/ml	83	8900	33
3. Normal (PHA 48 h)	0	$20{\cdot}0 \times 10^5$/ml	42	18500	—
(30×10^5/ml)*	200	$23{\cdot}0 \times 10^5$/ml	34	930	95
Human					
1. Molt	0	$7{\cdot}0 \times 10^5$/ml	< 10	110000	—
($6{\cdot}8 \times 10^5$/ml)*	125	$3{\cdot}3 \times 10^5$/ml	53	12000	89
	250	$2{\cdot}0 \times 10^5$/ml	72	8000	93
2. NC-37	0	$7{\cdot}2 \times 10^5$/ml	< 10	13600	—
($6{\cdot}2 \times 10^5$/ml)*	125	$4{\cdot}0 \times 10^5$/ml	44	1140	92
	250	$3{\cdot}5 \times 10^5$/ml	51	250	98
3. Normal (PHA 72 h)	0	$4{\cdot}0 \times 10^5$/ml	< 10	48000	—
(5×10^5/ml)*	200	$3{\cdot}9 \times 10^5$/ml	< 10	430	99

* Number of cells seeded initially.

inhibited the ^{3}H-TdR uptake of NC-37 cells, in accordance with the suggestion of Florentin, Kiger and Mathé (1973) that lymphocyte chalone might have T and B cell specificity.

Effects of ultrafiltrates upon normal lymphocytes

The effect of 50 μg/ml of ultrafiltrate of the aqueous extract of calf spleen upon the percentage of lymphoblasts determined morphologically was studied at various times after stimulation of 10^6 normal human lymphocytes by PHA *in vitro* in parallel with the determination of the inhibition of ^{3}H-TdR incorporation into acid insoluble DNA content of these cells. Lymphocyte morphology was evaluated by making thin smears of cultured cells and staining with Wright's stain. These results are summarized in Table II. In the absence of splenic ultrafiltrate, up to 75% of the normal lymphocytes were transformed into lymphoblasts by PHA. Further, not only was the incorporation of ^{3}H-TdR into acid insoluble DNA inhibited by incubation of PHA stimulated human lymphocytes with spleen ultrafiltrate, but so, in a parallel fashion, was the percentage of morphologically transformed lymphoblasts in the population of these cells *in vitro*. Clearly, the viability of normal lymphocytes incubated with and without spleen ultrafiltrates containing lymphocyte chalone activity did not differ one from the other.

TABLE II.—*Inhibitory Effects of Spleen "Chalone" Concentrate (30,000–50,000 dalton) 50 μg/ml upon the Percent Lymphoblastic Population and ^{3}H-thymidine Uptake by 10^6 PHA Stimulated Human Lymphocytes* in vitro

h after PHA	% Lymphoblasts: PHA alone	% Lymphoblasts: PHA and "chalone"	% ^{3}H-thymidine inhibition	Viability %
0	0	0	0	90+
24	14	2	—	90+
48	30	15	56	90+
72	60	23	51	90+
144	75	20	98	90+

Effect of ultrafiltrates upon primary cultures of leukaemic lymphocytes

EL-4 *primary* cultures were incubated in triplicate for 48 h with various con-

TABLE III.—*The Cytotoxicity and DNA Inhibition Produced by Incubating Various Concentrations of Spleen Ultrafiltered Extracts with Primary Cultures of* 5×10^5*/ml EL-4 Mouse Lymphocytes in Triplicate for* 48 *h* in vitro

Conc. (μg/ml)	Cell no. ($\times 10^5$/ml)	% viable cells	% inhibition of DNA
0	6·5	66	0
75	7·2	45*	54*
125	5·7	24*	81*
250	6·9	17*	98*
500	5·9	5*	99*

* Means found to be significantly different from 0 concentration control ($P < 0·05$).

TABLE IV.—*The Effects of Inoculum Size upon the Cytotoxicity of* 100 *or* 200 *μg/ml of Ultrafiltered Spleen Extracts upon L-*1210 *Mouse Leukaemic Lymphocytes after* 48 *h Incubation in Triplicate at* 37 °*C*

No. of cells seeded ($\times 10^5$/ml)	No. of viable cells ($\times 10^5$/ml) after incubation with spleen extract		
	0 μg/ml	100 μg/ml	200 μg/ml
9·5	14·0	5·3*	5·4*
5·3	6·5	3·6*	3·6*
2·3	6·4	6·3	3·7*
0·8	2·3	2·4	2·2

* Means found to be significantly different from 0 concentration control ($P < 0·05$).

centrations of the spleen extract, as shown in Table III. Although the mean number of cells was not reduced significantly, the percentage of these primary cultured cells remaining viable was reduced in direct proportion to the dose of spleen extract, as had been the case for the *established in vitro* cell line EL-4.

Effects of ultrafiltrates upon sparse and crowded cultures of leukaemic lymphocytes

We studied the cytotoxic effects of 100 and 200 μg/ml of spleen ultrafiltrate, using cultures which had been seeded initially with various numbers of L-1210 cells and incubated for 48 h at 37°C. These results are shown in Table IV. Concentrations between 5 and 10×10^5 cells/ml (concentrations used routinely in most laboratories) incubated with either 100 μg or 200 μg of the spleen extract contained a significant number of dead cells, as judged by the exclusion of vital dye. With $2·5 \times 10^5$ or less cells/ml, 100 μg/ml of the spleen extract no longer had cytotoxic effects, while 200 μg/ml still demonstrated a 40% cytotoxicity. However, this dose was not cytotoxic to cultures initially seeded with $0·8 \times 10^5$ lymphoblasts/ml and did not inhibit their proliferation. This dose did inhibit some 70% of the ^{3}H-TdR uptake into acid insoluble DNA by these cells, however. DNA synthesis of L-1210 lymphoblasts has recently been shown to be inhibited by spleen " chalone " concentrated in cultures containing 1×10^5 cells per ml (Garcia-Giralt and Macieira-Coelho, 1974) but, despite this, in this study neither cytotoxicity nor inhibition of the proliferation of these cells was observed.

Irreversible cytotoxicity of ultrafiltrate on leukaemic lymphocytes

L-1210 murine leukaemia cells ($2·5 \times 10^5$ per ml) were incubated in medium containing 200 μg/ml of ultrafiltered spleen extract. After different periods of incubation, these cells were washed twice with Hanks' solution, fresh medium was added, and the incubation continued for a total of 48 h. In the last 6 h of incubation, 1 μCi of ^{3}H-TdR was added before harvesting. The amount of ^{3}H-TdR incorporated into the acid insoluble DNA of these cells was determined by liquid scintillation counting. To be certain that the results would not be affected by cell loss in the manipulation of " chalone " removal, untreated cells were washed with Hanks' solution in a manner identical to the " chalone " treated cultures. The data in Table V show that the cytotoxic effects paralleled DNA inhibition throughout the period of incubation with " chalone " and that this effect was permanent. Cells incubated with the spleen extract for less than 6 h were found to grow at

TABLE V.—*L-1210 Lymphoblasts* $2{\cdot}5 \times 10^5$ *per ml were Incubated with* 200 μg/ml *of Lymphocyte Chalone for Various Times. The Cells were then Washed and Reincubated in Fresh Medium for a Total of* 48 *h. Viability was Judged from Vital Dye Exclusion. DNA Synthesis was Determined from the Amount of* ^{3}*H-TdR Incorporated into the Acid Insoluble Fraction, Using a* 6-*h Pulse after* 42 *h Incubation at* 37°*C*

Incubation time (h)	Viable cell no. ($\times 10^5$)	Ct/min (mean)	% DNA inhibition
0	6·1	19392	0
8	4·6	12643	35
16	4·4	8302	57
26	2·9	8029	59
48	2·9	8664	55

a rate subsequently similar to that of the control cells. However, irreversible cytotoxic effects were shown with those cells which had been incubated with the spleen extract for longer than 6 h.

Reversible effects of ultrafiltrate on normal lymphocytes

Normal human lymphocytes were incubated for 48 h in the appropriate medium with 100 or 200 μg/ml of the splenic ultrafiltrate. After this time, the cells were collected by centrifugation and rinsed once in Medium 199, then resuspended in fresh medium supplemented with 20% calf serum and gentamicin and glutamine. These cultures were then stimulated with PHA for 66 h, at which time the usual pulse of 1·0 μCi of ^{3}H-TdR was added and the incubation continued for another 6 h. The effects of rinsing preincubated spleen ultrafiltrate treated cells before PHA stimulation upon the incorporation of ^{3}H-TdR into acid insoluble DNA by these cells was determined. The results indicated that, while these concentrations of spleen ultrafiltrate should have inhibited over 98% of the incorporation of isotope into acid insoluble DNA with PHA stimulation, the rinsing of these cells reduced this inhibition to only 23% for 100 μg/ml and to only 32% for 200 μg/ml of treated cells. The control cells for this experiment had also been incubated for 48 h before rinsing without chalone, and thus all cultures had been incubated for a total of 5 days *in vitro*. Viability studies, using the Trypan blue exclusion technique, indicated that all of the cells, both control and chalone treated, were essentially 100% viable.

DISCUSSION

It would appear that 30,000–50,000 dalton fraction of aqueous extracts of lymphoid tissues from cow and pig could inhibit the proliferative activity of both mouse and human lymphoblastic established cell lines *in vitro* and was cytotoxic to these cells. There was no apparent cytotoxic effect on either normal lymphocytes or sparse cultures of leukaemic cells, although DNA synthesis in these cells was also inhibited. Perhaps the most important difference between crowded and sparse cultures was that most of the cells in sparse cultures were continually passing through the mitotic cycle for replication, that is, these cultures contained a large fraction of actively growing cells. Since DNA specific inhibitors of replication act by interfering with cells in the S phase of the cell cycle, this large growth fraction makes these cells highly susceptible to the killing effects of such drugs as cytosine arabinoside and methotrexate (Hryniuk, Fischer and Berlino, 1969; Clarkson, 1974; Skipper, Schabel and Willcox, 1967). In contrast, under crowded culture conditions a much smaller fraction of these cells was actively growing and the leukaemic cells became much less susceptible to S phase-specific drugs (Clarkson, 1974). However, crowded leukaemic cells in culture should be mostly in the G_1 phase of their mitotic cycle and are susceptible to the cytotoxic effect of the splenic ultrafiltrate containing lymphocyte chalone activity.

One explanation of our results could be that the functional end cells are

producing a factor that inhibits further the growth of their own kind. This is suggested by the data of Table IV (controls), which show that the relative cell yield decreased as the cell number initially seeded increased. Therefore, one might assume that in crowded cell populations *in vitro*, there is a combined effect of the " chalone " produced or released by the cells and exogenous " chalone ". In sparse cell populations less chalone would be produced by the cells and the total concentration of " chalone " would thus be less. This explanation is in agreement with the finding of Hersh, McCredie and Freireich (1974) of an inhibitor of blastogenesis in supernatants of cultured lymphoblasts grown under crowded conditions.

Since " chalone " concentration per cell under crowded conditions was 1/10th that per cell in the sparse cultures, and yet only the crowded cultures demonstrated cytotoxic response to the more dilute chalone, some arcane interaction between chalone and the cells in crowded culture may well be involved in this cytotoxicity. This cytotoxic consequence of cell crowding and chalone could not be demonstrated for normal lymphocytes and hence must be a manifestation of some unique quality of leukaemic cells. Nutritional competition appears unlikely, particularly in short-term (24 h) culture since the same medium will support PHA stimulated proliferation of the same number of normal lymphocytes for 72 h.

This lymphocytotoxicity of spleen ultrafiltrates was clearly unique for established cell lines of murine and human leukaemic lymphoblasts. These same preparations of spleen ultrafiltrate which were toxic for leukaemic lymphoblasts were found to be without cytotoxicity for normal human and murine lymphocytes, even after stimulation by PHA.

The incubation of splenic ultrafiltrates with murine leukaemic lymphoblasts for less than 6 h resulted in no cytotoxic effects upon these cells *in vitro*. However, the quantitative cytotoxicity effects of these extracts on L-1210 cells was directly correlated with incubation time between 8 and 16 h. This cytotoxic effect was maximal after 16 h incubation; almost one complete cell cycle (Skipper *et al.*, 1967). One interpretation of these results would be that splenic lymphocytotoxicity for leukaemic cells was specific for the G_1 phase of the cell cycle. If lymphocyte chalone concentration was maintained during this cycle, cells in S, G_2, and M phases eventually would enter the G_1 phase where they would be arrested and killed before S phase.

Since many chemotherapeutic agents are most active against rapidly growing cells (Hryniuk *et al.*, 1969; Clarkson, 1974) but not against dormant tumour cells, this finding of a leukaemia specific cytotoxicity or splenic ultrafiltrate for the more slowly growing crowded cell cultures (G_0 or G_1) appears to be of considerable interest, particularly since thymic ultrafiltrate cytotoxicity appears to be specific for T *vs* B cell established cell lines of human lymphoblasts. However, the relationship between the lymphocyte chalone and the cytotoxic factor, is only assumed and remains to be determined.

Gratitude is expressed to Drs E. Esber and C. Hunt for their helpful suggestions, and to Miss Marino for her assistance.

Supported in part by a grant in aid from the National Institutes of Health, CA12743.

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