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Acute Lymphoblastic Leukemia of Childhood Monitored by Bacteriocin and Flowcytometry*

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Abstract—*Bacteriocin and flowcytometric analysis of* 106 *blood samples from children with acute lymphoblastic leukemia were correlated with clinical stages of disease. Bacteriocins interacted selectively with malignant, and not with normal, lymphocytes causing cell cycle perturbation which was rapidly and objectively recorded by the flowcytometer. The patients were grouped as:* (*A*) *newly-diagnosed* (15); (*B*) *early induction* (11); (*C*) *remission with viral infection* (7); (*D*) *remission* (64); (*E*) *bone marrow relapsed* (5); (*F*) *extramedullary relapsed* (3); (*G*) *non-malignant pediatric controls* (8). *Bacteriocin reacted usually with groups A, B, C, E and not with groups D, F and G. Repeated testing correlated well with the clinical status. Blood from* 7 *patients in remission and from* 3 *normal individuals, each with transient viral infection, reacted with bacteriocin.*

A quantitative correlation between peripheral blood blasts or surface markers for ALL and bacteriocin reactivity was not established. Unexpected results were obtained only in 13% (*false-positive* 11% *and false-negative* 3%).

This test can be recommended for preliminary diagnosis and possibly prognosis of lymphoblastic leukemia and provides means of monitoring progress during chemotherapy.

INTRODUCTION

BACTERIOCINS are antibacterial proteins produced by many bacteria and will also interact and kill malignant mammalian cells [1–4]. The interaction with bacteriocins leads to the inhibition of cell proliferation in various ways; among which interference with DNA synthesis and the promotion of its degradation are of special interest [2, 5]. It was noted that inhibition of the proliferation of neoplastic cells by bacteriocin was highly selective, so that concentrations which inhibited neoplastic cells were harmless to normal bone marrow cells [6, 8]. The effect of bacteriocin can be determined by inhibition of ^{3}H-thymidine uptake, by the reduction in colony-forming units, by counting the affected cell cultures [2] and by using the flowcytometer (FCM) [5]. The latter provides an objective and speedy means of analysis of the bacteriocin effect. DNA histograms are generated by the FCM, thus monitoring perturbations in the cell cycle of such cells which interacted with and were affected by the bacteriocin [5]. Consequently, bacteriocins were used as a laboratory diagnostic tool to differentiate chronic lymphocytic leukemic (CLL) and lymphoma, from non-malignant cases [7–10] as well as tumorigenic from non-tumorigenic cells [12, 13].

Preliminary results indicated that acute lymphoblastic leukemic (ALL) cells were highly bacteriocin-sensitive. Therefore, a project was launched to determine the sensitivity to a bacteriocin of peripheral blood lymphocytes/lymphoblasts from ALL patients who were classified by conventional clinical criteria, into the following groups: (A) newly-diagnosed, (B) early induction, (C) remission with viral infection, (D) remission and well, (E) hematologically relapsed, (F) isolated central nervous system (CNS) relapsed, (G) controls (rheumatoid arthritis, thalassemia, hemophilia). The patients with viral infection were only transiently reactive to bacteriocin while the infection persisted. The

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bacteriocin-sensitive cells of all groups were screened for surface markers, but no correlation pattern became apparent.

Bacteriocin with the aid of FCM differentiated the various phases of illness and correlated well with the clinical status of the patients with ALL. Of 106 blood samples tested, only 13% non-accountable false reactions were encountered. A preliminary note was published recently [10].

MATERIALS AND METHODS

Bacteriocin production and potency evaluation

The bacterial strains used, the method of bacteriocin production, the titration of its potency and the definition of the inhibitory concentration 50 (IC_{50}), were described earlier [12]. Colicin HSC10 was used in this study, produced by *Escherichia coli* HSC10 [2].

Mammalian cells

Human peripheral blood, anticoagulated with heparin, was overlayed on Ficoll-Paque™ (Pharmacia Fine Chemicals, Dorval, Quebec). The lymphocytes were concentrated by density gradient centrifugation [11], removed, washed 3 times with veronal buffered saline, pH 7.5 (VBS), supplemented with Ca^{2+} (7.5×10^{-7}M) and Mg^{2+} (2.5×10^{-6}M) and resuspended in RPMI 1640 medium (Ontario Cancer Institute, Toronto, Ontario). (See Flow Sheet Fig. 1a.) The cells were counted in the Coulter-S +2 (Coulter Electronics, Inc., Hialeah, Florida).

Flowcytometer (FCM) and experimental procedure

Cell fluorescence was measured in the FCM described by Horan and Wheeless [14] and in our previous publications [5, 8]. The instrument was calibrated using a standard of human peripheral lymphocytes isolated from normal individuals [15]. The cells were prepared and counted as described above in Fig. 1(a) and then stained by the Krishan propidium iodide method [16] (Fig. 1b). The peak channel for the G_0/G_1 cells was adjusted to that of the standard. For the bacteriocin-treated cells (test) no further instrument adjustments were made. The coefficient of variation (c.v.) was calculated for each histogram. Positive reactivity was evaluated by values equal or greater than 10% in 'Pre-G_1' channels, either 1–31 or 16–31, and/or less than 10% in G_0/G_1 phase.

RESULTS

Recognition of malignancy in PBl from ALL patients

The inhibition and destruction of the malignant cells by the bacteriocin is indicated by changes in cell distribution in the cell cycle as shown in the DNA histograms in Fig. 2. It can be seen from Fig. 2(A), that the non-malignant lymphocytes were not sensitive to the bacteriocin, colicin HSC10. These exhibited similar cell distribution in the cell cycle (similar histograms), whether treated with bacteriocin (test) or without bacteriocin (controls). However, the test histograms for an ALL patient, shown in Fig. 2(B), indicated that the patient-lymphocytes were greatly affected by the bacteriocin, decreasing in number from the G_0/G_1 phase (channels 32 →) calculated as previously described [5]. The cells of the 'pre-G_1' phase of the histogram accumulated and they represent disrupted cells (debris, channels 1–15) and cells with diminished amounts of DNA (channels 16–31), due to leakage of nucleotides [2, 5].

Recognition of clinical stages in ALL patients

In Fig. 3, a summary of the analysis of 106 samples is shown. The accumulation of cells in the 'pre-G_1' channels and cell loss from the G_0/G_1 phase are given. Eight non-malignant pediatric controls and patients grouped according to their clinical state are presented: (A) newly-diagnosed; (B) early induction (1–14 days of treatment); (C) remission with viral infection; (D) remission and well; (E) and (F) with bone marrow and extramedullary relapse (not shown in Fig. 3, but detailed in Table 1); (G) non-malignant pediatric controls. Groups (A), (B) and (C) exhibited the greatest bacteriocin sensitivity (P values: (A) < 0.005; (B) < 0.025; (C) < 0.05) as compared to the non-malignant controls, (G). On the other hand, patients in remission (D) and the extramedullary CNS and skin relapsed patients, (F) (Table 1) did not exhibit this sensitivity and their P values were not significantly different from the controls (G). Normal adult lymphocytes gave essentially the same non-reactivity pattern as did the pediatric control cases [7, 8].

Reactivity to bacteriocin of PBl from patients with bone marrow and extramedullary relapse

The reactions of the patients in relapse, are given in Table 1. Patients with bone marrow relapse with and without circulating blasts were usually recognized by bacteriocin (4/6); whereas isolated extramedullary relapse was not recognized by bacteriocin (0/3).

Reactivity to bacteriocin of PBl from patients in early induction

A summary of the reactions to bacteriocin of PBl lymphocytes from 11 patients in early induction, the days of induction and % blasts, are given in Table 2. Generally, a reactivity with bacteriocin was found (9/11). One of the 2 non-reactive PBl was from patient (SB); this patient was on treatment and free of blasts. Thus, non-reactivity to bacteriocin was to be expected. The other non-reactive

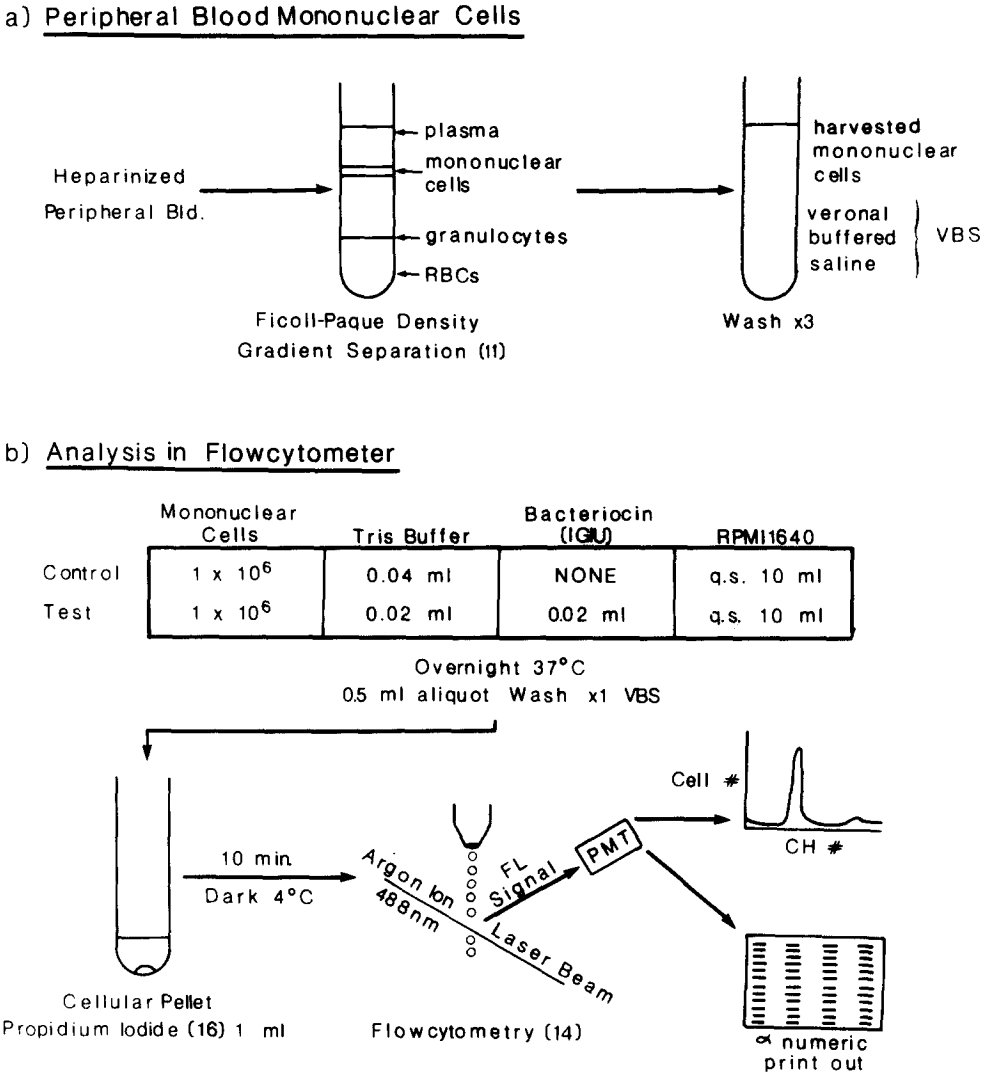

Fig. 1. Preparation of mononuclear cells from peripheral blood and the test conditions for flowcytometry.

patient (JE) was only 2 days on treatment, also free of blasts. It is of interest that in most instances of patients tested during induction, no blasts were found, nevertheless a positive reactivity with bacteriocin was noted. Two other patients with as many as 21 and 51% blasts in their PBl, who did react to bacteriocin, showed reactivity which was not as strong as would be expected if a correlation to the percentage of blasts was evident.

Repeated testing of PBl during the same or different phases of ALL

Upon repeated testing of patients for their reactivity to bacteriocin (51 tests), generally the clinical impression was confirmed. However, in some instances with patients in remission and hematologically well, non-accountable positive reactions occurred. It is of interest that of 7 patients in remission who had a viral infection, 6 reacted to bacteriocin (Table 4). This was a transient finding, observed also in normal adults with viral infection. One ALL patient and 3 adults, upon retesting, did not react to bacteriocin when the viral infection cleared.

Repeated testing of unexpected results in ALL patients

Repeated tests of the unaccounted positive reactions in patients considered in remission, are given in Table 3. It was found that patients MA, WMc, DM, and JM without peripheral blasts and clinically in remission, still reacted to bacteriocin. Thus, cells accumulated in the 'pre-G_1' channels. These results would be regarded as false-positive. However, when re-tested later, at various intervals, the reactivity cleared entering the normal reaction range which conformed with the clinical diagnosis of remission. Patient TR, however, remained positive to bacteriocin during the second test, 13 weeks

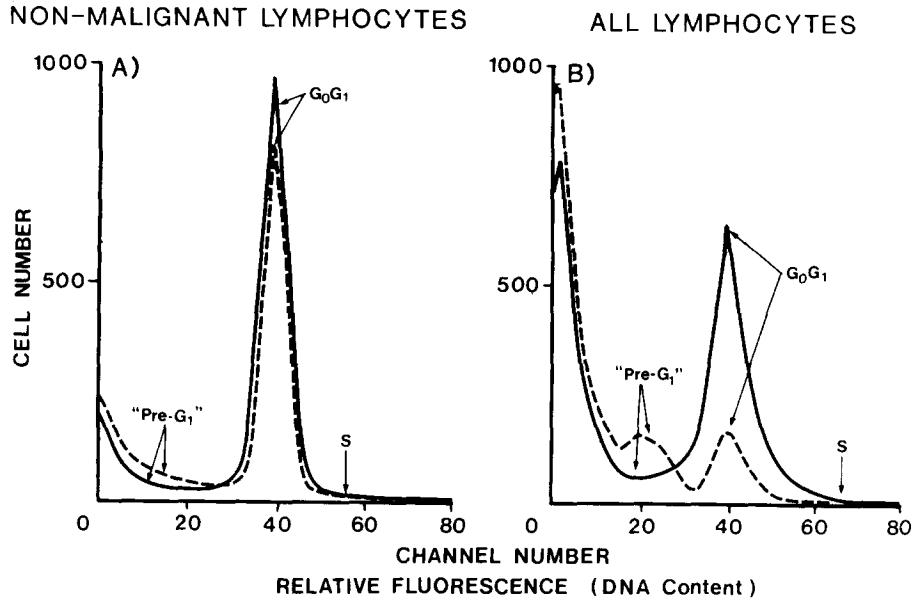

Fig. 2. Representative DNA histograms of human peripheral blood lymphocytes, analyzed in the flowcytometer. (A) Non-malignant and (B) acute lymphoblastic leukemia (ALL). The lymphocytes were treated for 24 hr *with the bacteriocin, colicin HSC_{10} at one* IC_{50}[2] (---); *or with Tris buffer* (0.03M, *pH* 7.8), *the colicin diluent, control* (——).

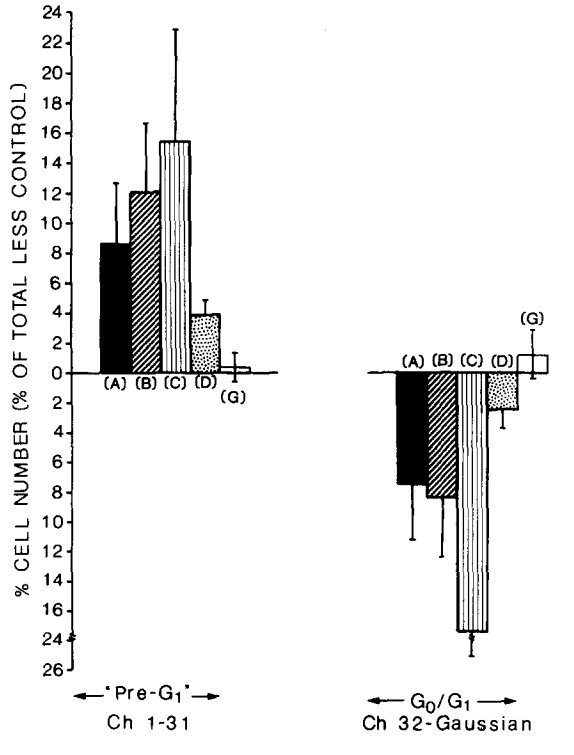

Fig. 3. Clinical grouping of the 106 *ALL patients and* 8 *non-malignant controls and their peripheral blood reactions to bacteriocin. Cell number* (%) *in 'pre-G_1'* (*Channels* 1–31 *of histogram*) *and G_0/G_1* (*Channels* 32 → *Gaussian*) *following* 24 hr *contact with bacteriocin, colicin HSC_{10}, at one* IC_{50} [2]; (*A*) ■ *average of* 15 *newly-diagnosed*; (*B*) *diagonal lines, average of* 11 *in early induction*; (*C*) *vertical lines, average of* 7 *with viral infection*; (*D*) *dots, average of* 64 *in remission*; (*E*) (*F*) *See Table* 1; (*G*) □ *average of* 8 *non-malignant patients. The vertical bar is the standard error.*

after diagnosis, though to a lesser degree than when first tested. This result can be regarded as a false-positive reaction which cannot be explained. However, when a third test was performed a month later, the reactivity disappeared in conformity with the clinical status of the patient. Two other patients (EM and AR) were unique in that the false-positive reaction during remission and, in absence of detectable blasts, were followed by hematological relapses. EM was retested during reinduction (7 days) and the reactivity to bacteriocin was increased further, while the clinician was still unable to detect blasts. Another relapse followed shortly afterwards. Still no blasts were found, whereas reactivity to bacteriocin was still present. This severely ill patient died somewhat later. Similarly AR relapsed repeatedly following our 'false-positive' reactivity to bacteriocin, and died soon after. The patients described in Table 3 did not have notation of viral infection when their clinical records were reviewed retrospectively.

Summary of unexpected results in ALL

A total of 14 unexpected reactions were encountered among 106 cases tested (13.2%). Seventy-one cases in remission, expected not to react with bacteriocin, gave 11 positive reactions (15%), as shown in Table 4. The reactions to bacteriocin, for this group, are also given, showing the average % cell distribution in the cell cycle. On the other hand, 33 cases were expected to react positively with bacteriocin. These cases consisted of newly-diagnosed, in early induction (1–14 days of treatment) and with viral infections. Only 3 negative results of 33 cases (one of them was a borderline reaction), were encountered (9%) (Table 4).

Table 1. Reactions in nine relapsed ALL patients

Type of relapse	Bacteriocin–peripheral blood interaction Distribution of cells in cell cycle (% of total less control) 'Pre-G_1' Ch* 1–31	Peak of 'pre-G_1' Ch 16–31	G_0/G_1 Ch 32–Gaussian	Reactivity
Bone marrow				
Circulating blasts	+13.3	− 5.3	−14.7	+
	− 7.8	+ 9.8	+ 7.6	+
No circulating blasts				
Bone marrow only	+13.9	+ 1.2	−14.1	+
	+12.3	− 4.4	−10.2	+
	+ 1.2	+ 0.2	− 0.3	−
Bone marrow + CNS	+ 0.3	−15.7	− 7.1	−
Extramedullary				
CNS*	+ 5.5	−12.8	− 7.3	−
Skin	+ 3.1	− 0.2	− 2.6	−
	− 2.7	+ 0.6	+ 2.4	−

*Ch—Channel; CNS, central nervous system; Reactivity +, relates to values of 10% or more in 'Pre-G_1', either in channels 1–31 or 16–31, and/or values less than 10% in G_0/G_1.

Table 2. Reactivity to bacteriocin of PBl from patients in early induction

Patient	Days of induction	Blasts %	Bacteriocin–peripheral blood interaction Distribution of cells in cell cycle (% of total less control) 'Pre-G_1' Ch* 1–31	Peak of 'pre-G_1' Ch 16–31	G_0/G_1 Ch 32–Gaussian	Reactivity
XP	1	51	+ 9.2	− 3.8	− 2.7	±
DM	1	0	− 4.3	+13.8	− 0.9	+
JE	2	0	− 0.1	− 2.7	− 0.1	−
SMc	2	0	+13.9	+ 2.9	−15.1	+
PT	3	0	+14.1	+11.1	− 6.7	+
MA	3	0	+ 6.1	+ 9.1	− 9.0	+
NT	4	0	+35.8	+ 5.5	−23.3	+
DS	6	21	− 0.9	+ 9.1	+15.4	+
CS	7	1	+13.1	−19	−13.7	+
EM	7	0	+43.3	+ 1.8	−33.5	+
SB	12	0	+ 2.4	− 0.2	− 4.2	−

*Ch—channel; reactivity +, as described in Table 1 and in Materials and Methods.

Surface markers for ALL and the correlation to bacteriocin reactivity

It was of interest to investigate whether bacteriocin recognizes any of the surface markers which are being used to diagnose ALL and its subtypes. The blast cells of 15 newly-diagnosed patients, which reacted to bacteriocin, were tested for their reaction to common ALL antigen (CALLA) using BA_3 antibody produced by Tucker LeBien, University Hospital, Minneapolis, Minn., U.S.A.; to Ia using 21W4 antisera produced by Michelle Letarte, Immunology Laboratory, Hospital for Sick Children, Toronto, Canada; and for B and T surface markers by looking for the presence of surface and cytoplasmic immunologlobulin and AET rosettes. For patients tested at a later phase of our study, a panel of monoclonal antibodies was also used. The blasts were also classified according to the FAB (French American British) morphologic classification as L_1, L_2 or L_3. Most of the cases were identified as non-T non-B ALL (11/15). The reactions to bacteriocin varied in degree, within this group. Three cases were grouped as pre-B and again the degree of sensitivity to bacteriocin varied among these patients. One patient only, among the 15 newly-diagnosed was of the T type, and the sensitivity to bacteriocin was not great. There was no correlation between the above ALL surface markers and the surface recognition site for bacteriocin. There was also no apparent correlation with the FAB classification but there were only a few cases with L_2 morphology and none with L_3.

Table 3. Repeat testing of seven ALL patients with 'false-positive' reactions to bacteriocin (no recorded viral infection)

Patient	Weeks after diagnosis	Clinical stage	PBl blast (%)	Bacteriocin-peripheral blood interaction Distribution of cells in cell cycle (% of total less controls) 'Pre-G_1' Ch* 1–31	Peak of 'pre-G_1' Ch 16–31	G_0/G_1 Ch 32–Gaussian	Reactivity	
MA	3	R*	0	+27	+27	−25	+	FP*
	4	R	0	+ 5	NA*	− 5	−	
WMc	44	R	0	+36	− 4	−40	+	FP
	47	R	0	0	− 3	− 7	−	
DM	46	R	0	+20	+ 6	−19	+	FP
	55	R	0	+ 8	− 8	− 1	−	
JM	81	R	0	+20	+ 1	− 7	+	FP
	86	R	0	+ 3	+ 7	+ 3	−	
TR	0	New*	67	+30	+5	−26	+	
	13	R	0	+ 3	+16	− 2	+	FP
	17	R	0	+ 7	−11	− 8	−	
EM	260	R	0	+14	− 4	−16	+	FP
	272	Relapse*	NA	N†*	NT	NT		
	273	ReInd.(7d)*	0	+43	+ 2	−34	+	
	288	Relapse	0	+14	+ 1	−14	+	
	302	Death						
AR	64	R	0	− 2	+ 2	+ 2	−	
	68	R	0	+19	−15	−19	+	FP
	79	Relapse	75	NT	NT	NT		
	80	ReInd.(6d)	0	NT	NT	NT		
	82	Relapse Death	70	NT	NT	NT		

*Ch—channel; R—remission; Relapse—bone marrow relapse; ReInd—reinduction (1–14 days on chemotherapy); New—newly-diagnosed; NT—not tested; NA—not available; FP—false-positive; Reactivity +, evaluated as described in Table 1 and in Materials and Methods.

Table 4. Unexpected results in ALL patients

	N	Bacteriocin–peripheral blood interaction Distribution of cells in cell cycle (% of total less control) 'Pre-G_1' Ch* 1–31	Peak of 'pre-G_1' Ch 16–31	G_0/G_1 Ch 32–Gaussian	Reactivity
False positives (in remission—well, 64) (in remission—viral, 7)	11/71	+13.0	+5.9	−8.6	+
False-negatives					
Newly-diagnosed	1/15	+ 7.6	−13.8	− 8.4	−
Early induction	1/11	+ 9.2*	− 3.8	− 2.7	±
Remission viral	1/7	+ 1.2	− 0.8	− 0.4	−

*Ch—channel; borderline false negatives; Reactivity +, evaluated as described in Table 1 and in Materials and Methods.

DISCUSSION

Our earlier laboratory studies using bacteriocins and flowcytometry have shown that some lymphocytic malignancies can be recognized [7–9]. Thus, the malignant cells which interact selectively with bacteriocin, leak DNA degradation products, due to DNA breakdown. This event is indicated by changes in the DNA histograms generated by the flowcytometer. Preliminary tests with peripheral blood lymphocytes from children with acute lymphoblastic leukemia, indicated a similar sensitivity to bacteriocin. It was therefore decided to launch a prospective screening program of unselected ALL patients at various stages of their illness. Preliminary notes of our findings were published [10, 17], not covering the extended observations and

details presented here. The reactions of the neoplastic cells to the bacteriocin seemed usually to reflect the clinical condition of the patient. A total of 106 blood samples were tested without knowledge of the clinical information. Medical charts were reviewed at the conclusion of the study. Patients in remission and clinically well, were not reactive to bacteriocin, similar to non-malignant controls. The reactive groups (Fig. 3), as expected, were newly-diagnosed cases (A), not yet on chemotherapy; usually those in early induction (B) seemingly before treatment was effective; in remission but with viral infection (C); and those with bone marrow (hematological) relapse (E). Such cases reacted usually to bacteriocin even when no circulating blasts were detected on the blood smear. Similarly, in early induction (Table 2), positive reactions to bacteriocin were noted in spite of lack of blasts in PBl. Possibly, subtle cytological changes may have occurred in the lymphocytes, not apparent by microscopy, but recognized by the bacteriocin. Also seen in Table 2, a patient on treatment for 12 days did not react to bacteriocin when presumably an effective stage of remission was achieved. It is of interest that isolated extramedullary relapses (skin and CNS), were not recognized by bacteriocin. Actually, since peripheral blood lymphocytes are tested, the reaction to bacteriocin should, in spite of isolated extramedullary relapse, indeed remain negative (Table 1).

It is to be noted that some unexpected reactions occurred, such as positive reactivity to bacteriocin, when in fact the patient was in remission according to the clinical findings. In 6 of such instances (Table 4), it was confirmed that the patients had a coincident viral infection. These findings, although seemingly unfavorable to the diagnostic potential of bacteriocin for the neoplastic state, do not negate its importance, since upon retesting the lymphocytes after the infection cleared, the reactivity to bacteriocin also had disappeared. This transient response was also noted both in 3 normal adults tested during viral infections and later after recovery (data not shown). Such a reversal in reactivity to bacteriocin is not observed with malignant cells, unless successful therapy has been instituted, as found for patients going into remission (Fig. 3). We have retested 24 ALL patients (51 samples) during the same or different phases of their disease, while on chemotherapy. Generally, the reactions to bacteriocin correlated well with the clinical stage, except 7 false-positive reactions (14%) (Table 3). It is of interest to note (Table 3), that upon retesting 5/7 cases (MA; WMc; DM; TR; JM) the reactivity disappeared. We wish to speculate that these patients may have had a possible viral infection, not recorded by the oncologist, and therefore reacted to bacteriocin. Two other patients (Table 3—EM; AR) were of special interest and are regarded by us as doubtful 'false-positive' cases, because they relapsed after the reactivity to bacteriocin was noted. Though reinduction therapy was instituted, the patients relapsed once more and died sometime later. Thus, in retrospect we might regard the diagnosis by bacteriocin as possibly being predictive. During remission but with sensitivity to bacteriocin, a few reactive lymphocytes were occasionally seen on the peripheral film but blasts were never detected (Table 3). Similarly no peripheral blasts were seen in some bone marrow relapses (Table 1) and patients in early induction (Table 2) which were reactive to bacteriocin. There is the possibility that the bacteriocin may recognize some early cellular or surface alterations, which cannot yet be detected clinically or morphologically. If this is so, it may provide a means of detecting a relapse before it becomes clinically obvious by other conventional methods. However, if our assumption of a viral infection for some of our unaccountable false-positive findings is not to be taken into account, we still remain with 11 false-positive (10%) and 2 false-negative reactions (2%) (Table 4) for the 106 blood samples studied. This represents only a 12% discrepancy with the clinical findings. An attempt to find a relation between surface markers of ALL and bacteriocin reactivity was not possible.

In conclusion. Bacteriocins, with the aid of the flowcytometer, detect and differentiate the malignant cells from the normal lymphocytes. The flowcytometer records rapidly and objectively the cell cycle perturbation caused by bacteriocin. This useful and simple screening method could serve as a preliminary diagnostic and prognostic tool for lymphocytic malignancies as well as provide the means of monitoring progress during chemotherapy.

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