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Bacteriocin and Flow Cytometry in Laboratory Diagnosis of Leukemic Peripheral Blood Lymphocytes and Bone Marrow Cells*

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Abstract—*The bacteriocin, colicin HSC10, produced by* Escherichia coli *HSC10, was studied as a laboratory tool for detection and differentiation of leukemic from normal lymphocytes in human peripheral blood. Flow cytometry studies detected DNA loss in bacteriocin-affected cells by computerized histograms. Differential analysis is given for the peripheral blood of 26 individuals using bacteriocin, cytochemistry and surface markers. Sensitivity to colicin was detected in 10 (83%) of the 12 patients with chronic lymphocytic leukemias and other leukemias with morphologically immature lymphocytes. Cells were lost from the G_0/G_1 phase and accumulated in the '$pre\text{-}G_1$' channels of the histogram, indicative of cells with reduced DNA content. The lymphocytes of 14 normals, however, were not or only slightly affected by the bacteriocin ($P < 0.001$). Similarly, normal bone marrow cells exposed to bacteriocin remained unaffected ($P > 0.2$). Thus, immaturity* per se *was not recognized by bacteriocin. The bacteriocin effect was more discriminatory than other laboratory tests reported here and in most cases differentiated malignant from normal cells.*

INTRODUCTION

BACTERIOCINS are antibiotic proteins produced by various bacteria [1, 2] which can be obtained under controlled laboratory conditions with ease and at low cost [3]. They inhibit bacterial growth [1, 4] but are also cytotoxic to mammalian cells [5–13]. A common feature in the mode of action of the different bacteriocins is their specific interaction with membrane receptors, followed by intracellular events which prevent cell division. However, the intracellular events may vary, depending on the bacteriocin [2, 4, 14, 15]. In our studies with mammalian cells such bacteriocins were chosen which inhibit DNA synthesis and cause its degradation [8], leading to cell death [6, 8, 16].

This investigation, using bacteriocins as a laboratory diagnostic tool, was stimulated by earlier work with bacteriocins which indicated a preferential affinity for neoplastic rather than for normal cells *in vivo* and *in vitro* [7, 8, 16]. Using the spleen colony assay of Till and McCulloch [17], lymphoma cells were inhibited at four increasing concentrations of bacteriocin, neither of which had an adverse effect on normal bone marrow (BM) cells [16]. This concurred with earlier observations for two established human adenocarcinoma cell lines from the colon and rectum [18] which were more sensitive to a bacteriocin than normal human embryonic intestinal cells [6]. Furthermore, murine leukemic cell lines were sensitive to bacteriocins to a greater extent than their less malignant counterparts [19].

The effects of bacteriocins on mammalian cells were also studied in the flow cytometer (FCM) and perturbations of the cell distribution in the cell cycle were detected with rapidity and objectivity [20, 21]. The analysis was based on measuring relative differences in fluorescence intensity of stained DNA in the individual cells under study. The flow cytometer was most suitable to detect them since the bacteriocin-affected mammalian cells were found to have decreased amounts of

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DNA [21], similar to bacteriocin-affected bacterial cells [4, 8, 22].

Thus in view of these earlier observations that (a) bacteriocins have an affinity for and affect adversely malignant rather than normal cells and (b) the FCM detects the bacteriocin effect with ease and rapidity, a laboratory diagnostic approach was attempted using bacteriocins and the FCM to recognize lymphocytic malignancies.

It is reported here that by such an approach peripheral blood lymphocytes (PBL) and BM from normal individuals were differentiated from chronic lymphocytic leukemia (CLL) patients (B and T cell types) and prolymphocytic (immature) leukemias. A preliminary note has already been published [23].

MATERIALS AND METHODS

Bacteriocin production and potency evaluation

The bacterial strains used, the methods of production, the titration of bacteriocin potency and the definition of a growth-inhibitory unit (GIU) were described earlier [8]. Colicin HSC10 was used in this study, produced by *Escherichia coli* HSC10.

Mammalian cells

(a) *Human PBL.* Peripheral blood, anticoagulated with ethylenediaminotetraacetate (EDTA), was overlayed on Ficoll-Paque™ (Pharmacia Fine Chemicals, Dorval, Quebec). The lymphocytes were concentrated by density gradient centrifugation [24], removed, washed three 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). The cells were counted in a Coulter-S counter (Coulter Electronics, Inc., Hialeah, FL).

(b) *BM cells.* These were obtained by aspiration, diluted in heparinized RPMI 1640 medium and treated as described above under (a) for PBL.

Flow cytometer (FCM)

Cell fluorescence was measured in the FCM described by Horan [25] and Horan and Wheeless [26]. This equipment is a two-parameter sorter (TPS-1) equipped with a 488-nm argon ion laser beam and a 513-nm high pass filter between the lens and photomultiplier tube (Coulter Electronics, Inc., Hialeah, FL). The instrument was calibrated using a standard of human peripheral lymphocytes isolated from normal individuals [27] and treated as described above, and stained by the Krishan propidium iodide method [28]. Then the channel for the G_0/G_1 peak in the DNA histogram was established. A DNA histogram of the lymphocytes to be tested to which no bacteriocin was added (control) was subsequently obtained and the peak channel for G_0/G_1 cells were adjusted to that of the standard. For the bacteriocin-treated cells (test) no further instrument adjustments were made. The coefficient of variation (CV) was calculated for each histogram [27].

Experimental procedure

The cells, prepared as described above, were suspended in RPMI 1640 medium supplemented with penicillin G (100 units/ml) and streptomycin (100 μg/ml) either in the presence or absence of 10% fetal calf serum (FCS) (Difco, Detroit, MI). Bacteriocin, diluted in tris (hydroxymethyl)aminomethane at 0.03M, pH 7.8 (Tris), at the required potency in terms of GIU [8] (test) or Tris only (control) was added. Incubation followed at 37°C for the required time. Aliquots with about 5×10^5 cells were stained by the Krishan method [28] with propidium iodide (Sigma Chemical Co., St. Louis, MO) for 10 min at 4°C in the dark, filtered through 88-μm monofilament nylon mesh (Small Parts Inc., Miami, FL) and analyzed by the FCM.

Calculations

These were made from numerical data and DNA histograms. The percentage of the total cell number was calculated for the 'pre-G_1' phase (channels 2–31), the peak of the 'pre-G_1' phase (channels 15–31) and the G_0/G_1 phase by integration of the cells under the peak (Gaussian). The final results of the tests were expressed following subtraction or addition of the percentage changes which occurred in the cell numbers of the controls, i.e. lacking the bacteriocin.

Cytochemistry

The acid phosphatase test [29] and the non-specific α-naphthyl acetate esterase test (ANAE) [30] were used.

Surface marker analysis

(a) E_N *rosettes.* Neuraminidase-treated sheep red blood cells (SRBC) for T lymphocyte rosettes were tested [31], with some modifications. Neuraminidase (type V: purified *C. perfringens*, Sigma Chemical Co., St. Louis, MO) 0.02 unit/ml was used. A minimum of 500 cells were counted.

(b) *Erythrocyte antibody complement (EAC) rosettes.* To detect complement receptors on B lymphocytes, sensitized SRBC were used [31] with some modifications. Bacto-trypsin (Difco Labs., Detroit, MI) 1.2% (w/v) was used. An equal volume of the trypsinized SRBC, 1:300 rabbit anti-SRBC (IgM 19S—Cordis Labs., Miami, FL) and 1:200 guinea pig complement (Wellcome Reagents Ltd., Beckenham, England) were mixed and incubated at 37°C for 30 min. A minimum of 500 cells were counted.

(c) *Surface membrane immunoglobulin (SmIg).* Lymphocytes were washed twice with Hanks Balanced Salt Solution, Ca^{2+} and Mg^{2+}-free (HBSS) (Grand Island Biological Co., Grand Island, NY), containing 5% FCS and 2 mg/ml purified sodium azide (Fisher-Scientific Co., Fair Lawn, NJ). The cell pellet was stained with fluorescein-isothiocyanate (FITC) anti-immunoglobulin (monospecific) (Meloy Labs., Springfield, VA), diluted 1:10 with VBS and kept on ice for at least 30 min. Dilution in 1 ml HBSS–FCS–sodium azide followed and the mixture was overlayed on a 2-ml FCS gradient and centrifuged at 300 *g* for 5 min at 4°C. The pellet was resuspended and washed twice in HBSS. The cells were examined using a fluorescence microscope (Wild-Leitz, GMBH, Wezlar, Germany) and the percentage of fluorescing cells for each SmIg was determined by counting at least 100 cells (H. R. Toben, Wayne State University, MI, personal communication).

RESULTS

Effect of bacteriocin concentration and exposure time on human CLL PBL, analyzed in FCM from DNA histograms

It can be seen in Fig. 1A that the DNA histogram of PBL from a CLL patient remained practically unchanged for 24 hr (---) when not treated with colicin (0-GIU), except for a marginal increase of cells in the 'pre-G_1' channels of the histogram. However, when the patient's lymphocytes were treated with 1 GIU colicin (Fig. 1B) a marked increase in the 'pre-G_1' cell number and a decrease in G_0/G_1 was evident at 24 hr interaction (---). If an increased concentration of 2 GIU colicin was used (Fig. 1C), more pronounced changes in the histograms became evident, resulting as expected in a dose response to the bacteriocin. When the cells were exposed for 48 hr to the bacteriocin the changes in the DNA histograms were further amplified (not shown). Thus the exposure of the patients' lymphocytes to only 1 GIU colicin HSC10 and for not more than 24 hr was adequate to bring about a distinct DNA histogram, different from that for the CLL cells not treated with bacteriocin but kept under equal test conditions.

A comparison of the DNA histograms of PBL from a normal individual and a CLL patient exposed to colicin HSC10 are shown in Fig. 2. It can be seen that the DNA histograms of the normal and the CLL PBLs, if not treated with bacteriocin, yielded similar histograms (—). However, if both were exposed to 1 GIU colicin, clear differences in the DNA histograms became evident: the histograms of the PBL from the normal individual were not significantly affected, whereas the CLL lymphocytes increased in the

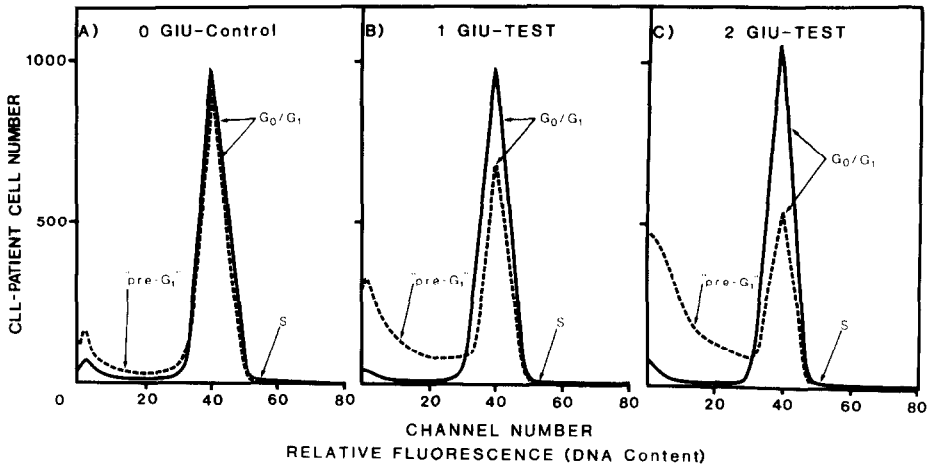

Fig. 1. Effect of bacteriocin concentration and exposure time on DNA histograms of PBL from a CLL patient, analyzed in the FCM. The cells were prepared, exposed to the bacteriocin, colicin HSC10, and stained as described in Materials and Methods. (A) Controls, CLL cells treated with Tris, the bacteriocin diluent, (0 GIU); (B) and (C) CLL cells treated with colicin at 0 hr (—); 24 hr (---). Equal cell numbers were scored for each histogram.

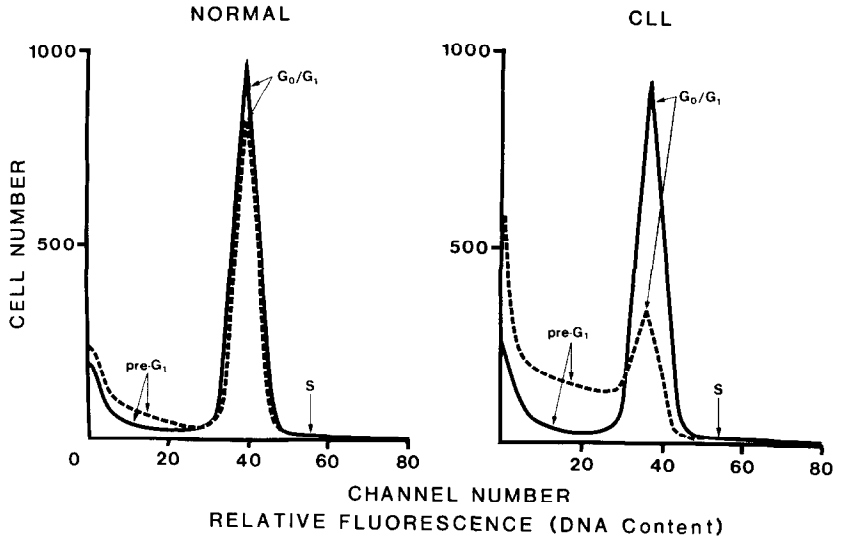

Fig. 2. Effect of bacteriocin on DNA histograms of PBL from a normal individual and a CLL patient, analyzed in the FCM. Procedural details as described for Fig. 1. Colicin HSC10 at 1 GIU and exposure time of 19 hr at 37°C. Controls (—); bacteriocin-treated (---). Coefficient of variation (CV) 4.4 and 3.8 (normal); CV 4.1 and 4.6 (CLL). Reproduced by courtesy of Elsevier IRCS.

'pre-G_1' channels and decreased in the G_0/G_1 phase. These observations indicate that the bacteriocin–cell interaction is a prerequisite for recognizing differences in the DNA histograms of normal and patient PBL.

Twenty-six histograms of human PBLs were analyzed. They comprised three groups, as shown in Fig. 3: (A) nine CLL (probably B type) patients; (B) two prolymphocytic leukemias (ProLL) and one CLL (probably T type) patient, and (C) fourteen normal individuals. The cells were exposed to colicin HSC10 and their distribution in the various phases of the cell cycle determined from the DNA histograms. Any fragility of a cell population to the procedural manipulation will become apparent in the histograms of the bacteriocin-untreated controls. The final results for the tests were expressed following subtraction or addition of the percent changes which occurred in the cell numbers of such bacteriocin-untreated controls. Thus the increase or decrease in the final cell number expressed for each phase of the DNA histogram is due to bacteriocin action and not to cell fragility *per se*. Impressive differences were found in the sensitivity of PBL from the two groups of patients and the group of normal individuals ($P < 0.001$). These differences are shown by increased cell numbers in the 'pre-G_1' phase (27.3%, Group A; 55%, Group B). In contrast, the normal lymphocytes were hardly affected by colicin, only a slight increase in cell number (4%) in the 'pre-G_1' and a decrease on average (3.5%) in the G_0/G_1 phase being observed for the 14 normals on average

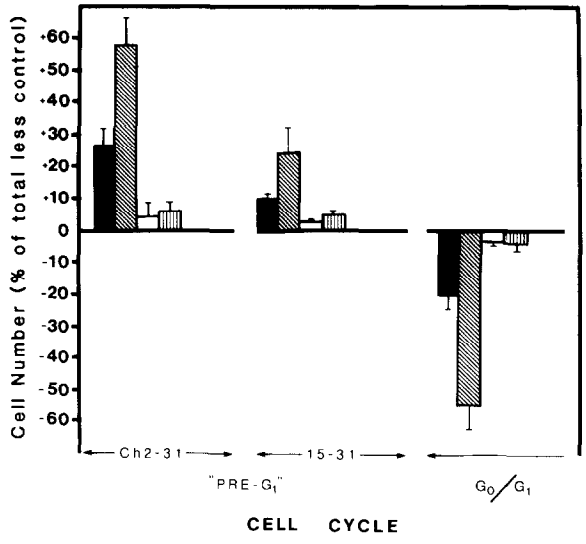

Fig. 3. Effect of bacteriocin on cells from PBL and BM analyzed in the FCM and calculated from DNA histograms. Procedural details as for Fig. 1. Percentages were derived as described in Materials and Methods and expressed as an average for each group. Colicin HSC10 at 1 GIU, 24 hr interaction at 37°C. ■ Group A—CLL, 9 patients; ▨ Group B—ProLL and T-type CLL, 3 patients; □ Group C—normals, 14 individuals. ▥ BM—normals, 3 individuals. Ch, channel; bar represents standard error.

(group C). It is to be noted that among the 12 patients investigated, 2 (17%) were atypical to the group, resulting in 'false-negative' histograms.

It was of interest to investigate whether the high sensitivity to the bacteriocin of the ProLL cells (Fig. 3, group B) was due to their immaturity or to some other marker. To answer

this, hemopoietic BM cells from normal individuals were treated as described in Materials and Methods. These cells were exposed to colicin and then analyzed in the FCM. The cell numbers found in the 'pre-G_1' and G_0/G_1 phase following bacteriocin interaction, averaged for the cases studied, are also given in Fig. 3. The normal BM cells were only slightly affected following exposure to colicin, a pattern similar to that of the fourteen normal PBLs ($P > 0.2$). These results indicate (a) that the extreme sensitivity of the ProLL cells was not likely due to immaturity *per se* and (b) that bacteriocin interaction affects the DNA histograms of the malignant cells but has practically no such effect on normal cells.

Various other tests carried out on lymphocytes from patients and normal individuals were compared to the results obtained with the FCM following bacteriocin interaction. It can be seen in Table 1 that the various tests, other than counts and morphology, did not give a specific pattern for showing the patients' lymphocytes as being unique and different from other lymphocytopathies. Thus the WBC count and percentage of lymphocytes of the nine CLL patients (Group A) and three other leukemias (Group B), though elevated relative to that of normal individuals (Group C) [32], consisted of fluctuating numbers. Morphological characteristics of PBL from Group A were consistent with typical CLL, whereas the lymphocytes of Group B patients were pleiomorphic and nucleolated, features not seen in typical CLL or normal individuals. The nonspecific esterase ANAE [30] in the patients of both groups fluctuated from 2 to 23% (Group A) and 2 to 88% (Group B), and the acid phosphatase [29] ranged from 1 to 24% (Group A) and 16 to 92% (Group B). Of the surface markers, E_N rosetting [31] was depressed in all the patients below the values found for T cells in normal PBLs and EAC rosetting was variable, as also were the various surface immunoglobulins (SIg), overlapping the normal range for PBLs [33]. In contrast, it is clearly seen in Table 1 that when lymphocytes were exposed to the bacteriocin, colicin HSC10, and tested in the FCM, a very distinct difference became apparent in the percentage of cells of the 'pre-G_1' and G_0/G_1 phases of the 9 CLL patients (Group A) and the 3 other leukemias (Group B), as compared to the lymphocytes from the 14 normal individuals (Group C).

DISCUSSION

Bacteriocins, antibacterial proteins produced by Gram-negative bacteria, are known to interact with and kill mammalian cells [5, 8, 16]. Similar observations were also made by researchers in Czechoslovakia [11–13] and Japan [9, 10].

We have noted that bacteriocins interact preferentially with malignant cells rather than with their normal counterparts [6, 7, 16, 19, 34–36]. Such observations led to the initiation of experiments using bacteriocins as tools to differentiate between malignant and normal human lymphocytes. The FCM [25, 26] provided an extremely rapid and objective analysis of cell distribution in the cell cycle and perturbations caused by bacteriocin were easily detected [20, 21, 23, 35, 36].

These changes resulted since, following bacteriocin interaction with sensitive mammalian cells, DNA degradation occurred [20, 21] similar to the degradation in sensitive bacteria [4, 8, 22]. An obvious possibility existed that the DNA histograms from CLL patients, independent of bacteriocin–cell interaction, could be distinct from normal PBL DNA histograms since it was known that some neoplastic cells have more DNA than normal cells [26] and that the karyotype of CLL lymphocytes may contain extra chromosomes [37]. Our results indicated that the DNA histograms were very similar for the malignant and normal lymphocytes (Figs 1 and 2). However, following exposure to colicin, only the malignant cells were affected, decreasing from the G_0/G_1 phase and accumulating in the 'pre-G_1' phase, indicative of cells with a reduced content of DNA.

These findings, however, did not exclude the possibility that the cells with apparent reduced DNA content were actually (a) in a resting phase (i.e. low-intensity fluorescence) or (b) were blocked to interact with the fluorochrome [38], thus still with a full complement of DNA.

The accumulated 'pre-G_1' cells nearest G_0/G_1 (channels 15–31) were 'gated' by fluorescence and 'sorted' and observed by fluorescence microscopy. Intact cells were found with diminished intensity of nuclear fluorescence [20, 21]. Furthermore, a decrease was found in the TCA-precipitable intracellular DNA following interaction with bacteriocin [21].

From the above we suggest that the malignant cells are recognized by the bacteriocins, which upon interaction cause DNA degradation and its reduced interaction with the fluorochrome. This seems to be a transient situation leading to the eventual cell disintegration. However, the normal cells are not thus affected by the bacteriocin; therefore the likelihood exists that an increase in nuclear fragility to PI staining [28] of the malignant cells following bacteriocin interaction may also contribute to the accumulation of 'pre-G_1' cells.

Table 1. Differential testing of CLL, other leukemias and normal PBL by

Cases No.	Age	Sex	Cell count WBC/mm^3 × 10^3*	Cell count Lymphocytes†	Lymphocyte morphology† Smudges/100 WBCs	Lymphocyte morphology† Small	Lymphocyte morphology† Clefted nuclei	Lymphocyte morphology† Nucleolated nuclei
Group A—9 Patients								
1	68	F	102.6	98	40	99	0	1
2	65	F	18.2	65	6	89	3	8
3	67	F	77.2	92	45	99	0	1
4	74	M	48.2	90	62	96	0	4
5	80	M	14.2	59	26	89	2	9
6	76	F	106.5	98	40	99	0	1
††7	71	M	38.7	86	33	94	4	2
††8	59	M	26.6	76	5	90	4	6
††9	60	M	78.0	79	17	98	0	2
Range			14–107	59–98	5–62	89–99	0–4	1–9
Group B—3 Patients								
10	51	F	23.8	79	1	51	27	22
11	70	M	28.0	87	4	8	4	88
12	70	M	151.0	99	5	6	0	94
Group C–Normals			7.5 ± 3.5‡‡	20–45%‡‡	0.7 ± 0.8§§	98 ± 1.9§§	1.3 ± 1.7§§	0.4 ± 0.5§§

Procedural details for DNA histograms as for Fig. 1; colicin HSC10 at IGIU, 20–24 hr interaction at 37°C. All values are expressed as a percentage except WBC counts and 'smudges'.
*Counted in Coulter-S.
†May–Grunwald–Giemsa-stained PBL films.
‡§‖¶**Test procedures as described in Materials and Methods.
††Treated patients.
‡‡See Ref. [32].
§§Data from Mount Sinai Laboratories.
‖‖See Ref. [33].

The question arose whether bacteriocins recognize a marker which is linked to immaturity. This was of interest since some malignancies of poorly differentiated lymphocytes (ProLL patients of group B in Fig. 3) and poorly differentiated lymphomas [39] seemed to be particularly sensitive to the bacteriocin. However, bone marrow from normals was not adversely affected by bacteriocin (Fig. 3). Such results seem to indicate that normal immature hematopoietic cells are not recognized by bacteriocins, but malignant developmental arrest is recognized.

The test as employed here is simple to perform and objective in character; only small numbers of cells ($< 10^6$) are required and after an overnight incubation the results become available in quantitative and graphic terms within minutes.

We have tested other malignant cells, both of murine and human origin, disseminated or localized in nature, for their sensitivity to bacteriocin. The bacteriocins showed a greater discriminatory capacity for the malignant cells than for their normal counterparts [6, 16, 19].

At this phase of our work it is not possible even to speculate what the specific 'malignancy marker' may be. However, bacteriocin recognizes a marker apparently in common for malignant cells of diverse origins.

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cell count, morphology, cytochemistry, surface markers and bacteriocin effect

Cytochemistry		Surface markers						Bacteriocin effect	
		Rosettes		Surface membrane immunoglobulin**				DNA histogram	
ANAE-positive‡	Acid phosphatase-positive§	E_N‖	EAC¶	SmIgG	SmIgA	SmIgM	SmIgD	'Pre-G_1' (channels 2–31)	G_0/G_1
4	5	4	38	42	5	9	6	47.2	−34.7
9	23	25	5	9	16	12	19	32.6	−41.0
23	24	7	6	66	4	9	50	47.3	−38.4
8	10	9	57	18	3	2	4	6.2	−5.3
12	18	33	11	6	5	0	5	10.7	−5.9
2	9	6	20	4	5	10	2	37.9	−14.0
4	19	3	3	6	6	14	13	28.4	−27.2
8	19	18	23	48	10	6	14	13.7	−9.2
3	1	6	14	11	11	13	5	22.0	−20.0
2–23	1–24	3–33	3–57	6–66	3–16	0–14	2–50	27–5	−22–5
88	92	42	1	8	7	10	19	33.7	−25.22
9	19	8	4	51	47	69	60	72.6	−69.3
2	16	8	1	70	44	88	82	46.6	−46.0
68	72§§	75.2 ± 5.3	21.6 ± 6.1	7.1‖‖	2.2‖‖	8.9‖‖	6.2‖‖	4 ± 0.9	−4 ± 1.2
									14 individuals

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