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Mitogenic response of neoplastic B cells: comparison of reactivity to *Staphylococcus aureus* Cowan I and anti-immunoglobulin antibodies

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SUMMARY

Neoplastic B cells from two patients with hyperleucocytic hairy cell leukaemia (HCL) and 19 patients with chronic lymphocytic leukaemia of B cell Type (B-CLL) were investigated to examine the mitogenic responses to the $F(ab')_2$ fraction of anti-human immunoglobulins (anti-Igs) and *Staphylococcus aureus* Cowan I (STA). Neoplastic cells from both HCL patients lacked surface Tac antigen. Mononuclear cells from the two HCL patients strongly responded to both anti-Igs and STA as measured by ^{3}H-thymidine incorporation *in vitro*. Although the mononuclear cells from two patients with B-CLL showed high response to STA, cells from none of the patients with B-CLL responded to anti-Igs. Mononuclear cells as well as T-cell-depleted fractions from the two HCL patients showed a strong proliferative response by anti-γ chain antibody (anti-γ) and the mononuclear cells from one of the patients were also induced to proliferate by anti-δ, whereas those from normal subjects responded only to a high concentration of anti-μ. Based on the difference in reactivity to anti-Ig, it is suggested that the HCL cells in this study originate from a subset equivalent to 'memory' B cells, whereas the B-CLL cells originate from a subset equivalent to 'virgin' B cells.

Keywords hairy cell leukaemia chronic lymphocytic leukaemia mitogenic response *Staphylococcus aureus* Cowan I anti-immunoglobulin

INTRODUCTION

Agents that make cross-linkage of the surface immunoglobulin (s-Ig), such as anti-immunoglobulin antibodies (anti-Ig) or *Staphylococcus aureus* Cowan I (STA), have been used to prepare models of the interaction of antigen with the s-Ig on the B lymphocytes (Sell & Gell, 1965; Falkoff, Zhu & Fauci, 1982). Culture with anti-Ig or STA under appropriate condition has been shown to cause B cell proliferation. The additional signals of T cell derived differentiation factors can induce these STA or anti-Ig activated B cells to differentiate into immunoglobulin producing cells (Falkoff *et al.*, 1982).

However, not all B cells may proliferate in response to STA or anti-Ig. Experiments with murine B cells have shown that anti-Ig is mitogenic for mature but not immature B cells (Sieckmann *et al.*, 1981). In human B cells, Chiorazzi, Fu and Kunkel (1980) reported that a high concentration of $F(ab')_2$ fraction of goat anti-human Ig antibody induced proliferation in the absence of T cells.

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Muraguchi *et al.* (1983) demonstrated that a high concentration of anti-Ig induces B cell proliferation which is independent of B cell growth factor (BCGF), whereas at a low concentration BCGF is required. STA has been shown to be a T-independent mitogen of human B cell (Forsgren, Svedjelund & Wigzell, 1976), and there have been several reports of human B cell subset which responds to STA (Romagnani *et al.*, 1980; Harada *et al.*, 1982).

On the other hand, leukaemic B cells have been used as a model system of a single B cell clone, which can be activated to proliferate or differentiate by various factors (Yoshizaki *et al.*, 1982). However, comparisons of the mitogenic responses of neoplastic B cells, which are presumably derived from different stages of normal B cell differentiation, have not been fully investigated. The present study deals with mononuclear cells from patients with hairy cell leukaemia (HCL) and patients with chronic lymphocytic leukaemia of B cell type (B-CLL) and is focused on their mitogenic responses to two s-Ig cross-linking agents, that is, STA and $F(ab')_2$ preparations of anti-Ig.

MATERIALS AND METHODS

Patients. Nineteen patients with B-CLL diagnosed according to the standard criteria and two patients with HCL, who were splenectomized after this study and whose histological findings of the spleen were compatible with the diagnosis, were subjected to this study. None of the patients had M-components in their sera. Six healthy subjects were included as controls.

Cell. Mononuclear cells were separated from heparinized venous blood by flotation in Ficoll-sodium diatrizoate (LSM, Litton Bionetics, Kensington, Maryland, USA) and washed three times in Hanks' balanced salt solution (HBSS) supplemented with 2% fetal calf serum (FCS) (Flow Laboratories, North Ryde, Australia). The cells were then divided into aliquots and froze in a medium consisting of RPMI 1640 (Flow Laboratories, North Ryde, Australia), 10% dimethyl sulfoxide, and 25% FCS. A programmable freezer (Cryo Med., Mt Clemens, MI, USA) was used to freeze cells at a rate of -1°C/min. The frozen cells were cryopreserved at -196°C in the vapour phase of liquid nitrogen until used, so that they could be examined under the same conditions. The cells were thawed rapidly at 37°C, diluted slowly and washed three times with HBSS supplemented with 2% FCS and resuspended in RPMI 1640 supplemented with 10% FCS. Viability of the recovered cells was greater than 90% as determined by trypan blue dye exclusion. T-cell-depleted fractions were obtained from the cells of the two HCL patients and the two healthy subjects by rosetting with neuraminidase-treated sheep erythrocytes and by cytotoxic treatment with OKT3 (Ortho Diagnostics, Raritan, NJ, USA) and rabbit complement. For normal T-cell-depleted fractions, monocytes were partly depleted by adhering to plastic dishes. E rosetting cells were less than 1% in each fraction obtained.

Immunological markers. S-Igs were determined by the direct immunofluorescent test using fluorescein-conjugated rabbit (Behring Institute, Marburg, FRG) and goat (Miles Laboratories, Elkhart, IN, USA) antisera to human immunoglobulin heavy and light chains. Immunoglobulins adsorbed by Fc-receptors were dissociated by the acid pH procedure described by Kumagai *et al.* (1975). Surface B1 antigen and Tac antigen were detected by the indirect immunofluorescence method using monoclonal anti-B1 antibody (Coulter Immunology, Hialeah, FL, USA) and anti-Tac antibody (kindly provided by Dr T Uchiyama, Kyoto University), and fluorescein-conjugated goat anti-mouse IgG (Meloy Laboratories Inc., Springfield, VA, USA) as the second antibody. E rosette formation was measured using neutraminidase-treated sheep erythrocytes (West *et al.*, 1977).

Reagents. STA was prepared by the method reported by Kessler (1975). The $F(ab')_2$ fragment of rabbit anti human Ig (μ, γ, α, heavy and light chains) (anti-Igs) and the $F(ab')_2$ fragment of goat antibodies each specific for human μ, γ, δ chains and the Fab portion of human IgG (anti-μ, anti-γ, anti-δ and anti-Fab) were purchased from N.L. Cappel Laboratories, Inc. (Cochranville, PA, USA).

Cell culture. Mononuclear cells were cultured in RPMI 1640 supplemented with 10% FCS, 8 μg/ml of gentamicin and 2 mM of L-glutamine. In stimulation tests, cells were cultured at 1×10^6/ml in

200 μl of medium in 96-well plates (Costar, Cambridge, MA, USA) in the presence or absence of STA (0·01% v/v) or anti-Igs (10 & 100 μg/ml).

The mononuclear cells and T-cell-depleted fraction from the two HCL patients and those from two healthy subjects were also cultured with anti-μ, anti-γ, anti-δ and anti-Fab.

In each experiment, cells were cultured 72 h, and pulsed with 0·5 μCi of ^{3}H-thymidine (^{3}H-TdR) (50 Ci/m mol, RCC Amersham, UK) per well during the last 4 h of the culture. The ^{3}H-TdR did not affect the viability of the cells at the end of the culture. The incorporation of ^{3}H-TdR was measured by scintillation spectrophotometry after harvesting with a multiple automatic sample harvester (Labo Mash, Labo Science, Tokyo, Japan).

RESULTS

The characterization of the surface markers of the mononuclear cells from the patients is summarized in Table 1. The white blood cell count of the patients varied from 11,000 to 500,000 per μl. In both cases of HCL, neoplastic cells carried surface immunoglobulin of $\gamma\lambda$ type, whereas in the majority of the B-CLL cases the neoplastic cells carried duel heavy chain determinants of $\mu\delta$ type. The percentage of B cells estimated by B1 was highest in all of the cases studied.

Because of faintness of the surface immunoglobulin of the neoplastic cells, in some cases the count of the s-Ig bearing cells have been underestimated with a much lower percentage than those of B cells estimated by B1. The neoplastic cells from both HCL cases were negative for Tac-antigen, which Korsmeyer *et al.* (1983) had reported to be positive in most HCL cases.

The results of proliferative responses to STA and anti-Igs are shown in Table 2. The mononuclear cells from the two HCL patients responded vigorously to both reagents. Although the mononuclear cells from two B-CLL patients responded strongly to STA, these cells did not respond to anti-Igs. The remaining 17 patients of B-CLL responded neither to STA nor anti-Igs. Some

Table 1. Surface markers of the mononuclear cells studied

		WBC count/μl	S-Ig type	E (%)	S-Ig (%)	B1 (%)
HCL Patients	1	500,000	$\gamma\lambda$	2·0	38·5	94·5
	2	120,000	$\gamma\lambda$	6·0	92·0	92·5
B-CLL Patients	1	60,000	$\mu\delta\lambda$	1·0	86·5	92·5
	2	37,000	$\mu\delta\lambda$	16·5	82·5	86·5
	3	14,000	$\mu\delta\lambda$	16·5	63·0	70·0
	4	13,000	$\mu\delta\lambda$	3·5	72·0	95·5
	5	13,000	$\mu\delta\lambda$	9·0	91·5	91·0
	6	13,000	$\mu\kappa$	2·0	54·0	90·5
	7	11,000	$\mu\delta\lambda$	5·0	55·0	95·5
	8	17,000	$\mu\delta\lambda$	6·5	93·0	94·5
	9	200,000	$\mu\delta\lambda$	1·0	75·5	95·0
	10	14,000	$\mu\delta\lambda$	9·0	90·0	91·0
	11	35,000	$\delta\lambda$	6·0	75·5	93·5
	12	85,000	$\mu\kappa$	2·0	81·5	97·5
	13	17,000	$\mu\delta\kappa$	19·0	84·5	78·0
	14	19,000	$\mu\delta\kappa$	7·0	86·5	89·5
	15	12,000	$\mu\kappa$	2·5	52·5	89·0
	16	42,000	$\mu\delta\lambda$	2·5	93·5	94·5
	17	21,000	$\gamma\mu\kappa$	15·5	78·0	83·0
	18	12,000	$\gamma\kappa$	15·0	75·5	74·5
	19	21,000	$\mu\delta\lambda$	9·0	89·5	93·0
Normal ($n=6$)				62·0 ± 4·0*	10·8 ± 2·6*	7·3 ± 1·9*

* mean ± s.d.

Table 2. Mitogenic response of mononuclear cells to STA and anti-Igs

		STA 0·01% 100 μg/ml		Anti-Igs 10 μg/ml		Anti-Igs 10 μg/ml		Medium
		ct/min*	SI	ct/min*	SI	ct/min*	SI	ct/min*
HCL Patients	1	33,279	30·6	34,107	31·3	26,081	24·0	1,087
	2	31,955	34·6	33,051	34·6	16,032	17·4	923
B-CLL Patients	1	21,871	120·2	328	1·8	377	2·1	182
	2	48,848	149·0	533	1·8	539	1·8	301
	3	4,058	10·0	1,065	2·6	820	2·0	403
	4	304	0·9	304	0·9	155	0·5	337
	5	152	0·3	690	1·3	412	0·8	516
	6	293	0·7	552	1·3	284	0·7	436
	7	223	0·8	573	2·1	247	0·9	268
	8	375	1·5	453	1·8	268	1·1	248
	9	68	0·5	272	1·9	90	0·6	144
	10	558	1·8	366	1·2	203	0·7	305
	11	531	1·2	689	1·6	503	1·2	428
	12	300	0·5	453	0·7	486	0·8	621
	13	2,654	3·1	1,291	1·4	752	0·9	857
	14	361	0·6	616	1·0	327	0·5	601
	15	208	0·3	502	0·8	472	0·8	601
	16	150	0·5	293	0·9	375	1·2	309
	17	2,935	4·3	711	1·0	629	0·9	683
	18	640	3·4	259	1·4	689	3·7	187
	19	380	0·4	221	0·5	221	0·5	403
Normal	1	25,340	14·8	3,488	2·0	1,801	1·1	1,711
	2	22,347	11·2	6,322	3·2	2,258	1·1	2,006
	3	8,800	9·5	1,851	2·0	1,496	1·6	925
	4	28,617	11·7	5,720	2·3	2,859	1·2	2,443
	5	20,045	11·5	4,722	2·7	2,871	1·6	1,749
	6	25,414	6·4	5,460	1·4	4,431	1·1	3,945

* Mean ct/min of triplicate cultures.
SI ct/min with mitogen ÷ ct/min without mitogen.

variations between the unresponding B-CLL patients might be due to the varying population of normal B cells present in these populations, because almost no response was observed in the patients with high leucocyte count. Mononuclear cells from the normal subjects responded considerably to STA and hardly at all to anti-Igs.

In an experiment using various anti-Ig reagents, the mononuclear cells from the two HCL patients responded strongly to anti-γ (100 μg/ml & 10 μg/ml) (Table 3). Cells from HCL Patient 1 also responded to anti-δ at the concentration of 100 μg/ml. On the other hand, mononuclear cells from the two normal subjects only responded to anti-μ at a concentration of 100 μg/ml. In another experiment with T-cell-depleted fractions, similar results were obtained, showing these responses to be independent of T cells (Table 4).

DISCUSSION

In recent years there has been a debate as to the origin of the neoplastic cells of HCL and B-CLL. The present study was designed to clarify the difference in the mitogenic responses of these cells to

Table 3. Mitogenic response of mononuclear cells to various anti-Ig reagents

		HCL Patient 1		HCL Patient 2		Normal 1		Normal 2	
		ct/min*	SI	ct/min*	SI	ct/min*	SI	ct/min*	SI
Anti-γ	100 μg/ml	51,381	52·7	34,507	29·7	704	0·5	1,099	1·1
	10 μg/ml	55,175	56·5	24,217	20·9	916	0·7	1,783	1·1
Anti-μ	100 μg/ml	2,668	2·7	1,007	0·9	15,572	11·1	10,998	6·7
	10 μg/ml	1,114	1·1	764	0·7	2,487	1·8	3,336	2·0
Anti-δ	100 μg/ml	8,039	8·3	1,428	1·2	1,302	0·9	1,947	1·2
	10 μg/ml	1,949	2·0	752	0·6	1,176	0·8	2,143	1·3
Anti-Fab	100 μg/ml	4,348	4·5	1,632	1·4	2,217	1·6	3,341	2·0
	10 μg/ml	2,705	2·8	1,316	1·1	1,155	0·8	1,944	1·2
Medium		975		1,161		1,405		1,631	

* Mean ct/min of triplicate cultures.
SI ct/min with mitogen ÷ ct/min without mitogen.

Table 4. Mitogenic response of T-cell-depleted fraction to various anti-Ig reagents

		HCL Patient 1		HCL Patient 2		Normal 1		Normal 2	
		ct/min*	SI	ct/min*	SI	ct/min*	SI	ct/min*	SI
Anti-γ	100 μg/ml	28,265	122·4	15,508	32·0	2,851	1·7	2,847	1·6
	10 μg/ml	23,243	100·6	9·793	20·2	2,039	1·2	1,877	1·1
Anti-μ	100 μg/ml	929	4·0	631	1·3	17,097	11·0	17,139	9·9
	10 μg/ml	239	1·0	520	1·1	3,171	1·9	3,676	2·1
Anti-δ	100 μg/ml	2,200	9·5	445	0·9	3,155	1·9	2,803	1·6
	10 μg/ml	848	3·7	nd	—	2,309	1·4	2,287	1·3
Anti-Fab	100 μg/ml	4,103	17·8	681	1·4	4,238	2·6	4,683	2·7
	10 μg/ml	1,597	6·9	nd	—	nd	—	2,984	1·7
Medium		231		485		1,635		1,739	

* Mean ct/min of triplicate cultures.
SI ct/min with mitogen ÷ ct/min without mitogen.
nd Not done.

STA or anti-Ig reagents. Using monoclonal antibodies directed against various B cell differentiation antigens, Jansen, Le Bien and Kersey (1982) and Zola *et al.* (1983) have demonstrated that in the majority of HCL cases the neoplastic cells show maturation arrest at a more mature stage compared with the neoplastic cells of B-CLL. Using monoclonal antibodies with variable specificities to hairy cells, Posnett, Chiorazzi and Kunkel (1982) reported that HCL is a neoplasm of activated B cells. Further, Korsmeyer *et al.* (1983) showed that Tac antigen, interleukin 2 receptor (Leonard *et al.*, 1982), was expressed on the neoplastic cells of most HCL cases. More recently, Tac antigen has been demonstrated on activated B cells as well as on activated T cells (Tsudo, Uchiyama & Uchino, 1984). However, we were not able to demonstrate Tac antigen on the neoplastic cells of the two HCL cases that we studied. These two HCL cases had a typical morphological appearance of hairy

cells and typical histological findings of the spleens, as well as marked leucocytosis and hypercellular marrow, as for most cases of HCL reported in Japan (Katayama *et al.*, 1984). The two cases could possibly be included in the atypical European cases reported by Cawley, Burns and Hayhoe (1980).

We previously reported that neoplastic cells from four HCL patients showed a vigorous proliferative response to STA (Tamaki *et al.*, 1985). In the current study, we also demonstrated that neoplastic cells from two out of 19 B-CLL patients also responded to STA similarly with those from two HCL patients. A similar result for the reactivity of B-CLL cells to STA was reported by Romagnani *et al.* (1981). The neoplastic cells from the two HCL patients also responded to anti-Igs as well as to STA. Using anti-μ-chain-conjugated polyacrylamide beads, Anderson *et al.* (1985) recently demonstrated that hairy cells showed little or no response to anti-Ig. In this study, it is certain that neoplastic hairy cells were induced to proliferate by anti-γ, whilst the normal mononuclear cells and T-cell-depleted fraction showed little change. In spite of the cross-linking activity to all s-Ig bearing cells, anti-Igs had no effect of inducing proliferation of B-CLL cells, even those from the two patients that responded to STA. With regard to the reactivity of B-CLL cells to anti-Ig, Fu, Chiorazzi and Kunkel (1979) have reported that these cells are not induced to proliferate by anti-Ig. Although Yoshizaki *et al.* (1982) reported that the neoplastic cells from a B-CLL case were induced to proliferate by anti-Ig in the presence of T cell factor, their case had M-protein in the serum and might not be a typical case of B-CLL. In experiments with murine B cells it is shown that mature B cells respond to anti-Ig whereas immature B cells do not (Sieckmann *et al.*, 1981). Further, it was recently reported that the B cells of mice being maintained in a pathogen-free environment failed to responsd or responded weakly to anti-Ig, whereas those from mice that were being subjected to heavy antigenic stimulation, without any known challenge at the time of the experiment, showed a markedly positive response (Boyd & Metcalf, 1984). Therefore, the difference in reactivity to anti-Ig between HCL cells and B-CLL cells shown in our experiment might come from a variety of maturation stages or be recognized as the original B cell subset between the two neoplastic cells.

In this study, we have shown a discrepancy in the reactivity to STA and anti-Ig of neoplastic cells from two B-CLL patients. It was recently reported that insolubilized anti-Ig induces proliferation of murine immature B cells to a certain degree (Mond *et al.*, 1983). In light of the results obtained with murine experiment, this discrepancy might arise from a difference in the solubility of the cross-linking agents. However, our date, even at a preliminary stage, showed that anti-Ig insolubilized by coupling with Sepharose 4B failed to induce proliferation of the B-CLL cells that responded strongly to STA.

With regard to the normal human B cell subset responding to anti-Ig, Muraguchi *et al.* (1983) used a low concentration of $F(ab')_2$ fraction of anti-μ and BCGF and showed that anti-Ig reacts with resting or small cell fraction of normal B cells which then proliferate in the presence of BCGF. On the other hand, the activated or large cell fraction of B cells proliferated directly by BCGF, which was totally unrelated to anti-μ activation.

In the current study, HCL cells showed a strong proliferative response to anti-γ at both high and low concentrations without T cell factors. This result suggests that these neoplastic cells originate from a B cell subset that is extremely sensitive to the proliferative signal by anti-Ig. The reactivity of the HCL cells from patient 1 to anti-δ could be explained as the possession of a small amount of δ-chain which was undetectable by the direct fluorescent method. Sensitivity to the proliferation signal of anti-Ig and absence of the Tac antigen suggests that, in contrast to the cases reported by Korsmeyer *et al.* (1983), the neoplastic cells from our HCL cases originated from the resting B cell subset. Further, by comparing with the data given by Boyd and Metcalf (1984), it is strongly suggested that the HCL cells employed in this study originated from a subset equivalent to 'memory' B cells, whereas the B-CLL cells originated from a subset equivalent to 'virgin' B cells.

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