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REGULATION OF IL-4 RESPONSIVENESS IN LYMPHOMA B CELLS

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Abstract—The responsiveness to IL-4 with and without costimulation with anti-IgM antibodies or phorbolester was studied in 35 cases of low grade non-Hodgkin lymphoma by analyzing enhancement of CD23 and HLA class II expression. The predominant phenotype responds directly to IL-4. Separate differentiation states can be distinguished according to coordinate or differential upregulation of CD23 and HLA class II molecules by IL-4 alone, and differences in responsiveness to anti-IgM antibodies. A particular subgroup of B-lymphoma cells defines a separate stage of B-cell differentiation. They fail to express high affinity binding sites for IL-4 and accordingly do not respond to IL-4-mediated signals. Cross-linking membrane IgM receptors or direct activation of protein kinase C via phorbolester induces IL-4 receptor expression and subsequent IL-4 reactivity.

Key words: B lymphocytes, CD23, CLL, HLA class II, IL-4, IL-4-receptor, membrane immunoglobulin.

INTRODUCTION

THERE is much evidence that important signals required in B-cell activation involve sIg cross-linking which leads to and is dependent upon subsequent activation of PKC [1]. Another signalling system implicated in early B-cell activation processes, using different, not yet clearly understood second messenger systems, is IL-4 [2–4]. The expression of functional IL-4 receptors without a requirement for prior activation is a hallmark of immunocompetent resting B cells [5, 6]. This finding, in addition to the definition of type 2 T-helper cells which secrete large amounts of IL-4 [7], has been taken as evidence that IL-4 may play an important role in the early events of B-cell activation, and may be even part of the cognate T-helper signal. The sIg as well as the IL-4-induced signal cascade result in activation of genes whose products seem to be involved in regulation of B-cell proliferation [8, 9] or T-cell, B-cell cooperation [10–14]. Both signals lead to upregulation of MHC class II expression on B cells [6, 15]. IL-4 induces enhanced CD23 [5, 16] expression whereas sIg cross-linking fails to do so. However, they are connected in that costimulation with anti-Ig and IL-4 leads to hyperinduction of CD23 expression [5].

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Lymphocytic lymphoma B cells are thought to be in a developmental stage closely related to immunocompetent, resting B cells [17]. Therefore we addressed the question how low grade NHL B cells can respond to the two B-cell activation pathways induced either by cross-linking of the sIg receptor or signals generated by IL-4. This is important for judging how a B-cell tumor which is expanded in most compartments of the immune system may interact with the normal immune cells. Functional distinctions among B-cell tumors may be representative for normal B cells in particular stages of activation or differentiation. Different B-cell tumors can be valuable tools for dissecting the signal cascades required for HLA class II and CD23 expression and the cooperation between IL-4 and sIg mediated signals.

MATERIALS AND METHODS

Reagents. Recombinant human IL-4 was purchased from Genzyme (IC Cemikalien, Munich, F.R.G.). Soluble goat $F(ab')_2$-anti-IgM antibodies were obtained from Jackson Laboratories (Dianova, Hamburg, F.R.G.), phorbolmyristate-acetate (PMA) from Sigma (Deisenhofen, F.R.G.). Monoclonal antibodies, phycoerythrin (PE)- and fluorescein-iso-thyocyanate (FITC)-labeled, were used: Leu12 (CD19), Leu1 (CD5), HLA-DR, HLA-DQ, Leu-M3 (CD14), Leu11c (CD16), Leu19 (CD56) (Becton-Dickinson, Heidelberg, F.R.G.) and IOB8 (CD23) (Immunotech, Dianova, F.R.G.). Purified mAb against IgM,

kappa- and lambda-light chains (Coulter, Krefeld, F.R.G.) were developed with a FITC- labeled $F(ab')_2$-fragment of rabbit anti-mouse IgG (Jackson, Dianova).

Lymphoma cells. Thirty-five patients were diagnosed as having a leukemic B-cell lymphoma of low malignancy, mainly CLL, immunocytoma and centrocytic lymphoma (case 23, 33, 35). Cell surface phenotyping revealed a typical staining pattern [18]: CD19, CD5, HLA class II-positive with low expression of a light chain restricted surface IgM. Only case 22, 23, 31 and 35 stained negative for the CD5 antigen. Even though most cases were also CD23-positive. This antigen showed a considerable variability in staining intensity. The cell size distribution of the lymphoma cells showed most of them only slightly larger than low density peripheral blood cells as judged by forward scatter analysis (Table 1c). No patient received any form of specific therapy.

Cell preparation and culture conditions. Mononuclear cells (PBL) were isolated from heparinized blood (Heparin NOVO, Novo Industry, Mainz, F.R.G.) on Ficoll–Hypaque (Pharmacia, Freiburg, F.R.G.) density gradients. Part of the cells were cryopreserved using a programmable cell freezer (Sylab, Austria). Peripheral lymphoma cells were T-depleted by rosetting with AET-treated sheep erythrocytes or anti-CD2, anti-CD4 and anti-CD8 coupled ox erythrocytes [19]. This depleted also most of NK cells and activated monocytes. FACS analysis revealed ⩾98% CD19-positive cells. Less than 1% stained with either T-cell-specific (CD3), monocyte-specific (CD14) or NK-specific (CD16, CD56) antibodies. Normal control B cells were purified from a spleen of a donor without hematological disease. T cells, NK cells and activated monocytes were depleted by rosetting with anti-CD2, anti-CD4 and anti-CD8 coupled ox erythrocytes. Monocytes were further depleted by avidin-coupled magnetic beads (Dynabeads M-280, Dianova) using biotinylated MY4 (CD14, Coulter) antibodies. This gave 95–98% CD19-positive cells. Purified B cells were cultured at 10^6/ml in RPMI 1640, containing 10% fetal calf serum (Gibco, New York) in 24-well tissue culture plates (Falcon, Heidelberg, F.R.G.).

Surface marker analysis. FACS analysis was done after staining with monoclonal antibodies using a FACscan (Becton-Dickinson, Mountain View, CA, U.S.A.). Ten thousand viable cells were analyzed using forward/sidescatter gating.

Determination of IL-4 receptors. Recombinant IL-4 was radiolabeled using the enzymobead radioiodination reagent (Bio Rad, München, F.R.G.) [20]. Equilibrium binding of ^{125}I-labeled IL-4 (^{125}I-IL-4) to human B-lymphoma cells was done according to Cabrillat [21]: purified lymphoma cells (10^6) were washed and suspended in 200 µl RPMI, containing 2% bovine serum albumin (BSA) and 0.2% sodium azide, with various concentrations of ^{125}I-IL-4 either in the presence or absence of a 6000-fold excess of cold IL-4. After incubation for 2 h at 4°C, cells were washed twice. Cell bound ^{125}I-IL-4 was separated from free ^{125}I-IL-4 by centrifugation through 200 µl silicon oil. The number of binding sites per cell was calculated from the linear portion of Scatchard plots.

RESULTS

Tables 1 a–c give an overview of the studied lymphoma cells. The data are presented as values of mean fluorescence intensity for the respective directly labeled mAb. We think it important to provide the raw data for the different stimuli, since it reveals the heterogeneity in baseline expression of CD23 and HLA class II and more importantly in responsiveness between the different lymphoma B cells.

Responsiveness to sIg and PMA-mediated signals

We analyzed the expression of HLA class II, CD23 molecules and increase in cell size upon stimulation with soluble $F(ab')_2$-anti-IgM antibodies or direct activation of PKC via PMA. A maximum response was usually seen between 10 and 30 µg/ml $F(ab')_2$-anti-IgM and 1–5 ng/ml PMA, after a culture period of 30–40 h (not shown). As judged from the FACS staining profiles, positive responses included usually more than 80% of the cells.

In contrast to normal control splenic B cells only 14 out of 35 lymphoma cases showed a reproducible, significant increase of HLA class II expression in response to soluble $F(ab')_2$-anti-IgM antibodies (Table 1b: cases 22–24, 26–35). The reactivity is not related to the intensity of basal HLA class II expression. An appreciable cell enlargement is only seen in three cases (patients 33–35) upon sIg cross-linking (Table 1c). Apart from these, most of the other studied lymphoma cells increased their cell size upon PMA stimulation but failed to do so with $F(ab')_2$-anti-IgM antibodies (data not shown). However, the majority of cases respond to PMA with an increase in HLA class II expression (Table 1b). Thus the events of the intracellular signalling cascade distal to PKC activation seem to function in most lymphoma cases.

Soluble $F(ab')_2$-anti-IgM antibodies induce no appreciable increase in CD23 expression on normal or on the studied lymphoma cells, whereas PMA is able to induce CD23 on the same cells (Table 1).

Responsiveness to IL-4-mediated signals

Since we could enhance HLA class II and CD23 expression in all studied lymphoma cells (via PMA or anti-Ig), we asked if the IL-4-induced signal cascade is also operating in the lymphoma cells and results in the expression of these particular gene products. Therefore we measured HLA class II and CD23 expression upon stimulation with IL-4.

Tables 1a and b show that 31 out of 35 lymphomas are responsive to IL-4 without prior activation. IL-4 can upregulate the expression of CD23 or HLA class II molecules. In most cases both antigens could be coordinately upregulated similar to normal control B cells (NC, Tables 1a, b). We did not observe any dose or kinetic differences between the lymphomas.

TABLE 1a. REGULATION OF CD23 EXPRESSION (MFI)* ON LEUKEMIC B CELLS

Case	Nil	Anti-IgM†	IL-4‡	Anti-IgM + IL-4	PMA§	PMA + IL-4
1	21	17	143	137	229	552
2	18	15	121	118	n.d.‖	n.d.
3	30	31	105	121	159	232
4	23	24	72	71	n.d.	n.d.
5	28	26	63	n.d.	n.d.	n.d.
6	22	19	59	55	120	234
7	20	18	55	52	66	131
8	10	10	32	29	127	262
9	8	5	27	25	115	284
10	9	9	30	n.d.	n.d.	n.d.
11	5	4	30	35	25	337
12	4	3	21	21	222	676
13	4	5	18	23	10	101
14	6	7	15	15	52	155
15	9	8	21	20	32	94
16	8	6	17	18	32	66
17	11	11	23	24	157	274
18	13	12	25	29	84	158
19	4	n.d.	7	n.d.	60	114
20	12	12	15	15	n.d.	n.d.
21	7	5	8	10	91	235
22	6	6	40	289	40	514
23	5	6	38	93	31	98
24	5	4	23	22	83	410
25	43	40	97	157	n.d.	n.d.
26	8	7	19	40	166	224
27	4	7	18	26	108	316
28	4	4	14	13	47	70
29	7	8	16	50	72	343
30	3	3	5	6	5	58
31	2	2	3	3	4	20
32	5	4	6	7	27	77
33	4	5	6	204	18	178
34	3	4	4	96	17	97
35	8	9	8	567	62	429
NC¶	7	6	34	191	30	649

Purified B cells were cultured at 10^6 cells/ml for 40 h. Expression of CD23 was determined by flow cytometry. The lymphoma cases were grouped for reproducible responsiveness to anti-IgM antibodies. Cases 1–21 show no anti-Ig reactivity, whereas cases 22–35 do respond. Cases 33–35 are separated from the latter group because of lacking IL-4 responsiveness.

* Mean fluorescence intensity, linear scale.
† $F(ab')_2$-anti-IgM 10 μg/ml.
‡ rIL-4 100 U/ml.
§ PMA 3 ng/ml.
‖ Not done.
¶ Purified B cells of a healthy control person.

The maximum effect is reached between 10 and 100 U/ml IL-4 after a 32–40 h culture period (data not shown). We never saw a significant size increase upon IL-4 in normal control B cells or in lymphoma cells (Table 1c).

However there is heterogeneity in the response pattern to IL-4. Most cases upregulate CD23 and HLA class II molecules coordinately, but five out of 35 cases show a dissociation in that they are consistently induced for elevated HLA class II expression, while failing to upregulate CD23 (Table 1: cases 19–21, 30, 31). Three out of 35 lymphomas (cases 33–35) show no response to IL-4 at all, neither for HLA class II nor for CD23 expression, even though both antigens can be upregulated in these cells by $F(ab')_2$-anti-IgM antibodies or PMA, respectively (Tables 1a, b).

Enhancement of IL-4-induced CD23 expression upon costimulation with anti-Ig or PMA

It has been reported that costimulation with insolubilized anti-IgM antibodies and IL-4 leads to

TABLE 1b. REGULATION OF HLA CLASS II EXPRESSION (MFI)* ON LEUKEMIC B CELLS

Case	Nil	Anti-IgM†	IL-4‡	Anti-IgM + IL-4	PMA§	PMA + IL-4
1**	319	324	500	486	730	806
2**	1811	2035	1755	1762	n.d.‖	n.d.
3**	482	504	685	654	602	629
4††	143	146	223	211	n.d.	n.d.
5††	334	365	478	n.d.	n.d.	n.d.
6**	685	626	1382	1553	1963	2350
7††	292	213	523	453	723	679
8††	150	154	193	173	194	153
9††	820	780	1055	1219	1176	1420
10††	576	531	873	n.d.	n.d.	n.d.
11**	1596	1470	1746	1610	2813	n.d.
12**	644	673	1346	1348	1295	1027
13**	1827	1860	1778	1700	1670	1786
14††	271	280	922	1037	764	982
15**	666	706	1071	1058	829	1044
16††	281	231	578	519	567	698
17**	1034	973	1778	1911	2458	2350
18††	791	874	906	1065	965	1186
19**	342	n.d.	637	n.d.	858	843
20**	973	1036	1486	1472	n.d.	n.d.
21††	362	312	710	677	1018	1252
22**	528	1640	1275	1685	1928	1009
23**	538	1446	615	973	1309	1298
24**	1197	1472	2207	2329	2548	2690
25††	644	638	679	730	n.d.	n.d.
26††	2048	2652	3046	2958	2661	2680
27**	1520	2060	2113	2288	2262	3128
28**	720	1241	1124	1134	1208	1176
29**	1370	2329	1554	1778	1395	1358
30**	445	1610	835	1554	1459	1433
31††	813	1091	1395	1472	1843	1778
32††	770	964	851	828	931	1000
33**	1461	2490	1440	2468	1345	n.d.
34**	306	1700	349	1346	757	874
35**	599	1472	610	1234	604	n.d.
NC¶††	1578	2585	2180	2210	2453	2848

Purified B cells were cultured at 10^6 cells/ml for 40 h. Expression of HLA class II was determined by flow cytometry. The lymphoma cases were grouped for reproducible responsiveness to anti-IgM antibodies. Cases 1–21 show no anti-Ig reactivity, whereas cases 22–35 do respond. Cases 33–35 are separated from the latter group because of lacking IL-4 responsiveness.

* Mean fluorescence intensity, linear scale.
† $F(ab')_2$-anti-IgM 10 μg/ml.
‡ rIL-4 100 U/ml.
§ PMA 3 ng/ml.
‖ Not done.
¶ Purified B cells of a healthy control person.
** HLA-DR.
†† HLA-DQ.

markedly enhanced CD23 expression on normal B cells [5]. Table 1a shows that costimulation with soluble $F(ab')_2$-anti-IgM can also enhance IL-4-induced CD23 expression. This enhanced CD23 expression operates in lymphoma cells which also respond to anti-Ig with enhanced HLA class II expression. Therefore the missing signal transmission in anti-Ig non-reactive lymphomas affects also co-stimulation of CD23 expression with IL-4 as well as stimulation of class II expression.

Again, direct stimulation of PKC by PMA caused a potentiating effect on IL-4-induced CD23 expression in all lymphomas, indicating that the signal cascade distal from PKC activation is regularly functioning. For HLA class II expression we did not observe any costimulatory enhancement by anti-Ig and IL-4.

TABLE 1c. REGULATION OF CELL DIAMETER (FSC)* ON LEUKEMIC B CELLS

Case	Nil	Anti-IgM†	IL-4‡	Anti-IgM + IL-4	PMA§	PMA + IL-4
33	84	108	84	n.d.	120	n.d.
34	87	111	90	115	136	123
35	75	100	76	103	143	n.d.
NC‖	78	114	81	117	119	116

Purified B cells were cultured at 10^6 cells/ml for 40 h. Cell size was determined by flow cytometry. Cases 33–35 lack IL-4 responsiveness.

* Forward scatter.
† $F(ab')_2$-anti-IgM 10 μg/ml.
‡ rIL-4 100 U/ml.
§ PMA 3 ng/ml.
‖ Purified B cells of a healthy control person.

TABLE 2. INDUCTION OF HIGH AFFINITY IL-4 RECEPTORS ON B LYMPHOMA CELLS

Case	Conditions	IL-4R/cells
34	Freshly isolated	Not detectable
33	Freshly isolated	Not detectable
33	18 h culture in medium	Not detectable
33	18 h culture with anti-IgM*	180
33	18 h culture with PMA†	190
21	18 h culture in medium	140
21	18 h culture with PMA†	380

Binding experiments were done as shown in Fig. 3. Numbers of IL-4 receptors were calculated from Scatchard plot analysis. Lymphoma cells were depleted of T cells and were 98% CD19-positive.

* $F(ab')_2$-anti-IgM was used at 10 μg/ml.
† PMA at 3 ng/ml.

Induction of IL-4 reactivity in IL-4 non-reactive lymphoma cells

Three out of 35 lymphoma cases (Nos 33–35), which stained negative for CD23, repeatedly failed to respond to IL-4 as judged by enhancement of HLA class II expression (Fig. 1, case 33) or CD23 expression (Table 1a). However, cross-linking sIg via $F(ab')_2$-anti-IgM antibodies induces expression of HLA class II molecules with coordinate upregulation of all three subgroups HLA-DR, -DQ and -DP (Fig. 1). CD23 expression can be induced by PMA. Co-stimulation of these cells with $F(ab')_2$-anti-IgM antibodies and IL-4 result in high expression of CD23 even though both stimuli alone failed to do so (Table 1a).

We then analyzed the time sequence of the two required signals in these lymphoma cells. Figure 2 demonstrates a two stage culture experiment with lymphoma case 33: during the first culture period the cells were cultured either with medium, IL-4, or $F(ab')_2$-anti-Ig antibodies for 18 h. Cells were then recovered, extensively washed and recultured

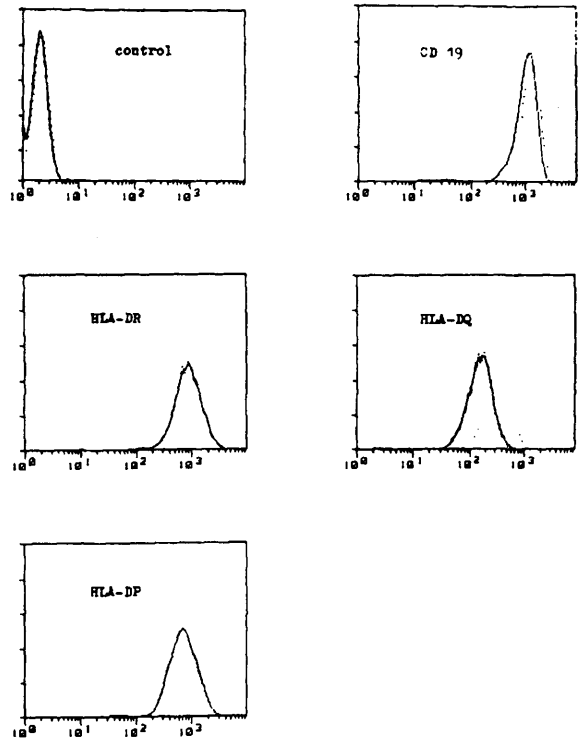

FIG. 1. Purified B lymphoma cells of case No. 33 were cultured at 10^6/ml with either medium (----), soluble $F(ab')_2$-anti-IgM at 10 μg/ml (. . . .) or 100 U/ml IL-4 (. . . . .). After 40 h the expression of HLA-DR, -DQ, -DP and CD19 was determined by flow cytometry.

with and without IL-4 and/or $F(ab')_2$-anti-IgM antibodies. The expression of the CD23 antigen was determined after 7 h and 23 h in the 2nd culture period. The result indicates that prestimulation with $F(ab')_2$-anti-IgM antibodies leads to responsiveness for the IL-4 signal. In the anti-Ig prestimulated cells, more than 80% of the cells show significant CD23 expression upon IL-4. In addition, the IL-4-induced CD23 expression can be detected much earlier on these cells. At 7 h after the IL-4 signal, anti-Ig

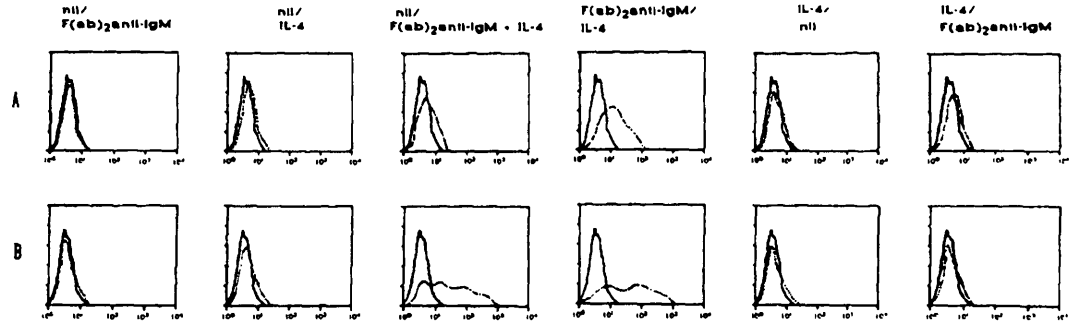

FIG. 2. Purified B cell tumor cells of case No. 33 were cultured in a 1st culture period of 18 h with medium, $F(ab')_2$-anti-IgM antibodies at 10 μg/ml, or IL-4 at 100 U/ml. Subsequently cells were washed three times and recultured with medium, $F(ab')_2$-anti-IgM 10 μg/ml, IL-4 100 U/ml or both. Seven hours (A) and 23 h (B) later the surface expression of CD23 was determined by flow cytometry. The solid line depicts cells cultured and treated in parallel always in culture medium. The dotted line corresponds to cells treated as given above the diagram: 1st culture period/2nd culture period.

prestimulated cells have increased their CD23 expression, while cells which get anti-Ig antibodies and IL-4 together at the beginning of the second culture period show no CD23 expression. After 23 h in the second culture, both groups have reached their maximal stimulation. There is no further CD23 increase up to 40 h in the second stage (data not shown). IL-4 prestimulation followed by $F(ab')_2$-anti-IgM induces no significant CD23 expression. This indicates that case 33 has no baseline IL-4 reactivity, and mIg cross-linking induces IL-4 reactivity in these cells. Cases 34 and 35 show a similar reactivity pattern.

Expression and induction of IL-4 receptors in lymphoma B cells

The lack of IL-4 responsiveness of cases 33–35 may be due to a failure of IL-4 receptor expression or may indicate a requirement for IL-4 receptor activation for signal transmission. We therefore determined the number of IL-4 receptors on the lymphoma cells by binding of radiolabeled IL-4. Figure 3 illustrates typical equilibrium binding data for ^{125}I-IL-4 to a IL-4 responsive lymphoma (case 1, Table 1b). Scatchard plot analysis yielded 750–800 specific binding sites per cell.

Recently it has been shown that insolubilized anti-IgM antibodies upregulate the number of high affinity IL-4 receptors on normal human B cells [22]. Similarly we demonstrate elevated numbers of IL-4 receptors after PMA stimulation. As shown in Table 2, unstimulated cells of B lymphoma case 21 express 190 specific IL-4 binding sites per cell. Upon stimulation

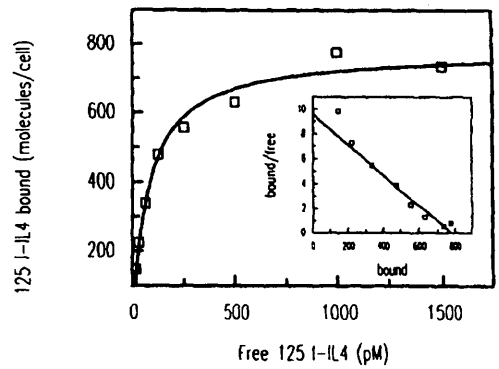

FIG. 3. Equilibrium binding of ^{125}I-IL-4 to purified lymphoma cells of case No. 1 as described in Materials and Methods. The inset illustrates Scatchard plot analysis of specific binding.

with PMA they upregulate their IL-4 receptor number to 380 per cell. This corresponds to the functional data, where these cells respond without prior activation to IL-4 with enhanced HLA class II expression and costimulation with PMA and IL-4 enhances IL-4 reactivity as judged by elevated CD23 expression (Tables 1a, b).

The cells of the IL-4 non-responsive lymphomas cases 33 and 34 repeatedly failed to show specific binding of ^{125}I-IL-4 under comparable conditions (Table 2). However, after stimulation of lymphoma case 33 for 18 h with either soluble $F(ab')_2$-anti-IgM or PMA, we can detect 180 and 190 IL-4 receptors per cell, respectively. This parallels the induction of IL-4 reactivity by these stimuli (Fig. 2). These data indicate that the IL-4 non-responsive lymphoma cells

fail to express IL-4 receptors. Cross-linking sIg with soluble anti-IgM antibodies or direct activation of PKC with PMA induces expression of IL-4 receptors rendering these cells responsive to the IL-4 signal.

DISCUSSION

It has been shown that certain neoplastic B cells or B-cell lines can respond to IL-4. Some CLL B cells upregulate CD23 expression upon stimulation with IL-4 [23–25], and some Burkitt lymphoma cell lines respond to IL-4 with coordinate expression of CD23 and HLA class II molecules [26]. We extend these observations of IL-4 responsiveness by comparing expression of CD23 and HLA class II molecules and determining the influence of sIg or phorbolester-mediated signals upon IL-4 responsiveness and IL-4 receptor expression. These signals and inducible gene products have been shown in a number of systems to be involved in B-cell activation/differentiation [8, 9, 24, 27–30] and T cell–B cell cooperation [10–14].

To avoid phenotypes produced by *in vitro* selection of a cell line, we used only cells freshly isolated from the peripheral blood. The lymphoma cases were not selected for reactivity in our activation system. They were studied in the order of their presentation and were required only to have a peripheral lymphoma cell count of over 10 000 cells/mm^3.

Our results indicate a remarkable heterogeneity in responsiveness to the employed signals which could not be related to the phenotype of the lymphoma cells. The majority of the lymphomas respond to IL-4 by coordinate upregulation of CD23 and HLA class II molecules as has been shown for some Burkitt lymphoma cell lines [26]. Thus, like normal tonsillar [5] or splenic B cells (NC Tables 1a–c), they express functional IL-4 receptors without a requirement for prior activation. The pattern of responsiveness is not dependent from the source of B cells, since we have not observed fundamental differences between B cells isolated from the blood, tonsils or spleen in healthy persons [unpublished].

The upregulation of CD23 can be shown in lymphoma cells which express low amounts of CD23 as well as in cells which already stain for CD23. Accordingly, high affinity receptors can be demonstrated by binding of radiolabeled IL-4. The various lymphoma cells express between 200 and 900 IL-4 receptors per cell (Fig. 3, Table 2 and [T. Lehrnbecher, unpublished data]) which corresponds well to the number of IL-4 receptors on normal, resting B cells [22]. Therefore most neoplastic B cells resemble normal, resting immunocompetent B cells, which may argue that these lymphoma cells represent a closely-related developmental stage.

CD23 expression has been used to define developmental B-cell stages, since it is not expressed on immature B cells and is lost after isotype switching of mature B cells [31–33]. This developmental analysis has been extended by analyzing the dissociation of the IL-4 response as measured by CD23 or HLA class II expression. Waldschmidt and coworkers have shown that immature, murine IgM^+/IgD^+ B cells respond to IL-4 by enhanced HLA class II expression, but fail to upregulate CD23. These authors even suggest that this response pattern would better define the self-renewing "sister-B-cell lineage" [32]. The lack of coordinated expression of CD23 and HLA class II molecules can also be seen in a group of the lymphomas which we studied. For example, cases 19–21, 30, 31 (Tables 1a–c) respond quite well with elevated HLA class II expression in response to IL-4. However, we could not see CD23 induction with IL-4 in these lymphoma cells even though CD23 could be upregulated with PMA. This is independent of the IL-4 dose employed (data not shown). These types of lymphoma cells may allow the dissection of the signal cascades required for MHC class II and CD23 expression.

sIg signalling results at least in part from stimulation of inositol phospholipid breakdown and activation of PKC [34–36]. Therefore phorbolesters can be used to "shortcut" the sIg-mediated signal and substitute for anti-Ig antibodies in certain activation events [37]. Accordingly, PMA did induce HLA class II molecules and acted, similar to soluble $F(ab')_2$-anti-IgM in normal splenic B cells, to enhance responsiveness to IL-4 as indicated by hyperinduction of CD23 in essentially all the lymphomas studied (Tables 1a–c). This is probably due to enhanced IL-4 receptor expression, which we demonstrated in these lymphomas in response to PMA (Table 2).

However, PMA displays an additional signal quality in relation to anti-IgM antibodies since PMA alone leads in normal as well as in the studied lymphoma cells to enhanced CD23 expression whereas soluble $F(ab')_2$-anti-IgM antibodies regularly fail to do so (Tables 1a–c). This is in contrast to a report by Ghaderi *et al.* [38] who find in 8 out of 9 CLL cases a decreased CD23 expression upon TPA stimulation. The difference may be related to the indirect rosetting technique used for measuring CD23 expression.

Three lymphoma cases (Nos 33–35) did not show any reactivity to IL-4. This was not due to a defect in the machinery for the expression of HLA class II or CD23 molecules, since HLA class II expression was inducible by membrane Ig cross-linking and CD23 by PMA (Tables 1a, b). In fact all three major

HLA class II subclasses HLA-DR, -DQ and -DP could be upregulated in these cells by cross-linking sIg (Fig. 1).

Binding studies with iodinated IL-4 failed to demonstrate any specific binding, indicating a lack of IL-4 receptor expression on cells of cases 33 and 34 (Table 2). However, signalling through sIgM by soluble $F(ab')_2$-anti-IgM antibodies induces specific IL-4 binding sites in a frequency usually found on resting normal B cells [22]. Also, direct activation of PKC via PMA resulted in expression of IL-4 receptors, pointing to the central role of PKC in the regulation of IL-4 receptor expression. Parallel to the expression of IL-4 receptors on the cell surface we can demonstrate IL-4 responsiveness by analyzing subsequent CD23 expression. This is also reflected in the kinetic data showing that anti-Ig preactivated B cells increase CD23 expression following addition of IL-4 earlier as compared to B cells receiving the anti-Ig and IL-4 signals at the same time (Fig. 2).

These experiments support the hypothesis that hyperinduction of CD23 upon concomitant anti-Ig and IL-4 signalling is due to enhanced expression of IL-4 receptors following the sIg-mediated signals, rather than receptor activation.

The IL-4 receptor-negative lymphomas showed no distinctive phenotype of surface markers usually used for phenotyping B-cell lymphoma cells. In particular CD5 expression did not distinguish them from the other B-cell lymphomas of low malignancy. However, all stained negative for CD23. They are infrequent in as much as we only found 3 in 35 studied cases. They seem to define a distinct differentiation stage of B cells because they not only lack IL-4 receptors and responsiveness but show distinctive functional behavior. They respond well upon sIg cross-linking with cell size enlargement, HLA class II expression and expression of functional IL-4 receptors, features most low grade lymphomas lack. Future studies will be required to show how they fit into the differentiation scheme of B cells and whether or not they correspond to a distinct, self-renewing B-cell lineage, which has been extensively characterized in the mouse system [39].

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REFERENCES

1. Cambier J. C. & Ransom J. T. (1987) Molecular mechanism of transmembrane signaling in B lymphocytes. *Ann. Rev. Immun.* **5,** 175.
2. Justement L., Chen Z., Harris L., Ranson J., Sandoval V., Smith C., Rennick D., Roehm N. & Cambier J. (1986) BSF1 induces membrane pro tein phosphorylation but not phosphoinositide metabolism, calcium mobilization, protein kinase C translocation, or membrane depolarization in resting murine B lymphocytes. *J. Immun.* **137,** 3664.
3. Mizuguchi J., Beaven M. A., Ohara J. & Paul W. E. (1986) BSF1 action on resting B cells does not require elevation of inositol phospholipid metabolism or increased Ca^{2+}. *J. Immun.* **137,** 2215.
4. Finney M., Guy G. R., Michell R. H., Gordon J., Dugas B., Rigley K. P. & Callard R. E. (1990) Interleukin 4 activates human B lymphocytes via transient inositol lipid hydrolysis and delayed cyclic adenosine monophosphate generation. *Eur. J. Immun.* **20,** 151.
5. Defrance T., Aubry J. P., Rousset F., Vanbervliet B., Bonnefoy J. Y., Arai N., Takebe Y., Yokota T., Lee F., Arai K., De Vries J. & Banchereau J. (1987) Human recombinant interleukin 4 induces Fcε receptors (CD23) on normal human B lymphocytes. *J. expl Med.* **165,** 1459.
6. Noelle R., Krammer P. H., Ohara J., Uhr J. W. & Vitetta E. S. (1984) Increased expression of Ia antigens on resting B cells: an additional role for B-cell growth factor *Prod. natn. Acad. Sci. U.S.A.* **81,** 6149.
7. Mosmann T. R., Cherwinski H., Bond M. W., Giedlin M. A. & Coffman L. (1986) Two types of murine helper clones. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immun.* **136,** 2348.
8. Gordon J., Rowe M., Walker L. & Guy G. (1986) Ligation of the CD23, p45 antigen triggers the cell-cycle progression of activated B lymphocytes. *Eur. J. Immun.* **16,** 1075.
9. Rabin E. M., Mond J. J., Ohara J. J. & Paul W. E. (1986) B cell stimulatory factor-1 prepares resting B cells to enter S phase in response to anti-IgM and lipopolysaccharide. *J. expl Med.* **164,** 517.
10. Matis L. A., Glimcher L. H., Paul W. E. & Schwartz R. H. (1983) Magnitude of response of histocompatibility-restricted T cell clones is a function of product of the concentrations of antigen and Ia molecules. *Proc. natn. Acad. Sci. U.S.A.* **80,** 6019.
11. Sanders V. M., Fernandez-Botran R., Urh J. W. & Vitetta E. S. (1987). Interleukin 4 enhances the ability of antigen-specific B cells to form conjugates with T cells. *J. Immun.* **139,** 2349.
12. Keegan A. D., Snapper C., van Dusen R., Paul W. E. & Conrad D. H. (1989) Superinduction of the murine B cell FcεRII by T helper cell clones: Role of IL-4. *J. Immun.* **142,** 3868.
13. Crow M. K., Kushner B., Jover J. A., Friedman S. M., Mechanic S. E. & Stohl W. (1989) Human peripheral blood T helper cell induced B cell activation results in B cell surface expression of the CD23 antigen. *Cell. Immun.* **121,** 99.
14. Stevens T. L. & Vitetta E. S. (1990) Antigen-specific sIa_{low} B cells do not secrete IgG2a in response to TH1 cells and antigen; responsiveness can be restored by IL-4. *J. Immun.* **144,** 869.
15. Godal T., Davies C., Smeland E. B., Heikkilä R., Funderud S., Steen H. B. & Hildrum K. (1985) Antibodies to surface IgM and IgD increase the expression of various class II antigens on human B cells. *Eur. J. Immun.* **15,** 173.

16. Hudak S. A., Gollnick S. O., Conrad D. H. & Kehry M. R. (1987) Murine B-cell stimulatory factor 1 (IL-4) increases expression of the Fc receptor for IgE on mouse B cells. *Proc. natn. Acad. Sci. U.S.A.* **84,** 4606.
17. Gordon J. (1984) Molecular aspects of immunoglobulin expression by human B cell leukemias and lymphomas. *Adv. Cancer Res.* **41,** 71.
18. Marti G. E. & Fleisher T. A. (1987) B cell CLL immunophenotypes. In *Chronic Lymphocytic Leukemia: Recent Progress and Future Directions* (Gale R. & Rai K., Eds). Alan R. Liss, Inc., New York.
19. Wilhelm M., Pechumer H., Rank G., Kopp E., Riethmüller G. & Rieber E. P. (1986) Direct monoclonal antibody rosetting. An effective method for weak antigen detection and large scale separation of human mononuclear cells. *J. Immun. Meth.* **90,** 89.
20. Park L. S., Friend D., Sassenfeld H. & Urdal D. L. (1987) Characterization of the human B cell stimulatory factor 1 receptor. *J. expl Med.* **166,** 476.
21. Carbillat H., Galizzi J.-P., Djossou O., Arao N., Yokota T., Arai K. & Banchereau J. (1987) High affinity binding of human interleukin 4 to cell lines. *Biochem. biophys. Res. Comm.* **149,** 995.
22. Zuber C., Galizzi J.-P., Valle A., Harada N., Howard M. & Banchereau J. (1990) Interleukin 4 receptors on normal human B lymphocytes: characterization and regulation. *Eur. J. Immun.* **20,** 551.
23. Ishida H., Kumagai S., Iwai K., Takahashi T., Kawabe T., Yodoi J., Arai N., Namiuchi S., Konaka Y. & Imura H. (1989) Heterogeneity in terms of interleukin 4 dependent regulation of Fcε receptor/CD23 expression on chronic B-lymphocytic leukemia cells. *Immun. Letts* **20,** 323.
24. Shields J. G., Armitage R. J., Jamieson B. N., Beverley P. C. L. & Callard R. E. (1989) Increased expression of surface IgM but not IgD or IgG on human B cells in response to IL-4. *Immunology* **66,** 224.
25. Hivroz C., Valle A., Brouet J. C., Banchereau J., Grillot & Courvalin C. (1989) Regulation by interleukin 2 of CD23 expression of leukemic and normal B cells: comparison with interleukin 4. *Eur. J. Immun.* **19,** 1025.
26. Rousset F., De Waal R., Slierendregt B., Aubry J.-P., Bonnefoy J.-Y., Defrance T., Banchereau J. & De Vries J. E. (1988) Regulation of Fc receptor for IgE (CD23) and class II MHC antigen expression on Burkitt's lymphoma cell lines by human IL-4 and IFN-γ. *J. Immun.* **140,** 2625.
27. Cambier J. C. & Lehmann K. R. (1989) Ia-mediated signal transduction leads to proliferation of primed B lymphocytes. *J. expl Med.* **170,** 877.
28. Gordon J., Cairns J. A., Millsun M. J., Gillis S. & Guy G. R., Interleukin 4 and soluble CD23 as progression factors for human B lymphocytes: analysis of their interactions with agonists of the phosphoinositide "dual pathway" of signalling. *Eur. J. Immun.* **18,** 1561.
29. Gordon J., Millsun M. J., Flores-Romo L. & Gillis S. (1989) Regulation of resting and cycling human B lymphocytes via surface IgM and the accessory molecules interleukin 4. CD23 and CD40. *Immunology* **68,** 526.
30. Oliver K., Noelle R. J., Uhr J. W., Kramer P. H. & Vitetta E. S. (1985) B-cell growth factor-1 is a differentiation factor for resting B cells and may not induce cell growth. *Proc. natn. Acad. Sci. U.S.A.* **82,** 2465.
31. Kikutani H., Suemura M., Owaki H., Nakamura H., Sato R., Yamasaki K., Barsumian E., Hardy R. & Kishimoto T. (1986) Fcε receptor, a specific differentiation marker transiently expressed on mature B cells before isotype switching. *J. expl Med.* **164,** 1455.
32. Waldschmidt T. J., Conrad D. & Lynch R. (1989) Expression of B cell surface receptors. II. IL-4 can accelerate the developmental expression of the murine B cell IgE Fc receptor. *J. Immun.* **143,** 2820.
33. Waldschmidt T. J., Conrad D. H. & Lynch R. G. (1988) The expression of B cell surface receptors. I. The ontogeny and distribution of murine B cell IgE Fc receptor. *J. Immun.* **140,** 2148.
34. Coggeshall K. M. & Cambier J. C. (1984) B cell activation. VIII: Membrane immunoglobulins transduce signals via activation of phosphatydilinositol hydrolysis. *J. Immun.* **133,** 3827.
35. Bijsterbosch M. K., Meade C. J., Turner G. A. & Klaus G. G. B. (1985) B-lymphocyte receptors and polyphosphoinositide degradation. *Cell* **41,** 999.
36. Nishizuka Y. (1984) The role of protein kinase C in cell surface signal transduction and tumor promotion. *Nature* **308,** 693.
37. Monroe J. G., Niedel J. E. & Cambier J. C. (1984) B cell activation. IV: Induction of cell membrane depolarization and hyper-I-A expression by phorbol diesters suggests a role for protein kinase C in murine B lymphocyte activation. *J. Immun.* **132,** 1472.
38. Ghaderi A., Richardson P., Cardona C., Millsum M. J., Ling N., Gillis S., Ledbetter J. & Gordon J. (1988) Stimulation of B-chronic lymphocytic leukemia populations by recombinant interleukin 4 and other defined growth promoting agents. *Leukemia* **2,** 165.
39. Herzenberg L. A., Stall A. M., Lalor P. A., Sidman C., Moore W. A., Parks D. R. & Herzenberg L. A. (1986) The Ly-1 B cell lineage. *Immun. Rev.* **93,** 81.