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EXPRESSION OF V_HIII-ASSOCIATED CROSS-REACTIVE IDIOTYPE ON HUMAN B LYMPHOCYTES

Association with Staphylococcal Protein A Binding and *Staphylococcus aureus* Cowan I Stimulation[1]

FAZEL SHOKRI, RIZGAR A. MAGEED, B. ROBERT MAZIAK, AND ROY JEFFERIS[2]

From the Department of Immunology, The Medical School, University of Birmingham, Birmingham, United Kingdom

It has been demonstrated that staphylococcal protein A (SPA) has an "alternative" binding site with specificity for human Ig H chain V region of the V_HIII subgroup. Because the major mitogenic component of *Staphylococcus aureus* Cowan I (SAC) is SPA, it is possible that SAC stimulates a subpopulation of B cells expressing Ig of the V_HIII H chain subgroup. In the present study, we have investigated further the relationship between SPA binding and the expression of V_HI- or V_HIII-associated cross-reactive idiotype (CRI) on the surface of tonsillar B lymphocytes enriched for the expression or nonexpression of the CRI, and we examined the Ig secreted by cell lines established from these populations of B cells by EBV transformation. The V_HIII CRI (D12)-enriched population yielded 21 cell lines, with 67% of them secreting SPA-reactive Ig; in contrast, only 6% (1 of 16) of V_HI CRI-expressing lines secreted SPA-reactive Ig. The CRI-negative B cell population yielded 54 cell lines, of which 20% secreted SPA-reactive Ig, as might be anticipated because a majority of V_HIII Ig^+ B cells will be CRI^-. SAC stimulation of CRI^+ and CRI^- populations showed preferential stimulation of the D12 population. These data support the proposal that SAC stimulation of human B cells is mediated through binding of SPA by its alternative binding site to Ig V regions of the V_HIII subgroup.

The interaction between SPA[3] and Ig has been extensively studied over the last four decades (1, 2). Initial studies demonstrated that IgG from most vertebrate species binds to SPA (3, 4). Structural, serologic, and gene sequencing studies have localized the IgG binding site of SPA to five repetitive and highly homologous domains, each consisting of 58 amino acids consecutively arranged from the amino-terminal region of the SPA molecule (5, 6). The interaction of SPA with Ig was initially thought to be restricted to the Fc region of IgG (1); however, subsequent studies revealed that SPA can also bind to human Ig of all major isotypes tested (7, 8). This binding site is distinct from the Fcγ site, because the binding cannot be inhibited by Fcγ fragments. The binding site(s) was localized to the $F(ab)_2$ fragments and, thus, termed the "alternative binding site," to indicate a distinction from the "classical Fcγ binding site." The alternative binding site allows immobilized SPA to function as a highly efficient mitogen for human B lymphocytes, through cross-linking of surface Ig (9, 10), and as a tool for the purification of some human IgM proteins (11, 12).

The observations that SPA binds only to a proportion of polyclonal and monoclonal human IgM (11–13) and a fraction of peripheral blood B cells from normal individuals and patients with CLL (14, 15) led to the assumption that particular residues within the variable domains of Ig are recognized by the alternative binding site (8). It was later demonstrated by Vidal and Conde (16) that the reactivity of Fab fragments to SPA was retained in some of the subfragments, such as Fv and the V_H domain, but not in the isolated L chain or the V_L domain. This suggests that the V_H region of Ig is involved in the alternative SPA binding site and that the L chain does not influence V_H recognition. Recent studies by Sasso et al. (13) established a clear association between the SPA alternative binding site and H chains from the V_HIII subgroup. Our studies of B cell stimulation with SAC revealed a preferential stimulation of B cells expressing rheumatoid factor-associated CRI from the V_HIII subgroup of H chains, compared with those from the V_HI subgroup (F. Shokri, R. A. Mageed, and R. Jefferis, manuscript in preparation). The expression of these rheumatoid factor-associated CRI is evidence of a restricted usage of germline genes, or minimally mutated germline genes, by clonally related B cells (17, 18). To further delineate the reactivity of SPA with B cells expressing different V_H regions, we have prepared B cell populations enriched for V_HI- and V_HIII-associated CRI, established EBV-transformed cell lines, and analyzed their Ig products for coincident expression of CRI and SPA binding activity. Our results indicate that the B cell population responsive to SAC expresses Ig using V regions of the V_HIII family and that anti-CRI antibodies allow further dissection of this B cell subpopulation.

MATERIALS AND METHODS

Murine mAb. The production and characterization of mAb G6 and G8 (V_HI-associated CRI), B6 and D12 (V_HIII-associated CRI), and C7

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[2] Please address all correspondence and requests for reprints to Roy Jefferis, Department of Immunology, The Medical School, University of Birmingham, Birmingham B15 2TT, UK.
[3] Abbreviations used in this paper: SPA, staphylococcal protein A; CLL, chronic lymphocytic leukemia; CRI, cross-reactive idiotype; SAC, *Staphylococcus aureus* Cowan I.

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($V_{K}III$ subgroup specific) have been described in detail elsewhere (19–22). mAb C6 has specificity for an epitope expressed on all $V_{K}IIIb$ L chains tested and also reacts with some proteins of the $V_{K}II$ subgroup; it was produced to the IgMRF paraprotein Ko, known to express a $V_{K}IIIb$ L chain (R. A. Mageed, unpublished observations). mAb specific for human IgM, IgG, IgA, κ and λ L chains, and the CD19 Ag on B cells (AF6 and BU1, 8a4, 2D7, 6e1, C4, and BU-12, respectively) were produced in the Department of Immunology, University of Birmingham (Birmingham, UK) (commercially available from Unipath-Oxoid, Bedford, UK). The anti-CD3 reagent (OKT3) was produced in the department from hybridoma cells obtained from the American Type Culture Collection; anti-CD14 antibody CRIS-6 was kindly provided by Dr. R. Vilella (Servei d'Immunologia, Hospital Clinic, Barcelona, Spain).

Antibodies were purified from ascitic fluids by ion exchange chromatography on DEAE-cellulose or affinity chromatography on protein A-Sepharose 4B, as previously described (23). All mAb are of the IgG1 isotype, except OKT3 and BU1, which are of the IgG2a isotype. $F(ab')_2$ fragments of the anti-CRI antibodies were prepared by pepsin digestion of the purified proteins in 0.1 M sodium acetate buffer, pH 4.0, with an enzyme to substrate ratio of 1:40 (w/w), at 37°C for 8 h. Undigested IgG was removed on a protein A-Sepharose 4B column. Purity of the $F(ab')_2$ preparation was confirmed by SDS-PAGE on a 10% gel.

Mononuclear cell preparation and enrichment for CRI-expressing B cells. Cells from an individual tonsil, obtained following tonsillectomy, were teased from the tissue, collected, washed, resuspended in RPMI 1640 medium (GIBCO, Paisley, Scotland), and overlaid on an equal volume of Ficoll-Paque (Pharmacia Fine Chemicals, Central Milton Keynes). Following centrifugation at 350 × *g* for 20 min at room temperature, mononuclear cells were collected from the interface, washed two times with RPMI medium, and resuspended in culture medium containing RPMI 1640 with L-glutamine (GIBCO), 10% heat-inactivated FCS (GIBCO), penicillin (200 IU/ml), streptomycin (100 μg/ml), and 2-ME (5×10^{-4} M).

Monocytes were removed by incubation of the mononuclear cells in a plastic tissue culture flask at 37°C for 1 h and harvesting of nonadherent cells by careful decantation. T cells were removed by rosette formation with 2-aminoethyl isothiouronium bromide-treated SRBC at 4°C for 2 h, followed by centrifugation over Ficoll-Paque. Cells at the interface were collected as purified B cells.

B cells enriched for expression of the G8 CRI were prepared after incubation of purified B cells (10^7 cells/ml) with G8-sensitized SRBC (0.5% suspension), at 4°C overnight, and centrifugation on Ficoll-Paque. Rosetted cells (G8-enriched population) were collected and treated with sterile distilled water for 45 s to lyse the SRBC. The cells were immediately washed three times with RPMI and resuspended in tissue culture medium. This procedure was repeated for D12-sensitized SRBC to prepare a D12-enriched population of B cells. Nonrosetted cells were collected as the G8/D12-depleted population. The viability of the positively and negatively selected populations was >95%, as determined by dye exclusion using trypan blue (0.5%).

Proliferation assay. B cells were incubated, in triplicate, at 10^5 cells/well in flat-bottomed 96-well tissue culture plates (Flow Laboratories, UK), in the presence or absence of the antibody BU1 (10 μg/ml), for 8 h, followed by a 72-h incubation with or without mitogen at 37°C, in an atmosphere of 5% CO_2 in a humidified incubator; SAC mitogen (Sigma, Poole, UK) was used as a 0.01% suspension and PMA (Sigma) at 5 ng/ml. All wells were pulsed for 18 h with [*methyl*-^{3}H]thymidine (0.5 μCi/well) (TRK 120; Amersham International, Amersham, UK). Cells were harvested onto glass fiber filters (Titertek; Flow), using a semiautomatic harvester (Skatron, Lierbyen, Norway). [^{3}H]Thymidine incorporation was determined by liquid scintillation counting (LKB, Stockholm).

EBV transformation and cloning of B cells. B cells of each positively or negatively selected population were resuspended in 10 times concentrated supernatant produced by EBV-infected B-95-8 marmoset cells (kindly provided by Drs. C. Gregory and M. Rowe, Department of Cancer Studies, University of Birmingham). After a 1-h incubation at 37°C, with periodic agitation, the cells were washed with RPMI and resuspended in culture medium supplemented with 20% heat-inactivated FCS and antibiotics. Cells were then seeded, at 100 and 200 cells/well, into 96-well tissue culture plates containing human fetal fibroblasts as feeder layer (provided by Drs. Gregory and Rowe). Culture supernatants from growing cell lines were tested for the expression of L and H chain isotypes and CRI. An estimate of the frequency of transformation was calculated from the equation:

$$\frac{\text{Number of growing lines}}{\text{Total number of cells seeded}} \times 100$$

Reverse passive hemagglutination. SRBC were sensitized with purified antibody, using chromium chloride, as previously described (24). The culture supernatant being tested (30 μl) was serially diluted in U-bottomed microtiter plates containing HEPES-buffered RPMI supplemented with 2% heat-inactivated FCS; sensitized SRBC (30 μl of a 0.35% suspension) were added and end points were read after a 2-h incubation at room temperature. Reactivity with SPA was similarly determined, using SPA-sensitized SRBC.

Inhibition of SPA binding by $F(ab')_2$ fragments of anti-CRI. Cell supernatants, diluted to a concentration twice the minimum hemagglutinating dose, were incubated with serial dilutions of $F(ab')_2$ fragments of B6 or D12, from a starting concentration of 50 μg/ml, at 37°C for 90 min. SPA-sensitized SRBC (30 μl of a 0.35% suspension) were added, and results were recorded following incubation at room temperature for 2 h. $F(ab')_2$ fragments of an antibody (G4) of irrelevant specificity were used as control.

Statistical analysis. Comparisons were made using the χ^2 test, and differences were considered significant when the *p* values were <0.05.

RESULTS

Positive selection of V_HI- and V_HIII-expressing tonsillar B cells. T cell and monocyte-depleted mononuclear cells were incubated successively with G8- and D12-coated SRBC, and rosetted cells were collected to yield G8 (V_HI)- and D12 (V_HIII)-enriched B cells, respectively. A purified mAb of irrelevant specificity (G4) was employed as a control to account for nonspecific rosette formation (Table I). The uncorrected values are given in Table I and the corrected values are quoted in the text. The unfractionated cells contained 6.5% and 13% G8$^+$ and D12$^+$ cells, respectively. The G8-enriched population represented a yield of 47% of the G8$^+$ B cells present in unfractionated cells; 37.5% were G8$^+$, whereas only 4.5% were D12$^+$. Similarly, the D12-enriched population represented a yield of 56%, with 45% of cells being D12$^+$, whereas 2% were G8$^+$. The third population of cells were essentially G8$^-$ and D12$^-$. No significant differences in the proportions of $V_{K}III$ or $V_{K}IIIb$ cells within each B cell population were observed.

EBV transformation and cloning of transformed B cells. Each of the three cell populations were infected with EBV and cultured in 96-well tissue culture plates, at 100 and 200 cells/well. A total of 138 growing cell lines were obtained after 3 weeks of culture, of which 124 produced Ig that appeared monoclonal by the criteria of several serologic markers expressed (see *Discussion*). The G8$^+$ population yielded 31 cell lines, of which 12 (39%) were G8$^+$ (Table II); similarly, the D12$^+$ population gave 40 lines, with 19 (47%) being D12$^+$, and the G8$^-$,D12$^-$ population gave 53 lines, with 3 (6%) being G8$^+$ and 1 (2%) being D12$^+$. Five (42%) of the G8$^+$ cell lines coexpressed the V_HI-associated CRI G6, whereas none were G6$^+$,G8$^-$. Similarly, 68% of the D12$^+$ lines coexpressed the V_HIII-associated CRI B6, and none were B6$^+$,D12$^-$. The proportion of κ- and λ-expressing cell lines obtained from each B cell population is essentially similar. The frequency of transformation for the G8$^+$, D12$^+$, and G8$^-$,D12$^-$ cell populations was 0.06%, 0.07%, and 0.11%, respectively.

Reactivity of Ig produced by B cell lines with SPA. Culture supernatants of cell lines established from the three B cell populations were tested for reactivity with SPA (Table III), and the results were subjected to statistical analysis. SPA was shown to be associated with the expression of the D12 CRI, and significant difference values of $p = 0.0002$ ($\chi^2 = 13.751$) and $p = 0.0001$ ($\chi^2 = 14.583$) were obtained for comparison of D12$^+$ with G8$^+$ or G8$^-$,D12$^-$ supernatants, respectively. No significant

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TABLE I

Phenotype of human tonsillar B lymphocyte populations separated on the basis of expression or nonexpression of H chain V region-associated CRI

Cell Population	Expression (%)[a]													
	IgM	κ	V_κIII	V_κIIIb	G6	G8	B6	D12	G4	IgG	SPA	CD3	CD14	CD19
G8$^+$	73	63	12.0	3.8	24	38	NT[b]	5	0.5	14	15	1	NT	88
D12$^+$	78	61	7.5	3.0	NT	2.0	25	45	0	19	39	0.5	NT	88
G8$^-$,D12$^-$	83	65	11.5	6.0	0.3	1.5	0.3	0	0.5	NT	NT	1	NT	88
Unfractionated (T-depleted)	75	66	12.0	3.5	4.5	7.5	8.0	14	1.0	NT	NT	2	1.3	81

[a] Data refer to percentage of B lymphocytes in each isolated cell population or in the unfractionated population forming rosettes with appropriately sensitized SRBC.
[b] NT, not tested.

TABLE II

Isotype and CRI profile of Ig secreted by cell lines established following EBV transformation of B lymphocyte populations reported in table I

Enriched Cell Population	No. of lines[a]												
	Total	IgM	IgG	IgA	λ	κ	V_κIII	V_κIIIb	G6	G8	B6	D12	SPA
G8$^+$	31	27	3	1	14	17	7	7	5	12	1	1	5
D12$^+$	40	37	3	0	18	22	11	10	NT[b]	1	13	19	17
G8$^-$,D12$^-$	53	46	6	1	23	30	5	5	NT	3	NT	1	NT

[a] The presence of Ig expressing the isotype or CRI was determined in a reverse passive hemagglutination assay, as described in *Materials and Methods*.
[b] NT, not tested.

TABLE III

SPA reactivity of Ig secreted by G8$^+$, D12$^+$, and G8$^-$,D12$^-$ cell lines

Cell Line	No. of lines[a]								
	Total	κ	λ	V_κIII	G6	B6	SPA	IgG	IgA
G8$^+$	16	12	4	4	5	0	1	0	0
D12$^+$	21	12	9	7	0	14	14	1	0
G8$^-$,D12$^-$	54	26	28	7	0	0	11	0	3

[a] The presence of Ig expressing the isotype, CRI, or SPA binding activity was determined by reverse passive hemagglutination.

TABLE IV

Inhibition of SPA binding to secreted Ig by anti-CRI antibodies B6 and D12

Sample	Minimum concentration of inhibitors required for complete inhibition (μg/ml)		
	B6	D12	G4
L3	25	3.1	NI[a]
L24	<0.1	0.2	NI
L27	6.25	25	NI
L37	25	1.6	NI

[a] NI, no inhibition was observed for G4 antibody at 50 μg/ml.

difference was observed when SPA binding of G8$^+$ and G8$^-$,D12$^-$ supernatants was compared (p = 0.118, χ^2 = 1.733). Of the 14 D12$^+$ cell lines also positive for B6, 13 bound SPA, giving a highly significant p value of $\ll$0.0001 (χ^2 = 22.502) for comparison with the G8$^+$ cell lines. Because six of the seven D12$^+$ cell lines not expressing the B6 CRI did not bind SPA, the data suggest that B6 expression is more directly associated with SPA binding than D12 expression.

Spatial relationship between the SPA binding site and CRI. F(ab')$_2$ fragments of B6 and D12 were evaluated for their ability to inhibit the binding of SPA to the Ig product of four of the D12$^+$,B6$^+$ cell lines. Inhibition was observed in each case (Table IV); however, the concentration of Ig giving complete inhibition varied for each cell line. No inhibition was obtained with the F(ab')$_2$ fragments of G4 antibody.

Proliferative response of G8- and D12-enriched B cells to SAC and PMA. The proliferative response of the D12-enriched population to SAC was more than 3-fold higher than that of the G8$^+$ population, whereas the proliferative response to PMA showed only a 0.5-fold increase (Fig. 1). The response of the G8$^-$,D12$^-$ population to SAC was higher than for the G8$^+$ but lower than for the D12$^+$ B cell populations. However, the response of the latter two populations to PMA was higher than that of the G8$^-$,D12$^-$ population. When all the populations of B cells were first confronted with anti-IgM antibody (BU1) and then stimulated with PMA, essentially equivalent responses were observed (Fig. 2).

DISCUSSION

We have recently demonstrated that supernatants from human B cells stimulated with SAC are relatively enriched for Ig expressing V_HIII-associated CRI, whereas Ig expressing V_HI-associated CRI were not detectable (F. Shokri, manuscript in preparation). Because SPA is considered to be the major B cell-stimulatory component of SAC (25, 26), it is possible that SAC may preferentially stimulate B cells expressing the V_HIII subgroup of H chain. To test this hypothesis, we have established cell lines from tonsillar B cells enriched for the expression or nonexpression of V_HI (G8)- and V_HIII (D12)-associated CRI. The Ig products of these lines have been studied for the frequency and coincident expression of CRI and reactivity with SPA. Of 768 wells seeded at 100 cells/well, 77 were positive for cell growth, in agreement with a frequency of approximately 0.1%. An additional 61 positive

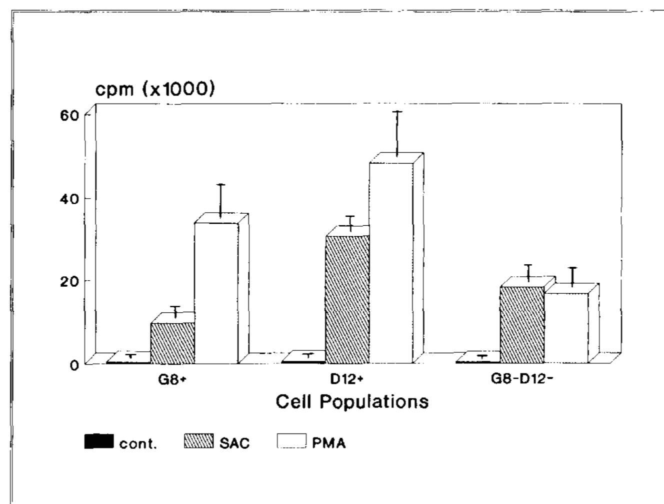

Figure 1. Proliferative response of positively and negatively selected human tonsillar B lymphocytes to SAC and PMA. *cont.*, control.

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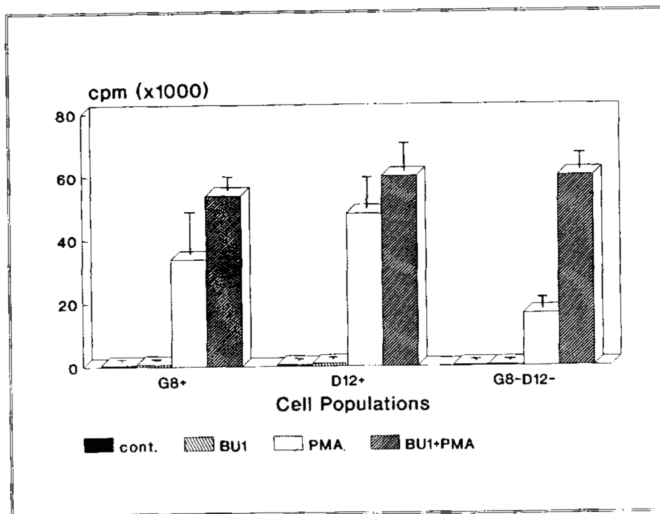

Figure 2. Proliferative response of positively and negatively selected human tonsillar B lymphocytes to a combination of anti-IgM antibody and PMA. *cont.*, control.

wells resulted from a total of 384 wells seeded at 200 cells/well. Of the total of 138 positive wells, 124 appeared to be monoclonal by the criteria of expression of a single H and L chain type, the nonoverlapping expression of the V_HI- and V_HIII-associated CRI G8 and D12, and a distinct reactivity pattern with SPA (nonexpression of the $G8^+$ CRI on SPA-reactive Ig products and expression of the D12 CRI on a large proportion of the SPA-reactive Ig). When the Ig produced by the established cell lines were tested for reactivity with SPA, a very clear and direct correlation was observed for expression of the D12 CRI and, more particularly, the B6 CRI. Thus, of 21 $D12^+$ cell lines, the Ig of 14 reacted with SPA, and all these lines, except one, coexpressed the B6 CRI. Of the 16 $G8^+$ lines, only one reacted with SPA (Table III). The negatively selected $G8^-$,$D12^-$ population gave rise to 11 (20%) lines secreting SPA-reactive Ig. These data can be rationalized with the demonstration of an alternative SPA binding site being associated with the structure of V_HIII subgroup V regions and our demonstration of the association of the D12 and B6 CRI with the V_HIII subgroup of H chains. Obviously, the concordance between SPA binding and CRI expression is not absolute, but this finding is readily rationalized.

Analysis of the frequency of occurrence of V_HIII paraproteins in multiple myeloma and hybridization experiments aimed at counting Ig V region genes indicates that V_HIII comprises the major family of Ig V_H genes. Each gene may be expected to encode for a different protein sequence, and not all of these gene products may bear the alternative site for SPA binding. Similarly, only a proportion of the genes will encode for products that express the CRI studied; thus, representation within a panel of paraproteins expressing the V_HIII subgroup of H chains was 35% for B6 (27) and D12 (R. A. Mageed, unpublished observations). Our data suggest that the SPA binding site can be expressed independently from the D12 or B6 CRI; however, there appears to be a concordance of D12 and, in particular, B6 expression and SPA binding. This interpretation has not considered the possible influence of the D and/or J region gene that may be expressed together with a given V_H gene segment. Neither does it speculate on the effect of somatic mutation on the expression of these reactivities, because a majority of the cell lines secreted IgM, suggesting the Ig to be more likely the product of primary B cells.

Localization of the SPA binding site to the V region of the Ig in spatial proximity to the B6 and D12 CRI was suggested by binding inhibition experiments. The IgM product of each of four $D12^+$,$B6^+$ cell lines was incubated with the $F(ab')_2$ fragment of B6 or D12 before the addition of SPA-sensitized SRBC, and in each case inhibition of agglutination was observed, although to variable extents in different lines. These findings support the view that SAC stimulation of human B cells is primarily mediated by cross-linking of IgR by SPA present on the surface of SAC. It follows, therefore, that we should expect SAC to preferentially stimulate B cells expressing Ig from the V_HIII subgroup of H chains. Evidence in support of this interpretation was obtained from SAC stimulation of the separated lymphocyte populations. The proliferative response of the D12-enriched population was 3-fold greater than that of the G8-enriched population, as might be expected for enrichment with V_HIII^+ lymphocytes. The response of the $G8^-$,$D12^-$ population was also higher than that of the $G8^+$ population, as expected because it contains proportionally more V_HIII-expressing cells that do not display the D12 CRI. Alternatively, the presence of B cells expressing other V_H subgroups reactive with SPA may partially account for this finding.

The response to PMA, however, differed from that to SAC, in that both $G8^+$ and $D12^+$ B cell populations responded similarly better than the negatively selected $G8^-$,$D12^-$ cell population. It is possible that positive selection by rosetting with antibody-coated SRBC might have influenced the proliferative response of the positively selected cell populations to PMA. Therefore, the influence of cross-linking of Ig receptors was investigated for all populations of B cells by first incubating with an anti-IgM antibody (BU1) and then stimulating with PMA. The proliferative response was found to be similar for all cell populations (Fig. 2). This indicates that the differences observed in the responses of the positively and negatively selected B cells to PMA are most likely due to selection of the CRI-expressing B cells through their Ig receptors. Cross-linking of IgR has been shown to increase the proliferative response of B cells stimulated with PMA (28, 29). This study provides evidence that SAC may selectively stimulate a subpopulation of B cells, namely those bearing Ig from the V_HIII subgroup of H chains. This provides a rational explanation for our earlier finding that Ig secreted after SAC stimulation contains a relatively high proportion of Ig bearing V_HIII-associated CRI. It is of interest to note that it has previously been reported that CLL cells that bear SPA-reactive IgM responded vigorously to SAC stimulation (14). We have confirmed this observation and extended it to show that the majority of such responder CLL cells express the V_HIII-associated CRI, rather than the V_HI-associated CRI (F. Shokri, P. A. Mageed, and R. Jefferis, unpublished observation). Our findings suggest that anti-CRI reagents can be employed to select subpopulations of B cells for functional studies.

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