Immunology 1992 **76** 535–540

Relative expression of surface IgM, IgD and the Ig-associating α(mb-1) and β(B-29) polypeptide chains

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Accepted for publication 26 March 1992

SUMMARY

Membrane immunoglobulins are associated with a transmembrane disulphide-linked heterodimer composed of an α-chain (mb-1) and a β-chain (B-29). The relative surface expression of all of the polypeptide chains comprising the Ig-αβ complex has been investigated using surface labelling co-precipitation analysis and two-colour flow cytometric analysis. The main conclusions are that mb-1 and B-29 are B-cell surface markers on immature and mature B cells, and that all components of the surface Ig-αβ complex are expressed in stoichiometrically equivalent amounts. Thus the complex was quantitatively precipitated from digitonin lysates of ^{125}I-surface-labelled cells with anti-B-29, anti-mb-1 or anti-Ig. Secondly, by two-colour FACS analysis there was a proportionality between the relative amounts of cell surface mb-1 or B-29 and surface IgM or IgD, but not other B-cell markers (class II, B220, FcRγ, FcRε). Finally there was an insignificant number of B cells expressing membrane Ig without α- and β-chains, and vice versa. Thus there appears to be a closely controlled relative synthesis and surface expression of all components of the B-cell receptor complex.

INTRODUCTION

Antigen-specific receptors of both B[1,2] and T[3] lymphocytes are associated with transmembrane molecules thought to be involved in the process of signal transduction. The Ig-associated molecules are composed of two disulphide linked components, the α-chain, a product of the mb-1 gene[4,5] and the β-chain, coded for by the B-29 gene.[6,7] Despite some original confusion, possibly due to technical variables,[8–10] this basic structure may associate with all major Ig classes, although there is a level of heterogeneity imposed by variable glycosylation of the α-chain.[11] Some, but not all, Ig classes have an absolute requirement for association with the αβ complex in order to be transported to the cell surface.[11] When α- and β-chains are available, however, they will associate with all membrane located immunoglobulin classes. An important issue is the quantitative relationship, or stoichiometry, of the associating subunits of the surface Ig-αβ complex, and this forms the basis of the work to be presented.

MATERIALS AND METHODS

Cell suspensions

Spleen and bone marrow cell suspensions were prepared from the Institute's colony of specific pathogen-free CBA × BALB/c female mice, 3–6 months old. The suspension was depleted of T cells by treatment with rat monoclonal antibody (mAb) anti-mouse Thy-1 (NIM-R1)[12] and red cells by ammonium chloride treatment. The small dense resting B cells were then recovered from the 85–75% interphase of Percoll gradients.[13]

Purified B cells were activated *in vitro* with lipopolysaccharide (LPS) (50 μg/ml) in medium RPMI-1640 supplemented with 5% (v/v) foetal calf serum, non-essential amino acids, glutamine, penicillin, streptomycin and 5×10^{-5} M mercaptoethanol.

Polyclonal antibodies

Rabbit and goat anti-mouse Ig and affinity-purified rabbit and goat antibodies to mouse κ-chains, μ-chains and δ-chains were prepared as described elsewhere.[14] In brief, rabbits and goats were immunized with purified IgM (κ) (TEPC 183) for the preparation of anti-κ-chains and anti-μ-chains and with purified IgD (κ) (TEPC 1017) for the preparation of anti-δ-chains. The serum from animals immunized with IgM (κ) TEPC 183 was passed sequentially over columns of IgG2a (κ) (Adj PC5), and IgG1 (κ) (MOPC 21) and IgM (λ) (MOPC 104E). Bound antibodies from the first two columns (IgG1 and IgG2a) were pooled and served as the anti-κ-chain reagent; the bound antibody from the third column served as the anti-μ-chain reagent. The serum from the animals immunized with IgD (κ) TEPC 1017 was sequentially passed over columns of IgG2a (κ) (Adj PC5), IgG1 (κ) (MOPC 21), IgM(λ)(MOPC104E) and IgD(κ)(TEPC 1017). Prior to passage through the final TEPC 1017 IgD column, 10% of the sample volume of normal mouse

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serum was added to the experimental serum. The bound antibody from the fourth (IgD) column served as the anti-δ-chain reagent. Bound antibodies were dissociated from the columns with 0·2 M glycine, pH 2·4, immediately neutralized by the addition of solid Tris, dialysed against phosphate-buffered saline, concentrated and finally passed through columns of guinea-pig serum and 1 M acetic acid-treated rabbit Ig (to remove non-specific binding material and rheumatoid factor, respectively). Their isotype specificity was confirmed by ELISA assays, using a panel of purified mouse Ig heavy chain immunoglobulin classes. In addition, these affinity-purified antibodies stained only the B cells when used for staining cryostat sections or cell suspensions of spleen and thymus.

A rabbit anti-rat Thy-1, reactive with murine Thy-1 was given to us by Dr A. Williams (Sir William Dunn, School of Pathology, Oxford, U.K.).

Polyclonal rabbit antibodies to human mb-1 and murine B-29 were produced by immunization with peptides coupled to thyroglobulin and specific affinity-purified antibodies were eluted from peptide-bovine serum albumin-Sepharose columns with 4 M $MgCl_2$. The human mb-1 peptide had the sequence: gly-thr-tyr-gln-asp-val-gly-ser-leu-asn-ileu-ala-asp-val-gln, representing amino acids 202–216 inclusively, as deduced from the human mb-1 cDNA sequence.[4] The murine B-29 peptide had the sequence: cys-gly-glu-val-lys-trp-ser-val-gly-glu-his-pro-gly-gln-glu, representing amino acids 215–229 inclusively, as deduced from the mouse sequence.[16]

Polyvalent, affinity-purified goat anti-mouse Ig (absorbed rat Ig), goat anti-rat Ig (absorbed mouse Ig) and goat anti-rabbit Ig coupled to fluorescein and phycoerythrin were purchased from Southern Biotechnology Associates (Birmingham, AL).

Monoclonal antibodies

The rat mAb to mouse lymphocyte glycoproteins used in the study were: anti-mouse κ-chains (OX20) from Dr A. Williams (University of Oxford); anti-mouse δ-chains (NIM-R9; R. M. E. Parkhouse, unpublished data); anti-mouse μ-chains (B7.6); anti-class II (NIM-R4);[17] anti-CDw32 or FcRγ (2.4G2);[18] anti-CD23 or FcRε low (B3B4);[19] an antibody to a B-cell-specific isoform of CD45 (B220).[20,21]

Surface labelling and co-precipitation

Small, dense resting B cells were labelled with iodine by the lactoperoxidase method and lysed in: (i) 1% w/v Nonidet P-40 (Shell Chem. Co., U.K.) containing 50 mM iodoacetamide and inhibitors of proteolysis [1 mM phenyl methyl sulphonyl fluoride; 1-chloro-4-phenyl-3-1-toluene-P-sulphonaminobutane-2-ene (TPCK) (50 μg/ml); 7-amino-1-chloro-3-tosyl-amidoheptan-2-ene (TLCK) (25 μg/ml); aprotinin (Sigma, Poole, Dorset, U.K.; 100 Kallikrein U/ml);[22] or (ii) a similar buffer, but with 1% (w/v) digitonin (Sigma) replacing the Nonidet P-40.[23] Co-precipitation was done with rabbit antibodies, and the resulting soluble complexes were absorbed to protein A-Sepharose (Pharmacia, Uppsala, Sweden), eluted with 2% (w/v) sodium dodecyl sulphate, and characterized by one-dimensional or two-dimensional (non-reducing/reducing) SDS–PAGE.

Simultaneous staining of surface markers and intracellular determinants of the Ig-associating α- and β-chains

In order to achieve surface staining viable cells were first reacted (20 min, room temperature) with rat mAb or fluorescein isothiocyanate (FITC)-coupled affinity-purified antibody in phosphate-buffered saline containing sodium azide (2 mg/ml) and bovine serum albumin (2 mg/ml) (PBA). The cells were then washed twice in PBA. Bound rat mAb was revealed by FITC-coupled, affinity-purified goat anti-rat Ig, absorbed mouse Ig (20 min, room temperature), and the cells were washed twice in PBA. The FITC-stained cells were then fixed, permealysed and similarly counter-stained with rabbit antibody followed by phycoerthrin (PE)-coupled affinity-purified goat anti-rabbit Ig. Surface stained cells were fixed in 2% (w/v) paraformaldehyde (10 min, 4°) permealysed by addition of Triton X-100 to a final concentration of 0·05% (w/v) (10 min, 4°) and then washed twice with PBA. Negative controls included FITC goat anti-rabbit Ig for the surface staining and normal rabbit Ig/phycoerythrin goat anti-rabbit Ig for the intracellular staining. Samples were then analysed on the Becton-Dickinson FACSCAN (Becton-Dickinson, Mountain View, CA).

RESULTS

Sequential co-precipitation of surface Ig and surface Ig-associated molecules mb-1 and B-29

Co-precipitation of surface Ig and Ig-associated molecules from digitonin lysates of ^{125}I surface labelled B cells with rabbit anti-mouse Ig, anti-mb-1 or B-29, followed by two-dimensional SDS–PAGE yielded comparable patterns of radioactive components. An example is given in Fig. 1, where the analysis of material co-precipitated by the anti-mb-1 reagent is presented.

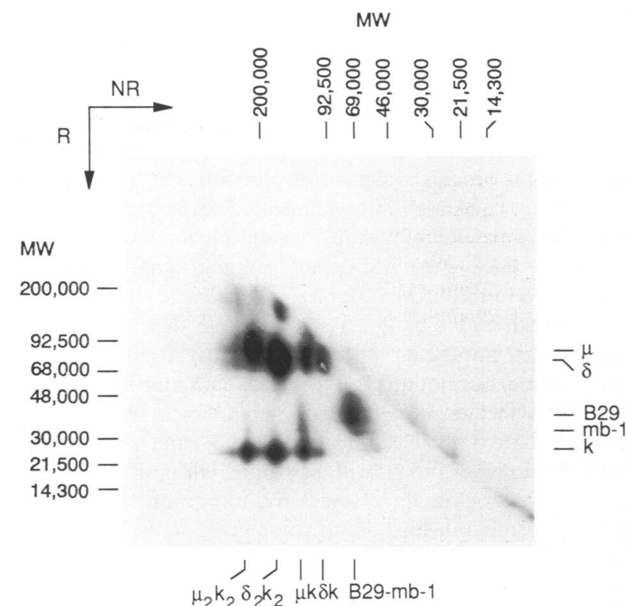

Figure 1. Two-dimensional SDS–PAGE of the surface immunoglobulin complex. Purified splenic B cells were surface labelled with ^{125}I, lysed in digitonin, and co-precipitation was done with rabbit anti-mb-1 and protein A-Sepharose. The co-precipitated material was dissociated with 2% (w/v) SDS, and then analysed by two-dimensional SDS–PAGE. The directions of the first, non-reducing (NR), and second, reducing (R) dimensions, the positions of the molecular weight markers and the molecular assignments are given on the figure. Similar results were obtained with rabbit anti-mouse Ig and rabbit anti-B-29 (data not shown).

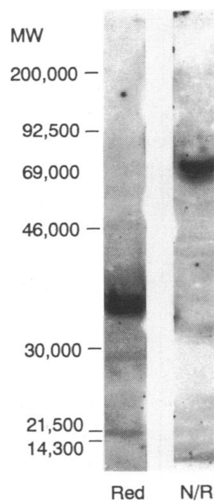

Figure 2. Identification of mb-1 in B-cell lysates by immuno-electrotransfer (Western blot). A digitonin lysate of purified splenic B cells was submitted to SDS–PAGE under reducing (R) and non-reducing (NR) conditions. The separated material was then electrotransferred form the polyacrylamide gel to nitrocellulose. The nitrocellulose was 'blocked' [3% (w/v) bovine serum albumin in PBSD] and then incubated with rabbit anti-mb-1 followed by a goat anti-rabbit Ig-peroxide conjugate and substrate (Tetramethyl benzidine; Sigma)

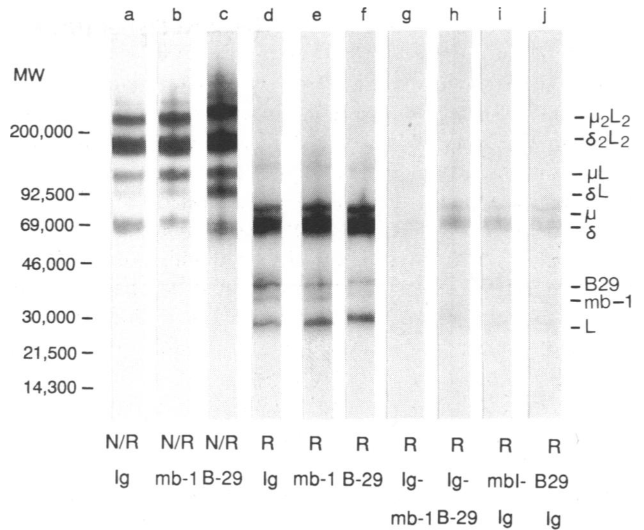

Figure 3. Sequential co-precipitation of the immunoglobulin complex. Analysis by SDS–PAGE. Purified splenic B cells were surface labelled with ^{125}I, lysed in digitonin and co-precipitation was done with rabbit antibodies and protein A-Sepharose as in Table 1. The co-precipitated material was dissociated with 2% (w/v) SDS, and then analysed by one-dimensional SDS–PAGE under non-reducing (a–c) and reducing (d–j) conditions. Co-precipitations were direct, with anti-mouse Ig (a, d), anti-mb-1 (b, e) or anti-B-29 (c, f) or sequential; thus, the supernatant from the anti-mouse Ig co-precipitation was reprecipitated with anti-mb-1 (g) or with anti-B-29 (h); the supernatant from the anti-mb-1 co-precipitation was reprecipitated with anti-mouse Ig (i); the supernatant from the anti-B-29 co-precipitation was reprecipitated with anti-mouse Ig (j).

Table 1. Sequential co-precipitation of surface Ig and surface Ig-associated molecules mb-1 and B-29

Co-precipitation	Radioactivity (c.p.m.)
N Rbt serum	2984
Anti-Mlg	18,297
Anti-mb-1	17,156
Anti-B-29	18,265
Anti-Mlg/anti-mb-1)	2822
Anti-Mlg/anti/B-29	1539
Anti-mb-1/anti/Mlg	3991
Anti-B-29/anti-Mlg	3735
Anti-B-29/anti-mb-1	2740

Details in the legend to Fig. 3. Single co-precipitations were done with anti-Mlg, anti-mb-1 and anti-B-29 as indicated. The supernatants from such co-precipitations were then reprecipitated as indicated in the table by the second of the two antibodies listed. The radioactivity in the co-precipitated material was estimated by counting.

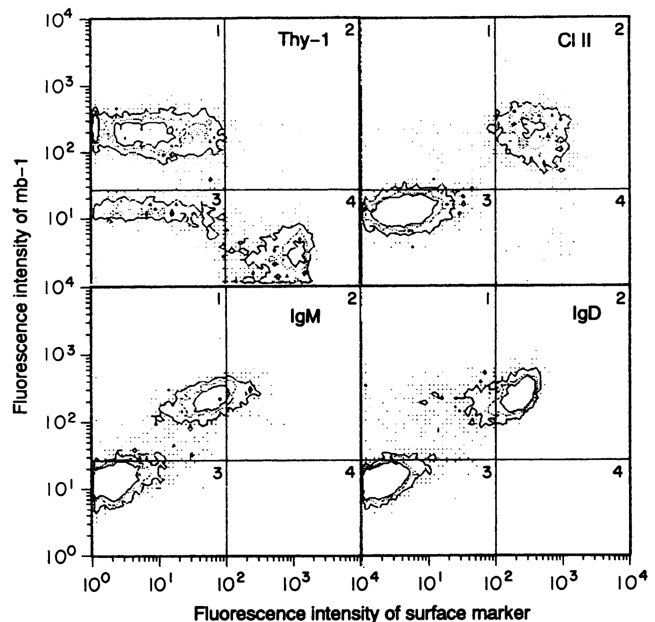

Figure 4. Correlation between expression of mb-1 and membrane Ig in murine B cells. Spleen cells were surface stained with FITC-rabbit anti-Thy-1, FITC mAb NIMR4 anti-class II, FITC affinity-purified goat anti-mouse μ-chain and FITC affinity-purified goat anti-mouse δ-chain. Cells were fixed, permealysed and stained with rabbit anti-mb-1 followed by phycoerythrin affinity-purified goat anti-rabbit Ig and examined in the FACSCAN.

As can be seen, the surface IgM and IgD associate with a heterodimer composed of the mb-1 (α) and B29 (β) chains. As has been noted earlier,[22] both IgM and IgD are found in the disulphide-linked H_2L_2 and HL units with the H_2L_2 subunits quantitatively predominating.

Using SDS–PAGE/Western blot with detergent lysates of B cells, rabbit anti-mb-1 antibody clearly identified the 32,000–34,000 MW α-chain component (reducing conditions) and the $\alpha\beta$ heterodimer (non-reducing conditions) (Fig. 2). Similar

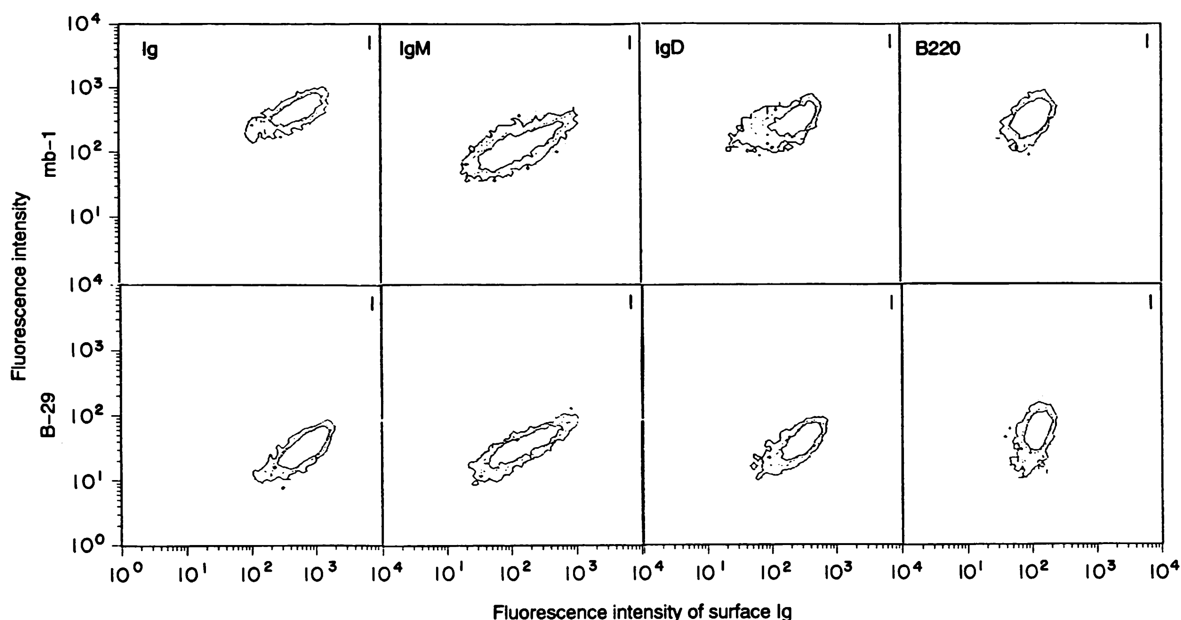

Figure 5. Correlation between expression of mb-1 and B-29 and expression of membrane Ig in murine B cells. Spleen cells were treated with rat mAb anti-Ig (κ) (0X20), anti-μ-chain (B7.6), anti-δ-chain (NIM-R9) and anti-B220 and then stained with FITC affinity-purified goat anti-rat Ig, absorbed mouse Ig. Cells were fixed, permealysed, stained with rabbit anti-mb-1 and rabbit anti-B29 followed by phycoerythrin affinity-purified goat anti-rabbit Ig, and examined in the FACScan.

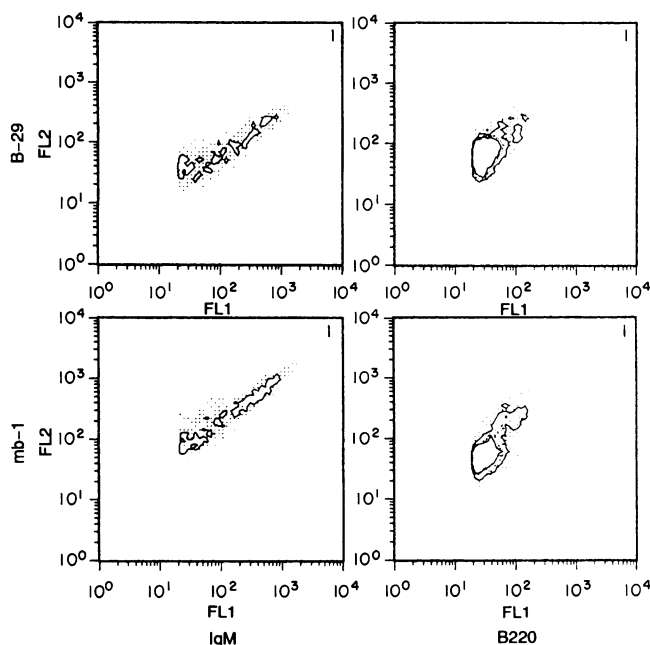

Figure 6. Correlation between expression of surface IgM and mb-1 and B-29 in immature (bone marrow) B cells. Bone marrow B cells were surface stained with FITC affinity-purified goat anti-mouse-μ-chain. Cells were fixed, permealysed and stained with rabbit anti-mb-1 and rabbit anti-B-29 followed by phycoerythrin affinity-purified goat anti-rabbit Ig, and examined in the FACScan.

Western blot analysis with the rabbit anti-B-29 was negative, perhaps as a result of lower affinity antibody.

Radioactive surface labelled B-cell digitonin lysates were then submitted to the series of single and sequential co-precipitations listed in Table 1. For example, after precipitation with anti-mouse Ig, the supernatant was further reacted with anti-mb-1 or anti-B-29. The resulting precipitates were collected for counting and analysis by one-dimensional SDS–PAGE in order to determine whether either surface or Ig or its associating α- and β-chains were present in excess. The conclusion from the co-precipitation data (Table 1) and SDS–PAGE analysis (Fig. 3) is that essentially all surface Ig is associated with mb-1 and B-29 and vice versa.

Correlation between expression of membrane Ig, mb-1 and B-29 in murine B cells

Two-colour FACS analysis of spleen cells stained for mb-1 combination with anti-T-cell (Thy-1) and anti-B-cell (IgM, IgD and class II) markers showed that the rabbit anti-mb-1 reagent was B-cell specific and, moreover, that there was a proportional relation between expression of surface IgM and IgD on the one hand, and mb-1 on the other. There was no such correlation between mb-1 expression and another B-cell marker, class II (Fig. 4). The same conclusions were reached when expression of B-29 was similarly evaluated. Once again expression of both mb-1 and B-29 was correlated and the amount of surface Ig, but not the B-cell marker B220 (Fig. 5), nor FCRγ and FcRε (data not shown). The FACS analysis also showed that < 1% of B cells (equal to control background level) were mb-1 or B-29 negative.

Expression of mb-1 and B-29 on neonatal and activated B cells

Normal bone marrow B cells were stained for surface IgM and B220 and then mb-1 or B-29. As with adult splenic B cells, the neonatal bone marrow cells exhibited a correlation between expression of the associating αβ complex and surface IgM, but not B220 (Fig. 6). There was no significant population of cells

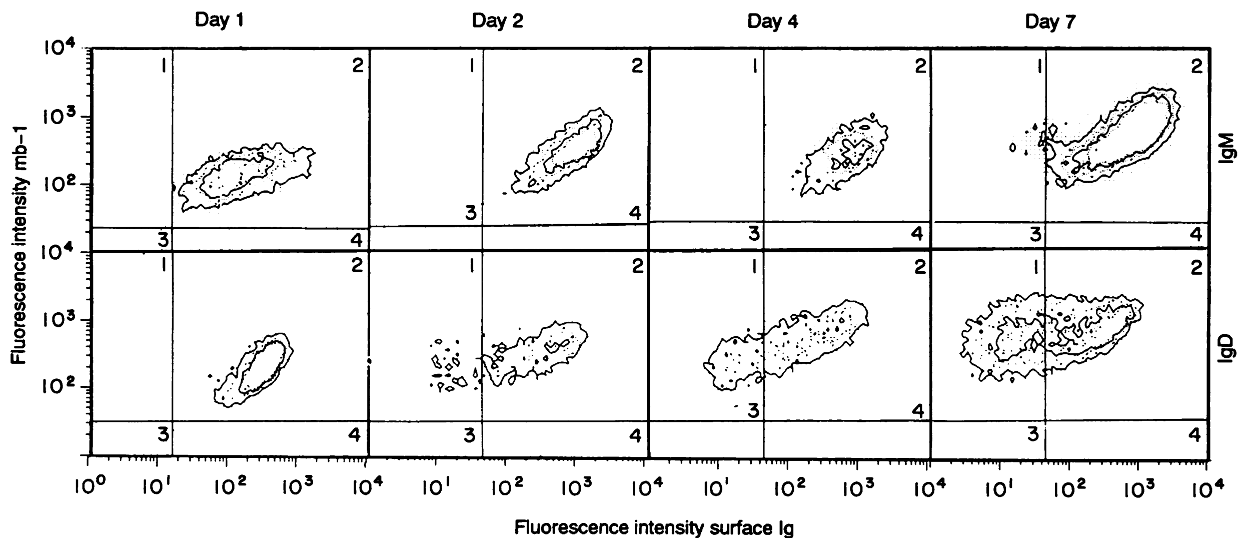

Figure 7. Expression of mb-1 on LPS-activated murine B cells. Splenic B cells were activated with LPS and samples were taken for analysis on Days 1, 2, 4 and 7. The cells were surface stained with FITC affinity-purified goat anti-mouse μ-chain (upper panel) or FITC affinity-purified goat anti-mouse δ-chain (lower panel). The cells were then fixed, permealysed and stained with rabbit anti-mb-1 followed by phycoerythrin affinity-purified goat anti-rabbit Ig. For FACScan analysis Day 1 cells were small lymphocytes whereas at later times the larger, activated cells were selected for analysis.

positive for only one of the two markers. Similarly, B cells activated with lipopolysaccharide continued to express the Ig-associated complex along with surface Ig, even after long periods of activation (Fig. 7).

DISCUSSION

It is remarkable that surface Ig, although long recognized as the antigen receptor of B cells, has only recently been defined as just one component of a complex multi-molecular receptor unit. Retrospectively this is all the more surprising since the amino acid sequence information had clearly shown that membrane Ig lacked a significant cytoplasmic domain and was therefore inherently incapable of a direct signalling function during activation; an adaptor, or associated molecule, now seems a painfully obvious solution. Ironically, therefore, the much later characterized T-cell antigen receptor was first shown to be incorporated into such a complex functional receptor unit. The subsequent discovery of the murine membrane Ig-associating mb-1 (α-chain)-B-29 (β-chain) heterodimer was due to well conceived analysis of a surface Ig-negative mutant cell line,[24] and the use of relatively non-dissociating detergents that preserved weak intermolecular interactions in cell lysates.[23] This has now been followed by the recent biochemical description of the human homologue,[25] confirmed using antibodies to relatively conserved C-terminal peptides deduced from nucleotide sequences.[15] The same antibodies to human mb-1 have been used in the present study. Their cross-reaction with the murine homologue has permitted a biochemical and fluorocytometric analysis of the relative levels of expression of all components of the B-cell surface Ig-$\alpha\beta$ receptor complex.

The main conclusions emerging from the work are that mb-1 and B-29 are B-cell markers, and that all components of the B-cell surface Ig-$\alpha\beta$ complex are expressed in stoichiometrically equivalent amounts. Thus, in co-precipitation experiments with ^{125}I cell surface labelled lysates of B cells, anti-Ig or anti-mb-1 (α-chain), or anti-B-29 (β-chain) precipitated all components of this complex, without leaving a significant residue of uncombined Ig, α-chain or β-chain subsequently precipitable by the homologous antibody. Secondly, and by two marker FACS analysis, there was a clear proportionality between the relative amounts of cell surface Ig and cell surface mb-1 or B-29. Moreover, this was observed in immature (bone marrow), mature (splenic) and LPS-activated B cells. Indeed, insignificant numbers of Ig positive-$\alpha\beta$ negative, or $\alpha\beta$ positive-Ig negative B cells were detected in cell suspensions from normal murine lymphoid tissue. At the same time, however, the relative expression of mb-1 and B-29 was completely unrelated to the expression of a series of B-cell markers (class II, B220, FcRγ and FcRε). All of this points to closely controlled relative synthesis and surface expression of the components of the B-cell receptor complex, perhaps via shared promoter/enhancer sequences.

How synthesis, assembly and transport of various elements of the Ig receptor complex are controlled will be fascinating problems to explore. Such studies may well suggest important, and even unsuspected, functional roles for the interacting polypeptides. At present it must be confessed that there are no compelling experiments defining function, although control of intracellular transport, B-cell activation and perhaps even a function in antigen processing, remain popular areas of speculation.

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