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Expression and Dynamics of Membrane Immunoglobulins*

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Membrane Immunoglobulins on B Lymphocytes

Burnet's "clonal selection" theory (1) proposed that cells destined to produce antibody specific for a given antigen have receptors for that antigen on their membranes. Furthermore, interactions of antigens with these receptors are considered to lead either to clonal expansion and an immune response or clonal deletion and tolerance. This hypothesis stimulated an extensive search for the presence of antibody molecules, the logical candidates for such receptors, on the membranes of lymphocytes. Indirect evidence for the existence of these membrane molecules came from experiments demonstrating that anti-allotype antibodies induced blast transformation and DNA synthesis in rabbit lymphocytes (2, 3). However, the actual direct demonstration of membrane immunoglobulins on lymphocytes was achieved a few years later by a variety of methods including immunofluorescence (4–6), binding of radiolabelled anti-immunoglobulin antibodies followed by autoradiography (7), and demonstration of rosettes in the mixed antiglobulin reaction (8, 9).

Membrane immunoglobulins (mIg) were identified as markers of B lymphocytes in several ways. First, analysis of cell populations with various antisera directed at a variety of B and T lymphocyte markers indicated that membrane immunoglobulin positive cells were strictly limited to those cells expressing B cell markers and were unrelated to cells of thymic origin (5, 10–14). In addition, selective depletion of antibody-producing function without affecting T lymphocyte functions could be accomplished by removal of mIg-bearing lymphocytes (10, 15, 16).

In order to function as membrane receptors in a clonal selection model, B lymphocyte membrane immunoglobulins must be actively synthesized by the cells bearing them and not be acquired passively from the body fluids. This property has been amply verified by the observations that mIg on rabbit lymphocytes demonstrated allelic exclusion (4) and that induction of blast transformation by anti-allotype antisera was specific to the allotype on the lymphocyte surface and not to other allotypes present in the incubation medium or circulating in the serum (2, 3). Furthermore, lymphocytes "stripped" of their membrane proteins by treatment with proteolytic enzymes regenerate their membrane immunoglobulins in vitro without the exogenous addition of any immunoglobulins (11, 17, 18). Finally, and most convincingly, membrane immunoglobulins can be biosynthetically labelled with both radioactive amino acids and sugars (19, 20).

Evidence for the role of mIg as the cellular receptor for antigen came from direct demonstration of antigen binding both by immunofluorescence (4, 11) and by the "antigen suicide" technique involving selective elimination of antigen reactive cells by binding to antigen radiolabelled to high specific activity (21–23) as well as from inhibition of binding by anti-immunoglobulin antibodies (11, 16, 24–27). Also crucial to their role as antigen receptors is the fact that all the mIg molecules on a single cell show specificity for one and only one antigen (7, 28).

The constraints of a selective immune system would require that the antigen receptor immunoglobulin molecules possess specificities and binding properties identical to those of the antibody molecules secreted by their plasma cell progeny. This relationship between mIg and secreted immunoglobulin has been demonstrated at the level of antigen specificity (29–31), antibody avidity (32, 33), and idiotypes, the antigenic determinants related to the antibody combining site (34–43).

Although little is known concerning the mechanisms by which interactions with membrane immunoglobulins transmit signals to the interior of the cells bearing them, much has been inferred from data on the structure, orientation, and mode of attachment of these molecules. Unlike its 19 S secreted counterpart, membrane IgM, the major class of mIg, is an 8 S monomeric molecule (19, 44–46) with its antigen-binding Fab portions oriented to the outside and its Fc portion in contact with (19), but not deeply buried within, the cell membrane (44, 46–49). Further studies involving detergents and lactoperoxidase-catalyzed iodination of inside-out plasma membrane vesicles have revealed that mIg are integral membrane proteins (50) that interact with the membrane, which they do not entirely traverse (51), via noncovalent forces (46, 52, 53) involving their hydrophobic Fc portions (54–56).

Further attempts to account for the unique structural properties of membrane Ig have concentrated on possible differences between the amino acid sequences of membrane and secreted molecules. Analysis of C-terminal

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amino acids by carboxypeptidase digestion (57) as well as peptide mapping (58) in conjunction with molecular weight analyses (59–62) point to the presence of specific hydrophobic membrane insertion sequences in mIg. Confirmation of this notion comes from studies at the DNA level, which reveal the presence of different terminating sequences for secreted and membrane IgM with the latter rich in codons for hydrophobic amino acids (158).

Once inserted in the membrane, mIg molecules have a turnover rate that seems to vary greatly depending on the system studied and the method used. Studies employing a variety of approaches have yielded rapid mIg turnover times as short as 45 to 60 minutes (64, 65), intermediate times ranging from 4 to 12 hours (18, 52, 66, 67), and slow times of approximately 10 to 80 hours (20, 50, 68).

Aside from differences in specificity from one B cell to another, there is also considerable variation in the pattern of mIg expression with respect to the Ig class or combination of classes present on single B cells. While the majority of peripheral B lymphocytes bear both membrane IgM and IgD (66, 69–78), there are few cells that express IgM or IgD alone (70, 74–79) or mIgG (75, 80–85). Cells that express mIgM alone are far more common in neonatal spleen as well as in neonatal and adult bone marrow than in adult peripheral lymphoid organs (85). Sole expression of mIgG or IgA is seen only in adult lymphoid tissues (85). A current area of controversy, based mainly on limitations of technology, concerns the ability of B cells to express more than two classes of membrane Ig.

Definition of functional subsets of B lymphocytes based on the particular immunoglobulin class or combination of classes on their membranes has been derived from studies that involve either selective enrichment or depletion of particular B cell subsets in reconstitution experiments or use of class specific antisera to block the function of cells bearing a particular mIg class and thus part or all of a given response. The precursor B lymphocytes of the primary IgM and IgG responses are generally $mIgM^+$ IgD^+ cells (23, 84, 86–93) with a contribution to the latter by IgG-bearing cells as well (23, 84, 86, 87) and a small contribution to both by cells bearing mIgM alone (23, 79). Precursors of the secondary IgM response are mainly $mIgM^+$ IgG^+ cells with some possible contribution from IgD-bearing cells as well (83, 88, 94). In contrast, memory cells for the IgG secondary response express predominantly one class of membrane Ig, namely IgG (16, 23, 79, 82, 83, 88, 94, 95) and probably arise from cells expressing both IgM and IgG, which can be easily detected shortly after secondary challenge (94–96) and possibly mIgD as well (23, 79, 97). An additional system of B cell classification suggests that cells bearing mIgM alone are capable of responding to one subclass of thymus-independent antigens, while $mIgM^+$ IgD^+ cells are capable of responding to a different subclass of thymus-independent antigens as well as to thymus-dependent antigens (89–91, 93).

An additional approach to understanding the role of membrane immunoglobulin has been to study the effects of anti-immunoglobulin antibodies on B lymphocyte function. Membrane immunoglobulins are subject to the phenomenon of antigenic modulation, a process that involves cross-linking of mIg by antibody, capping, and subsequent endocytosis or shedding of the mIg-anti-Ig complexes (10, 17, 98–101). The ability to regenerate mIg after exposure to antibody has ceased is dependent on a functioning protein synthetic apparatus (99) and is strikingly greater in adult lymphocytes than in cells from newborns (17, 101). This difference in behavior between newborns and adults may somehow relate to the greater ease with which immature cells are made tolerant (101).

Important information on the effects of interactions with mIg on B cell differentiation has been derived from experiments in which anti-immunoglobulin antibodies could block specific antibody production in an in vitro immune response (87) as well as differentiation to immunoglobulin-secreting cells in mitogen-activated lymphocyte cultures (102–105). On the other hand, if mIg truly functions as a membrane receptor molecule, interactions with ligands such as anti-immunoglobulin antibodies should also result in positive stimulation of B lymphocytes. In fact, both induction of proliferation with lymphocytes from various species (2, 3, 105–110) as well as induction to differentiation under select conditions have been demonstrated (94, 111).

Membrane IgD

Since immunoglobulin D (IgD) is present in very low concentrations in the serum (112) while mIgD is found on a large proportion of B lymphocytes far exceeding the small number of plasma cells with intracytoplasmic IgD (113), it is likely that IgD functions mainly as a membrane receptor and, as mentioned in the previous section, is almost always accompanied by mIgM (69–73, 75–78). Comparison of mIgM and IgD on single cells has revealed that these two classes of membrane immunoglobulins share immunoglobulin light chain types (70, 71), idiotypes (41, 43, 114), and specificity for antigen (31, 75). Despite these similarities, mIgM and mIgD are two distinct classes of immunoglobulin as demonstrated by independent "capping" of the two with class specific antibodies (70, 71).

Although mIgM and mIgD appear together on most adult peripheral B lymphocytes, this is not the case for immature B cells. Neonatal murine B lymphocytes bear mIgM alone; mIgD first appears several days after birth, independent of T cell influence and antigen stimulation (75, 93, 115). The neonatal pattern of mIgD expression also holds for lymphocytes from the bone marrow, which contains a population of $mIgM^+$ IgD^- lymphocytes much larger than that of the peripheral lymphoid organs and also a significant number of $mIgM^+$ IgD^+ cells (75, 115–117).

There has been a great deal of speculation concerning the function of mIgM and mIgD with presumably iden-

tical combining sites on the same cell. One hypothesis proposes that mIgM acts as a "tolerizing" receptor while mIgD acts as a "triggering" receptor (118). This notion is supported by the observation that cells naturally low in mIgD expression, e.g., neonatal peripheral B lymphocytes (119) or cells from which mIgD has been removed (120, 121) show an increased susceptibility to tolerance induction. A second proposal contends that signals for proliferation are delivered through mIgM and signals for differentiation through mIgD. Experiments demonstrating anti-IgM antibody-induced B cell proliferation (105, 107, 109, 110) with concurrent inhibition of differentiation (122) and anti-IgD antibody-induced differentiation to antibody secreting cells (105, 123) support this dual signal concept. Another proposal for the required presence of mIgD, in addition to mIgM, is that it enables B lymphocytes to receive T cell signals (124). The fact that anti-IgM antibodies block the in vitro response to thymus-dependent and thymus-independent antigens, while anti-IgD antibodies block the response to only thymus-dependent antigens, supports the notion that IgD is involved in transmission of T cell signals (86, 90, 91, 93). A final postulated role for mIgD is that it is required for the generation of more memory cells rather than only differentiated antibody-secreting cells from memory cells already present (125, 126). It has been shown that mIgD-positive memory B cells give rise to mIgD-negative memory cells and that the differentiated progeny of the latter secrete antibodies of higher avidities than those of the former (97). Furthermore, the ability to generate IgD-negative memory cells from IgD-positive cells is dependent on signals from helper T cells and thus also seems to argue in favor of an association between mIgD and interactions with T lymphocytes (127).

Simultaneous Expression of mIgM and IgD

Since most peripheral B lymphocytes bear both mIgM and IgD, the question of the quantitative regulation as well as of the simultaneous synthesis of these two classes becomes relevant. Among normal human peripheral blood B lymphocytes, a polyclonal population, there is a great deal of heterogeneity with respect to the IgM to IgD ratios on single cells ranging from predominance of IgM to predominance of IgD (123). This range of values is reflected in monoclonal populations of lymphocytes from patients with chronic lymphocytic leukemia (CLL) that bear mIgM and IgD in different ratios in individual cases (123, 128, 129) and in samples taken at various times from the same patient (128). Further studies on monoclonal B cell proliferations, such as Waldenstrom's macroglobulinemia, have shown that within even a single clone of leukemic cells there is marked heterogeneity in the IgM/IgD ratio (31).

This ratio of mIgM to IgD has been useful in characterizing the overall B cell populations in the various lymphoid organs. In bone marrow cells, mIgM predominates over mIgD [by immunofluorescence we found a μ/δ ratio of 2.0 in Balb/c bone marrow lymphocytes (Pernis and Tonda, unpublished data)] while in the peripheral lymphoid organs, this relationship is reversed as follows: spleen, $\mu/\delta = 1.0$; lymph nodes, $\mu/\delta = 0.2$–0.5; Peyer's patches, $\mu/\delta = 0.05$ (115, 130). Within the mature murine spleen and lymph nodes themselves, however, there is marked heterogeneity in mIg expression from cell to cell. Furthermore, a large population of cells with low to intermediate Ig density, which is comprised of large amounts of non-mIgM, i.e., mIgD relative to mIgM, is generally present and is believed to constitute a mature subset of cells. Evidence for the maturity of these cells is provided by their absence in the lymphoid organs of neonatal mice and CBA/N mice, which have an X-linked mutation resulting in absent or delayed development of more mature B cell populations (131, 132). The absence of this cell population in CBA/N defective mice is reflected by the large increase in the μ/δ ratio in their peripheral lymphoid organs when compared to normal control mice (133). Another strain of mice that shows a similar abnormality in the μ/δ ratio in its peripheral lymphoid organs is the NZB, which display characteristic abnormalities in immune function including failure to respond to polyclonal B cell activators in vitro and a high incidence of auto-immune disease (134). Correlation between abnormalities of mIg expression and immune function in these mice suggests that normal immune function may depend heavily on expressions of particular ratios of mIgM to mIgD.

Further alterations of the μ/δ ratio come with cell maturation. Characteristically, mIgD is lost as B lymphocytes differentiate to antibody-secreting cells. Studies on clones of leukemic cells as well as polyclonal populations of normal lymphocytes have revealed that while mIgD is never expressed on IgG- and IgA-secreting cells, the more highly differentiated B cell progeny, it may be present on a proportion of IgM-secreting cells, their less differentiated counterparts (31, 113). This identical phenomenon of an increased μ/δ ratio due to loss of mIgD with maturation has also been observed in in vitro cultures of both murine and human lymphocytes stimulated with mitogens (123, 135, 136) and in antigen-binding splenic B cells after immunization with the antigen to which they are specific (137, 138). Furthermore, the rapidity with which mIgD is lost after stimulation (139) as well as the fact that treatment with anti-IgD antibodies in vivo has a strong adjuvant effect on the response to antigen administered concurrently suggests that removal of mIgD may be an absolute requirement for, and not a coincidental secondary result of, the process of B cell differentiation (140–142).

The finding that the majority of normal peripheral B lymphocytes bear mIgM and mIgD suggests that lymphocytes are capable of simultaneous synthesis of μ and δ mRNA molecules. Stronger evidence is provided by the presence of some monoclonal lymphomas such as chronic lymphocytic leukemia in which 100% of the cells express both mIgM and IgD. If simultaneous synthesis of μ and δ were not the case, it would be expected that at least a

proportion of the cells should express one class or the other, but not both (103, 129). In addition, analysis of bone marrow lymphocytes of patients with Waldenström's macroglobulinemia reveals that the neoplastic clone consists of lymphocytes with mIgM and mIgD as well as IgM-containing plasma cells with either mIgM plus mIgD or mIgM alone (31). This suggests that B lymphocyte clones begin with IgM synthesis alone, activate IgD synthesis without inactivating IgM synthesis, and finally differentiate to IgM-secreting plasma cells that have lost the ability to synthesize IgD. This implies that there should be lymphocytes capable of simultaneous synthesis of the two classes of mRNA, especially in view of the high proportion of lymphocytes expressing both mIgM and mIgD.

Another line of evidence for simultaneous synthesis of μ and δ mRNA molecules comes from the isolation in vitro of cell lines derived from human and murine B lymphocytes that simultaneously express mIgM and mIgD (143, 144). The stable double expression of these two mIg classes through multiple rounds of cell division argues against the possibility of active synthesis of only one mRNA class and the persistence of a long-lived mRNA of the opposite class. In addition, clones derived from $mIgM^+$ IgD^+ human cell lines resemble each other as well as the parental line with respect to Ig expression (145); this suggests that the various cells in the parental populations have not undergone any μ to δ or δ to μ switch, but rather are capable of simultaneous synthesis of both classes.

A completely separate source of information on Ig gene expression comes from molecular biology. Recent findings on the organization of immunoglobulin heavy chain genes are compatible with the notion of simultaneous synthesis of IgM and IgD. The sequence of heavy chain constant region (C_H) genes from the 5′ to 3′ end of the DNA appears to be as follows: $C\mu$, $C\delta$, $C\gamma_3$, $C\gamma_1$, $C\gamma_{2b}$, $C\gamma_{2a}$, $C\epsilon$, and $C\alpha$ (146–147). Most current data suggest that the switch from IgM production to IgG or IgA involves deletion of all C_H genes between the expressed heavy chain variable (V_H) region gene and the newly expressed C_H gene. Thus, γ_3 producers will have deleted their μ and δ C_H-genes and so on (146–151). This type of mechanism results in irreversible commitment to a particular phenotype. Similar studies for IgM and IgD expression have only recently become possible because of the newly available δ-chain probe derived from murine and rat IgD myelomas. Although differential RNA splicing has been ruled out as the mechanism for the heavy chain switch from IgM to IgG or IgA (152, 153), this still remains a likely possibility for co-expression of IgM and IgD (151, 154, 155). In fact, the presence of mRNA splicing mechanisms has previously been implicated in the processing of both heavy chain (63, 156) and light chain (157) primary transcripts. Furthermore, the existence of a differential mRNA splicing mechanism for the regulation of expression of two Ig molecules has already been suggested in the synthesis of membrane-bound and secreted IgM (158). In the case of IgM and IgD, the observation that the DNA segment coding for the first domain of the δ chain is separated from the terminal exon of the μ chain by only 2466 base pairs, a distance shorter than that between any other two C_H gene segments (159) and the fact that there is no V_H to $C\delta$ DNA switch rearrangement in $mIgM^+$ IgD^+ B cells (160) suggests that the simultaneous expression of $C\mu$ and $C\delta$ with a single V_H region is mediated by alternative splicing of primary transcripts containing the V_H, $C\mu$ and $C\delta$ genes.

Internalization or Shedding of Cross-Linked Membrane IgM and IgD in Human B Lymphoid Cells

This section summarizes some recent work on this subject performed in our laboratory. Experiments were performed on human peripheral blood B lymphocytes and clones of human B lymphoblastoid cell lines. Fluorochrome-conjugated anti-immunoglobulin antibodies were prepared as described by Ferrarini et al. (113). Cells were exposed in culture to class-specific anti-immunoglobulin antibodies to determine whether there were any differences in the fate of cross-linked mIgM and mIgD. In order to distinguish between membrane immunoglobulin molecules that had been internalized from those that had remained on the membrane, the following approach was employed: First, cells were both treated with fluorescein (FITC)-conjugated anti-immunoglobulin antibodies (Ab 1) and washed at 4°C. Following incubation for 2 to 6 hours at either 0°C or 37°C, the cells were restained in suspension with rhodamine (TRITC)-conjugated antibodies (Ab 2) directed at the first immunofluorescent reagent, Ab 1, which was either rabbit, goat, or monoclonal mouse immunoglobulins. Membrane Ig molecules that remained on the membrane throughout all treatments would be stained with both fluorochromes, while those that were internalized would be inaccessible to staining by Ab 2 and thus would be positive for fluorescein only.

Most of the experiments that involved human B lymphoblasts were performed on MW-E and BL, two clones of the HLA-DRw 1-2 and HLA-DRw 2-4 phenotypes, respectively, derived from B lymphoblastoid cell lines. While MW-E was comprised of approximately 15% cells bearing both mIgM and mIgD, 22% bearing mIgD alone and the remaining cells being mIg-negative, BL consisted of 71% cells expressing both mIgM and mIgD and 24% expressing mIgM alone. Both clones expressed only kappa (and no lambda) light chains.

Treatment of lymphoblasts with anti-immunoglobulin antibodies revealed differences in the responses of mIgM and mIgD to cross-linking (P. Roth and B. Pernis, unpublished observations). MW-E cells treated with rabbit anti-μ antibodies at 37°C displayed IgM-containing cytoplasmic vacuoles in 29% of cells, whereas cells treated

with rabbit anti-δ antibodies contained IgD vacuoles in only 1% to 2% of cells. Anti-μ treated BL cells contained IgM-positive vacuoles in nearly 100% of cells in marked contrast to the 1% to 2% of cells containing IgD-positive vacuoles after anti-δ treatment. Furthermore, intracytoplasmic Ig vacuoles, when present, also stained positively for rabbit Ig as well as kappa light chains. These results suggest that after cross-linking, mIgM-anti-μ complexes are internalized via repeated rounds of capping, endocytosis, and re-expression of mIgM as suggested previously by the work of Kearney et al. (122). Membrane IgD, on the other hand, is predominantly shed from the membrane after cross-linking as suggested by the finding of few cells with intracytoplasmic vesicles that could be stained with anti-IgD in cells exposed to rabbit or goat antibodies directed against this isotype. Cells so treated also showed very few, if any, intracytoplasmic vesicles with rabbit or goat immunoglobulins, a confirmation of the fact that most of the IgD-anti-IgD complexes formed on the membrane had not been internalized. Since the foreign immunoglobulins were not found on the cell membranes either, one must conclude that most of the cross-linked IgD had been shed.

Similar experiments performed on normal human peripheral blood B lymphocytes demonstrated internalization of mIgM following anti-μ treatment in virtually all cells, but differed from lymphoblasts in that internalization of IgD after anti-δ treatment was observed in a significant proportion of cells. These differences between the B lymphocytes and lymphoblasts may reside in the fact that certain properties of membrane dynamics are different in dividing blasts and resting cells. Furthermore, experiments with human peripheral B lymphocytes deal with a polyclonal population of cells, while those on the B lymphoblasts involve only single clones of cells. There may be clonal differences in the process of internalization or shedding of cross-linked IgD.

It appears, therefore, that there are differences in the dynamics of the two main isotypes of membrane immunoglobulins after cross-linking with the corresponding antibodies and that, furthermore, different patterns may be found in different cells. The functional significance of these different patterns is unknown, but it is likely to be connected with different dynamic interactions that the cross-linked membrane immunoglobulins may establish with other components of the B lymphocyte membrane. For instance, it has been established that cross-linked membrane IgM interacts with the Fc receptor for IgG that is present on the membrane of most B lymphocytes while cross-linked IgD does not show this reaction (161, 162). It appears likely, therefore, that differences in the dynamics of mIgM and mIgD, after these receptors are cross-linked by antigens, will eventually provide a reason for the different roles of these membrane immunoglobulins in the immune response as well as for the remarkable genetic and biochemical conditions that insure the simultaneous synthesis and expression on the membrane of single B lymphoid cells of two classes of receptors for antigen with identical combining sites.

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