

**ANTIGEN-INDUCED CO-CAPPING OF IgM AND IgD-LIKE
RECEPTORS ON MURINE B CELLS***

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The major receptors for antigen on murine B lymphocytes are currently thought to consist of monomeric IgM and an immunoglobulin molecule previously proposed to represent the murine homolog of human IgD (1-7). Whether other classes of immunoglobulin function as receptors remains controversial (1, 2). In particular, it now appears that very few human or murine B cells bear IgG on their surface (1-8).

Studies of the murine IgD-like molecule have been hindered by the lack of specific antisera. However, we have recently reported the presence of an allootypic determinant on the murine IgD-like molecule and have shown that some commonly available alloantisera contain antibodies to this molecule. Since the IgD-like molecule was found to be structurally and serologically distinct from the known serum immunoglobulins, a new immunoglobulin heavy chain gene (Ig-5) was proposed (7). The production of a specific antiserum to this molecule was also recently described by Abney et al., who immunized rabbits with IgD-like immunoglobulin purified from lymphocyte membranes by affinity chromatography (9).

It has previously been reported that individual human leukemias bore identical idiotypes on surface IgM and IgD (10-12). Raff et al. (13) and Nossal and Layton (14) have shown that all the immunoglobulin detected by polyspecific anti-immunoglobulin antisera on individual antigen-binding cells was able to be capped by antigen. Taken together, these results infer that an individual B cell is capable of the simultaneous expression of one variable region gene product attached to two different constant region gene products.

In this paper, we report the direct demonstration that (a) most splenic B cells from mice aged 11-52 wk bear both μ - and δ -like heavy chains, (b) splenic B cells from neonatal mice possess surface μ -chains only, (c) when treated with specific antisera, these molecules cap independently, and (d) the two receptor types on individual cells can be co-capped by antigen.

Materials and Methods

Mice. C57BL/6 and its allotype congenic partner strain (7) C57BL/6.Ig^cN14F4 were from Walter and Eliza Hall Institute stocks. Mice of either sex were used.

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Antigens and Antisera. Antisera made in C57BL/6 mice against CBA/CaH WEHI spleen cells were produced and tested as previously described (7). The antisera used contained antibodies to H-2^k and Ia^k antigens and also to IgD-like surface immunoglobulin (7). When tested against C57BL/6.Ig^e cells, only antibodies to IgD are detected (7). Fluorescein (FITC)-conjugated anti- μ -serum was purchased from Meloy Laboratories Inc., Springfield, Va., and the heavy chain specificity confirmed by radioimmunoassay by Dr. N. L. Warner. Rabbit antiserum to mouse IgG was rendered specific for γ -chains by repeated absorption with the 19S fraction of serum from old NZB mice coupled to Sepharose 4B. The IgG fraction of this serum (isolated by protein A-Sepharose) was conjugated (15) with tetramethyl rhodamine isothiocyanate (TRITC, Baltimore Biological Laboratories, Cockeysville, Md.) using 10 μ g TRITC (dissolved in dimethyl sulfoxide) per mg IgG. Conjugates were purified by DEAE-Sephadex A50 with linear salt gradient elution. The OD₃₅₅:OD₂₈₀ ratio was 0.5, and the conjugate was used at 0.5 mg/ml.

4-hydroxy-3-iodo-5-nitrophenylacetic acid coupled to polymerized flagellin (NIP-POL) was produced as previously described (14).

Enrichment of Antigen-Binding Cells. The separation of antigen-specific B cells on haptenated gelatin adsorbents was as previously described (14).

Fluorescence Procedures. Treatment of cells with antisera, washing, and examination for fluorescence was as previously described (14). Typically 200 cells were counted per slide.

Results and Discussion

Lymphoid cells from 11-wk-old C57BL/6 or C57BL/6.Ig^e mice were treated with fluorescein-conjugated goat antiserum to mouse μ -chain (FITC-anti- μ), washed, and then held with C57BL/6 anti-CBA spleen serum ("anti- δ "). The cells were washed once again and then treated with tetramethyl rhodamine-conjugated rabbit antiserum to mouse γ -chains (TRITC-anti- γ). All manipulations were at 4°C. After a final wash cells were examined for fluorescence (Table I).

In both control (C57BL/6) and experimental (C57BL/6.Ig^e) groups, approximately 43% of spleen cells showed green membrane fluorescence, indicating the presence of surface IgM. When cells from C57BL/6.Ig^e mice were treated with FITC-anti- μ , followed by C57BL/6 anti-CBA and TRITC-anti- γ , a considerable fraction bore red membrane fluorescence (Table I), indicating the presence of δ -chains. When C57BL/6 cells were examined for red fluorescence, only 1–2% of these control cells were positive, indicating that very few B cells bore detectable surface IgG. This result was consistent with previous biochemical and immunofluorescence studies (1, 2, 7, 8).

Considerable attention was given to the specificity of staining, particularly to the problem of binding of complexes and aggregates to Fc receptors. The finding that significant numbers of red staining cells were only found when the target cells were of the appropriate Ig-5 allotype indicates that nonspecific staining due to binding to the Fc receptor did not occur. The anti- μ -serum was also tested for binding to the Fc receptor by use of the T-cell tumor line S-49. This line possesses a very strong Fc receptor, but does not possess enough surface immunoglobulin to be detected by immunofluorescence (16). This tumor line has proven very useful as a sensitive reagent for testing for the presence of IgG aggregates. When tested against S-49, the FITC-anti- μ serum produced no visible membrane staining. These controls, together with the previously published data (7), indicate that staining was specific.

Significant numbers of cells positive for IgD-like surface immunoglobulin were found in spleen, lymph node, and Peyer's patches (Table I). A few positive

TABLE I
Organ Survey of Surface Immunoglobulin in 11-Wk-Old C57BL/6.Ig^e Mice

	Percent positive			
	$\mu^+\delta^-$	$\mu^+\delta^{**}$	$\mu^-\delta^{**}$	$\mu^-\delta^-$
Spleen	7.0	35.7	5.6	51.8
Thymus	0.2	0.2	0.2	100
Lymph node	2.2	17.6	2.2	78.0
Peyer's patch	1.8	36.5	4.8	56.9
Bone marrow	5.0	2.3	0.5	92.2

Cells were treated with 1:15 (vol/vol) dilution of FITC-anti- μ for 30 min at 4°C, washed, and treated with 1:10 (vol/vol) dilution of C57BL/6 anti CBA serum (4°C, 30 min). Cells were washed again and treated with TRITC-anti- γ (0.5 mg/ml final concentration) for 60 min at 4°C. After a final wash, cells were examined for fluorescence. At least 200 cells were counted (500 for thymus).

Cells from control (C57BL/6) mice were treated in an identical fashion. The percentage of μ -positive cells was similar to that of C57BL/6.Ig^e, but only approximately 1% showed red staining.

* Figures include any cells bearing γ -chains.

cells were found in bone marrow, but none in thymus. Most of the positive cells in all organs were also stained for surface IgM. Preliminary results of velocity sedimentation studies indicate that of the Ig-positive cells in the rapidly sedimenting group, about half are " μ only" and the remainder are doubles, confirming the suggestion of Goodman et al. (17). In bone marrow, a larger fraction (68%) of positive cells bore μ -chains only, although significant numbers of doubles were also seen.

There was considerable variation among cells in the intensity of staining with the anti- δ -like serum. A similar observation was made by Abney et al. (9). It must therefore be stressed that the figures obtained will be influenced by the sensitivity of the method, and the "cut off" point (fluorescence significantly above background) is somewhat arbitrary. Since the control values (C57BL/6 treated with C57BL/6 anti-CBA followed by TRITC anti- γ) have not been subtracted, cells with red membrane fluorescence could have borne either γ - or δ -chains. However, the number of γ -bearing cells in C57BL/6 never exceeded 2%, so it is reasonable to conclude that any significant excess over this number is due to δ -bearing cells.

When a similar experiment was performed with 9-day-old mice, approximately 21% of spleen cells were positive for μ -chains, but only 1% were stained with C57BL/6 anti-CBA (Table II). However, a few δ -positive cells were found in lymph node. All these cells were also positive for μ -chains. Cells from 1-yr-old mice were also examined. In spleen, 32% of all cells were $\mu^+\delta^+$, 4% were $\mu^-\delta^+$, and 2% $\mu^+\delta^-$, and in lymph node the figures were very similar, although $\mu^+\delta^-$ cells were scarce (1-2%). Peyer's patch cells from 1-yr-old mice were also mostly doubles, although the intensity of red fluorescence was subjectively greater. Thoracic duct cells from 24-wk-old mice contained 27% $\mu^+\delta^+$, 2.1% $\mu^-\delta^+$, and <0.5% $\mu^+\delta^-$ (less than 0.3% of 2,500 cells counted bore detectable γ -chains in control experiments). These results confirm and extend those of Abney et al. (9) and provide additional evidence for the specificity of staining.

It has already been shown (18) that surface IgM and IgD cap independently on human lymphocytes. Similar results have been reported in the mouse (7, 9). As a control for the experiments to follow, an additional experiment was performed to demonstrate independent capping in the systems used in this study. Because sandwich conditions are much more powerful in inducing capping, the order of

TABLE II
Organ Distribution of Surface Immunoglobulin in 9-Day-Old and 23-Day-Old C57BL/6Ig^c Mice*

	Percent positive			
	$\mu^+\delta^-$	$\mu^+\delta^+\ddagger$	$\mu^-\delta^+\ddagger$	$\mu^-\delta^-$
9-day old				
Spleen	20 (17)	1 (1)	0.2 (0.2)	79 (82)
Thymus	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	100 (100)
Lymph node	7 (13)	5 (1)	0.1 (0.1)	88 (86)
Bone marrow	3 (3)	0.1 (0.1)	0.1 (0.1)	97 (97)
23-day old				
Spleen	21.5	12.0	0.5	66.5
Thymus	0.1	0.1	0.1	100
Lymph node	5.9	13.4	1.1	79.7
Peyer's patch	5.6	30.0	0.6	64.4
Bone marrow	3.8	0.2	0.2	96

At least 200 cells were counted. In the case of thymus, approximately 1,000 cells were scanned and no positive cells were seen. Staining protocol as for Table I.

* Control C57BL/6 values are in parentheses.

‡ Figures include any cells bearing γ -chains.

TABLE III
Independent Capping of Surface IgM and IgD-Like Immunoglobulin

δ -caps, μ -rings	Both chains capped	δ -caps only
76%	2.9%	21%

Spleen cells from C57BL/6.Ig^c mice (aged 17 wk) were fractionated on NIP-gelatin as previously described; 51% were shown to bind detectable amounts of TRITC-NIP-POL. Cells were treated with C57BL/6 anti-CBA serum (1:10 vol/vol) for 30 min at room temperature, washed, and held with TRITC-anti- γ (0.5 mg/ml) in an identical fashion. All media and reagents following this step contained 10 mM sodium azide. Cells were washed and resuspended in FITC-anti- μ , held at 4°C for 30 min, washed, and examined. 100 cells with red caps were examined for distribution of green fluorescence.

addition of reactants was reversed from the previous experiments (legend to Table III). The δ -like receptors were capped by treatment with C57BL/6 anti-CBA serum followed by TRITC-anti- γ . After the second layer, cells were washed in medium containing 10 mM sodium azide to prevent further capping. All subsequent washes and incubations were in medium containing 10 mM sodium azide. Cells were treated with FITC-anti- μ and were washed and examined after 30 min at 4°C. Control experiments showed that 10 mM sodium azide prevented capping. The great majority of cells (Table III) showed red caps with green rings, confirming the previous findings.

To test whether the μ - and δ -like chains had similar antigen-binding specificity, spleen cells from 12-wk-old C57BL/6Ig^c mice were fractionated on NIP-gelatin dishes as previously described (14). Their receptors were capped by treatment with 30 μ g/ml of NIP-POL (30 min at 4°C followed by 5 min at 37°C), and cells were then fixed with 1% paraformaldehyde (15 min at 4°C). This protocol has previously been shown to prevent further capping (14). The cells were then treated with FITC-anti- μ followed by C57BL/6 anti-CBA and TRITC-anti- γ (conditions as for Table I). The fractionated cells contained 41% which bound detectable amounts of TRITC-conjugated NIP-POL as previously described (14). Cells were examined for the presence of green caps. Of 50 cells with

green (μ) caps, 49 showed corresponding red caps and one was negative for red fluorescence. In a second experiment, all of 50 cells with green caps also had red caps. No cells were found with red caps that did not also have green caps. In a third experiment, NIP-specific cells from mice aged 17 wk were treated with NIP-POL under capping conditions, then labeled with C57BL/6 anti-CBA, TRITC-anti- γ , and FITC-anti- μ . All steps after antigen-induced capping were carried out in 10 mM sodium azide. Of the cells with red caps, 96% had green caps, 3% had red caps but a residual green ring, and 1% were negative for μ (a lower percentage than in previous experiments).

Our results show that most B cells from mice aged 11–52 wk bear both IgM and IgD-like surface immunoglobulin. The finding that both receptors on individual cells can be capped by the same antigen indicated that both immunoglobulin classes have combining sites which recognize this antigen.

Certain human lymphatic leukemias have been shown to bear identical idiotypes on surface IgM and IgD (10–12), and a biclonal myeloma was recently found to possess identical variable region sequences on γ - and α -chains (19). Thus, the concept may be proposed that a given lymphocyte is capable of the simultaneous expression of one variable region gene product attached to two different constant region gene products. This notion would be compatible with the idea that B cells are capable of an IgM to IgG “switch.”

Summary

Double fluorescence studies indicated that most mature lymphocytes of 11–52-wk-old mice possess both IgM and IgD-like surface immunoglobulins, while spleen cells from neonatal mice possess surface IgM only. These molecules cap independently with class-specific antisera, but co-cap when capping is induced by antigen. It is proposed that the two heavy chains on individual lymphocytes possess similar or identical antigen-combining sites.

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