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Specificity of the murine IgD receptor on T cells is for N-linked glycans on IgD molecules

ASHOK R. AMIN*†, S. M. LAKSHMI TAMMA‡, JOEL D. OPPENHEIM§, FRED D. FINKELMAN¶, CLAUDINE KIEDA||, RICHARD F. COICO‡, AND G. JEANETTE THORBECKE*†**

Departments of *Pathology and §Microbiology and †Kaplan Cancer Center, New York University School of Medicine, New York, NY 10016; ‡Department of Microbiology, City University of New York Medical School, New York, NY 10031; ¶Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD 20814; and ||Centre de Biophysique Moleculaire, Centre National de la Recherche Scientifique, Orleans, France

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ABSTRACT **IgD receptors on murine T cells have been reported in this issue [Tamma, S. M. L., Amin, A. R., Finkelman, F. D., Chen, Y.-W., Thorbecke, G. J. & Coico, R. F. (1991) *Proc. Natl. Acad. Sci. USA* 88, 9233–9237] to bind either the first or third constant region of the heavy-chain of IgD molecules—findings that could not be satisfactorily explained by IgD amino acid sequences. We now find that boiled IgD molecules or low-M_r fragments from protease-digested IgD still inhibit binding of IgD-coated erythrocytes to IgD receptors. This inhibitory activity can be absorbed with the murine IgD-binding lectin from *Griffonia simplicifolia* 1 (GS-1) immobilized on Sepharose. N-linked glycans, obtained from N-glycanase-treated IgD and purified by binding to GS-1-Sepharose, also inhibit rosette formation of T-helper cells bearing receptors for IgD with IgD- or mutant IgD-coated erythrocytes. Deglycosylated IgD, produced by treatment with N-glycanase, no longer binds to the lectin and fails to inhibit IgD rosetting. Binding of intact IgD to T cells is also competitively inhibited by *N*-acetylgalactosamine, galactose, *N*-acetylglucosamine, and neoglycoproteins containing these sugars. These results clearly show that N-linked glycans, present in both the first and third constant regions of the δ heavy-chain domains, are prerequisites for binding of IgD to IgD receptors.**

Within 1–2 hr after exposure to oligomeric, aggregated, or antigen-crosslinked monomeric secreted IgD, ≈25–35% of splenic murine T-helper cells (1) and 10–15% of human peripheral blood T cells (2) exhibit receptors for IgD (IgD-R), forming rosettes with IgD-coated erythrocytes. Interleukin 2 (IL-2), interleukin 4 (IL-4), and interferon γ also up-regulate IgD-R on $CD4^+$ polyclonal or cloned T cells (3–5). We established the isotypic specificity of these receptors on the T-helper cells bearing receptors for IgD (Tδ) cells by showing that dimeric IgD, at concentrations ≥120 μg/ml (1 μM), competitively inhibits Tδ rosetting, whereas IgM, IgG1, IgG2, IgG3, IgA, or IgE fail to do so (3, 5). The possible physiological significance and role of these IgD-R on T cells in immunoregulation has been discussed (3, 6).

More recent observations in our laboratory (7) have shown that IgD-R can bind two entirely nonoverlapping parts of murine IgD molecules, the first and third constant region of the δ heavy-chain domains ($C_δ1$ and $C_δ3$, respectively), which are only 26% homologous at the amino acid level (8). Moreover, these two domains can compete with each other in binding to the IgD-R on T cells. Because we failed to identify homologous protein sequences in these domains to explain these findings, we investigated the role of carbohydrates in IgD–IgD-R interaction. This approach seemed particularly relevant due to our observation that mutant IgD molecules, containing either only the $C_δ1$ or only the $C_δ3$ domain, each bind to the lectin *Griffonia simplicifolia* 1 (GS-1) (7), a lectin shown to specifically bind N-linked glycans from murine IgD (9).

The results show that the binding of IgD to its receptor can be blocked by (*i*) low-M_r fragments from protease-digested IgD molecules, (*ii*) N-linked glycans isolated from IgD, and (*iii*) Gal, GalNAc, and/or GlcNAc and neoglycoproteins containing these sugars, but not by (*i*) deglycosylated IgD (DG-IgD); (*ii*) low-M_r fragments from IgM, IgA, or IgG2a; (*iii*) Man, Fuc, melibiose, lactose, and Glc or neoglycoproteins containing their derivatives; or (*iv*) most Gal/GalNAc-rich bacterial polysaccharides tested.

MATERIALS AND METHODS

All methods and reagents were as in ref. 7, except as noted below.

Reagents. Gal, GalNAc, GlcNAc, and Man were from Sigma; melibiose was from Pfanstiehl Chemicals; and lactose was from Fluka. The purified polysaccharides from *Pneumococcus* and *Klebsiella* (10) were made available by M. Heidelberger (New York University School of Medicine). Purified GS-1 was donated by EY Laboratories.

Preparation of Neoglycoproteins. Monosaccharide-substituted bovine serum albumin (BSA) (neoglycoproteins) were prepared as described (11, 12): *p*-nitrophenyl glycosides (Sigma) were reduced into *p*-aminophenyl glycosides, converted into glycosidophenyl isothiocyanates, and coupled to BSA up to ≈20 sugar residues per mol.

Enzymatic Treatment of IgD. *Pronase (Calbiochem)/proteinase K (Sigma).* Low-M_r IgD fragments were prepared by Pronase (see Table 1, F1) or proteinase K (F2) digestion of purified TEPC-1033 IgD. Ten milligrams of IgD dissolved in 1 ml of 0.1 M Tris·HCl, pH 7.4, was digested with 2 mg of Pronase or proteinase K for 12 hr at 37°C, 2 mg of the same enzyme was added, and the digestion was continued for 12 hr, followed by a third addition of 2 mg of enzyme and another 12-hr digestion period. The lowest M_r components (<5000) of these digests were obtained by gel filtration on Sephadex G-25, in which the position of the retained small molecules was determined with tryptophan as standard. The retained fractions were reduced to 1 ml by lyophilization and passed over Sephadex G-10. The retained fractions from these columns were pooled and lyophilized. The low-M_r fraction

Abbreviations: IgD-R, IgD receptor(s); DG-IgD, deglycosylated IgD; RFC, rosette-forming cell(s); IL-2 and IL-4, interleukin 2 and 4, respectively; Tδ, T-helper cell bearing receptors for IgD; GS-1, lectin from *Griffonia simplicifolia* 1; BSA, bovine serum albumin.

**To whom reprint requests should be addressed at: Department of Pathology, New York University Medical School, 550 First Avenue, New York, NY 10016.

††Michael Heidelberger (deceased June 25, 1991) intended to communicate this paper and on his behalf I am honored to do so, but sadly for all of us who admired him and prized his friendship.

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from IgG2a was similarly prepared by Pronase digestion of UPC-10 myeloma protein (see Table 1, F3). Unfractionated fragments, obtained by complete digestion of TEPC-1017 IgD, 3A6 hybridoma IgA (F4), and TEPC-187 IgM (F5) with proteinase K, were found to be <5000 in molecular weight on analysis by 4–20% SDS/PAGE.

N-Glycanase. IgD was treated with N-glycanase (peptide: N-glycosidase F) as described (9). Ten milligrams of purified TEPC-1017 IgD was treated with N-glycanase, according to the manufacturer's specifications (Genzyme). A positive control of transferrin was also treated similarly. All samples were then tested for glycans by dot-blot analysis by the staining method with digoxigenin succinyl-ε-amidocaproic acid hydrazide (Boehringer Mannheim Biochemica glycan detection kit) to monitor the reaction. The partially DG-IgD and the released asparagine-linked oligosaccharides were purified as follows: the reaction mixture was passed through a PD-10 desalting column, according to the manufacturer's specifications (Pharmacia). The protein and (salt plus oligosaccharide) fractions were pooled individually and lyophilized. The DG-IgD was concentrated and fractionated on a Superose 6 fast protein liquid chromatography column to isolate the intact IgD molecules. This DG-IgD was passed over the GS-1-Sepharose column, and the fall-through fraction was passed over Extracti-Gel-D to remove traces of detergent according to the manufacturer's specifications (Pierce) and extensively dialyzed against Dulbecco's phosphate-buffered saline before use. The control IgD was treated similarly without enzyme. The released glycans (in the salt fraction) were purified by passage over a GS-1-Sepharose column. The glycans were eluted with glycine·HCl, pH 3.0. The neutralized sample was lyophilized. After dissolving in 2 ml of distilled water, the carbohydrate content was estimated by the anthrone method. The DG-IgD was analyzed for its reactivity with peroxidase-conjugated GS-1 and anti-IgD in immunodiffusion gels and dot-blot assays. The released glycans were shown to inhibit immunoprecipitin reactions between IgD and GS-1 in immunodiffusion gels.

RESULTS

Low-M_r IgD Fragments Block IgD Interaction with IgD-R. Initially, we analyzed the consequences of heat denaturation and complete proteolytic enzymic digestion of IgD vis-a-vis IgD-R binding. TEPC-1033 IgD, after boiling for 10 min, no longer reacted with polyclonal goat anti-IgD antiserum (data not shown) but still competitively inhibited IgD-rosette formation with TEPC-1017 IgD-coated sheep erythrocytes (Table 1). Complete Pronase or proteinase K digestion of IgD resulted in fragments of M_r <5000, which could still competitively inhibit IgD-RFC. Low-M_r IgM, IgA, or IgG2a fragments, produced by an identical procedure, failed to inhibit significantly IgD-RFC (Table 1), even at concentrations of 150 μg per assay (data not shown). These findings demonstrate not only that the inhibition was specific for IgD fragments but also that contaminating enzymes and/or reagents used to prepare the IgD fractions did not cause the IgD-RFC inhibition.

We have also demonstrated that a GS-1 with specificity for Gal and GalNAc binds exclusively to murine IgD and to no other murine immunoglobulin isotype or any other proteins in ascites (9). Like the IgD-R (3, 5), it is, therefore, completely specific for IgD among all mouse immunoglobulin isotypes. Completely protease-digested IgD retained its ability to precipitate with GS-1 on double diffusion in agar, whereas neither intact nor digested IgM or IgA showed any precipi-

Table 1. Competitive inhibition of IgD rosetting by low-M_r IgD fragments with affinity for GS-1

Blocking agent*	IgD-RFC inhibition,† %		
	Exp. 1	Exp. 2	Exp. 3
Undigested TEPC-1033/1017 IgD (50 μg)	69 ± 1	70 ± 4	74 ± 2
Boiled IgD (10 min at 100°C)	54 ± 1		
Low-M_r fragments of Pronase-digested IgD (F1)	75 ± 3	58 ± 1	
Low-M_r fragments of proteinase K-digested IgD (F2)	55 ± 3	51 ± 1	55 ± 5
Low-M_r fragments of Pronase-digested UPC-10 IgG2a (F3)	7 ± 6		
Low-M_r fragments of proteinase K-digested 3A6 IgA (F4)			2 ± 5
Low-M_r fragments of proteinase K-digested TEPC-187 IgM (F5)			2 ± 5
Combined low-M_r fragments of Protease-digested IgD (F1 + F2)		62 ± 4	
Boiled low-M_r IgD fragments (F1 + F2)		45 ± 8	
Low-M_r fragments of IgD (F1 + F2) after absorption with GS-1-Sepharose		2 ± 3	
Low-M_r fragments of IgD (F1 + F2) after absorption with BSA-Sepharose		49 ± 3	

*In Exp. 1 and 2, blocking agents (prepared from TEPC-1033 IgD) were added at 50 μg per rosetting mixture of splenic T cells and IgD-coated sheep erythrocytes (300 μl). In Exp. 3, boiled fragments (10 min at 100°C), prepared from 75 μg of TEPC-1017 IgD, IgM, or IgA, were added to the rosetting mixtures. Because digestion was complete and all fragments were <5000 in M_r, the fragments in Exp. 3 were not further fractionated before assay. In Exp. 1 and 2, low-M_r fragments were prepared as described. Absorption of low-M_r fractions with GS-1 was achieved by passage over GS-1-coupled Sepharose 4B.

†Splenic T cells were induced to express IgD-R by overnight incubation with IL-4 (10 units/ml). Control IgD-RFC values (i.e., no blocking agent added to assay cell plus IgD-RBC mixtures) were 35 ± 6% (Exp. 1), 34 ± 3% (Exp. 2), and 26 ± 2% (Exp. 3) (n = 3 or 4). Results are expressed as mean ± SD. Percentages of IgD rosette inhibition were calculated with the following formula: 100 − 100 × (% IgD-RFC above BSA-RFC background in blocked samples/% IgD-RFC above BSA-RFC background on control sample).

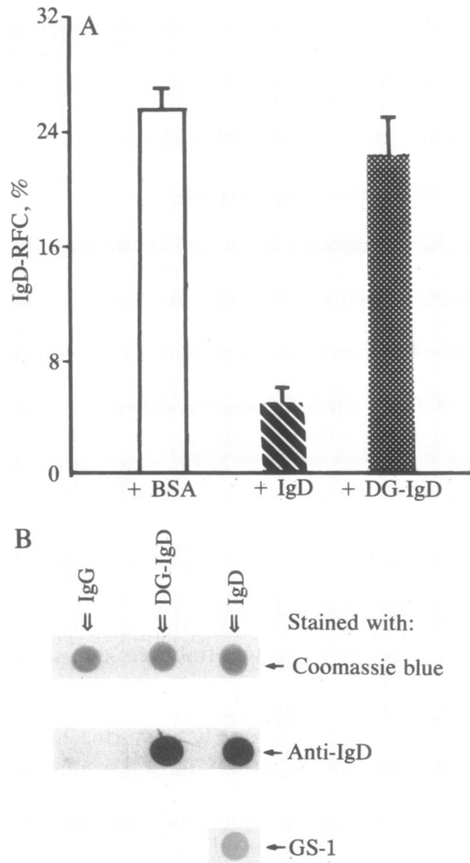

FIG. 1. Inability of DG-IgD to inhibit IgD rosetting. Splenic T cells were isolated and induced to express IgD-R as described. (*A*) Competitive blocking of IgD-RFC was done by using 50 μg of partially DG-IgD (IgD lacking N-linked glycans) and control IgD per assay, respectively. (*B*) One microgram of IgD, DG-IgD, and IgG was dot blotted onto nitrocellulose paper in sets of three. Set 1 was stained with Coomassie blue R-250, set 2 was probed with phosphatase-conjugated sheep anti-mouse IgD (Pel-Freez Biologicals), and set 3 was probed with peroxidase-conjugated GS-1.

tation. To investigate the possible contribution of IgD-associated carbohydrates in the IgD digests to the rosette-inhibition activity, we determined the ability of the lectin to absorb this activity. Absorption with Sepharose-bound GS-1, which binds secreted murine IgD via its N-linked carbohydrates (9), fully removed the IgD-inhibitory activity from the Pronase and proteinase K digests, whereas BSA-Sepharose did not (Table 1).

N-Linked Carbohydrate Moieties Are Involved in the Interaction of IgD with IgD-R. N-linked sugars were removed from IgD through treatment with N-glycanase. This DG-IgD, which did not bind to GS-1, still interacted with anti-IgD (Fig. 1*B*) but failed to significantly inhibit IgD rosetting (Fig. 1*A*). DG-IgD caused 12 ± 13% inhibition, as compared with 74 ± 2% inhibition by monomeric B1-8.δ1 IgD (data not shown) and 80 ± 4% inhibition by dimeric TEPC-1017 IgD. The carbohydrates released during hydrolysis by N-glycanase were purified on GS-1-Sepharose and tested for their capacity to block rosetting (Table 2). A dose-dependent inhibition of IgD rosetting was obtained, not only when the indicator erythrocytes were coated with intact IgD but also when they were coated with mutant molecules Gen.24 or KWD6 (Table 2). Approximately 10–15 μg of N-glycans per ml was needed to obtain 50% inhibition. As previously shown by their reactivity with GS-1, both the mutant IgD proteins contain N-linked glycans (7).

Monosaccharides Can Competitively Inhibit the Binding of IgD to its Receptor. Because GS-1 binds GalNAc as well as Gal (13), we examined whether these sugars could also inhibit IgD-RFC. GalNAc caused a highly significant, dose-related inhibition of rosette formation (Fig. 2). However, assuming an average molecular size of 10 sugar residues for the isolated mixture of N-glycans from IgD, this monosaccharide was much less effective (0.1 mM) with respect to 50% inhibition of IgD rosetting than the IgD-associated N-glycans (5 μM). Gal and GlcNAc were less effective than GalNAc. Significant competitive inhibition of IgD-RFC was not seen with Man, Glc, or the disaccharides lactose, melibiose, β-D-Gal-(1→3)-D-GalNAc or α-D-Gal-(1→4)-D-Gal (data not shown).

Neoglycoproteins, such as α-D-GalNAc–BSA, α-D-GlcNAc–BSA, and α-D-Gal–BSA, when added at a concentration of 3 μM (as protein) to Tδ cells, blocked IgD rosetting by 76.1%, 43%, and 39.8%, respectively, as compared with dimeric TEPC-1017 IgD, which causes 50% inhibition at 0.15 μM (7). On the other hand, α-D-lactose–BSA, α-D-Man–BSA, α-D-Man-6-PO_4–BSA, α-L-Fuc–BSA, and β-D-Glc–BSA did not significantly inhibit at the same concentration; these results are consistent with those obtained with the uncoupled monosaccharides. Moreover, the three inhibiting neoglycoproteins precipitate with GS-1 on double diffusion in agar at 4°C, whereas the other neoglycoproteins do not.

Various Gal-/GalNAc-rich purified polysaccharides of bacterial origin were also tested for their ability to inhibit IgD rosetting. These polysaccharides included pneumococcal

Table 2. Competitive inhibition of murine IgD-RFC by purified N-linked glycans obtained from IgD

Sheep erythrocyte coat	Blocking agent*	IgD-RFC† (% block) Exp. 1	Exp. 2
TEPC-1017 IgD	None	28 ± 2	25 ± 2
	IgD (50 μg)	4 ± 1 (86)	3 ± 0.4 (88)
	Glycan (5 μg)	9 ± 0.3 (68)	8 ± 0.5 (69)
	Glycan (2.5 μg)	ND	19 ± 2 (27)
Gen.24	Buffer	24 ± 0.3	ND
	Glycan (5 μg)	9 ± 1 (63)	ND
KWD6	Buffer	29 ± 1	ND
	Glycan (5 μg)	12 ± 1 (59)	ND

Splenic T cells were induced to express IgD-R by overnight incubation with IL-4 (10 units/ml). ND, not done.

*Blocking agents were all derived from TEPC-1017 IgD. Low-M_r fractions containing N-glycans from IgD, prepared as described, were passed over GS-1-Sepharose. Adherent glycans were eluted with glycine·HCl, pH 3.0, neutralized to pH 7.0 with 1 M Tris, and lyophilized. Equal amounts of similarly neutralized glycine·HCl were used as control buffer.

†BSA-RFC (<2%) was subtracted. Percentage block was calculated as described for Table 1 (n = 3).

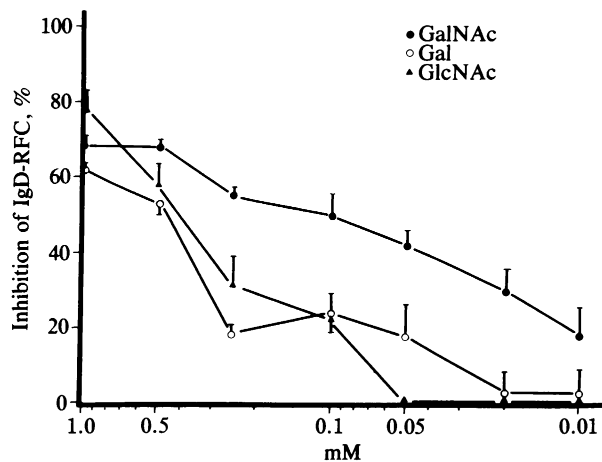

FIG. 2. Carbohydrate specificity of IgD-R. BALB/c splenic T cells were isolated and induced to express IgD-R with IL-4 (10 units/ml) as described (7). Various carbohydrates were added as blocking agents at the doses indicated. Percentages of inhibition were calculated as described for Table 1. IL-4-induced Tδ cells, rosetted with IgD-coated erythrocytes in the absence of any blocking agent, showed a mean IgD-RFC value of 25 ± 2% (n = 3).

polysaccharides S1, S4, S8, S11a, S13, S14, S15, and S29, and *Klebsiella* polysaccharides K11, K12, K16, K18, K21, K22, K23, K24, K25, K31, K38, K41, K51, K53, K56, K74, and K83 (10). Two of these polysaccharides gave a substantial degree of inhibition: K11 caused 29 ± 9% and 64 ± 13%, and K25 caused 30 ± 1% and 50 ± 8% inhibition of IgD-RFC, each at 25 and 100 μg per RFC mixture, respectively. None of the other polysaccharides inhibited by >10% at 25 μg per rosette mixture.

DISCUSSION

In view of the high content of N-linked glycans in murine IgD (14, 15) and the previous demonstration that N-linked glycans of IgD are solely responsible for the binding of IgD to the Gal/GalNAc IgD-specific GS-1 (9), N-linked glycans from IgD were tested for their ability to inhibit IgD rosetting and found active at very low concentrations. Assuming a 10–14% content of carbohydrate for IgD, the effectiveness of the glycans on a wt/vol basis as compared with intact IgD suggests that it alone could be responsible for the rosette inhibition. A change in tertiary structure could, of course, have contributed to the absence of inhibitory activity in DG-IgD. In addition, we cannot exclude the possibility that the protein-backbone structure contributes to the stabilization of IgD–IgD-R complexes after initial binding of the glycans by the receptors.

Thus, the present observations show that the IgD-R functions as a lectin in its interaction with IgD. Further studies are needed to determine whether the IgD-R is at all related to the Fcε receptor II (CD23) or to endothelial leukocyte adhesion molecule 1 (ELAM-1) and gp90^{MEL} of the selectin family, which are also lectin-like molecules (16–19). Some of the carbohydrate ligands of selectins have been identified (20). Results from recent studies also indicate that transfection of a specific α(1,3)fucosyltransferase cDNA into nonmyeloid cell lines results in the *de novo* expression of functional ligands for ELAM-1-mediated cell adhesion (21). Unpublished findings in our laboratory have shown that the Gly-Arg-Gly-Asp-Ser pentapeptide, the sequence of fibronectin recognized by an integrin (22), does not inhibit IgD rosetting.

Among the immunoglobulin-specific receptors the IgD-R are therefore unique because there is an absolute requirement for the presence of carbohydrate on its ligand for binding. Fcγ receptor does not have a strict requirement for glycans on IgG for its binding (23). Although Fcε receptor II exhibits a lectin-like domain in its structure important for the interaction with IgE, it does not predominantly recognize carbohydrate moieties of IgE (24, 25). It is of interest to note that Ca^{2+} is required in the interaction between IgE and Fcε receptor II (26), as it is for the interaction between IgD-R and IgD (27) and for a variety of C-type lectins (28). In view of recent observations that human T cells also express receptors for IgD (2), similar studies as done here for murine IgD will be needed to determine whether human IgD-R also exhibit lectin-like properties. Human IgD fails to inhibit murine T-cell IgD rosetting and vice versa (R.F.C., unpublished work). Functional studies for human Tδ cells have not yet been reported. However, the observation that IgD enhances human T-cell proliferation responses to mitogen (29) may be relevant in this respect.

The specificity of the glycan recognition by murine IgD-R appears to involve Gal, GalNAc, and/or GlcNAc because these three monosaccharides significantly inhibit IgD rosetting, whereas Man and Glc do not. This result agrees with the fact that murine IgD is very rich in Gal residues (14). The lectin specificity of murine IgD-R resembles that of GS-1, allowing us to use GS-1 in the isolation of IgD-associated glycans that inhibit IgD rosetting. On further analysis of the specificity, none of the Gal- or GalNAc-containing disaccharides examined inhibits IgD rosetting, suggesting that none of the linkages represented in these disaccharides can mimic the structure as well as does free Gal. αGal(1→6)Glc (melibiose) has high affinity for GS-1, but fails to inhibit IgD rosetting, whereas βGal(1→4)Glc (lactose) does not appear to have high affinity for either GS-1 or IgD-R. Because one monosaccharide molecule, such as Gal, can be linked to another one in at least 16 different ways, it is not surprising that the two disaccharides used do not present the right configuration for recognition by IgD-R. Inspection of the structures of the bacterial polysaccharides used (10, 30) fails to reveal a common repetitive disaccharide configuration peculiar to the two polysaccharides that partially inhibit IgD rosetting at 300 μg/ml, K11 and K25.

Because the IgD-R, as well as the lectin GS-1, recognize only IgD among all murine immunoglobulins, the N-linked glycans available on the IgD molecule appear totally specific for IgD. This specificity is apparently reflected in the functional properties of IgD because it and not other immunoglobulins augment antibody production (3). Surface IgD on B cells seems not only to be a receptor for antigen but also to double as a ligand for IgD-R on T-helper cells. A lectin-like property of IgD-R on Tδ cells, such as described here, highlights it as a candidate adhesion molecule, as does its ability to recognize and bind the $C_\delta1$ region of B-cell surface IgD (31). The binding of IgD by IgD-R would strengthen the cognate T cell–B cell interaction, irrespective of the possible additional secondary signals that may be generated in the T-helper cells (via IgD-R) or B cells (via surface IgD) due to receptor–receptor crosslinking and resulting cross-talk between cells. The ultimate effect is enhanced production of all isotypes, except of IgD itself (32), as well as augmented primary and secondary responses (3).

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