Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9233–9237, October 1991
Immunology

IgD receptors on murine T-helper cells bind to Fd and Fc regions of immunoglobulin D

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Communicated by H. Sherwood Lawrence, July 18, 1991‖

ABSTRACT **Receptors for immunoglobulins on animal cells invariably show specificity for Fc regions of the protein and are hence called Fc receptors. The present study shows that immunoglobulin D receptors present an exception to this rule. Binding of IgD-coated erythrocytes to murine IgD-receptor-bearing T-helper cells is competitively inhibited by IgD, by its Fabδ fragments, and by deletion mutants of IgD lacking (*i*) the first constant domain of the δ heavy chain (KWD1), (*ii*) that region plus the δ heavy-chain-hinge region (KWD6), or (*iii*) the third constant domain of the δ heavy chain (Gen.24). KWD1, Gen.24, or KWD6 mutants bind to T-helper cells bearing receptors for IgD independently of each other. Furthermore, Gen.24 and KWD6 mutants also competitively inhibit binding of each other in cross-blocking experiments. These results show that the IgD receptor binds to the Fdδ and the Fcδ and cannot readily be explained by sequence homology between the two parts of the IgD molecule.**

IgD is expressed on the majority of mature B lymphocytes and is found in low quantity in serum (1). Its role in the humoral immune response is not known. We have recently shown the presence of IgD receptors (IgD-R) on T cells from mice harboring IgD-secreting plasmacytomas, TEPC-1017 or TEPC-1033, or injected with IgD produced by these plasmacytomas. Such mice exhibit significantly enhanced antibody responses of all isotypes except IgD (2, 3). The augmented ability to produce antibodies can be transferred from IgD-treated to normal mice by $CD4^+$, Lyt 1^+, $CD8^-$ T cells (4), the same subset of T cells that also exhibit IgD-R as shown by their capacity to form rosettes with IgD-coated sheep erythrocytes (IgD-SRBC); these cells bearing receptors for IgD have been called Tδ cells (5). Results from these studies indicate that IgD is a cell-membrane receptor involved in T–B cell interaction (6, 7).

Immunoglobulin isotype-specific Fc receptors (i.e., FcγR, FcεR, FcμR, and FcαR) appear on cells such as macrophages, granulocytes, and lymphocytes. Some of these receptors trigger various functions, such as phagocytosis, antibody-dependent cytotoxicity, and the secretion of potent mediators (8, 9). We previously established the isotypic specificity of IgD-R-bearing T-helper (Tδ) cells by showing that IgD, at concentrations ≥120 μg/ml (1.0 μM), competitively inhibits Tδ-cell rosetting, whereas IgM, IgG1, IgG2a, IgG3, IgA, or IgE fail to do so (5, 6). Exposure of T cells to oligomeric secreted IgD (TEPC-1017 or -1033) or to antigen-crosslinked monomeric secreted IgD (such as B1-8.δ1, a monoclonal antibody of IgD isotype) or to B-cell surface IgD crosslinked by anti-IgD or anti-immunoglobulin causes up-regulation of IgD-R on these cells, both *in vitro* and *in vivo* (10). B cells with crosslinked surface IgM do not cause such IgD-R up-regulation. Interleukin 2, interleukin 4, and interferon γ also up-regulate IgD-R on $CD4^+$ polyclonal or cloned T cells (6, 11, 12).

We sought to identify the heavy-chain domains of IgD involved in the interaction with the IgD-R on T-helper cells, and we report that IgD-R on T-helper cells are not exclusively Fc receptors but also bind equally well to the Fd domain of IgD.

MATERIALS AND METHODS

Mice and Cell Lines. Six- to eight-week-old BALB/c and CB6F1 mice were obtained from Charles River Breeding Laboratories. Two IgD-secreting plasmacytomas TEPC-1017 and -1033 (13) were maintained i.p. in pristane-primed BALB/c mice. The hybridoma B1-8.δ1, secreting IgD specific for the hapten 4-hydroxy-3-nitrophenyl-acetyl, was from K. Rajewsky (Institute for Genetics, University of Cologne, Cologne, F.R.G.): this hybridoma was maintained i.p. in CB6F1 mice. The T-cell hybridoma (2H10) of helper phenotype and specific for cytochrome *c* was maintained in Click's/RPMI 1640 (1:1) medium; this hybridoma was provided by R. H. Schwartz (National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Reagents. Hδ^a/1 and AMS-15 are monoclonal antibodies specific for Fcδ and Fdδ, respectively (14, 15). Rabbit anti-mouse IgD and Fab were prepared as described (5). Purified lectin from *Griffonia simplicifolia* 1 (GS-1) was donated by EY Laboratories. Purified $F(ab')_2$ fragments of IgG were from W. O. Weigle (Scripps Institute for Medical Research, La Jolla, CA). Recombinant interleukin 4 (rIL-4) produced by myeloma transfectants (16) was used as a source of rIL-4, standardized by comparison with rIL-4 obtained from M. Howard (DNAX). Peptides KINLGCLVIGSQPLKI derived from the first constant region of δ heavy-chain ($C_δ1$) and SSWLLCEVSGFFPENI from the third constant region of δ heavy chain ($C_δ3$) were prepared by solid-phase synthesis (17) with a model 430A Applied Biosystems peptide synthesizer; their purity was determined by HPLC.

Purification of Splenic T cells. T cells were prepared by sequential depletion of adherent cells at 37°C in Petri dishes (1400-1; Nunclon, Rockilde, Denmark) and of B cells by negative selection (18) at 4°C on Petri dishes coated with

Abbreviations: SRBC, sheep erythrocytes; RFC, rosette-forming cell(s); IgD-R, IgD receptor(s); rIL-4, recombinant interleukin 4; BSA, bovine serum albumin; $C_δ$, constant region of the δ heavy chain; GS-1, lectin from *Griffonia simplicifolia* 1; Tδ, T-helper cells bearing receptors for IgD.

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‖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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affinity-purified anti-mouse immunoglobulin (remaining Ig^+ cells <1%).

Purification of IgD. Purification of IgD was done by slightly modifying the method of Finkelman *et al.* (13). IgD was also purified by affinity chromatography on an IgD-specific GS-1-Sepharose column and eluted with galactose (19). Mutant IgD molecules Gen.24, KWD1, and KWD6 were affinity purified over a goat anti-mouse IgD-Sepharose column, as monitored by double diffusion in agarose gel, SDS/PAGE, and ELISA.

Enzymatic Treatment of IgD. Purified TEPC-1017 IgD was digested for 5 hr at 37°C with immobilized papain, as prescribed by the manufacturer (Pierce). Fcδ fragments were subsequently isolated by affinity chromatography with the Fcδ-specific, $H\delta^a$/1-Sepharose column. Fabδ fragments of naturally degraded purified IgD were also isolated by passage through this column (see Fig. 2). SDS/PAGE was done under reducing and nonreducing conditions.

Rosette-Forming Cell (RFC) Assay. Splenic T cells (2.5×10^6) were incubated at 37°C for 18 hr in 1 ml of RPMI 1640 medium/2% fetal calf serum with rIL-4 (10 units·ml^{-1}) or crosslinked IgD (100 μg/ml) overnight. These resulting Tδ cells were used as IgD-R bearing cells in RFC assays. T cells were examined for their expression of receptors for IgD by a rosetting method (5). Purified IgD-, Gen.24-, KWD1-, KWD6-, or bovine serum albumin (BSA)-coated SRBC were prepared by the $CrCl_3$-coupling method (20). T cells surrounded by more than three indicator cells (minimal definition) were scored as rosettes and recorded as percent RFC. It should be noted, however, that most rosettes were represented as daisy- or morula-like structures, with occasional RFCs showing this minimal definition. Percentages of cells rosetting with BSA–SRBC were subtracted (<5 ± 1 for normal T cells and <24 ± 6 for hybridoma T cells). Rosette-inhibition assays were done with rIL-4- or IgD-induced Tδ cells as IgD-R^+ T cells. IgD-R^+T cells were incubated with various purified test proteins—i.e., IgD, Gen.24, KWD1, Fabδ, Fcδ, or KWD6—on ice for 30 min and then rosetted with IgD–SRBC in 300 μl. Percentages of IgD rosette inhibition were calculated with the formula: 100 − 100 × (% IgD-RFC above BSA–RFC background in blocked sample/% IgD-RFC above BSA–RFC background in control sample).

RESULTS

IgD-R Independently Recognize $C_\delta 1$ and $C_\delta 3$ Regions. Mouse IgD, unlike human IgD, lacks the second constant region of the δ heavy-chain domain (21). It therefore has $C_\delta 1$, C_δ-hinge, and $C_\delta 3$ domains. To determine the portion of the IgD molecule recognized by IgD-R, we examined the ability of mutant IgD molecules lacking one or more heavy-chain domains to bind to $CD4^+$ Tδ cells in rosetting assays when used to coat SRBC. Their ability to inhibit rosetting of $CD4^+$ T hybridoma or splenic Tδ cells with IgD-coated SRBC was also tested. Although all mutant IgD molecules used have intact variable region heavy-chain and light-chain domains, KWD1 lacks the $C_\delta 1$ domain (22), whereas KWD6 lacks both $C_\delta 1$ and C_δ-hinge (J. D. Owens, F.D.F., J. D. Mountz, and J. F. Mushinski, unpublished work) (Table 1). These deletions of heavy-chain domains were demonstrated by RNA

Table 1. Properties of mutant IgD molecules and of Fabδ

Property	DNA/mRNA or protein				
	TEPC-1017 Gen.24	KWD1	KWD6	TEPC-1017 IgD	TEPC-1017 Fabδ
2H + 2L protein,* kDa	100	90	85	135	66–70
mRNA size,† kb	1.15	1.1	1.05	1.75	
DNA/mRNA reactivity					
V_H probe	+	+	+	+	
$C_\delta 1$ probe	+	−	−	+	
$C_\delta H$ probe	±	+	−	+	
$C_\delta 3$ probe	−	+	+	+	
Protein reactivity‡					
Rabbit anti-IgD	+++	+++	++++	+++	+++
Rabbit anti-Fab	++	++	++	++	+++
$H\delta^a$/1 (mAb to Fcδ)	−	+++	+++	++	−
mAb AMS-15	++++	±	±	++++	ND
GS-1-peroxidase	++	++	+	+++	+
RFC,§ %					
Splenic Tδ cells	28 ± 3	15 ± 2	23 ± 2	21 ± 2	ND
2H10 cells	57 ± 8	37 ± 4	46 ± 3	69 ± 5	ND
Inhibition of IgD-RFC,¶ % ± SD					
100 μg	ND	ND	ND	91 ± 5	ND
40–50 μg	82 ± 6	94 ± 4	ND	88 ± 8	ND
20–25 μg	88 ± 2	90 ± 1	ND	69 ± 4	50 ± 2
10–12.5 μg	60 ± 8	73 ± 3	ND	17 ± 2	12 ± 2
5–6 μg	43 ± 6	63 ± 5	ND	0	0

V_H, variable region heavy chain; mAb, monoclonal antibody. ND, not done.

*Assayed by SDS/PAGE.

†Size was assayed by Northern blots on RNA from mutant protein-producing cells with domain-specific oligomer cDNA probes (22).

‡Reactivity was assayed by agglutination of chromic chloride-treated coated SRBC and/or by immunoblotting and ELISA.

§Each indicated protein was used to coat erythrocytes; the coated erythrocytes were then tested for their ability to detect IgD-RFC. BSA–RFC backgrounds, already subtracted, were 5 ± 1% (T cells) and 24 ± 6% (2H10 cells).

¶For IgD-RFC method, see text. Assay cells rosetted with TEPC-1017 IgD-SRBC without blocking agents gave 30–38% rosettes. Other immunoglobulin isotypes showed no inhibitory effect in this assay. Amounts of protein added per assay volume (300 μl) are given in the table.

blotting and by ELISA with monoclonal anti-δ antibodies (Table 1). In contrast, Gen.24, produced by a spontaneous variant of TEPC-1017 IgD-producing cells, lacks $C_\delta 3$ while possessing $C_\delta 1$ and part of the C_δ-hinge (23). All mutant IgD molecules were found to bind GS-1, which was shown to bind specifically to the N-linked glycans of murine IgD (19).

We found that not only intact dimeric (TEPC-1017) and monomeric (B1-8.δ1, data not shown) IgD blocked IgD rosetting approximately equally, but the mutant IgD molecules examined also blocked to the same extent (Table 1), especially when the amount for ≈50% inhibition by these molecules is compared on a molar basis. Considering that TEPC-1017 is present as a dimer (260 kDa, ref. 13), its molar effectiveness is quite comparable to that of Gen.24 (100 kDa) and KWD1 (90 kDa). In addition, all of the mutant proteins mediated rosette formation by $CD4^+$ splenic Tδ cells or by 2H10 $CD4^+$, IgD-R^+ T-hybridoma cells, although KWD1-coated erythrocytes gave a somewhat lower percentage of RFC than KWD6 or Gen.24 mutants (Table 1). To confirm the data obtained with KWD6 molecules, we attempted to isolate Fcδ molecules but failed to obtain Fcδ fragments sufficiently homogeneous in size to confidently be used in inhibition studies. The heterogeneous preparation obtained, which reacted with Hδ^a/1, did not react with rabbit anti-Fab, and had an average size of 40 kDa, inhibited IgD rosetting only 23 ± 1% at 120 μg/ml.

Fd and Fc Regions of IgD Compete for Binding to IgD-R. The previous results show that KWD6 and Gen.24 mutants, in spite of their lack of $C_\delta 1$ plus C_δ hinge and $C_\delta 3$ domains, respectively, each have a determinant recognized by the IgD-R. The identity of these determinants was further examined in the experiments shown in Fig. 1 by RFC cross-blocking experiments. KWD6 and Gen.24 mutants were equally effective in blocking rosetting with Gen.24-coated SRBC, whereas KWD6 was quantitatively more effective than Gen.24 in blocking the rosetting with KWD6-coated SRBC, although blocking was obtained with both. These results show that the ($C_\delta 1$ plus C_δ-hinge) and $C_\delta 3$ domains of IgD can independently bind to the IgD-R and competitively inhibit each other for binding to the same receptor.

Common Binding Site for IgD-R Is Not Directly Determined by Amino Acid Sequence Homology. The tailpiece of the murine-secreted IgD is considerably longer (21 residues) than that of human IgD (24). All of the mutant molecules presumably share this C-terminal amino acid sequence. To determine whether these residues played any role in the binding of these molecules to the IgD-R, we prepared Fabδ fragments. We isolated Fabδ fragments by passing spontaneously degraded purified IgD over an Fcδ-specific Hδ^a/1-Sepharose affinity column. As shown in Fig. 2, two IgD fragments of ≈66–70 kDa as well as a 90-kDa fragment were present in a stored preparation of TEPC-1017 IgD. Hδ^a/1-Sepharose bound the 90-kDa and the 130-kDa intact IgD but not the 66- and 70-kDa fragments. Reduction of the 66- and 70-kDa fragments generated immunoglobulin light chains (25 kDa) and 32- and 36-kDa heavy-chain fragments. Immunoblotting of these unreduced IgD fragments showed that they reacted with both rabbit anti-Fab and rabbit anti-IgD (Fig. 2), while by ELISA they reacted with AMS-15 antibody (Fdδ-specific) but not with Hδ^a/1 (data not shown). The Fabδ fragments were >95% pure, as estimated by ELISA (data not shown).

As was true for the mutant proteins, Fabδ fragments, but not IgG Fab molecules (data not shown), bound to IgD-R, as shown by their ability to inhibit Tδ rosette formation with SRBC coated with intact IgD (Table 1). However, on a molar basis, Fabδ was less effective in inhibiting IgD rosetting than the corresponding Gen.24 molecule. This discrepancy can possibly be explained by the lower avidity of single δ chain versus double δ chain containing molecules. The effectiveness of the Fabδ in rosette inhibition shows that the C-terminal amino acid residues of the secreted form of IgD are not necessary for binding to IgD-R.

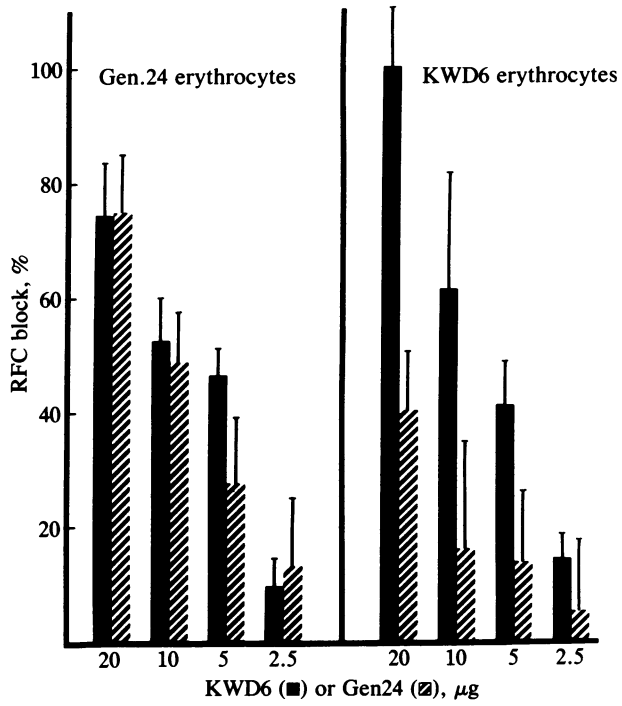

FIG. 1. Cross-inhibition of rosette formation by Gen.24 and KWD6 IgD. Splenic T cells were prepared and induced to express IgD-R by rIL-4 exposure, as described. Indicator cells were prepared by coupling the mutant myeloma protein Gen.24 and the mutant IgD hybridoma KWD6 to SRBC. IgD-R^+ T cells (2.5×10^5 in 0.1 ml) were incubated with or without a range of concentrations of either Gen.24 or KWD6 (20, 10, 5, and 2.5 μg) for 30 min on ice and then rosetted with Gen.24- and KWD6-coated erythrocytes with the blocking agents to determine efficiency of cross-inhibition of rosetting by these mutant IgD molecules. Results are expressed as mean % ± SD (n = 3). Without inhibitors, IgD-RFC values were 27–32% after subtracting 3–5% BSA–RFC values.

Another possibility to explain cross-inhibition by $C_\delta 3$- and $C_\delta 1$ plus hinge-containing molecules could be through some common determinants in these regions of IgD, such as amino acid sequence homology or common carbohydrate moieties. $C_\delta 1$ and $C_\delta 3$ at positions 28–40 and 24–36, respectively, show a significant degree of homology (6/13 amino acid residues) (21). These two peptides together with their neighboring residues (16-mer) were synthesized and used as inhibitors of IgD rosetting. No inhibition was seen at concentrations as high as 300 μg/ml (data not shown).

DISCUSSION

The Fcγ receptor principally recognizes the $C_\gamma 2$-hinge region of IgG (for review, see ref. 25), whereas Fcε receptor I recognizes residues 301–376 roughly centered in the interface between the second and third constant regions of the ε heavy-chain (for review, see ref. 26). The Fcμ receptor recognizes the third constant region of μ heavy chain (27, 28).

The present results show that IgD-R reacts with two entirely nonoverlapping parts of the IgD molecule—the $C_\delta 1$ and $C_\delta 3$ domains. The cross-inhibition of rosetting between the mutant IgD molecules KWD6 and Gen.24 indicates that regions of both $C_\delta 1$ and $C_\delta 3$ are involved in the interaction with the same receptor. In addition, Fab fragments of IgD can inhibit rosetting of Tδ cells with IgD-coated erythrocytes. This observation rendered unlikely the possibility that the tailpiece of IgD was responsible for the cross-inhibition of

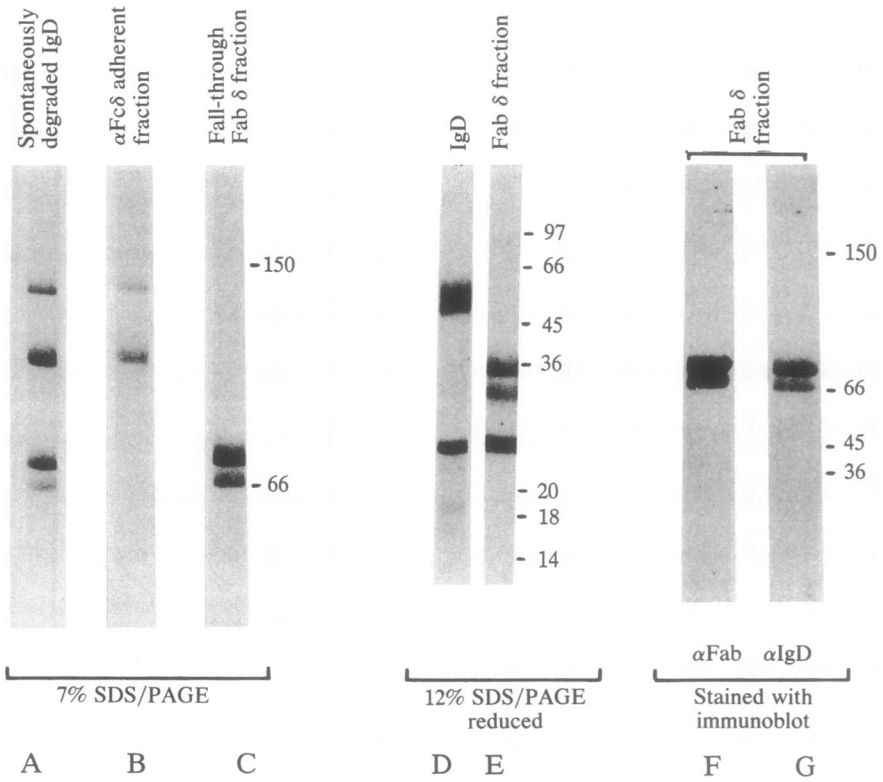

FIG. 2. SDS/PAGE analysis of affinity-purified, naturally degraded TEPC-1017 IgD fragments. Partially degraded IgD fragments were subjected to SDS/PAGE (7%) under nonreducing conditions (lanes A–C). Lanes: A, naturally degraded TEPC-1017 IgD molecules of 130, 100, 70, and 66 kDa; B, 4 M $MgCl_2$ eluate of anti-Fcδ (Hδ^a/1)-adherent TEPC-1017 IgD fragments of 130 and 100 kDa; C, Hδ^a/1 nonadherent Fabδ fragments of 70 and 66 kDa; D, intact TEPC-1017 IgD molecules subjected to gel electrophoresis under reducing conditions showing heavy (54 kDa)- and light (25 kDa)-chain molecules; E, same as lane C but under reducing conditions; F and G, 9% SDS/PAGE, Fabδ fragments, immunoblotted, and probed with anti-Fab (lane F) and anti-IgD (lane G), respectively. Positions of molecular mass markers are in kDa.

rosetting seen between Gen.24 and KWD6. Moreover, these results could not readily be explained by homology in the $C_\delta 1$ and $C_\delta 3$ polypeptide-backbone structures. Oligopeptides, corresponding to the most homologous region of $C_\delta 3$ with $C_\delta 1$, did not inhibit rosetting. In addition, this region also shows strong homology with the murine fourth constant region of μ heavy-chain (21), whereas IgM fails to interact with IgD-R (5). The results show, on the other hand, that the mutant molecules and the Fabδ share with IgD the ability to bind to GS-1, a lectin previously shown to specifically bind N-glycans isolated from IgD, while unable to bind deglycosylated IgD. This result suggests that further studies on the role of carbohydrate moieties in the binding of IgD to IgD-R are required.

The mechanism by which Tδ cells augment B-cell responses has not been resolved. It has been suggested that soluble IgD-binding factors, which are released by Tδ cells, may contribute to their immunoaugmenting properties (29). The current IgD-R specificity data are consistent with our previously hypothesized functional role for Tδ cells in regulating humoral immune responses (6). We have speculated that Tδ cells interact more efficiently with IgD$^+$ B cells subsequent to antigen-induced crosslinking of membrane IgD molecules. This speculation was from our observations that (*i*) both primary and secondary antibody responses are augmented by injections of IgD before the primary injection of antigen (2), and (*ii*) B cells with crosslinked surface IgD induce up-regulation of IgD-R on T cells *in vivo* and *in vitro* (10). Crosslinking of surface IgD with the $C_\delta 3$-specific monoclonal antibody Hδ^a/1 also causes such IgD-R up-regulation. Because this antibody might be expected to sterically hinder interaction of IgD-R with the $C_\delta 3$ portion of IgD, a role for $C_\delta 1$ in this IgD-R up-regulation is likely. In agreement with these findings, our results show that the portion of the IgD molecule available on the surface of B cells can bind to IgD-R of T cells, pointing to the possibility that Fabδ–antigen complexes, released from the surface of B cells by cleavage of the IgD molecule, could function in regulation of the immune response by up-regulating IgD-R on T cells. Because the idiotype of the IgD molecules would be present in such complexes, a T-cell-mediated idiotype-specific influence on the immune response, as has been proposed (30), could be an integral part of the immunoregulatory effect.

We conclude that, at least in the mouse, the receptor on T cells for IgD is not limited to the Fc region; therefore, these receptors should be referred to as IgD-R, rather than as Fcδ receptors.

S.M.L.T. and A.R.A. contributed equally to this work. We are grateful to Gregory Stanton from EY Laboratories for the generous donation of GS-1 lectin. This work was supported by U.S. Public Health Service Grants AI22645, RR03060, AG04860, RR07123, and A1-26113; Professional Staff Congress–City University of New York Award 668227; and Uniformed Services University of the Health Sciences Research Protocol RO 8308. A.R.A. was the recipient of a postdoctoral fellowship from the National Cancer Institute (Training Grant CA-09161).

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