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Immunology

Release of IgD-binding factor by T cells under the influence of interleukin 2, interleukin 4, or cross-linked IgD

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ABSTRACT **Helper T cells with receptors specific for IgD have immunoaugmenting properties. We have now detected soluble IgD-binding factor in cell supernatants immobilized on nitrocellulose paper by their ability to bind ^{125}I-labeled IgD. IgD-binding factor is released by normal splenic T cells stimulated with recombinant interleukin 2, recombinant interleukin 4, or crosslinked IgD in amounts paralleling the induction of IgD receptors on the cells. IgD receptors are constitutively produced by antigen-specific helper T-cell hybridomas 2H10 and A3.4C6. Incubation of these hybridoma cells with recombinant interleukin 2 increases release of IgD-binding factor while reducing expression of IgD receptors. Specificity of the binding factor for IgD is established by (*i*) competitive inhibition; (*ii*) the ability of the binding factor to bind radiolabeled IgD and not monoclonal IgE, IgG2a, or polyclonal IgG; and (*iii*) the removal of the binding factor on passage through an IgD-Sepharose column and recovery in a subsequent acid eluate.**

T-cell factors with affinity for the immunoglobulin isotypes IgG, IgE, and IgA have been reported (1–4). In general, release of these immunoglobulin-binding factors (Ig-BF) can be induced by incubating lymphocytes with immunoglobulin of the corresponding isotype (5–7). Monoclonal antibodies to Fc_γ (8) or Fc_ε (9, 10) receptors usually also react with IgG-BF or IgE-BF, respectively. In addition, IgE-BF exhibits sequence homology with Fc_ε receptor (11), suggesting that binding factors are related to cell surface receptors.

Our laboratory previously reported that pretreatment with IgD enhanced antibody responses in normal, but not in athymic mice (12). This augmenting effect is adoptively transferred by Lyt-1^+2^-, L3T4^+ T cells from IgD-treated mice to normal mice (13), a result that led to the identification of a subset of helper T cells with receptors for IgD (Tδ) (14). Crosslinking of soluble IgD or of B-cell surface IgD is required for upregulating receptors for IgD on T cells both *in vivo* and *in vitro* (15). Receptors for IgD are also induced on T helper cells by lymphokines such as interleukin 2 (IL-2), interleukin 4 (IL-4) (16), and interferon γ (17, 18). Similarly, antigen-specific cloned T cells show an increased incidence of IgD rosette-forming cells (RFC) after incubation with IgD, antigen, or recombinant IL-2 (rIL-2) (17).

Adachi and Ishizaka (19) recently provided evidence that incubation of T cells with IgD induces the production of IgD-BF, which inhibits IgD rosette formation of lymphocytes. Our experiments were designed to determine the relationship between the induction of Tδ cells and the production of IgD-BF after incubation with rIL-2 or recombinant rIL-4 (rIL-4) *in vitro* or after crosslinking of IgD *in vivo*. Recognizing a possible confusion between IgD and IgD-BF in rosette-inhibition assays, we assayed for the latter by the capacity of T-cell culture supernatants, immobilized on nitrocellulose, to bind ^{125}I-labeled IgD (^{125}I-IgD).

MATERIALS AND METHODS

Mice. BALB/c and $CB6F_1$ mice were obtained from Charles River Breeding Laboratories. C57BL/6 mice were purchased from The Jackson Laboratory.

Purification of IgD. IgD-secreting plasmacytoma TEPC-1017 (20) was the source of IgD. IgD was purified over goat anti-IgD-Sepharose 4B (12).

Cell Lines. T-cell hybridomas A3.4C6 (17) and 2H10 (21) are of helper phenotype, are specific for sperm whale myoglobin and pigeon cytochrome *c*, and were maintained in RPMI 1640 medium and Click's/RPMI 1640 medium, respectively.

Reagents. Hδa/1, a monoclonal IgG2a derived from a mouse of b allotype, is specific for IgD of the a allotype (22). Purified goat anti-mouse IgD (23), univalent Fab/Fc fragment of Hδa/1 obtained by elastase digestion (24), rabbit anti-mouse immunoglobulin and anti-Fab, and purified monoclonal rat IgG2a antibody (2.4G2) to mouse B-cell and monocyte Fc receptors (25) were prepared as described (24). IgG2a (from hybridoma AC5 ascites) was purified over recombinant protein A-Sepharose 4B (26) (Beckman). Affinity-purified mouse IgG was obtained from Pel-Freez Biologicals, and purified mouse monoclonal IgE was from A. Nisonoff. Recombinant human IL-2 was obtained from Cetus (Emeryville, CA). Recombinant murine IL-4 (27) and purified monoclonal anti-IL-4 (11B11) (28) were donated by W. E. Paul.

Purification of Splenic T Cells. Splenic T cells were depleted of adherent cells at 37°C in Petri dishes (1400-1, Nunclon, Rockilde, Denmark) and subsequently panned (29) at 4°C on Petri dishes coated with affinity-purified anti-mouse immunoglobulin (remaining Ig$^+$ cells <1%).

IgD RFC Assay. Splenic T cells and T-hybridoma cells were examined for receptors for IgD (14). Affinity-purified IgD- or bovine serum albumin (BSA)-coated sheep erythrocytes (SRBC) were prepared by the $CrCl_3$ method of coupling (30). Cells surrounded by more than three indicator cells were scored as rosettes and recorded as percent RFC. Percentages of cells rosetting with BSA–SRBC were subtracted (<2% for normal T cells and up to 20% for hybridoma T cells).

IgD-BF and Cell Extracts. T-hybridoma cells (5×10^4 cells per ml) were grown overnight, washed thoroughly, and incubated (2.5×10^6 cells per ml) in serum-free RPMI 1640 medium. Splenic T cells were incubated in serum-free Eagle's minimal essential medium with or without rIL-2 or rIL-4 at a

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Abbreviations: Tδ, helper T cells bearing receptors for IgD; SRBC, sheep erythrocytes; RFC, rosette-forming cell(s); rIL-2 and rIL-4, recombinant interleukin 2 and 4, respectively; Ig-BF, immunoglobulin-binding factor; IgE-BF, IgG-BF, and IgD-BF represent specific Ig-BFs; ^{125}I-IgD, ^{125}I-labeled IgD; BSA, bovine serum albumin.

density of 2.5×10^6 cells per ml at 37°C for 8–14 hr. The culture cell-free supernatants were collected, supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM ε-amino-1-caproic acid, and 0.02% (wt/vol) NaN_3, concentrated 2- to 4-fold by vacuum ultrafiltration, and stored at −70°C. For some experiments IgD-BF was purified by absorption onto mouse IgD-conjugated Sepharose 4B, followed by elution with 0.2 M glycine·HCl (pH 3.0) at 4°C and concentration to the original volume. Cell extracts were prepared as described (31) by treating 10^8 cells for 1 hr at 0°C with 0.5% Nonidet P-40 in 0.01 M Tris·HCl/0.15 M NaCl/1.5 mM $MgCl_2$ buffer, pH 7.4. Leupeptin, EDTA-Na_2, pepstatin, and PMSF were added at concentrations of 0.5 μg/ml, 1 mM, 0.7 μg/ml, and 0.2 mM, respectively.

Dot Blotting and Measurement of IgD-BF. Nitrocellulose dot assays were quantitated essentially as described (32, 33). Briefly, nitrocellulose sections of equal sizes (1.8 mg ± 0.1) were cut around spots with a paper punch, and radioactivity was counted in a γ counter. Equal-sized pieces were cut from parts of the nitrocellulose to which no sample had been applied. Values were expressed as ng of ^{125}I-IgD bound per spot after correction for paper background (from 0.13 ± 0.004 to 0.31 ± 0.02 ng per spot) on the basis of paper size and weight as reported (34).

In some cases the amount of IgD-BF was estimated by densitometry; the autoradiographs of nitrocellulose filters were scanned using an LKB ultroscan XL-densitometer, and spot densities were converted into gated peak areas (mm^2) as calculated by aid of a computer program.

Conditions for immunoblotting of the IgD-BF were basically as reported (35).

RESULTS AND DISCUSSION

Iodination of IgD and Measurement of IgD Binding on Dot Blots. Affinity-purified IgD, IgE, IgG2a, and IgG were iodinated (36) for 4–10 min (IgD) and 15 min (other isotypes) with Iodo-beads (Pierce) according to the manufacturer's specifications. The extent of radiolysis of IgD was examined by $NaDodSO_4$/PAGE. Only intact ^{125}I-IgD molecules (0.5–0.8 μCi/μg; 1 Ci = 37 GBq) were used for binding studies. Standardization of the nitrocellulose disc binding assay was done by binding ^{125}I-IgD to different amounts of affinity-purified goat anti-IgD spotted on nitrocellulose paper. Fig. 1*a* shows an excellent correlation between the amount of goat anti-IgD immobilized on the nitrocellulose and the amount of ^{125}I-IgD bound per spot.

Demonstration of IgD-BF Released from T Cells Incubated with rIL-2. In previous experiments rIL-2 was found to induce functional IgD receptors on T cells (16); release of IgD-BF by such cells was therefore studied (Fig. 2). Splenic T cells were incubated overnight in serum-free medium containing rIL-2 at 100–10,000 units per ml. Cells were assayed for IgD-RFC (Fig. 2*b*). Concentrated cell-free supernatants (100–200 μl) representing 8–9 × 10^6 T cells were spotted (in triplicate) onto nitrocellulose paper. There was a progressive increase in the IgD-BF in cell-free supernatants from cells incubated with 10^3–10^4 units of rIL-2 per ml (Fig. 2*a*). The percentages of IgD-RFC induced by 10^3 and 10^4 units per ml were not significantly different from that induced by 100 units per ml, although 100 units per ml failed to induce a significant release of IgD-BF. T-cell viability after incubation with rIL-2 at 10^4 units per ml was significantly reduced but was not affected by 100–1000 units per ml.

Similar experiments were performed with the concentrated cell-free supernatants from 10^6 T hybridoma A3.4C6 or 2H10 cells. In contrast to splenic T cells, these T-hybridoma cells showed constitutive expression of IgD receptors and spontaneous release of IgD-BF. Although percentages of IgD-RFC decreased after exposure to rIL-2 at ≥100 units per ml

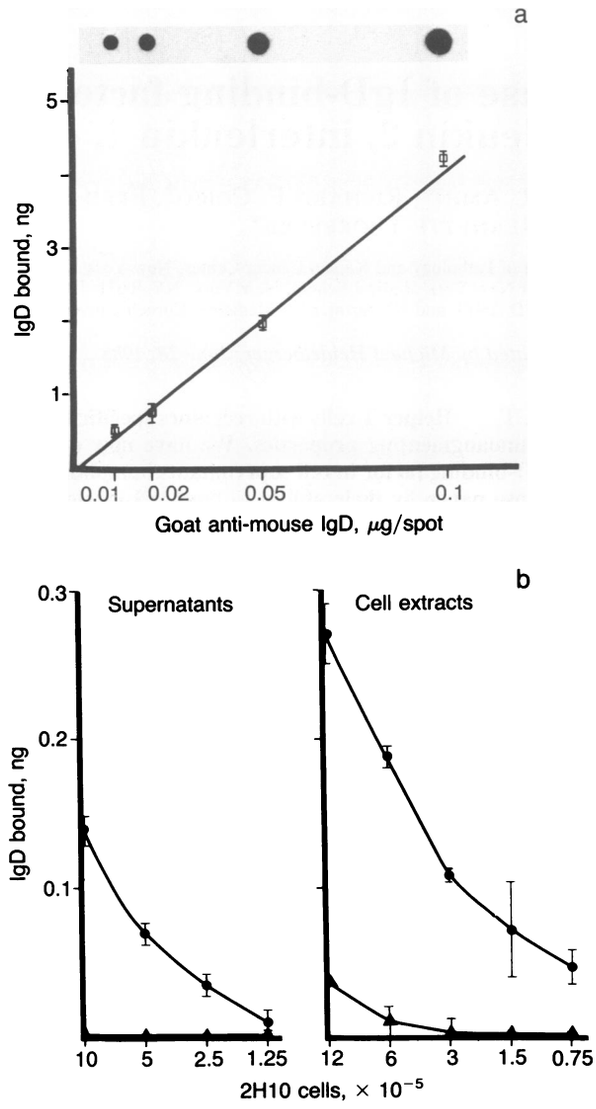

FIG. 1. (*a*) Quantitative nitrocellulose disc dot-blot assay showing the binding of ^{125}I-IgD in ng per spot at various concentrations of goat anti-mouse IgD. Test samples were spotted in triplicate onto 0.2-μm-pore nitrocellulose paper (Schleicher & Schuell) with a Bio-Rad dot-blot apparatus. The nitrocellulose was blocked with 3% BSA in phosphate-buffered saline (PBS) and probed with ^{125}I-IgD (6 × 10^4 cpm/cm^2) in PBS/3% BSA/0.1% (vol/vol) Tween 20 (Sigma). Filters were washed with PBS/0.1% Tween 20 and air dried. Corresponding autoradiographs of spots after overnight exposure at −70°C, prepared before radioactivity in each spot was counted, are shown. (*b*) Relationship between cell number and amount of IgD-BF released into cell-free supernatants after overnight incubation or detected in Nonidet P-40 cell lysates (cell extracts). Hybridoma cells studied were 2H10 (●) and the negative control fusion partner thymoma BW5147 (▲). Cells were cultured overnight in RPMI 1640 medium containing 1% (vol/vol) Nutridoma-SP (Boehringer Mannheim), 1.5 mM sodium pyruvate, 15 mM Hepes, 1 mM oxalacetic acid, insulin at 20 units per ml, and 5 × 10^{-5} M 2-mercaptoethanol.

(Fig. 2*d*), the quantity of ^{125}I-IgD bound by aliquots of cell-free supernatants from these cells increased (Fig. 2*c*)—i.e., they contained more IgD-BF, particularly with A3.4C6. The hybridoma parent cells, BW5147, and the cytotoxic T-cell line CTLL were negative both for IgD-RFC (16, 17) and for release of IgD-BF. As with other characteristics of T-cell hybridomas that are unstable on prolonged *in vitro* propagation, the ability of 2H10 and A3.4C6 cells to make IgD receptors and IgD-BF was lost after repeated passage.

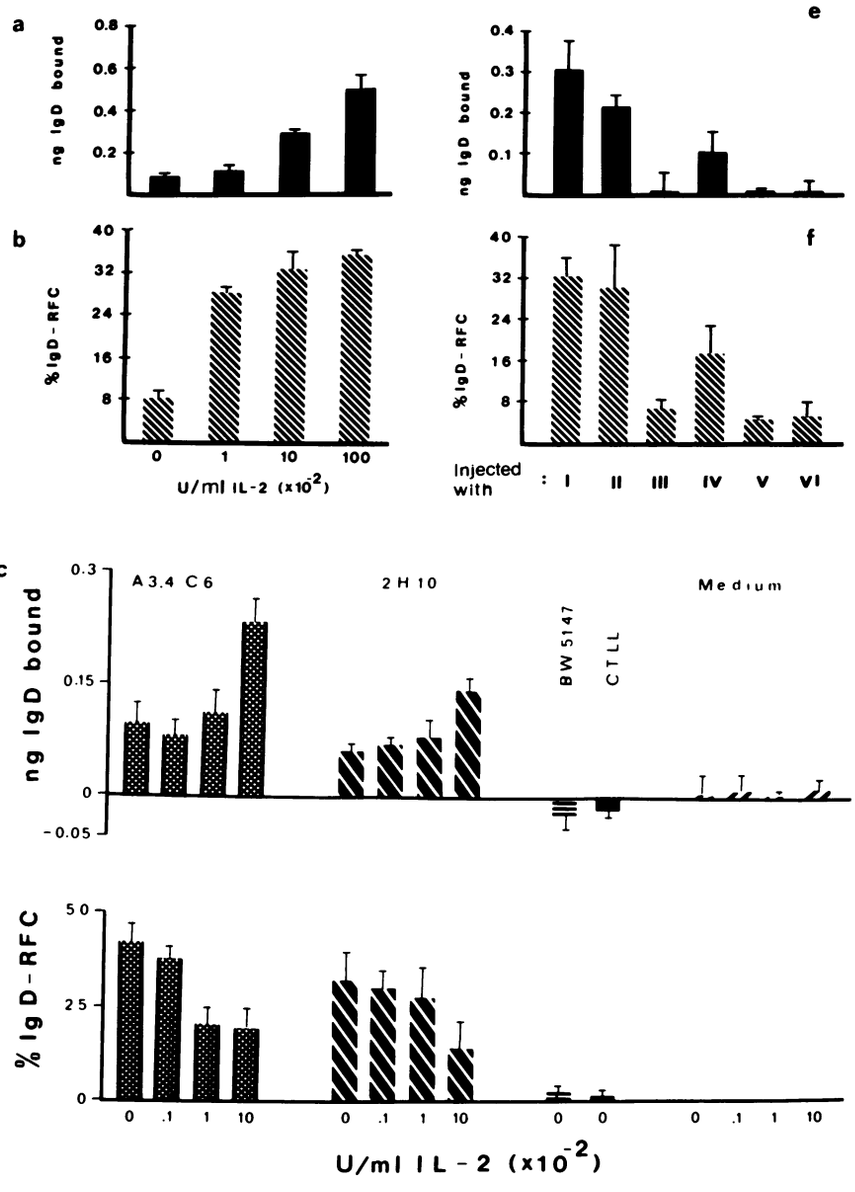

FIG. 2. (*a* and *b*) Effect of various concentrations of rIL-2 (100–10,000 units per ml) on release of IgD-BF into the cell-free supernatants (*a*) and on IgD-receptor expression (*b*) by BALB/c splenic T cells. (*c* and *d*) Effect of various concentrations of rIL-2 (10–1000 units per ml) on release of IgD-BF into the cell-free supernatants (*c*) and on IgD-receptor (*d*) expression by T-cell hybridomas A3.4C6 and 2H10. Negative control cells examined were BW5147 thymoma and CTLL1, a Ly-2$^+$ cytotoxic T-cell line. Medium containing rIL-2 but no cells was an additional negative control. (*e* and *f*) Requirement for crosslinking of IgD for the induction of Tδ cells *in vivo* and the subsequent release by these cells of IgD-BF *in vitro*. Groups of three mice were injected i.v. on day 1 with Hδa/1 (100 μg) (I); Hδa/1 (100 μg) plus 2.4G2 (500 μg) (II); 2.4G2 (500 μg) (III); Hδa/1 Fab/Fc (100 μg) (IV); Hδa/1 Fab/Fc (100 μg) plus 2.4G2 (500 μg) (V); and none (VI). Splenic T cells were prepared on day 0, and aliquots were either incubated in Eagle's minimal essential medium overnight and their cell-free supernatants assayed for IgD-BF (*e*) or analyzed for IgD-RFC on day 0 (*f*). The data of *f* have been published (15) but are presented here for comparison.

Demonstration of IgD-BF in Cell Extracts. The ability of Nonidet P-40 extracts and cell-free supernatants from similar numbers of 2H10 cells to bind ^{125}I-IgD in spot tests was compared (Fig. 1*b*). As for goat anti-IgD, a positive relationship existed between amount of cell-free supernatant or cell extract spotted on nitrocellulose and amount of ^{125}I-IgD bound (Fig. 1*b*). The bound IgD, as calculated per cell, was ≈3-fold higher in cell extracts than in cell-free supernatants.

Induction of Tδ Cells and IgD-BF by rIL-4. We had reported that Tδ cells could be induced by exposure to rIL-4 (16), but the specificity for IgD of the induced receptors required further confirmation. In agreement with our preliminary findings (16) splenic T cells incubated for 18 hr at 37°C without or with rIL-4 (500 units per ml) showed 9.9 ± 1.8% and 44.7 ± 1.2% IgD-RFC, respectively. When assayed for RFC with IgG, IgG2a, IgE, or IgD (each at 100 μg/ml), we detected 32.1 ± 14.8%, 37.7 ± 3.4%, 35.8 ± 8.4%, and 9.8 ± 5.1% IgD-RFC, respectively. Addition of anti-IL-4 antibody (11B11) during culture of T cells with rIL-4 at 100 units per ml inhibited the IgD-RFC percentages to 12.7 ± 4.6% (at 2.5 μg/ml) and 8.0 ± 7.5% (at 5 μg/ml) as compared with 38.6 ± 1.3% IgD-RFC for T cells incubated with rIL-4 alone. These results show that rIL-4 induces receptors specific for IgD on splenic T cells. The results are comparable to our previously reported findings with rIL-2-mediated induction of IgD-specific receptors on T cells (17). Hence, we sought to determine whether a similar correlation existed for rIL-4.

Splenic T cells were incubated overnight in serum-free medium with or without rIL-4 at 5000 units per ml. Supernatants equivalent to 10^6 T cells were spot tested for IgD-BF, and the amount of ^{125}I-IgD binding was quantitated by densitometry of autoradiographs. This method, although producing less reproducible results than direct radioactivity measurement of the nitrocellulose spots, detected a significant (P = 0.02) increase in ^{125}I-IgD binding by cell-free supernatants from cells with rIL-4 (1.14 ± 0.22 mm^2) as compared with the cell-free supernatants from cells without rIL-4 (0.47 ± 0.24 mm^2). The medium alone gave similar background readings regardless of the rIL-4 concentration added (0.33 ± 0.05 mm^2). Spot-tested rabbit anti-mouse Fab (0.1 μl) gave a reading of 1.53 ± 0.24 mm^2. Thus, as also reported for IgE-BF (37), rIL-4 increases both receptors for IgD and release of IgD-BF.

Injection of Anti-IgD Induces Release of IgD-BF by T Cells. Exposure of T cells to crosslinked IgD increases IgD receptors on T cells (15). To determine whether such Tδ cells release IgD-BF, splenic T cells were prepared from mice 16–20 hr after injection of allo anti-IgD, Hδa/1, or a Fab/Fc

univalent monomer prepared from Hδa/1. Some groups of mice also received the monoclonal anti-Fcγ receptor antibody 2.4G2. Percentages of Tδ cells correlated highly with the ng of ^{125}I-IgD bound to spotted cell-free supernatant samples from these cells (Fig. 2 *e* and *f*). The bivalent anti-IgD Hδa/1 induced Tδ and IgD-BF, whether or not injected with 2.4G2, whereas the univalent fragment of Hδa/1 was less effective. In addition, the effect of Hδa/1 Fab/Fc was abolished by the simultaneous injection of 2.4G2, suggesting, as discussed (15), that this effect was from an indirect IgD crosslinking dependent on the presence of cells with Fc_γ receptors. Splenic T cells from BALB/c, $CB6F_1$ and C57BL/6 mice, injected with goat anti-IgD or with Hδa/1, were similarly examined (data not shown). Again, Tδ-cell induction correlated precisely with detectability of IgD-BF in cell-free supernatants. Thus, these experiments support a direct relationship between presence of Tδ cells and release of IgD-BF. However, further experiments are needed to verify the molecular relationship between IgD-BF and receptors for IgD.

T cells with upregulated IgD receptors have immunoaugmenting effects (13, 14). The correlation between expression of such receptors and release of IgD-BF suggests a biological importance of IgD-BF. Indeed, it has recently been suggested that IgD-BF measured by inhibition of IgD rosetting augments antibody formation *in vitro* (38), but the relationship between that IgD-BF and the one described here remains to be established.

Partial Characterization of IgD-BF. Cell-free supernatants from normal T cells incubated with rIL-2 and from 2H10 and A3.4C6 hybridoma cells were applied to IgD-Sepharose 4B. The column effluents and acid eluates were spot tested for their ability to bind ^{125}I-IgD (Fig. 3*a*). In all three cases the IgD-BF was removed by passage over IgD-Sepharose and could be eluted with glycine·HCl, pH 3.0. Specificity of the IgD-BF from 2H10 cells was studied and is indicated in Table 1. Both semi-purified and crude IgD-BF bound ^{125}I-IgD but did not bind any other immunoglobulin preparations tested, including polyclonal ^{125}I-labeled IgG, monoclonal ^{125}I-labeled IgG2a, and ^{125}I-labeled IgE. Moreover, IgD but no other immunoglobulin isotypes studied competitively inhibited ^{125}I-IgD binding to IgD-BF. A similar binding specificity was shown for cell-free supernatants obtained from 2H10 cells, and from splenic T cells incubated with rIL-2 (Table 1), and by cell-free supernatants from splenic T cells incubated with rIL-4 (>90% inhibition by unlabeled IgD as compared with <5% inhibition by unlabeled IgE or IgG2a). Note that competitive inhibition of ^{125}I-labeled immunoglobulin binding to anti-Fab antibody was always more complete than the inhibition of ^{125}I-IgD binding to IgD-BF by similar amounts of unlabeled protein (1000× excess, Table 1), perhaps indicating that the IgD-BF binds IgD with relatively low affinity.

Cell-free supernatants taken after 1 and 3 hr of incubation of 2.5×10^6 2H10 cells per ml in serum-free medium was concentrated 16- to 20-fold and subjected to 12% NaDodSO$_4$/PAGE under reducing conditions. An immunoblot prepared from this gel, probed with ^{125}I-IgD, showed heterogeneity of the IgD-BF with respect to molecular size (Fig. 3*b*). Control cell-free supernatants from BW5147 showed no ^{125}I-IgD-binding bands, whereas cell-free supernatants from 2H10 cells showed multiple bands of ≈14, 20, 24, 33, 42, 62, and 80 kDa. Reduced and nonreduced samples showed a similar pattern of IgD-binding bands (data not shown). The nonreduced sample of goat anti-IgD had the expected band at 150 kDa (Fig. 3*b*, lane 4). Comparison of immunoblots obtained with cell-free supernatants prepared after 1- and 3-hr incubation (Fig. 3*b*, lanes 1 and 2, respectively) shows an intensity increase of the 14- and 20-kDa bands, suggesting that these smaller components could result from proteolytic

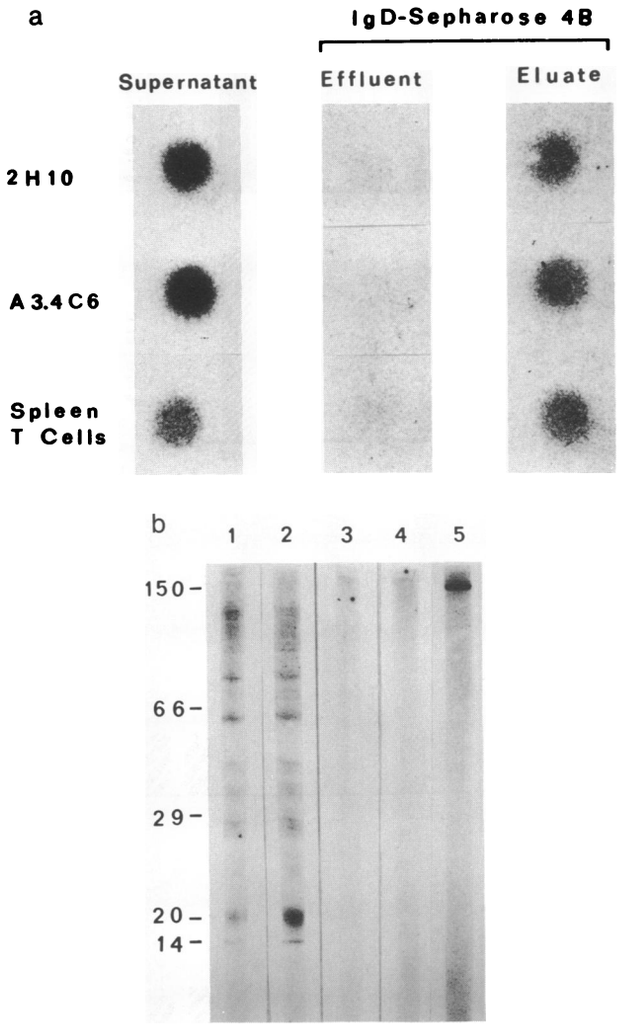

FIG. 3. (*a*) Affinity purification of IgD-BF from cell supernatants of T-cell hybridomas and splenic T cells on IgD-Sepharose 4B. Concentrated supernatants of T hybridomas (≈5×10^5 cells) and rIL-2-treated splenic T cells (≈2×10^6 cells) were spotted onto nitrocellulose paper. The effluents and neutralized glycine·HCl eluates (containing ≈2–5% of applied protein) from the IgD-Sepharose were similarly examined after concentration by diafiltration (final cell equivalent was twice the original). Nitrocellulose paper was incubated with ^{125}I-IgD and washed, and autoradiographs were prepared. Although there clearly was loss of total binding activity, the only detectable activity was in the eluate. (*b*) Immunoblot of 12% NaDodSO$_4$/PAGE of IgD-BF-containing cell-free supernatants from T-cell hybridoma 2H10 and parental BW5147 lymphoma-concentrated cell-free supernatant equivalent to 4–5 × 10^7 cells. Lanes 1–4 were run under reducing conditions. Represented are cell-free supernatants from 2H10 cells obtained at 1 hr (lane 1) and 3 hr (lane 2) and cell-free supernatants from BW5147 cells obtained at 3 hr (lane 3) and 1 μg of goat anti-mouse IgD (lane 4). Lane 5 is the pattern obtained with goat anti-mouse IgD under nonreducing conditions. The relative mobilities of reference proteins of different molecular masses (kDa) are indicated at left.

breakdown. Preliminary unpublished results suggest the presence of 40- and 80-kDa IgD-binding molecules in Nonidet P-40 extracts of 2H10 cells.

Size heterogeneity, similar to that seen here for IgD-BF, has been reported for murine Fc receptors for other immunoglobulin isotypes, including those for IgG (39) and IgE (40). Binding activity is retained by molecules as small as 19–23 kDa for IgG (39) and 14 kDa for IgE (40). Proteolytic fragments of human IgE-BF, as small as 14–16 kDa, retain IgE-binding activity (37, 41–43).

Table 1. Specificity of IgD-BF

Spotted sample	Relative binding of ^{125}I-labeled immunoglobulin to spotted sample, %*				Block of binding of ^{125}I-IgD to spotted sample, %†			
	IgD	IgE	IgG	IgG2a	IgD	IgE	IgG	IgG2a
Purified IgD-BF								
Splenic T cells	14 ± 3	<1	<1	<1				
2H10 cells	64 ± 17	<1	2 ± 1	<1				
SN from 2H10 cells	50 ± 18	2 ± 3	<1	<1	69 ± 5	19 ± 18	<1	<1
					(78 ± 9)	ND	ND	(<1)
SN from splenic T cells					78 ± 12	13 ± 7	<1	<1
Anti-mouse IgD‡					(100 ± 0)	ND	ND	(8 ± 3)
Anti-mouse Fab§					90 ± 4	94 ± 1	95 ± 1	100 ± 0

ND, not done; SN, cell-free supernatant(s).

*IgD-BF obtained from 2H10 cells and from rIL-2 (1000 units per ml)-incubated splenic T-cell SN were purified by affinity chromatography on IgD-Sepharose 4B column. These purified IgD-BF and SN from 2H10 cells were spotted on nitrocellulose paper in triplicate. Goat anti-mouse IgD‡ (0.02 μg) and rabbit anti-mouse Fab serum§ (0.1 μl) were also spotted. Spots were probed with ^{125}I-labeled IgD, IgE, IgG, and IgG2a (5 × 10^5 cpm/ml for each). Quantitation was by densitometry of autoradiographs. Results are recorded as percentages of ^{125}I-labeled immunoglobulin of the indicated isotype bound to the spotted IgD-BF, relative to the same ^{125}I-immunoglobulin bound to spotted anti-Fab.

†Nitrocellulose spots were probed with 0.5–1.0 μg/ml of ^{125}I-IgD with or without a 1000-fold excess of the unlabeled isotype indicated. Quantitation was by densitometry except for the experimental results in parentheses, where bound IgD was determined by γ scintillation counting.

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