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Functional studies of IgD-receptor-positive murine T delta cells and soluble IgD-binding factor

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FUNCTIONAL STUDIES OF IGD-RECEPTOR-POSITIVE MURINE T DELTA CELLS AND SOLUBLE IGD-BINDING FACTOR

by

YI JUN YANG

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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ABSTRACT

FUNCTIONAL STUDIES OF IGD-RECEPTOR-POSITIVE MURINE T DELTA CELLS AND SOLUBLE IGD-BINDING FACTOR

by

YI JUN YANG

Adviser: Professor Richard F. Coico

The biological role of immunoglobulin D (IgD) has remained enigmatic since it was discovered two decades ago. IgD is found in very low concentration in the serum and is co-expressed with IgM on the majority of mature B lymphocytes. Evidence from previous studies has shown that receptors specific for IgD (IgD-R) are expressed by human $CD4^+$ and $CD8^+$ T cells whereas IgD-R appear to be restricted to the $CD4^+$ T cell population in the mouse. IgD-R are induced in both human and murine T cells by their exposure to cross-linked IgD or lymphokines such as IL-4, IL-2, and IFN-gamma. IgD-R^+ T cells (Tδ) are also found to release IgD-Binding factor (IgD-BF) by similar induction. Previous in vivo functional studies of murine Tδ cells have demonstrated that up-regulation of these receptors correlates with increased helper activity as measured by enhanced primary and secondary antibody responses to various

antigens.

The goal of this study is to gain some insight into the mechanism by which IgD augments antibody responses. By establishing an *in vitro* assay system, we have been able to reproduce the previous *in vivo* studies. Furthermore, our results showed that IgD-induced augmentation of humoral antibody response was mediated by physical contact of B cells with antigen-specific TCR bearing Tδ cells, but not by soluble factors released by Tδ cells. Our study also revealed that Tδ cells facilitated antigen presentation by IgD-bearing B cells. Preliminary studies on characterizing IgD-BF and IgD-R demonstrated heterogeneous species of IgD-BF and IgD-R with molecular weights ranging from 20 kDa to 69 kDa, as recognized by both IgD and E11. In conclusion, our studies suggest that IgD plays an important regulatory role in humoral antibody responses by: (1) upon being cross-linked by antigen, inducing IgD-R and functioning as adhesion molecules that facilitate T-B interaction; and (2) upon B cell activation, terminating the T-B interaction by down-regulating expression, the negative effect of which is further augmented by the release and degradation of IgD-BF.

FOR MY DEAR FATHER

my grandmother, mother, aunt and my beloved husband

Without their sustained support and love,
I will never have been able to
complete this task.

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I would like to thank Dr. Susan Bonitz. She has gone through this work and has given me many suggestive comments. I also wish to thank Ms. Kathy Gaskins for her skillful help in editing and typing this work.

In the years I spent in CUNY, I have been befriended by many of my colleagues. I want to thank all of them for their support.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

ABBREVIATIONS

DPBS	-- Dulbecco's buffered saline
ICAMs	-- intracellular adhesion molecules
IgD-BF	-- IgD binding factor
IgD-R	-- IgD receptor
LFA	-- lymphocyte function-associated antigen
mAb	-- monoclonal antibody
mIg	-- membrane-bound immunoglobulin
mϕ	-- macrophage
PFC	-- plaque forming cell
RFC	-- rosette forming cell
Rx	-- treatment
SRBC	-- sheep red blood cell
Tδ	-- IgD-receptor positive cell
TD	-- thymus dependent
TI	-- thymus independent
TNP	-- trinitrophenylated hemocyanin

INTRODUCTION

Structural Studies of IgD

Immunoglobulin D (IgD) was initially described as a human serum Ig present in very low concentrations relative to other Ig classes.[1] IgD has a molecular weight of 175 kDa.[2] Similar to other immunoglobulin classes, it consists of two heavy chains and two light chains.[1] However, IgD is structurally unique in that it has an unusually long hinge region as well as a high carbohydrate content of 11.3 to 12.65%.[3] Because of its long hinge region, which is susceptible to proteolysis, IgD is an unstable molecule with a short half-life of 2-3 days in comparison with 23, 6, and 5 days for IgG, IgA, and IgM, respectively.[3]

IgD is present as both membrane-bound and secreted forms, which lack cysteine residues in the hinge region. However, in the membrane-bound IgD (mIgD) form, a cysteine residue is present in the spacer region,[4] which allows the creation of a disulfide bond. Nevertheless, mIgD can exist as a monomer (two heavy and two light chains), or as a half-monomer (one heavy and one light chain).[5,6] On the cell surface, there seems to be an equilibrium between the monomer and half-monomer of mIgD. The secreted form of IgD contains a cysteine residue in the secreted terminus and is released in dimeric form.[4] The membrane-bound form of IgD

is 2000 to 8000 daltons heavier than monomers of the secreted forms. This is due to the presence of an added hydrophobic transmembrane sheath of amino acids near the C-terminal end of the Fc portion of the molecule.[7]

The structure of IgD in human and mouse is surprisingly different. Whereas the constant region of human IgD has three domains, mouse IgD has only two heavy chain constant region domains, namely, Cδ1 and Cδ3.[8] Other differences between human and mouse IgD are seen at the serum concentration level. The concentration of IgD in human serum ranges from 0.3 to 30 μg/ml as compared with 0.2 to 4.0 μg/ml in mouse serum.[9,10]

Expression of IgD

A high density of IgD molecules, as the predominant surface immunoglobulin, exists on B lymphocytes.[11,12] The majority of mature, resting B lymphocytes co-express membrane IgM and IgD. Because these membrane IgM and IgD possess the same idiotypes on any given B cell,[13,14,15,16,17,18] they will co-cap upon introduction to an antigen bearing repeated epitopes with which they can interact. In contrast, IgD and IgM will cap independently upon exposure to specific anti-IgD or anti-IgM antibody.[18] In both human and murine systems, cloning of the respective μ-δ loci has revealed a complex transcriptional unit, 5'VDJ-Cμ-Cδ 3', that can generate both μ and δ mRNA.[19,20,21,22,23] Although the

transcriptional level of RNA encoding μ chains is two to three times greater than that for δ chains, membrane IgD is present in approximately a two-fold excess over IgM on the mature B cell surface,[24,25] probably due to a more rapid turnover rate of IgM.[26]

During ontogeny, the earliest Ig-producing B lineage cell, the pre-B cell, produces μ H chains in the absence of L chain synthesis or L chain gene rearrangement.[27,28] The vast majority of μ chains remain within the cell and are degraded because they cannot dissociate from the H chain binding protein.[29,30] As differentiation continues, rearrangement and expression of a conventional L chain gene occurs, leading to expression of IgM monomers as antigen receptors on the surface of the newly formed B lymphocyte. With further differentiation, most B cells then co-express IgD together with IgM chains.[17,31,32]

The expression of IgM and IgD can be followed during the 21-day gestation period of the mouse. IgM^+ cells appear in the fetal liver on day 13 of gestation, and appear in the fetal spleen and bone marrow from days 15 to 17 of gestation.[33] In the newborn mouse, >90% of splenic B cells express only IgM.[34] IgD first appears on the murine B-cell surface approximately 3 to 5 days after birth.[32] The proportion of IgD-bearing cells increases with age and reaches adult levels at 6 to 10 weeks of age.[35]

In the adult mouse, approximately 90% of IgM^+ cells are

also IgD^+. There tends to be a slightly higher percentage of IgD^+ cells in the lymph nodes and Peyer's patches than in the spleen. Whereas most bone marrow B cells express IgM, only 30 to 40% of bone marrow B cells express membrane IgD.[32] Throughout life, as B cells develop from Ig-negative precursors in the bone marrow, they go through a sequence similar to that described during ontogeny, expressing surface IgM first, and then gradually increasing their complement of IgD. After B cell activation by an antigen or mitogen, the expression of mIgD decreases.[1] Depending on the nature of the antigen and the form in which it is presented, a B lymphocyte may then differentiate to a plasma cell that secretes IgM, IgG, IgA, IgE, or IgD antibody, or to a memory cell with the same specificity that is capable of responding to a subsequent challenge with that antigen. However, memory cells do not bear IgD.[26,36]

One of the more salient features of B cell activation is that the decrease of mIgD expression, but not of mIgM expression, accompanies blast transformation following antigenic or mitogenic stimulation.[37,38,39,40] The results of a study in CBA mice showed that the decrease in IgD occurs because IgD is not replaced at an accelerated rate 5 days after immunization, whereas surface IgM and IgG are replaced at an accelerated rate.[41] Further investigations suggested that the loss of IgD occurs during initial entry into the cell cycle when stimulated B cells leave G_0 and transit G_1.[36]

The loss of IgD appears to be the result of accelerated shedding after encountering antigen stimulation without subsequent accelerated replacement. Nevertheless, it appears that low concentrations of IgD in human serum are not due to the shedding of mIgD because evidence exists that human B cell membrane IgD is primarily associated with kappa light chains whereas secreted serum IgD appears to be predominantly associated with lambda light chains.[42] The mechanism for the differentiation stage-specific expression of IgD has been further characterized in the mouse, using both cell lines[43,44] and normal mitogen-activated B cells[26,45] with similar results. The Cδ gene was transcribed at equivalent rates in IgM^+ immature B cells, IgM^+/IgD^+ mature B cells, and IgM^+/IgD^- activated B cells. However, δ mRNA was found only in mature B cells, indicating a posttranscriptional regulation of this isotype. Further analysis of the molecular events involved in the differential expression of the μ and δ heavy chain genes in mature B cells strongly suggests that the differential choice of a cleavage and polyadenylation-addition site and subsequent processing of the mRNAs regulates the relative levels of the respective mRNA species.[46]

In contrast to the mouse, human δ mRNAs are found at all differentiation stages from IgM^+ pre-B cells to IgM-secreting plasmablasts. These mRNAs are translated to yield the membrane and secretory forms of IgD. However, the δ-

chains synthesized in pre-B cells are degraded. Therefore, it appears that IgD can be stably expressed and secreted only in more mature cell types,[47,48] implicating some form of posttranslational regulation. Another significant difference between human and murine cells is that the δ mRNA that will be translated into the secretory form of IgD represents a significant proportion of the δ mRNA only in human cell lines.[48] Thus, differential regulation at the mRNA level may account for the difference in IgD serum concentration between human and mouse cells. However, exceptions have been found in both murine and human B cell plasmacytomas secreting only IgM, where δ mRNA is barely detectable, suggesting that δ mRNA production may be precluded by transcription termination before Cδ.[43] Therefore, at different stages of differentiation, IgD expression seems to be regulated by distinctly different mechanisms.

Biological Role of IgD

Although IgD was discovered more than two decades ago, the biological role of IgD has remained enigmatic despite continuing efforts aimed at defining its role in B cell development and immune responses. Its biological function is generally described in negative rather than positive terms: it is not secreted following antigenic or mitogenic stimulation of IgD$^+$ B cells, it does not appear to

neutralize antigen, and it does not fix complement. These negative functional properties have been used to support the view that IgD is primarily a cell-surface antigen receptor.[49]

However, the importance of IgD is underscored by the conservation of the δ heavy chain gene in phylogenetically divergent species.[50] Indeed, it has been suggested that the δ heavy chain gene system originated early in evolution and branched from the α chain shortly after divergence of the gene systems for α and μ.[51,52] Several studies have demonstrated that IgD and IgM are functionally distinct when expressed on the surface of an immature as well as a mature B cell.

Controversy regarding possible differences in the signaling properties of IgD and IgM still exists. Recent studies with murine CH33 B lymphomas phenotypically characterized as IgM^+, IgD^+ or IgM^+, $IgD^+/^-$ cells, have suggested that IgD may deliver a qualitatively different signal. In CH33 B lymphomas transfected with an allotypically different construct of IgD, antibody-mediated cross-linking of IgM but not IgD mediated growth inhibition of these cells.[53] Qualitatively different signals have also been demonstrated for IgD and IgM in studies with similarly transfected WEHI-231 B lymphomas. Cross-linking of IgD but not IgM had an inductive effect on the expression of *myc*, suggesting that membrane IgD may elicit a stimulatory

response.[54]

Ontogenetic studies have suggested that IgD may play a role in resistance to tolerance induction. In the newborn mouse, >90% of splenic B cells express only IgM. Such cells are very susceptible to tolerance induction by certain antigens. In addition, immature B cells are highly susceptible to functional inactivation following cross-linking of IgM. Fully mature B cells, co-expressing IgM and IgD, are less sensitive to tolerance induction and can be stimulated to enter the S phase of the cell cycle by cross-linking of either IgM or IgD.[55,56,57]

The role of T cells in tolerance induction was elucidated by the evidence that immature B cells are very susceptible, but mature B cells are highly resistant to tolerance induction in the presence of T cells. However, in the absence of T cells, tolerance susceptibility was identical in IgD^+ and IgD^- cells.[58] Similarly, recent studies have demonstrated that although treatment of adult splenic B cells with anti-μ antibodies induced significant unresponsiveness, treatment with anti-δ alone generally failed to reduce the subsequent response to either lipopolysaccharide (LPS) or fluoresceinated Brucella abortus[59]. However, cross-linking of IgD before treatment with anti-μ or simultaneously cross-linking of IgM and IgD are synergistic with respect to tolerance induction.

Tryphostin, a protein tyrosine kinase inhibitor,

inhibits tolerance induction by anti-μ whereas it facilitates tolerance induction by anti-δ,[59] suggesting that different signaling pathways are taken by these two surface Igs. This is consistent with studies which have shown that protein tyrosine kinases are key elements in transduction of signals generated through various membrane receptors, including IgM and IgD. These studies propose that upon ligation of membrane Ig, phospholipase C (PLC) is activated by mIg-induced tyrosine phosphorylation (evidenced by the fact that several different tyrosine kinase inhibitors block mIg-stimulated phosphatidylinositol breakdown).[60,61,62] Activated PLC then catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate, yielding two intracellular second messengers: diacylglycerol, which activates protein kinase C; and inositol 1,4,5-triphosphate, which causes the release of calcium from intracellular stores.[53,54,63,64]

Despite several common substrates phosphorylated on tyrosine, cross-linking of IgM versus IgD resulted in two isotype-specific substrates with molecular weights of 32 kDa and 33 kDa. The 32 kDa protein was phosphorylated only in response to anti-IgM antibody whereas the 33 kDa protein was phosphorylated only by anti-IgD treatment. Indeed, these isotype-specific proteins could be the homologues of a recently reported isotype-specific Ig complex.[65] When membrane IgM was recovered, it was accompanied by a

heterodimer consisting of two glycoproteins with molecular weights of about 34 kDa and 39 kDa, known as IgM-α and Ig-ß, respectively. Although mIgD was also found to be in a similar Ig complex containing the same Ig-ß, the IgD-α was approximately 1-2 kDa larger than IgM-α.[66] Therefore, it is suggested that the signaling pathways triggered by cross-linking of IgM and cross-linking of IgD are different, although initial events such as mobilization of Ca^{2+} and stimulation of inositol phospholipid metabolism may be similar.

How can we explain the finding that transcriptional regulation of the δ heavy chain gene appears to be uniquely programmed for down-regulation following stimulation of the B cell? Does this "transient" expression of mIgD allow the B cell to be transiently sensitive to IgD-specific regulatory signals? Previously reported studies in our lab have shown that murine and human T lymphocytes express membrane receptors specific for IgD and also release soluble IgD-BF.[67,68,69] The term "Tδ" has been coined to define such an IgD-R^+ T cell. In the murine system, functional studies of T cells with up-regulated IgD-receptor (IgD-R) have suggested that such cells play a role in the regulation of humoral immune responses.

The search for IgD-R^+ cells began with studies of IgD plasmacytoma-bearing mice.[70] BALB/c mice injected with IgD-secreting tumors or with isolated IgD protein showed

significantly enhanced in vivo humoral immune responses.[96] Experiments exploring the cellular basis for this effect led to the identification of IgD-R expressed on $CD4^+$ T cells.[67] Human Tδ cells, in contrast with murine Tδ cells, include both $CD4^+$ and $CD8^+$ T lymphocytes.[68] Unlike Ig receptors for other Ig classes, which are specific for the Fc domain of the Ig molecule, studies carried out by Tamma et al.[71] and Amin et al.[72] have shown that murine IgD-R recognizes the Cδ1 or Cδ3 domains of IgD. Moreover, the binding specificity of IgD-R to IgD appears to be linked to IgD-associated carbohydrates because deglycosylated IgD fails to bind IgD-R.[72] In addition, Amin et al.[72] demonstrated that N-acetylgalactosamine is capable of blocking the binding of IgD-R to IgD-coated target cells, supporting the idea that IgD-R has lectin-like properties.

It has been suggested that Tδ cells play a pivotal role in normal humoral immune responses through their recognition of B cell mIgD. Several lines of evidence argue that IgD facilitates responses of B cells to T-dependent antigens. Although some of the data conflict, it appears that, while $mIgM^+$, IgD^+ B cells respond to both thymus-independent (TI) and thymus-dependent (TD) antigens,[73] cells that express mIgD at a low density may be less able than mIgD-rich B cells to mount a primary response to TD and possibly type I TI antigens.[74,75] A recent report on IgD-deficient mice shows that although such mice are capable of mounting responses to

both TI and TD antigens, the levels of antigen-specific immunoglobulin in primary and secondary responses of such mice to a TD antigen (4-hydroxy-3-nitrophenyl acetyl coupled to chicken gamma-globulin) are lower on day 6 after immunization. This probably reflects a delayed onset of the carrier-specific response in IgD-deficient mice compared with control mice[76].

There is good evidence that the B cells resident in the splenic marginal zones, which appear to be responsible for the antibody response to carbohydrate antigens, express little or no IgD.[77] However, B cells that reside in the mantle zone of the tonsil, which is thought of as the remains of the primary follicle pushed outward by the expanding germinal center,[78] preferentially express IgD.[79] Interestingly, $CD4^+$ T cells have also been observed in the mantle area.[80]

Another highly thymus-dependent process, namely, Ig heavy chain class switching, which occurs during B cell differentiation, is also reported to be affected by mIgD expression. Experiments using mice rendered IgD-deficient by treatment of anti-δ antibody from birth or by transgenic techniques indicate that such mice are either deficient in secondary IgG and IgE responsiveness[81] or deficient in affinity maturation (i.e., production of the high-affinity antibody IgG_1 is delayed by 3-4 days). Such a deficiency clearly imposes disadvantage in such IgD-deficient mice in

the defense against pathogens.[76] The role of IgD in the generation of IgG memory is also demonstrated by the fact that monomeric IgD, when injected at the time of priming, significantly inhibits the secondary responses mediated by IgG-producing cells.[82]

In light of the investigations on IgD thus far, a dual role may be assigned to the function of B cell mIgD in the immune system: (1) as an antigen-specific B cell membrane protein; and (2) as a ligand for IgD-specific receptors expressed by T cells as well as soluble IgD-BF released by such cells.

Given this latter hypothetical role for IgD, the following question emerges: does the phenomenon of antigen-stimulated down-regulation of B cell mIgD serve, in part, as a regulatory mechanism to prevent ongoing excessive stimulation of B cells by IgD-R^+ T cells? Information regarding the functions of Tδ cells as well as the molecular nature of soluble IgD-BF and membrane IgD-R will certainly facilitate a better understanding of the role of IgD in the immune system. While previously published findings from <u>in vivo</u> studies have provided some insight into the possible role of Tδ cells in the regulation of antibody responses, a detailed analysis of the mechanism underlying this phenomenon will require <u>in vitro</u> studies of these cells. Thus, the major objectives of this study are: (1) to examine the functional properties of Tδ cells <u>in vitro</u>; (2) to

determine the molecular features of IgD-BF as well as its structural relationship with IgD-R; and, (3) to begin to explore the roles of IgD-BF and IgD-R in the regulation of normal humoral immune response.

MATERIALS AND METHODS

Mice and cell lines: Four to eight-week old female BALB/c mice and athymic BALB/c mice were purchased from Charles River Breeding Laboratories (Wilmington, MA). The IL-2-dependent CTLL line was a gift from Dr. Jeanette Thorbecke (NYU Medical Center, New York, NY). This line was maintained in RPMI 1640 (GIBCO, Grand Island, NY), supplemented with 10% heat-inactivated FCS (Hyclone Laboratories, Logan, UT), 10 mM L-glutamine, 50 U/ml of penicillin G, 50 ug/ml of streptomycin, and 10% rat spleen cell Con A supernatant, prepared as previously described.[83] The pigeon cytochrome c-specific, CD4 T hybridoma (2H10) cells were kindly provided by Dr. Ron H. Schwartz (NIAID, NIH, Bethesda, MD), and maintained in Click's/RPMI medium. The $CD4^+$, Ia^s-specific T hybridoma line, IDIE7, was kindly provided by Dr. Vincent Tsiagbe (NYU Medical Center, New York, NY). It was produced by fusion of splenic T cells derived from SJL/J mice (Jackson Labs, Bar Harbor, ME) with BW5147 thymoma cells.

Reagents: Sheep erythrocytes (SRBC) were purchased from Colorado Serum Co. (Denver, CO). Goat anti-mouse IgG, goat anti-mouse IgM, and goat anti-mouse IgG+IgM were obtained from Boeringer Mannheim Biochemicals (Indianapolis, IN). Affinity-purified mouse IgG, rat IgG, goat gamma globulin,

mouse anti-rat Ig, FITC-conjugated mouse anti-rat Ig (FITC-MaR Ig), alkaline phosphatase-conjugated goat anti-mouse IgG, and alkaline phosphatase-conjugated goat anti-mouse IgM were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Affinity-purified mouse IgM was obtained from Bionetics (Kensington, MD). Recombinant mouse IL-1ß was purchased from R & D Systems (Minneapolis, MN). Killed Brucella abortus (BA) was provided by the U.S Department of Agriculture. P-nitrophenyl phosphate was obtained from Sigma (St Louis, MO). Goat anti-mouse IgD was kindly provided by Dr. Fred Finkleman (Dept. Medicine, Uniformed Services University of the Health Sciences, Bethesda, MD). Monoclonal antibody specific for Thy1.2 and low toxic rabbit complement were purchased from Cedarlane Laboratories Limited (Hornby, Ontario, Canada). Alkaline phosphatase-conjugated goat anti-rat Ig (AP-GaR Ig) was obtained from Southern Biotechnology Associates (Birmingham, AL). Recombinant IL-2 and IL-4 were obtained from Genzyme (Boston, MA). Sepharose-4B was obtained from Pharmacia (Piscataway, NJ).

IgD source and purification: Oligomeric-IgD-secreting plasmacytoma TEPC-1017 were maintained by i.p. transfer in Pristane-primed BALB/c mice. Following ammonium sulfate precipitation, IgD was then purified from IgD-containing peritoneal fluid by affinity chromatography using Griffonia

simplicifolia-1 (GS-1) Sepharose, eluted with galactose, and dialyzed against DPBS.[84] Mutant IgD KWD1 and KWD6 (aggregated and non-aggregated forms), monomeric IgD B1.8δ and KWD8, as well as purified Fabδ were kindly provided by Dr. Ashok R. Amin (Dept. of Pathology, NYU Medical Center, New York, NY). Briefly, Fabδ was prepared as follows: 5-10 mg/ml TEPC-1017 IgD was incubated with immobilized trypsin to generate Fabδ basically as described by Nisonoff et al.[85] The Fabδ fragments were purified by column chromatography on Superose-12 (FPLC, Pharmacia) in accordance with manufacturer's instructions.

Immunizations: BALB/c mice were employed for all experiments.

For measuring antibody production, animals were primed with antigens 72 hr prior to the day of the experiment. In some experiments, animals were immunized i.v. with 10^8 SRBC in 0.1 ml of saline with or without 50-100 μg IgD. Alternatively, mice were immunized with TNP-BA received 80 μg of antigen (Ag) i.v. with or without 50 - 100 μg IgD.

For cell proliferation assays, when SRBC was used as the antigen in an experiment, mice were immunized i.v. with 10^8 SRBC in 0.1 ml of saline alone or together with 100 μg of TEPC-1017-derived IgD 3 days prior to the day of experiment. SRBC were prepared by being washed twice with Dulbecco's buffered saline (DPBS) or saline and adjusted to

$1x10^9$/ml. When goat gamma globulin was used as the antigen, animals were primed i.p. with 100 μg of goat gamma globulin every 10 days, over a period of 80 days, followed by a final boost i.v. of 100 μg of goat gamma globulin with or without 100 μg of IgD 3 days prior to the day of experiment.

TNP haptenization of BA and SRBC: Conjugation of TNP to B. abortus was carried out as previously described.[86] Briefly, 2 ml of a USDA-supplied suspension of killed B. abortus was combined with 2 ml of 20 mg/ml TNBS in 2% K_2CO_3 and stirred at room temperature for 2 hr, then at 4°C overnight. The mixture was washed with freshly made glycylglycine buffer and stored in 0.1% NaN_3.

Preparation of TNP-SRBC was as described previously.[87] Briefly, 20 mg of TNBS (2,4,6-trinitrobenzene sulfonic acid) was added to 7 ml of 0.005 M cacodylate buffer, pH 6.9. Next, 1 ml of packed SRBC were suspended in this solution slowly while stirring, followed by washing with glycylglycine and SA buffer. TNP-conjugated-SRBC was adjusted to 10% in SA buffer.

Modified Mishell-Dutton assay: Spleen cell suspensions were prepared in DPBS. Cells were adjusted to $5x10^6$/ml in Iscove's modified Dulbecco's medium supplemented with 10% FCS, 10 mM L-glutamine, 50 U/ml of penicillin G, 50 μg/ml of streptomycin, and $5x10^{-5}$ M 2-mercaptoethanol. The cells

were cultured in 24-well plates (Becton Dickinson, Lincoln Park, NJ) at 37°C, 5% CO_2 for 4 days. Cells were harvested and resuspended in Hanks' balanced salt solution (HBSS) for plaque-forming cell (PFC) assays. In certain experiments, double-chamber cultures were established in which an equal number of cells, $5x10^6$, were cultured in upper and lower chambers, separated by a membrane with a pore size of 0.45μ (Becton Dickinson). Cells from the upper wells were collected for PFC assays. Where indicated, a 1% suspension of SRBC was added at a dose of 50 μl/culture. Alternatively, TNP-BA was used at doses ranging from 5 to 10 μg/culture.

PFC Assay: IgM anti-SRBC antibody-forming cells derived from *in vitro* cultures were assayed by the slide modification of the technique of Jerne et al.[88] Indirect PFC were performed by the addition of goat anti-μ to the agar and rabbit anti-mouse Ig to the complement as previously reported.[89] Anti-TNP PFC were assayed with TNP-SRBC.

Spleen cell fractionation: Splenic T cells were purified by negative selection on goat anti-mouse IgG+IgM-coated tissue culture grade Petri dishes (Dynatech Laboratories, Chantily, VA) as described by Wysocki et al.[90] Splenic B cells were prepared by complement-mediated cytolytic elimination of T

cells as previously described.[91] Adherent cells were removed or enriched by coating the wells of 24-well plates with $5x10^6$ whole spleen cells and then incubating at 37°C, 5% CO_2 for 4 hr, followed by extensive removal (trituration) of non-adherent cells.

Quantitation of antibody by ELISA: Ninety-six well plates (Becton Dickinson) were coated with goat anti-mouse IgG or IgM (for quantitation of mouse IgG or IgM), purified IgD-BF (for direct assay of anti-IgD-BF), IgD, IgG, or IgM (for indirect assay of anti-IgD-BF) in carbonate buffer, pH 9.6, at 4°C overnight. The wells were then subsequently washed 3X with DPBS containing 0.1% Tween-20 and blocked for 1.5 hr at 37°C with the same buffer containing 1% BSA. All subsequent washing steps were performed with DPBS-Tween-BSA buffer. The wells were then sensitized for 1.5 hr at 37°C with titrated culture supernatants (for assay of mouse Ig), or IgD-BF (for indirect assay of anti-IgD-BF). For detecting rat-anti-IgD-BF antibody, the specific antibody was added as an additional layer. Wells then were probed with alkaline phosphatase-conjugated goat anti-mouse Ig, or alkaline phosphatase- conjugated mouse anti-rat Ig, followed by the addition of substrate (p-nitrophenyl phosphate). A BioRad ELISA reader (BioRad Labs, Life Science Group, Richmond, CA) was used to measure absorbance at 414 nm.

Immunoblot assays: Immunoblots were prepared by spotting nitrocellulose with purified IgD-BF or control proteins (e.g., BSA) followed by three 10-min cycles of washing and blocking with DPBS-Tween and DPBS-Tween-BSA. Enzymatic reactions were developed with 5-bromo-4-chloro-3-indoyl phosphate P-toluidine + p-nitroblue tetrazolium (BCIP-NBT) substrate (BioRad).

Induction of IgD-receptor-positive Tδ cells: In vivo induction of Tδ cells was achieved by i.v. injection of 50-100 μg purified IgD 72 hr prior to the day of the experiment. In vitro induction of Tδ cells was achieved by panning $3x10^7$ splenic T cells for 2 hr at 37°C in 100-mm Petri dishes (Fisher 8-757-12, Fisher Scientific, Pittsburg, PA) coated with 50 μg of purified TEPC-1017-derived IgD as previously described.[91]

Quantitation of IL-2: IL-2 dependent CTLL cells were washed twice with RPMI 1640 media supplemented with 10% FCS, 10 mM L-glutamine, 50 ug/ml of streptomycin, and 50 U/ml of penicillin G. These cells were then seeded onto wells of 96-well plates (Becton Dickinson) at a final density of $1x10^4$/ml, in a total volume of 0.2 ml. 100 μl of serially diluted sample supernatants from cell proliferation assays (see below) were then added to the wells. These cells were cultured at 37°C, 5% CO_2 for 18 hr. This was followed by a 1

μCi/well ^{3}H-thymidine (DuPont NEN Research Products, Boston, MA) pulse for 6 hr. Cells were harvested with a PHD cell harvester (Cambridge Technology, Boston, MA). Incorporation of ^{3}H-thymidine was measured by a 1219 RackBeta Spectral liquid scintillation counter (Pharmacia LKB Nuclear, Gathersburg, MA). The quantity of IL-2 in each sample was determined by probit analysis (described later), with recombinant IL-2 (rIL-2) as a standard control.

Cell proliferation assays: When SRBC were employed as the antigen in an experiment, whole spleen cells were prepared in DPBS, and adjusted to $5x10^6$/ml in proliferation assay medium (RPMI 1640 supplemented with 10% FCS, 10 mM L-glutamine, 50 U/ml penicillin G, 50 μg/ml streptomycin, and 1.5 mM 2-mercaptoethanol). 200 μl of cells were then seeded onto wells of 96-well plates with the addition of 10 μl of 0.1% SRBC. Alternatively, both purified T cells and B cells were adjusted to $1x10^6$/ml in proliferation assay medium and 100 μl of each were then seeded onto wells of 96-well plates in the presence of 10 μl of 0.1% SRBC.

When goat gamma globulin was used as the antigen, purified T and B cells were adjusted to $5x10^5$/ml and $1x10^6$/ml, respectively, in proliferation assay medium. 100 μl each of the T and B cells were then seeded onto wells of 96-well plates, followed by the addition of one of the following antigens at 2.5 μg/ml: goat anti-mouse IgM, goat

anti-mouse IgD, or goat gamma globulin. In some experiments, various doses of E11 (rat anti-mouse IgD-BF mAb) were also added to cultures. After 3 days of culture, 50 μl of supernatant was collected from each well and stored frozen for IL-2 quantitation. For all experiments, cells were cultured at 37°C, 5% CO_2 for 3 days, followed by an additional 18 hr pulse with 1 μCi/well ^{3}H-thymidine prior to harvesting.

Preparation of IgD-BF: IgD-BF was prepared from two sources: serum-free supernatants (SN) from 2H10 T hybridoma cells stimulated for 18 hr with 1000 U/ml of recombinant IL-2 (rIL-2) or, serum-free SN from IDIE7 T hybridoma cells stimulated for 18 hr with 10 U/ml of recombinant IL-4 (rIL-4). Briefly, $5x10^4$/ml T hybridoma cells were grown overnight, washed thoroughly, and incubated for 24 hr at $2.5x10^6$/ml in serum-free RPMI 1640 medium supplemented with rIL2 or rIL-4. Cells were then incubated at 37°C, 5%CO_2. The cell-free culture SN were collected, supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 Mm ε-amino-1-caproic acid, and 0.02% (wt/vol) NaN_3. They were then concentrated 2- to 4-fold by vacuum ultrafiltration and stored at -70°C.

IgD-BF was purified by absorption onto mouse IgD-conjugated-Sepharose, followed by elution with 0.2 M glycine-HCl, pH 3, at 4°C. It was concentrated to the

original volume by using an XM100A Diaflo membrane (Amicon, Lexington, MA) and then monitored as described previously.[70] Briefly, IgD-BF was subjected to 12% SDS-PAGE under reducing conditions, and then transferred to a nitrocellulose filter followed by incubation with ^{125}I-IgD as the probe, washing, and finally autoradiography.

Preparation of anti-IgD-BF monoclonal antibody: Female Sprague-Dawley rats were immunized with IgD-BF as described below. The 20 kDa molecular weight species of IgD-BF was cut from SDS-PAGE gels and used as a source of antigen, which was emulsified in complete Freund's adjuvant for the first 3 i.p. injections. This was repeated 3 more times over a 6-week period with the exception that incomplete Freund's adjuvant was used each time to emulsify the gel containing IgD-BF. Three days prior to the fusion of lymphoid B cells, a final IgD-BF boost was given by i.v. injection of purified IgD-BF without adjuvant. Serum anti-IgD-BF titers were monitored throughout the immunization procedure by ELISA and immunoblot assays with 2H10-derived IgD-BF. Spleens and lymph nodes from immunized rats were fused with Ig-non-secreting NS-1 myeloma cells using the polyethyleneglycol-fusion method.[92] Cells from wells testing positive for rat Ig, assayed by ELISA using AP-mouse anti-rat Ig, were expanded, and their SN examined for anti-IgD-BF Ab using an ELISA.

A B cell hybridoma line, E11, that produces monoclonal rat IgG1, kappa type, was cloned, and re-cloned by standard limiting dilution techniques. E11 hybridoma cells were grown *in vitro* and then adapted for *in vivo* growth in Pristane-primed athymic BALB/c mice. Purification of E11 was performed following ammonium sulfate precipitation of IgG from ascites using affinity chromatography with Sepharose-4B (Pharmacia) coupled to mouse anti-rat Ig (Jackson Immunoresearch Lab).

IgD-rosette-forming cell (RFC) assays: IgD-receptor expression was monitored using a rosetting technique.[67] Briefly, splenic T cells or T hybridoma cells were mixed with indicator cells prepared by coupling affinity-purified mouse IgD to SRBC using the $CrCl_3$ coupling method.[93] 100 μl of 1% IgD-SRBC was mixed with 0.1 ml of 2.5×10^5 assay cells at 37°C for 30 min. Cells were then centrifuged at 500 r.p.m. (200 X G) for 5 min and were incubated at 4°C for 2 hr or overnight. Immediately before scoring RFC, the lymphocytes were stained using a 1% toluidine blue solution. Lymphocytes surrounded by more than 3 indicator cells (minimal definition) were scored as rosettes and the results were expressed as percent RFC. BSA-SRBC were used as control indicator cells.

SDS-PAGE and Western Assays: Preformed SDS-gradient gels

(Amersham, Arlington Heights, IL) were employed to estimate the molecular weight of IgD-BF. Briefly, samples were subjected to electrophoresis under reducing conditions and 100 V according to manufacturer's instruction, followed by transblotting to a nitrocellular filter under standard conditions as previously described.[94] When electrophoresis was complete, the gel was equilibrated in transfer buffer for 1 hr, followed by assembly
of the transfer sandwich: in layers of wet filter paper, gel, nitrocellular membrane, and filter paper in the same buffer, (avoiding air bubbles). Proteins were transferred electrophoretically from gel to membrane at 30 V in a tank filled with transfer buffer at 4°C for 14 hr.

Immunoprecipitation of IgD-R and IgD-BF with E11- and IgD-Sepharose: 2H10 cells were washed twice in serum-free RPMI-1640 medium, then incubated overnight at 37°C, 5%CO_2 in the same medium at a density of 10^7 cells. Supernatants were then collected and supplemented with protease inhibitors: 5 μg/ml leupeptin, 0.2 M PMSF, and 0.7 μg/ml pepstatin together with 0.05% NP40 (Boerhinger Mannheim, Indianapolis, IN) and then stored at -70°C. Cell pellets were incubated for 1 hr in lysing buffer supplemented with the protease inhibitors listed above. Lysed cells were centrifuged for 15 min in a microcentrifuge and the lysate material was stored at -70°C.

Prior to immunoprecipitation, both SN and lysates were absorbed separately for 1 hr each with: BSA-Sepharose, goat anti-BSA agarose (Sterogene Bioseparations, Octavia, CA), and mouse IgG-Sepharose. These pre-absorption steps were repeated one additional time prior to overnight immunoprecipitation of SN and lysate material with either IgD-Sepharose or E11-Sepharose. Sepharose beads were washed extensively with DPBS and resuspended in 100 μl of SDS sample buffer. Samples of the resuspended Sepharose beads were then subjected to SDS-PAGE under reducing conditions. Western blotting was then performed using E11 (or control rat Ig) as the probe. For detecting proteins recognized by E11, the Enhanced Chemiluminescence (ECL) Western blotting analysis system and horseradish peroxidase-linked mouse anti-rat Ab (Amersham) were employed. Eluates from the Sepharose beads were also used for biological function studies.

Cell staining and FACS analysis: 1×10^6 cells were washed 2X with DPBS containing 0.01% NaN_3, incubated with the appropriate amount of biotin-conjugated IgD - for IgD staining, prepared by using a kit obtained from Pierce Chemical Company (Rockford, IL) - or with E11 - for E11 staining - for 30 min on ice, and washed 3X with DPBS containing 0.01% NaN_3. Cells were then stained with 1 μg of FITC-conjugated goat anti-mouse antibody and incubated for

another 30 min on ice. Finally, cells were washed 3X, resuspended in DPBS, and subjected to FACS analysis using a Coulter ELITE cell sorter (Coulter Electronics, Hialeah, FL). In some experiments, the ability of competitive inhibitors (e.g., IgD, IgG) to block the binding of biotin-IgD (or E11) to Tδ cells was examined. Blocking agents or control proteins were added simultaneously with the biotin-conjugated IgD.

Data analysis: Mean and standard deviations were calculated for quantitative data. Student t tests were employed for evaluation of significance, which is determined at the 5% level. All experiments were repeated $\geq$ three times. In all PFC assays, duplicate cultures were set up for cells from each individual animal or each group of cells, and triplicate slides were set up, therefore, n=6. In all proliferation assays, a minimum of five cultures were set up for each individual animal or each group of cells, therefore, n= or > 5.

Probit analysis: A standard IL-2 preparation supplied by the Cetus was used. The following steps were used to generate data based on probit analysis.[95] (1). The ^{3}H-thymidine incorporation was converted to a percentage of maximum cpm. (2). The converted data were plotted on probability graph paper, or converted directly to log

values. (3). The X axis was converted to $\log_2$ dilutions of control growth factor sample. Zero equals the undiluted sample, and the dilutions along the X axis were labeled 1 through 5. (4). The Y axis coordinate was expressed intercepting the 50% growth factor control in units of activity by dividing the $Factor_{50}$ of the sample by the $Factor_{50}$ of the control.

Preparation of solutions:

SA buffer (5X): 167.6 g NaCl, 5.04 g $NaHCO_3$, 6.0 g barbital sodium (Merck), 9.2 g barbital N.F., 0.4 g $MgCl_2.6H_2O$. All reagents were dissolved in 4 liter of H_2O on a magnetic hot plate.

Glycylglcine buffer: 88 mg glycylglycine was dissolved in 140 ml 1X SA buffer (see above). The buffer should be freshly prepared.

DPBS-Tween buffer: 0.01 ml Tween-20 (Sigma, St Louis, MO) was added to 100 ml Dulbeccos phosphate-buffered saline (DPBS) (GIBCO, Grand Island, NY).

DPBS-Tween-BSA buffer: 1 g BSA (Sigma, Grand Island, NY) was dissolved in 100 ml DPBS-Tween buffer.

Carbonate coating buffer: 1.59 g Na_2CO_3 (0.015 M) and 2.93 g $NaHCO_3$ (0.035 M) were dissolved in 900 ml H_2O, adjusted to pH 9.6 with 1M NaOH or 1M HCl, and H_2O was added to 1 liter. This was prepared fresh every other week because the buffer absorbs CO_2, resulting in a pH change.

Toluidine Blue (1%): 0.588 g sodium barbitol was dissolved in 10 ml H_2O; sodium acetate was dissolved in 10 ml H_2O and 2 g Toluidine Blue was dissolved in 180 ml H_2O. The above reagents were prepared separately, then combined and stored in a dark bottle.

RESULTS

Part I. Functional effects of in vivo pre-treatment of IgD on in vitro antibody responses to antigen.

1. TEPC-1017 derived IgD in vivo pre-treatment enhances in vitro antibody responses. Studies were performed to determine whether the humoral immunoaugmenting effect of IgD detected in BALB/c mice *in vivo* were reproducible *in vitro*. These studies were undertaken because our *in vivo* studies had established that IgD-pre-treatment induces the appearance of increased numbers of splenic IgD-R$^+$ T cells (Tδ cells), which mediate the immunoaugmenting effects.[68,96,97,98,99,100] We initially set out to determine whether similar treatment *in vivo* would predispose spleen cells to mount enhanced primary antibody responses *in vitro*.

The effects of IgD-pre-treatment *in vivo* can be seen in Figure 1a. In this experiment, mice were given a single injection of purified, oligomeric TEPC-1017 three days prior to the establishment of *in vitro* spleen cell cultures stimulated with TNP-BA. The results were consistent with previously reported studies of the effects of such IgD-pre-treatment. Thus, frequencies of IgD-R$^+$ Tδ cells were significantly increased as compared with T cells from control mice, demonstrated as percentage of IgD-rosette-forming cells (IgD-RFC). As shown in Figure 1a, primary

anti-TNP PFC responses were significantly enhanced as compared with those seen in cultures of spleen cells derived from control mice. Similar effects of TEPC-1017 IgD were observed in early secondary anti-TNP responses generated in vitro (Fig. 1b). In vitro immunization of spleen cells from IgD-pre-treated mice using the particulate antigen, namely, SRBC, also yielded enhanced in vitro antibody responses. However, the magnitude of the IgD-induced augmenting effects on anti-SRBC PFC responses was lower than when anti-TNP was used (Fig. 1b).

Culture supernatants from similarly treated SRBC-stimulated spleen cells were analyzed by ELISA to quantitate IgM and IgG levels. As shown in Figure 2, both IgM and IgG levels were elevated as compared with those found in culture supernatants from SRBC-stimulated control cells, although the level of IgM secretion was significantly higher (2-3 fold) than that of IgG. As expected, direct IgM+IgG in vitro anti-SRBC PFC responses of IgD pre-treated spleen cells were also enhanced (Fig. 2).

2. TEPC-1017 derived IgD in vitro pre-treatment enhances in vitro antibody responses. Previous studies have demonstrated that Tδ cells, induced either in vivo or in vitro, are capable of augmenting antibody responses when passively transferred to recipients together with antigen.[100] In addition, we have shown that when Tδ cells from IgD pre-

treated mice are re-exposed to IgD in vitro, their immunoaugmenting activity is further increased. However, this enhanced functional activity is not accompanied by further increases in the frequency of IgD-R$^+$ Tδ cells.

T cells panned on IgD-coated dishes induced the expression of IgD-R as determined by IgD-RFC assays, showing an increase in Tδ cell frequencies from 5 ± 1 % in control cells to 32 ± 3 % in IgD-induced cells. Enumeration of Tδ cell frequencies in spleen cells from IgD pre-treated mice yielded similar results. Furthermore, there was no significant increase in the number of IgD-R$^+$ cells seen in these spleen cells following panning on IgD-coated dishes.[97] The data shown in Figure 3 indicate that essentially identical results are obtained with similarly treated whole spleen cells when antibody responses are generated in vitro rather than in vivo. Thus, antibody responses of control cells are enhanced, and those of cells from mice pre-treated with IgD in vivo are further augmented by in vitro treatment with IgD.

3. Mutant IgDs with deficiency in C1 or C3 domains and Fab_2 fragments retain the ability to augment antibody responses. Previous studies have demonstrated the lectin properties of IgD-R.[72] Because the interaction between IgD and IgD-R occurs via the recognition of carbohydrates on IgD by a lectin-like portion of IgD-R, the amino acid sequence of IgD

appears not to be directly involved. Similar to the wild-type oligomeric TEPC-1017 IgD, aggregated mutant IgDs such as KWD1 and KWD6 increase the frequency of IgD-R$^+$ Tδ cells. This increase occurs although KWD1 lacks the Cδ1 domain, and KWD6 lacks both the Cδ3 and Cδ-hinge domains. Similarly, Fab_2 fragments produced by enzyme digestion also have the ability to induce Tδ cells.[71]

In agreement with these findings, our data demonstrate the augmenting effect of such IgD molecules on antibody responses. These effects can be seen in Figure 4a, where a similar level of immunoaugmentation to that obtained by pre-treatment with wild-type IgD (oligomeric TEPC-1017) is reached by pre-treatment of aggregated mutant KWD1, KWD6, or Fab_2 fragments. However, aggregation of oligomeric TEPC-1017 IgD does not show a significant further augmenting effect as compared with naturally occurred TEPC-1017.

4. Cross-linking of IgD-R is required for the immunoaugmenting effect of IgD. Several lines of evidence have indicated that upregulation of IgD-R by IgD is dependent on cross-linking of the IgD receptors. Cross-linking of IgD on B cells with anti-IgD both *in vitro* or *in vivo* causes upregulation of IgD-R on T cells, where Tδ cell frequencies increase from background levels of 1-2 % to 20-30 %.[101]

Although the monomeric IgD B1.8δ1 by itself failed to

induce any increase of Tδ cells, it induced IgD-R when used in combination with the antigen that it recognizes.[101] Recent studies of the Cδ heavy chain domains associated with the upregulation of IgD-R have also shown the need to use aggregated forms of IgD for optimal induction of Tδ cells.[82] Monomeric IgD, given either in vivo or in vitro, failed to upregulate IgD-R on T cells or augment primary and secondary antibody responses.[82]

In our in vitro studies, we have seen similar effects with monomeric IgD. As shown in Figure 4b, spleen cells from mice pre-treated with non-aggregated forms of KWD1 or KWD6 IgD do not show enhanced antibody responses to antigen as compared with cells from control mice. However, cells that were pre-treated with oligomeric IgD (TEPC-1017) showed highly enhanced responses as expected.

5. Monomeric IgD inhibits the immunoaugmentation induced by oligomeric IgD pre-treatment. It is postulated that Tδ cells induced by IgD or antigen exposure augment antibody responses by interacting with B cells, via the interaction between IgD-R and IgD.[97,99] Therefore, monomeric IgD, which cannot cross-link IgD-R but can recognize IgD-R, should be able to hinder the interaction between Tδ cells and B cells. As predicted, recent studies have demonstrated this fact. Monomeric IgD, when given prior to or given simultaneously with antigen or oligomeric IgD, prevented the induction of

IgD-R on T cells by either antigen or oligomeric IgD, respectively. Accordingly, the immunoaugmenting effect of oligomeric IgD was also abolished by simultaneous *in vivo* monomeric IgD treatment.[82]

The blocking effects of monomeric IgD were also tested in our *in vitro* studies. Two monomeric forms of IgD (KWD8 and B-1.8δ1), were tested in a dose-dependent fashion. It was found that either type reduces the antibody responses to a level lower than that of the control cultures (shown as 100% of PFC/culture in Fig. 4c). However, when two different doses of oligomeric IgD (TEPC-1017) were given in the *in vitro* cultures, opposite effects on antibody responses of IgD-retreated spleen cells were obtained (Fig. 4c).

Furthermore, when monomeric IgDs from various source were used, differences between their blocking effects were detected. As shown in Figure 4d, immunoaugmention induced by *in vivo* IgD pre-treatment was reduced by non-aggregated mutants IgD KWD1 and KWD6, as well as by Fabδ fragments prepared from wild-type IgD. The following results were observed when various types of IgDs were compared for their ability to reduce augmenting effects of *in vivo* IgD pre-treatment on antibody responses: GS-1 bound mutant IgDs (which possess the carbohydrate moieties shown to be required for binding to IgD-R) were better than GS-1 unbound mutant IgDs; KWD1 was better than KWD6; Fabδ fragments were

similar to oligomeric IgDs, comparable to GS-1 unbound KWD1, better than GS-1 unbound KWD6, but not as good as GS-1 bound mutant IgDs.

Based upon these results, we would suggest that although the carbohydrates on IgD molecules are probably the major component recognized by IgD-R, the amino acid sequence of Cδ may also play a minor role in binding of IgD-R. The inhibitory effect of TEPC-1017 is actually the net results of in vitro induction and inhibition, resulting in a reduced blocking effect compared to GS-1-bound KWD1 and KWD6 (see Fig. 5 for a detailed description). Non-aggregated Fabδ fragments show less blocking effects than GS-1 bound mutant IgDs, suggesting that either the Cδ3 sequence is more important than the Cδ1 sequence in the interaction of IgD-R and IgD, or that less glycan is linked to the Cδ1 domain as compared to the Cδ3 domain.

6. Additional IgD in vitro demonstrates a dose-dependent dual effect on immunoaugmentation induced by in vivo IgD treatment. It has been previously shown that although in vitro treatment of T cells with oligomeric IgD resulted in a dose-dependent increase in IgD-RFC, a supraoptimal dose of the same IgD molecule caused lower than peak values after maximal values had obtained.[62] A similar inhibitory effect of a supraoptimal ligand concentration has been observed in our current studies with respect to antibody responses. IgD

was added to cultures in various doses and the frequency of antibody- secreting cells was measured. As showed in Figure 5, when oligomeric IgD (TEPC-1017) was added to the *in vitro* cultures, it augmented antibody responses of spleen cells from control mice. This effect was observed from the lowest dose (1 μg) to the second highest dose of IgD tested (50 μg). However, the highest dose of IgD (100 μg) showed no effect on the *in vitro* antibody responses.

The effects of IgD on the cultures of cells pre-treated with IgD are markedly different from that of culture cells from control mice. Whereas doses of 10 μg and 50 μg of IgD showed an augmenting effect on antibody responses, the highest IgD dose tested (100 μg) reduced the augmented responses induced by *in vivo* IgD-pre-treatment. The lowest dose (1 μg) did not show any significant effect. These results may be explained as follows: suboptimal doses of IgD in the *in vitro* cultures will upregulate or further upregulate the density of IgD-R on Tδ cells. However, after maximal density of IgD-R has been reached, a further increase of IgD concentration cannot induce more IgD-R due to the so-called "ceiling effect". Instead, it will bind to the IgD-R, thus blocking the interaction between IgD and IgD-R. In the control group, the inductory effect of IgD-R is greater than the inhibitory one, resulting in the former effect; whereas in the IgD pre-treated group, the inhibitory or blocking effect dominated. Thus, the final effect of IgD

on the *in vitro* cultures may be viewed as the net balance between induction of IgD-R expression and competitive inhibition of the binding of these receptors to mIgD.

7. IgD in the in vitro cultures plays its role mainly on the first day of culture. To determine how IgD affects *in vitro* cultures over time, large doses (100 μg) of TEPC-1017 derived IgD were added to the cultures on day 0, day 1, and day 2. As expected, large doses of IgD *in vitro* had a different effect on various pre-treated cells. Whereas IgD showed its greatest effect when added to the cultures on day 0, it failed to show significant effects on later days (Fig. 6).

Interestingly, previous *in vivo* studies have also shown that the augmenting effects of IgD are seen only when it is administered at a specific time. It was found that *in vivo* pre-treatment of IgD augmented the secondary antibody response only when it was given on the day of priming and not when it was administered 1 or 2 days after the primary antigen injection.[97] Moreover, monomeric IgD did not show any inhibitory effect on secondary antibody responses when injected at the time of secondary antigen priming.[82] Based upon additional studies,[82] it appears that an interaction between IgD-R and IgD is required during the cognate interaction between T and B cells, which occurs early after encountering antigen, for immunoaugmention induced by IgD.

Based upon the kinetics of effect of *in vitro* treatment of IgD, our results support this conclusion. IgD molecules on the cell surface will be down-regulated rapidly after B cells are activated by antigen stimulation, which makes the interaction mediated by IgD and IgD-R impossible.

8. T cells from mice pre-treated with IgD in vivo are responsible for the augmentation of in vitro antibody responses; adherent cells are required in the in vitro culture. Previous studies have shown that the immunoaugmenting effect of IgD is transferable by injection of Tδ cells (induced *in vivo* or *in vitro*), strongly suggesting that the IgD-augmented antibody response is mediated directly by these helper T cells.[100] This idea is further supported by results obtained from our *in vitro* culture system. Initially, special attention had been paid to optimizing the conditions of the *in vitro* culture. Despite the use of different culture conditions (e.g., media supplements, incubation times, etc.), optimizing conditions to facilitate reproducibility of these assays was difficult. However, in light of the findings from these studies, it now appears that the precise culture conditions are less important with respect to the reproducibility of these assays than the accessory cell/cytokine requirements. In subsequent sections, we will discuss findings supporting a role for mIgD and IgD-R in antigen presentation by resting B

cells. Here, we will focus on the requirement for adherent cells and/or IL-1 in our cultures. It should be noted, however, that these experiments were performed both to demonstrate the immunoaugmenting effects of IgD *in vitro* and to begin to assess the possible role played by IgD in antigen presentation by B cells.

Early in the 1980s it became clear that B cells are capable of presenting soluble antigens to T cells.[102,103] Several lines of evidence have demonstrated that resting or activated B cells can present antigen to previously activated T cells,[104,105,106] and that activated B cells can activate resting T cells.[107] However, resting B cells are not capable of presenting antigen to resting T cells.[107,108] As a major cytokine secreted by macrophages and many other cell types,[109] IL-1 has been found to markedly enhance the antigen-presentation ability of resting B cells,[110] perhaps by activating T cells.[111,112] Spleen cells employed in our experiments were primed with antigen on day -3 relative to the establishment of cultures. Therefore, the subsequent *in vitro* activation of B cells should be considered as a continuum of the ongoing primary antibody response. As such, the B cells involved are newly stimulated from their previous resting state. Most of the B and T cells are resting cells.

In agreement with the studies cited above, our data demonstrate that adherent cells are necessary components in

our *in vitro* cultures to successfully reproduce the Tδ cell-mediated IgD-induced immunoaugmention. As shown in Figure 7, spleen cells failed to show the immunoaugmenting effect of IgD induction after they were depleted of adherent cells (mainly macrophages), whereas this effect was observed when adherent cells were supplemented. It was also found that IL-1 can replace the function of adherent cells in these cultures. Although the background responses of control cells were non-specifically enhanced by IL-1, the responses of IgD-pre-treated cells was of a significantly greater magnitude under the same conditions.

Because of the unavoidable depletion of adherent cells during the procedure of T cell purification, any *in vitro* antibody-producing assays involving such a procedure hereafter were supplemented with adherent cells or the appropriate amount of recombinant IL-1. The data shown in Figure 8 demonstrate that results from *in vitro* studies are identical to that of *in vivo* functional studies, in terms of the critical role that Tδ cells play in the augmentation of antibody responses. In this experiment, T and B cells were purified from mice primed with antigen alone (T and B cells) or from mice pre-treated with both antigen and IgD (Tδ and "Bδ" cells). All possible combinations of cells from these sources were prepared and cultured, followed by PFC assays. Co-cultures of T and B cells as well as those of T and Bδ cells failed to augment antibody responses as compared with

whole spleen cells from control mice. However, co-cultures of Tδ and B cells as well as Tδ and Bδ cells demonstrated significantly augmented responses. The lower responses of the combination of Tδ and B cells compared to that of whole spleen cells from IgD-pre-treated mice may be due to the suboptimal conditions to which T and B cells are subjected during the cell fractionation. Alternatively, these lower responses may be due to the presence of suppressor T cells in such a high density of cells, as seen in Figure 9.

9. Augmentation of antibody responses induced by IgD to a specific antigen requires simultaneous expression of IgD-R and antigen-specific TCR by T cells. Although it is now clear that IgD augments antibody responses through Tδ cells, one question that had not yet been addressed is the following: is upregulated IgD-R the sole factor required for the augmentation by Tδ cells? Two experiments were performed to answer this question. As shown in Figure 9a, cultures of whole spleen cells (group A) from mice primed with antigen alone (-) or primed with antigen plus IgD (+) were supplemented with T cells (group B) from mice pre-treated with antigen plus IgD or IgD alone, in various doses. T cells from mice primed with IgD plus antigen enhanced the responses of whole spleen cells in a dose-dependent fashion compared to mice primed with antigen alone. It was noted that the enhanced responses of whole

spleen cells from mice primed with IgD plus antigen occurred at low T cell doses (1.5×10^6, 0.5×10^6), whereas there was an inhibition of responses at the highest T cell dose (2.5×10^6). In contrast, T cells from mice pre-treated with IgD alone failed to augment the response of B cells from all sources. Moreover, they inhibited the immunoaugmentation induced by *in vivo* treatment of IgD in high T cell doses (1.5×10^6, 2.5×10^6).

Results consistent with these findings were obtained when purified T and B cells were employed in the assays (Fig. 9b). The results showed that only T cells from mice primed with antigen plus IgD were capable of enhancing responses of B cells, because T cells from mice primed with antigen alone or treated with IgD alone failed to show a significant effect as compared to that of control spleen cells. T cells from mice pre-treated with IgD alone, however, showed an inhibiting effect. The responses of B cells from mice pre-treated with IgD appear to be greater than those of B cells from control mice when T cells are added from mice primed with antigen alone or IgD alone. This effect may be due to T cell contamination following purification of B cells.

The consistently shown inhibiting effect of T cells at high doses is probably due to the effect of suppressor T cells. Because immune responses are regulated by T helper and T suppressor cells, the final outcome of our *in vitro*

antibody responses will be the net balance between help and suppression. On the other hand, the "activation" status of spleen cells before the addition of purified T cells is very important. If splenic T cells are not previously induced by IgD, the addition of Tδ cells will result in a high augmenting effect that may shield the effect of suppressor T cells. However, if splenic T cells are previously induced by IgD, the additional Tδ cells cannot further enhance the responses that have already been augmented by IgD.

Thus, in the case of T cells expressing IgD-R and specific TCR, help is dominant for spleen cells from control mice and help is still dominant for spleen cells from IgD pre-treated mice with the addition of low numbers of T cells. However, suppression is dominant for spleen cells from IgD pre-treated mice in high doses of T cells because the number of suppressor T cells will also increase as the T cell dose increases. In the case of T cells expressing IgD-R but without antigen-specific TCR, help is not provided to spleen cells. Therefore, no effect of T cells is seen using spleen cells of control mice. A non-specific suppressor effect of T cells is dominant in spleen cells from IgD pre-treated mice. In summary, when T cells with antigen-specific TCR but without upregulated IgD-R are added, T cells help B cells respond to antigen. Augmentation is seen when these T cells co-express up-regulated IgD-R.

10. Augmentation of antibody responses induced by IgD pre-treatment is not mediated by soluble factors released by T cells. Previous reports on IgD-binding factor (IgD-BF) have suggested that IgD-R$^+$ splenic T cells release IgD-binding factors following stimulation by antigen, cross-linked mIgD, IL-2, or IL-4.[69,113,114] The release of IgD-BF from a T hybridoma cell line (2H10) has also been reported.[69] Further more, as IgD-BF increases in the culture supernatant, IgD-R on the T cells has been shown to be down-regulated, suggesting a certain relationship between these molecules.[69] Functional studies on IgD-BF suggest that Tδ cells augment antibody responses by releasing these soluble factors, because a purified IgD-BF enhances antibody production *in vitro*.[114]

To clarify the mechanism by which IgD augments antibody responses, we tested supernatants from IgD-induced splenic cell cultures for their ability to enhance antibody responses. As shown in Figure 10, cell-free supernatants collected from spleen cell cultures with or without IgD pre-treatment were added concurrently to cultures to a final concentration of 10%. Responses of control cultures were not significantly affected by these cell-free supernatants that had been subjected to IgD pre-treatment.

A special double-chamber culture plate was then employed, where contact between cells in the upper chamber and those in the lower chamber was prevented by a membrane

with a pore size of 0.45 μ. This set-up was chosen to facilitate the ability of soluble factors to diffuse between the chambers through the membrane pores (Fig. 11). When spleen cells from control mice and cells from IgD pre-treated mice were cultured in the upper and lower chamber, respectively, no enhancement was seen in cells in the upper chamber. Thus, our results strongly suggest that the immunoaugmentation induced by IgD is not mediated by soluble factors such as IgD-BF released by Tδ cells. The discrepancies between results of our studies and those of other groups may be due to factors such as contamination, dose-dependent action, functional distance, and short half-life time of protein molecules, as will be explained in the discussion.

Part II. Functional effects of in vivo IgD-pre-treatment on antigen presentation by B cells.

1. In vivo IgD-pre-treatment enhances antigen-specific proliferation of T cells. Class II MHC molecules (Ia), as well as many adhesion molecules such as proteins of the integrin family, play a role in the phenomenon known as antigen presentation. The loss of Ia that occurs upon culturing of macrophages results in a significant reduction in their ability to present antigen to T cells, whereas the acquisition by macrophages of Ia induced by exposure to

lymphokines such as interferon-gamma results in a proportional increase in their accessory cell function.[115] It has been demonstrated that TCR stimulation results in a rapid, transient increase in the avidity of T cell LFA-1 for ICAM-1, as measured by the binding of T cells to immobilized ICAM-1.[116] Because our speculation that IgD molecules on B cells function not only as antigen receptors, but also function as ligands for IgD-R on T cells, we wished to determine whether upregulated IgD-R expressed on T cells had a similar function to those adhesion molecules mentioned above.

Cultures of splenic cells from control mice versus those from IgD-treated mice were established and cell proliferation was measured as an indication of activation. As shown in Figure 12, spleen cells from control mice showed a low level of proliferation following in vitro stimulation with SRBC. However, spleen cells from IgD-treated mice gave significantly higher levels of proliferation (2-fold) in the absence of SRBC, and even greater proliferation was observed (more than 8-fold) in the presence of antigen. Results of the latter group (minus Ag cultures) may be explained by previous studies, which showed that repetitive injection of IgD in mice resulted in polyclonal antibody responses against environmental antigens.[117] The enhanced proliferation by spleen cells from IgD pre-treated mice in the absence of antigen appears to result from environmental

antigens that those cells encountered during *in vitro* culture.

Next, we wanted to know whether such effects could be blocked by IgD competitive inhibition. Whereas IgD enhances proliferation of spleen cells derived from control mice in proportion to its dose in a culture, it inhibits proliferation of those cells derived from IgD-pre-treated mice in high doses (50 μg/culture) but shows no effect in low doses (5 μg/culture) as shown in Figure 12.

To characterize the type of cells responsible for such enhanced proliferation induced by IgD, purified T and B cells were employed. As shown in Figure 13, T and B cells from either IgD-pre-treated or control mice were given a lethal dose of gamma irradiation and cultured in the mixture with non-irradiated B or T cells, respectively, with stimulation by SRBC. Figure 13 shows that when B cells are irradiated, T cells derived from IgD pre-treated mice (as responder cells) give significantly higher proliferative responses to antigen in comparison to T cells derived from control mice. However, when T cells are irradiated and B cells are used as responder cells, whether from mice pre-treated with or without IgD, the B cells failed to show any significant differences in their response to antigen stimulation. Moreover, when B cells were used as the irradiated partner, whether from control mice or mice pre-treated with IgD, they showed no significant influence on

the responder T cells. In parallel with the activation of such T cells in response to antigen stimulation, our results demonstrate a markedly higher percentage of IgD-RFC in T cells from IgD pre-treated mice in comparison to those from control mice, as indicated by the IgD-rosetting assay.

2. In vivo IgD-pre-treatment facilitates antigen presentation of B cells to T cells. Several well characterized adhesion molecules such as MHC II, CD4, LFA-1, and I-CAM-1 have been found to facilitate contact between the antigen-presenting cells (APC) and the CD4 T lymphocytes.[118] The immune-competent cells respond to antigenic stimulation by proliferation and differentiation. Thus, the above mentioned evidence that in vivo IgD-pre-treatment enhances proliferation of splenic cells to in vitro antigen stimulation may suggest that IgD and IgD-R could also function as paired adhesion molecules. Therefore, we wished to study the role that IgD and IgD-R play in the process of antigen presentation. To produce antigen-specific T cells in vivo, mice were primed i.p. with eight doses (100 μg/dose) of goat gamma globulin over a period of 80 days, followed by an i.v. boost of a final dose (100 μg) of the same antigen alone (control) or with purified 100 μg IgD. T cells were then fractionated from those IgD pre-treated mice and control mice, with portions of the latter group induced in vitro with IgD, followed by 4

days of culture with stimulation by the same antigen in various forms - goat anti-IgD, goat anti-IgM, and normal goat gamma globulin. Gamma-irradiated B cells, as APC, were fractionated from naive mice.

In comparison to the control group, T cells pre-treated with IgD either *in vivo* or *in vitro* gave significantly higher responses to antigen presented by B cells (Fig. 14). However, the T cells subjected to IgD *in vitro* gave an even higher response, which paralleled the expression of IgD-R on their surface, as indicated by the percentage of IgD-RFC. The Ig isotypes recognized by the goat antibody also influenced the outcome of these experiments. Goat anti-IgD antibody gave a stronger stimulation than goat anti-IgM, and the normal goat gamma globulin gave a very poor stimulation.

Part III. Characterization of monoclonal rat anti-IgD-binding factor mAb (E11) and its function; role of IgD-binding factor in vitro.

1. E11 is specific for IgD-BF released from T hybridoma cell lines (2H10 and 1D1E7).

IgD-BF has been reported to be composed of two molecular weight species, 78- and 37-kDa proteins, as reported by Adachi and Ishizaka.[113] More heterogeneous IgD-BF species, ranging from 20-80 kDa (with a major species of 20-25 kDa), have been reported by Amin et al.[69] Studies with other Ig-specific BFs, including IgE-,

IgA-, and IgG-BF have demonstrated their ability to positively and/or negatively influence synthesis of the Ig isotype to which they bind.[119,120,121] Like other Ig-BFs, IgD-BF may also contribute to the immunoaugmenting effects of Tδ cells that have been observed in vivo by Coico et al.[67,98,100] Studies by Adachi and Ishizaka using purified B cells primed in vivo with Ag have suggested that murine IgD-BF may have immunoaugmenting activity,[114] which is not in agreement with our results (Figs. 10 and 11).

To investigate these discrepancies, an IgG1 monoclonal antibody (E11) specific for mouse IgD-BF was developed by immunizing rats with a 20-kDa fragment of IgD-BF derived from the supernatant of an IgD-R$^+$ pigeon cytochrome C-specific 2H10 T hybridoma cell line. The specificity of this monoclonal antibody was tested by indirect and direct ELISA. Results of the indirect ELISA, which measured both the IgD specificity of IgD-BF and the IgD-BF specificity of E11, are shown in Figure 15. IgD-coated wells showed positive dose-dependent ELISA results as compared to wells coated with IgG or IgM. Additional control wells were coated with IgD and then probed with E11 alone (i.e., in the absence of IgD-BF in the ELISA sandwich). Results of controls were uniformly negative.

E11 mAb also reacted with IgD-BF derived from another IgD-R$^+$ T hybridoma line (1D1E7). 1D1E7 cells released increased quantities of IgD-BF (based on IgD-Sepharose

chromatography profiles, data not shown) following an overnight incubation in serum-free medium supplemented with IL-4. Thus, supernatants were prepared from such IL-4-stimulated 1D1E7 cells, concentrated 10-fold, and used as a source of crude IgD-BF. ELISA wells were then coated overnight with 100 μl of this supernatant, and the wells were then probed with serially diluted purified E11 mAb. The results of the ELISAs indicated a dose-dependent reaction, in contrast to negative results obtained using 10-fold concentrated, IL-4-containing, serum-free medium (Fig. 16).

2. E11 also recognizes IgD-R on normal splenic T and T hybridoma cells. Because studies have suggested that IgD-BF may be the released IgD-R shed from T cells by certain types of stimulation,[69] we attempted to determine the structural relationship between these two molecules. A hybridoma that constitutively expresses IgD-R, 1D1E7 T, was employed in these experiments. As shown in Figure 17, the IgD-RFC of such cells were effectively blocked by E11 in a dose-dependent fashion. In contrast, control rat IgG failed to block IgD-rosettes. Similar results were obtained using normal splenic T cells induced overnight with IL-4 (Fig. 18).

We wished to obtain more direct evidence on the ability of E11 to recognize IgD-R on T cells. This was obtained by

indirect staining of 1D1E7 and normal splenic T cells by E11 using FITC-conjugated-mouse-anti-rat antibody as probe. IgD-R expressed on 1D1E7 cells can also be upregulated by IgD, IL-2, or IL4, as indicated by IgD-RFC (Fig. 19). In parallel to their expression of IgD-R, these cells showed an increased indirect staining after induction by IgD (Fig. 20a), IL-2 (Fig. 20b), and IL-4 (Fig. 20c). However, the fusion partner of this hybridoma (i.e., BW5147 thymoma cells that are IgD-R negative) failed to show increased staining with E11 after the same treatments (Fig. 20d, e, and f). Normal splenic T cells induced by IgD (21d) also showed increased staining by E11 as compared with uninduced cells (Fig. 21c), which demonstrated that without IgD-pre-treatment, splenic T cells stained showed only background level of staining (21a, 21b) by E11.

As shown in Figure 22, 2H10 cells are directly stained by biotinylated IgD in a dose-dependent fashion. Its specificity was also demonstrated by the fact that only IgD, but not IgG, blocked the IgD staining (Fig. 23). Furthermore, the blocking effect of E11 was tested and the results are shown in Figure 23. Biotinylated IgD, as expected, increased the staining of such cells (Fig. 23a), whereas E11 and IgD blocked the staining (Fig. 23d and e, respectively). The specificity of such a blocking effect was further demonstrated by data shown in Figure 23b and c, which showed that both mIgG and normal rat gamma globulin

failed to block the staining. The fact that E11 blocks the direct staining of the 2H10 T hybridoma by biotinylated IgD also suggested that E11 recognizes IgD-R.

3. Partial characterization of IgD-BF. The E11 mAb was used to characterize IgD-BF derived from 2H10 hybridoma cells. Cell-free and serum-free supernatants from 2H10 hybridoma cells were pre-absorbed with BSA-, goat anti-BSA-, and mouse IgG-Sepharose, then incubated with E11-, IgD-, and normal rat Ig-Sepharose. Eluates from IgD- and E11-, and normal rat Ig-Sepharose were subjected to gradient SDS-electrophoresis under reducing conditions. An immunoblot of this gel was then probed with purified E11. Following incubation with a peroxidase conjugated anti-rat Ab and an appropriate substrate, the specific bands were detected by chemiluminescence autography.

Experiments were performed to assess whether E11 could be used to immunoprecipitate membrane IgD-R from 2H10 cells. As shown in Figure 24, after immunoprecipitation with E11-Sepharose, a 2H10 membrane lysate contained proteins with molecular weights of 69 kDa, 46 kDa, 40 kDa, and 20 kDa (lane 4); whereas a culture supernatant contained only one protein with a molecular weight of 69 kDa (lane 5). By immunoprecipitating a membrane lysate with IgD-Sepharose, proteins with molecular weights of 69 kDa, 30 kDa (lane 7), and 20 kDa (lane 9) were detected; whereas

immunoprecipitation of culture supernatants containing IgD-BF resulted in detection of proteins with molecular weights of 69 kDa, 46 kDa, 40 kda, and 20 kDa (lane 8). Thus, membrane lysates consistently showed proteins of 69 kDa and 20 kDa recognized by both IgD and E11; whereas a culture supernatant showed only one protein of 69 kDa recognized by both IgD and E11.

In addition, the membrane lysate and culture supernatant showed proteins that did not overlap when probed with E11 or IgD. Moreover, samples of membrane lysate stored at -70°C for about one month (lane 9) showed no signal at the position of the 30 kDa protein and a weaker signal at the position of the 20 kDa protein precipitated by IgD-Sepharose. Thus, our results suggest that the IgD-BF consists of proteins with heterogeneous sizes, which may be explained, in part, by its susceptivity to degradation during storage. As controls, a culture supernatant and a cell lysate derived from the same cell line, as well as a plain culture medium, when subjected to immunoprecipitation by normal rat Ig-sepharose (lane 2, lane 3, and lane 1, respectively), showed negative signals at the specific molecular weights mentioned above.

4. Effects of E11 and IgD-BF on antibody responses.

(1). E11 augments in vitro primary antibody responses.

Spleen cell cultures were established as described in Figure

1b using SRBC as the antigen. E11 was present in some of the cultures as indicated. As shown in Figure 25, E11 augmented the primary antibody responses of spleen cells from mice without *in vivo* treatment.

(2). Dual effect of E11 in vitro on secondary antibody responses. E11 showed a very similar effect to IgD on early secondary antibody responses of spleen cells. As shown in Figure 26, E11 augmented the responses of cells from control mice in a dose-dependent fashion in the *in vitro* cultures. While it inhibited the IgD-induced immunoaugmentation at the high dose (20 μg), it further enhanced the responses at the low dose (10 μg). In contrast, normal rat gamma globulin did not affect the responses.

(3). E11 has a dual effect upon the process of antigen presentation of B cells to T cells. Similar experiments were established as described for Figure 14 to assess the effects of E11 on antigen presentation. Figure 27a shows that E11 augmented antigen-stimulated proliferation of control T cells at a low dose (10 μg/culture) but exhibited little or no effect at a high dose (100 μg/culture). However, E11 demonstrated a very different effect on both *in vivo* (Fig. 27b) and *in vitro* (Fig. 27c) IgD-pre-treated T cells where it inhibited antigen-stimulated T cell proliferation at a high dose (100 μg/culture) but enhanced

responses at a low dose (10 μg/culture). Normal rat gamma globulin as a control showed no significant effect in all cases.

Having demonstrated that E11 was not only specific for IgD-BF but also recognized IgD-R, it is not a surprise that E11 behaves as IgD does with respect to T cell proliferative responses following Ag presentation. Similarly, they induce the expression of IgD-R when they cross-link IgD-R on T cells, whereas, on the other hand, they appear to be capable of interrupting the interactions of T and B cells during Ag presentation by binding to IgD-R.

(4). IgD-BF eluted from IgD- and E11-Sepharose fails to augment antibody responses. Preliminary experiments were performed to determine the effects of IgD-BF on *in vitro* antibody responses. IgD-BF, eluted from E11- or IgD-conjugated-Sepharose, was employed in functional studies. Cultures were established as described in Figure 1b using SRBC as the antigen. IgD-BF from these two sources were supplemented to the cultures in two different doses. As shown in Figure 28, while none of the eluates affected the responses of spleen cells from control mice, they all showed an inhibiting effect on cells obtained from IgD-pre-treated mice. Eluates from E11-Sepharose seemed to have a greater inhibitory effect than eluates from IgD-Sepharose. As a control, eluates from normal rat gamma globulin failed to affect the response significantly.

DISCUSSION

Previous *in vivo* studies in mice have revealed that oligomeric IgD injections augment both *in vivo* primary and secondary antibody responses to either T-dependent or T-independent antigens in normal but not in athymic mice.[67,96,97,98,99] Moreover, such an immunoaugmenting effect can be adoptively transferred by IgD-receptor positive $CD4^+$ T cells but not by $CD8^+$ T cells or B cells from IgD-treated mice to normal mice.[100] Therefore, $CD4^+$ T cells have been proposed to play a central role in such an immunoaugmentation induced by IgD.

Indeed, it has been found that murine $CD4^+$ T cells, but not $CD8^+$ T cells or B cells, can be induced to express a higher density of IgD-receptors upon cross-linking of such receptors on their surfaces by oligomeric IgD, antigens, and cytokines such as IL-2, IL-4, or INF-γ. Monomeric IgD, being unable to cross-link IgD-R, cannot induce Tδ cells, nor can it augment immune responses.

The interaction between IgD-R and IgD occurs via the recognition of N-glycans on Cδ1 and Cδ3 domains of IgD by IgD-R.[72] In fact, IgD-R has recently been characterized as having lectin-like properties. Therefore, mutant IgD and Fabδ retain their immunoaugmenting ability despite their lack of either a Cδ1 hinge or Cδ3 regions. That an interaction between surface IgD on B cells and IgD-R on T

cells is necessary for immunoaugmentation is further demonstrated by the fact that addition of monomeric IgD to dimeric or aggregated IgD blocks IgD-receptor upregulation on T cells in vitro and in vivo.[82] Immuoaugmentation induced by oligomeric IgD is also abolished by simultaneous injection of monomeric IgD.[82]

Our present in vitro studies successfully reproduce the previous in vivo studies carried out with IgD. Thus, IgD in vivo and in vitro treatments significantly enhance in vitro primary and early secondary antibody responses to antigens such as TNP-BA and SRBC. Oligomeric IgD or aggregated IgD augments antibody responses in vitro whereas monomeric IgD does not, and treatment of aggregated mutant monomeric IgD as well as Fabδ fragments develop the immunoaugmenting properties.

Evidence from our current studies have provided insight into the mechanism by which IgD augments antibody responses. Our experimental design was based upon the rationale that IgD is lost from B cell surfaces within days after activation by a specific antigen.[122] Thus, animals in all experiments were primed with Ag and given IgD three days prior to the establishment of experiments to determine the role of interaction between IgD and IgD-R in the immunoaugmentation induced by IgD. The functional role of Tδ cells had been ascertained by co-cultures of T and B cells purified from mice with or without IgD pre-treatment.

Our results showed that T cells from IgD-pre-treated but not those from control mice significantly augmented the antibody responses of B cells. Moreover, the mechanism by which the Tδ cells enhance the immune responses did not appear to be via a direct effect of soluble factors released from such cells. The addition of supernatants collected from IgD-pre-treated splenic cell cultures failed to enhance the antibody responses of normal splenic cell cultures. Instead, our evidence from double-chamber cultures where cell-cell contact was prohibited, whereas communication of soluble factors was allowed, strongly suggests that contact between Tδ cells and B cells is required for the IgD-induced immunoaugmentation.

The critical role of interaction between IgD and IgD-R during the cognate interaction between Tδ and B cells for immunoaugmentation was further supported by the blocking effect of IgD. In vitro addition of monomeric IgD molecules such as KWD8 and B1.8 δ1, as well as other monomeric mutant IgD molecules such as KWD1 and KWD6, significantly inhibited the augmenting effect induced by oligomeric IgD treatment in vivo. Addition of oligomeric IgD in vitro in low doses further enhanced the immunoaugmentation induced by in vivo IgD treatment, whereas addition of high doses blocked the augmentation. These results reflect a dual role of IgD in the antibody responses, which are actually the net balance between further induction of IgD-R and blocking of IgD-R

available as a ligand for IgD. A similar effect of oligomeric IgD is also seen on the proliferation of spleen cells.

An examination of the IgD structures shows that KWD1 lacks Cδ1, KWD6 lacks both Cδ1 and the Cδ-hinge, and Fabδ lacks the Cδ3 region. The fact that these molecules block immunoaugmentation induced by oligomeric wild-type IgD suggests that the N-glycans linked to different Cδ regions, the density of the N-glycans on the whole IgD molecule, or both the N-glycans and the protein sequences of the Cδ regions are important for the interaction between IgD and IgD-R.

In one of our experiments, GS-1 bound monomeric IgD molecules were shown to significantly block immunoaugmentation as compared with the weak blocking effects of GS-1 unbound IgDs, suggesting further that the carbohydrates on IgD are important in binding to IgD-R. Further studies showed that in both cases KWD1 showed a greater ability than KWD6 to block the immunoaugmentation, suggesting that: (1) the type of carbohydrates distributed on the Cδ-hinge, or; (2) the total amount of carbohydrates linked to the IgD molecule; and/or (3) the protein sequence of the Cδ-hinge also plays a role in binding to IgD-R. Moreover, Fabδ fragments showed less blocking ability than GS-1 bound KWD6, but greater blocking ability than GS-1 unbound KWD6, suggesting that the carbohydrates associated

with IgD molecules are more important than the protein domains in binding of IgD to IgD-R.

To investigate the specificity of Tδ cells in immunoaugmentation, we co-cultured B cells with T cells from mice treated with IgD alone, or mice treated with IgD plus antigen. The results strongly suggest that T cells have to possess both IgD-R and specific TCR to demonstrate in vitro immunoaugmentation of B cell responses to the same antigen recognized. We have demonstrated that when Tδ cells are primed with the same antigen they can consistently augment antibody responses of B cells. Furthermore, similar cultures of whole spleen cells from antigen-primed mice or antigen plus IgD-treated mice failed to show immunoaugmentation upon addition of T cells from mice treated with IgD alone. This suggests that T cells bearing IgD-R but without TCR specific for the same antigen recognized by B cells, cannot effectively collaborate with B cells as measured by enhanced Ag-specific Ig production.

The significance of antigen presentation by B cells to T cells has been suggested by several lines of evidence. A mechanism of T-B collaboration via antigen bridging has been postulated by classic studies using a hapten-carrier system, which demonstrated that a collaboration between T and B cells resulted from the proximity of carrier primed T cells and the hapten primed B cells brought by such an antigen.[123,124] Moreover, B cells, like macrophages, have

been shown to process and present an antigen to T cells in a specific or non-specific way. In a non-specific presentation, although the capacity of activated B cells to present antigen is comparable to that of B lymphoma cells or macrophages after stimulation with LPS, it might at least in part be explained by their 10-fold greater capacity to take up antigen.[125,126,127] In contrast, as one might expect, B cells have been found to be an extremely efficient APC when their surface Ig functions to specifically bind Ag with high-affinity binding, thereby focusing the Ig receptor to the antigen.[128,129]

Evidence from our studies shows that when macrophages are depleted from the cultures, B cells fail to show the immunoaugmentation induced by IgD. Such a result is consistent with previous reports. Resting B cells have been found to be incapable of activating resting T cells.[108,130] It should be noted that the majority of T and B cells employed in our experiments are "resting" cells. Although the animals have been primed with antigen three days prior to the day of culture initiation, the few T and B cells specific for antigen have had insufficient time to expand and display all the requisite functional properties ascribed to activated cells.

There are several reasons for the poor ability of resting B cells to present antigens. Normal T cell activation requires two signals: specific signals come from

the complex of peptide fragments of a protein antigen bound to self MHC molecules (present on the APC surface), which are then recognized by a MHC-restricted T cell receptor complex.[131,132] Non-specific costimulator signals are provided by the APC during antigen presentation. These costimulators can be adhesion molecules causing physical interaction, which facilitate signal transduction on the part of T cells, or cytokines released from the APC that cause T cell activation and division.[133]

IL-1 (secreted and membrane-bound forms) has been demonstrated to be a requisite costimulator for $CD4^+$ murine T cells. Both secreted and membrane bound IL-1 forms consist of two species, IL-1α and IL-1ß. IL-1ß is the predominate species in the secreted form, and IL-1α in the membrane-bound form. IL-1 is not only secreted by APC such as macrophages and activated B cells but also produced by many other cells.

In general, the APCs do not constitutively express a costimulatory activity. Thus, macrophages harvested from lymphoid organs or from the peritoneal cavity do not express IL-1 mRNA or protein.[134,135] Likewise, resting B cells are IL-1 negative.[136] Furthermore, several adhesion molecules have been shown to be involved in the capacity of B cells to activate T cells. MHC class II (Ia) antigens increase on the B cell surface by as much as 10-fold after activation.[137] Moreover, an inverse relationship between the quantity of Ia

expressed on accessory cells and the concentration of antigen required for the induction of a T cell response has been reported.[138] Thus, if B cells are non-activated, they do not express IL-1 or a high enough level of adhesion molecules on their surface, and hence will not be able to present antigen specifically or non-specifically to resting T cells. These resting T cells in turn will not be able to help B cells to differentiate and proliferate in response to an antigen. Macrophages supplemented to such cultures may actually facilitate B cells in that they initiate the antigen presentation by activating T cells, leading to secretion of IL-1. After the number of activated T cells increases, such T cells can interact with resting B cells, which become activated and then take over the role of antigen presentation.

Because supplementation of IL-1 alone recovers the antibody response, we suspect that this factor is the limiting factor for activation of T cells in our <u>in vitro</u> system. Previous studies have demonstrated that T cell proliferation is directly proportional to the combined amount of MHC class II molecules and membrane IL-1 expressed on the macrophage surface.[138] This indicates that the metabolic effects transduced by these two stimulatory molecules on T cells are additive. Therefore, with the compensatory effect of IL-1, resting B cells that are bearing Ag-specific Ig can start to present antigens. This

can occur even though these resting B cells express low amounts of adhesion molecules on their surface. Moreover, spleen cells from control mice show an enhanced antibody response under the influence of IL-1.

Although it is clear that IgD treatment can induce an augmented antibody response in spleen cells, knowledge as to whether such an immunoaugmentation results from enhanced proliferation of B cells or enhanced differentiation of B cells into antibody-secreting cells is lacking. To solve this puzzle, proliferation assays using IgD-treated cells were established. Initial studies with whole spleen cells demonstrated an enhanced proliferation induced by IgD treatment. Assays employing purified T and B cells clearly showed that T but not B cells are responsible for proliferative activity. Therefore, IgD treatment appears to induce differentiation of B cells to produce Ig (a qualitative change) without inducing the proliferation of B cells.

A surprising finding is that IgD appears to enhance the Ag-induced proliferation of T cells. We believe that this effect on T cells is directly related to the ability of IgD-R to facilitate interactions between IgD^+ B cells presenting antigen to Tδ cells. Because we have shown that IgD can induce IgD-R on $CD4^+$ T cells, and we speculate that IgD also functions as a ligand for IgD-R, we thereby conclude that IgD plays a role in both the antigen-presenting process and

the induction of Tδ cells. Previously reported studies have revealed that adhesion molecules are required for physical interactions,[139,140] and in some cases signal transduction between APC and T cells.[141,142,143,144] IgD-R, as the ligand for IgD, may possess signal transduction properties similar to other well characterized adhesion molecules. However, these studies are beyond the scope of the current investigation.

Several lines of evidence have demonstrated that adhesion molecules are involved in the regulation of the antigen presenting process. In addition to Ia molecules, the expression of ICAM-1 and its ligand LFA-1 have been found to be upregulated on B cells after T cells are activated by anti-CD2, anti-CD3 mAb.[116,145] Upon the initiation of interaction between T and B cells, these molecules become capable of supporting the induction of T cell proliferation.[145,146,147] Moreover, recent evidence has indicated that LFA-1 molecules express a transient high-affinity state upon activation of T cells[116,145] as well as B cells.[148]

Adhesion molecules also play a role in intracellular signaling. Studies on a B lymphoma transfectant expressing Ia molecules show that cells with Ia molecules truncated in the cytoplasmic domain are severely impaired in both antigen presentation and in anti-Ia-induced intracytoplasmic signaling.[141] This signal, which can be mimicked by dibutyryl cAMP, induces expression of a newly defined B cell

accessory molecule B7, resulting in effective antigen presentation.[141] Investigations on normal B lymphocytes resulted in a similar conclusion.[142] Thus, monoclonal anti-Ia antibodies enhance anti-μ-induced B cell proliferative responses by increasing the anti-μ-induced expression of the c-myc gene and of a key enzyme (ornithine decarboxylase) in polyamine biosynthesis .[142] Cross-linking of ICAM-1, using anti-CD54 mAb, has been shown to potentiate the effect of anti-CD2 stimulation[143] and modulate cytokine production during PHA stimulation,[144] thus inducing cell aggregation or proliferation.[149]

The putative intracytoplasmic signals transduced by the interaction of IgD and IgD-R have not yet been investigated. A common receptor-mediated signal transduction mechanism involving the hydrolysis of phosphatidylinositol 1,4,5-triphosphate (1,4,5-IP_3) has been observed in the activation responses of a wide variety of cells including platelets, neutrophils, hepatocytes, and lymphocytes.[150,151] In all these systems, receptor stimulation results in the activation of an intracellular enzyme, phospholipase C, which cleaves the phosphodiester linkage of phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the formation of inositol 1,4,5-triphosphate (1,4,5-IP_3) and 1,2-diacylglycerol (DG). These molecules, in turn, function as intracellular "second messengers" to induce an increase in Ca^{2+} and activation of protein kinase C (PKC),

respectively. The PKC is found to represent a family of at least five closely related enzymes that share structural features and requirements for Ca^{2+}, phospholipid, and DG.[152] Several proteins such as human CD3 γ and δ chains,[153] and the murine CD3 δ and ϵ chains,[154] have been identified to be phosphorylated following the activation of PKC. Likewise, the candidates for the signals transduced upon the interaction between IgD and IgD-R may well be the components of the pathway mentioned above. However, to clarify the activation pathway, extensive biochemical investigations on identifying modification of proteins following interaction of IgD-IgD-R will be required.

Indeed, recent studies indicate that upregulation of IgD-R occurs upon triggering of T cell activation by cross-linking cell surface molecules using anti-CD3, anti-CD2, and anti-Thy-1.[155] IgD-R is also upregulated by pharmacologically active compounds that increase intracellular cAMP and by PMA/DG plus ionomycin. Furthermore, induction of IgD-R by IgD appears to require tyrosine kinase activity, perhaps in an intracellular Ca^{2+}-dependent manner.[155]

Evidence obtained from our experiments by employing goat-anti-Ig as antigen strongly supports the conclusion that IgD-R plays a role in antigen presentation. This approach was first used to document the role of B cells as APCs.[156] We reasoned that if membrane IgD is cross-linked

(by goat anti-IgD antibody) and such cells are then used to present goat Ig to goat Ig-specific T cells, then interactions between these cells may be facilitated by the co-expression by T cells of the appropriate TCR and IgD-R. It should be noted that in all of our experiments, we carried out parallel rosetting assays to confirm the upregulation of expression of IgD-R by Tδ cells. Using this system, we found that upregulation of IgD-R on T cells results in a more efficient antigen presentation by B cells. We speculate that this effect may be due to a strengthened interaction between T and B cells mediated by IgD and IgD-R.

As previously reported, antigen bound on a surface Ig will be endocytosed, disintegrated in the cytoplasm, form a complex with MHC II molecules, and then finally presented on the APC.[157] Surface Ig will not be degraded, instead, they will be recycled to the surface of cells.[158] Thus, IgD molecules may increase the cohesiveness of B cells in two ways: (1) as an antigen-Ig complex; or (2) as recycled molecules after being endocytosed. As noted earlier, it is not clear from our results whether IgD and IgD-R, like other adhesion molecules, can also transduce intracytoplasmic signals during antigen presentation.

In agreement with previous studies, which have shown that B cells themselves can induce expression of IgD-R by T cells using goat-anti-IgD to cross-link mIgD, our data also demonstrate a significantly greater response of T cells to

goat-anti-δ than to goat-anti-μ. This probably results from further induction of IgD-R on T cells by IgD molecules cross-linked by goat-anti-IgD. Other possibilities also exist. Different signals transduced by IgD vs. IgM may result in different responses of T cells. The difference of membrane density between these two Igs on mature B cells may also explain these results because mIgD is typically expressed at a higher density as compared with mIgM.

T cells have been found to modulate humoral responses in several different ways. Fc receptors for immunoglobulin (FcR) on T lymphocytes have found to be associated with classical helper and suppressor regulatory pathways. Other than IgD-R, Fc receptors for IgA,[159] IgM,[160] IgG,[161] and IgE,[162] which are all membrane glycoproteins that bind Ig molecules via their Fc region, have been identified on the surface of T cells. FcαR$^+$ T cells appear to be involved in IgA regulation. The outcome of this regulation (help vs. suppression) depends on the phenotype of T cells bearing FcαR. Increased numbers of FcαR$^+$ T cells of a suppressor phenotype have been isolated from mice with an IgA myeloma.[159] In addition, these cells have been shown to suppress IgA isotype-specific responses.[163] On the other hand, FcαR$^+$ T cells separated from human peripheral blood have been shown to specifically enhance IgA responses in pokeweed mitogen(PWM)-driven B cell cultures.[164]

T cells are also found to release these immunoglobulin-

binding factors (Ig-BF) which participate in regulation of antibody synthesis. Alloantigen-activated T cells and T cell hybridomas, which express FcγR, were induced to release these receptors as IgG-binding factors that subsequently suppressed IgG synthesis.[120,165] Extensive studies by Ishizaka have shown that FcεR$^+$ T cells regulate IgE responses via production of IgE-BF, which exhibits either enhancing or suppressing properties depending on the different degrees of IgE-BF glycosylation.[166]

ConA-activated FcαR$^+$ T cells or T cell hybridomas were induced by IgA to release IgA-BF, which suppressed IgA synthesis in PWM-triggered murine spleen cell cultures.[167,168] Studies on IgA-BF released from a T helper cell line derived from mouse Peyer's patches, which express FcαR and constitutively produce IgA-BF, show that these IgA-BF either suppress or enhance antigen-dependent IgA responses, and that the response was dependent on the amount of factor added to <u>in vitro</u> cultures.[169] IgD-BF has also been reported to be released by IgD-R$^+$ murine splenic T cells induced by dimeric IgD, IL-4, anti-IgD antibodies.[113,114] Furthermore, IgD-BF is released by normal splenic T cells induced by recombinant IL-2, recombinant IL-4, and cross-linked IgD in amounts paralleling the induction of IgD receptors on the cells. Induction of two hybridoma cell lines with rIL-2 increases release of IgD-BF while reducing expression of IgD-R on these cells. Thus, these experiments

suggest a direct relationship between IgD-R and IgD-BF.

To further explore the biochemical characteristics of mouse IgD-BF and its relationship with IgD-R, we employed a monoclonal antibody (E11) specific for IgD-BF. The specificity of this mAb to mouse IgD-BF has been confirmed by direct and indirect ELISA assays (see results). Our results also show that E11 recognizes IgD-R expressed on the surface of T cells, suggesting a structural relationship between IgD-R and IgD-BF. Thus, E11 blocks formation of IgD-RFC by hybridoma cells (1D1E7), which constitutively express IgD-R, as well as by normal splenic T cells induced to express IgD-R by IL-4. FACS analyses showed that E11 stains 1D1E7 cells induced by dimeric IgD, IL-2, and IL-4 as well as normal splenic T cells induced by dimeric IgD; E11 also blocks staining of hybridoma cells (2H10) by dimeric IgD.

Solid-phase immunoprecipitation of IgD-R from cell membrane lysates and IgD-BF released into cell cultures of 2H10 was carried out by employing IgD or E11-conjugated Sepharose. These experiments revealed electrophoretic patterns showing partial similarity between these two proteins, which are recognized by both IgD and E11. Thus, both IgD-R and IgD-BF contain protein fragments of 69 kDa and 20 kDa. However, we don't know whether IgD-Sepharose can be used to precipitate fragments other than those listed above because some bands precipitated by IgD do not appear

to be recognized by E11.

Our results show that the molecular weight of IgD-BF is somewhat different from those reported previously.[69] IgD-BF are 37 and 78 kDa as determined by gel-filtration analysis of such factors derived from Ag-stimulated splenic T cells.[113] However, other studies[69] showed that IgD-BF may be more heterogeneous with respect to molecular weight, existing as species of 80, 62, 42, 33, 24, 20, and 14 kDa, with a major species in the 20-25 kDa range,[69] It is not a surprise to find the size heterogeneity because this has been reported for murine Fc receptors for other immunoglobulin isotypes, including those specific for IgG[170] and IgE.[171]

Functional studies using E11 <u>in vitro</u> to assess its effects on antibody responses of splenic cells show a similar effect of this mAb to that of IgD. Thus, addition of E11 to the <u>in vitro</u> cultures: (1) augments primary antibody responses; (2) dose-dependently augments early secondary antibody responses of control spleen cells; and, (3) augments secondary antibody responses of IgD-pre-treated spleen cells in low doses, but inhibits in high doses. E11 also influences B cell antigen presentation. Addition of E11 to <u>in vitro</u> cultures significantly augments responses of control T cells to the activation of B cells in a dose-dependent fashion, and augments the response of IgD-pre-treated T cells in low doses, but inhibits in high doses.

Such a result is expected because cross-linking of IgD-R on T cells by E11 results in upregulation of these receptors, which in turn facilitates the interaction between T and B cells. However, E11 can also hinder the interaction between IgD-R and IgD-BF. Therefore, just as we have proposed for a disparate, dose-dependent functional outcome using IgD in this system, the net effect of E11 will be determined by the balance of IgD-R induction by this mAb and interference of IgD-IgD-R interactions by this mAb.

Previously reported studies by Adachi and Ishizaka have demonstrated an immunoaugmenting effect of IgD-BF by addition of IgD-BF purified using mouse IgD-Sepharose.[172] However, our data disagree with this finding. IgD-BF, derived from 2H10 cell cultures and isolated by solid-phase immunoprecipitation using both IgD-Sepharose and E11-Sepharose, was tested in our in vitro culture system for its effect on antibody responses. Our results showed that IgD-BF failed to show any significant effect on antibody production but reduced the immunoaugmentation induced by IgD-pre-treatment in a dose-dependent fashion.

The discrepancy between our results and others may be explained as follows: (1) the reported augmenting effect of IgD-BF (purified by IgD-Sepharose chromatography) may actually be the result of contaminating IgD leached off the Sepharose; indeed, our current results show that low doses of IgD will enhance antibody responses in vitro by inducing

the IgD-R on T cells; (2) the effect of IgD-BF on antibody responses may be dose-dependent, just like that of previously reported IgA-BF;[169] however, the mechanism is not clear; (3) the distance that the IgD-BF can travel and function properly after its release from Tδ cells may also influence the results; and (4) the IgD-BF employed in our experiment is derived from a hybridoma cell line (2H10), which is derived from a strain of mouse other than that used for our in vitro assays (i.e., BALB/c). However, we know that murine IgD does not show strain specificity;[3] thus IgD-BF derived from different sources should be able to recognize IgD molecules on cells from various strains. Indeed, the dose-dependent blocking effect of IgD-BF which we have observed supports this speculation. Finally, the half-life of the IgD-BF is unlikely to be a critical factor in explaining the lack of function of IgD-BF in our assay system, especially if the IgD-BF, purified from a time-consuming purification process works in the previous reported studies. Moreover, our IgD-BF is unlikely to be contaminated by IgD eluted from the IgD-Sepharose because it does not show any effect on control cells, the response of which should be enhanced by low doses of IgD should such a contamination occur.

Results from double-chamber assays also fail to support the conclusion that IgD-BF is the factor responsible for IgD-induced immunoaugmention. Instead, it may inhibit such

a response by interrupting the interaction between IgD and IgD-R. In control cell cultures, the interaction of IgD and IgD-R may only account for a minor portion of the factors facilitating T and B cell interactions among many other factors, which may explain why IgD-BF does not affect responses of control cells.

Attempts to purify large amounts of IgD-BF failed using different sources and approaches. We speculate that IgD-BF released from IgD-R$^+$ T cells is a very labile protein molecule which may undergo spontaneous degradation or proteolytic enzyme-mediated degradation after its release. This may also explain its appearance as multiple species, as well as the different IgD-BF molecular sizes reported from different groups.

In summary, the combined results of previous studies as well as those of our current investigation, allow us to propose a modified hypothetical scheme based on the work published by Coico et al.[97] for the function of IgD under physiological conditions:

1. IgD and IgD-R play a role in antigen presentation. T cell IgD-R are upregulated by previous antigen encounters or by lymphokines such as IL-2, IL4, or INF-γ, and further upregulated as a consequence of antigen presentation by antigen cross-linked IgD. Thus, antigen presentation by B cells will also be facilitated by interactions between IgD on B cells and IgD-R on T cells. It is not clear whether

the interaction of IgD and IgD-R gives rise to the intracytoplasmic signal or merely strengthens the contact between T and B cells.

2. Cognate T and B cell interactions are essential to the immunoaugmentation mediated by Tδ cells. Tδ cells augment antibody responses by signaling B cells through cell-cell contact but not by releasing soluble factors. Such a contact is mediated by IgD and IgD-R because it can be interrupted by either monomeric or dimeric IgD. Augmention of specific antibody responses requires that Tδ cells simultaneously possess specific antigen-receptor (TCR) on their surface. Moreover, Tδ cells enhance the differentiation of B cells, which then develop into Ab-secreting cells. However, the collaboration of T and B cells leads to the proliferation of T cells. Since it is known that IgD molecules are down-regulated following B cell activation, the interaction between IgD and IgD-R must take place as an early event in humoral immune responses.

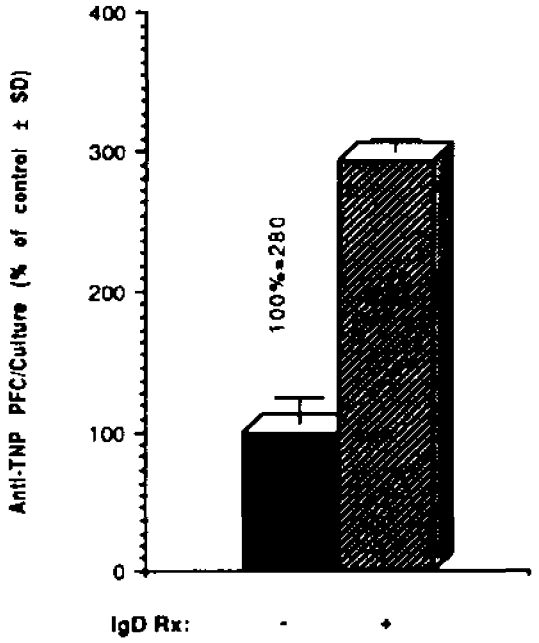
3. Consequences of T and B cell activation. Upon activation of T cells, lymphokines such as IL-2, IL-4, and INF-γ are produced. These can function as autocrine factors. If T cells with upregulated IgD-R were to continue to interact with B cells, excess B and T cell stimulation might occur. Thus, the down-regulation of IgD may actually facilitate a physiologic break in cognate interaction. IgD-BF, released from the Tδ cells may also play a role in

turning off IgD-IgD-R-mediated cognate interactions. Although experiments were not designed to formally test whether IgD-BF can bind to membrane IgD, if we accept this as a possibility, then IgD-BF may bind to IgD and interrupt the interaction between IgD and IgD-R. IgD-BF appears to be quickly degraded, which ensures that subsequent immune responses involving interactions between IgD and IgD-R go on without interference. Because IgD and IgD-BF appear to have a structural relationship, as shown in our current studies, and, because the decrease of IgD-R is accompanied by an increase of IgD-BF in cell culture supernatants, we speculate that IgD-BF is the released form of IgD-R. If this is the case, then the release of IgD-R itself functions as a regulatory mechanism for B cell activation. Similarly, since the release of IgD-BF from Tδ cells is associated with decreased IgD-R expression, antigen-specific T cell proliferative responses facilitated by IgD-R-IgD interactions between Tδ cells and B cells (i.e., Ag-specific APCs), would also be down-regulated.

We have also initiated a biochemical characterization of the IgD-BF and membrane IgD-R. Our preliminary data showed two protein bands, 69 kDa and 30 kDa common to the soluble and the membrane protein derived from Tδ cells, which are recognized by both IgD and IgD-BF-specific mAb (E11), suggesting that the former is the released form of the latter. Similar to other Ig-BFs such as IgG- and IgE-

BF, as well as published work on IgD-BF, the heterogeneous size species (69 kDa, 46 kDa, 40 kDa, 30 kDa and 20 kDa) detected in our experiments strongly suggests that IgD-BF is a labile protein. Therefore, a full characterization of these proteins will be necessary to define the relationship between them. In addition, more work is needed to further clarify the role of IgD and to identify the mechanism by which it functions.

Figure 1a. IgD pre-treatment in vivo enhances in vitro primary antibody responses to TNP-BA. Where indicated, BALB/c mice were injected i.v. on day -3 with 50 μg of TEPC-1017-derived IgD. In vitro cultures of spleen cells from IgD-treated and control mice were established on day 0 at a density of $5x10^6$/ml (1 ml) in the presence of 10 μg of TNP-BA. Cells were incubated at 37°C, 5% CO_2 for 4 days and then harvested for a TNP-specific IgM PFC assay. Results of the PFC assay are expressed as % of control ± SD ($p < 0.01$). IgD-rosette assays on spleen cells prior to the establishment of cultures were also undertaken and shown as % of IgD-RFC. Rosetting with BSA-coated SRBC yielded backgrounds of 3-5% (mean = 4%). This was subtracted from IgD-RFC values.



% IgD-RFC 7 ± 1 27 ± 1

Figure 1a

Figure 1b. IgD pre-treatment in vivo enhances in vitro early secondary antibody responses to both TNP-BA and SRBC. Mice were injected i.v. on day -3 with 80 μg of TNP-BA or 10^8 SRBC with or without 100 μg of TEPC-1017 IgD. Spleen cells from control (no IgD pre-treatment) or IgD pre-treated mice were cultured on day 0 at $5x10^6$/ml in the presence of 5 μg/ml of TNP-BA (TNP-BA-primed cells) or 50 μl/ml of 0.1% SRBC (SRBC-primed cells) for 4 days, and then assayed for TNP-specific or SRBC-specific IgM PFC, respectively. Data are expressed as % of control ± SD ($p < 0.01$).

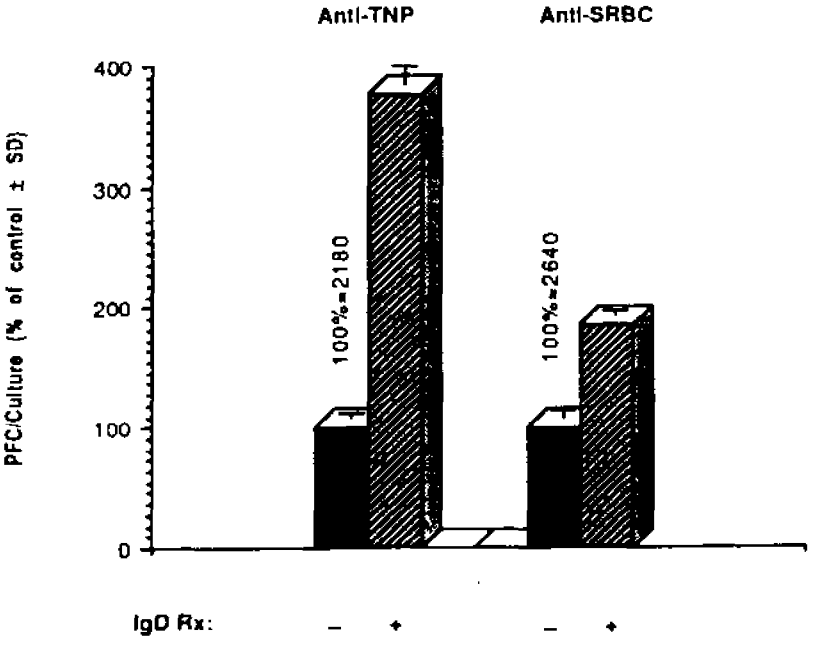

Anti-TNP
Anti-SRBC
PFC/Culture (% of control ± SD)
400
300
200
100
0
100%=2180
100%=2640
IgD Rx:
–
+
–
+

Figure 1b

Figure 2. Pre-treatment with IgD in vivo augments early secondary IgM- and IgG-anti-SRBC responses in vitro. Mice were primed with SRBC together with or without IgD and their spleen cells were cultured in vitro as described in Figure 1b. Culture supernatants were collected and cells were harvested on day 4, followed by ELISA assays for IgM and IgG production and for IgM-anti-SRBC PFC responses, respectively. Data are expressed as % of control ± SD ($p < 0.01$)

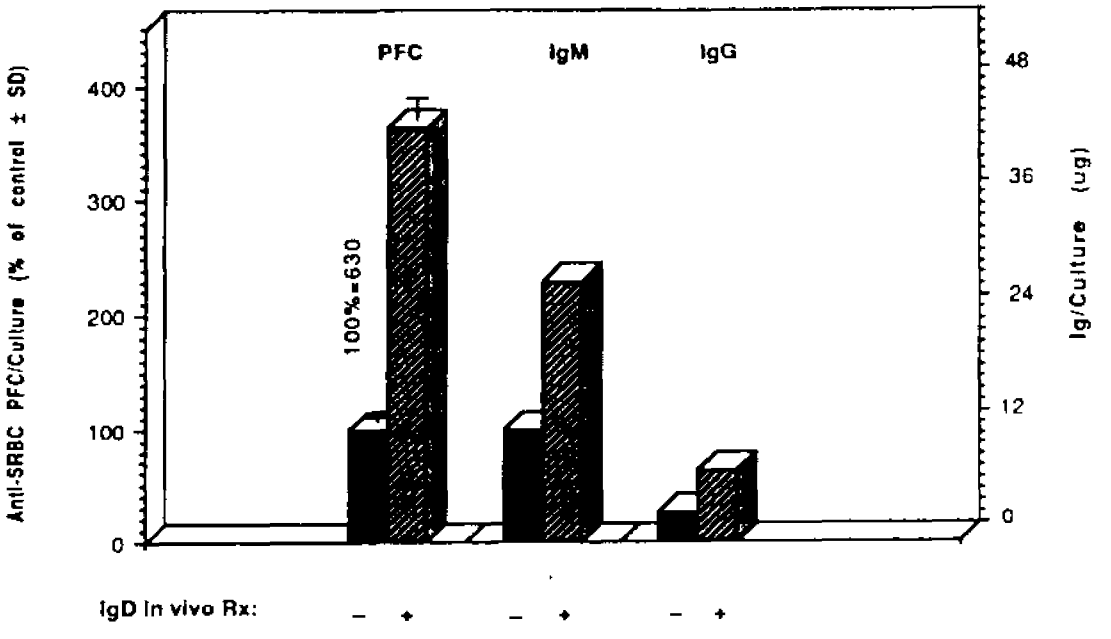

PFC
IgM
IgG
400
300
200
100
0
100%=630
48
36
24
12
0
Anti-SRBC PFC/Culture (% of control ± SD)
Ig/Culture (ng)
IgD In vivo Rx:
– + – + – +

Figure 2

Figure 3. In vitro exposure of spleen cells to IgD potentiates their early secondary antibody responses to SRBC. Mice were primed with SRBC as described in Figure 1b with or without 100 μg of TEPC-1017 IgD. Spleen cells from control or IgD pre-treated mice were panned on IgD-coated Petri dishes for 2-4 hr at 37°C, 5% CO_2 and then harvested, washed twice, and cultured as described in Figure 1b. Cells from mice that were SRBC-primed only and not panned on IgD-coated dishes served as controls. SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.05$).

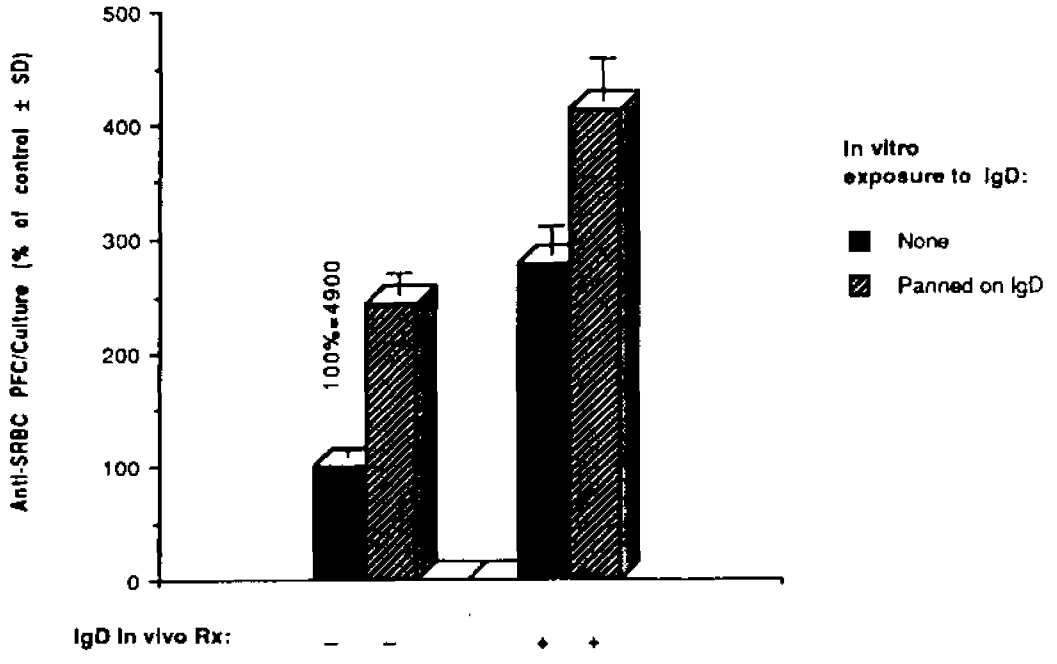

Anti-SRBC PFC/Culture (% of control ± SD)
500
400
300
200
100
0
100%=4900
IgD In vivo Rx:
–
–
+
+
In vitro
exposure to IgD:
None
Panned on IgD

Figure 3

Figure 4a. Immunoaugmentation of in vitro early secondary antibody responses to SRBC by in vivo pre-treatment with aggregated mutant IgD versus aggregated Fabδ from TEPC-1017-derived IgD. Mice were injected i.v. on day -3 with 10^8 SRBC together with 100 ug of mutant IgD molecules or Fabδ as indicated. Spleen cells were cultured in the presence of SRBC on day 0 and assayed for anti-SRBC PFC responses as described in Figure 1b. Data are expressed as % of control ± SD ($p < 0.01$).

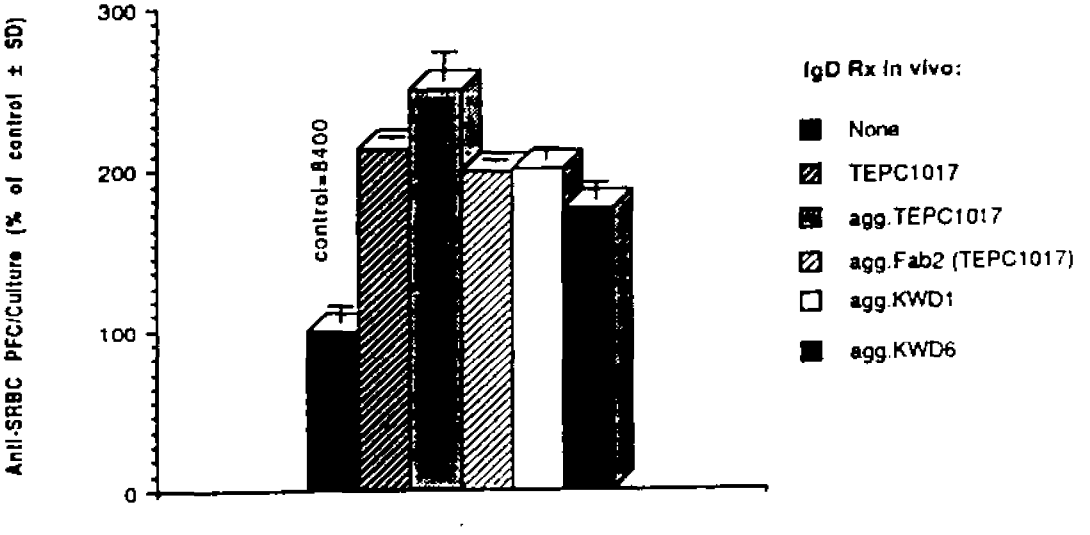

Anti-SRBC PFC/Culture (% of control ± SD)
300
200
100
0
control=8400
IgD Rx in vivo:
None
TEPC1017
agg.TEPC1017
agg.Fab2 (TEPC1017)
agg.KWD1
agg.KWD6

Figure 4a

Figure 4b. Non-aggregated mutant IgD molecules fail to augment in vitro early secondary antibody responses to SRBC. Mice were injected i.v. on day -3 with 10^8 SRBC with or without 100 μg of IgD from different sources as indicated. Spleen cells were cultured on day 0 in the presence of SRBC as described in Figure 1b, followed by SRBC-specific IgM PFC assays on day 4. Results are expressed as % of control ± SD ($p < 0.01$).

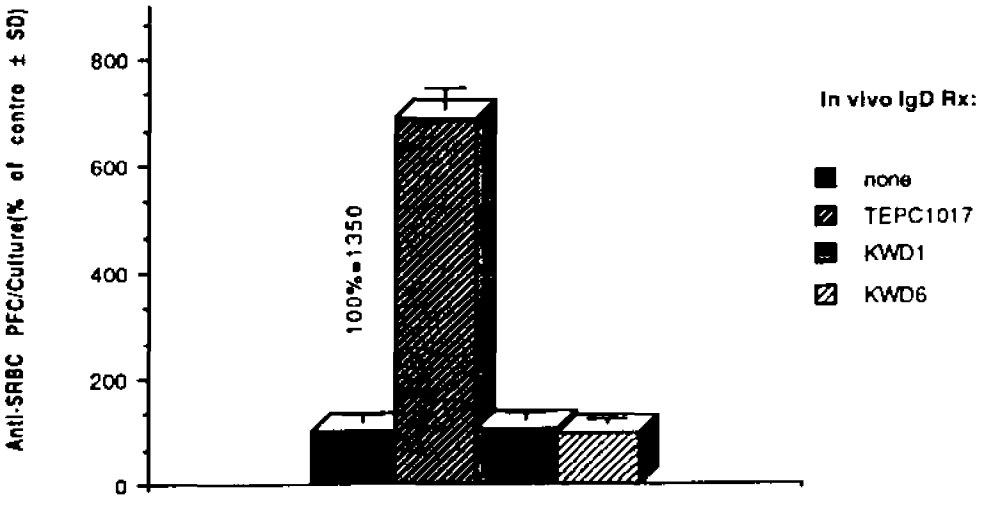

Anti-SRBC PFC/Culture(% of contro ± SD)
800
600
400
200
0
100%=1350
In vivo IgD Rx:
none
TEPC1017
KWD1
KWD6

Figure 4b

Figure 4c. Comparison of monomeric vs. oligomeric IgD on in vitro early secondary antibody responses to SRBC. Mice were primed with SRBC ± TEPC-1017 IgD as described in Figure 1b. Spleen cells were cultured in modified Mishell-Dutton cultures supplemented with or without 200 or 10 μg of monomeric KWD8, B1-8.δ1 IgD, or oligomeric TEPC-1017 IgD. Cells from SRBC-primed mice not treated in vivo with IgD served as controls (data shown as 100%). SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.01$).

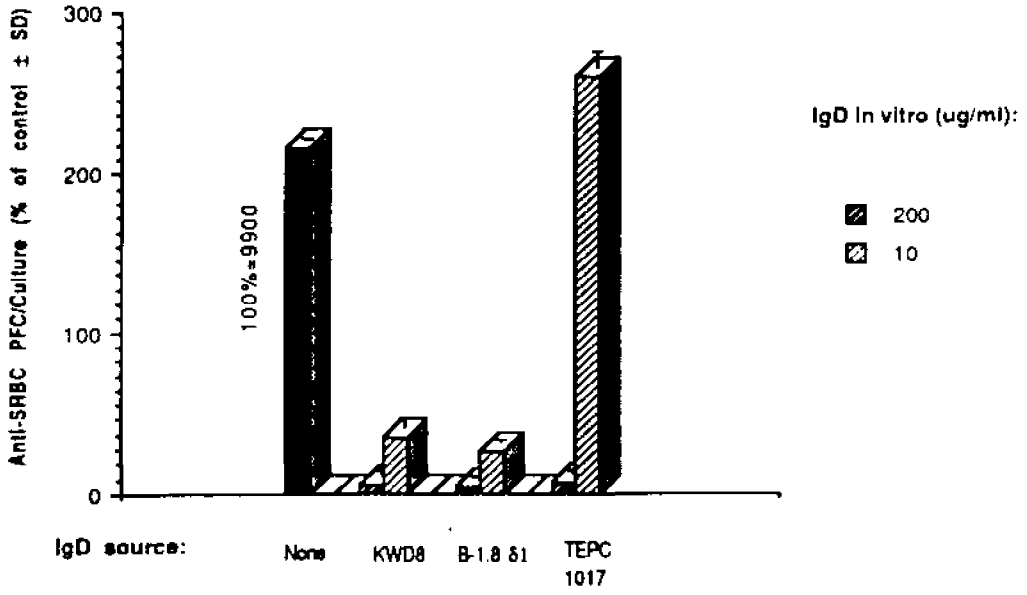

Anti-SRBC PFC/Culture (% of control ± SD)
300
200
100
0
100%=9900
IgD source:
None
KWD8
B-1.8 δ1
TEPC 1017
IgD in vitro (ug/ml):
200
10

Figure 4c

Figure 4d. Blocking effects of mutant IgDs and Fabδ on in vitro antibody responses. Mice were primed in vivo and their spleen cells cultured in the presence of SRBC as described in Figure 1b. Cultures were supplemented on day 0 with or without 100 μg of GS-1 bound or GS-1 unbound monomeric KWD1 or KWD6 IgD. Where indicated, cultures were supplemented with 100 μg of oligomeric TEPC-1017 IgD or TEPC-1017 IgD Fab fragments prepared as described in Materials and Methods. For comparison, the 100% response (8,400 PFC/culture) reflects that generated in vitro by whole spleen cells from mice primed with SRBC, but without IgD pre-treatment. SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.05$).

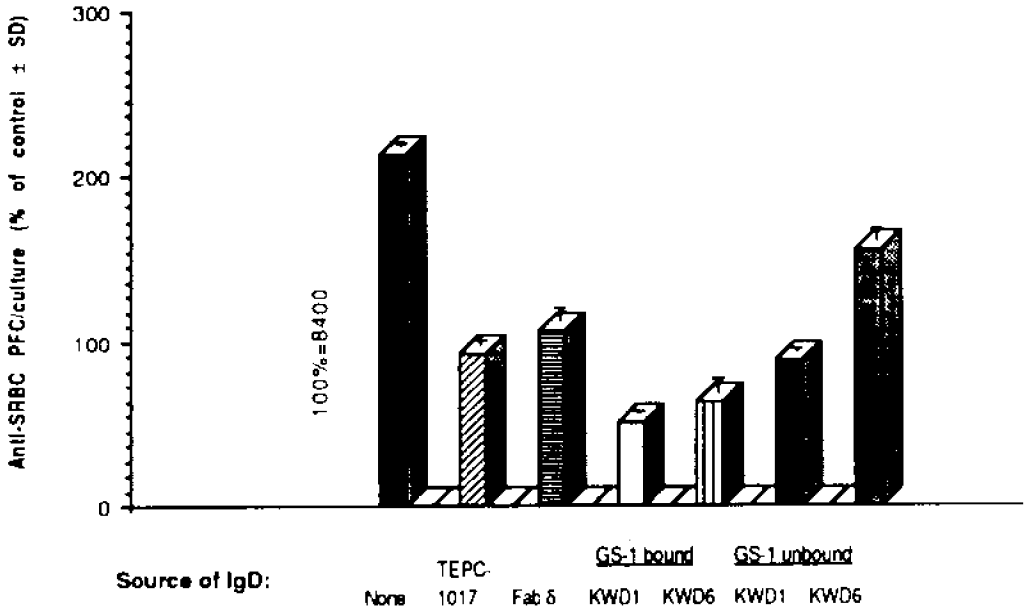

Anti-SRBC PFC/culture (% of control ± SD)
300
200
100
0
100%=8400
Source of IgD:
None
TEPC-1017
Fab δ
GS-1 bound
KWD1
KWD6
GS-1 unbound
KWD1
KWD6

Figure 4d

Figure 5. Dose-dependent effects of IgD in vitro on anti-SRBC responses of control vs. in vivo IgD-pre-treated spleen cells. SRBC-primed spleen cells from control and IgD-treated mice were cultured as described in Figure 1b. Various doses of TEPC-1017 IgD were added to cultures on day 0. SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.05$).

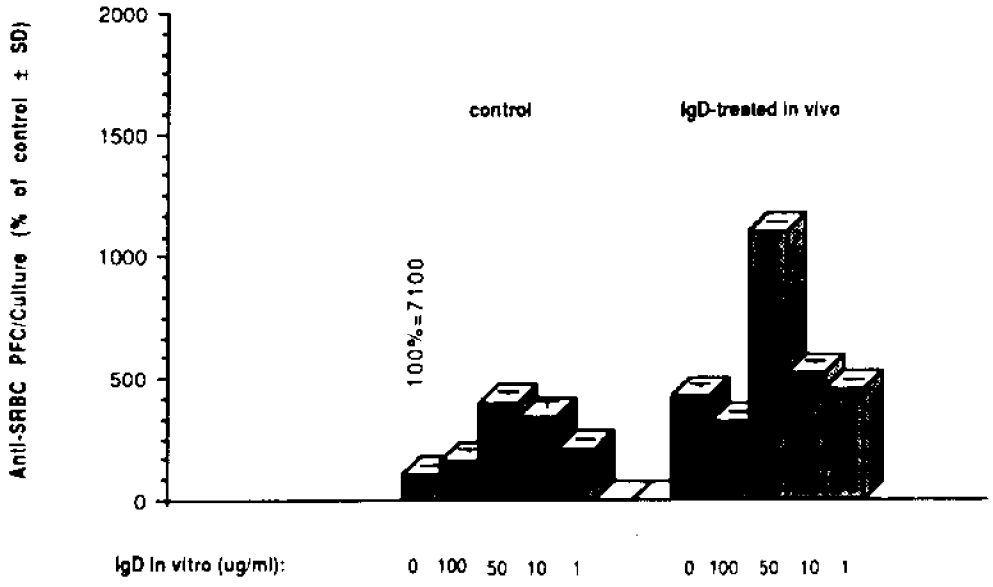

Anti-SRBC PFC/Culture (% of control ± SD)
2000
1500
1000
500
0
control
IgD-treated in vivo
100%=7100
IgD in vitro (ug/ml):
0 100 50 10 1
0 100 50 10 1

Figure 5

Figure 6. Time course of IgD effect on in vitro cultures of SRBC-primed control vs. in vivo IgD-treated spleen cells. SRBC-primed spleen cells from control and IgD-treated mice were cultured as described in Figure 1b. Cultures were supplemented with 100 μg of TEPC-1017 IgD on days 0, 1, and 2. SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.05$).

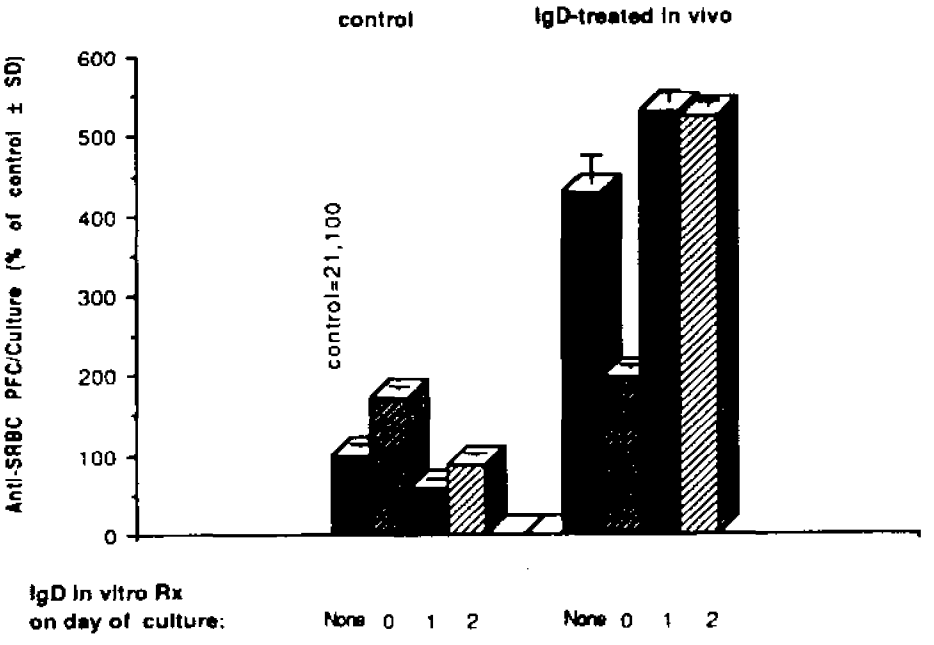

control
IgD-treated in vivo
Anti-SRBC PFC/Culture (% of control ± SD)
600
500
400
300
200
100
0
control=21,100
IgD in vitro Rx
on day of culture:
None 0 1 2
None 0 1 2

Figure 6

Figure 7. Requirement for adherent cells by IgD-treated spleen cells in the generation of augmented anti-SRBC responses in vitro can be replaced by IL-1. Mice were treated *in vivo* with SRBC ± TEPC-1017 IgD on day -3 as described in Figure 1b. On day 0, spleen cells from such mice were depleted of adherent cells by incubating whole spleen cells in 100-mm Petri dishes as described in Materials and Methods. Whole spleen cells from untreated, unprimed control mice were used as a source of adherent cells (macrophages), which were prepared by culturing $5x10^6$ cells in wells of 24-well plates for 4 hr at 37°C, 5% CO_2. Non-adherent cells were removed by trituration. Where indicated, macrophage-depleted spleen cells from control and IgD pre-treated mice were cultured with SRBC in the presence or absence of "macrophages" or 500 pg/ml of recombinant murine IL-1ß. SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.01$).

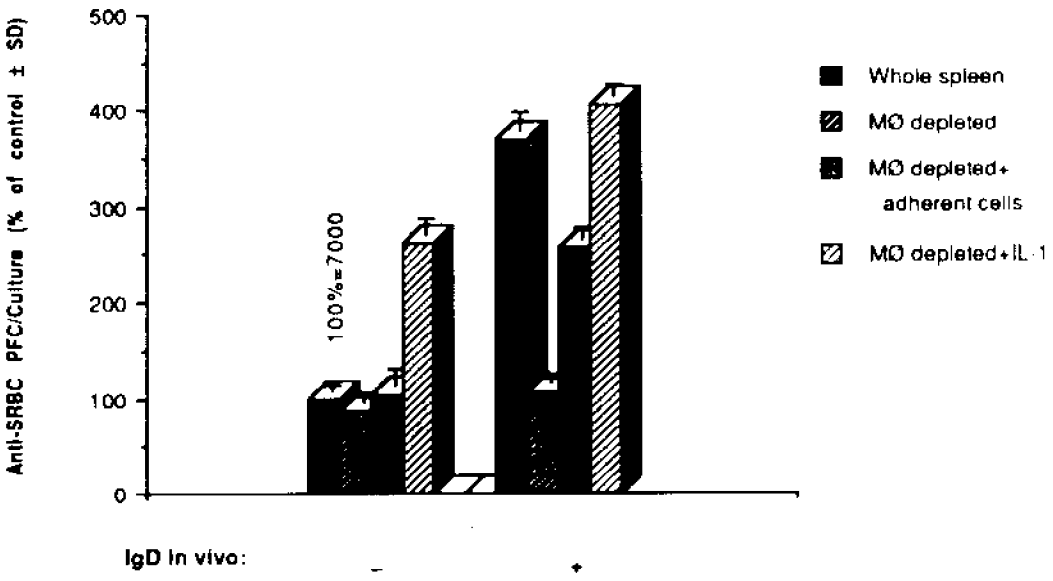

Anti-SRBC PFC/Culture (% of control ± SD)
500
400
300
200
100
0
100%=7000
IgD in vivo:
-
+
Whole spleen
MØ depleted
MØ depleted+ adherent cells
MØ depleted+IL-1

Figure 7

Figure 8. In vivo Tδ cells mediate humoral immunoaugmenting effects in vitro. Spleen cells from SRBC-primed mice, injected with or without IgD (see Fig. 1b) were fractionated into T and B cell populations (controls) and Tδ and Bδ cell populations, respectively (see Material and Methods). Equal numbers of T and B cells ($5x10^6$ total) isolated from control or IgD-treated mice were co-cultured as indicated in the presence of SRBC for 4 days. SRBC-specific IgM PFC assays were performed on day 4. Anti-SRBC antibody responses of unfractionated spleen cells from control and IgD-treated mice are also given. Results are expressed as % of control ± SD ($p < 0.01$).

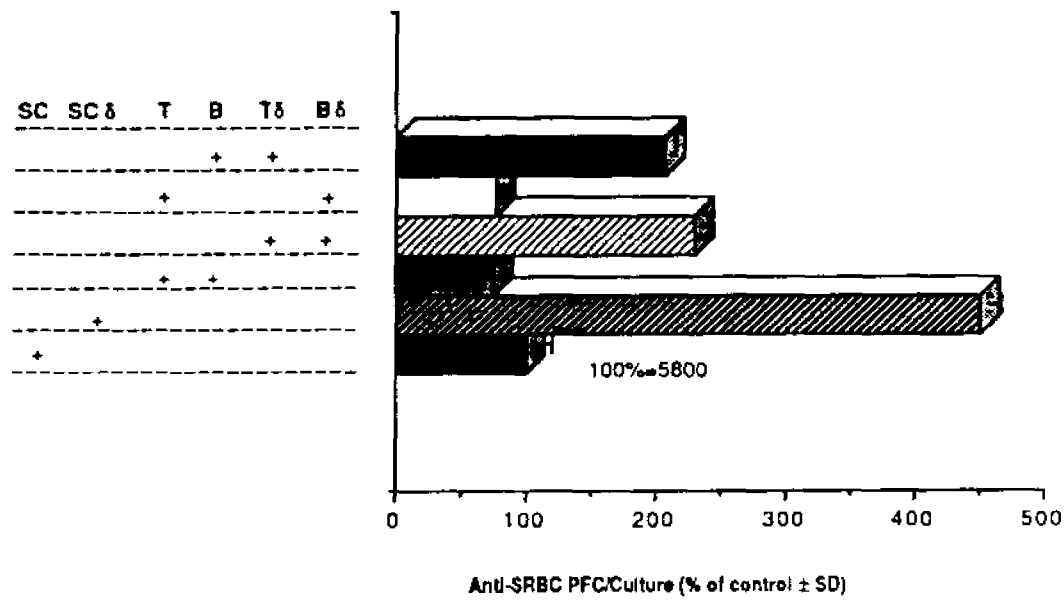

SC
SCδ
T
B
Tδ
Bδ
100%=5800
0
100
200
300
400
500
Anti-SRBC PFC/Culture (% of control ± SD)

Figure 8

Figure 9a. Augmenting effect of IgD pre-treated T cells on anti-SRBC antibody responses by SRBC-primed whole spleen cells is dependent upon T cell antigen priming in vivo.

Mice of group "A" were injected i.v. on day -3 with 10^8 SRBC alone or together with 100 μg of TEPC-1017 IgD. A separate group of mice, group "B", were treated with IgD alone, or with both SRBC and IgD on day -3. On day 0, whole spleen cells from group "A" were cultured in the presence of SRBC as described in Figure 1b with the addition of various doses of purified splenic T cells derived from group "B" as indicated. Responses of spleen cells from mice primed with SRBC alone (i.e., without additional T cells added) served as controls. SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.05$).

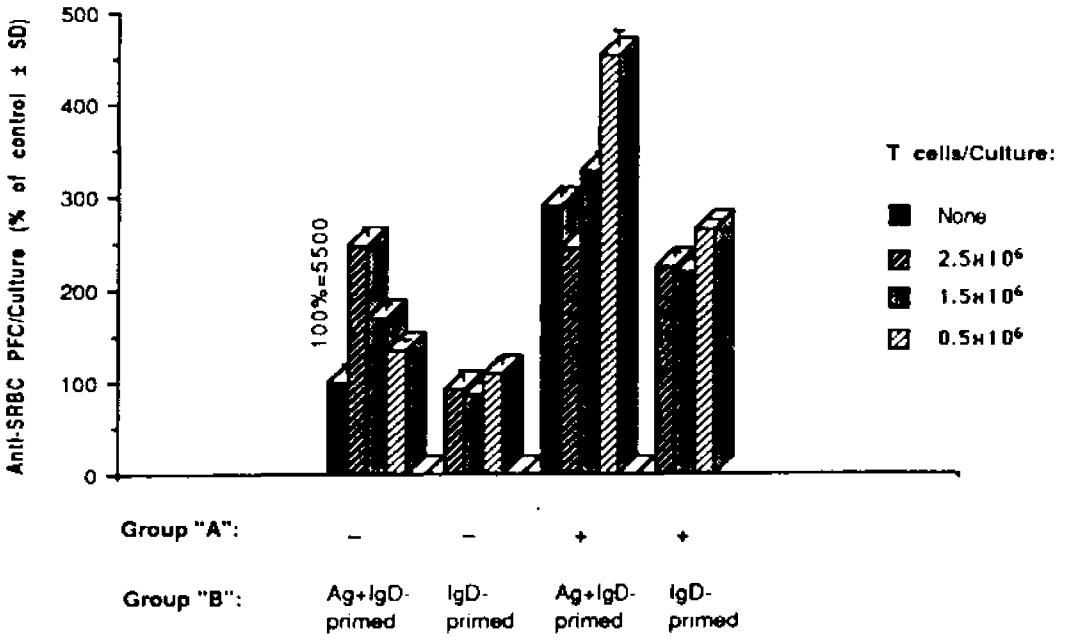

Anti-SRBC PFC/Culture (% of control ± SD)
500
400
300
200
100
0
100%=5500
T cells/Culture:
None
2.5x10^6
1.5x10^6
0.5x10^6
Group "A":
–
–
+
+
Group "B":
Ag+IgD- primed
IgD- primed
Ag+IgD- primed
IgD- primed

Figure 9a

Figure 9b. Augmenting effect of IgD pre-treated T cells on anti-SRBC antibody responses by SRBC-primed B cells is dependent upon T cell antigen priming in vivo. T cells were prepared from spleens of mice primed i.v. on day -3 with 10^8 SRBC alone, SRBC + 100 μg of TEPC-1017 IgD, or IgD alone. B cells were isolated from separate groups of mice primed i.v. on day -3 with SRBC alone or with both SRBC and IgD. Co-cultures of these isolated splenic T and B cells were established on day 0 in wells pre-coated with adherent spleen cells as described in Materials and Methods. For comparison, the 100% response (4,300 PFC/culture) reflects that generated in vitro by whole spleen cells from mice primed with SRBC alone. SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.01$).

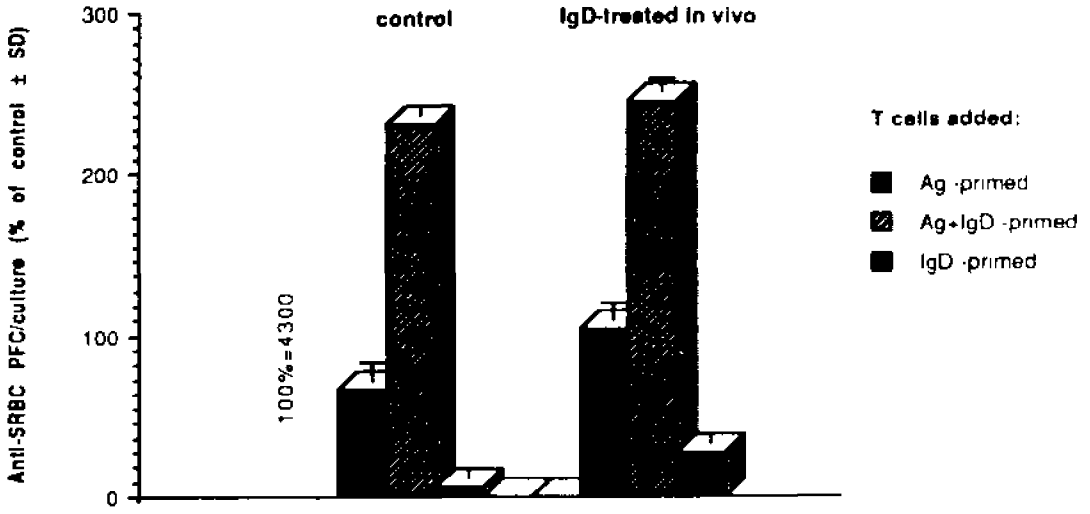

Anti-SRBC PFC/culture (% of control ± SD)
300
200
100
0
100%=4300
control
IgD-treated in vivo
T cells added:
Ag -primed
Ag+IgD -primed
IgD -primed

Figure 9b

Figure 10. Cell-free supernatants (SN) derived from spleen cell cultures from IgD-treated mice fail to enhance in vitro antibody responses. Mice were primed on day -3 with SRBC ± TEPC-1017 IgD *in vivo* as described in Figure 1b. Spleen cells were cultured in the presence of SRBC on day 0. Cell-free SN which were added to these cultures, as indicated, were prepared from 4-day-old Mishell-Dutton cultures of control spleen cells or IgD-pre-treated spleen cells stimulated with SRBC. SN were added to the present cultures at a final concentration of 10%. SRBC-specific IgM PFC assays were performed on day 4. Data are expressed as % of control ± SD ($p < 0.01$).

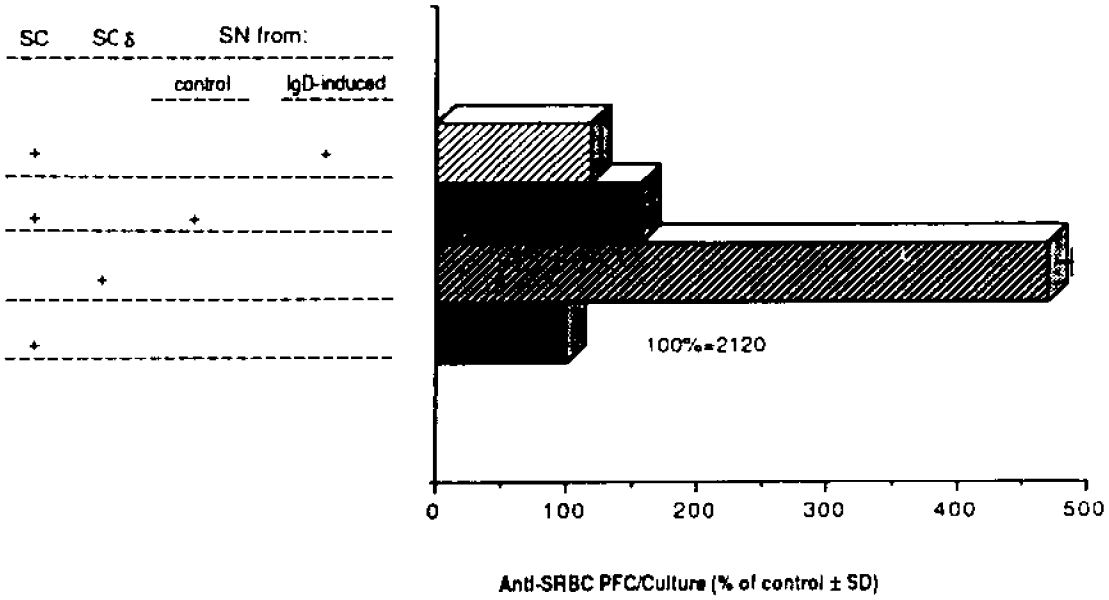

SC
SC δ
SN from:
control
IgD-induced
100%=2120
0
100
200
300
400
500
Anti-SRBC PFC/Culture (% of control ± SD)

Figure 10

Figure 11. Cognate interaction between control and IgD-treated spleen cells is required for augmentation of antibody responses. Spleen cells from control (-) and IgD-treated (+) mice were co-cultured under conditions in which cell-cell contact was precluded using cell culture insert wells (Becton-Dickinson). Cells were seeded into upper and lower wells separated by membranes with a pore size of 0.45 μ in the presence of 50 μl of 0.1% SRBC as indicated. SRBC-specific IgM PFC assays were performed using cells harvested from upper wells on day 4. Results are expressed as % of control ± SD ($p < 0.01$).

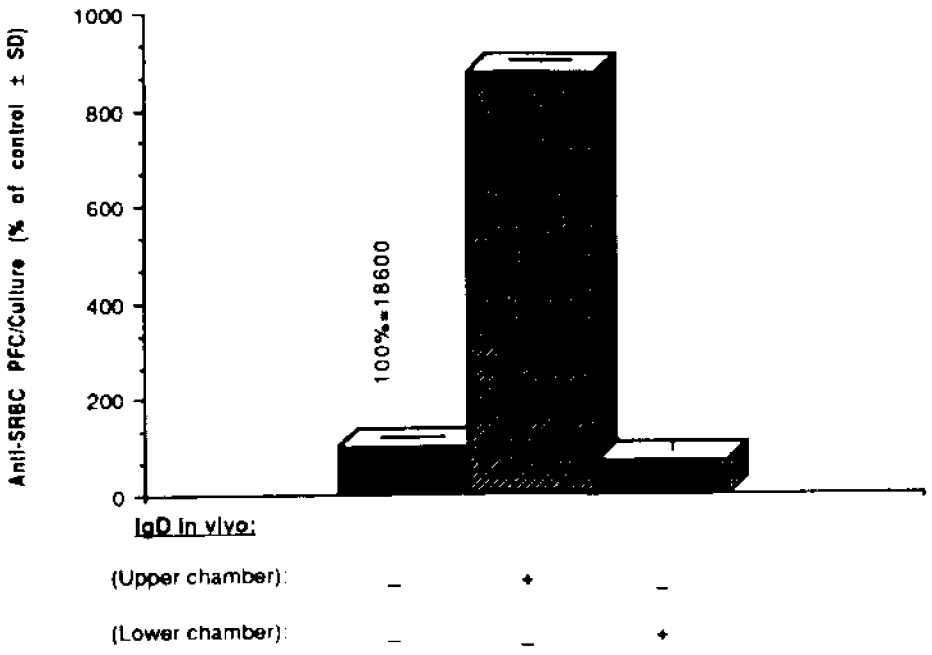

Anti-SRBC PFC/Culture (% of control ± SD)
1000
800
600
400
200
0
100%=18600
IgD in vivo:
(Upper chamber): – + –
(Lower chamber): – – +

Figure 11

Figure 12. IgD in vivo pre-treatment enhances in vitro proliferation of spleen cells to the stimulation by antigen. BALB/c mice were injected i.v. on day -3 with 10^8 SRBC alone or together with 100 μg of TEPC-1017 IgD. In vitro cultures of spleen cells were established on day 0 at a density of $2x10^5$/well in a total volume of 0.2 ml, with or without the presence of 10 μl of 0.1% SRBC, and with or without IgD in two different doses as indicated. Cells were incubated at 37^0C, 5% CO_2 for 72 hr and then pulsed with 1 μCi/well of ^3H-thymidine for an additional 18 hr. Cells were harvested and ^3H-thymidine incorporation was determined. Cultures of non-IgD-pre-treated spleen cells in the absence of SRBC served as controls. Results are expressed as % of control (mean cpm $\pm$ SD, $p < 0.05$).

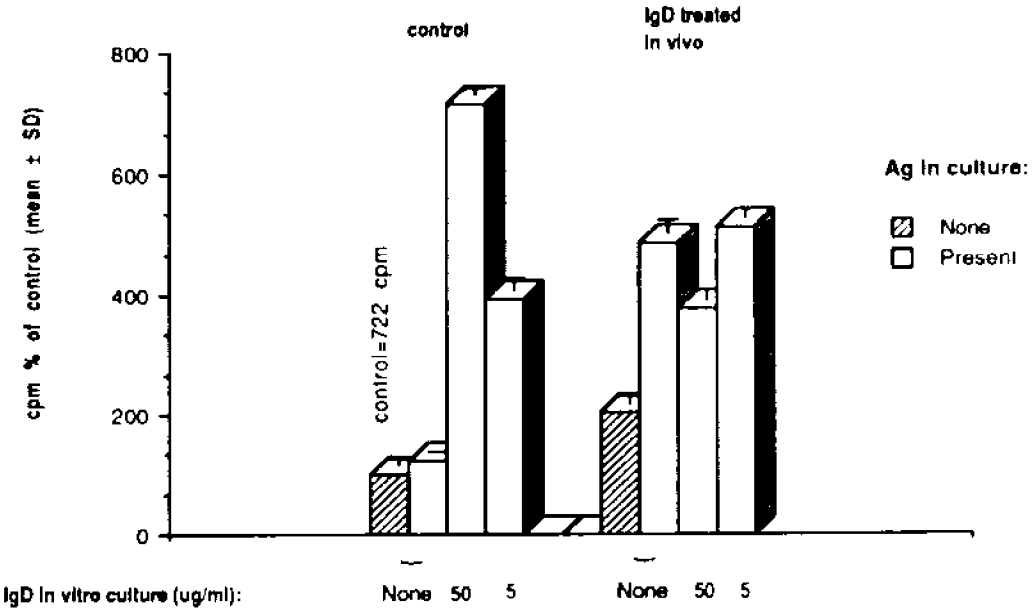

control
IgD treated
In vivo
800
600
400
200
0
cpm % of control (mean ± SD)
control=722 cpm
Ag in culture:
None
Present
IgD in vitro culture (ug/ml):
None 50 5
None 50 5

Figure 12

Figure 13. Enhanced proliferation of spleen cells induced by in vivo IgD treatment in response to the stimulation of antigen is responsible by T cells. Mice were treated in vivo on day -3 as described in Figure 12. T and B cell populations were fractionated from spleen cells and treated with or without (responder cells) gamma-irradiation (10,000 Rads) on day 0. Equal numbers of T and B cells ($5x10^4$) treated differently in vivo and in vitro were cultured in combinations as indicated, in a total volume of 0.2 ml, with or without 10 μl of 0.1% SRBC. Cell proliferation was determined by measuring ^{3}H-thymidine incorporation. Mixed cultures of T and irradiated B cells from non-IgD-pre-treated mice served as controls. Results are expressed as % of control (mean cpm ± SD, $p < 0.05$). IgD rosetting assays were also performed on T cells without gamma-irradiation prior to the establishment of cultures and results are shown as % of IgD-RFC (BSA-RFC, which were ranged from 2-3% were subtracted).

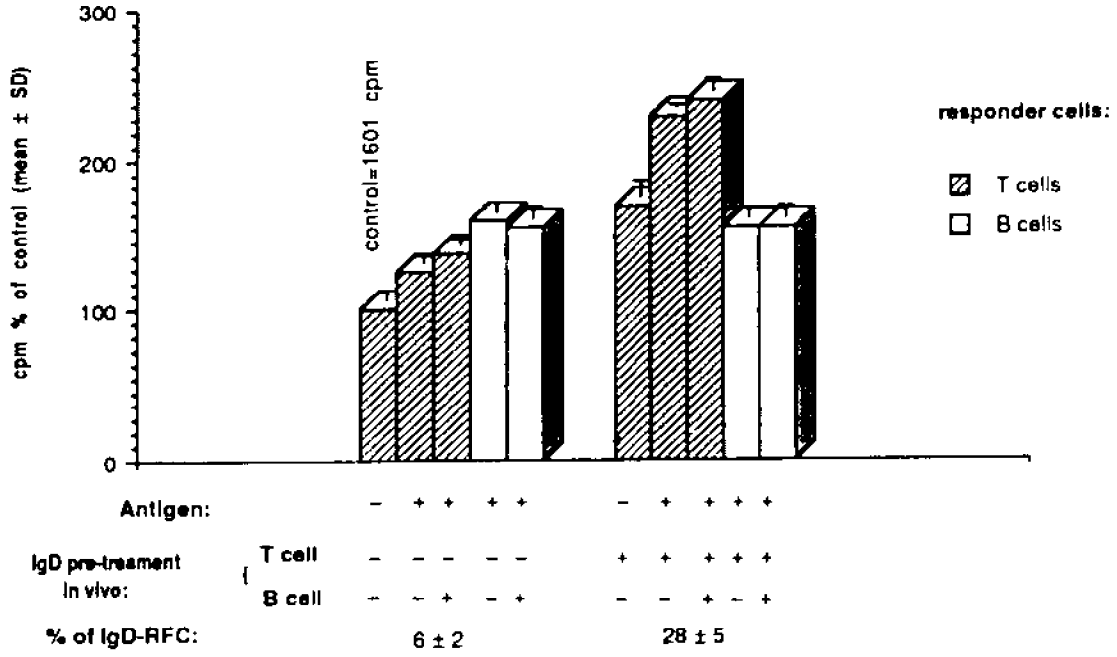

300
200
100
0
cpm % of control (mean ± SD)
control=1601 cpm
responder cells:
T cells
B cells
Antigen:
IgD pre-treament In vivo:
T cell
B cell
% of IgD-RFC:
6 ± 2
28 ± 5

Figure 13

Figure 14. IgD pre-treatment facilitates antigen presentation of B cells to T cells. Mice were primed i.p. with 100 ug of normal goat gamma globulin, every 10 days, over a period of 80 days. Three days prior to the day of experiment, mice were given a final boost i.v. of the same antigen alone or with 100 ug of TEPC-1017 IgD. Portions of T cells fractionated from mice primed with goat gamma globulin alone were induced with IgD *in vitro* by being panned on 100 μg IgD-coated Petri dishes at 37°C, 5% CO_2 for 4 hr. B cells were purified from naive mice, then gamma-irradiation (10,000 rads). Equal numbers of T and B cells (5×10^4) were then mixed as indicated, in a total volume of 0.2 ml/well, in the presence or absence of affinity-purified goat anti-mouse IgM Ab, goat anti-mouse IgD Ab, or normal goat gamma-globulin, at a final concentration of 0.5 μg/0.2 ml. Following 3 days of culture at 37°, 5% CO_2, cells were pulsed with ^{3}H-thymidine for an additional 18 hr. Cultures of T cells from mice primed with goat gamma globulin alone and irradiated B cells from naive mice served as controls. Data are expressed as % of control (mean cpm ± SD, $p < 0.05$). Data of IgD-rosetting assay on T cells are also shown (BSA-RFC values were <2% and this has been subtracted).

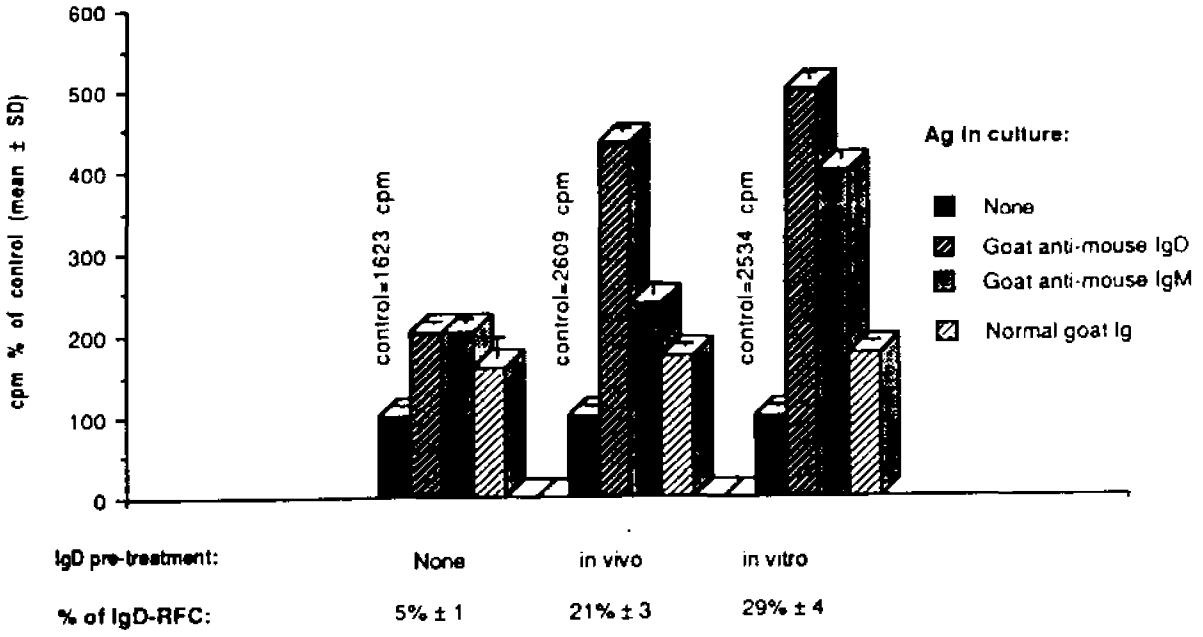

600
500
400
300
200
100
0
cpm % of control (mean ± SD)
control=1623 cpm
control=2609 cpm
control=2534 cpm
Ag in culture:
None
Goat anti-mouse IgD
Goat anti-mouse IgM
Normal goat Ig
IgD pre-treatment:
None
in vivo
in vitro
% of IgD-RFC:
5% ± 1
21% ± 3
29% ± 4

Figure 14

Figure 15. Indirect ELISA for murine IgD-BF probed with E11 mAb. ELISA wells were coated overnight with 1 μg of purified IgD in carbonate buffer, pH 9.6, or alternatively with 1 μg of mouse IgG or IgM. Wells were washed and blocked with PBS containing 0.01% Tween-20, 1% BSA (PBS-Tween-BSA), then seeded with serially diluted, IgD-Sepharose-purified IgD-BF derived from IL-4-stimulated 2H10 cells. After a 2 hr incubation, wells were washed with PBS-Tween-BSA, and 10 μg of purified E11 was added for 1 hr. Wells were washed and then probed with AP-goat anti-rat Ig. Quantitation of ELISA results was performed using a BioRad ELISA reader at 414 nm.

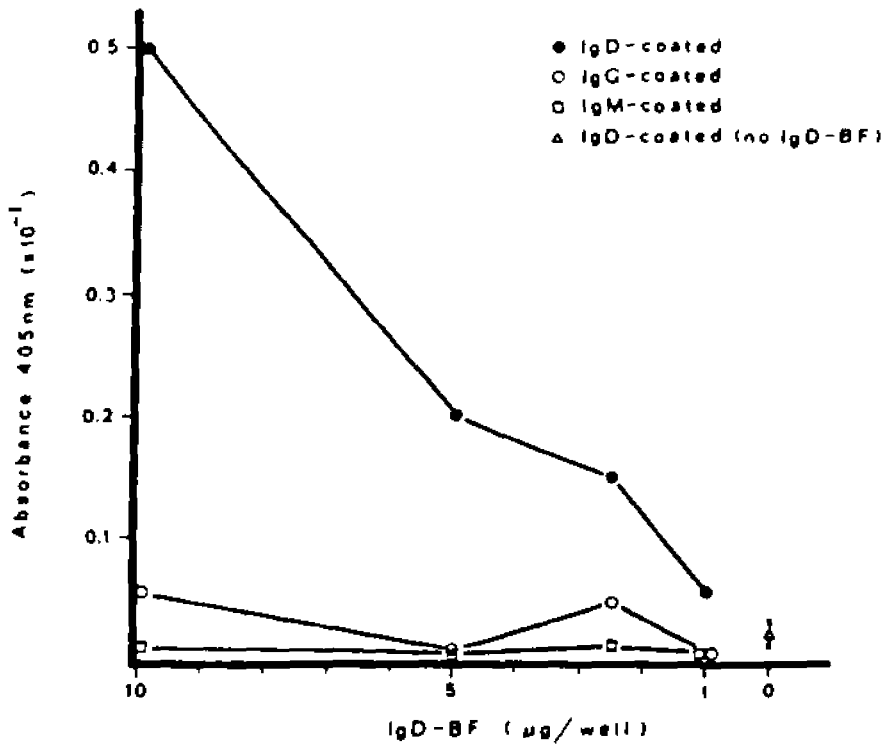

IgD-coated
IgG-coated
IgM-coated
IgD-coated (no IgD-BF)
Absorbance 405nm (x10⁻¹)
0.5
0.4
0.3
0.2
0.1
10
5
1
0
IgD-BF (µg/well)

Figure 15

Figure 16. Direct ELISA for murine IgD-BF probed with rat anti-mouse mAb E11. ELISA plate wells were coated overnight with 100 μl of IgD-BF-containing SN (closed circles) or control medium (open circles). Wells were then washed and blocked as described in Figure 15. Next, various amounts of E11 mAb were added to each well for 1 hr and the wells were then washed and probed with AP-goat anti-mouse Ig as described in Figure 15.

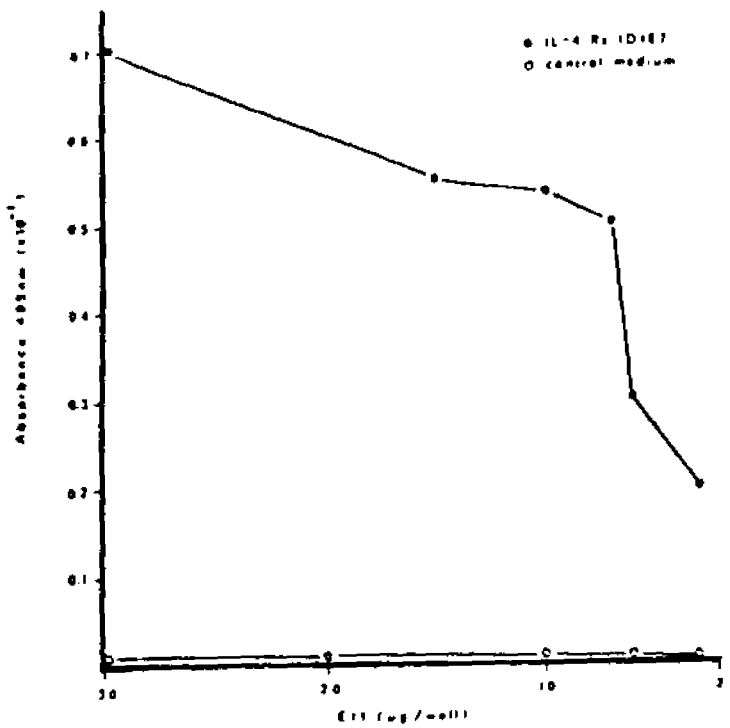


Figure 16

Figure 17. IgD rosetting of 1D1E7 T hybridoma cells is inhibited by rat anti-mouse-IgD-BF mAb E11. 1D1E7 cells were rosetted with IgD-SRBC in the presence and absence of purified E11 mAb or normal rat IgG. Competitive inhibition of IgD-RFC with 50 μg of TEPC-1017 IgD completely blocked IgD-RFC (data not shown). BSA-RFC backgrounds (5-7%) have been subtracted.

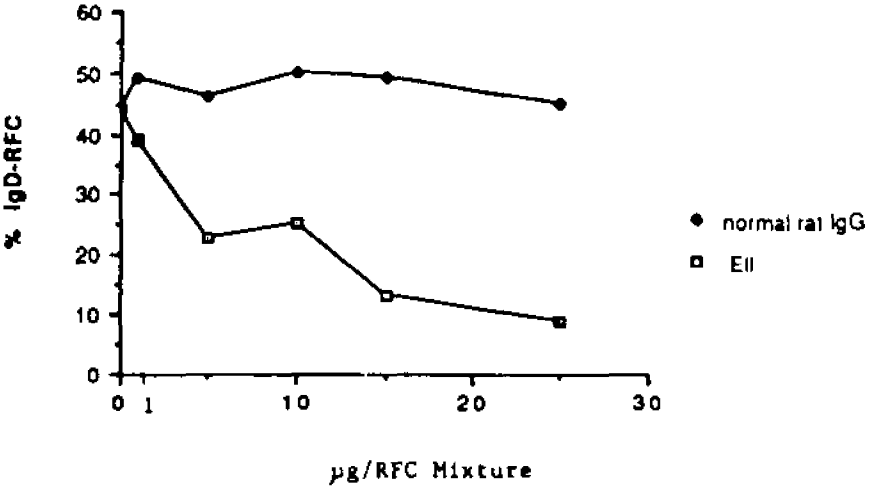

60
50
40
30
20
10
0
% IgD-RFC
0
1
10
20
30
μg/RFC Mixture
normal rat IgG
EII

Figure 17

Figure 18. IgD rosetting of normal spleen cells is inhibited by rat anti-mouse-IgD-BF mAb E11. Spleen cells from BALB/c mice were incubated for 18 hr in medium with 100 U/ml of rIL-4 at 37^0C, 5% CO_2. Cells were then washed twice with plain medium and rosetted with IgD-SRBC in the presence and absence of purified E11 mAb or normal rat IgG. Competitive inhibition of IgD-RFC with 50 μg of TEPC-1017 IgD completely blocked IgD-RFC (data not shown). BSA-RFC backgrounds (5-7%) have been subtracted.

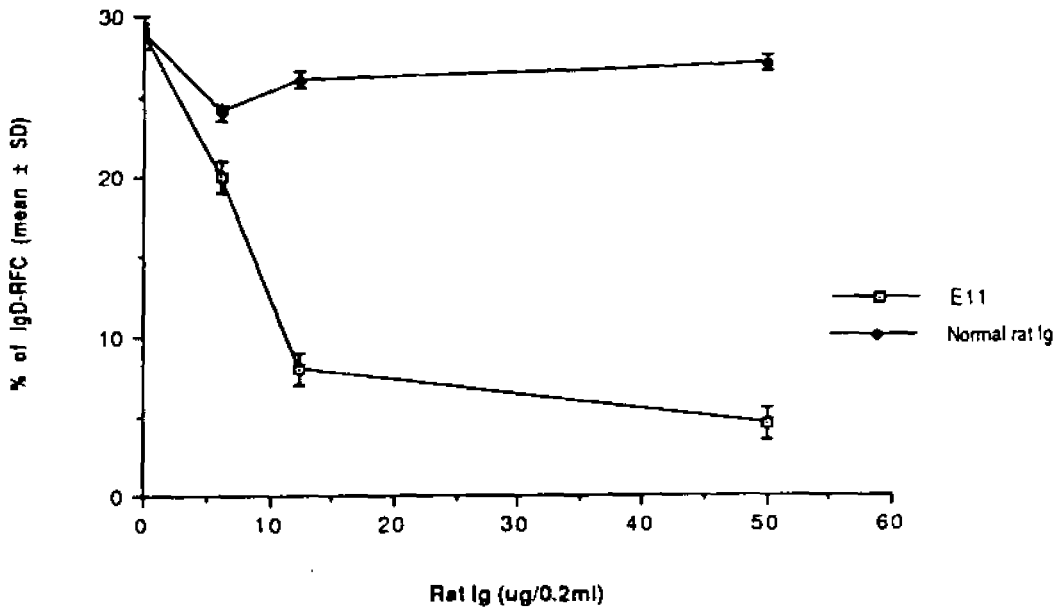

30
20
10
0
% of IgD-RFC (mean ± SD)
0
10
20
30
40
50
60
Rat Ig (ug/0.2ml)
E11
Normal rat Ig

Figure 18

Figure 19. Upregulation of IgD-R expression by 1D1E7 T hybridoma cells following stimulation with IgD, IL-2, or IL-4. 1D1E7 T hybridoma cells were incubated for 18 hr in medium containing 5 μg/ml of IgD, 1000 U/ml of rIL-2, or 100 U/ml of rIL-4. Following this incubation period, IgD-RFC assays were performed. BSA-RFC backgrounds (5-7%) have been subtracted.

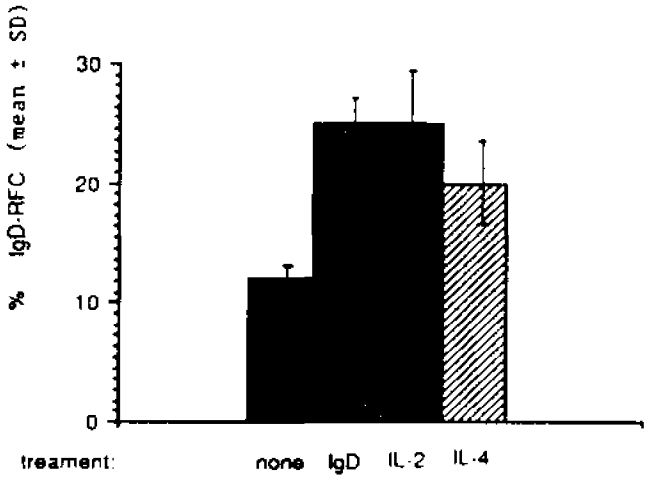


Figure 19

Figure 20. Effect of IgD, IL-2, and IL-4 treatment on E11 staining of IgD-R$^+$ 1D1E7 T hybridoma cells and IgD-R$^-$ BW5147 thymoma cells. 1D1E7 T hybridoma cells and BW5147 thymoma cells were grown separately in medium alone (controls) or stimulated for 18 hr with 5 μg/ml of IgD (Fig. 20 a and d), 1000 U/ml of rIL-2 (Fig. 20 b and e), or 100 U/ml of rIL-4 (Fig. 20 c and f). 10^6 cells were washed twice with PBS+0.01% NaN_3 and incubated for 1 hr at room temperature with 7.5 μg of purified E11 mAb. Following two washes, cells were stained with mouse anti-rat Ig-FITC and then analyzed cytofluormetrically using a Coulter Elite cell sorter.

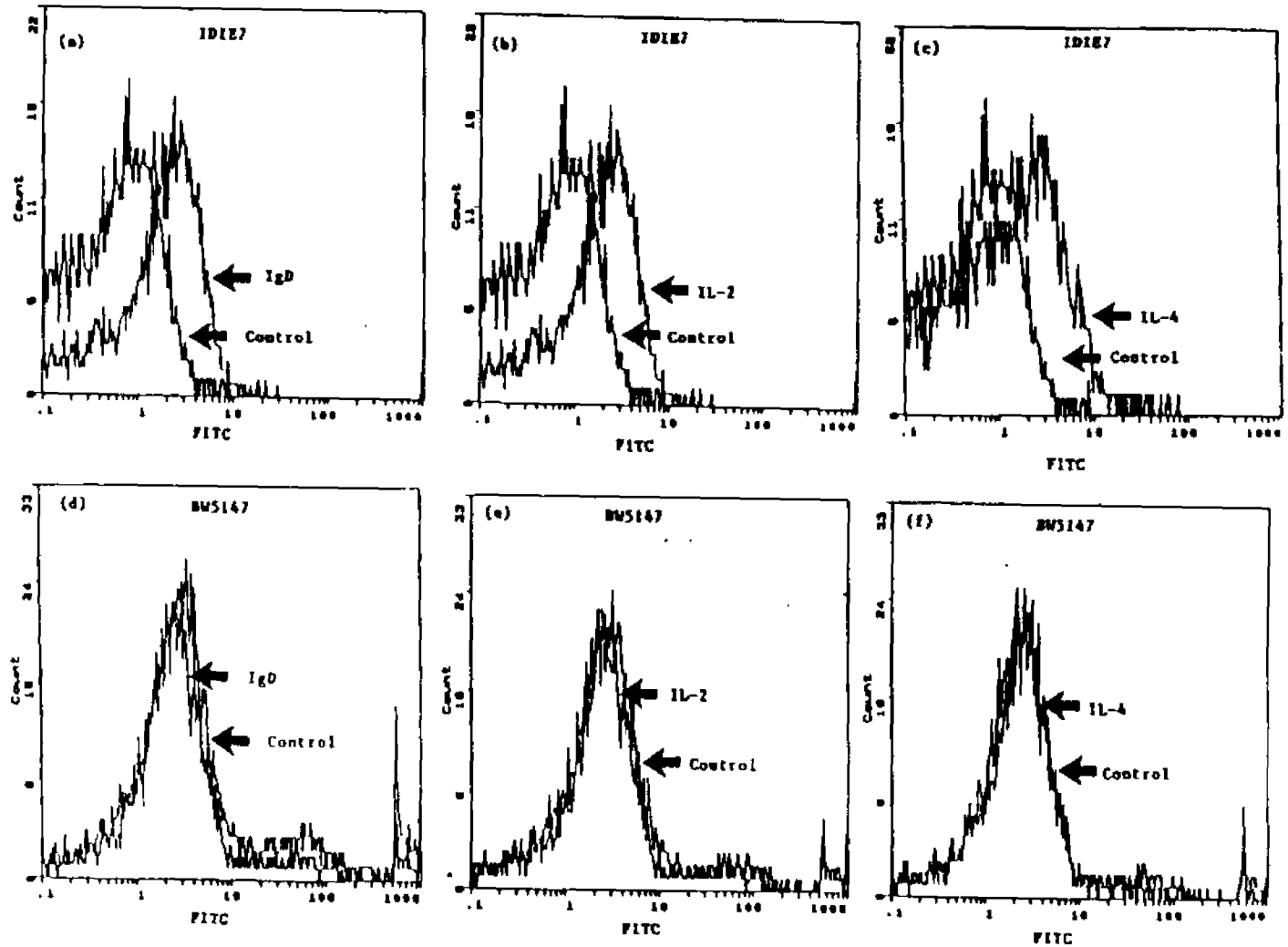

(a)
IDIE7
IgD
Control
FITC
(b)
IDIE7
IL-2
Control
FITC
(c)
IDIE7
IL-4
Control
FITC
(d)
BW5147
IgD
Control
FITC
(e)
BW5147
IL-2
Control
FITC
(f)
BW5147
IL-4
Control
FITC

Figure 20

Figure 21. Effect of IgD treatment on E11 staining of normal murine spleen cells. Normal spleen cells from BALB/c mice were either untreated (a, c) or induced (b, d) to express IgD-R by being panned on Petri dishes coated with TEPC-1017 IgD (50 μg/dish) for 2 hr, at 37^0C and 5% CO_2. Cells were collected and washed twice with PBS+0.01% NaN_3, then stained with E11 and analyzed as described in Figure 20. Panels a and b represent for background stains by Mouse anti-rat Ig-FITC, c and d represent for indirect E11 stains.

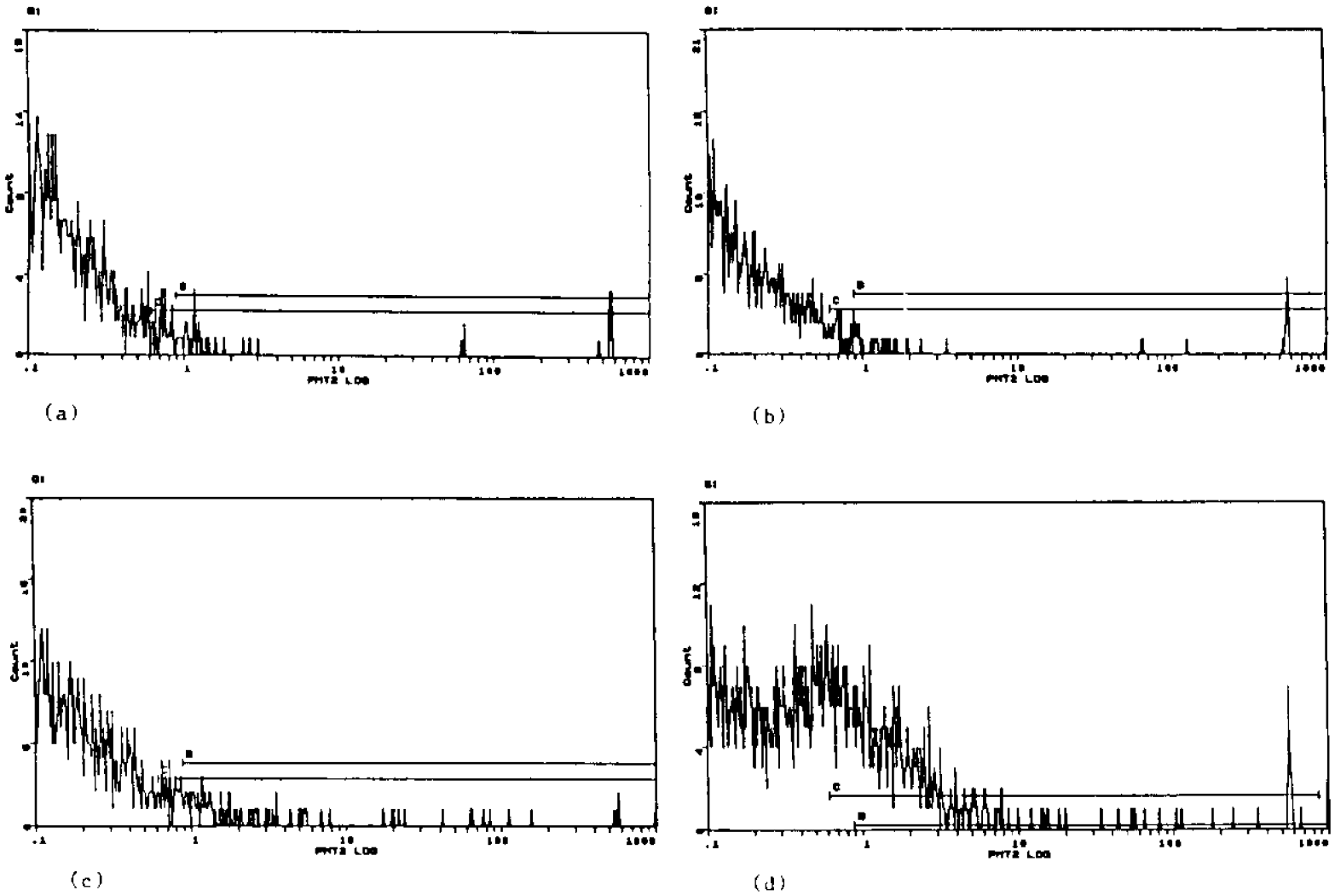


Figure 21

Figure 22. 2H10 T hybridoma cells can be stained by biotin-conjugated IgD in a dose-dependent fashion. 2H10 cells were incubated for 30 min on ice in medium alone or (a) or with 5 μg of biotin-conjugated TEPC-1017 IgD (b), 10 μg (c), 30 μg (d), and 50 μg (e). Following 2 washes with PBS+0.01% NaN_3, cells were stained with avidin-conjugated FITC and then analyzed cytofluormetrically using a Coulter Elite cell sorter.

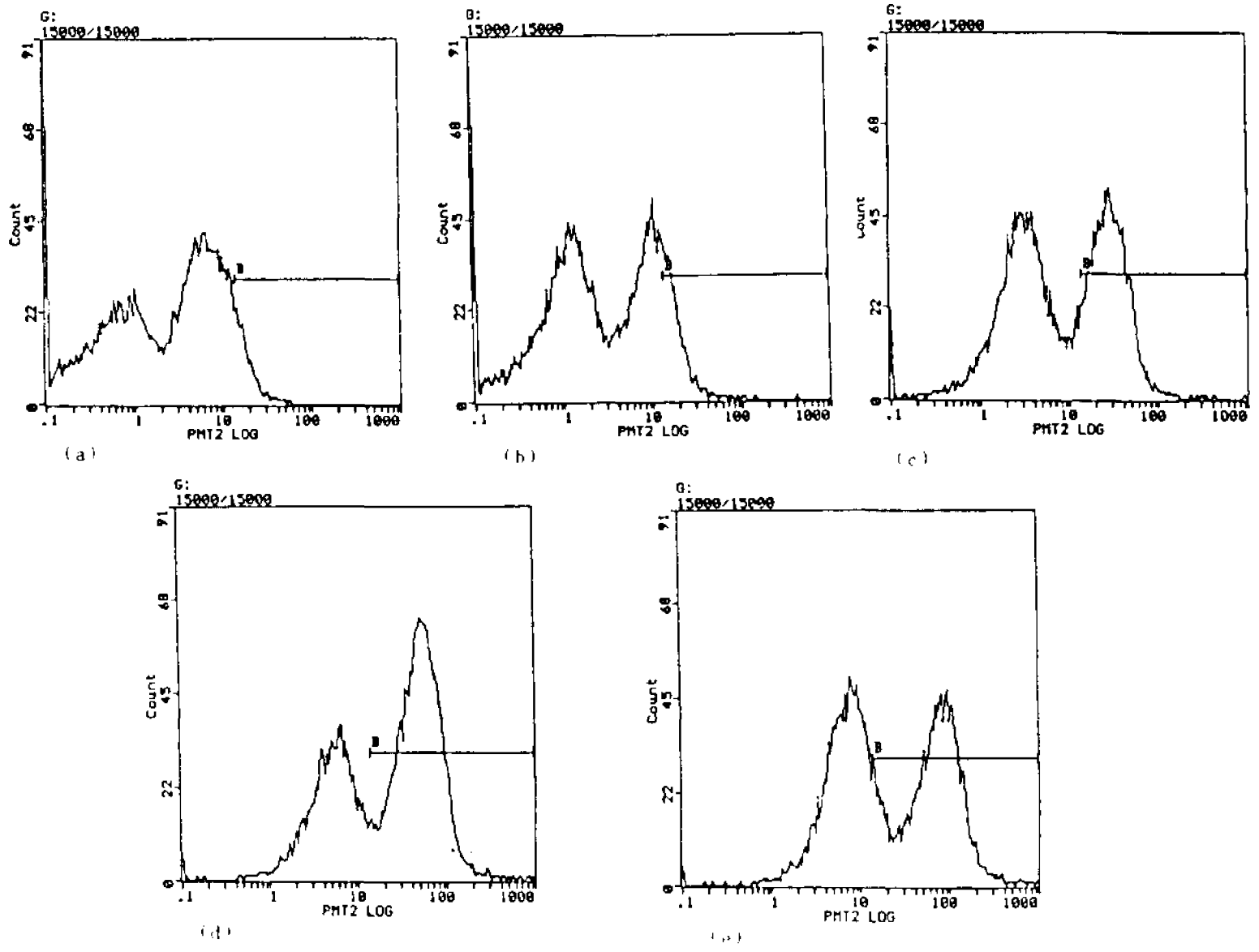

G:
15000/15000
Count
PMT2 LOG
(a)
B:
15000/15000
Count
PMT2 LOG
(b)
G:
15000/15000
Count
PMT2 LOG
(c)
G:
15000/15000
Count
PMT2 LOG
(d)
G:
15000/15000
Count
PMT2 LOG

Figure 22

Figure 23. Blocking effect of E11 and IgD on IgD staining of 2H10 T hybridoma cells. 2H10 T hybridoma cells were incubated for 30 min on ice with biotin-conjugated IgD alone (a) or biotin-conjugated IgD together with 250 μg of each of the following purified proteins: mouse IgG (b), normal rat gamma globulin (c), E11 (d), and TEPC-1017 IgD (e). Cells were then stained with avidin-conjugated FITC and analyzed cytofluormetrically using a Coulter Elite cell sorter.

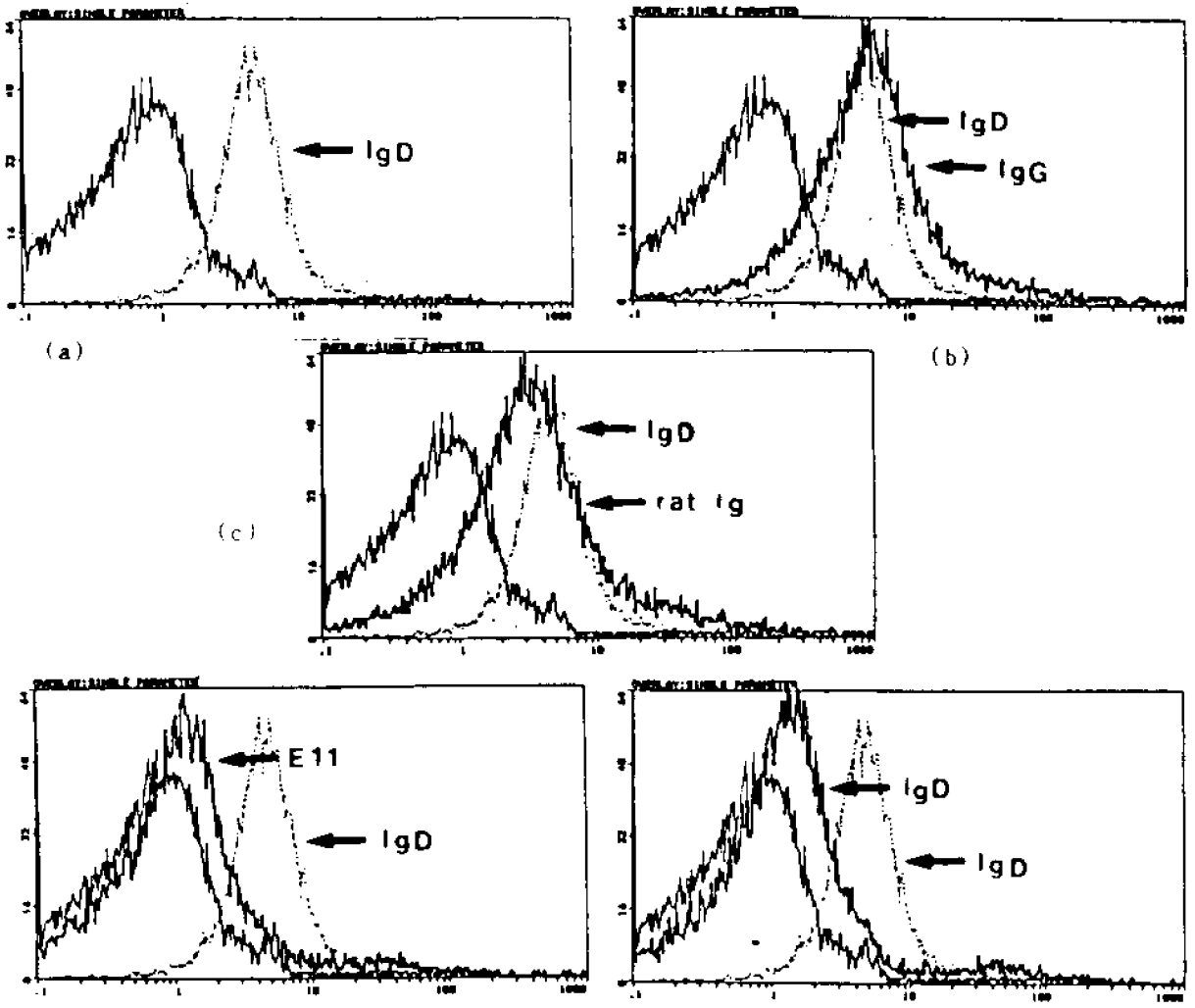


Figure 23

Figure 24. Partial identification of IgD-BF and IgD-receptor of T cells. IgD-BF and IgD-R were immunoprecipited using IgD- and E11 Sepharose beads from cell-free and serum-free culture supernatants or serum-free membrane lysates of 2H10 cells, as described in materials and methods. Eluates from these beads and normal rat-Ig-Sepharose beads (as a negative control) were then subjected to SDS-PAGE under reducing conditions. Western blotting was then performed using E11 (or control rat Ig) as the probe. The Enhanced Chemiluminescence (ECL) Western blotting analysis system and horseradish peroxidase-linked mouse anti-rat Ab were used to detect proteins recognized by E11. Represented are, controls: plain culture medium (lane 1), culture supernatants eluated from normal rat-Ig-Sepharose (lane 2) and cell lysates eluated from normal rat-Ig-Sepharose (lane 3); E11-Sepharose precipitated: membrane lysates run immediately (lane 4) and after being stored at -70°C for one month (lane 6), and culture supernatants (lane 5); IgD-Sepharose precipitated: membrane lysates run immediately (lane 7) and after being stored at -70°C for one month (lane 9), and culture supernatants (lane 8). The relative mobilities of reference proteins of different molecular weight (kDa) are indicated at right.

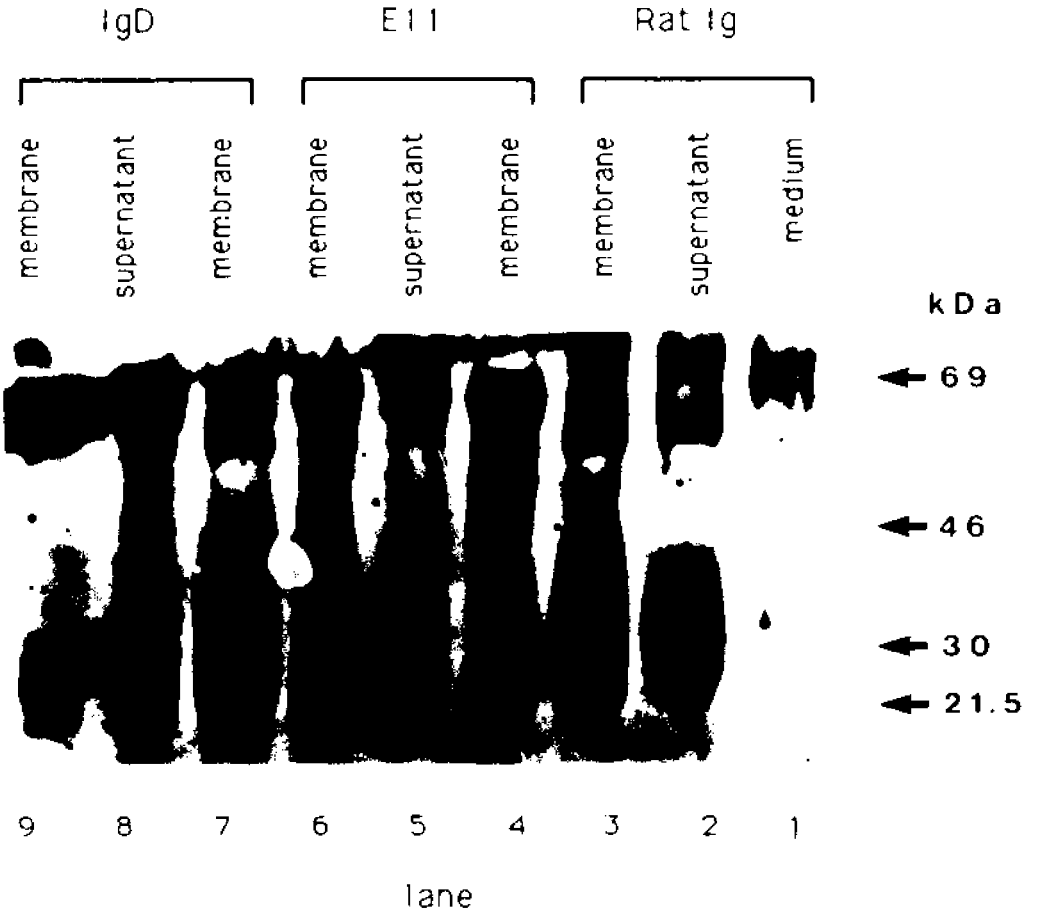

IgD
E11
Rat Ig
membrane
supernatant
membrane
membrane
supernatant
membrane
membrane
supernatant
medium
kDa
69
46
30
21.5
9
8
7
6
5
4
3
2
1
lane

Figure 24

Figure 25. Augmenting effect of E11 on in vitro primary anti-TNP antibody responses. In vitro cultures of spleen cells from BALB/c mice were established at a density of $5x10^6$/ml (1 ml) in the presence of TNP-BA. Where indicated, E11 was also added at 10 μg/ml on the day of culture establishment. Cells were incubated at 37^0, 5% CO_2 for 4 days and then harvested for TNP-specific IgM PFC assays. Results of PFC assays are expressed as % of control ± SD ($p < 0.01$).

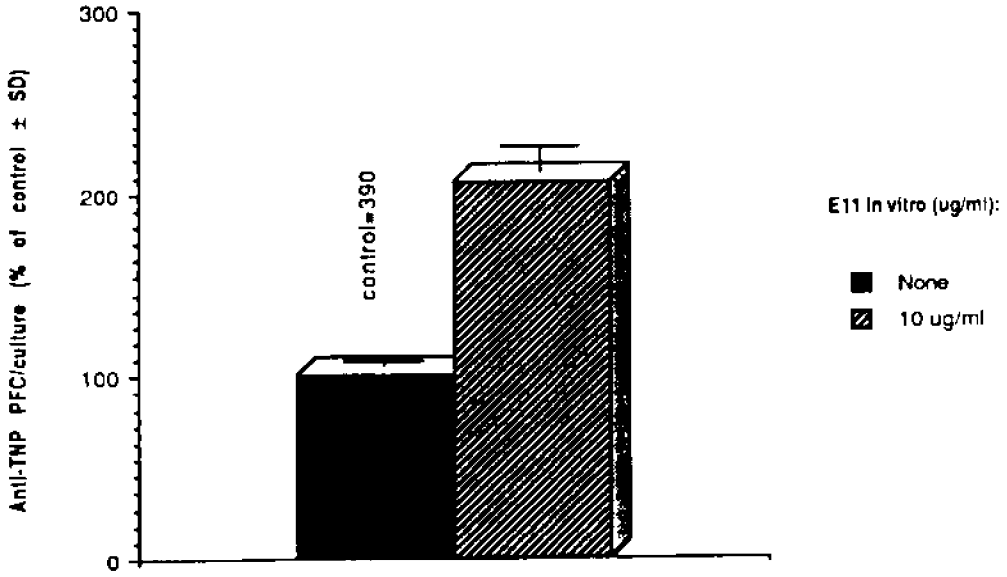

300
200
100
0
Anti-TNP PFC/culture (% of control ± SD)
control=390
E11 in vitro (ug/ml):
None
10 ug/ml

Figure 25

Figure 26. Augmenting effect of E11 on in vitro early secondary anti-SRBC antibody responses. BALB/c mice were primed with SRBC with or without IgD and cultures of spleen cells were established in the presence of SRBC as described in Figure 1b. E11 or normal rat Ig were also added to the cultures on the same day as culture establishment at various concentrations as indicated. SRBC-specific PFC were assayed after 4 days of culture and results are expressed as % of control ± SD ($p < 0.05$)

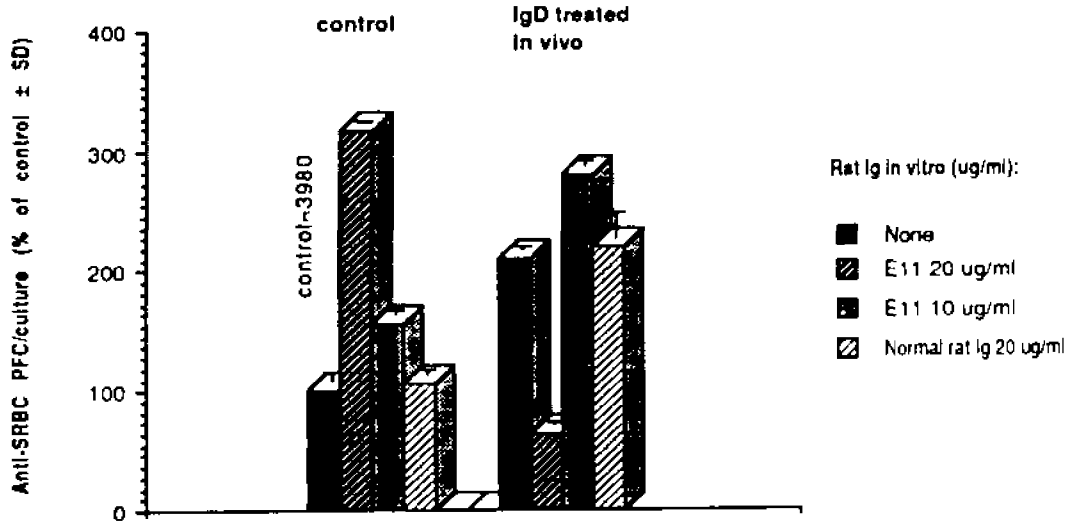

control
IgD treated
in vivo
Anti-SRBC PFC/culture (% of control ± SD)
400
300
200
100
0
control=3980
Rat Ig in vitro (ug/ml):
None
E11 20 ug/ml
E11 10 ug/ml
Normal rat Ig 20 ug/ml

Figure 26

Figure 27. Dual effect of rat anti-mouse-IgD-BF antibody (E11) on antigen presentation of B cells to T cells. Mice were primed with goat gamma globulin and pre-treated with or without IgD as described in Figure 14. Cultures were established by mixing irradiated B cells (10,000 Rads) from naive mice: (a) with T cells from mice primed with goat gamma globulin alone, (b) with T cells from mice primed with goat gamma globulin and IgD *in vivo*, (c) or with T cells from mice primed with goat gamma globulin and IgD pre-treatment *in vitro*, in the presence of goat anti-mouse IgM, goat anti-mouse IgD, or normal goat gamma globulin, as indicated. E11 and normal rat Ig were also added to some of the cultures in various doses as indicated. Proliferation of cells as determined by measuring their incorporation of ^{3}H-thymidine after 4 days. Cultures of irradiated B cells in combinations of the a, b, or c group of T cells in the absence of both antigen and rat Ig served as controls for each group, respectively (data shown as 100%). Data are expressed as % of control (mean cpm ± SD, $p < 0.05$). IL-2 assays were performed on supernatants collected from cultures after 3 days and the IL-2 concentration of each sample was determined by probit analysis.

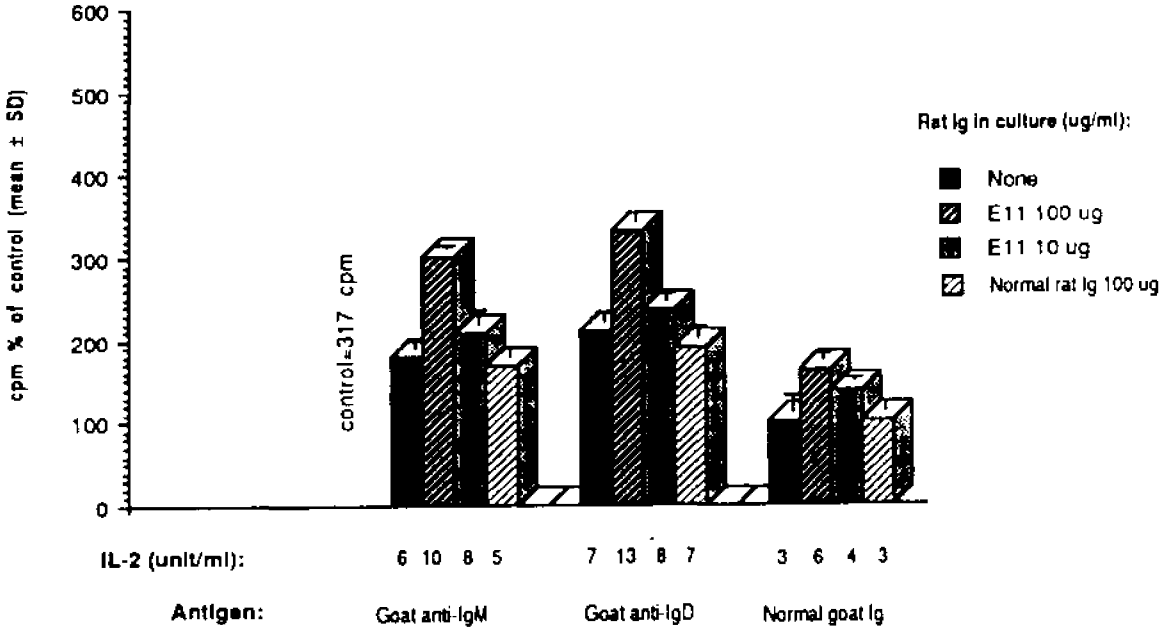

600
500
400
300
200
100
0
cpm % of control (mean ± SD)
control=317 cpm
Rat Ig in culture (ug/ml):
None
E11 100 ug
E11 10 ug
Normal rat Ig 100 ug
IL-2 (unit/ml):
6 10 8 5
7 13 8 7
3 6 4 3
Antigen:
Goat anti-IgM
Goat anti-IgD
Normal goat Ig

Figure 27a

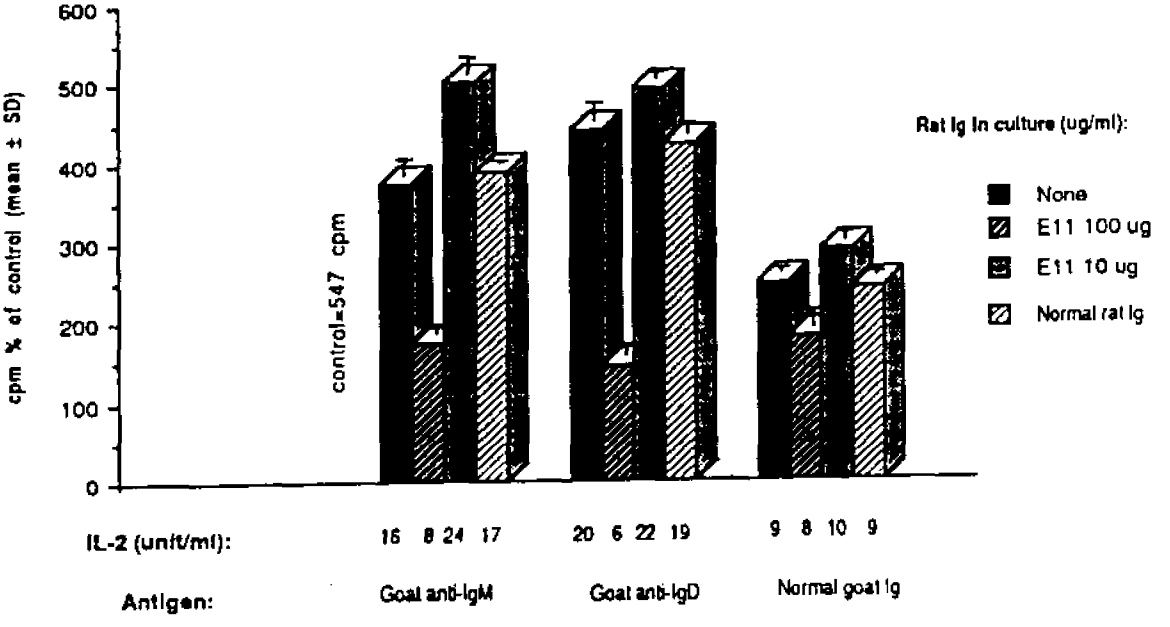

600
500
400
300
200
100
0
cpm % of control (mean ± SD)
control=547 cpm
Rat Ig in culture (ug/ml):
None
E11 100 ug
E11 10 ug
Normal rat Ig
IL-2 (unit/ml):
16 8 24 17
20 6 22 19
9 8 10 9
Antigen:
Goat anti-IgM
Goat anti-IgD
Normal goat Ig

Figure 27b

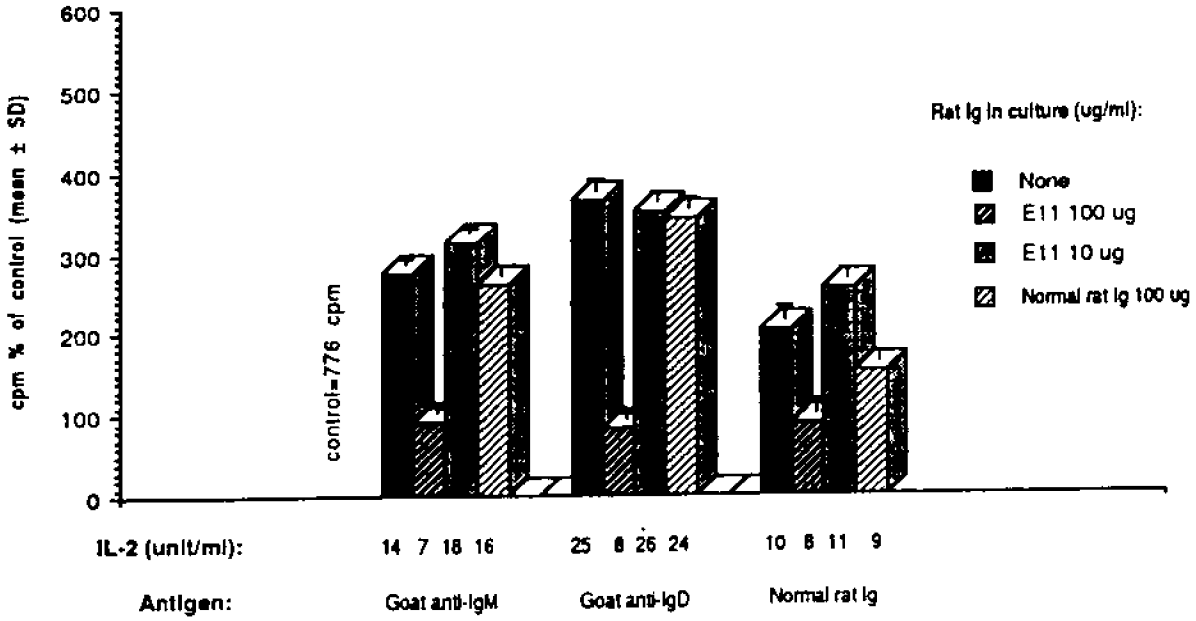

600
500
400
300
200
100
0
cpm % of control (mean ± SD)
control=776 cpm
Rat Ig in culture (ug/ml):
None
E11 100 ug
E11 10 ug
Normal rat Ig 100 ug
IL-2 (unit/ml):
14 7 18 16
25 8 26 24
10 8 11 9
Antigen:
Goat anti-IgM
Goat anti-IgD
Normal rat Ig

Figure 27c

Figure 28. Effect of eluate from IgD-conjugated Sepharose on in vitro early secondary antibody responses. BALB/c mice were primed with SRBC together with or without IgD on day -3 and their spleen cells were cultured as described in Figure 1b. As indicated, proteins eluted from IgD-, E11-, or normal rat Ig-conjugated Sepharose-4B were supplemented in various doses to the cultures on the same day as culture establishment. SRBC-specific PFC were assayed following 4 days of culture and the results are expressed as % of control ± SD ($p < 0.01$).

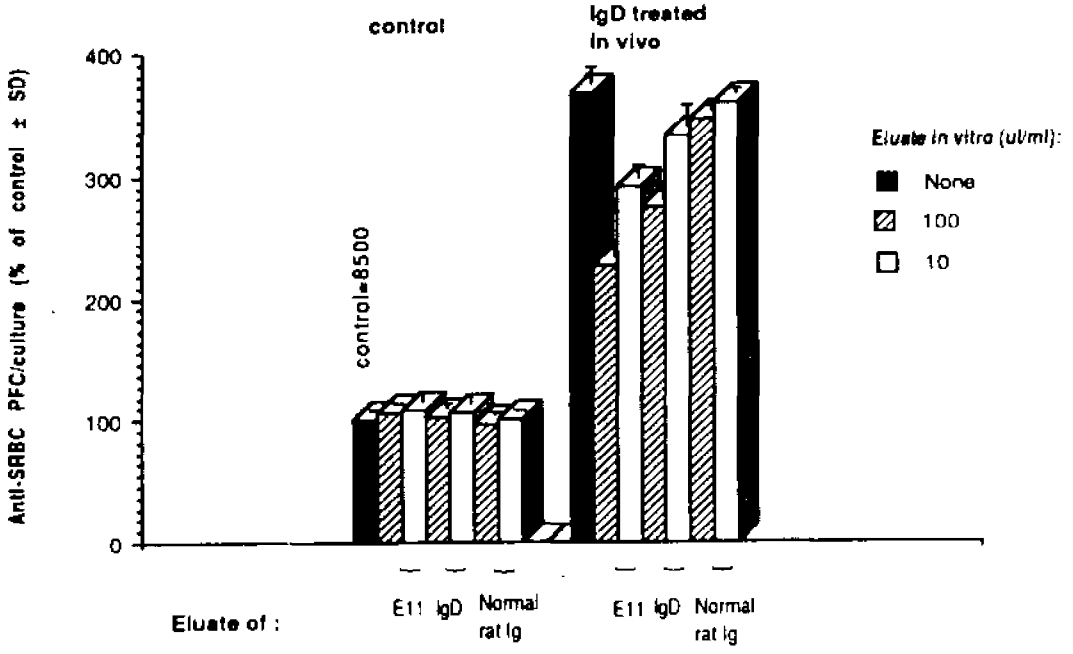

control
IgD treated
in vivo
Anti-SRBC PFC/culture (% of control ± SD)
400
300
200
100
0
control=8500
Eluate in vitro (ul/ml):
None
100
10
Eluate of :
E11
IgD
Normal rat Ig
E11
IgD
Normal rat Ig

Figure 28

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