

AUGMENTATION OF IN VITRO HUMORAL IMMUNE RESPONSES IN THE MOUSE BY AN ANTIBODY TO IgD*

BY FRED D. FINKELMAN, VIRGIL L. WOODS, SHANNON B. WILBURN, JAMES
J. MOND, KATHRYN E. STEIN‡, ALICE BERNING, AND IRWIN SCHER

From the Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Md. 20014; the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md. 20205; and the Department of Immunology, Naval Medical Research Institute, Bethesda, Maryland 20014

The identification of IgD in normal human serum (1) and its subsequent discovery of on the surface of most primate (2–5) and rodent (6–9) B lymphocytes generated questions regarding the function of this Ig isotype. In studying this problem, Pernis (10) injected rhesus monkeys with a rabbit antiserum specific for human IgD (RaH δ),¹ which cross-reacts with monkey IgD. These monkeys developed substantial increases in serum IgM and IgG levels, which were entirely accounted for by antibodies specific for constituents of rabbit serum. These experiments suggested that anti-IgD can have an adjuvant effect on the humoral immune response to simultaneously injected foreign antigens (10), but is not a polyclonal activator of B lymphocyte terminal differentiation. Pernis's interpretation has been supported by other studies that demonstrated that the injection of an IgG fraction of RaH δ or affinity-purified RaH δ into rhesus monkeys could considerably enhance the IgG response to a subsequently injected antigen, but did not lead to a polyclonal increase in serum IgM or IgG levels (11, 12).

The adaptation of this model to the mouse has been made possible by the production of hybridoma-derived monoclonal anti-mouse δ -chain antibodies (13). One such antibody is an IgG_{2a} of the b allotype secreted by hybridoma 10-4.22 (14) that has specificity for the Fc fragment of mouse δ -chain of the a allotype (4.22aM δ^a) (15). We report here the effects of 4.22aM δ^a on the IgM and IgG anti-2,4,6-trinitro-

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¹ *Abbreviations used in this paper:* AECM, aminoethylcarbonylmethylated; 4.22aM δ^a , IgG_{2a} antibody of the b allotype secreted by hybridoma 10-4.22 that has specificity for the Fc fragment of mouse δ -chain of the a allotype; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; HFA, Hanks' balanced salt solution supplemented with 10% fetal bovine serum and 0.2% NaN₃; NIH, National Institutes of Health; OVA, ovalbumin; RaH δ , rabbit antiserum specific for human IgD; Ra4.22Id, rabbit antibody against the idiotypic determinants of 4.22aM δ^a ; RaKLH, rabbit anti-keyhole limpet hemocyanin; RaM δ , rabbit anti-mouse δ -chain; RaMy, rabbit anti-mouse γ -chain; RaM μ , rabbit anti-mouse μ -chain; sIgD, surface IgD; sIgM, surface IgM; SRBC, sheep erythrocytes; TNP, 2,4,6-trinitrophenyl; TNP-Ficoll, TNP-AECM-Ficoll; TNP-SRBC 2,4,6-trinitrophenylated SRBC.

phenyl (TNP) response to TNP-conjugated sheep erythrocytes (SRBC) (TNP-SRBC) and TNP-conjugated aminoethylcarbonylmethylated (AECM)-Ficoll (TNP-Ficoll). Our results indicate that although 4.22aM δ^a by itself stimulates neither B cell proliferation nor terminal differentiation when injected into BALB/c mice, it substantially enhances the IgG anti-TNP response and modestly enhances the IgM anti-TNP response to subsequently injected TNP-Ficoll or TNP-SRBC. We have found that the degree of enhancement of the IgG anti-TNP response is highly dependent on the dose of TNP-SRBC or TNP-Ficoll used and on the time interval between injection of 4.22aM δ^a and antigen, and that even in the case of the T-independent antigen TNP-Ficoll this enhancement appears to be T lymphocyte dependent.

Materials and Methods

Animals. Male BALB/c mice were obtained from Microbiological Associates, Walkersville, Md. Male athymic (nu/nu) mice and their phenotypically normal (+/+ and nu/+) littermates were obtained from the Small Animal Section, Division of Research Services, National Institutes of Health (NIH), Bethesda, Md. Male C.B20 mice, which are congenic to BALB/c but possess the *Igh* genes of C57BL/Ka, were bred from mice originally obtained from Dr. Michael Potter, NIH. All mice were at least 8 wk old when used.

Antigens. TNP₆₅AECM Ficoll was purchased from Biosearch, St. Raphael, Calif. TNP-SRBC with a high hapten density were prepared as previously described (16).

Antibodies. The preparation of the following antibodies has been described elsewhere: isotype-specific affinity purified rabbit anti-mouse γ -chain (RaM γ) and rabbit anti-mouse μ -chain (RaM μ) (17), affinity-purified rabbit anti-keyhole limpet hemocyanin (RaK μ), and 4.22aM δ^a (18). The monoclonal IgG_{2a} secreted by the plasmacytoma CBPC-101, which does not bind to mouse cells, was purified from the ascites fluid of C.B20 mice that bore this tumor by elution from protein A-Sepharose. Both 4.22aM δ^a and CBPC-101 bear the b allotypic determinants characteristics of C57BL/6 IgG_{2a} immunoglobulins. A rabbit antibody against idiotypic determinants of 4.22aM δ^a (Ra4.22Id) was prepared by immunizing a rabbit with 4.22aM δ^a , absorbing the antiserum with normal BALB/c serum-Sepharose, and immunospecifically purifying the absorbed antiserum on a column of 4.22aM δ^a -Sepharose. The identification and purification of two mouse IgD κ plasmacytoma proteins, TEPC-1017 and TEPC-1033, and the production of an affinity-purified isotype-specific rabbit anti-mouse δ -chain (RaM δ) antibody will be described in detail in a separate paper (F. Finkelman, S. Kessler, F. Mushinski, and M. Potter. Manuscript in preparation.). RaM μ and RaM γ were labeled with Na¹²⁵I (Amersham Corp., Arlington Heights, Ill.) by the chloramine T technique (19); and 4.22aM δ^a and F(ab')₂ fragments of affinity-purified RaM δ , RaM μ , RaM γ , and RaK μ were labeled with fluorescein isothiocyanate (FITC) to molar fluorescein:protein ratios of from 2:1 to 5:1 as previously described (20).

Measurement of Serum Immunoglobulin Levels. Serum IgG and IgM were quantitated by the radial immunodiffusion technique. IgG₁, IgG_{2a}, and IgG_{2b} levels in 10- μ l serum samples were measured with radial immunodiffusion plates purchased from Meloy Laboratories Inc., (Springfield, Va.). In other experiments, radial immunodiffusion plates prepared with RaM γ that was sequentially affinity purified on columns of mouse IgG₁ and IgG_{2a} plasmacytoma proteins that were bound to Sepharose were used to measure total IgG levels. Serum IgM levels were measured with radial immunodiffusion plates prepared with isotype-specific RaM μ antiserum.

Quantitation of Immunoglobulin on Mouse Spleen Cells. Single cell suspensions were prepared from spleens of BALB/c mice that had been injected with 200 μ g of CBPC-101 or 4.22aM δ^a , treated with erythrocyte lysing buffer, and suspended in Hanks' balanced salt solution that contained 10% fetal bovine serum and 0.2% NaN₃ (HFA). Aliquots of 2 \times 10⁶ spleen cells in 100 μ l of HFA were stained for 30 min at 0°C with 0.5 μ g of FITC-labeled CBPC-101, 4.22aM δ^a , or affinity-purified F(ab')₂ fragments of RaM δ , RaM μ , RaM γ , or RaK μ ; washed; and analyzed for surface fluorescence with a fluorescence-activated cell sorter (FACS II; Becton, Dickinson FACS Systems, Mountain View, Calif.) as previously described (20, 21).

Measurement of Spleen Cell Proliferation. Spleen cell suspensions were prepared from mice

injected with 4.22aM δ^a or CBPC-101. Triplicate cultures of 5×10^5 cells each were incubated in flat-bottomed wells of microtiter plates in 0.2 ml of modified Mishell-Dutton medium with 1 μ Ci of [3 H]thymidine/well for 4 or 24 h. Cells were harvested with a Mash II harvester (Microbiological Associates) and [3 H]thymidine incorporation was analyzed by scintillation spectroscopy.

Titration of Anti-TNP Levels in Mouse Sera. IgG and IgM anti-TNP antibody levels in mice immunized with TNP-SRBC or TNP-Ficoll were titered with a solid-phase microtiter plate radioimmunoassay (22). TNP-Ficoll coated U-bottomed Wells in Flexvinyl microtiter plates (Cooke Engineering Co., Alexandria, Va.) were washed with 0.1% ovalbumin (OVA) in Tris. Negative control plates were prepared without TNP-Ficoll. Positive control plates were prepared by exposing wells to 100 μ g/ml of IgG $_1$, IgG $_{2a}$, IgG $_3$, or IgM instead of TNP-Ficoll. Individual sera were diluted to 1:40, 1:160, 1:640, and 1:2,560 in OVA-Tris buffer. Duplicate 25- μ l aliquots of each serum dilution were pipetted into TNP-Ficoll-coated wells and incubated for 30 min at room temperature, after which, wells were washed, refilled with 25 μ l of 125 I-RaM γ or 125 I-RaM μ (10^5 cpm/ml) in OVA-Tris buffer, incubated for 1 h at room temperature, and washed again. Individual wells were cut from plates and counted in a gamma counter. In preliminary experiments, $\sim 8 \times 10^4$ cpm of 125 I-RaM μ bound to IgM-coated wells, and $\sim 6 \times 10^4$, $\sim 4 \times 10^4$, and $\sim 1.5 \times 10^4$ cpm of 125 I-RaM γ bound to IgG $_1$ -, IgG $_{2a}$ -, and IgG $_3$ -coated wells, respectively. Less than 10^3 cpm of either reagent bound to wells coated either with OVA only or with TNP-Ficoll and OVA but which were not exposed to mouse serum. Less than 10^3 cpm of 125 I-RaM μ bound to mouse IgG-coated wells and, similarly, $<10^3$ cpm of 125 I-RaM γ bound to mouse IgM-coated wells. In general, TNP-Ficoll-coated wells exposed to an immune mouse serum bound 5–10 times as much 125 I-RaM μ and 10–20 times as much 125 I-RaM γ as OVA-coated wells exposed to the same dilution of the same serum. Serum titers were defined as the reciprocal of the serum dilution which was calculated by linear regression analysis of logarithms of serum dilutions to bind a given number of cpm of 125 I-RaM μ or 125 I-RaM γ (titer point). Titer points were chosen to allow for titration of all serum samples analyzed without extrapolation, and, when possible, to intersect titration curves of individual sera plotted on log-log paper at the steepest part of their slopes. Serum titers obtained this way provide only for relative measurement of serum antibody levels of simultaneously analyzed sera.

Results

Effects on Cell Surface Immunoglobulin. 1, 3, 5, or 7 d after 10-wk-old BALB/c mice were injected intravenously with 200 μ g of 4.22aM δ^a or CBPC-101, their spleen cells were obtained and stained with FITC-labeled RaM δ , RaKLH, 4.22aM δ^a , CBPC-101, RaM μ , or RaM γ . Fig. 1 shows the fluorescence profiles obtained by FACS II analysis of cells taken 3 and 7 d after in vivo injection of 4.22aM δ^a or CBPC-101 and stained with FITC-labeled RaM δ , RaKLH, or RaM μ . 3 d after the 4.22aM δ^a injection, the intensity of spleen cell staining for surface IgD (sIgD) is greatly diminished and approaches the low value of cells stained nonspecifically with FITC-RaKLH. Similar profiles were obtained from cells derived from mice given 4.22aM δ^a 24 h previously that were stained with either FITC-RaM δ or FITC-4.22aM δ^a . Cells from mice injected with 4.22aM δ^a stained no more brightly with FITC-RaM γ , which binds to 4.22aM δ^a , than did cells from CBPC-101-injected mice (data not shown). Thus, decreased FITC-RaM δ staining of cells from 4.22aM δ^a -treated mice is a result of modulation of sIgD rather than blocking of δ -chain determinants by 4.22aM δ^a . Spleen cells from mice injected 5 or 7 d previously with 4.22aM δ^a had considerably more sIgD than did cells from spleens removed 1 or 3 d after the 4.22aM δ^a injection, but considerably less than did spleen cells from control mice. Fig. 1 also shows that 3 and 7 d after in vivo 4.22aM δ^a treatment, the frequency of spleen cells that bore detectable surface IgM (sIgM) was diminished: 28–29% as compared to 44% in mice that had received CBPC-101. Because the total number of cells that bore cell surface I-region

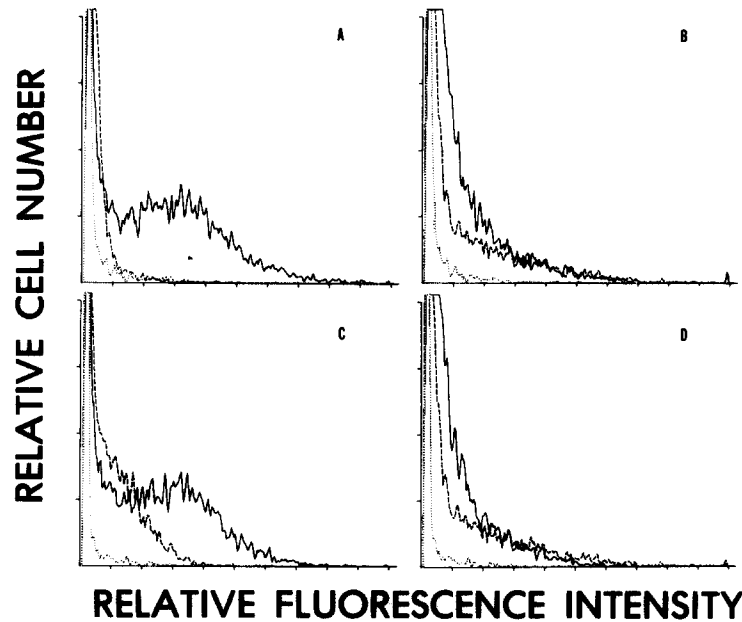


FIG. 1. Two groups of six BALB/c mice were injected with 200 μ g of CBPC-101 (—) or 4.22aM δ^a (---)/mouse. Three mice from each group were killed 3 d later and their spleen cells were pooled and stained with FITC-RaM δ (A) or FITC-RaM μ (B). These cells were then analyzed for fluorescence intensity with a FACS. To provide a measure of nonspecific staining, spleen cells from CBPC-101-treated mice were stained with FITC-RaKLN (\cdots) and similarly analyzed. In the fluorescence profiles shown, the fluorescence intensity of stained cells increases along the abscissa, while the ordinate depicts the number of cells at each level of fluorescence intensity. (C) and (D) show fluorescence profiles of spleen cells obtained from mice injected with 4.22aM δ^a or CBPC-101 7 d before killing and stained with FITC-RaM δ (C) or FITC-RaM μ (D) as well as with FITC-RaKLN.

associated (Ia) antigen or a receptor for the third component of complement was not diminished by this treatment, (data not shown) this result suggests that some B cells lose sIgM as well as sIgD after treatment with 4.22aM δ^a .

Persistence of Injected 4.22aM δ^a . Sera from six mice injected with 200 or 800 μ g of 4.22aM δ^a were obtained 5 and 7 d after the 4.22aM δ^a injection and were analyzed for their ability to form a precipitin line with Ra4.22Id (100 μ g/ml) in Ouchterlony plates. Sera from all six mice injected 5 d or 7 d previously with 800 μ g of 4.22aM δ^a , sera from all six mice injected 5 d previously with 200 μ g of 4.22aM δ^a , and three of six sera from mice injected 7 d previously with 200 μ g of 4.22aM δ^a generated precipitin lines with Ra4.22Id antibody.

Effects of In Vivo 4.22aM δ^a on the Humoral Immune Response to TNP-Ficoll and TNP-SRBC. BALB/c mice were injected intravenously with 100 μ g of 4.22aM δ^a or CBPC-101 and were given 0.2 ml of 10% TNP-SRBC intravenously or 100 μ g of TNP-Ficoll intraperitoneally 24 h later. Serum samples obtained before and 5, 7, 10, and 13 d after immunization were assayed for their IgG and IgM anti-TNP titers by radioimmunoassay. Fig. 2 shows log-log plots of counts per minute of 125 I-RaM μ bound to serial fourfold dilutions of sera obtained 5 d after TNP-SRBC immunization and similar fourfold dilutions of sera drawn 7 d postimmunization. Each point represents the geometric mean of the counts per minute

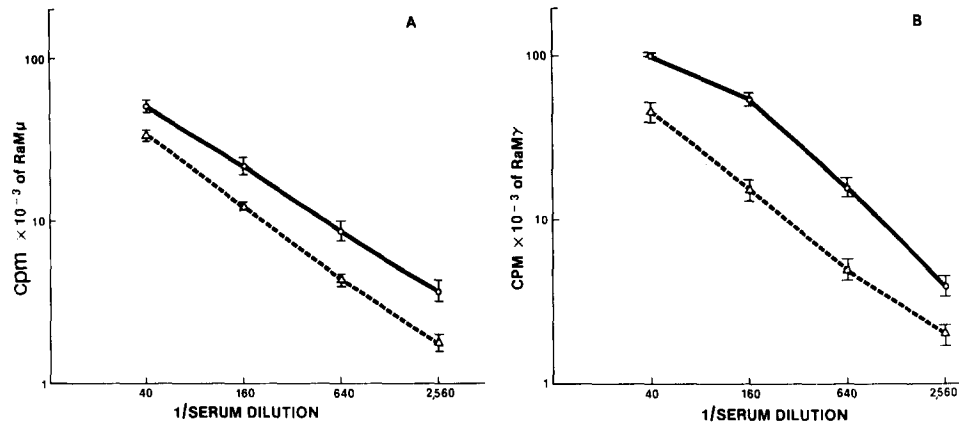


FIG. 2. Effect of 4.22aM δ^8 on the anti-TNP response in TNP-SRBC-immunized mice. Two groups of six BALB/c mice were given 100 μ g of 4.22aM δ^8 (O) or CBPC-101 (Δ)/mouse 1 d before injection with 0.2 ml of 10% TNP-SRBC. Sera obtained 5 d after immunization were assayed for IgM anti-TNP antibody (A), whereas sera obtained 7 d after immunization were assayed for IgG anti-TNP antibody (B). 40-, 160-, 640-, and 2,560-fold dilutions of individual sera were analyzed by solid-phase microtiter plate radioimmunoassay for the counts per minute of 125 I-labeled RaM μ or RaM γ that were bound to mouse antibody that was adherent to TNP-Ficoll-coated wells. Each point represents the geometric mean \times SE of counts per minute of 125 I-antibody bound by the specified dilution of sera from six mice.

TABLE I
Effects of 4.22aM δ^8 , CBPC-101, or No Antibody on the Primary Anti-TNP Response to TNP-SRBC in BALB/c Mice

Antibody injected	Serum titer*	
	IgM (day 5)	IgG (day 7)
None	130 (1.51)	133 (1.20)
CBPC-101	328 (1.11)	171 (1.28)
4.22aM δ^8	679 (1.20)	1,560 (1.23)

Three groups of six BALB/c mice were given no antibody, 200 μ g of CBPC-101, or 200 μ g of 4.22aM δ^8 /mouse, followed by immunization with 0.2 ml of 10% TNP-SRBC 1 d later.

* Geometric mean. Standard error in parentheses.

of 125 I-RaM μ or RaM γ bound by a given dilution of serum from 6 mice. Sera from mice that received 4.22aM δ^8 had approximately five times as much IgG anti-TNP antibody and approximately twice as much IgM anti-TNP antibody as mice that received CBPC-101. It should be noted that similar relative differences in titer between sera from 4.22aM δ^8 - and CBPC-101-treated mice would be calculated if the IgG anti-TNP titer point were chosen anywhere between 4×10^3 and 4.5×10^4 cpm and if the IgM anti-TNP titer point were chosen anywhere between 4×10^3 and 3×10^4 cpm.

To assure that the enhancement seen in 4.22aM δ^8 -treated mice did not actually reflect suppression by the control immunoglobulin, CBPC-101, three groups of six mice each were injected with 200 μ g of CBPC-101, 200 μ g of 4.22aM δ^8 , or no antibody, followed by immunization 24 h later with TNP-SRBC. As shown in Table I, 5-d postimmunization sera from mice given 4.22aM δ^8 had a geometric mean IgM

TABLE II
Effects of 4.22aM δ^a on Primary Anti-TNP Response to TNP-SRBC in C.B20 Mice

Antibody injected	Serum titer*	
	IgM (day 5)	IgG (day 7)
4.22aM δ^a	175 (1.29)	374 (1.43)
CBPC-101	149 (1.23)	399 (1.28)

Two groups of six C.B20 mice were given 200 μ g of 4.22aM δ^a or CBPC-101/mouse, followed by immunization with 0.2 ml of 10% TNP-SRBC 1 d later.

* Geometric mean. Standard error in parentheses.

anti-TNP titer approximately twice that of mice given CBPC-101 and approximately five times that of mice given no antibody; whereas sera obtained from 4.22aM δ^a -treated mice 7 d after immunization contained approximately ninefold more IgG anti-TNP antibody than mice given CBPC-101 or no antibody. In another experiment, the ability of 4.22aM δ^a to enhance anti-TNP antibody production by C.B20 mice immunized with TNP-SRBC was analyzed. Because 4.22aM δ^a binds IgD of the a allotype (found in BALB/c mice) but not of the b allotype (found in C57BL/Ka mice) and C.B20 mice are a BALB/c congenic strain that has C57BL/Ka Ig heavy-chain genes, 4.22aM δ^a would be expected to enhance anti-TNP antibody production in these mice if the enhancing factor were something other than anti- δ -chain activity. C.B20 mice treated with 4.22aM δ^a made no more IgM or IgG anti-TNP antibody than did mice treated with CBPC-101 (Table II).

We next studied the kinetics of anti- δ -chain enhancement of anti-TNP antibody responses in mice immunized with TNP-Ficoll or TNP-SRBC 24 h after injection with 4.22aM δ^a or CBPC-101. These experiments demonstrated that IgM anti-TNP titers were higher on day 5 than on day 7, whereas IgG titers were higher on day 7 than on days 5, 10, or 13 (data not shown). At all times studied, IgG anti-TNP titers in the mice that received 4.22aM δ^a exceeded those of CBPC-101 recipients by a factor of approximately five, whereas the enhancement of IgM anti-TNP antibody production by 4.22aM δ^a was approximately twofold.

We next examined the effects of varying the doses of antigen and 4.22aM δ^a on anti- δ -chain enhancement of anti-TNP antibody responses. Mice were injected with 25, 100, or 800 μ g of 4.22aM δ^a or CBPC-101 24 h before TNP-SRBC immunization. 5- and 7-d sera were titered for IgM and IgG anti-TNP antibody, respectively. Both IgG and IgM titers were higher in sera obtained from mice that had received 4.22aM δ^a instead of CBPC-101 at all three antibody doses tested (data not shown), and the variation observed with different antibody doses was not substantial. Enhancement of the IgG anti-TNP response by 4.22aM δ^a does appear, however, to be highly dependent upon the dose of antigen administered. As is seen in Fig. 3A, the IgG anti-TNP response decreased with decreasing amounts of TNP-SRBC and this effect was particularly pronounced in mice that had received 4.22aM δ^a rather than CBPC-101. Mice given 4.22aM δ^a before immunization with 0.2 ml of 10, 1, or 0.1% TNP-SRBC made 9.2-, 2.5-, and 1.1-fold more IgG anti-TNP antibody, respectively, than did mice immunized with the same quantity of antigen after having received CBPC-101. Similarly, the IgG anti-TNP response to 100 μ g of TNP-Ficoll was enhanced more by 4.22aM δ^a (9.4-fold) than the response to 10 μ g or 1 μ g of TNP-

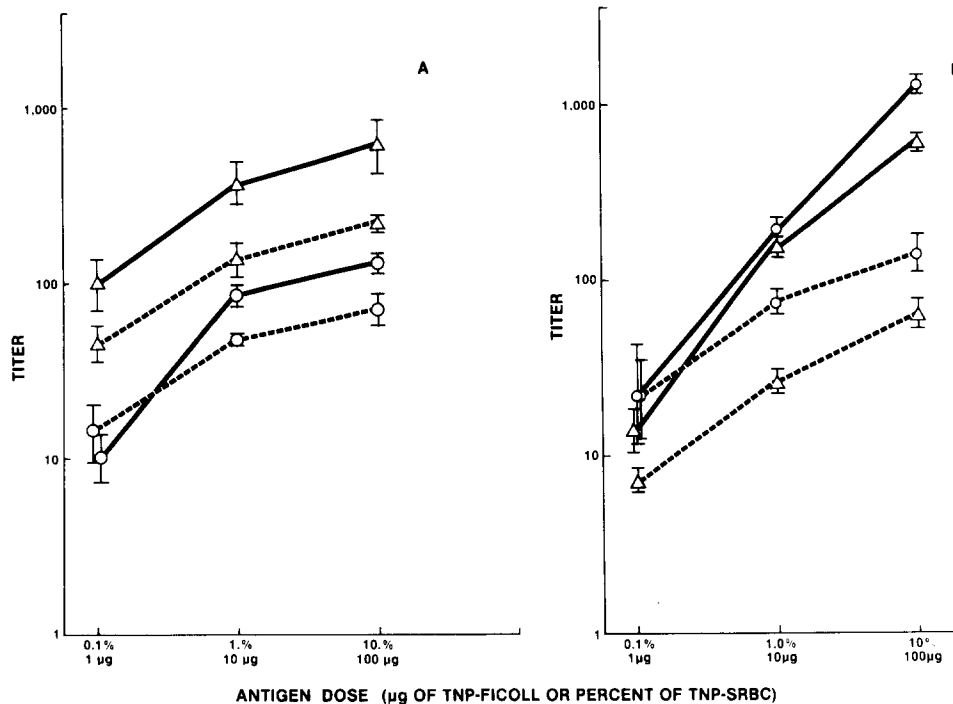


FIG. 3. Effect of antigen dose on 4.22aM δ^8 enhancement of the anti-TNP humoral immune response. Two groups of 36 BALB/c mice were given 200 μ g of 4.22aM δ^8 (—) or CBPC-101 (---) per mouse. 1 d later, six mice in each group were immunized with 1, 10, or 100 μ g of TNP-Ficoll (Δ) or 0.2 ml of 0.1, 1, or 10% TNP-SRBC (\circ)/mouse. Sera obtained 5 and 7 d after immunization were titrated for IgM anti-TNP levels (A) and IgG anti-TNP levels (B), respectively. Each point represents the geometric mean \times \pm SE of the titers of six individual sera.

Ficoll (5.8-fold or 1.9-fold, respectively). Although a similar trend was seen in 4.22aM δ^8 enhancement of the IgM anti-TNP response (Fig. 3B) the effects of variation of antigen dose on enhancement of IgM anti-TNP was much less striking than that seen on IgG anti-TNP levels. The lack of any enhancement by 4.22aM δ^8 of either the IgG or IgM anti-TNP response of 0.2% TNP-SRBC is particularly meaningful, as it indicates that 4.22aM δ^8 in the absence of other antigens does not induce any detectable anti-TNP humoral immune response (i.e., it is not a polyclonal activator).

The effect of varying the interval between administration of 4.22aM δ^8 or CBPC-101 and antigen was studied in experiments in which sera were obtained 5 and 7 d after TNP-SRBC administration for determination of IgM and IgG anti-TNP titers, respectively. Administration of 4.22aM δ^8 1 d before antigen enhanced the IgG anti-TNP response more than when 4.22aM δ^8 was given simultaneously with or 3 d before antigen (Fig. 4). Furthermore, no significant 4.22aM δ^8 enhancement was seen when 4.22aM δ^8 was given 7 or 14 d before antigen or 3 d after antigen. The effect of varying the interval between 4.22aM δ^8 and TNP-SRBC administration on the IgM anti-TNP response was less striking.

The possibility that T lymphocytes might play a role in 4.22aM δ^8 enhancement of humoral immune responses was studied by giving 200 μ g of 4.22aM δ^8 or CBPC-101

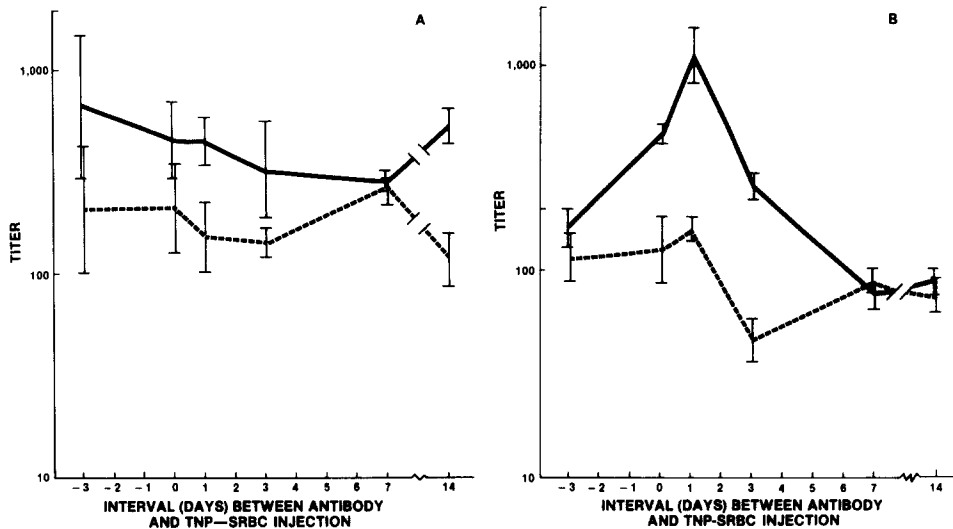


FIG. 4. Effect of varying the interval between administration of $4.22aM\delta^8$ and TNP-SRBC on $4.22aM\delta^8$ enhancement of the humoral anti-TNP response. Groups of six BALB/c mice were given $200\ \mu\text{g}$ of CBPC-101 (---) or $4.22aM\delta^8$ (—)/mouse 14, 7, 3, or 1 d before immunization with $0.2\ \text{ml}$ of 10% TNP-SRBC or were given CBPC-101 or $4.22aM\delta^8$ simultaneously with, or 3 d after, TNP-SRBC immunization. IgM and IgG anti-TNP titers were obtained for sera drawn 5 d (A) and 7 d (B) after immunization, respectively.

TABLE III

Effect of $4.22aM\delta^8$ on *nu/nu* and Normal Anti-TNP Responses to TNP-SRBC and TNP-Ficoll

Experiment	Mice	Antibody	Titer*			
			IgM		IgG	
			TNP-SRBC	TNP-Ficoll	TNP-SRBC	TNP-Ficoll
1	Normal‡	$4.22aM\delta^8$	538 (1.26)	499 (1.62)	949 (1.62)	1,510 (1.35)
		CBPC-101	355 (1.43)	259 (1.32)	144 (1.55)	275 (1.59)
	<i>nu/nu</i>	$4.22aM\delta^8$	0	466 (1.19)	0	489 (1.33)
		CBPC-101	0	636 (1.27)	0	407 (1.64)
2	<i>nu/nu</i>	$4.22aM\delta^8$				209 (1.29)
		CBPC-101				207 (1.10)

In experiment 1, groups of six athymic (*nu/nu*) mice or their phenotypically normal littermates were given $200\ \mu\text{g}$ of CBPC-101 or $4.22aM\delta^8$ /mouse 1 d before immunization with $100\ \mu\text{g}$ of TNP-Ficoll or $0.2\ \text{ml}$ of 10% TNP-SRBC. Sera obtained 5 and 7 d after immunization were titered for IgM and IgG anti-TNP antibody levels, respectively. Experiment 2 is a partial repeat of experiment 1, with only athymic mice and TNP-Ficoll.

* Geometric mean. Standard error in parentheses.

‡ *nu/+* or *+/+* littermates of *nu/nu* mice.

to congenitally athymic (*nu/nu*) mice or their phenotypically normal (*nu/+* and *+/+*) littermates, and immunizing them with $0.2\ \text{ml}$ of 10% TNP-SRBC or $100\ \mu\text{g}$ of TNP-Ficoll 24 h later. As expected, the phenotypically normal mice generated strong IgM and IgG anti-TNP antibody responses to both TNP-SRBC and TNP-Ficoll. These IgM responses were enhanced slightly, whereas these IgG responses were

enhanced considerably by 4.22aM δ^a (Table III). Also as expected, nu/nu mice made no detectable anti-TNP antibody in response to TNP-SRBC, but did produce IgM and IgG anti-TNP antibodies when immunized with TNP-Ficoll, the titer of which, in fact, exceeded that produced by their normal littermates. However, in this, as in a subsequent experiment (Table III, experiment 2), no enhancement by 4.22aM δ^a was seen. These findings indicate that directly or indirectly, T cells play a role in anti-IgD enhancement of in vivo humoral responses.

Effect of 4.22aM δ^a on Serum Immunoglobulin Levels and Spleen Cell Proliferation. To determine if the enhanced antibody responses induced by 4.22aM δ^a were accompanied by an increase in serum immunoglobulin, two groups of six mice were each given either 200 μ g of 4.22aM δ^a or CBPC-101 24 h before immunization with TNP-SRBC. Sera obtained 5 d after immunization were analyzed by radial immunodiffusion for IgM and IgG_{2a}, whereas 10-d sera were analyzed for IgG₁ and IgG_{2b}. Mice injected with 4.22aM δ^a showed a more substantial rise in serum IgG₁ levels (+79 \pm 13%) than mice injected with CBPC-101 (+22 \pm 10%); changes in other isotype levels were comparable for both groups. In a second experiment in which mice were given 200 μ g of either 4.22aM δ^a or CBPC-101 without subsequent antigenic challenge, no substantial differences were seen in IgM or total IgG serum levels between the two groups. These data are consistent with our finding that 4.22aM δ^a does not enhance anti-TNP antibody production stimulated by low doses of TNP-SRBC (i.e., anti- δ -chain by itself has little or no activity as a polyclonal activator of antibody secretion).

Spleen cells from mice given either 4.22aM δ^a or CBPC-101 1, 3, 5, or 7 d before were analyzed for their ability to incorporate [³H]thymidine during 4- and 24-h in vitro incubations. 4.22aM δ^a had no effect on spleen cell [³H]thymidine incorporation 1, 3, and 5 d after injection and a twofold stimulatory effect 7 d after injection. In a second experiment, even this slight stimulatory effect at day 7 was not seen.

As another measure of the effect of 4.22aM δ^a on spleen cell proliferation, splenic fragments from mice given 4.22aM δ^a or CBPC-101 3 or 7 d before killing were fixed, sectioned, stained with hematoxylin and eosin, and examined microscopically for mitotic figures. Less than 1% of splenic lymphoid follicle cells from either 4.22aM δ^a - or CBPC-101-treated mice showed mitotic figures.

Discussion

The intravenous administration of anti- δ -chain antibody removes almost all sIgD from mouse splenic B lymphocytes but leaves most sIgM on the majority of these cells intact. When antigen is given simultaneously, or 1 day after, anti- δ -chain antibody, a marked enhancement of antigen-specific IgG responses and a smaller increase in IgM responses are observed. This enhancement occurs in the absence of detectable anti- δ -chain-stimulated polyclonal splenic B cell proliferation or terminal differentiation. These data are similar to previous studies of the effects of anti- δ -chain on specific antibody responses in the rhesus monkey and rat (10–12, 23, 24). Our results differ, however, from experiments by Dresser and Parkhouse (25), which suggested that anti-mouse δ -chain antisera inhibit the response to simultaneously, or subsequently, injected antigens. These experiments were unlike ours in that anti-SRBC rather than anti-TNP antibody responses were examined, a conventional rabbit anti-mouse δ -chain antiserum was used instead of a monoclonal mouse anti-mouse δ -chain antibody, and splenic plaque-forming cells rather than serum antibody levels were measured. In

preliminary experiments, our affinity-purified RaM δ antibody enhances IgG responses as well as or better than 4.22aM δ^a and does not suppress anti-TNP responses at doses varying from 6 μ g to 2 mg. In addition, both 4.22aM δ^a and RaM δ can enhance the IgG anti-SRBC response of mice immunized with TNP-SRBC 1 d after treatment. Furthermore, the numbers of direct and indirect anti-SRBC plaque-forming cells are substantially enhanced by simultaneous administration of anti- δ -chain antibody with SRBC (26).

In addition to establishing that anti- δ -chain antibody has an adjuvant effect on specific antibody production to TNP-Ficoll and TNP-SRBC, our data indicate that this adjuvant effect is not observed when suboptimal, but immunogenic, antigen doses are used. Furthermore, this adjuvant effect is observable in nu/+ but not nu/nu mice and, hence, appears to be T cell dependent. The T cell dependence of anti- δ -chain antibody enhancement of the TNP-Ficoll response is somewhat surprising, as this antigen is widely thought to be T independent and elicits a strong humoral immune response in nu/nu mice (27). However, recent studies by Kettman and Dodd (28) and Mond et al. (29) indicate that T cell help is required for an optimal *in vitro* antibody response to TNP-Ficoll by B cells from normal mice.

To be consistent with our data, models of anti- δ -chain antibody enhancement should account for: (a) the requirement for T cell help, (b) the requirement for a relatively high antigen dose, and (c) the wide range of anti- δ -chain antibody concentrations that produce enhancement. We feel that these observations can best be explained within the confines of the two-signal theory of B cell stimulation; that is, that B lymphocytes are optimally stimulated to differentiate into antibody-secreting cells by the triggering of both sIg receptors and other receptors specific for T helper substances and/or polyclonal activators (30). Anti- δ -chain antibody could have an adjuvant effect on antigen-induced B lymphocyte terminal differentiation by increasing the amount or effect of T cell help on B cells, by interfering with T cell suppression or T cell dependent anti-idiotypic suppression of B cells, or by changing the quantity and quality of the binding of ligand to B cell sIg. These proposed effects of anti- δ -chain antibody upon a humoral immune response are not mutually exclusive, but, for convenience, can be expressed as three separate models. The first model proposes that T cells would respond to 4.22aM δ^a by generating helper factors specific for 4.22aM δ^a which would be focused onto B cells polyclonally by binding to 4.22aM δ^a that is associated with sIgD. However, our failure to detect 4.22aM δ^a associated with spleen cells by sensitive immunofluorescence techniques decreases the likelihood of this hypothesis.

A second model notes that anti-TNP antibody responses are limited by antibodies or suppressor substances specific for idiotypic determinants on anti-TNP antibodies (24). Because the binding of these idiotypic-specific substances to B cell sIg is required, presumably, for their suppressive effects, the loss of sIgD might interfere with their binding and, thus, enhance antigen-specific antibody responses. However, this model requires that sIgM, which is still present in large amounts on most B cells after the 4.22aM δ^a injection, is not able to bind idiotypic-specific suppressor substances and/or is not able to transmit a suppressive signal to the same degree as sIgD or as sIgM together with sIgD.

A third model proposes that the binding of anti- δ -chain antibody (or antigen) to sIgD partially activates B lymphocytes. This partial activation would be insufficient

to induce terminal differentiation, but might make the B cell respond more readily to T cell helper factors or other stimulators of B cell terminal differentiation. Several lines of evidence suggest that anti- δ -chain antibody may partially activate B lymphocytes. First, although 4.22aM δ^a does not induce B lymphocyte proliferation in vitro, both affinity-purified RaM δ and goat anti-mouse δ -chain antibody cause in vitro B lymphocyte proliferation (31). Second, treatment of murine B lymphocytes in vivo or in vitro with monoclonal or affinity purified anti- δ -chain antibody causes these cells to express considerably more I-region associated (Ia) determinants on their cell surfaces as detected by immunofluorescence using a FACS (32). This phenomenon is of particular interest because Ia appears to play a role in the interaction of B cells, T cells, and macrophages and could be a receptor for T helper factors (33, 34). Third, Parker et al. have found that 4.22aM δ^a (or RaM μ) bound to polyacrylamide beads greatly enhances concanavalin A helper-factor-induced in vitro differentiation of murine B lymphocytes into Ig-secreting cells ([35]; and D. Parker. Personal communication.).

The concept that anti- δ -chain antibody partially activates B lymphocytes fits well with the observation that 4.22aM δ^a enhancement of humoral immune responses requires the presence of both T lymphocytes and considerable doses of antigen. Both antigen and T lymphocytes would be necessary for the generation of carrier- and/or idiotype-specific T helper factors that are required to induce terminal differentiation of the partially activated B lymphocytes. The lack of 4.22aM δ^a enhancement of the antibody response to suboptimal antigen doses leads us to hypothesize that fairly high levels of T help are required to produce this enhancement and that such amounts of T help are produced only when high quantities of antigen are used. Alternatively, large antigen doses may be required for optimal focusing of antigen-specific helper factors onto B cells that have lost sIgD (i.e., helper factors may bind to antigen that has itself bound to B lymphocyte surface Ig, or antigen may itself be incorporated into helper factor). Reports that some anti- δ -chain antibodies block the in vitro antibody response to TNP-Ficoll and SRBC (36, 37) might raise the possibility that T cell help is limiting in these in vitro systems, and suggest that, under these circumstances, partial activation of B lymphocytes by anti- δ -chain antibody might actually suppress terminal differentiation. According to this concept, anti- δ -chain antibody mimics the effect of large antigen doses, which can suppress B cell terminal differentiation in the absence of T help but stimulate antibody secretion when sufficient T help is present (12, 37, 38).

If anti- δ -chain antibody enhances antigen-specific antigen responses by partially activating B lymphocytes, as suggested by our third model, it can be questioned why partial activation is not achieved to the same extent by antigen alone as by the combination of antigen and anti- δ -chain antibody, because both bind to sIgD. One possibility is that the combination of anti- δ -chain antibody plus antigen might enhance the partial activation of B cells induced by antigen alone simply by increasing the quantity of ligand bound to B cell sIg. However, the observation that 25 μ g of 4.22aM δ^a enhances the IgG anti-TNP response to a large dose of TNP-SRBC as well as 800 μ g of this antibody does not support this hypothesis, because more sIgD will be bound by 800 μ g of anti- δ -chain antibody than by 25 μ g.

A second possibility is that anti- δ -chain antibody enhances the B cell partial activation produced by antigen alone by increasing the ratio of sIgD:sIgM bound by

ligand. We find this hypothesis attractive as it is consistent with observations that the binding of ligand to sIgM is more tolerogenic to a cell than the binding of the same ligand to sIgD (39–41).

Summary

Heterologous anti- δ -chain antibodies have an adjuvant effect on specific in vivo humoral immune responses to simultaneously, or subsequently, injected antigens in the rat and rhesus monkey. We have used a hybridoma-secreted antibody that binds murine δ -chain of the a allotype (4.22aM δ^a) to study this phenomenon in the mouse and to investigate the mechanism of this effect. Injection of 4.22aM δ^a into BALB/c mice removes almost all surface IgD (sIgD) from splenic B lymphocytes. sIgD does not reappear until the serum level of 4.22aM δ^a decreases 5–7 d after injection. 4.22aM δ^a fails to induce detectable proliferation or to raise total serum Ig levels substantially above control values. However, 4.22aM δ^a injected 24 h before antigen elicits an approximately twofold enhancement of serum IgM and a 3- to 10-fold enhancement of serum IgG anti-trinitrophenyl (TNP) antibodies in response to immunization with optimal doses of TNP-Ficoll or TNP-sheep red blood cells (TNP-SRBC). 4.22aM δ^a injected 1 wk before or 3 d after TNP-SRBC, however, has no effect on IgG anti-TNP levels. The adjuvant effect of anti- δ -chain antibody was markedly decreased when suboptimal antigen doses were used. Furthermore, even in the case of TNP-Ficoll, a relatively T-independent antigen, the ability of 4.22aM δ^a to enhance the anti-TNP antibody response was T cell dependent. Our data suggest that the binding of anti- δ -chain antibody to cell sIgD may partially activate B lymphocytes and make them more capable of differentiating into antibody-secreting cells when stimulated by antigen-specific T cell help.

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