

**A DICHOTOMY BETWEEN THE EXPRESSION OF IgD ON B  
CELLS AND ITS REQUIREMENT FOR TRIGGERING SUCH  
CELLS WITH TWO T-INDEPENDENT ANTIGENS\***

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The majority of adult B lymphocytes in the mouse bear two immunoglobulin isotypes, IgM and IgD ( $\mu^+\delta^+$  cells) (1). A small population of IgM-bearing cells lacks, or expresses very low levels of IgD ( $\mu$ -predominant [ $\mu\mu$ ] cells) (1). These cells are believed to constitute a less mature subset of B cells analogous to neonatal B cells (2). Based on the time during ontogeny when responses to T-independent (TI) and T-dependent (TD) antigens appear (3, 4) and the ability to block in vitro responses with anti- $\mu$  or anti- $\delta$  (5, 6, D. Mosier, personal communication), it has been suggested that the precursors of two TI-1 responses, trinitrophenyl (TNP)-Brucella (TNP-BA) and TNP-lipopolysaccharide (TNP-LPS) are  $\mu\mu$  cells (5, 6), whereas the precursor for a TD response, TNP-sheep erythrocytes (TNP-SRBC), bears both IgM and IgD (6). However, the possibility cannot be excluded that IgD is present on some or all of the TI precursors, but that it is not obligatory for triggering.

In the present experiments we have examined the phenotypes of TI and TD precursors by treating cells with C' and either anti- $\mu$  or anti- $\delta$  before stimulation with antigen. Our results suggest that the majority of B cells that respond to TNP-BA, TNP-LPS, and TNP-SRBC bear IgD, even though in the case of the two TI antigens, IgD is not required for triggering.

**Materials and Methods**

*Animals.* C57BL/6 (B6), BALB/c, and (C57BL/6  $\times$  DBA/2)F<sub>1</sub> (BDF<sub>1</sub>) female mice, 2-4 mo of age, were obtained from The Jackson Laboratory, Bar Harbor, Maine.

*Antisera.* Anti-Ig5<sup>b</sup> (alloanti- $\delta$ ) was obtained from Sera-Labs, Crawley Down, Sussex, England, and is the product of a hybridoma (H6/31.HLK) (7). Rabbit anti-mouse  $\delta$  (RA $\delta$ ) (1), rabbit anti-mouse- $\mu$  (RA $\mu$ ) (8), rabbit anti-mouse Ig (RAMIg) (8), and rabbit anti-TNP-ovalbumin (RA-TNP) (9) were prepared and assayed as described previously.

*Specificity of the Alloanti- $\delta$ .* NP-40 lysates of radioiodinated splenocytes were prepared as described previously (8). The alloanti- $\delta$ , RAMIg, and RA-TNP (control) were coupled to Sepharose and added to aliquots of the cell lysate. After incubation at 37°C and 4°C, the Sepharose was removed by centrifugation and the supernates treated with RAMIg and *Staphylococcus aureus*. Complexes were eluted, reduced, and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8).

*Deletion of B-Cell Subsets.* Complement-mediated cytolysis after treatment with each antise-

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rum was performed as described previously (10). For killing with anti- $\delta$  serum and for cells cultured in vitro, sodium azide was not used.

*In Vitro Response after Negative Selection.* Aliquots of  $10^6$  viable cells in Richter's improved minimal essential medium with zinc and insulin (IMEMZO) (Associated Biomedic Systems, Inc., Buffalo, N. Y.) with supplements (11) were plated in replicate (10 per sample) in microtiter dishes (Microtest II, Falcon Labware, Div. of Becton, Dickinson, Oxnard, & Co. Calif.) (final vol: 0.2 ml per well) with TNP-SRBC (12) or TNP-BA (11). To each well containing TNP-BA was added  $1.6 \times 10^6$  irradiated (1,500 rads) splenocytes from unprimed B6 mice. Preliminary experiments established that the TNP-BA response is TI under these conditions. Each well containing TNP-SRBC received  $1.6 \times 10^6$  irradiated splenocytes from mice primed with SRBC (11) as a source of T-helper cells (13). After 4 d in culture (14), cells in individual wells were washed and assayed for the presence of direct plaque-forming cells (PFC) to TNP-horse erythrocytes (Colorado Serum Co., Denver, Colo.) (TNP-HRBC) (15-17). Percent inhibition of response was calculated on the basis of the number of input cells before depletion.

*In Vivo Response after Negative Selection.* After complement-mediated cytolysis, cells were washed and 0.2 ml injected into syngeneic low dose primed (13) (for the TNP-SRBC response), irradiated recipients. Four animals per group were used. The extent of inhibition of the response was assessed by comparing the response of treated cells with the average response of two doses of control cells. The lower dose was equivalent to the number of experimental cells injected whereas the higher dose was equivalent to the starting number of experimental cells before cytolysis. The number of cells used was the minimum required to attain a linear dose-response curve. 24 h after reconstitution, the animals were challenged with 0.2 ml 10% TNP-SRBC or 50  $\mu$ g TNP-LPS (18, 19) (LPS from *Escherichia coli* 055B5 [Difco Laboratories, Detroit, Mich.]). 5 d later, the animals were sacrificed and total PFC in the spleens against TNP-HRBC, HRBC, and SRBC were determined.

## Results

*Specificity of the Alloanti- $\delta$  Serum.* The alloanti- $\delta$  serum used in these studies is the product of the hybridoma H6/31.HLK (7). The findings of Pearson et al. (7) indicate that this serum is specific for an allotypic determinant on  $\delta$ -chains of mice bearing the  $Ig^b$  allele. It has not been proven, however, that this alloanti- $\delta$  serum is devoid of cross-reactivity for  $\mu$ -chains, i.e., it could have specificity against a determinant common to both  $\mu$ - and  $\delta$ -chains. We therefore tested this possibility by treating aliquots of lysate from iodinated B6 spleen cells with Sepharose coupled to RAMIg, alloanti- $\delta$  or RA-TNP (control). The Sepharose was removed by centrifugation and the Ig remaining in each sample was precipitated under saturating conditions with RAMIg and *S. aureus*, solubilized, and analyzed by SDS-PAGE. Pretreatment of lysates with Sepharose bound to RAMIg resulted in the loss of both IgM and IgD (Fig. 1). When lysates were treated with Sepharose bound to alloanti- $\delta$ , however, virtually all of the IgM, but less than 15% of the IgD was recovered from the supernate. Thus, there was no evidence for the binding of IgM by Sepharose alloanti- $\delta$  and it was concluded, therefore, that the alloanti- $\delta$  serum was specific for  $\delta$ -chains.

The specificity of alloanti- $\delta$  was further examined by assessing the C'-mediated cytolytic activity of the serum against spleen cells from mice carrying  $Ig^b$  and  $Ig^a$  alleles. As shown in Fig. 2, at optimal dilutions, alloanti- $\delta$  killed approximately one-half of B6, but not BALB/c, spleen cells, thus confirming (7) the alloreactivity of the serum.

*Effect of Deleting Cells Bearing IgM or IgD on the In Vitro Response.* As shown in Table I, depletion of cells bearing either IgM or IgD resulted in a profound inhibition of the in vitro responses to both TNP-BA and TNP-SRBC. In two of three experiments, treatment with anti- $\delta$  and C', but not with anti- $\mu$  and C' produced a greater inhibition

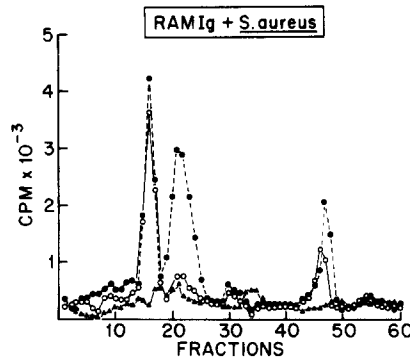


FIG. 1. Effect of treating lysates from radioiodinated C57BL/6 splenocytes with Sepharose coupled to RAMIg, RA-TNP-ovalbumin, or alloanti- $\delta$  on the subsequent recovery of IgM and IgD. Aliquots of lysate from radioiodinated cells were incubated with Sepharose-Ig, the immunoabsorbant removed by centrifugation, and the absorbed samples then treated with saturating amounts of RAMIg + *S. aureus*. Aliquots of each eluate were analyzed by SDS-PAGE. Pretreatment with  $\circ$ , Sepharose-anti- $\delta$ ;  $\bullet$ , Sepharose-anti-TNP;  $\blacktriangle$ , Sepharose-anti-Ig.

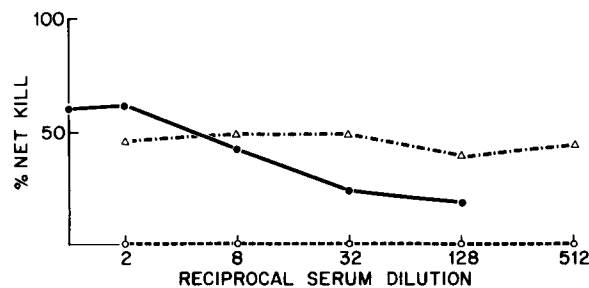


FIG. 2. Cytotoxicity of rabbit anti- $\mu$  and alloanti- $\delta$  on C57BL/6 and BALB/c splenocytes. Spleen cells were washed twice and resuspended in Eagle's minimal essential medium supplemented with 5% fetal calf serum. To  $10^6$  cells was added dilutions of alloanti- $\delta$ , RA $\mu$  (B6 only), normal mouse serum, normal rabbit serum, or medium, bringing the final vol to 0.05 ml per tube. After a 30-45-min incubation at 4°C, 0.05 ml of 40-50% rabbit complement (Pel-Freeze Biologicals, Inc., Rogers, Ark.) was added to each sample. Suspensions were incubated at 37°C for 30 min and then assessed for the presence of viable and dead cells by trypan blue exclusion. The results are expressed as the percent net kill where percent net kill =  $100 \times (\text{dead cells in experimental sample} + [\text{total cells in control sample} - \text{total cells in experimental sample}]) / \text{total cells in control sample} - 100$ .  $\bullet$ , anti- $\mu$ ;  $\Delta$ , allo-anti- $\delta$ (C57BL/6J);  $\circ$ , allo-anti- $\delta$ (BALB/c).

of the TD than of the TI response, suggesting that a portion of the cells contributing to the TI response express IgM, but little or no IgD.

*Effect of Deleting Cells Bearing IgM or IgD on the Adoptive Antibody Response.* We have also investigated the effect of deleting IgM or IgD-bearing cells on the adoptive antibody response to TI and TD antigens. TNP-LPS rather than TNP-BA was used as the representative TI antigen because the latter may be TD in vivo. The response to TNP-LPS, however, has been shown to be TI both in vivo and in vitro (18, 19). The dose of TNP-LPS used in these experiments was less than that required for polyclonal activation, i.e. it induced no increase above background in PFC to either HRBC or SRBC at the time of peak response to TNP-LPS.

As shown in Table II, after deletion of  $\delta^+$  or  $\mu^+$  cells, the adoptive responses to both

TABLE I  
*Percent Loss of the Primary In Vitro Responses to TNP-BA or TNP-SRBC after Treatment of C57BL/6 Splenocytes with C' and RA $\mu$  and/or Alloanti- $\delta$*

Exp	Cells treated with C' and antibody against	Inhibition of responses*	
		TNP-BA	TNP-SRBC
		%	
1	$\delta$	70	86
	$\mu$	94	91
	$\delta + \mu$	91	99
	Ig	94	—‡
2	$\delta$	65	90
	$\mu$	93	89
	$\delta + \mu$	95	94
	Ig	86	85
3	$\delta$	74	50
	$\mu$	69	91
Average	$\delta$	70	75
	$\mu$	85	90
	$\delta + \mu$	93	97
	Ig	90	85

\* The mean response to TNP-BA in control cultures was  $194 \pm 65$  (SEM) PFC per  $10^6$  viable treated cells, that to TNP-SRBC was  $32 \pm 11$  (SEM) PFC per  $10^6$  viable treated cells

‡ Not done

TNP-SRBC and TNP-LPS in syngeneic hosts were significantly inhibited. Pretreatment with RA $\delta$  appeared to more effectively inhibit both TI and TD responses than did treatment with alloanti- $\delta$ , suggesting that the latter may be less effective in C'-mediated cytolysis. Nevertheless, studies performed with both RA $\delta$  and alloanti- $\delta$  in vivo support the conclusions reached by using alloanti- $\delta$  in the in vitro system, namely, that both IgM and IgD are expressed by the majority of cells which respond to TI, as well as those which respond to TD, antigens

### Discussion

The present studies demonstrate that a major portion of the in vitro or adoptive responses to two TI antigens (TNP-BA and TNP-LPS) are elaborated by cells bearing IgM and IgD. Thus, these two responses were almost completely eliminated by treatment with anti- $\mu$  and C' and reduced approximately 70% by treatment with anti- $\delta$  and C'.

Previous findings indicated that the in vitro TI response to TNP-BA could not be blocked by anti- $\delta$  but could be completely abolished by anti- $\mu$  (5, 6). In contrast, the TD response to TNP-SRBC could be blocked by either anti- $\delta$  or anti- $\mu$  (6). The latter finding is consistent with the present observation that either anti- $\delta$  or anti- $\mu$ , together with C', can delete the precursors of the TNP-SRBC response. Thus, we conclude that the major precursors of the primary in vitro IgM response to TD antigens are  $\mu^+\delta^+$  and that both receptors are required for triggering by antigen. The major precursors of the TNP-BA and TNP-LPS responses are also  $\mu^+\delta^+$  cells, but only the IgM receptor is required for triggering by antigen. A possible explanation underlying

TABLE II  
*Percent Loss of the Primary Adoptive Responses to TNP-LPS and TNP-SRBC after Treatment of BDF<sub>1</sub> or C57BL/6 Splenocytes with C' and Anti- $\mu$  or Anti- $\delta$*

Response	Strain	Inhibition of response after treatment with C' and antibody directed against *	
		$\delta$	$\mu$
%			
TNP-LPS	BDF <sub>1</sub>	—‡	96
	BDF <sub>1</sub>	89	83
	C57BL/6	73	—
	C57BL/6	52	—
	Average	71	90
TNP-SRBC	BDF <sub>1</sub>	96	99
	BDF <sub>1</sub>	88	—
	C57BL/6	49	83
	C57BL/6	70	85
	Average	76	85

\* BDF<sub>1</sub> cells were treated with rabbit anti- $\delta$  and C57BL/6 cells were treated with alloanti- $\delta$ . The mean response to TNP-LPS was  $847 \pm 243$  (SEM) PFC per  $10^7$  injected cells in BDF<sub>1</sub> mice and  $2,196 \pm 1,061$  (SEM) PFC in C57BL/6 mice although the mean response to TNP-SRBC was  $19,577 \pm 2309$  PFC per  $10^7$  injected cells in BDF<sub>1</sub> mice and  $9,072 \pm 714$  PFC in C57BL/6 mice.

‡ Not done.

this latter finding is that antigens that have an appropriate mitogenic portion may bypass the requirement for IgD. This could occur if such antigens bind to both IgM and mitogen receptors. This explanation has some support in the literature. Thus, TNP-Ficoll, a partially TI antigen (20) which is relatively nonmitogenic, requires IgD on its precursor cells for triggering (5). In addition, Forni and Coutinho (21) have recently demonstrated a physical association between IgD and LPS receptors on the B-cell surface. It is possible, therefore, that binding of antigen to IgM and either IgD or the LPS receptor can deliver a triggering signal to the B-cell precursor.

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