

B-CELL TOLERANCE

IV. Differential Role of Surface IgM and IgD in Determining  
Tolerance Susceptibility of Murine B Cells\*

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During ontogeny IgD appears later than IgM on splenocytes of neonatal mice (1) and at a time when mice develop a markedly increased immune responsiveness (2). Based on these observations, it was suggested that IgD serves as a "triggering" isotype for induction of immune responses, whereas surface IgM functions as a tolerizing receptor (3). To test this hypothesis, the susceptibility of adult splenocytes (which are predominantly  $\mu^+\delta^+$  [4-6]) and neonatal splenocytes (which bear predominantly IgM [ $\mu\text{p}^+$ ; 1, 4-6]) to tolerance induction were compared. The results indicate that neonatal splenic B cells responsive to thymus dependent (TD) antigens are exquisitely susceptible to tolerance induction compared with those from adult mice (7-9). However, cells from *both* adult and neonatal mice were highly susceptible to tolerance induction when thymus independent (TI) antigen was used as immunogen (8). These results suggest that the major precursor for the TD response is a  $\mu^+\delta^+$ -cell which appears late in ontogeny and is resistant to tolerance induction and that the  $\mu\text{p}^+$ -cell is the major precursor for the TI response and is highly susceptible to tolerance induction. Other differences between responders for TI and TD antigens have been described previously (10-12). To test this concept, adult splenocytes were treated with papain under conditions in which IgD, but not five other surface molecules, was removed (13). Such treated splenocytes were shown to be markedly susceptible to tolerance induction, resembling TD responders from neonatal animals. This experiment was interpreted as indicating that IgD confers resistance to tolerance induction on  $\mu^+\delta^+$ -cells. To prove this interpretation, it is necessary to show that specific removal of IgD with anti- $\delta$  also results in increased susceptibility to tolerance induction and that treatment with anti- $\mu$  does not have a similar effect.

In the present studies, we have removed surface IgM or IgD by antibody-induced capping and assessed the tolerance susceptibility of the treated cells. Our results demonstrate that removal of IgD, but not IgM, from TD responders increases their susceptibility to tolerance induction.

Materials and Methods

*Experimental Plan.* BDF<sub>1</sub> (C57BL/6 × DBA/2 F<sub>1</sub>) splenocytes were treated with antibrain-

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associated Thy-1 (BA $\theta$ ) and complement (C') to remove T cells (7). The B cells were then treated with normal rabbit Ig or antibody to " $\mu$ " or " $\delta$ " under capping conditions; the capping was assessed by immunofluorescence; treated cells were exposed to tolerogen trinitrophenyl-human immunoglobulin (TNP<sub>17</sub>HGG) for 24 h (7) in the presence of the same antibody used for capping; cells were washed and incubated for 4 days with (TNP) on either a TI (*Brucella*) or a TD (sheep red blood cell [SRBC]) immunogen (8); direct plaque-forming cell responses to TNP and SRBC were assessed at the end of the incubation period (7).

*Antisera.* The preparation and specificity of rabbit antimouse Ig, rabbit anti- $\mu$ , rabbit anti-BA $\theta$ , and goat antirabbit Ig (GARIG) have been described previously (14). Rabbit antimouse- $\delta$  (15, 16) was absorbed with thymocytes and was judged to be monospecific by criteria described previously (16). These include: (a) a single peak on sodium dodecyl sulfate polyacrylamide gel electrophoresis after reaction with a lysate of iodinated splenocytes; (b) immunofluorescent staining of the predicted numbers of cells from various lymphoid tissues; (c) inability to stain splenocytes after their treatment with either anti- $\kappa$  or allotypic anti- $\delta$  (6); (d) independent capping of surface molecules on splenocytes with anti- $\delta$  and anti- $\mu$ . Chromatographically purified fractions from the antisera or a 30% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> precipitate of anti- $\delta$  were dissolved in phosphate-buffered saline pH 7.3 (PBS). The IgG fraction from GARIG was conjugated with fluorescein isothiocyanate (FITC; Sigma Chemical Co., St. Louis, Mo.) and chromatographed on DEAE-cellulose (DE52) to give a fraction with molar fluorescein-protein ratio of 2.6.

*Capping and Blocking of Cell Surface Immunoglobulin.* B cells were incubated for 30 min at 4°C with normal rabbit serum (NRS) or the rabbit antisera described above. Cells were washed and exposed to GARIG (17) for 90 min at 37°C in complete medium to achieve capping. After further washing, cells were cultured for 24 h in the presence of Ig fractions of the same antiserum used to induce capping (400  $\mu$ g Ig/ml) and were simultaneously exposed to varying concentrations of tolerogen (TNP<sub>17</sub>HGG).

*Immunofluorescent Staining.* Viable cells were prepared and treated with NRS or antisera specific for " $\mu$ ," " $\delta$ ," or Ig as described previously (17). After washing with balanced salt solution (BSS)-azide, the cells were resuspended at  $3 \times 10^7$  cells/ml in BSS-azide containing FITC-GARIG (0.1 mg/ml). After 10 min at 4°C, the cells were washed in BSS-azide and fixed in 1% paraformaldehyde in PBS. The cells were examined at  $\times 1,000$  with a Leitz Ortholux no. 2 fluorescence microscope. For each sample, 100–200 cells were scored for fluorescence without knowledge of the identity of the sample.

## Results

Before studies of function, the optimal conditions for modulating surface IgM and IgD on B cells from mouse spleens were determined by immunofluorescence studies. As can be seen in Table I, treatment with antibody was highly effective in removing the majority of the surface Ig to which the antibody was directed. It is presumed that the surface Ig remained modulated during tolerance induction because the concentrations of antibody in the incubation medium were similar to those that induced capping. These concentrations of antibody were chosen because they were also highly effective at blocking a primary immune response *in vitro* (18).

Fig. 1 shows a representative experiment designed to determine the effect of treating splenic B cells with antiserum on their susceptibility to tolerance induction. As seen in the left panel, only anti- $\delta$  increased the susceptibility of the TD responders to tolerance induction. Addition of IgM and IgG to the anti- $\delta$  serum did not abrogate this effect. In contrast, anti- $\delta$  had no effect on the tolerance susceptibility of TI responders (right panel). The dose-response curve of TD responsive precursors to tolerogen after treatment with anti- $\delta$  resembled the dose-response curve for untreated TI responders.

Treatment with anti- $\mu$  markedly *decreased* the tolerance susceptibility of TI

TABLE I  
Removal of Surface Ig from B Cells by Treatment with Anti- $\mu$   
or Anti- $\delta$ \*

Capping	Antiserum used for			
	Staining			NRS
	$\alpha\mu$	$\alpha\delta$	$\alpha Ig$	
				% positive
None	80	90	96	8
$\alpha\mu$	6	80	92	—
$\alpha\delta$	89	1	94	4
$\alpha\delta$ Absorbed with IgM and IgG	79	4	96	—
NRS	90	83	94	3

\* Splenic cells were treated with Ba $\theta$  + c'. The dead cells were removed before staining.

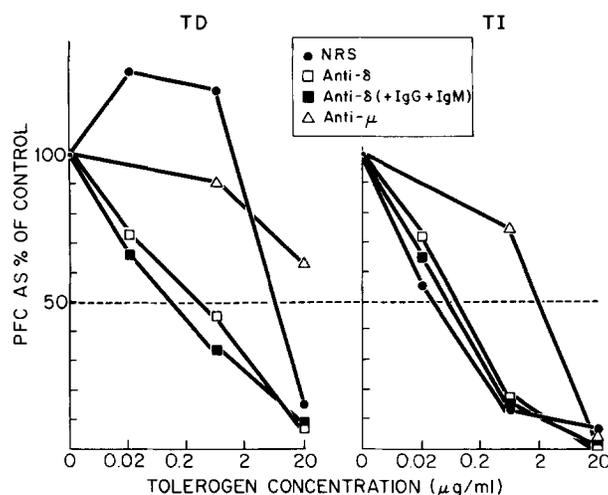


FIG. 1. The effect of removing IgM or IgD receptors on the ability to tolerize splenic B cells responding to TD (left panel) and TI (right panel) forms of TNP immunogen. The tolerogen was TNP<sub>17</sub>HGG (7). The control SRBC plaque-forming cells (PFC) were unaffected by varying the dose of tolerogen as described previously (7, 8). The TNP responses are expressed as direct PFC/10<sup>6</sup> viable recovered cells. TD control responses were: NRS, 810;  $\alpha\mu$ , 1,098;  $\alpha\delta$ , 1,149;  $\alpha\delta$  (+IgG+IgM), 1,286; TI control responses were: NRS, 412;  $\alpha\mu$ , 440;  $\alpha\delta$ , 517;  $\alpha\delta$  (+IgG+IgM), 435. This is a representative experiment of three that were done. Each point represents the average of the responses of duplicate cultures.

responders and also decreased slightly the tolerance susceptibility of TD responders (as judged by the 50% tolerance level).

### Discussion

The present studies provide further support for our earlier hypotheses (3, 8) cited above. Thus, removal of IgD with anti- $\delta$  was shown to increase susceptibility to tolerance induction of treated-TD responders but had no effect on TI responders, confirming earlier results with papain (13). In addition, it was shown that treatment with anti- $\mu$  did not similarly increase the tolerizability

of TD responders. This is a critical point because it could be argued that diminishing the concentration of *either* isotype would result in an increased susceptibility to tolerance induction. The present studies indicate that different reactivities are conferred on a  $\mu^+ \delta^+$ -cell by IgM and IgD with regard to tolerance induction, i.e., these two isotypes can be responsible for conveying different signals to the same cell. The absence of a similar effect of anti- $\delta$  on TI responders argues that they lack IgD or that, if it is present, it does not determine tolerance susceptibility.

It was also observed that treatment of B cells with anti- $\mu$  decreased the susceptibility of TI responders to tolerance induction. This finding implies that interaction of tolerogen with IgM receptors is a necessary event for tolerance induction in TI responders and that removing such receptors by stripping and blocking with anti- $\mu$  prevents this interaction. It is provocative that the stripping induced with anti- $\mu$  antibody itself does not give a tolerogenic signal.

In contrast to TI responders on which there may be only one functioning isotype, TD responders appear to require *both* IgM and IgD receptors for induction of an antibody response (18). Thus, our working hypothesis is that IgM receptors can deliver either a tolerogenic or triggering signal whereas IgD receptors may be able to deliver only a triggering signal. The events that determine which signal will be conveyed by IgM receptors remain to be elucidated.

*Note Added in Proof.* Experiments performed by Scott et al. (*J. Exp. Med.* 1977. 146:1473) using allotypic anti- $\delta$  to inhibit tolerance induction are in complete agreement with our studies using heterologous anti- $\delta$ .

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