

## B-CELL TOLERANCE

### III. Effect of Papain-Mediated Cleavage of Cell Surface IgD on Tolerance Susceptibility of Murine B Cells\*

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Murine B cells from bone marrow (1) and neonatal spleen (2, 3) are more readily tolerized than splenic B cells from adults. The maturational decrease in susceptibility to tolerance induction is accompanied by the acquisition of a variety of surface molecules including IgD (4), Lyb3 (5), MLS (6), and the C' receptor (7). In addition, mature B cells, in contrast to immature ones, can replace surface Ig molecules which have been removed by treating the cells for prolonged periods of time with divalent antibody to Ig, antigen, or proteolytic enzymes (8, 9).

We have previously hypothesized that the decreased susceptibility of mature B cells to induction of tolerance may be a result of the acquisition of surface IgD (2). To test this possibility, IgD has been removed from mature B cells under conditions in which a variety of other surface markers remain intact. The susceptibility of these altered cells to *in vitro* induction of tolerance has been examined. The results suggest that there is a direct correlation between the loss of surface IgD from mature cells and an increase in susceptibility to the induction of tolerance.

#### Materials and Methods

**Animals.** BDF<sub>1</sub> (C57BL/6 female × DBA/2 male F<sub>1</sub>) and A/J mice, 8- to 10-wk of age (The Jackson Laboratory, Bar Harbor, Maine) were used in this study. Animals to be used as the source of helper T cells were carrier-primed by intravenous injection of sheep erythrocytes (SRBC)<sup>1</sup> (Colorado Serum Co., Denver, Colo.; 0.2 ml of 0.01% suspension) 7 days before sacrifice (10). Spleen cell suspensions from primed animals were irradiated (1500 R; <sup>137</sup>cesium source) to remove B-cell activity before cultivation.

**Antigens.** Human gamma globulin (Cohn fraction II, Sigma Chemical Co., St. Louis, Mo.) was haptenated by using 2, 4, 6, trinitrobenzene sulfonic acid (TNBS, J. T. Baker Chemical

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<sup>1</sup> *Abbreviations used in this paper:* BSS, balanced salt solution; CR, complement receptor; CRL, complement receptor-bearing lymphocytes; GARiG, goat anti-rabbit Ig; GVB, gelatin veronal buffer; HRBC, horse erythrocytes; PBS, phosphate-buffered saline; PFC, plaque-forming cells; RAMiG, rabbit anti-mouse Ig; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS polyacrylamide gels; SRBC, sheep erythrocytes; TCA, trichloroacetic acid; TD, thymus dependent; TI, thymus independent; TNBS, trinitrobenzene sulfonic acid; TNP, trinitrophenyl.

Co., Phillipsburg, N. J.) to a substitution ratio of 17:1 (TNP<sub>17</sub>HgG) (10, 11) as determined spectrophotometrically. Heat killed *Brucella abortus* (National Animal Disease Laboratory, Ames, Iowa) (12) and SRBC (13) were haptenated as previously described.

**Induction of Unresponsiveness.** TNP-specific unresponsiveness was induced as described previously (2). Briefly, normal or papain-treated mouse spleen cells were incubated for 24 h at a density of  $10^7$ /ml in the presence of graded concentrations of TNP<sub>17</sub>HgG. Cells were then washed 3 times and resuspended at a density of  $10^7$  cells/ml in complete medium and plated in 35-mm Falcon dishes, (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) 0.5 ml per dish. To each dish were added,  $8 \times 10^6$  irradiated SRBC primed spleen cells in 0.1 ml complete medium and either TNP-SRBC (0.1 ml of 0.1% suspension) or TNP-*Brucella* (0.1 ml of 0.01% suspension) in balanced salt solution (BSS).

Antigen doses were chosen on the basis of their capacity to stimulate optimal direct plaque-forming cell (PFC) responses in control experiments. TNP-SRBC and TNP-*Brucella* were determined to be thymus-dependent (TD) and thymus-independent (TI), respectively, as described elsewhere (12). During the immune response phase, cultures were incubated (14) to obtain primary PFC responses to TNP-SRBC. The number of direct PFC was determined 4 days after immunogenic challenge.

**Assay.** PFC to SRBC were determined by a microscope slide modification (15) of the hemolysis in gel technique (16). Anti-TNP PFC were determined by using TNP horse erythrocytes (HRBC) (17). HRBC (Colorado Serum Co.)-specific PFC were subtracted from TNP-HRBC PFC to yield TNP-specific PFC. Data points represent means of triplicate cultures pooled immediately before assay.

**Papain Digestion.** Mouse spleen cells were treated with papain (Sigma Chemical Co., 2 times recrystallized from papaya latex, 25 mg/ml) under conditions described previously (18). Cells were suspended ( $10^7$ /ml) in minimal essential medium containing 10% fetal calf serum and varying amounts of papain (0–150  $\mu$ g/ml). Cysteine (final concentration 0.1 mM) was added to the suspension and cells were incubated for 30 min at 37°C. Control cells received cysteine alone. At the end of the incubation period cultures received iodoacetamide to a final concentration of 0.1 mM. Cells were then pelleted and washed 3 times in BSS.

**Preparation and Analysis of Radioiodinated Cells.** Splenocytes were iodinated as described previously (19). After iodination, cells were suspended in complete medium and incubated for 20 min at 37°C to remove remaining free <sup>125</sup>I. Cells were washed and in some instances treated with papain. After treatment, cells were lysed in 0.5% Nonidet P-40 (Shell Chemical Co., New York). Fetal calf serum was added to the lysates to a final concentration of 10%. Samples were dialyzed for 16 h against 1,000 vol of phosphate-buffered saline (PBS) at 4°C and centrifuged at 10,000 *g* for 30 min. Protein-associated radioactivity was determined by precipitating a small sample of the lysate in 5% trichloroacetic acid (TCA) (20). Portions of each sample were treated with saturating amounts of rabbit anti-mouse Ig (RAMIg) (21), or C57BL/6 anti-CBA (anti-H-2<sup>k</sup>, Ia<sup>k</sup>) serum. Complexes were adsorbed to fixed *Staphylococcus aureus*. (Cowans-I strain) (22), pelleted, washed, and antigen-antibody complexes eluted from the bacteria by boiling for 1–2 min in 1% sodium dodecyl sulfate (SDS) containing 8 M urea and 0.1 M 2-mercaptoethanol pH 8.6. When anti-Lyb-2 serum (23) was used, lysates were first treated with RAMIg and goat anti-rabbit Ig (GARIg) (21) to remove surface Ig. The supernates were then treated with anti-Lyb-2 and goat anti-mouse Ig. Precipitates were dissolved in 1% SDS, 8 M urea. The eluates and dissolved precipitates were electrophoresed for 16 h at 4 mA/gel on 7.5 SDS-polyacrylamide gels (SDS-PAGE) (20). Internal markers consisting of <sup>3</sup>H- $\mu$  and <sup>3</sup>H-L (K) chains were added to each sample before electrophoresis (24). Gels were fractionated and counted by using a Beckman LS350 counter with appropriate discriminators. (Beckman Instruments, Inc., Cedar Grove, N. J.)

**Preparation of the C57BL/6 Anti-CBA Serum.** C57BL/6 mice were injected intraperitoneally at weekly intervals for 7 mo with a mixture of spleen and lymph node cells from CBA/J mice. The mice were bled bimonthly and the pooled serum was assayed for its ability to bind H-2 and Ia molecules from lysates of radioiodinated splenocytes from a variety of mouse strains. These included CBA, C57BL/6, A/J, B10.HTT, B10.AQR, and B10.T(6R). The latter three strains were a gift from Dr. Jan Klein. Optimal amounts of the serum were added to dialyzed lysates and the immune complexes were adsorbed to killed *S. aureus*. The bound material was eluted and electrophoresed as described above. The data presented in Fig. 1 indicate that the antiserum recognizes H-2K<sup>k</sup>, Ia<sup>k</sup>, and Ic<sup>k</sup> molecules as assessed by both strain distribution and the molecular weight of

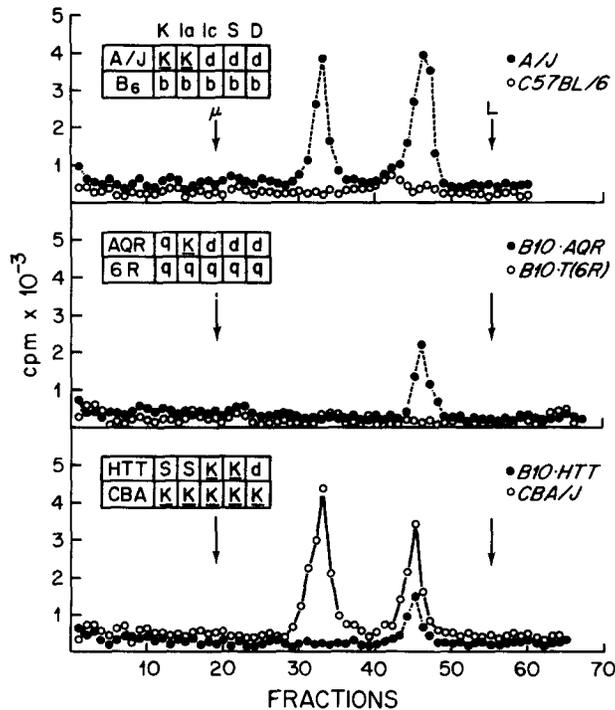


FIG. 1. SDS-polyacrylamide electropherogram of cell membrane-associated proteins precipitable with C57BL/6 anti-CBA serum.

the antigen. The serum does not contain anti- $\delta$  antibodies as described previously for this strain combination (25).

**Preparation of Anti-Lyb-2.** Anti-Lyb-2 was the generous gift of Doctors E. A. Boyse, J.-S. Tung, and F. W. Shen, from the Sloan-Kettering Institute. The serum was raised in C3H.I mice against I/St ascites tumor I.29 (H-2<sup>d</sup>). As shown elsewhere, the serum recognizes a molecule on B cells (22) which has an apparent mol wt of 44,000.<sup>2</sup>

**Complement Receptor-Bearing Lymphocytes.** Complement receptor-bearing lymphocytes (CRL) were assayed according to published procedure (26) with the following modifications. Sensitization of SRBCs was conducted in 0.01 M EDTA-GVB (gelatin veronal buffer). Rosettes were formed by incubation of 2.5% erythrocyte antibody-complement complexes in 0.04 M EDTA-GVB with an equal volume of washed cells in PBS ( $3-6 \times 10^6$  cells/ml).

**Assay of Chromium Release and [<sup>3</sup>H]Leucine Incorporation.** Chromium release from papain-treated or control cells was assayed as described by Wigzell (27). Cr<sup>51</sup> was obtained from Amersham/Searle Corp. Arlington Heights, Ill. Protein synthesis was analyzed by incubation of papain-treated and control cells for 24 h in complete medium containing [<sup>3</sup>H]leucine (Amersham/Searle). After incubation, cells were harvested, lysed, and precipitated with RAMIg and GARIg. Protein associated radioactivity was determined by precipitating a small sample of the lysate in 5% TCA. Precipitates were washed, dissolved, and counted in a Beckman LS350 counter.

## Results

**Removal of Cell Surface Molecules by Papain.** Prior studies have established that treatment of murine splenocytes with an appropriate concentration of papain results in cleavage of cell surface IgD with little or no removal of cell surface IgM (18). In the following experiments, we have evaluated the effect of

<sup>2</sup> Tung, J.-S., E. S. Vitetta, and E. A. Boyse. Manuscript in preparation.

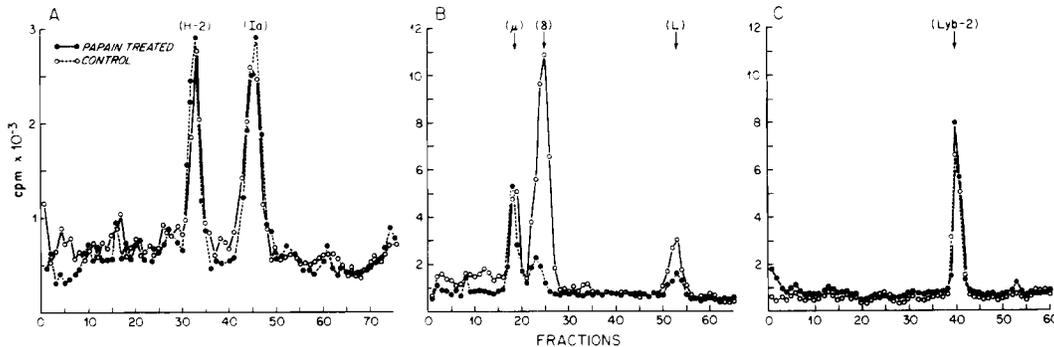


FIG. 2. SDS-polyacrylamide electropherograms of cell membrane-associated H-2 (A), Ia (A), IgM (B), IgD (B), and Lyb-2 (C) from normal-(-) and papain-(---) treated splenocytes.

such a concentration of papain on several other cell surface markers to determine if IgD is unusual in its susceptibility to cleavage by papain.

As shown in Fig. 2 B, treatment of cells with 150  $\mu\text{g/ml}$  papain resulted in the removal of approximately 90% of surface IgD as assessed by the loss of the  $\delta$ -chain peak on SDS-gels after electrophoresis of the dissolved immunoprecipitate. Under the same conditions, IgM, H-2, Ia, and Lyb-2 were not cleaved (Figs. 2 A, B, and C). Table I summarizes the results of similar experiments which indicate that only IgD is cleaved by treatment of the cells with papain. In three experiments, the effect of papain on the complement receptor was studied. The mean percent CRLs among total splenic leukocytes in controls was  $15 \pm 5.2$  SD, while the mean in papain treated populations was  $11 \pm 3.7$ . The difference is not statistically significant.

Thus, among the six surface antigens studied (IgM, IgD, H-2, Ia, Lyb-2, and CR) only IgD was removed by papain under the conditions used in these experiments.

**Effect of Papain on Cell Viability and Biosynthesis.** Treatment of cells with papain could affect intracellular functions in addition to altering surface proteins. As shown in Table II, when cells treated with papain were labeled in vitro for 24 h with [ $^3\text{H}$ ]leucine, treated cells incorporated as much precursor into total protein and Ig as control cells. Moreover,  $\text{Cr}^{51}$  release from treated and control cells was similar, suggesting that papain treatment affected neither viability nor synthesis of protein.

**Effect of Papain on Immune Responsiveness.** The effect of treating cells with papain on their subsequent immune responsiveness was studied as a necessary prelude to evaluating such treatment on induction of tolerance. Thus, cells were exposed to SRBC or TNP-*Brucella* immediately after treatment with various combinations of the reagents used in the papain digestion procedure. When SRBC were the antigen, cultures were supplemented with SRBC-primed irradiated splenocytes. Cultures were incubated for four days and direct anti-TNP and anti-SRBC PFCs determined. No effect on antibody responsiveness to TNP-*Brucella* or SRBC was observed. It was therefore possible to determine the effect of papain treatment of B cells on induction of tolerance.

**Effect of Papain on Tolerance Susceptibility and the Relationship to Removal of Surface IgD.** Adult splenocytes were treated with 0-100  $\mu\text{g/ml}$  papain,

TABLE I  
Relative Susceptibility of Surface Molecules on Splenocytes to  
Removal by Papain\*

Surface molecule	Number of experiments	Percent loss after papain treatment	
		Average	Range
IgD	5	92.8	87.4-98.6
IgM	5	3.6	0.8-10.6
H-2	3	0	0
Ia	2	0	0
Lyb2	2	2.1	0-4.2

\* Removal was assessed by treating radioiodinated cells with papain and comparing gel peaks of immunoprecipitates from treated and control cells after SDS-PAGE.

TABLE II  
Effect of Papain on Cell Metabolism

Parameter measured	Papain, 150 $\mu$ g/ml	Control
Protein synthesis*, cpm	$1.7 \times 10^6$	$1.6 \times 10^6$
Immunoglobulin synthesis†	$3.6 \times 10^5$	$2.3 \times 10^5$
%Cr <sup>51</sup> release‡	45	44

\* Papain-treated and control cells were cultured for 24 h in complete medium containing [<sup>3</sup>H]leucine. After 24 h, cells were centrifuged, lysed, and precipitated in 5% TCA acid. Values represent counts incorporated per  $5 \times 10^7$  cells.

† See footnote\*. Equal aliquots of the lysates were treated with RAMIg and an excess of GARIG. Precipitates were washed, dissolved, and electrophoresed on SDS-PAGE. cpm in H-chain peaks were determined. Values represent counts from H-chain peaks of  $5 \times 10^7$  cells.

‡  $\frac{\text{cpm in medium}}{\text{cpm in cell pellet} + \text{cpm in medium}} \times 100$

washed, and either radioiodinated or cultured for 24 h with varying doses of tolerogen (TNP<sub>17</sub>HgG). After cultivation, cells were washed and further incubated with TNP on either a TD carrier (SRBC) or a TI carrier (*Brucella*). In the former case, SRBC-primed irradiated splenocytes were added as a source of helper T cells. As increasing amounts of  $\delta$  were cleaved by papain (Fig. 3, top panel), tolerance susceptibility of cells responsive to TNP on the TD carrier increased proportionately (Fig. 3, lower left panel). Susceptibility to tolerance of cells responsive to TNP on the TI carrier remained at a constant high level regardless of the amount of IgD which had been cleaved (Fig. 3, lower right panel).

Fig. 4 summarizes five experiments. As can be seen, there appears to be a direct relationship between the percentage of intact IgD remaining on the cells and the amount of tolerogen required to suppress the response to TNP-SRBC (TD) antigen by 50% ( $T_{50}$ ) when the data are plotted on a log-log scale. As described above, removal of IgD has no effect on susceptibility to induction of tolerance of TI antigen responsive cells.

These results suggest that acquisition of IgD confers resistance to induction of tolerance upon TD antigen-reactive cells but not TI antigen-reactive cells.

*Regeneration of IgD and Tolerance Susceptibility.* The possibility that pa-

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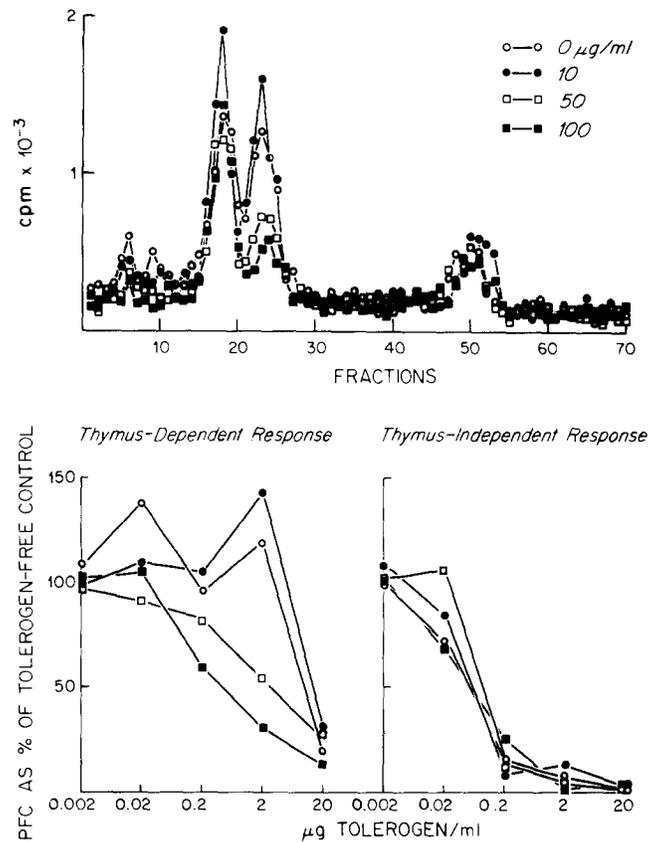


FIG. 3. SDS-polyacrylamide electropherogram (top panel) of cell membrane-associated immunoglobulins from murine splenocytes treated with varying concentrations of papain. Lower left panel: dose responses to tolerogen of papain-treated murine splenic B cells which are responsive to TD immunogen. Lower right panel: dose responses to tolerogen of papain-treated murine splenic B cells which are responsive to TI immunogen. Response to tolerogen-free controls (direct PFC/10<sup>6</sup> viable recovered cells) were as follows: Lower left; 0 papain 280, 10 μg papain 168, 50 μg papain 180, 100 μg papain 169. Lower right; 0 papain 695, 10 μg papain 690, 50 μg papain 557, 100 μg papain 654.

pain inactivated an IgD<sup>+</sup> cell population was considered. By using papain-treated splenocytes, the regeneration of IgD was evaluated as well as the loss of susceptibility to induction of tolerance. Adult spleen cells were treated with 100 μg papain and a portion of the cells radioiodinated. The remaining cells were exposed to tolerogen immediately, or incubated for 24 h without antigen. Cells incubated for 24 h without antigen were then either exposed to TNP<sub>17</sub> HgG to assess tolerance susceptibility or were radioiodinated, immunoprecipitated, and analyzed by SDS-PAGE to assess IgD regeneration. The results are shown in Fig. 5. Cells exposed to TNP<sub>17</sub>HgG immediately after treatment with papain (Fig. 5 A) were rendered susceptible to induction of tolerance as predicted by their loss of IgD (Fig. 5 C). Cells allowed to regenerate surface proteins for 24 h recovered approximately two-thirds of their IgD (Fig. 5 B) and virtually all of

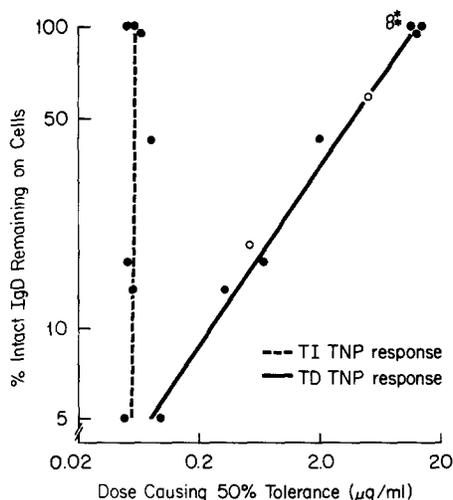


FIG. 4. Relationship of percent IgD remaining on murine splenocytes after papain treatment to the tolerogen dose required to suppress plaque responses 50% ( $[T_{50}]$  [●]). For TD response regression line, (—),  $r = 0.98$ . For TI regression line, (---),  $r = 0.66$ . Data points obtained from regeneration experiments by using cells before and after IgD regeneration (○) and controls for those points (○\*) were not used to construct regression line.

their resistance to induction of tolerance (Fig. 5 D). When the percentage of intact  $\delta$  was plotted against  $T_{50}$ , the two points fell near the regression line in Fig. 4, further supporting the concept of a relationship between cell surface IgD and susceptibility to tolerogen.

#### Discussion

The most striking observation to emerge from the present studies is that a cell surface molecule appears to regulate the susceptibility of B cells to tolerance induction. Thus, treatment of splenic B cells from adult mice with papain results in a marked increase in the susceptibility of those cells responsive to TD antigens to tolerance induction. Indeed, as a result of the proteolytic treatment, adult cells behaved like neonatal cells with regard to susceptibility to induction of tolerance in cells responsive to TD antigens. The simplest interpretation is that this alteration in adult cells results from cleavage of molecules from the cell surface. An alternative possibility, that the change in susceptibility to tolerance is not related to events on the cell surface, is regarded as unlikely since no evidence was obtained that treatment with papain under the defined conditions employed had significant effects on protein synthesis, cell viability, or immune responsiveness. Thus, treatment with papain did not affect incorporation by the cells of [ $^3\text{H}$ ]leucine into cellular protein or release of  $\text{Cr}^{51}$  from labeled cells. More pertinent, when cells were cultured for 4 days in the presence of antigen, both untreated and papain-treated cells responded similarly to both TNP-*Brucella* and TNP-SRBC. Taken together, the above results suggest that papain treatment has no effect on the subsequent antibody responsiveness of B cells, probably because cell surface components are regenerated *in vitro* rapidly

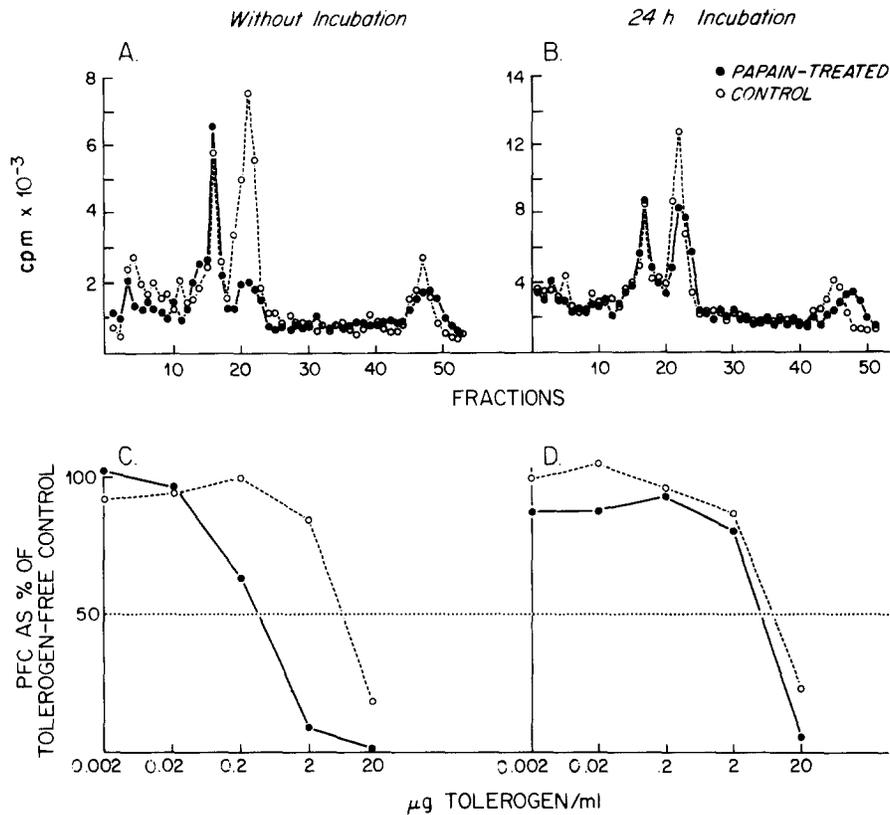


FIG. 5. Relationship between the regeneration of IgD on papain-treated cells and tolerance susceptibility. IgD cleavage and tolerance susceptibility were analyzed immediately after papain treatment (A, C) or after incubation for 24 h at 37°C to allow regeneration (B, D). Responses of tolerogen-free controls (direct PFC/10<sup>6</sup> viable recovered cells) were as follows: in C, control = 127, papain treated = 119; in D, control = 206, papain treated = 242.

enough to allow a normal response. Alternatively, those components removed by papain may be unnecessary for subsequent function.<sup>3</sup>

Treatment with papain under the conditions used in these experiments markedly affected only cell surface IgD and not five other known surface molecules. Thus, H-2, Ia, Lyb-2, and CR appeared unaffected by the treatment and IgM was reduced by a maximum of 10%. When the concentration of papain was varied, different proportions of cell surface IgD were cleaved from the cell. There was a striking correlation between the proportion of IgD cleaved and the ease of tolerance induction in the TD antigen-reactive cell pool. Indeed, if 90% of cell surface IgD was removed, then the dose of antigen that could tolerize to the 50% level was reduced 100-fold. Tolerance induction in TI responders was unaffected

<sup>3</sup> To clarify this point, the experimental protocol should be recalled (2). Since the tolerogen was added immediately after papain treatment, it is presumed that the assessment of the tolerance susceptibility of cells is occurring before the regeneration of molecules removed by papain. After 24 h, most surface molecules would be regenerated and no effect on the subsequent immune response to SRBC is detectable, as mentioned.

by papain treatment as predicted by the previous suggestion (12) that TI responders may bear no IgD.

As can be seen in Fig. 4, the relationship between the radiolabeled surface IgD cleavage and susceptibility to tolerance induction appears to be a complex one. Thus, B cells are a heterogeneous population with regard to their density of surface IgD, presence of other isotypes on the same cell, and affinity of receptors. Subpopulations may differ in their threshold requirements for tolerance induction. Finally, the radioiodination procedure may not give precise relative quantifications of IgD on different cell populations.

The possibility was considered that IgD-bearing cells were killed or permanently inactivated by treatment with papain even though the studies mentioned above of Cr<sup>51</sup> release and macromolecular synthesis argued against this possibility. Therefore, experiments were performed in which papain-treated cells were allowed to regenerate their receptors and the return of cell surface IgD and resistance to induction of tolerance were examined. Regeneration of 60–100% of normal amounts of surface IgD in 24 h suggested that cells previously denuded of IgD were alive and metabolically active. Restoration of the predicted degree of tolerance resistance (Figs. 4 and 5) provides additional correlative evidence that acquisition of IgD results in loss of susceptibility to the induction of tolerance.

These results suggest, but do not prove, that IgD is the surface molecule that determines, in large measure, susceptibility to induction of tolerance of B cells responsive to TD antigens. We cannot exclude the possibility that another unidentified molecule, which is removed (or altered) by papain and is subsequently regenerated in the same manner as IgD, is responsible for the changes in tolerance susceptibility. Moreover, since we were unable to selectively remove IgM from cells, while leaving IgD intact, it could be argued that the loss of either isotype might render cells more susceptible to the induction of tolerance. A more definitive test of the role of IgD might be to determine the effect of both anti- $\mu$  and anti- $\delta$  treatment of B cells on susceptibility to tolerance induction. These experiments are in progress.

### Summary

Under defined conditions, papain removes IgD from cells while leaving IgM, H-2, Ia, Lyb-2, and complement receptor intact. The effect of such treatment with papain on the induction of tolerance in murine splenic B cells was determined in an *in vitro* system. Treatment of the cells with papain has no effect on subsequent antibody responsiveness presumably because surface receptors regenerate before and during incubation with immunogen. Removal of increasing amounts of IgD results in increasing susceptibility of thymus-dependent responsive cells to tolerance induction. The tolerance susceptibility of thymus-independent responsive cells, which we have previously suggested are immature cells that bear only IgM, is unaffected by cleavage of IgD. If cells are incubated for 24 h after treatment with papain, cell surface IgD and tolerance resistance return. These results indicate that a surface molecule affects susceptibility of B cells to induction of tolerance and suggest that this molecule may be IgD.

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