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B Cell Responses to a Peptide Epitope. IX. The Kinetics of Antigen Binding Differentially Regulates Costimulatory Capacity of Activated B Cells

Lalitha Vijayakrishnan,1* Krishnamurthy Natarajan,1* Venkatasamy Manivel,* Sheikh Raisuddin,4 and Kanury V. S. Rao2*

We explore the possible mechanism by which association rates of Ag with activated B cells influences the ability of the latter to selectively recruit Th subsets. Our system used cocultures of Ag-activated B and T cells, where the Ag was a synthetic peptide, G41CT3. Restimulation was with either peptide G41CT3 or its analogue, G28CT3. Peptide G28CT3 has been previously shown to display a higher on rate, relative to the homologous peptide G41CT3, of binding to G41CT3-activated B cells. This difference in on rates was eventually exerted at the level of IFN-γ, but not of IL-10, induction from T cells, with peptide G28CT3 proving more effective. However, various treatment regimens rendered peptide G41CT3 as potent as peptide G28CT3 at eliciting IFN-γ responses from the above cultures. This included simultaneous treatment of B cells with peptide G41CT3 and the protein tyrosine kinase inhibitor tyrphostin. Alternatively, pretreatment of B cells with a peptide representing only the B cell epitope constituent of peptide G28CT3 (G28) was also equally effective. Subsequent experiments revealed that IFN-γ production from activated T cells resulted from an engagement of CD28 by B7-1 on the B cell surface. Finally, the extent of cell surface B7-1 up-regulation on activated B cells was dependent on the on rate of Ag binding to the membrane-bound Ig receptor. Thus, cumulative results suggest that the kinetics of Ag binding to activated B cells can differentially regulate intracellular signaling. This influences selective costimulatory molecule expression, with its consequent effects on relative Th subset activation. The Journal of Immunology, 2000, 164: S605–S614.

In contrast to naive, resting B cells, it is now accepted that Ag-activated B cells are potent APCs (1–5). This difference has generally been attributed to the presence of costimulatory molecules on activated B cells, which are absent on naive B cells (1–6). Activation of B cells is initiated by the B cell Ag receptor (BCR),3 a multimeric receptor complex of membrane-bound Ig (sIg) noncovalently associated with heterodimers of IgA and IgB (7). Engagement of sIg by Ag is translated into intracellular signals by the cytoplasmic domains of IgA and IgB molecules (8–10). A tyrosine phosphorylation cascade, with subsequent recruitment of second messengers downstream (8–11), rapidly follows ligation of BCR. Although the intervening pathways remain to be worked out, evidence suggests that at least one culmination point for processes initiated by BCR activation is in the up-regulation of cell surface-costimulatory molecules (12, 13). These latter processes are particularly relevant for T-dependent responses, because they reveal the etiological basis by which activated B cells acquire the potential to function as APCs. Thus, in T-dependent responses, engagement of sIg by Ag not only results in its internalization, processing, and presentation of a peptide-MHC class II complex on the cell surface (14–17) but also delivers the necessary signals for up-regulation of costimulatory molecules, most notably B7-2 (12). Signals delivered by costimulatory molecules are critical in ensuring full activation of T cells in the course of a cognate B-T interaction (18, 19).

Another facet that is now becoming apparent is that a sIg-Ag engagement does not always lead to a productive outcome. Depending on the circumstances and the status of the B cell, BCR ligation may result in stimulation, anergy, or even apoptosis (9, 20). These observations have led to the inference that the nature of the ligand-receptor interaction, among other factors, can potentially direct the physical recruitment of signaling pathways differently inside the lymphocyte and thereby define the nature of the subsequent immune response (9).

Despite the proven capacity of activated B cells to serve as APCs, their role in influencing CD4 T cell responses, particularly with respect to Th1 vs Th2 subsets, is less clear. Although in principle activated B cells have been shown to be capable of interacting with both subsets (21–23), a variety of studies have suggested a preference for activating Th2 cells (24–26). However, accumulating evidence now reveals that triggering of CD4 effector subsets is not strictly APC restricted. Rather the dominating influence of a variety of additional factors is being increasingly noted. One such factor appears to be the density of ligand presented, in the context of MHC class II, on APC. Murray et al. (27) have observed that whereas a high ligand density promoted Th1 differentiation, low ligand densities biased toward Th2 responses. In addition to density (27, 28), the affinity (or off rates) of peptide-MHC and peptide-TCR interactions has been identified as another regulatory parameter (29–31). Perhaps not unrelated to these two factors are the observations that the duration of TCR stimulation also has an important bearing on the outcome (32, 33). Another important regulator of T cell responses is the relative expression of

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2 The contributions of L.V. and K.N. should be considered equal.

3 Abbreviations used in this paper: BCR, B cell Ag receptor; sIg, membrane-bound surface Ig; PTK, protein tyrosine kinase; TNP, trinitrophenyl.

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the various costimulatory molecules on APC surface (30). Of particular note here are the B7 molecules, B7-1 and B7-2, which appear to impose selectivity, although in as yet unknown ways, in terms of CD4 T cell subset generation and stimulation (13, 34–38). Finally, the local cytokine milieu is also known to influence the outcome of a T cell response (39). Collectively, these findings indicate that both generation and regulation of CD4 effector subsets are more complex processes than hitherto suspected. The nuances are only now beginning to be revealed.

The above observations of extraneous influences on APC-mediated T cell responses also appear to extend to B cell APCs. For instance, display of high ligand density on activated B cells has been shown to preferentially stimulate Th1-like immunity (40). More recently, Pasare et al. (41) have demonstrated that increased, targeted delivery of Ag to activated B cells generates a Th1 bias in the immune response. Thus, in light of these findings and given the unique property vis-à-vis other professional APCs of Ag specificity, it can be anticipated that variations in Ag-binding properties of activated B cells may also impose a regulatory effect on subsequently elicited T cell responses.

Our recent studies have identified yet another variable that regulates both quantitative and qualitative aspects of T cells stimulation, in the course of a cognate interaction with activated B cells (42, 43). In these experiments, two single amino acid-substituted analogues of a model peptide Ag PS1CT3, were used, namely, peptides G41CT3 and G28CT3 (42, 43). All three peptides were shown to constitute T-dependent Ags, yielding primary and secondary IgG responses directed exclusively against a common tetrapeptide sequence between positions 4 and 7 (sequence, DPAF) (42). In other words, the substitutions performed in peptides G41CT3 (at position 10) and G28CT3 (at position 1) did not affect the fine specificity of B cells induced in the IgG response (42). Interestingly, however, peptide G28CT3 proved markedly more immunogenic than peptide G41CT3 (42). This difference in immunogenicity resulted from the greater ability of peptide G28CT3 to induce a Th cell recall response by peptide-primed B cells (42). Subsequent investigations revealed that the superior ability of peptide G28CT3 to elicit a B cell-mediated Th recall response was solely attributed to its increased on rate of binding, relative to peptide G41CT3, to B cells primed against the DPAF epitope (42). More recently, we observed that peptides G28CT3 and G41CT3 also differentially influence the relative extents of Th1 vs Th2 activation by peptide-primed B cells (43). Thus, a coculture of peptide G41CT3-primed B cells and peptide CT3-primed T cells yielded increased levels of the Th1-type cytokines in the presence of peptide G28CT3, relative to that when peptide G41CT3 was included as challenge Ag (43). These differences, again, could be solely ascribed to the increased on rate of binding of peptide G28CT3 (through the DPAF epitope) to the sIg receptor on G41CT3-primed B cells (43). Further, consistent with an on-rate-driven effect, the ratio of IFN-γ to IL-10 induced was dependent on the concentration of peptide G28CT3 used, with an IL-10 dominance at low peptide concentrations (43). Although various possible explanations for these findings were offered, experimental evidence in favor of any of these was lacking (43).

In this report, we explore the possible mechanistic basis for the variable influence of BCR-Ag association kinetics on CD4 T cell subset stimulation. Evidence presented here suggests that the kinetics of Ag binding to activated B cells can differentially regulate intracellular signaling pathways. This in turn influences selective costimulatory molecule expression on the B cell surface, with its consequent effects on relative Th subset activation.

Materials and Methods

Materials

Heavy chain-specific, HRP-labeled secondary Abs, the protein kinase inhibitors tyrphostin 51, genistein, and calphostin were all purchased from Sigma (St. Louis, MO). Rat mAbs against the mouse molecules CD80 (clone 1G10), CD86 (clone GL1), and CD28 (clone 37.51) and isotype controls were obtained from PharMingen (San Diego, CA). ELISA kits for the measurement of cytokines were purchased from Genzyme (Cambridge, MA), and magnetic beads for the purification of B and T cells were purchased from Dynal (Osd, Norway). Peptides used in this study were synthesized, purified, and characterized as described earlier (42, 43).

Animals and immunizations

Female BALB/c mice (6–8 wk of age) were obtained from the small animal breeding facility at the National Institute of Nutrition (Hyderabad, India). Immunizations were generally given i.p. with a dose of 50 μg/mouse in CFA (except where indicated otherwise). For polyclonal sera, mice were bled from the retroorbital plexus at indicated times.

Enrichment of B and T cell populations

This was achieved essentially as described before (43, 44). Briefly, B cells, mice were immunized with peptide G41CT3 with a 50-μg/mouse dose of peptide G41CT3 in CFA given i.p. Three weeks later, the spleens were removed and depleted of RBCs by lysis with ammonium chloride, and adherent cells were then depleted by panning on plastic plates at 37°C for 1 h. Nonadherent cells were collected and diluted to a cell concentration of 5 × 10⁶ cells/ml. From this, T cells were depleted by two rounds of incubation with Dynabeads anti-mouse Thy-1.2 (4 × 10⁶ beads/ml, Dynal) as recommended by the manufacturer. The resultant enriched B cells were then treated with mitomycin C at a final concentration of 50 μg/ml at 37°C for 20 min. Cells were washed thoroughly in culture medium before use.

For enriched T cells, mice were immunized with peptide CT3 and boosted 7 days later. At 3 days after the boost, inguinal lymph node cells were collected and first depleted of RBCs and adherent cells as described above. Nonadherent cells were collected and diluted to a cell concentration of 5 × 10⁶ cells/ml. B cells were depleted from this population by two rounds of incubation with Dynabeads mouse anti-B220 (4 × 10⁶ beads/ml, Dynal) following the recommended protocol of the manufacturer. As previously described, the above protocol yields a cell purity of 85–90% for both B and T cells, as assessed by flow cytometry.

Treatment of B cells with protein kinase inhibitors

Enriched B cells from G41CT3-primed mice were incubated at 5 × 10⁶ cells/well in wells of a 24-well culture plate in the presence of 37 μM final concentrations of peptide G41CT3, G28CT3, or CT3. These concentrations have been optimized earlier (43, 44). In addition, the protein kinase inhibitors genistein, tyrphostin, or calphostin were also included at the indicated final concentrations. The total volume of each culture was 3 ml, and except where indicated, incubation was for 16 or 37°C in a humidified atmosphere of 5% CO₂. The culture medium was RPMI 1640 containing, in addition to antibiotics, 10% FCS and 50 μM 2-ME. Subsequently, cells were washed extensively in HBSS to free them of residual peptides and inhibitors. Aliquots of 2.5 × 10⁶ B cells were mixed with an equal number of enriched T cells from CT3-primed mice, in a total volume of 200 μl culture medium (see above), and plated in wells of a 96-well culture plate. After 48 h, a time point standardized earlier (43), supernatants were collected for the estimation of cytokines by ELISA.

Pulsing of G41CT3-primed B cells with peptide G28

Enriched B cells (5 × 10⁶ from G41CT3-primed mice were pulsed with a final 37 μM concentration of peptide G28, either in the presence or the absence of 0.3 μM tyrphostin, for 16 h at 37°C in a humidified atmosphere of 5% CO₂. At the end of this period, cells were washed in HBSS. Subsequent coculture with CT3-primed T cells and cytokine estimation in culture supernatants was as described above.

Coculture of primed B and T cells in the presence of anti-CD28

Enriched B cells from G41CT3-primed mice (2.5 × 10⁶ cells/well) were cocultured with enriched T cells from CT3-primed mice (2.5 × 10⁶ cells/well) in multiple wells of a 96-well culture plate. The total volume of each culture was 200 μl. Increasing concentrations of either peptide G41CT3 or G28CT3 were also included, along with a final concentration of 2 μg/ml anti-CD28 mAb (clone 37.51, PharMingen). Control wells included cells incubated with either peptide only or peptide with nonspecific rat IgG at 2 μg/ml.
Enriched B cells from G41CT3-primed mice were pulsed as described above, harvested, and washed in HBSS. Aliquots of 5 × 10^6 cells each were then incubated with either anti-B7-1 or anti-B7-2 mAbs in a total volume of 2 ml culture medium. Final concentrations of anti-B-7 mAbs used were 10 μg/ml to ensure saturation, and incubation as for 4 h at 4°C. At the end of this period, cells were washed and cultured with CT3 primed T cells and a final concentration of 37 μg/ml peptide G41CT3, followed by cytokine estimation as described above.

**Estimation of cytokines**

A sandwich ELISA protocol using commercially available kits (Genzyme) measured all cytokines. The recommended protocol of the manufacturer was strictly followed in all cases. Quantitation was against a standard curve obtained for individual cytokine standards provided by the manufacturer. Background values obtained in cultures where no Ag was added was subtracted in all cases.

**Inhibition of BCR internalization**

Naive B cells were enriched from splenocytes of nonimmune BALB/c mice as described above. These were diluted to a concentration of 5 × 10^6 cells/ml and chilled on ice, and 1-ml aliquots were incubated with a final concentration of 10 μg/ml FITC-labeled anti-mouse IgG (Fab fragment). Incubation was at 4°C for overnight and in either the presence or absence of B7-7 mAbs. Cells were then washed with ice-cold RPMI containing, where necessary, the indicated concentration of the relevant inhibitor. Subsequently, the cells were warmed to room temperature, and aliquots were monitored for residual surface-bound fluorescence at regular intervals of 5 min, for a total period of 30 min.

**Fab anti-mouse IgG, its derivatization, and measurement of binding properties**

Monovalent Fab was generated by papain digestion of goat anti-mouse IgG (Sigma), followed by elution over a protein G-Sepharose column to remove Fc fragments. Completeness of digestion was ascertained by PAGE under nonreducing conditions. An aliquot of the resulting Fab fragment, at a final concentration of 2 mg/ml, was incubated with an 80-fold molar excess of typhostin or 60 μg/ml of genistein. Cells were then washed with ice-cold RPMI containing, where necessary, the indicated concentration of the relevant inhibitor. Subsequently, the cells were warmed to room temperature, and aliquots were monitored for residual surface-bound fluorescence at regular intervals of 5 min, for a total period of 30 min.

**Anti-Ig stimulation of splenic B cells and FACS analysis**

Splenic B cells were enriched from splenocytes of nonimmune BALB/c mice as described above. These were diluted to a concentration of 5 × 10^6 cells/ml and chilled on ice, and 1-ml aliquots were incubated with a final concentration of 10 μg/ml FITC-labeled anti-mouse IgG (Fab fragment). Incubation was at 4°C for overnight and in either the presence or absence of a final concentration of 0.3 μM typhostin or 60 μg/ml of genistein. Cells were then washed with ice-cold RPMI containing, where necessary, the indicated concentration of the relevant inhibitor. Subsequently, the cells were warmed to room temperature, and aliquots were monitored for residual surface-bound fluorescence at regular intervals of 5 min, for a total period of 30 min.

**Results**

Protein kinase inhibitors differentially modulate cytokine production from peptide-stimulated B-T cocultures

Both peptide analogues used in these studies, G28CT3 and G41CT3 (Fig. 1), have been previously described (42, 43). They represent single-amino acid-substituted analogues of a parent peptide PS1CT3 in which position 1 contains a histidine residue and position 10 contains an aspartic acid residue (42). As described earlier (42), peptide PS1CT3 represents a hybrid peptide containing a hepatitis B virus-derived B cell epitope (residues 1–15, segment PS1) and a promiscuous T cell epitope (residues 18–38, segment CT3) from an Ag of the malaria parasite *Plasmodium falciparum*. Separating the B and T cell epitopes is a spacer of two glycine residues at positions 16 and 17 (Fig. 1). As already discussed, these two peptides differ in their on rates of binding to G41CT3-primed B cells, leading to altered Th subset recruitment profiles from CT3-primed T cells (42, 43).

Our intent was to explore the underlying mechanisms by which BCR-Ag association rates can influence Th subset recruitment (43). For this, we first examined the effect of protein kinase inhibitors on peptide-stimulated recall responses from cocultures of G41CT3-primed B cells and CT3-primed T cells. It may be recalled here that peptide CT3 represents the common T epitope segment (residues 18–38) of the Ags described in Fig. 1. We have shown earlier that peptide CT3 elicits comparable recall responses from T cells primed with either G28CT3 or G41CT3 (42). For the present study, three protein kinase inhibitors were selected, tyrphostin, genistein, and calphostin. Whereas tyrphostin and genistein are inhibitors of protein tyrosine kinases (45, 46), calphostin is inhibitory to a hepatitis B virus-derived B cell epitope (residues 1–15, segment PS1) and a promiscuous T cell epitope (residues 18–38, segment CT3) from an Ag of the malaria parasite *Plasmodium falciparum*. Separating the B and T cell epitopes is a spacer of two glycine residues at positions 16 and 17 (Fig. 1). As already discussed, these two peptides differ in their on rates of binding to G41CT3-primed B cells, leading to altered Th subset recruitment profiles from CT3-primed T cells (42, 43).
enhancing G41CT3-dependent IFN-γ production is mediated through BCR activation.

We have already proved earlier that the differences in on rates of binding of peptides G28CT3 and G41CT3 to G41CT3-primed B cells are a direct reflection of the rapidity with which the DPAF epitope in these analogues can accommodate within the paratope of the sIg receptor (43). These differences could conceivably translate into altered levels of Ag uptake, eventually leading to differing densities of ligand presented in association with MHC class II. Ligand density differences are known to variably modulate T cell responses (27, 28, 40). Alternatively, as has recently been shown for T-independent responses (49), Ag-binding characteristics may variably influence BCR-mediated intracellular signaling, resulting in different outcomes.

To distinguish between the above possibilities, we devised a pulse-chase strategy where G41CT3-primed B cells were first pulsed with a peptide representing only the B cell epitope segment (residues 1–15, peptide G28) of peptide G28CT3. After this, the B cells were cocultured with CT3-primed T cells in the presence of peptide G41CT3. It was rationalized that a G28 pulse would facilitate BCR triggering, but without leading to any Ag presentation. Indeed, we have earlier confirmed that peptide G28 is devoid of a T cell epitope and that its inclusion as a challenge Ag in coculture of G41CT3-primed B cells and CT3-primed T cells does not lead to either T cell proliferation (42) or cytokine induction (43). Thus, the protocol outlined above was expected to permit a resolution between the relative contributions of BCR triggering alone and Ag presentation to the effect under study.

In initial experiments, G41CT3-primed B cells were incubated with peptide G28, in either the presence or the absence of tyrophostin, for a period of 16 h. Cells were then washed and cultured with CT3-primed T cells alone and Ag presentation to the effect under study.

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In initial experiments, G41CT3-primed B cells were incubated with peptide G28, in either the presence or the absence of tyrophostin, for a period of 16 h. Cells were then washed and cultured with CT3-primed T cells alone and Ag presentation to the effect under study.
Indeed, the levels of IFN-γ stimulation from cocultures of these B cells with CT3-primed T cells. G28CT3, in the absence of any pretreatment, are also shown for comparison obtained from a parallel set of cells in which the challenge Ag was peptide CT3-primed T cells in the presence of G41CT3 as the recall Ag. Results obtained from a parallel set of cells in which the challenge Ag was peptide G28CT3, in the absence of any pretreatment, are also shown for comparison. Results depicted are from one of four experiments.

with no further effect on IFN-γ levels in cultures containing G28-pulsed B cells (Fig. 4). A pulse with peptide G28 alone, in the absence of peptide G41CT3 challenge, did not yield any detectable levels of cytokines (Fig. 4). Similarly, peptide G41CT3 challenge in the absence of a G28 pulse yielded the expected low levels of IFN-γ. Levels of IL-10, however, remained unaffected either on G28 pulsing or tyrphostin treatment of the B cells (Fig. 4). Thus, the results in Fig. 4 indicate that a pretreatment of G41CT3-primed B cells with peptide G28 markedly augments the ability of peptide G41CT3 to subsequently stimulate IFN-γ, but not IL-10, production from cocultures of these B cells with CT3-primed T cells. Indeed, the levels of IFN-γ obtained here were comparable with those elicited by the higher binding rate peptide G28CT3 (Fig. 4). Thus, the differences exerted due to differences in Ag binding kinetics, at least in terms of IFN-γ elicitation from primed T cells, appear to originate from effects on BCR triggering and not on Ag presentation.

Anti-CD28 Abs augment IFN-γ production from peptide-stimulated cultures

A productive interaction between Ag-primed B and T cells requires two signals. The first is provided by specific peptide-MHC class II ligand cognately engaged by the TCR, and the second is the range of noncognate costimulatory signals provided by the APC to the T cell during cognate interactions (18, 19). Both the quantity and the quality of costimulatory signals provided play a significant role in determining the outcome of T cell stimulation, as well as biases in favor of either Th1 or Th2 cytokines (30). One such set of costimulatory interactions that regulate both T cell stimulation and cytokine production is that between the B7 family of proteins on APC and CD28/CTLA-4 on T cells (13, 34). Whereas B7-CD28 interactions have been shown to be stimulatory, engagement of CTLA-4 is thought to down-regulate T cell activation (50, 51).

To explore whether the effects of Ag-B cell association rates influence at the level of a cognate B-T interaction, the effect of addition of anti-CD28 mAb was examined on cocultures stimulated with varying doses of both analogue peptides. Levels of IFN-γ and IL-10 thus obtained in supernatants are shown in Fig. 5. An interesting effect of anti-CD28 on peptide G28CT3-stimulated IFN-γ induction is evident (Fig. 5A). Although maximal levels attained remained unaffected, the efficiency of peptide G28CT3 nevertheless increased significantly in the presence of anti-CD28. Six-fold lower concentrations of peptide G28CT3 were sufficient to achieve half-maximal responses, relative to that when anti-CD28 was absent (Fig. 5A). The effect of anti-CD28 addition was markedly more profound, in terms of IFN-γ induction, on G41CT3-stimulated cultures, where both qualitative and quantitative shifts were seen (Fig. 5A). Indeed, in the presence of anti-CD28, the IFN-γ induction dose response profile was identical with that observed for anti-CD28-supplemented, G28CT3-stimulated cultures (Fig. 5A). Thus, in the presence of anti-CD28, peptides G28CT3 and G41CT3 do not display any differences in their ability to induce IFN-γ secretion from cocultures of G41CT3-primed B cells and CT3-primed T cells. This strongly suggests that, at least at one level, differences in BCR-Ag binding rates exert their influence at the level of T cell costimulation.

In contrast to effects on IFN-γ, no qualitative differences in IL-10 responses could be observed in anti-CD28-supplemented cultures stimulated with either peptide G41CT3 or peptide G28CT3 (Fig. 5B). In both cases, addition of anti-CD28 resulted in a modest but comparable enhancement in IL-10 levels and with no significant effect on the dose-response profile (Fig. 5B).

B7-1 (CD80) and B7-2 (CD86) differentially regulate cytokine production from CT3-primed T cells

As discussed above, the data in Fig. 5 implicate differential engagement of CD28 in cultures stimulated with either G28CT3 or G41CT3. The counterreceptor for CD28 can be either B7-1 or B7-2 (34), and it has been suggested earlier that whereas B7-1 promotes Th1 responses, B7-2 directs Th2 responses (35). Further, Agrewala et al. (52) have recently shown that, in a coculture of activated B and T cells, anti-B7-1 selectively inhibited production of the Th1-type cytokines but not those from Th2 cells. Thus, it was likely that the observed differences in the profile of cytokines

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In contrast to effects on IFN-γ, no qualitative differences in IL-10 responses could be observed in anti-CD28-supplemented cultures stimulated with either peptide G41CT3 or peptide G28CT3 (Fig. 5B). In both cases, addition of anti-CD28 resulted in a modest but comparable enhancement in IL-10 levels and with no significant effect on the dose-response profile (Fig. 5B).

FIGURE 4. Prepulsing of B cells with peptide G28 selectively stimulates IFN-γ responses. G41CT3-primed B cells were pretreated with peptide G28 in the presence or absence of tyrphostin (Tyr) as described in Materials and Methods. After pretreatment, these cells were cultured with CT3-primed T cells in the presence of varying doses of both analogue peptides. Levels of IFN-γ and IL-10 thus obtained in supernatants are shown in Fig. 5. An interesting effect of anti-CD28 on peptide G28CT3-stimulated IFN-γ induction is evident (Fig. 5A). Although maximal levels attained remained unaffected, the efficiency of peptide G28CT3 nevertheless increased significantly in the presence of anti-CD28. Six-fold lower concentrations of peptide G28CT3 were sufficient to achieve half-maximal responses, relative to that when anti-CD28 was absent (Fig. 5A). The effect of anti-CD28 addition was markedly more profound, in terms of IFN-γ induction, on G41CT3-stimulated cultures, where both qualitative and quantitative shifts were seen (Fig. 5A). Indeed, in the presence of anti-CD28, the IFN-γ induction dose response profile was identical with that observed for anti-CD28-supplemented, G28CT3-stimulated cultures (Fig. 5A). Thus, in the presence of anti-CD28, peptides G28CT3 and G41CT3 do not display any differences in their ability to induce IFN-γ secretion from cocultures of G41CT3-primed B cells and CT3-primed T cells. This strongly suggests that, at least at one level, differences in BCR-Ag binding rates exert their influence at the level of T cell costimulation.

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B7-1 (CD80) and B7-2 (CD86) differentially regulate cytokine production from CT3-primed T cells

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FIGURE 5. Anti-CD28 modulates cytokine production from B-T cocultures. Enriched B cells from G41CT3-primed mice were cocultured with CT3-primed T cells in the presence of varying concentrations of either peptide G28CT3 (○, ●) or peptide G41CT3 (△, ▲). Each of the peptide stimulated groups also consisted of replicates sets where anti-CD28 was either included (●, ▲) or excluded (○, △). At the end of the culture period, supernatants were collected and assayed for the levels of IFN-γ (A) or IL-10 (B). Results from one of three experiments are shown, and individual values are presented.
induced in response to the two analogue peptides may represent a consequence of differential involvement of the two B7 proteins during costimulation.

To confirm whether the two B7 molecules do, in fact, differentially regulate cytokine production in our system, we used the earlier described protocol of prepulsing the B cells with peptide G28. G41CT3-primed B cells were pulsed with peptide G28, following which they were treated with anti-B7-1, anti-B7-2, or nonspecific (NS) rat IgG before their inclusion in culture with CT3-primed T cells (Materials and Methods). Levels of IFN-γ and IL-10 that resulted in the culture supernatants are shown as mean (±SE) of at least triplicate wells. Data represent one of three independent experiments.

Differential BCR triggering thresholds for up-regulation of cell surface B7-1 and B7-2

Our findings that peptide-dependent B cell-mediated differences in cytokine induction from CT3-primed cells may reflect differential engagement of CD28 by the B7 molecules prompted us to examine the effect of Ag-binding rates on relative B7-1 and B7-2 expression on B cells. Unfortunately, however, the extremely low frequency of Ag-specific B cells precluded a direct analysis of the effects of G28CT3 vs G41CT3 binding. To circumvent this problem, we adopted an alternate system wherein splenic B cells were triggered with the Fab fragment of anti-mouse IgG. The utility of this system was greatly facilitated by our finding that TNP derivatization of the Fab led to a near 4-fold reduction in association rates in association binding to mouse IgG, but with no concomitant effect on dissociation rates of the resultant complexes (Fig. 7).

Splenocytes enriched for slgG+ B cells were incubated with varying concentrations of either Fab anti-mouse IgG, or its TNP derivative along with a constant concentration of culture supernatant from activated T cells (Materials and Methods). Subsequently, the levels of induced surface B7-1 and B7-2 were analyzed by flow cytometry (Materials and Methods). As shown in Fig. 8, both Fab and its TNP derivative were equally proficient at up-regulating B7-2 in a dose-dependent manner (Fig. 8, A and B). However, B7-1 induction was severely attenuated in the presence of TNP-derivatized Fab, relative to that obtained in the presence of the unmodified molecule (Fig. 8, C and D). Because the only distinction in the two Ab preparations resides in their nonidentical association rates with mouse IgG, we infer that differences in the extent of B7-1 up-regulation seen in Fig. 8, C and D, is a direct consequence of this.
We also examined the effect of tyrphostin on B cells stimulated with TNP-derivatized Fab (Materials and Methods). As shown in Fig. 9A, no potentiating effect of tyrphostin treatment on B7-2 expression was detected. In contrast, treatment with tyrphostin resulted in a further, although modest, increase in B7-1 levels.

Extent of cell surface B7-1 expression correlates with ability to elicit IFN-γ responses from T cells

The ability to differentially modulate B7-1 levels on B cells with chemically derivatized Fab anti-IgG also facilitated a direct examination of the role of B7-1 in induction of IFN-γ responses from T cells. For this, enriched splenic B cells were incubated with varying concentrations of either Fab anti-mouse IgG, or its TNP derivative and subsequently cocultured with allogeneic T cells derived from C57BL/6 mice. Production of IL-10 and IFN-γ in culture supernatants was then determined; the results are presented in Fig. 10. As is clear, dose-dependent induction of IL-10 was equally sensitive to both analogues (Fig. 10A). However, lacking a T cell epitope, it was unable to induce either proliferation (42) or cytokine responses (43) from CT3-primed T cells. In other words, peptide G28 binds G41CT3 for binding to G41CT3-primed B cells (42, 43). However, lacking a T cell epitope, it was unable to induce either proliferation (42) or cytokine responses (43) from CT3-primed T cells. Therefore, peptide G28 binds G41CT3-primed B cells, but without leading to Ag presentation. Thus, the positive effect of a G28 pulse of G41CT3-primed T cells in potentiating IFN-γ production from CT3-primed T cells clearly implies a regulatory role for BCR triggering as opposed to effects resulting from any alterations in Ag presentation. Additional evidence supporting the absence of a role for Ag presentation could also be gleaned from experiments with tyrphostin-treated B cells. Although tyrphostin inhibited BCR internalization and, consequently, Ag uptake it nevertheless promoted IFN-γ responses from peptide G41CT3-stimulated cocultures. A notable effect of pre-pulsing B cells with G28 was that it eliminated the subsequent on-rate-dependent differences in IFN-γ induction due to G41CT3 and G28CT3 challenge, rendering the former analogue as potent as the latter. These cumulative results, therefore, potentially localize the origin of these on-rate-dependent differences to derive from altered BCR activation.

An immediate consequence of BCR ligation by Ag is the initiation of the intracellular signal transduction machinery mediated, at least in part, by cellular PTKs (8–10). Although some of the intermediates involved have now been identified (8–11), the biochemical pathways that eventually confer the “activated” phenotype to these B cells remains to be elucidated. Our initial observations that tyrphostin potentiates IFN-γ induction from G41CT3-stimulated cultures, whereas genistein inhibits, was somewhat puzzling. Both of these compounds are known inhibitors of PTKs, and also block BCR internalization (48). However, some resolution of this paradox is afforded by our more recent results revealing profoundly influence, both quantitatively (42) and qualitatively (43), the outcome of a cognate B-T interaction. Our findings that a pulse of G41CT3-primed B cells with peptide G28 rendered them more sensitive to peptide G41CT3-stimulated induction of IFN-γ responses from CT3-primed T cells proved particularly revealing. We have shown earlier that peptide G28, but not its analogue of scrambled sequence, competed effectively with both peptides G28CT3 and G41CT3 for binding to G41CT3-primed B cells (42, 43). However, lacking a T cell epitope, it was unable to induce either proliferation (42) or cytokine responses (43) from CT3-primed T cells. In other words, peptide G28 binds G41CT3-primed B cells, but without leading to Ag presentation. Thus, the positive effect of a G28 pulse of G41CT3-primed T cells in potentiating IFN-γ production from CT3-primed T cells clearly implies a regulatory role for BCR triggering as opposed to effects resulting from any alterations in Ag presentation. Additional evidence supporting the absence of a role for Ag presentation could also be gleaned from experiments with tyrphostin-treated B cells. Although tyrphostin inhibited BCR internalization and, consequently, Ag uptake it nevertheless promoted IFN-γ responses from peptide G41CT3-stimulated cocultures. A notable effect of pre-pulsing B cells with G28 was that it eliminated the subsequent on-rate-dependent differences in IFN-γ induction due to G41CT3 and G28CT3 challenge, rendering the former analogue as potent as the latter. These cumulative results, therefore, potentially localize the origin of these on-rate-dependent differences to derive from altered BCR activation.

**Discussion**

In the present report, we examined the basis of our earlier findings (42, 43) that the kinetics of Ag recognition by activated B cells can

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**FIGURE 9.** Tyrphostin treatment enhances B7-1 but not B7-2 levels in TNP-Fab stimulated B cells. For experimental details, refer to Materials and Methods. Histograms depict staining for B7-2 (A) and B7-1 (B) in B220<sup>+</sup> gated cells stimulated with TNP-derivatized Fab in the presence (thin line) or absence (dashed line) of tyrphostin. The thick line represents staining of cells treated with tyrphostin, but in the absence of TNP-Fab. The latter was superimposable with B7 staining obtained for a parallel culture of cells that were not treated with either tyrphostin or TNP-Fab. Although modest, the tyrphostin-mediated enhancement in B7-1 levels in B cells was, nevertheless, significant. A Kolmogorov-Smirnov statistical analysis (Cell Quest, Becton Dickinson Immunocytometry Systems) yielded a value for D of 0.25–0.30 in three separate experiments. In contrast, the mean value for the tyrphostin-untreated groups between the three experiments was 0.10 ± 0.03.

**FIGURE 10.** Fab anti-mouse IgG and its TNP derivative differentially induce IFN-γ production from cocultures of allogeneic B and T cells. Enriched sIgG<sup>+</sup> splenic B cells from BALB/c mice were activated with indicated doses of Fab anti-mouse IgG (C), TNP-Fab (D), or a combination of TNP-Fab and 0.3 μM tyrphostin (E) for 16 h as described in Materials and Methods. Subsequently, cells were washed, and aliquots of 2.5 × 10<sup>5</sup> B cells were mixed with an equal number of enriched T cells obtained from C57BL/6 mice, in a total volume of 200 μl culture medium (Materials and Methods). Production of IL-10 (A) and IFN-γ (B) was monitored in culture supernatants collected 48 h later. Results shown are from one of two separate experiments, and values are mean ± SE of triplicate determinations. Cocultures where untreated B cells were used gave values for IL-10 and IFN-γ that were <120 pg/ml.
both genestein and tyrphostin, that of phospholipase C

K. V. S. Rao, unpublished results). Thus, the differences in results
hibited only by genistein, but not by tyrphostin (K. Natarajan and

Materials and Methods

in study, Kouskoff et al. have noted that the correlation between re-
made above, that differences in the kinetics of Ag association by B

this study, Kouskoff et al. have noted that the correlation between re-
consequence, whereas inhibition by genistein implicates the involve-
ment of BCR-stimulated PTKs, results with tyrphostin suggest that
selective modulation of PTKs recruited on BCR activation may
fluence the outcome.

Also significant were our findings that, in similarity with G28
pulsing, tyrphostin treatment of G41CT3-primed B cells also ren-
dered peptide G41CT3 as competent as peptide G28CT3 at elic-
ting IFN-γ responses from B-T cocultures. Further, there was no
evidence for either synergistic, additive, or competitive influences
when both treatments were simultaneously included. This implies,
but does not confirm, that these two treatments probably act on a
common biochemical pathway. These collective results may,
therefore, be interpreted to suggest that the peptide on rate-medi-
ad differences, which are initially exerted on relative BCR acti-
activation, translate into differences at the level of intracellular signals
that are generated, at least with respect to PTK involvement.

That the BCR is in fact susceptible to Ag-regulated differential
signaling has been recently demonstrated, albeit for T-independent
responses (49). Using a panel of M13-phage-displayed peptide li-
gands with varying affinities for B cells bearing a transgenic sIg
receptor, Kouskoff et al. (49) reported that BCR-mediated activa-
tion, as judged by a variety of parameters, is variably influenced by
the affinity of Ag for BCR. Indeed some, but not all, of the early
BCR-dependent signaling such as Ca++ mobilization and tyrosine
phosphorylation of Syk and IgA, but not Lyn, were highly depen-
dent on affinity of BCR for peptide (49). These latter results are
particularly significant in the present context. They reveal that
the spectrum of PTKs recruited on BCR activation is dependent on its
Ag-binding properties. This lends credence to our own argument,
made above, that differences in the kinetics of Ag association by B
cells may variably modulate intracellular signaling. Indeed, in their
study, Kouskoff et al. have noted that the correlation between re-
sponse and affinity of BCR for Ag was not perfect (49). They have
consequently suggested that, rather than equilibrium affinity, the
kinetics of BCR-ligand interaction may constitute the principal
determinant of the outcome of activation responses (49).

Activation of T cells requires two signals to be provided by an
APC. Whereas one derives from the peptide-MHC class II complex,
a range of accessory cell surface molecules, also known as
costimulatory molecules, provides the second. Of the latter, the
most prominent of the currently identified interactions, particularly
with respect to cytokine choices, is that between the B7 molecules
on APC and CD28 on T cells (13, 34, 38). Although subject to
debate (34, 38), it has been suggested that B7-1 directs Th1 re-
sponses whereas B7-2 promotes a bias in favor of Th2 responses
(35). In the context of B-T interactions, Agrewala et al. (52) have
recently demonstrated that production of IFN-γ by T cells was
dependent on the presence of B7-1 on B cells. As shown, the
present system examined here was also subject to a similar cate-
gorization. Thus, whereas anti-B7-2-blocked IL-10 production
from peptide-stimulated cocultures of G41CT3-activated B cells
and CT3-primed T cells, anti-B7-1 selectively abrogated IFN-γ
responses. Interestingly, results from such experiments also sug-
gested that differences in on rates of peptide G28CT3 and G41CT3
association with G41CT3-activated B cells are eventually exerted
at the level of costimulation resulting from B7-CD28 interactions.
Two lines of evidence supported such an inference. First was the
fact that inclusion of anti-CD28 in the cocultures rendered the
efficiency of peptide G41CT3 comparable with that of peptide
G28CT3 with respect to elicitation of T-dependent IFN-γ. Parity
was established not only in terms of peak IFN-γ levels induced but
also at the level of the respective dose-response profiles. This in-
fluence of anti-CD28 in establishing equipotency between the two
peptides implies that differences in peptide-B cell association rates
eventually reflect at the level of CD28 activation on T cells. Indi-
rect substantiation for such an inference was also afforded by our
findings that the potentiatory effect of G28, vis-a-vis G41CT3-
ducible IFN-γ secretion, could be neutralized by treatment of B
cells with anti-B7-1. Thus, collectively, these results strongly sug-
gested the possibility that differences in BCR-ligand association
rates may eventually regulate at the level of relative involvement
of B7-1 vs B7-2 in CD28 engagement, leading to altered T cell-
elicited cytokine profiles. In this connection, it has been shown that
binding of a T-dependent Ag to activated B cells eventually leads

FIGURE 11. Fab anti-mouse IgG and its TNP derivative do not differ in their ability to enhance MHC class II expression on activated B cells. Enriched
sIgG B cells were cultured with varying doses of either Fab anti-mouse IgG, TNP-Fab, or a combination of TNP-Fab and 0.3 μM tyrphostin as described
in Materials and Methods for a period of 48 h. Under these conditions, resting sIgD B cells yield a 5- to 10-fold enhancement in surface I-A levels on
activation with anti-IgM. Following this, cells were washed and surface levels of I-A were determined by flow cytometry. Although the figure shows data
obtained for treatment with 20 μg/ml Ab, essentially similar results were obtained over a dose range of 5–40 μg/ml. Histograms are for a gated B220+ popu-
lation of cells. In both panels, the dashed line represents anti-I-A staining in unstimulated cells, whereas the thick line represents that for cells treated
with either Fab (A) or TNP-Fab (B). In addition, the thin line in B depicts staining for cells treated with TNP-Fab plus tyrphostin. Data are from one of two
independent experiments.
to up-regulation, albeit with different kinetics, of the two B7 molecules on the cell surface (12, 13). Thus, our deduction that binding properties of Ag with BCR could varyably influence at the level of costimulation was not altogether an improbable one.

Although the low frequency of Ag-specific B cells in splenocytes precluded a direct verification of our inference above we could, nevertheless, demonstrate “proof of principle” by comparing between Fab anti-IgG and its TNP derivative as surrogate Ags for sIgG+ splenic B cells. Whereas the native Fab was fully competent at inducing enhanced cell surface expression of both B7-1 and B7-2, its TNP-derivatized analogue proved significantly less effective at inducing B7-1 although B7-2 levels remained unchanged. In light of the fact that these two Ab preparations principally differ in their rates of association with IgG, these results confirm the existence of differential BCR-triggering thresholds for induction of B7-1 and B7-2, with the former being more sensitive to BCR-Ag association rates. Although modest, the positive effect of tyrphostin treatment on B7-1 levels in B-cells activated with TNP-derivatized Fab also supports our earlier inference of on-rate-dependent differences in engagement of intracellular PTKs. Finally, this system also permitted a direct verification of our proposal that BCR-Ag association rates directly regulate expression of B7-1 on B cells which, in turn, impacts on Th subset recruitment.

The intracellular biochemical pathways, which mediate between BCR activation and B7 expression are currently unknown and await delineation. Also of interest would be a resolution of how Ag-binding rates modulate BCR-initiated signaling. Here, one plausible explanation derives from the “strength of signal” hypothesis proposed for T cell activation (13). At high rates of association, it can be anticipated that, at any given time, a large proportion of BCRs would be ligand occupied. This would result in simultaneous triggering of multiple BCRs, generating a high intensity intracellular signal. In contrast, low rates would also imply low BCR occupancy rates, leading to the generation of a low intensity signal. Again in similarity with the proposal for T cells (13), the intensity of intracellular signal produced may dictate different outcomes, particularly in the present context, with respect to cytokine responses and migrating properties of auto-antigen specific to cells. J. Exp. Med. 182:335.


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