THE JOURNAL OF IMMUNOLOGY

B Cell Responses to a Peptide Epitope. VI. The Kinetics of Antigen Recognition Modulates B Cell-Mediated Recruitment of T Helper Subsets

Lalitha Vijayakrishnan, Venkatasamy Manivel and Kanury V. S. Rao

J Immunol 1998;161;4661-4670

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

B Cell Responses to a Peptide Epitope. VI. The Kinetics of Antigen Recognition Modulates B Cell-Mediated Recruitment of T Helper Subsets

Lalitha Vijayakrishnan, Venkatasamy Manivel, and Kanury V. S. Rao[1]

The ability of Ag-primed B cells to recruit distinct Th subsets was examined using two analogous synthetic peptides, G41CT3 and G28CT3, as model Ags. With sequence differences at only two positions, these peptides were identical both with respect to fine specificity of Abs induced and ability to prime T cells. Lymph node cell populations primed with peptide G41CT3, when challenged with the homologous Ag, yielded predominantly Th2 cytokines. In contrast, a challenge with the heterologous Ag, G28CT3, resulted in a markedly increased production of Th1 cytokines. These distinctions derived from altered APC function of Ag-primed B cells due to differential kinetics of recognition of the two Ags by surface Ig receptors, as confirmed by binding studies with a panel of anti-G41CT3 mAbs. A concentration-dependent circular dichroism study revealed differences in the nature of intermolecular associations for these two peptides. Furthermore, the on-rate of peptide G28CT3 binding to Ab also increased with increasing peptide concentration, implying a dependence on intermolecular interactions. This, in turn, correlated well with the ability of peptide G28CT3 to preferentially activate either Th1 or Th2 cells. Thus, the relative proportion of Th1 vs Th2 cells recruited by Ag-primed B cells is governed by the on-rate of Ag binding to surface Ig receptors, with higher on-rates promoting Th1 recruitment. Further, even subtle changes in solution behavior of an Ag can markedly influence the kinetics of recognition by B cells. ***The Journal of Immunology*****, 1998, 161: 4661–4670.**

A cognate interaction between Ag-primed B and T cells is critical for the sustenance and progression of a T-dependent humoral response (1–3). Ags that bind specifically to surface Ig (sIg)[2] receptors on B cells are endocytosed and processed, and appropriate fragments are subsequently presented in the context of MHC class II molecules for recognition by the TCR on Ag-activated T cells (4). A consequence of this physical interaction is the proliferation of both partners and, in addition, differentiation of B cells into Ab-secreting plasmacytes and memory B cells (1–4).

Earlier studies describing the minimal requirements of ligand density on the APC surface (5, 6), and the more recent reports identifying minimal threshold levels of TCR triggering (7, 8) as a prerequisite for T cell activation collectively suggest that the APC activity of B cells can be modulated by its ability to bind Ag. Thus, concentration of free Ag, efficiency of Ag capture, and the efficiency with which the Ag is processed are likely to play important roles in defining the ability of Ag-activated B cells to present Ag (4). Our own results have revealed that the APC function of a B cell, with its consequent effects on Th recruitment capacity, is regulated by a complexity of parameters that include not only affinity of sIg receptor for epitope on Ag (9), but also the kinetics of this interaction (10) and competitive processes between sIg receptor and secreted Ig for Ag (11). Indeed, it is the collective outcome of these processes that eventually determines the hierarchy of immunodominance observed against B cell epitopes displayed on multideterminant Ags (9–13).

The discrimination of effector CD4 T cells into the Th1 and Th2 subsets (14, 15) offers an Ag-presenting B cell the option of selectivity in terms of Th recruitment. While, in principle, such B cells are known to be capable of interacting with both subsets (16–18), recent data suggest a preference for activating Th2 cells (19–21). However, the studies of Murray et al. (22) demonstrating a key role for ligand density on an APC for determining the relative extents of Th1 vs Th2 differentiation and activation add yet another level of intricacy to these issues. This is particularly true given our earlier findings that the efficiency of an Ag-activated B cell as APC, also a consequence of ligand density, is a variable that is under the influence of a variety of factors (9–11). Thus, it is likely that parameters that regulate Ag recognition by B cells can also influence the relative extent of Th cells recruited by it.

In this report, we have attempted to address these issues using two model synthetic peptide immunogens, peptides G28CT3 and G41CT3. Both peptides shared an identical amino acid sequence except for variations in two positions. We have earlier shown that these peptides prime a common pool of Th cells equally well and that they induce B cells of identical fine specificities, although peptide G28CT3 is more immunogenic (10). Enhanced immunogenicity of peptide G28CT3 could be ascribed to the higher on-rate of binding to specific B cells relative to peptide G41CT3, which in turn ensured a more efficient B cell-mediated recruitment of T cell help (10). Thus, peptides G41CT3 and G28CT3 appeared to represent useful models for examining the influence of Ag-binding parameters on selective engagement of Th subsets by activated B cells. We show here that on-rates of Ag binding to B cells can regulate the relative proportion of Th1 vs Th2 cells that are recruited by Ag-specific B cells, with a bias toward increased Th1 recruitment at higher on-rates. Further, subtle changes in Ag sequence, which do not induce any obvious structural changes, can

Immunology Group, International Centre for Genetic Engineering and Biotechnology, New Delhi, India

Received for publication March 4, 1998. Accepted for publication June 29, 1998.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Kanury V. S. Rao, Immunology Group, International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India.

[2] Abbreviations used in this paper: sIg, surface Ig; LNCs, lymph node cells; CD, circular dichroism; BCR, B cell Ag receptor; TFE, trifluoroethanol.

0022-1767/98/$02.00

nevertheless dramatically influence the kinetics of Ag recognition by B cells.

Materials and Methods

Materials

Heavy chain-specific horseradish peroxidase-labeled secondary Abs, goat anti-mouse IgG1, and goat anti-mouse IgG2a (both heavy chain specific) were purchased from Sigma Chemical (St. Louis, MO). Magnetic beads for panning of T and B cells were obtained from Dynal (Oslo, Norway). ELISA kits for measurement of cytokines were purchased from Genzyme (Cambridge, MA) and F-moc-amino acid derivatives for peptide synthesis from Novabiochem (Laufelfingen, Switzerland).

Peptide synthesis

Peptides were synthesized by the solid phase method (23, 24) on a Millipore 9050 automated peptide synthesizer (Millipore, Bedford, MA) using the F-moc chemistry (25). Crude peptides were purified to at least 95% purity by reverse phase HPLC on a C-18 column (15 μ, δ Pak, 19 $\times$ 300 mm; Waters, Milford, MA) using an aqueous gradient of 0 to 70% acetonitrile in 0.1% trifluoroacetic acid. Identities of all peptides were ascertained by amino acid analysis.

Animals and immunizations

Female BALB/c mice (6–8 wk old) 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 as an emulsion in CFA (except where otherwise indicated). For polyclonal sera, mice were bled from the retro-orbital plexus at the indicated times.

Generation of anti-G41CT3 mAbs

Female BALB/c mice immunized with a single dose (50 μg/mouse) of peptide G41CT3 in CFA were then subsequently boosted i.v. with 50 μg each of soluble peptide in PBS. Three days later, the highest responder from the group was taken for the generation of IgG-secreting hybridomas. Polyethylene glycol-mediated fusion to hypoxanthine-aminopterin-thymidine-sensitive myeloma derivative SP2/O-Ag 14, maintenance of derived lines, and limiting dilution cloning were essentially as described earlier (26). The secretion of Ab in culture supernatants was screened by ELISA against peptide-coated wells, and only wells showing relatively higher Ab levels (ELISA readings of $>$1.0) were selected for subcloning and subsequent analysis. All of the five IgG mAbs thus obtained were found to be specific for the DPAF segment (residues 4–7) on peptide G41CT3, as determined by mapping against an overlapping G41-derived peptide panel using a protocol that has been previously described (10).

Enzyme-linked immunosorbent assays

Plates were coated with 2 μg of peptide/well in 100 μl of PBS (pH 7.2) at 37°C for 3.5 h. Subsequently, they were blocked with 300 μl/well of a 5% solution of fat-free dry milk powder in PBS at 37°C for 1 h. Then, 100 μl of the appropriate dilution of mouse antiserum was added and incubated at 37°C for 1 h. After washing, bound Ab was detected with horseradish peroxidase-labeled secondary Ab (37°C, 1 h) followed by color development with *o*-phenylenediamine as chromogen. Absorbance was measured at 490 nm.

For competitive ELISA experiments, antisera were used at dilutions representing 50% of titer value. Twofold higher concentrations of antiserum and competitor peptide were mixed in equal volumes and incubated for 10 min at room temperature. This was then added to duplicate wells at 100 μl/well. The remaining procedure was as described above.

Enrichment of B cell populations

For enrichment of T cells, lymph node cells (LNCs) from either immune or nonimmune mice were first depleted of RBCs by lysis with ammonium chloride, and subsequently adherent cells were removed by panning on plastic plates at 37°C for 1 h. Nonadherent cells were collected and diluted to a cell concentration of 5×10^7 cells/ml. B cells were depleted from this population by two rounds of panning with Dynabeads mouse anti-B220 (4×10^8 beads/ml; Dynal) following the recommended protocol of the manufacturer.

To obtain enriched B cells, mice immunized with peptide G41CT3 were boosted 3 wk later with a 50 μg/mouse dose of G41CT3 in PBS given i.v. Three days later, the spleen was removed and depleted of RBCs, following which adherent cells were removed by panning as described above. Nonadherent cells were diluted to 5×10^7 cells/ml, and T cells were depleted by two rounds of panning with Dynabeads anti-mouse Thy1.2 (4×10^8 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. When unprimed B cells were required, an identical protocol was followed except that the splenocytes were taken from nonimmune mice. The purity of B cells obtained by such a protocol was found to be between 85 and 90% upon analysis by flow cytometry.

PEPTIDE	SEQUENCE
PS1CT3	HQLDPAFGANSTNPDGGDIEKKIAKMEKASSVFNVVNS
G28CT3	**G**QLDPAFGANSTNPDGGDIEKKIAKMEKASSVFNVVNS
G41CT3	HQLDPAFGANSTN**G**DGGDIEKKIAKMEKASSVFNVVNS

FIGURE 1. The amino acid sequence of peptides G41CT3 and G28CT3. Both of these peptides represent single amino acid, glycine-substituted variants of the parent peptide PS1CT3, for which the sequence is also included for comparison. Peptide G28CT3 is an analogue in which the amino-terminal histidine residue in PS1CT3 has been substituted with glycine. In peptide G41CT3, the proline residue at position 14 has been substituted with glycine. The single-letter code for amino acids is used to describe the sequences, and the glycine substitutions relative to peptide PS1CT3 are indicated in bold letters. A two-glycine residue spacer between the B and T cell epitopes that has been used in these peptides (10) is underlined.

Lymphocyte cultures and cytokine estimations

BALB/c mice were immunized s.c. at the tail base with 50 μg/mouse of peptide G41CT3 as an emulsion in CFA. Seven days later, the mice were sacrificed and inguinal lymph nodes removed. LNCs, at 5×10^5 cells/well, were cultured in duplicate in 200 μl of RPMI 1640 containing 10% FCS, gentamicin (2 mg/L), and 2-ME (0.05 mM) in wells of a 96-well plate. In addition, the indicated peptide at a final concentration of 30 μM (previously identified as optimal; Ref.10) was also included as challenge Ag. Cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide for 48 h, at which point supernatants were removed for quantitation of cytokines. Preliminary experiments identified this time point as optimal for the determination of all relevant cytokines. In experiments employing enriched B and T cells, cocultures were set up with 2×10^5 cells/well of each culture.

The cytokines IL-2, IL-4, and IFN-γ were measured in a sandwich ELISA protocol using commercially available kits (Duoset, Genzyme), as was also IL-10 (Intertest-10x, Genzyme). The recommended protocol of the manufacturer was followed in all cases, and quantitation was against a standard curve obtained for individual cytokine standards provided by the manufacturer, after subtracting background in culture wells where no Ag was added.

Determination of on-rates

Equal volumes of the appropriate anti-G41CT3 mAb and the relevant peptide were mixed at room temperature, and time-dependent Ab binding in terms of quenching of tryptophan fluorescence was continuously monitored over a 100-min period in a Shimadzu RF-1501 spectrofluorometer. The excitation wavelength used was either 280 or 290 nm, depending on the Ab, and emission was measured at 330 nm. The final Ab concentration employed was 200 nm (except where indicated), whereas the peptide concentration employed was 20 μM (except where indicated). The extent of fluorescence quenching was used to determine unbound Ab concentrations as a function of time; values for k_{on} (association rate constant) were calculated as described earlier (10). Values for k_{on} reported are the mean ($\pm$SD) of three independent determinations.

Results

Peptides G41CT3 and G28CT3

Both peptides G41CT3 and G28CT3 have been described earlier (10), and their sequences are given in Figure 1. They represent single amino acid-substituted analogues of a model peptide, PS1CT3, for which the sequence has also been included in Figure 1 for the sake of comparison. The first 15 residues of peptide PS1CT3 (segment PS1) represent a known B cell epitope derived

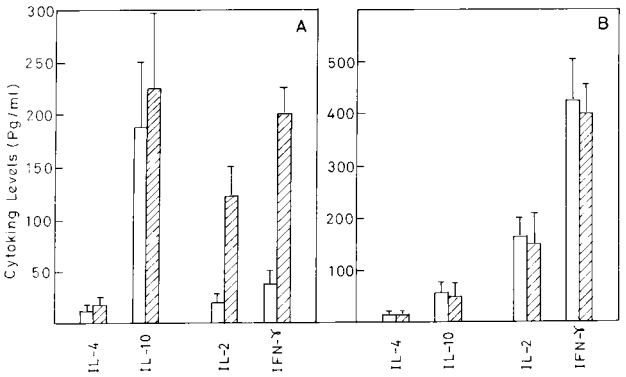

FIGURE 2. Peptides G28CT3 and G41CT3 induce qualitatively different T cell recall responses from G41CT3- but not CT3-primed LNCs. Either G41CT3 (*A*)- or CT3 (*B*)-primed LNCs were cultured in the presence of an optimal concentration (*Materials and Methods*) of either peptide G41CT3 (open bars) or peptide G28CT3 (stippled bars). Subsequently, production of indicated cytokines in the culture supernatants was measured as described in *Materials and Methods.* Values presented are the mean (±SD) levels obtained from three independent experiments after subtracting the basal values obtained from culture supernatants to which no peptide was added.

from the pre-S1 sequence of the hepatitis B surface Ag (27). The carboxyl-terminal 21-residue sequence (segment CT3) corresponds to the promiscuous Th cell epitope identified from the circumsporozoite protein of the malaria parasite, *Plasmodium falciparum* (28). Separating the B and T cell epitopes is a spacer of two glycine residues at positions 16 and 17 (Fig. 1). Peptide G41CT3 is an analogue of peptide PS1CT3 in which the proline residue at position 14 was substituted by glycine, whereas the amino-terminal histidine residue was substituted for glycine in peptide G28CT3 (Fig. 1). We have already shown earlier that, akin to peptide PS1CT3, both peptides G41CT3 and G28CT3 exist predominantly in a random structure in solution as indicated by their circular dichroism (CD) spectra (10).

Differential induction of cytokines by peptides G28CT3 and G41CT3 from G41CT3-primed LNCs

In earlier studies, we showed that, similar to peptide PS1CT3, both peptides G41CT3 and G28CT3 induce a T-dependent primary B cell response in BALB/c mice (10). In all three cases, the primary IgG response was directed, at least predominantly, against a tetrapeptide segment between positions four and seven (sequence DPAF, Fig. 1), indicating that the amino acid variations in peptides G41CT3 and G28CT3 do not affect the fine specificity of B cells induced in the primary IgG response (10). Interestingly, however, peptide G28CT3 was markedly more immunogenic than peptide G41CT3. This difference in immunogenicity could be ascribed to the greater ability of peptide G28CT3 to induce a Th cell recall response by peptide-primed B cells (10). For example, either total LNCs or enriched splenic B cells from peptide G41CT3-primed mice gave markedly elevated T cell proliferative responses with peptide G28CT3 as the challenge Ag relative to that when challenged with peptide G41CT3. Thus, it was of interest to determine whether peptides G41CT3 and G28CT3 also showed differences in relative activation of Ag-specific Th subsets.

Our initial experiments probed for the induction of cytokines diagnostic of Th1- and Th2-type responses in ex vivo cultures of LNCs from G41CT3-primed mice, when either peptide G41CT3 or peptide G28CT3 was added as the challenge Ag. As shown in Figure 2*A*, recall of G41CT3-primed LNCs with peptide G41CT3 yielded a cytokine spectrum that was heavily dominated by IL-10, indicative of a predominantly Th2-type response. In contrast, however, a challenge with peptide G28CT3 resulted in a five- to six-fold enhancement in both IL-2 and IFN-γ levels, whereas the Th2-type cytokines IL-4 and IL-10 were relatively unaffected (Fig. 2*A*). These results suggest that whereas peptide G41CT3 induces a dominant recruitment of Th2 effectors from G41CT3-primed LNCs, recruitment in the presence of peptide G28CT3 is comparably distributed between both Th1 and Th2 subsets from the same pool.

Table I. *Peptides G41CT3 and G28CT3 induce differential cytokine profiles from cocultures of G41CT3-primed B cells and CT3-primed T cells*[a]

Expt.	Recall Ag	Cytokine Levels (pg/ml)			
		IL-4	IL-10	IL-2	IFN-γ
1	G41CT3	<10	46	30	25
	G28CT3	<10	43	150	150
2	G41CT3	<10	161	45	63
	G28CT3	<10	160	95	307
3	G41CT3	<10	197	80	100
	G28CT3	<10	146	320	680

[a] B cells enriched from splenocytes of G41CT3-immunized mice were cocultured with enriched T cells derived from lymph nodes of mice primed with peptide CT3 in the presence of optimal concentrations of either peptide G41CT3 or peptide G28CT3, as described in *Materials and Methods.* The resulting cytokines from culture supernatants were measured by ELISA *(Materials and Methods)* and the mean of duplicate wells determined. Results of three separate experiments are shown.

In parallel experiments, we also examined peptide-modulated Th recall in LNCs primed with a synthetic peptide representing only the T cell epitope encoded within the peptides shown in Figure 1 (segment CT3, the carboxyl-terminal 21 residues). The results from these set of experiments are shown in Figure 2*B*. Contrary to the results obtained with peptide G41CT3-primed LNCs, both peptides G28CT3 and G41CT3 induced comparable levels of all cytokines tested from the CT3-primed LNC population (Fig. 2*B*). Since the principal difference in the two sets of experiments described in Figure 2, *A* and *B*, was either the inclusion or exclusion of the B cell epitope segment (segment G41) in the priming immunogen, we thought it possible that the results depicted in Figure 2*A* could represent a consequence of Ag-activated B cells functioning as APCs. Such an inference would also be consistent with our prior observations on lymphocyte proliferative responses in G41CT3-primed LNCs with these same peptides (10).

Differential cytokine induction by peptides G41CT3 and G28CT3 is due to Ag presentation by activated B cells

The experiments described above suggested that the observed differences in the cytokine patterns as a result of challenge with either peptide G41CT3 or G28CT3 of G41CT3-primed LNCs could be due to B cells functioning as APCs. We examined this possibility in a relatively more defined experiment in which purified CT3-primed T cells were cocultured with mitomycin C-treated enriched splenic B cells from mice primed with peptide G41CT3. These cultures were then challenged with optimal concentrations of either peptide G41CT3 or G28CT3, and the resulting cytokines produced measured. As shown in Table I, the results obtained here were entirely consistent with those shown in Figure 2*A* in that significantly higher levels of both IL-2 and IFN-γ were induced by G41CT3-primed B cells when G28CT3 was added as challenge Ag as opposed to when G41CT3 was employed. On the other hand, IL-4 and IL-10 levels were comparable with both of the challenge peptides (Table I). In separate experiments, we observed that pretreatment of G41CT3-primed B cells with chloroquine (300 μM,

Table II. *Unprimed B cells do not show peptide-mediated differences in Th recall*[a]

Expt.	Recall Ag	Cytokine Levels (pg/ml)			
		IL-4	IL-10	IL-2	IFN-γ
1	G41CT3	<10	15	150	273
	G28CT3	<10	15	250	354
2	G41CT3	<10	114	50	590
	G28CT3	<10	86	85	675
3	G41CT3	<10	31	60	80
	G28CT3	<10	40	85	120

[a] The experimental protocol employed here was identical to that described for Table I except that enriched B cells from splenocytes of unprimed BALB/c mice were used in this case.

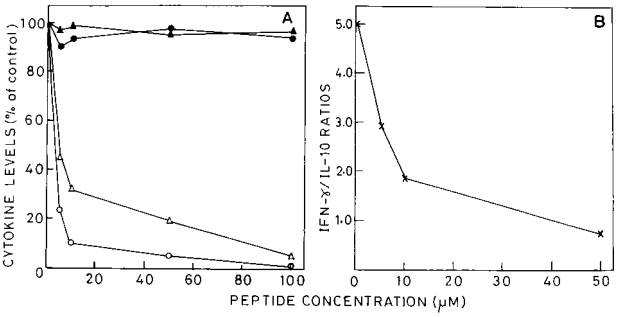

FIGURE 3. Peptide G28CT3-induced T cell recall responses from G41CT3-primed B cells is mediated by BCR-Ag interactions. Cocultures of enriched B cells from G41CT3-primed mice and enriched T cells from CT3-primed mice were challenged with peptide G28CT3 as described in Table I (*A*). In addition, indicated concentrations of either peptide G28 (open symbols) or a control peptide of scrambled G28 sequence (closed symbols; Ref. 10) were also added. Subsequently, supernatants were collected for the measurement of IFN-γ (○, ●) or IL-10 (△, ▲). *B*, A replot of the relevant data shown in *A* as IFN-γ/IL-10 ratios with increasing peptide G28 concentrations.

20 min at 37°) before inclusion in such cultures inhibited both IFN-γ and IL-10 production to barely detectable levels (<15 pg/ml). This was true regardless of whether peptide G28CT3 or G41CT3 was used as the challenge Ag (data not shown), implying that Ag processing and, consequently, its intracellular uptake were necessary for the differences observed in Table I. Essentially similar results were also obtained when G41CT3-primed B cells were first fixed with 0.1% paraformaldehyde before inclusion in the coculture (data not shown), further supporting the view that Ag uptake and processing are necessary. We also confirmed in these experiments that the cytokines measured were a product of the $CD4^+$ T cells in selective depletion studies. Thus, while depletion of $CD8^+$ T cells from CT3-primed T cells had no significant effect on the results shown in Table I, selective $CD4^+$ depletion virtually abrogated the response, reducing the levels of all indicated cytokines to those that were barely detectable (data not shown).

To determine whether Ag priming of B cells was necessary for the observed effects, we performed experiments in which unprimed splenic B cells, enriched from naive mice, were cocultured with T cells purified from CT3-primed LNCs before challenge with either peptide G41CT3 or G28CT3. As shown in Table II, no significant peptide-mediated differences in cytokine induction could be observed in such experiments.

Although the data in Tables I and II strongly implied an effect of Ag presentation mediated by B cells, this conclusion remained to be rigorously established, particularly considering that the B cell preparation employed in Table I was only between 85 and 90% pure. Consequently, the possibility of interference from contaminating cell populations could not be ignored. To ascertain this possibility, the enriched B cell preparation from Table I was further purified by two rounds of panning against anti-mouse IgG-coated plates, yielding a B cell preparation between 93 and 96% in purity with no detectable contamination from either macrophages or dendritic cells. However, the results obtained from this additionally purified B cell preparation were not significantly different from those shown in Table I (IFN-γ/IL-10 ratios between 5 and 7 in three experiments). This finding further supports the conclusion that the results in Table I were a consequence of Ag-activated B cells functioning as APCs.

The observation that differential cytokine induction by the two analogous peptides was dependent on the inclusion of Ag-primed, but not unprimed, B cells in the coculture implicated the involvement of B cell Ag receptor (BCR)-mediated uptake of Ag. To confirm this conjecture, peptide G28CT3 challenge of B-T cocultures was performed as described in Table I, except that varying concentrations of a peptide representing the B cell epitope segment of peptide G28CT3 (residues 1–15, segment G28) were also included. The latter peptide was added as a competitive inhibitor of peptide G28CT3 binding to specific BCR (10). Since peptide G28 lacked the T cell epitope, inhibition of peptide G28CT3 recognition by B cells was also expected to negatively influence T cell recall responses. As shown in Figure 3*A*, peptide G28, but not its scrambled analogue, induced a striking dose-dependent inhibition of both IFN-γ and IL-10 secretion to nearly basal levels at higher G28 concentrations. This clearly suggests that the induction of both IFN-γ and IL-10 seen in Table I represents the outcome of BCR-mediated uptake of peptide by Ag-specific B cells, followed by its presentation to CT3-specific T cells. Also notable was the observed effect of competitor peptide (G28) concentration on IFN-γ/IL-10 ratios as depicted in Figure 3*B*. The overwhelming dominance of the Th1 cytokine was found to diminish as peptide G28 concentration increased, implying a relationship between efficacy of Ag uptake and the relative extent of Th1 vs Th2 stimulation.

Collectively, therefore, the results shown in Tables I and II and Figure 3 provide evidence to suggest that the observed differential influence on Th subset stimulation by peptides G41CT3 and G28CT3 is mediated by activated Ag-specific B cells functioning as APCs and that these differences probably arise from differences at the level of BCR-Ag interactions.

Peptides G41CT3 and G28CT3 also induce qualitatively distinct anamnestic IgG responses from G41CT3-primed B cells

Differential engagement of the Th1 vs the Th2 subsets by activated B cells has been shown to alter the isotype profile of Abs secreted by such B cells. This is a result of differential effects of Th1- and Th2-type cytokines on B cells. Thus, while IFN-γ selectively induces secretion of the IgG2aisotype of Abs, IL-4 promotes the production of the IgG1 isotype (29, 30).

We have shown so far that the relative proportion of Th1 vis a vis Th2 cells recruited by G41CT3-primed B cells can be influenced by the challenge Ag. It was logical, therefore, to expect that such Ag-driven differences in Th subset recruitment by primed B cells should also, in turn, reflect the level of differences in the proportion of IgG1 vs IgG2a Abs secreted as the outcome of the B-T cognate interactions. To verify this, we immunized BALB/c mice with a single dose of peptide G41CT3, and the spleens were collected 3 wk later. Primed splenocytes were then adoptively transferred into fresh, irradiated recipients, which were then challenged with soluble forms of either peptide G41CT3 or G28CT3.

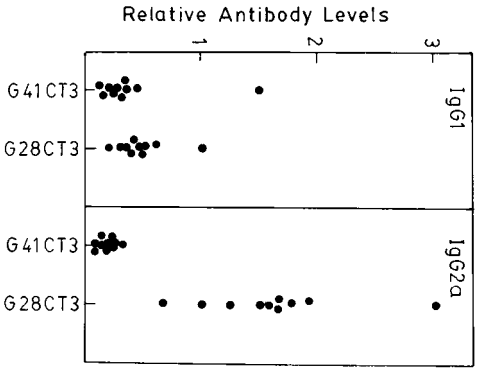

FIGURE 4. Increased production of the IgG2a isotype of Abs on challenge of G41CT3-primed B cells with peptide G28CT3. Day 21 splenocytes (5×10^7/mouse) from G41CT3-primed mice were injected i.v. in 0.5 ml of PBS into the tail vein of irradiated (550 rad) BALB/c recipients. At 16 h after transfer, the hosts were challenged i.v. with soluble forms of either peptide G41CT3 or G28CT3 (50 μg/mouse) in sterile PBS. An additional control Ag, V3 MNCT3, was also included; V3 MN represents a 15-amino acid residue sequence derived from the V3 loop of the MN isolate of HIV-1 (sequence RKRIHIGPGRAFYTT). Blood was collected 6 days after antigenic challenge, and relative levels of peptide-specific IgG1 and IgG2a isotype of Abs were determined by quantitative ELISA using total mouse IgG as standard. A parallel set in which naive splenocytes were transferred did not yield any IgG response on antigenic challenge, and neither did the group that was challenged with peptide V3 MNCT3, at least at the time point tested. Values shown are for individual mice from one of two independent experiments. Finally, as shown earlier (10), in these experiments the total anti-peptide IgG obtained was three- to fourfold higher with peptide G28CT3 than with peptide G41CT3 as challenge Ag.

Relative levels of anamnestic IgG1 and IgG2a titers were then measured from sera collected 6 days later; the results are summarized in Figure 4. While the IgG1 levels were relatively uninfluenced by challenge Ag, serum IgG2a levels were markedly enhanced in mice boosted with peptide G28CT3 relative to those boosted with peptide G41CT3 (Fig. 4). These results further confirm, at the functional level, our contention that G41CT3-primed B cells recruit a larger proportion of Ag-specific Th1 cells when recalled with peptide G28CT3 as opposed to a restimulation with peptide G41CT3. Further, this altered recruitment represents a productive B-T interaction, with its expected consequences on the secreted Ab isotype distribution.

Peptides G41CT3 and G28CT3 differ in their on-rates for binding to anti-G41CT3 mAbs

Results described thus far indicate that G41CT3-primed B cells recruit a larger proportion of Ag-specific Th1 cells in the presence of peptide G28CT3 as opposed to peptide G41CT3. This, however, was not true in the case of either unprimed B cells or CT3-primed LNCs. Furthermore, we have also previously shown (10) that peptides G41CT3 and G28CT3 do not differ significantly in terms of processability upon uptake by APCs. Collectively, these data suggested that peptide-dependent differences in specific Ag uptake by the primed B cell population may account for the observed differences in interactions with the Th subsets. It was therefore of interest to examine the binding of both peptide G41CT3 and G28CT3 to the sIg receptors on G41CT3-primed B cells. However, purification in sufficient quantities and culture of Ag-specific B cells becomes difficult, particularly in a primary response, given their low frequencies of occurrence. This is especially true of peptide G41CT3, which has been shown to be poorly immunogenic (10). Consequently, we decided to employ mAbs generated against peptide G41CT3 as surrogates for the sIg receptor on G41CT3-primed B cells. Admittedly, however, the known low frequency of fusion renders it unlikely that a panel of mAbs generated will be representative of the actual repertoire of G41CT3-primed B cells generated in vivo. Nevertheless, results obtained with such mAbs should prove helpful in identifying a trend, if any exists.

Table III. *Affinity and on-rates of peptide G41CT3 and peptide G28CT3 binding to anti-G41CT3 mAbs*[a]

	IC_{50} (μM) with Peptide:		k_{on} ($M^{-1}s^{-1}$) $\times 10^{-6}$ with Peptide:	
mAb	G41CT3	G28CT3	G41CT3	G28CT3
G41.4	84 ± 16	110 ± 22	0.7 ± 0.2	10.4 ± 1.2
G41.8	8 ± 3	18 ± 4	0.5 ± 0.1	8.6 ± 0.3
G41.14	80 ± 7	75 ± 10	2.2 ± 0.3	19.7 ± 0.5
G41.15	107 ± 12	109 ± 14	1.0 ± 0.1	3.0 ± 0.2
G41.16	4 ± 2	3 ± 1	0.5 ± 01	1.6 ± 0.2

[a] IC_{50} values (competitor concentration required to achieve 50% inhibition of Ab binding) of individual anti-G41CT3 mAb binding to either peptide was determined by competitive inhibition ELISA, whereas k_{on} (association rate constant) values were obtained from fluorescence quenching measurements *(Materials and Methods)*. For both cases, values presented are the mean (±SD) of at least three independent experiments.

mAbs of the IgG class were obtained from mice immunized with peptide G41CT3 after boosting with soluble Ag 28 days later. A total of five highly reactive (*Materials and Methods*) stabilized clones were obtained, all of which were found to be specific for the tetrapeptide sequence DPAF between positions four and seven, as identified by fine specificity mapping against overlapping hexapeptides, described earlier (data not shown; Ref. 10). The low number of hybridomas obtained against peptide G41CT3 is probably a consequence of its poor immunogenicity (10).

All five of these mAbs were evaluated for their relative affinities for the homologous peptide G41CT3 and the analogue, peptide G28CT3, by competitive inhibition ELISA. In addition, we also measured the on-rates of binding of either peptide to each of these mAbs in a fluorescence quenching assay under pseudo first order conditions, as described in *Materials and Methods*. The results from both these sets of experiments are summarized in Table III. All mAbs showed comparable affinities for both peptide G41CT3 and G28CT3, except mAb G41.8 in which the relative affinity for peptide G41CT3 was marginally higher (Table III). Interestingly, however, despite the largely comparable affinities, dramatic differences in on-rates for binding to the two peptides could be observed. In all cases, on-rates for mAb binding to the heterologous peptide G28CT3 was markedly higher than that for the homologous Ag G41CT3, with differences ranging from 3- to as much as 17-fold for mAb G41.8.

Thus, although all obtained anti-G41CT3 mAbs bind both peptides with comparable affinities, the kinetics of this interaction nevertheless differ, with peptide G28CT3 universally displaying a greater facility for recognition by the paratope of mAbs tested.

Peptides G41CT3 and G28CT3 do not differ in secondary structural characteristics

An attempt to rationalize the observed differences in the kinetics of recognition of the two peptides was made by examining the secondary structural properties by CD spectroscopy. Earlier, such studies had already revealed an equally random distribution of conformations in aqueous solution for both peptides (10). Nevertheless, we probed further for any subtle variations in either the secondary structure or propensities for it, for which purpose we employed the secondary structure-enhancing solvent, trifluoroethanol (TFE; Ref. 31). Either the whole immunogen molecule

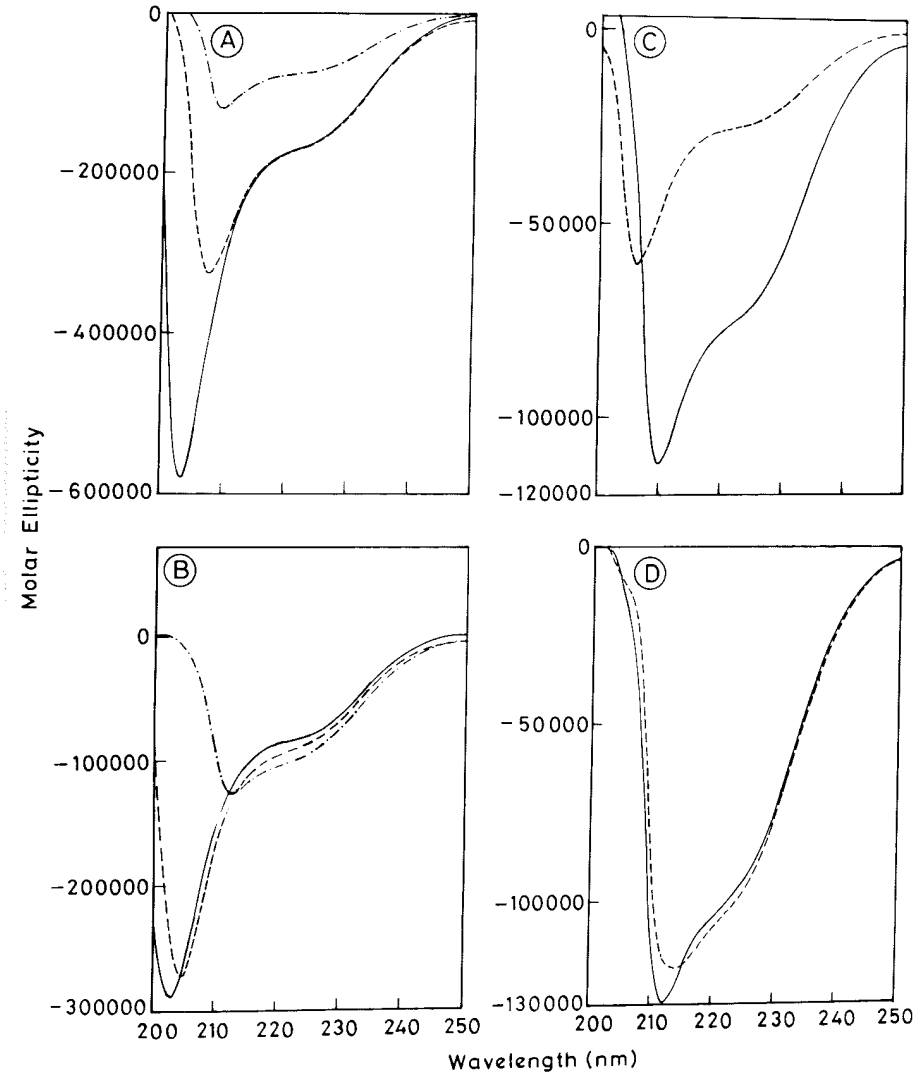

FIGURE 5. Concentration dependence of the CD spectra of peptides G41CT3 and G28CT3. Either peptide G41CT3 (*A*) or peptide G28CT3 (*B*) was dissolved in phosphate buffer (pH 7.2) at a final concentration of either 6 (—), 20 (- - -), or 60 (-·-) μM, and the CD spectra were obtained on a JASCO model J710 spectropolarometer (JASCO, Tokyo, Japan) over a wavelength range of 200 to 250 nm. A step resolution of 0.1 nm and a scan speed of 200 nm/min were employed. Spectra shown are those averaged over a total of 30 accumulations. Molar ellipticity units were calculated using the appropriate software provided by the manufacturer. In addition, spectra for peptides G41CT3 (*C*) and G28CT3 (*D*) were also recorded at 60-μM peptide concentrations either in the absence (—) or presence (- - -) of added sodium chloride at a final concentration of 0.5 M.

(i.e., G41CT3 or G28CT3) or synthetic peptides representing only the B epitope segments (residues 1–15, G41 or G28) or the T cell epitope segment (residues 18–38, peptide CT3) were carefully examined for their CD spectra in the presence of varying concentrations of TFE. However, such studies again proved unproductive in that no marked distinctions could be discerned. Thus, while both peptides G41CT3 and G28CT3 showed increased, but comparable, helical content with increasing TFE concentrations, this increase proved to result entirely from the CT3 segment (data not shown). The B epitope segments (G41 and G28), however, were equally resistant toward inducibility into any secondary structure, even at TFE concentrations as high as 50% (data not shown).

Peptides G41CT3 and G28CT3 show altered solution behavior

In contrast to the results described above, an analysis of the CD spectra as a function of increasing peptide concentration proved interesting. Such studies revealed a shift in the minima of the negative peak to longer wavelengths (Fig. 5, *A* and *B*). Such a rightward shift at higher concentrations is usually indicative of the presence of intermolecular associations. This association, however, did not represent peptide aggregation—at least in the sense that it is generally understood—in that a simple dilution was sufficient to regenerate the profile obtained at lower concentrations for both peptides. Thus, dilution from a 60-μM peptide solution to concentrations of either 20 or 6 μM readily reproduced the spectra depicted in Figure 5, *A* and *B*, for the corresponding concentrations. The intermolecular associations observed at higher concentrations, therefore, apparently represent weak interactions.

We next probed for the nature of these interactions by examining the effect of adding salt on the right shift seen at higher peptide concentrations. For this experiment, solutions of peptide G41CT3 and G28CT3 at a concentration of 60 μM were prepared either in the presence or absence of a final concentration of 0.5 M NaCl, and the CD spectra were recorded. As shown in Figure 5*C*, the addition of salt partially disrupts the right shift seen for peptide G41CT3, reducing the wavelength of the occurrence of the minima to 206

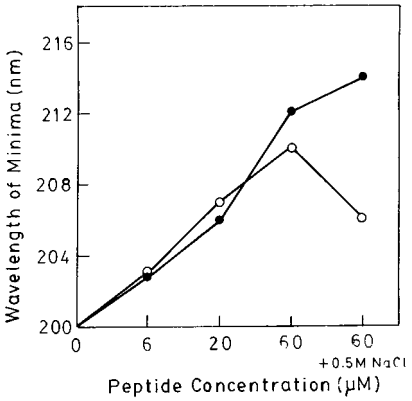

FIGURE 6. A graphic representation of the data presented in Figure 4. The shifts in minima of the negative peaks observed for peptides G41CT3 (open circles) and G28CT3 (closed circles) under the various conditions described in Figure 4 are illustrated here in a graph format.

nm. Thus, the intermolecular interactions between peptide G41CT3 molecules seen at higher concentrations are at least partially salt sensitive, implying that a significant proportion of these interactions are of a hydrophilic nature. In contrast, salt had no disruptive effect on the CD spectrum of a 60-μM concentration of peptide G28CT3 (Fig. 5*D*). Rather, a further rightward shift of 2 nm was observed (Fig. 5*D*). We infer from this that, contrary to peptide G41CT3, the intermolecular associations observed for peptide G28CT3 are of a predominantly hydrophobic nature. The cumulative data from Figure 5 are graphically summarized in Figure 6.

Thus, although peptides G41CT3 and G28CT3 do not display any differences either in secondary structure or propensities for it, they nevertheless show subtle alterations in the way the individual molecules of these peptides interact with each other when present at moderately high concentrations.

The kinetics of Ag recognition is also dependent upon peptide concentration

The biophysical studies described in Figures 5 and 6 were performed in an attempt to rationalize the differences in recognition of the two peptides G41CT3 and G28CT3 by anti-G41CT3 mAbs. The only distinction, with respect to behavior in solution, that became apparent from these studies was in the nature of the intermolecular interactions that these two peptides are involved in at higher concentrations. It was of interest, therefore, to determine whether these differences could account for the observed alteration in binding behavior. For this purpose, we selected mAb G41.14 as a representative anti-G41CT3 mAb. A 100-nM concentration of mAb G41.14 was incubated in the presence of 2, 6, or 20 μM final concentrations of either peptide G41CT3 or peptide G28CT3, and the rate of binding was measured as quenching of tryptophan fluorescence over a period of 60 min. The resulting slopes obtained were then plotted against peptide concentrations; the linear regression fits are shown in Figure 7. An interesting divergence became evident from these results. While binding rates were only marginally affected with increasing G41CT3 concentrations, indicative of a true pseudo first order reaction, peptide G28CT3 binding rates were dramatically influenced (Fig. 7). Surprisingly, at 2-μM concentrations, binding rates were comparable for both peptides. However, peptide G28CT3 became markedly more efficient relative to peptide G41CT3 at the 6-μM concentration, which was further enhanced when the peptide concentration was increased to 20 μM (Fig. 7).

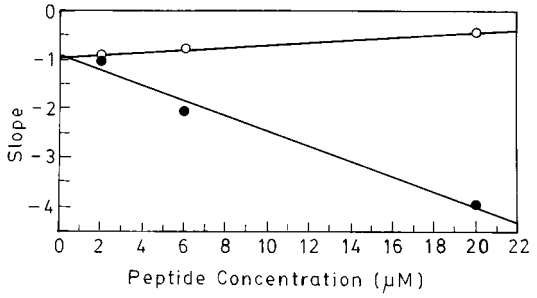

FIGURE 7. Concentration dependence of the rates of Ab-Ag interactions. A 100-nM final concentration of mAb G41.14 in PBS was mixed with either 2, 6, or 20 μM final concentrations of either peptide G41CT3 (open circles) or peptide G28CT3 (closed circles), and rates of binding were measured as described in *Materials and Methods*. The figure shows a linear regression plot of slopes obtained at each of these concentrations vs the concentration employed.

The observed concentration-dependent differences between rates of binding of the peptide analogues to mAb G41.14 correlate with the observed differences in Figure 5, *B* and *D*, supporting the possibility that the altered nature of intermolecular association could account for the differential binding rates of peptides G41CT3 and G28CT3 to anti-G41CT3 mAbs.

The ability of peptide G28CT3 to induce B cell-mediated Th1 stimulation correlates with its concentration-dependent differences in kinetic behavior

Our above-mentioned finding (Fig. 7) that the on-rate of peptide G28CT3 binding to an anti-G41CT3 mAb was dependent on peptide concentration also prompted us to assess the effect of peptide dose on relative activation of Th subsets. For this assessment, G41CT3-primed B cells along with CT3-primed T cells were cultured in the presence of varying amounts of either peptide G41CT3 or G28CT3, and levels of IL-10 and IFN-γ, as prototypic Th2 and Th1 cytokines, respectively, were measured in the culture supernatants. The results of such an experiment are shown in Figure 8*A*. When peptide G41CT3 was employed as recall Ag, the relative dominance of IL-10 over IFN-γ remained invariant over all the concentrations tested (Fig. 8*A*). This was, however, not the case for peptide G28CT3 in which a pronounced IL-10 dominance at low

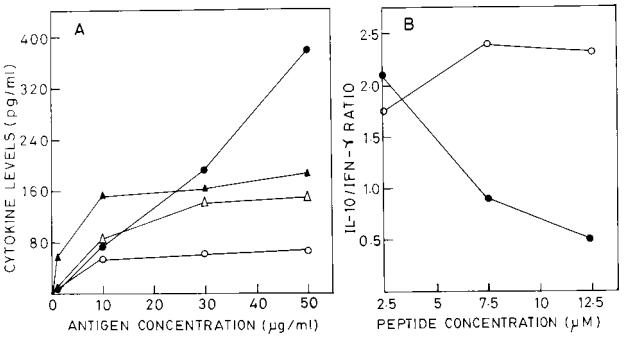

FIGURE 8. Concentration dependence of cytokine profiles induced in response to either peptide G41CT3 or peptide G28CT3. The protocol employed was similar to that described for Table I, except that peptide concentrations were varied as indicated. *A*, Levels of IL-10 (△, ▲) and IFN-γ (○, ●) produced in culture supernatants in response to varying challenge concentrations of either peptide G41CT3 (△, ○) or G28CT3 (▲, ●). *B*, A replot of the same data presented for IL-10/IFN-γ ratios as a function of molar concentration with peptide G41CT3 (○) or peptide G28CT3 (●). A representative of three independent experiments is shown.

concentrations inverted to IFN-γ dominance as the peptide concentration was increased (Fig. 8*A*). These results clearly show that whereas peptide G28CT3 induced a dose-dependent switch in the dominance of Th2 to the Th1 type of cytokines, peptide G41CT3 was unable to do so over the concentration range evaluated. Unfortunately, higher concentrations could not be tested because both peptides proved toxic at doses beyond those described (data not shown).

The dose-dependent Th2 to Th1 switch induced by peptide G28CT3 was strongly reminiscent of the dose-dependent alterations in its biophysical properties (Figs. 5 and 6), with its consequent influence on the on-rate of peptide binding to an anti-G41CT3 mAb (Fig. 7). This is exemplified particularly in Figure 8*B* where we have replotted the data in Figure 8*A* as ratios of IL-10 to IFN-γ at doses relevant to those employed in Figure 7. The trends observed for both peptides in Figure 8*B* are clearly consistent with those obtained for concentration-dependent kinetic behavior (shown in Fig. 7). Thus, while the IL-10 to IFN-γ ratios elicited by peptide G41CT3 remain relatively unaffected over the concentration range depicted (Fig. 8*B*), this is also true of its binding kinetics (Fig. 7). In contrast, the pronounced bias toward the Th1-type cytokines seen with increasing peptide G28CT3 concentration (Fig. 8*B*) correlates exceedingly well with the concentration-dependent influence on peptide G28CT3 binding kinetics noted in Figure 7. Collectively, these studies clearly establish a strong link between the facility of B cell epitope recognition and the ability of Ag-primed B cells to differentially stimulate the Th1 vs the Th2 subset of Ag-primed Th cells.

Discussion

T cell help for B cells clearly resides in Ag presentation by B cells and the subsequent display of class II-bound peptides recognized by specific T cells that ensures cytokine release targeted to Ag-specific B cells. Although B cells are known to interact with both subsets (16–18), a number of studies have shown that Ag-presenting B cells optimally stimulate the Th2 subset of effector CD4 cells (19–21). Indeed, it is now generally accepted that while B cell APCs are biased toward a Th2 response, non-B cell APCs such as macrophages and dendritic cells drive toward development of Th1 cells (19, 21).

Interesting, new insights have recently been provided that further our understanding of APC-driven differential activation of CD4 effector subsets. For instance, Murray et al. (22) have demonstrated that ligand density available on an APC surface for T cell recognition plays an important role in promoting relative differentiation of Th subsets. A high ligand density was found to favor Th1 differentiation, whereas a low ligand density is biased toward Th2 differentiation (22). Notably, a display of high ligand density by B cells has also been shown to preferentially stimulate Th1-like immunity (32). Recent results also suggest that the selective generation of Th1 vs Th2 effectors may be regulated by the strength of signal delivered by TCR upon engagement by the MHC class II-peptide complex on APCs (33). It is therefore now becoming apparent that differentiation/stimulation of CD4 effector subsets is not rigidly APC restricted, but is also subject to influence by extraneous parameters. The basis for ligand density-dependent modulation of CD4 effector pathways has been suggested to reside in differences in thresholds of activation required for Th1 vs Th2 cells (34).

Our present studies provide further evidence to support a role for ligands in determining APC-mediated selectivity in Th subset activation. Here, we employed a pair of analogous peptides, which although differing in sequence at two positions were nevertheless previously shown to be invariant with respect to both the fine specificity of B cells induced and their ability to prime T cells for a proliferative response (10). Notably, however, peptide G28CT3 proved superior to peptide G41CT3 at inducing a T cell recall response by G41CT3-primed B cells (10). In this report, we have extended these observations by demonstrating that, in addition to quantitative differences, peptides G41CT3 and G28CT3 also induce qualitatively distinct T cell recall responses from a common pool of Ag-specific T cells. Thus, for example, while an in vitro challenge of G41CT3-primed LNCs with the homologous peptide G41CT3 yielded predominantly Th2-type cytokines, a marked enhancement of Th1 activation was observed from the same culture when the recall Ag employed was the heterologous peptide, G28CT3. Subsequent experiments verified that these differences in recall abilities was a consequence of G41CT3-primed B cells serving as APCs. Particularly relevant here was our demonstration that enriched G41CT3-primed B cells recruited a significantly higher proportion of Th1 cells from a CT3-primed T cell pool in the presence of peptide G28CT3 rather than peptide G41CT3. Further, our observations that neither unprimed B cells nor CT3-primed LNCs were susceptible to peptide-mediated influences collectively imply an interaction between primed B cell and the B cell epitope on Ag as the putative causatory factor for the altered Th recruitment profiles. Finally, the physiologic validity of our in vitro findings was amply demonstrated in adoptive transfer experiments showing that anamnestic responses of G41CT3-primed B cells contained an enhanced proportion of the IgG2a isotype of Abs, relative to IgG1, on boosting with peptide G28CT3 as opposed to boosting with peptide G41CT3.

The Ag-presenting efficacy of primed B cells is dictated both by the efficiency of Ag capture through sIg receptors and the efficiency of processing. We have shown earlier that peptides G28CT3 and G41CT3 do not differ significantly in terms of processability subsequent to uptake by APCs (10). This, therefore, left the possibility that the observed differences in Ag-mediated recruitment of Th subsets could result from differential recognition of the two peptide Ags by sIg receptors on G41CT3-primed B cells. Because of the difficulties associated with obtaining large amounts of G41CT3-primed B cells, we employed anti-G41CT3 mAbs as surrogates for sIgG receptors to probe for differences in binding properties of peptides G41CT3 and G28CT3. Interestingly, while all mAbs tested displayed comparable affinities for both peptides, the kinetics of this binding proved to be markedly different. Significantly higher on-rates of binding were obtained for peptide G28CT3 relative to peptide G41CT3, although the magnitude of this difference varied from 3- to 17-fold depending on the mAb. We believe that the uniformity of these results permits an extrapolation to suggest that similar differences could also apply to the sIgG receptors on G41CT3-primed B cells.

Although no significant inherent differences in secondary structural propensities could be detected between the two peptides, we were able to identify concentration-dependent alterations in solution behavior. CD studies in a high ionic strength solution revealed that the intermolecular associations engaged in by molecules of the two peptides G41CT3 and G28CT3 at higher concentrations were of a qualitatively distinct nature. The salt disruptability of associations seen for peptide G41CT3 was indicative of interactions that are predominantly hydrophilic (or ionic?). In contrast, the stabilizing effect of salt on the CD spectrum of peptide G28CT3 revealed the occurrence of intermolecular associations that are composed almost exclusively of hydrophobic interactions.

The sole distinction in biophysical properties, detectable as qualitative differences in the nature of their intermolecular associations, prompted us to suspect that these differences could also

account for differences in the binding behavior of the two peptides. Such a presumption was subsequently confirmed by the results of an experiment wherein we measured binding rates of both peptides to an anti-G41CT3 mAb as a function of varying peptide concentration. The rate of peptide G28CT3 binding to the mAb was found to be profoundly influenced by peptide concentration, with a marked increase at higher concentrations. In contrast, the binding rate for peptide G41CT3 was relatively insensitive to concentration effects with, in fact, a marginal decrease at higher concentrations. The concentration dependence of differences in on-rates of mAb binding to the two peptides clearly implicates the operation of concentration-dependent differences in B cell epitope presentation, which, in turn, may be explained by invoking our results on the variant nature of the intermolecular association of peptides G41CT3 and G28CT3. It is possible that the differences in such weak interactions may be sufficient to alter the kinetics of Ag-Ab interactions, although too weak to influence equilibrium binding parameters such as affinity.

Having demonstrated a direct correlation between the kinetics of Ag binding and the concentration-dependent behavior of peptide G28CT3 in solution, it remained for us to ascertain whether these factors could, in fact, be responsible for the altered B cell-mediated Th subset recruitment profiles by peptide G28CT3 compared with peptide G41CT3. To verify this, we examined the effect of varying peptide concentrations on the ability of G41CT3-primed B cells to elicit Th1- vs Th2-type cytokines from a population of CT3-primed T cells. The results of these experiments proved extremely interesting. While relative predominance of the Th2 cytokines was independent of the dose of peptide G41CT3, a clear transition from a Th2 to Th1 dominance was observed when peptide G28CT3 concentrations were increased. A comparison of the peptide concentrations required for the switch and also of IL-10/IFN-γ ratios, with changes in kinetic and biophysical behavior, all as a function of increasing peptide concentration, revealed striking similarities. This strongly suggests an etiologic relationship between alterations in solution properties of peptide G28CT3, the kinetics of its binding, and its ability to elicit altered cytokine profiles from a primed T cell population in the presence of G41CT3-activated B cells.

The mechanism by which the altered nature of intermolecular associations in peptide G28CT3 promotes binding remains an issue of particular interest. One possibility is that such interactions render the B cell epitope more accessible to recognition, thereby increasing the probability of productive collisions leading to a binding interaction. Alternatively, intermolecular interactions may also facilitate Th1 recruitment by ensuring simultaneous, multiple occupancy of recognition sites within either sIg receptors or Ab molecules, leading in the former case to an increased strength of the intracellular signal generated. A more definitive conclusion must, however, await detailed analysis.

Our demonstration here of Ag-induced variability in B cell-mediated Th subset activation profiles adds yet another level of intricacy to our current understanding of the processes that mediate between immune recognition and the qualitative nature of an immune response. It will be particularly interesting to evaluate the significance of these findings in polarizing effector responses in infectious disease situations (35). It would also be interesting to elucidate the mechanism by which the alterations in Ag binding kinetics by B cells modulate their Ag-presenting properties. At present, several alternate possibilities may be invoked. For example, at an empirical level, an increased rate of binding may simply correspond to increased Ag uptake, rapidly leading to a high density of ligand presentation. As discussed earlier, high ligand densities have been proposed to favor Th1 activation (22, 34). A related factor that also needs consideration stems from the recent demonstration that the nature of Ag-BCR interaction directly influences intracellular targeting of Ag to lysozomes, for eventual processing and presentation (36). While this has been shown to quantitatively influence T cell activation (36), qualitative distinctions, if any, remain to be examined. Finally, the possibility that Ag binding kinetics may differentially regulate the extent of B cell activation—with its consequent effects on up-regulation of one or more cell surface accessory molecules involved in cognate B-T interaction (37–41)—also cannot be excluded. Discriminating between these and perhaps other alternatives is a current emphasis in our laboratory.

In summary, we have demonstrated here that relative recruitment of Th1 vs Th2 subsets of Ag-activated T cells by Ag-primed B cells is not invariant but, rather, is modulated by the kinetics of Ag recognition through its sIg receptor. While the default pathway is biased toward Th2 recruitment, higher on-rates of binding promote increased Th1 activation. Also of note is our finding that even subtle alterations in solution structure, resulting from minor changes in amino acid sequence, can so markedly influence the outcome of a cognate interaction between Ag-activated B and T cells. These findings may also bear implications for molecular mimicry, wherein homologous B cell epitopes could differentially skew Th effector responses with the potential for pathogenic consequences if one of the epitopes happens to derive from a self Ag.

Acknowledgments

We thank Drs. R. P. Roy and S. Kumaran (National Institute of Immunology, New Delhi, India) for allowing us to use their CD spectropolarometer and spectrofluorometer. We also thank Dr. Dinakar Salunke (National Institute of Immunology) for many discussions on protein structure.

References

1. Kishimoto, T., and T. Hirano. 1988. Molecular regulation of the B lymphocyte response. *Annu. Rev. Immunol. 6:485.*
2. Noelle, R. J., and E. C. Snow. 1991. T helper cell-dependent B cell activation. *FASEB J. 5:2770.*
3. Clark, E. A., and J. A. Ledbetter. 1994. How B and T cells talk to each other. *Nature 367:425.*
4. Lanzavecchia, A. 1990. Receptor-mediated antigen uptake and its effect on antigen presentation to class II-restricted T lymphocytes. *Annu. Rev. Immunol. 8:773.*
5. Demotz, S. J., H. M. Grey, and A. Sette. 1990. The minimal number of class II MHC-antigen complexes needed for T-cell stimulation. *Science 249:1028.*
6. Harding, C. V., and E. R. Enanue. 1990. Quantitation of antigen-presenting cell MHC class II/peptide complexes necessary for T cell stimulation. *Nature 346: 574.*
7. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T cell receptors by a few peptide-MHC complexes. *Nature 375:148.*
8. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science 273:104.*
9. Agarwal, A., S. Sarkar, C. Nazabal, G. Balasundaram, and K. V. S. Rao. 1996. B cell responses to a peptide epitope. I. The cellular basis for restricted recognition. *J. Immunol. 157:2779.*
10. Vijayakrishnan, L., S. Sarkar, R. P. Roy, and K. V. S. Rao. 1997. B cell responses to a peptide epitope. IV. Subtle sequence changes in flanking residues modulate immunogenicity. *J. Immunol. 159:1809.*
11. Vijayakrishnan, L., V. Kumar, J. N. Agrewala, G. C. Mishra, and K. V. S. Rao. 1994. Antigen-specific early primary humoral responses modulate immunodominance of B cell epitopes. *J. Immunol. 153:1613.*
12. Tuteja, N., A. Agarwal, L. Vijayakrishnan, B. P. Nayak, S. K. Gupta, V. Kumar, and K. V. S. Rao. 1997. B cell responses to a peptide epitope. II. Multiple levels of selection during maturation of primary responses. *Immunol. Cell Biol. 75:245.*
13. Agarwal, A., and K. V. S. Rao. 1997. B cell responses to a peptide epitope. III. Differential T helper thresholds in the recruitment of B cell fine specificities. *J. Immunol. 159:1077.*
14. Mosmann, T. R, and R. L. Coffman. 1989. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol. 7:145.*
15. Carter, L. L., and R. W. Dutton. 1996. Type 1 and type 2: a fundamental dichotomy for all T-cell subsets. *Curr. Opin. Immunol. 8:336.*
16. Chang, T.-L., L. M. Shea, S. Urioste, R. C. Thompson, W. H. Bloom, and A. K. Abbas. 1990. Heterogeneity of helper/inducer T lymphocytes. III. Response of IL-2 and IL-4 producing (Th1 and Th2) clones to antigens presented by different accessory cells. *J. Immunol. 145:2803.*

17. Noelle, R. J., J. Daum, W. C. Bartlett, J. McCann, and D. M. Shepherd. 1991. Cognate interactions between helper T cells and B cells. V. Reconstitution of T helper/inducer cell function using purified plasma membranes from activated Th1 and Th2 T helper cells and lymphokines. *J. Immunol. 146:1118.*
18. Hodgkin, P. A., and A. Basten. 1995. B cell activation, tolerance and antigen presentation functions. *Curr. Opin. Immunol. 7:121.*
19. Gajewski, T. F., M. Pinnas, T. Wong, and F. W. Fitch. 1991. Murine Th1 and Th2 clones proliferate optimally in response to distinct antigen presenting cell populations. *J. Immunol. 146:1750.*
20. Saoudi, A., S. Simmonds, I. Huitinga, and D. Mason. 1995. Prevention of experimental allergic encephalomyelitis in rats by targeting auto-antigen to B cells: evidence that the protective mechanism depends on changes in cytokine responses and migrating properties of auto-antigen-specific T cells. *J. Exp. Med. 182:335.*
21. Stockinger, B., T. Zal, A. Zal, and D. Gray. 1996. B cells solicit their own help from T cells. *J. Exp. Med. 183:891.*
22. Murray, J. S., C. Pfeiffer, J. Madri, and K. Bottomly. 1992. Major histocompatibility complex (MHC) control of CD4 T cell subset activation. II. A single peptide induces either humoral or cell-mediated responses in mice of distinct genotypes. *Eur. J. Immunol. 22:559.*
23. Merrifield, R. B. 1986. Solid phase synthesis. *Science 232:341.*
24. Stewart, J. M., and J. D. Young. 1984. *Solid Phase Peptide Synthesis,* 2nd Ed. Pierce Chemical Co., Rockford, IL.
25. Atherton, E., and R. C. Sheppard. 1989. *Solid Phase Peptide Synthesis: A Practical Approach.* IRL Press, Oxford, U.K..
26. Coligon, J. E., A. M. Kruisbeck, D. M. Margolies, E. M. Shevach, and O. M. Stoker. 1991. *Current Protocols in Immunology.* John Wiley and Sons, New York, p. 2.5.1.
27. Neurath, A. R., and S. B. H. Kent. 1988. The pre-S region of hepadna virus envelope proteins. *Adv. Virus Res. 34:64.*
28. Sinigaglia, F., M. Guttinger, J. Kilgus, D. M. Doran, H. Matile, H. Etlinger, A. Trezciak, D. Gillessen, and J. R. C. Pink. 1988. A malaria T cell epitope recognized in association with most mouse and human MHC class II molecules. *Nature 336:778.*
29. Snapper, C. M., and W. E. Paul. 1988. Interferon-γ stimulates IgG2a secretion by murine B cells stimulated with bacterial lipopolysaccharide. *J. Immunol. 140: 2121.*
30. Croft, M., and S. L. Swain. 1991. B cell responses to T helper subsets. II. Both the stage of T cell differentiation and the cytokine secretion determines the extent and nature of helper activity. *J. Immunol. 147:3679.*
31. Sonnichsen, F. D., J. E. Van Eyk, R. S. Hodges, and B. D. Sykes. 1992. Effect of trifluoroethanol in protein secondary structure: an NMR and CD study using a synthetic Actin peptide. *Biochemistry 31:8790.*
32. Murray, J. S., J. P. Kasselman, and L. T. Schountz. 1995 High density antigen presentation of an immunodominant minimal peptide on B cells is MHC-linked to Th1 like immunity. *Cell. Immunol. 166:9.*
33. Tao, X., S. Constant, P. Jorritsma, and K. Bottomly. 1997. Strength of TCR signal determine the costimulatory requirements for Th1 and Th2 $CD4^+$ T cell differentiation. *J. Immunol. 159:5956.*
34. Constant, S. L., and K. Bottomly. 1997. Induction of TH1 and TH2 $CD4^+$ T cell responses: the alternative approaches. *Annu. Rev. Immunol. 15:297.*
35. Romagnini, S. 1997. The Th1/Th2 paradigm. *Immunol. Today 18:263.*
36. Aluvihare, V. R., A. A. Khamlichi, G. T. Williams, L. Adorini, and M. S. Neuberger. 1997. Acceleration of intracellular targeting of antigen by the B cell antigen receptor: importance depends on the nature of antigen-antibody interaction. *EMBO J. 16:3553.*
37. Ullman, K. S., J. P. Northrop, C. L. Verweij, and G. R. Crabtree. 1990. Transmission of signals from the T lymphocyte antigen receptors to the genes responsible for cell proliferation and immune function: the missing link. *Annu. Rev. Immunol. 8:421.*
38. Kuchroo, V. K., M. P. Das, J. A. Brown, A. M. Ranger, S. S. Zamvil, R. A. Sobel, H. L. Weiner, N. Nabvi, and L. H. Glimcher. 1995. B7-1 and B7-2 co-stimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell 80:707.*
39. Lederer, J. A., V. L. Perez, L. DesRoches, S. M. Kim, A. K. Abbas, and A. H. Lichtman. 1996. Cytokine transcriptional events during helper T cell subset differentiation. *J. Exp. Med. 184:397.*
40. Kundig, T. M., A. Shahinian, K. Kawai, H.-W. Mittrucker, E. Sebzda, M. F. Bachmann, T. W. Mak, and P. S. Ohashi. 1996. Duration of TCR stimulation determines co-stimulatory requirements of T cells. *Immunity 5:41.*
41. Shaw, A. S., and M. L. Dustin. 1997. Making the T cell receptor go the distance: a topological view of T cell activation. *Immunity 6:361.*