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B Lymphocytes as Antigen-Presenting Cells for CD4⁺ T Cell Priming In Vivo

Stephanie L. Constant

The contribution of B lymphocytes as APCs for CD4⁺ T cell priming remains controversial, based on findings that B cells cannot provide the requisite ligating and costimulatory signals for naïve T cells to be activated. In the current study, we have examined Ag-specific T:B cell collaboration under circumstances in which B cells take up Ag through Ig receptors in vivo. This results in their activation and an ability to effectively stimulate naïve CD4⁺ T cells both in vitro and in vivo. The aim of this work was to establish some of the key molecular interactions, as well as kinetics, between Ag-specific T and B cells that enable this priming to take place. Our approach was to amplify the starting pools of both Ag-specific T and B cell populations in vivo to track directly the events during initial T:B cell collaborations. We show that the induction of optimal levels of T cell priming to a protein Ag requires the involvement of Ag-specific B cells. The interaction that results between Ag-specific T and B cells prevents the down-modulation of B7 costimulatory molecules usually observed in the absence of appropriate T cells. Moreover, this prevention in down-modulation is independent of CD40:CD40 ligand contact. Finally, we present data suggesting that once Ag-specific T and B cells interact, there is a rapid (1–2-h) down-regulation of antigenic complexes on the surface of the B lymphocytes, possibly to prevent them from engaging other T cells in the vicinity and therefore focus the initial interaction.


The initial priming of an Ag-specific CD4⁺ T cell in vivo requires that two signals be provided to the T cell: 1) a ligating signal through the TCR, provided by MHC class II/peptide complexes, and 2) a costimulatory signal, in the form of CD28 ligation by B7 molecules. In vivo both of these signals are provided by APC. The conditions under which APC induce successful T cell priming have been shown to be quite stringent, with the type of APC and its state of activation being a key factor. However, it has now also been shown that, far from simply being the recipient of APC-derived signals, the T cell being primed provides signals of its own to the APC, which can either enhance or down-regulate the interaction (1). Thus, there is cross-talk between interacting T cells and APC. This in turn can give rise to situations in which the T cell becomes successfully primed or to situations that render the T cell unable to respond to subsequent stimulation, a state known as T cell anergy or tolerance.

The contribution of B lymphocytes as APC for CD4⁺ T cells is still controversial, with evidence of a capacity to induce both priming and anergy in T cells. However, which of the two states is induced in the T cell seems to be dependent on the activation status of both the T and B lymphocyte during their initial encounter. For example, in vitro studies using various combinations of resting and already activated populations of T and B cells suggest that when both populations are resting, any interaction between them will be nonproductive (2–4). Similar results have also been obtained using in vivo models of Ag presentation in which either resting or activated Ag-bearing B cells were transferred into naive recipient mice (5, 6). The main explanation put forward for such results is that resting B cells, unlike their activated counterparts, do not express high enough levels either of MHC class II/peptide complexes or of costimulatory molecules to correctly engage and activate a resting T cell. Without activation, the T cell in turn cannot provide the signals needed by the B cell to prolong and successfully complete their interaction (7). One of these signals includes an appropriate CD40L:CD40 interaction between the T:B cell (8). Since CD40L is only expressed on already activated T cells, a failure to initially activate the T cell ultimately affects both the T and B lymphocyte during their interaction. Under some circumstances, this can lead to anergic B, as well as T cells.

A different outcome is observed when the population of CD4⁺ T cells being stimulated is already in a state of activation. Studies both in vitro (9, 10) and in vivo (11) show that previously activated T cells can be stimulated to proliferate and differentiate using B cells as APC. It has been suggested that this is the result of activated T cells having a less stringent requirement for high levels of MHC class II/peptide complexes and costimulatory signals (12). Furthermore, activated T cells express CD40L, which will help the B cell enhance its own activation status. Therefore, it follows from these data that a T cell will first need to be activated by another APC type, bearing adequate levels of both antigenic complexes and costimulatory molecules, before a subsequent productive interaction with a B lymphocyte can take place.

Evidence in support of B cells being able to stimulate resting naïve cells is less well documented, but is gradually emerging. It has been argued that B cells will never be able to prime naïve T cells because it would be difficult to have a situation in vivo in which a B cell can become activated in the absence of T cell help.

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3 Abbreviations used in this paper: CD40L, CD40 ligand; CFSE, 5(and 6)-carboxyfluorescein diacetate succinimidyl ester; HEL, hen egg lysozyme; MCC, moth cytochrome c; pMCC, MCC peptide.

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However, there are several ways, other than through the help of T cells, that B lymphocytes can be activated to express high levels of MHC class II and B7 costimulatory molecules. These include exposure to bacterial components, such as LPS, and cross-linking of Ig receptors (IgR) by anti-Ig Ab. For example, Parker and coworkers showed that the coadministration of activating concentrations of divalent mouse anti-δ with the foreign protein, F(ab')2 fragment of rabbit IgG, led to Ag-primed T cells (13). This was not the case when the foreign Ag was administered in the absence of activating Ab, supporting the idea that B cells require an initial activation step resulting from IgR cross-linking to be competent APC for naïve T cells. Since then, other groups have reported the effectiveness of T cell priming by B cells using the combined Ag/anti-Ig approach (14).

Yet a third approach for inducing B cell activation in vivo is by direct uptake of cognate Ag through the IgR of Ag-specific B cells. One of the difficulties in trying to study T cell priming by Ag-specific B cells in vivo is the very low frequency of B cells with an IgR specific for a given Ag. In our laboratory, our approach for studying cognate T:B cell interactions has been to amplify the initial pool of Ag-specific B cells. We have reported previously how the delivery of a soluble protein Ag to mice bearing a transgenic IgR specific for that protein leads to a pool of B cells that express high levels of antigenic complexes and costimulatory ligands, thus fulfilling the requirements as APC for successful T cell priming (15). In the current study, we have examined Ag-specific T:B cell collaboration during an initial encounter with Ag. We show that B cells can induce Ag-specific T cell priming in vivo and we describe some of the key molecular interactions, as well as kinetics, between the T and B cell, which enable this priming to take place.

Materials and Methods

Mice

The Ig transgenic mice (receptor specific for lysozyme) (16) were derived from founders obtained from C. Goodnow (John Curtin School of Medical Research, Canberra, Australia), and were backcrossed onto B10.BR mice. Screening for the presence of the Ig transgene was conducted by staining peripheral B lymphocytes for the presence of the IgC-δ haplotype (DS-1 Ab from PharMingen, San Diego, CA). The TCR transgenic mice (TCR specific for pigeon cytochrome c) (17) were originally provided to us by J. Kaye (Scripps Institute, La Jolla, CA) and are maintained as heterozygotes on a B10.BR genetic background. Screening for the presence of the TCR transgene was conducted by staining peripheral T lymphocytes for the presence of the Vβ3 TCR chain (KJ25 Ab; PharMingen). The TCR×Ig transgenic mice were generated by crossing TCR and Ig transgenic mice and screening for the presence of both transgenes, as described above. The TCR×Ig×CD40L−/− mice were generated by backcrossing TCR×Ig transgenic mice onto CD40L−/− mice (originally obtained from R. Flavell, Yale University School of Medicine, New Haven, CT) (18) until homozygous for the knockout gene. Homozygous offspring were identified by PCR of genomic tail DNA, as described by Xu et al. (18). B10.BR mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Antigens

The TCR from the transgenic mice recognizes residues 81–103 derived from tobacco hornworm moth or pigeon cytochrome c in the context of I-Ek MHC class II. For our experiments, we used the moth cytochrome peptide (pMCC) synthesized and purified, as described previously (19). The conjugate Ag, MCC-HEL, was generated by conjugating pMCC to lysozyme protein, as described previously (15). For experiments involving cytochrome c native protein, pigeon cytochrome c was used since moth cytochrome c protein is not available commercially. Lysozyme and pigeon cytochrome c proteins were purchased from Sigma (St. Louis, MO).

All proteins and conjugates were dialyzed extensively against PBS before use to remove any peptide fragments present in the preparations.

Preparation of CD4+ T cell and B cell populations

Combined lymph node and splenic CD4+ T cells were isolated from TCR transgenic mice by negative selection, as described previously (3), using mAbs to CD8 and MHC class II, followed by incubation with anti-mouse and anti-rat Ig-coated magnetic beads (Advanced Magnetics, Cambridge, MA). B lymphocytes were isolated from splenocytes, as described previously (15). B cells used for T cell stimulation assays were treated with 50 µg/ml mitomycin C before use. Purity of both cell preparations was always >95%, as determined by FACS analysis.

For some experiments, purified TCR transgenic CD4+ T cells were labeled with the intracellular fluorescent dye, 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR), based on the method of Lyons et al. (19). Briefly, the cells were suspended at 5 × 10^6/ml in prewarmed PBS containing a final concentration of 10 µM CFSE and incubated for 30 min at 37°C. The cells were then washed once in PBS/0.5% BSA and twice in PBS before transfer.

In vivo administration of Ag and CFSE-labeled CD4+ T cells

Mice received 100 µg (for CFSE experiments) or 1 mg (for in vitro experiments) of pMCC, lysozyme, or MCC-HEL Ags in 200 µl PBS by i.v. administration into the lateral tail vein. For in vivo T cell stimulation experiments, CFSE-labeled TCR transgenic CD4+ T cells (1 × 10^7 per mouse) were transferred in 200 µl PBS into the lateral tail vein at 2–4 h after administration of Ag. At various times after administration, spleens were removed from injected mice, and CD4+ T cells or B cells were isolated for staining or in vitro assays of stimulation.

Analysis of CFSE-labeled cells after transfer

Splenocytes were isolated and RBC were removed by centrifugation over Ficoll, as described previously (3). The cells were then incubated in PBS/0.5% BSA containing anti-CD4 MACS magnetic microbeads (Miltenyi Biotec, Sunnyvale, CA), and the CD4+ cells were isolated using a MiniMACS system, according to the manufacturer’s protocol (Miltenyi Biotec). The resulting cells were then costained with RED670- conjugated anti-CD4 (Life Technologies, Gaithersburg, MD) and PE-labeled anti-V83 (PharMingen) to identify the TCR transgenic CD4+ T cells. For each group, 50,000 CD4+ Vβ3+ cells were gated and collected by flow cytometry, and then examined for CFSE content using the FL1 channel.

Staining for expression of B7-2

Splenic B lymphocytes were stained for the presence of B7-2 using purified anti-B7-2 mAb (PharMingen), followed by PE-labeled goat anti-rat IgG (heavy and light chain specific) purchased from Caltag Laboratories (Burlingame, CA). The cells were then stained with FITC-labeled anti-CD20 (PharMingen) to specifically gate on B lymphocytes. Cells were also stained without anti-B7-2 to control for any nonspecific staining by the PE anti-rat IgG. All staining was conducted in the presence of 1 mg/ml purified mouse IgG to absorb out any cross-reactivity between rat and mouse Ig.

Stimulating CD4+ T cells in vitro

CD4+ T cells purified from TCR transgenic mice were cultured in triplicate at 2 × 10^5/well in 96-well flat-bottom plates with various numbers of APC loaded in vivo with Ag (see figure legends for details). For some experiments, anti-CD28 (PharMingen) was included in the cultures at a final concentration of 2.5 µg/ml. Proliferation was measured after 72 h by the overnight addition of 1 µCi/well [3H]thyymidine.

Results

Uptake of Ag via Ig receptors makes B cells competent APCs for CD4+ T cell priming in vitro and in vivo

We have described previously an in vivo system in which Ag-specific B cells become loaded with Ag within a few hours of i.v. administration of a soluble protein Ag and become competent APCs for the priming of Ag-specific CD4+ T cells in vivo (15). Using Ig transgenic mice with a B cell receptor specific for HEL protein, we demonstrated that within 4 h of administering a MCC-HEL protein conjugate, the Ig transgenic B cells in the spleen had up-regulated their expression of B7-2 molecules (Fig. 1A). Furthermore, these B cells were able to stimulate naive cytotoxicity-specific TCR transgenic CD4+ T cells in vitro in a dose-dependent manner (Fig. 1C). This was in contrast to B cells from Ig transgenic mice injected with soluble pigeon cytochrome c protein.
therefore, uptake of Ag is independent of the Ig receptor), in which neither B7-2 up-regulation (Fig. 1B) nor cytochrome c-specific T cell stimulation (Fig. 1C) was observed. At no time was B7-1 staining detected on any groups of B cells, as reported previously (15). Our conclusions from these findings were that B lymphocytes can be competent APCs for priming CD4\(^+\) T cells as long as the Ag is taken up via the Ig receptor.

We next took advantage of a technique that allows the course of T cell stimulation to be visualized in vivo (19), therefore enabling us to confirm that the Ag-loaded B cells in our system could indeed prime T cell in situ. Cytochrome c-specific CD4\(^+\) T cells were prelabeled using an intracellular fluorescent dye (CFSE) and then transferred into recipient Ig transgenic mice that had been injected with either PBS, HEL protein, or MCC-HEL protein conjugate. Three days later, splenocytes were isolated and the CD4\(^+\) T cells were purified and stained with PE anti-V\(\beta\)3 and RED670 anti-CD4 to identify transgenic cells. FACS plots show the CFSE profiles (FL1 channel) after gating on the transgenic populations and the percentages of the cells that have undergone division. These data are representative of more than five individual experiments.

We had reported previously that the extent of Ag-specific CD4\(^+\) T cell stimulation induced by APC loaded with the conjugate in vivo was equivalent whether T cell-depleted splenocytes or FACS-sorted B cells were used (15). This suggested that the uptake of this Ag in vivo was predominantly by the Ag-specific B cells, rather than other APC types. We were able to test this directly in vivo by transferring CFSE\(^-\) cytochrome c-specific T cells into Ig transgenic or nontransgenic recipients that had been pulsed with MCC-HEL conjugate 3 h earlier. Clearly, significant priming of the naive CD4\(^+\) T cells was evident only when the appropriate Ag-specific B cells were present (Fig. 3A compared with Fig. 3B). These results fit well with our earlier in vivo and in vitro observations that
other types of APC, such as dendritic cells, have a poor capacity to present this soluble protein (20). When a peptide form of cytochrome c (pMCC) was used, T cell priming was equivalent in both Ig transgenic and nontransgenic recipients (Fig. 3, C and D), which agrees with the findings that when an Ag does not require uptake and processing, presentation is more likely to be through APC such as dendritic cells (20).

**Ag-loaded B cells lose their Ag-presenting capacity over time**

During the course of these studies, we noted that the ability of the Ag-loaded B lymphocytes in our in vivo system to stimulate naive CD4\(^+\) T cells was kinetics dependent. Following in vivo Ag administration, the expression of B7-2 (Fig. 4A) and the ability of the B cells to prime Ag-specific T cells was evident by 4–6 h (Fig. 4B) and then gradually decreased over time, such that by 24 h the B cells no longer expressed B7-2 molecules (Fig. 4C) and were no longer able to stimulate T cells in vitro (Fig. 4D). Fewer than 10% of transferred CFSE\(^+\) CD4\(^+\) T cells were induced to proliferate at this 24-h time point (data not shown).

One possibility that we considered was that the B cells that had taken up Ag were being induced to apoptose, resulting in their physical loss from the pool of splenic APC. However, based on B

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**FIGURE 3.** The presence of Ag-specific B cells induces optimal CD4\(^+\) T cell priming to protein Ag in vivo. Ig transgenic (A and C) or B10.BR (B and D) mice received an i.v. injection of MCC-HEL conjugate (A and B) or pMCC (C and D) Ag, followed 3 h later by an i.v. administration of 1 \times 10^7 TCR transgenic CD4\(^+\) T cells labeled with CFSE dye. Three days later, splenocytes were isolated and the CD4\(^+\) T cells were purified and stained with PE anti-V\(\beta\)3 and RED670 anti-CD4 to identify transgenic cells. FACS plots show the CFSE profiles (FL1 channel) after gating on the transgenic populations and the percentages of the cells that have undergone division. These data are representative of three individual experiments.

**FIGURE 4.** Ag-loaded B cells lose their Ag-presenting capacity over time. Ig transgenic mice were given an i.v. injection of MCC-HEL conjugate Ag, and 4 h (A) or 24 h (C) later, splenocytes were isolated and stained with purified anti-B7-2 mAb, followed by PE-labeled goat anti-rat IgG and then FITC-labeled anti-B200. FACS plots show the B7-2 expression after gating on B220\(^+\) cells. Solid lines represent B7-2 staining relative to staining with secondary mAbs alone (dotted lines). B cells were isolated from the same splenocytes, and different numbers were used to stimulate MCC-specific TCR transgenic CD4\(^+\) T cells in vitro. Bar graphs show the MCC-specific proliferation induced by B cells after loading with Ag in vivo for 4 h (B) or 24 h (D). These data are representative of more than five individual experiments.
B cells. These data are representative of three individual experiments. mAb had no stimulatory effect on the T cells in the absence of Ag-loaded C. 24-h Ag-loaded B cells plus 2.5 MHC/Ag complexes, but down-regulate their expression of B7-2 up cognate Ag in vivo most likely retain their expression of B7-2 molecules to up-regulate. In our current studies, we have generated anti-CD28 Ab. The anti-CD28 mAb had no stimulatory effect on the T cells in the absence of Ag-loaded B cells. These data are representative of three individual experiments.

FIGURE 5. Anti-CD28 restores capacity of 24-h Ag-loaded B cells to prime CD4+ T cells. Ig transgenic mice received an i.v. injection of MCC-HEL conjugate, and 4 or 24 h later, B cells were isolated, and different numbers were used to stimulate MCC-specific TCR transgenic CD4+ T cells in vitro. Bar graphs show the T cell proliferation measured after stimulation with A, 4-h Ag-loaded B cells; B, 24-h Ag-loaded B cells; and C, 24-h Ag-loaded B cells plus 2.5 μg/ml anti-CD28 Ab. The anti-CD28 mAb had no stimulatory effect on the T cells in the absence of Ag-loaded B cells. These data are representative of three individual experiments.

cell counts at various times after Ag injection, we saw no significant change in the number of splenic B cells over time (data not shown). An alternative possibility was that the B cells were still present in the spleen 24 h after Ag uptake, but that they had lost their functional ability to stimulate naïve CD4+ T cells either because they no longer express the appropriate MHC class II/Ag complexes, or they express insufficient numbers of B7-2 molecules, or both. To test this directly, splenic B cells isolated 24 h after in vivo Ag administration were used to stimulate Ag-specific CD4+ T cells in the absence or presence of additional costimulatory signals, provided by anti-CD28 Ab. As shown in Fig. 5, the addition of costimulation to cultures of B cells pulsed in vivo for 24 h restored their ability to stimulate T cells to levels almost equivalent to those induced by B cells pulsed in vivo for 4 h. These data suggest that over the course of 24 h, B cells that have taken up cognate Ag in vivo most likely retain their expression of MHC/Ag complexes, but down-regulate their expression of B7-2 molecules. The likelihood of this explanation was further confirmed by using rIL-2 to bypass the requirement for costimulation or by using previously activated Ag-specific CD4+ T cells (which are less dependent on costimulatory signals) as a source of responders (data not shown).

Presence of appropriate TCR maintains B7-2 expression on Ag-loaded B cells

We next investigated the reason for the observed down-regulation of B7-2 molecules on 24-h Ag-loaded B cells, and whether we could induce a situation in which expression was maintained. One hypothesis we considered was that, following the uptake of Ag, the now activated B cells need to be provided with additional signals to maintain their activated status beyond 4–6 h. We postulated that the source for these signals could be T cells and that the engagement of TCRs of the appropriate specificity with MHC/Ag complexes on the surface of B cells might provide those signals. To address this possibility, the Ig transgenic mice were bred onto mice with a TCR transgenic for cytochrome c, thus providing an in situ source of both T and B cells specific for the MCC-HEL conjugate being used in our studies. When the conjugate Ag was administered to the TCR×Ig transgenic mice and the expression of B7-2 on B cells monitored over time, significant levels of B7-2 were still detectable after 24 h (Fig. 6D), unlike the B cells of Ig transgenic mice with polyclonal TCRs in which expression was back to baseline levels (Fig. 6C). Moreover, when we examined the CD4+ T cells from these mice for the presence of the early activation marker, CD69, only CD4+ cells from the TCR×Ig transgenic mice were found to be activated (data not shown), suggesting some kind of interaction had taken place in situ between the T and B cell populations.

Up-regulation and maintenance of B7-2 on in vivo Ag-loaded B cells are CD40L independent

These data suggest that, in order for Ag-loaded B cells to retain their expression of B7-2 molecules, a cognate interaction with T cells bearing a relevant TCR must take place within at least 24 h of Ag uptake. Several reports have implicated a requirement for CD40/CD40L interactions between B and T cells to induce or enhance a state of activation in the two cell subsets (reviewed in Ref. 8). Interestingly, many of these reports suggest that such an interaction is needed for the initial up-regulation of B7 molecules on B cells, and that this in turn results in enhanced Ag presentation by the B cell, and therefore enhanced stimulation of cognate T cells. Since CD40L is only expressed on activated T cells, an argument put forward by several groups is that resting B cells will only be capable of stimulating previously primed CD4+ T cells because these are the only T cells that will be able to induce B7 molecules to up-regulate. In our current studies, we have generated a situation in which B7 up-regulation on B cells appears to be independent of the presence of cognate T cell interactions (Fig. 1A), although prolonged up-regulation does correlate with the presence of T cells of the appropriate specificity (as observed with the TCR×Ig transgenic mice in Fig. 6D). As mentioned above, the CD4+ T cells in the TCR×Ig transgenic mice were found to be in a state of activation 24 h after Ag administration, and therefore could be involved in providing CD40L signals to the B cells for the maintenance of B7-2 expression. To assess directly the involvement of CD40L in both the induction and the maintenance of B7-2 expression on Ag-loaded B cells, TCR×Ig transgenic mice were backcrossed onto CD40L knockout mice and Ag was administered for either 4 or 24 h, as previously described. Interestingly, when compared with B cells from TCR×Ig transgenic mice with intact CD40L (Fig. 7, B and E), the absence of CD40L had no effect on either the initial up-regulation (Fig. 7C) or the maintenance (Fig. 7F) of B7-2 expression on Ag-loaded B cells. We would conclude...
from these results that in situations in which B cells are activated as a result of taking up Ag via their Ig receptor, the up-regulation and prolonged expression of B7-2 are CD40L independent. Interestingly, when we used our transfer system to examine how cytochrome c-specific CD4\textsuperscript{+} T cells with a CD40L knockout phenotype would respond to Ag presented by B7-bearing Ag-specific B cells, we were only occasionally able to recover any (<1%) of the transferred CFSE\textsuperscript{1} T cells. This was regardless of the type of Ag used (MCC-HEL conjugate or pMCC). These data suggest that although CD40L:CD40 interactions are unnecessary for the expression of B7 on B cells that have internalized Ag through their IgR, these interactions are clearly still critical for the maintenance and expansion of CD4\textsuperscript{+} T cells being primed.

**FIGURE 6.** Presence of appropriate TCR maintains B7-2 expression on Ag-loaded B cells. MCC-HEL conjugate Ag was administered i.v. to Ig transgenic (A and C) and TCR\texttimes{}Ig transgenic (B and D) mice, and either 4 h (A and B) or 24 h (C and D) later, splenocytes were isolated and stained with purified anti-B7-2 mAb, followed by PE-labeled goat anti-rat IgG and then FITC-labeled anti-B200. FACS plots show the B7-2 expression after gating on B220\textsuperscript{+} cells. Solid lines represent B7-2 staining relative to staining with secondary mAbs alone (dotted lines). These data are representative of more than five individual experiments.

**FIGURE 7.** Up-regulation and maintenance of B7-2 on Ag-loaded B cells are CD40L independent. MCC-HEL conjugate Ag was administered i.v. to Ig transgenic (A and D), TCR\texttimes{}Ig transgenic (B and E), and TCR\texttimes{}Ig\times{}CD40L\textsuperscript{-/-} transgenic (C and F) mice. Splenocytes were isolated either 4 h (A, B, and C) or 24 h (D, E, and F) later, and stained with purified anti-B7-2 mAb, followed by PE-labeled goat anti-rat IgG and then FITC-labeled anti-B200. FACS plots show the B7-2 expression after gating on B220\textsuperscript{+} cells. Solid lines represent B7-2 staining relative to staining with secondary mAbs alone (dotted lines). These data are representative of five individual experiments.

**TCR interaction with Ag-loaded B cells induces rapid down-regulation of antigenic complexes**

The final question we addressed was whether the maintenance of B7-2 expression on the B cells at 24 h after Ag administration also correlated with a maintained ability to prime Ag-specific CD4\textsuperscript{+} T cells, or whether these B cells were still deficient in T cell stimulation (see Fig. 4). Ag was administered to TCR\texttimes{}Ig transgenic mice and their B cells were isolated at 4 and 24 h after administration to be used as APCs for naive cytochrome c-specific T cells. B cells from the Ig transgenic mice gave the usual results of T cell stimulation being induced after 4, but not 24 h of Ag loading (Fig.
A). Surprisingly, however, B cells from TCR×Ig transgenic mice were unable to induce high levels of T cell stimulation, regardless of whether they were used at 4 or 24 h after Ag administration (Fig. 8B). This suggests that, despite the expression of B7-2 both at 4 and 24 h after Ag administration, B cells from the TCR×Ig mice were somehow impaired in their ability to stimulate naive CD4+ T cells. To assess whether these B cells had at any time been capable of T cell priming, we performed a kinetics experiment in which B cells were isolated at 1, 2, 3, and 4 h after Ag administration and used as APCs for cytochrome c-specific CD4+ T cells. As shown in Fig. 9, the B cells from the TCR×Ig transgenic mice can present Ag within 1–2 h following Ag administration before their ability to stimulate decreased. Since B7-2 expression is still present at 4 h after Ag loading, the likelihood is that MHC/peptide complexes are becoming down-regulated. We propose that, once an appropriate interaction between a cognate T and B cell has been established, there follows a rapid down-regulation of antigenic complexes on the surface of the B cells, thus preventing interaction with other CD4+ T cells. Although MHC class II/peptide complexes are no longer available to the T cell, TCR engagement having been initiated, signals are still being provided by the B cell via B7;CD28 and CD40;CD40L contact points. In cases in which T cells of the appropriate specificity are not available to the B cell, antigenic complexes are maintained on the surface for at least 24 h (Fig. 5) before down-regulation eventually occurs at about 48 h (unpublished observations).

Discussion

In the current work, we have described several different events that take place when Ag-specific T and B cells first interact. Using both in vitro and in vivo approaches, we showed that the uptake of Ag through IgR results in B cells that have an activated phenotype (up-regulation of surface B7-2 and MHC class II) and are competent APC for naive CD4+ T cells. The contribution, if any, of other APC types during this priming is difficult to assess in the current studies. We found that presentation of a foreign soluble protein by APC in the absence of appropriate Ag-specific B cells was extremely limited (Fig. 3), suggesting a major contribution to priming by the B cells. In support of a role for B cells alone being

FIGURE 8. Presence of appropriate TCR does not restore capacity of 24-h Ag-loaded B cells to prime CD4+ T cells. Ig and TCR×Ig transgenic mice received an i.v. injection of MCC-HEL conjugate, and 4 or 24 h later, B cells were isolated, and different numbers were used to stimulate MCC-specific TCR transgenic CD4+ T cells in vitro. Bar graphs show the T cell proliferation measured after stimulation with B cells from Ig transgenic (A) and TCR×Ig transgenic (B) mice after 4 h (solid bars) or 24 h (hatched bars) of Ag loading. These data are representative of more than five individual experiments.

FIGURE 9. TCR interaction with Ag-loaded B cells induces rapid down-regulation of antigenic complexes. TCR×Ig transgenic (A, B, and C) and Ig transgenic (D) mice received an i.v. injection of MCC-HEL conjugate. B cells were then isolated 1 h (A), 2 h (B), 3 h (C), or 4 h (D) after Ag loading, and different numbers were used to stimulate MCC-specific TCR transgenic CD4+ T cells in vitro. Bar graphs show T cell proliferation results. These data are representative of three individual experiments.
capable of T cell priming, we have reported previously that FACSPurified populations of Ag-specific B cells loaded with Ag in vivo induced levels of T cell priming equivalent to those of Ag-loaded T cell-depleted splenocytes (15). In contrast, Jenkins and coworkers, using a very elegant in vivo model in which they could visualize specific B and T cell interactions in lymph nodes, have recently reported that during the first 24 h after immunization with protein Ag, T cells become clustered around dendritic cells and that the interaction between Ag-specific T and B cells does not take place until at least 48 h after Ag administration (21). What is not clear in their study is whether the CD4+ T cells that cluster with the dendritic cells are actually being primed or whether this occurs later during their interaction with the Ag-specific B cells. We (20) and others (22–24) have shown that the uptake and processing of soluble proteins by mature dendritic cells in secondary lymphoid organs are extremely limited, suggesting that their primary role as APC will be to present peptide fragments of Ags that require neither internalization nor degradation. For our studies, we have been particularly stringent about excluding as much contaminating free peptide from our MCC-HEL conjugate as possible, since we have found this to be a problem when trying to interpret some of our earlier studies (15). We have estimated that of the total 100 μg of conjugate Ag being given, <0.01% is available as free peptide. It is possible that the conjugate Ag used by Jenkins’ group has a higher percentage of contaminating peptide, which could explain the involvement of dendritic cells in their model. An alternative explanation is that their use of adjuvant during immunization, as compared with our adjuvant-free approach, favors the involvement of dendritic cells, thus making it difficult to compare directly the two studies. Furthermore, using a transfer system in which numbers of Ag-specific B cells are more limiting than in Ig transgenic mice might also contribute to a more stringent requirement for dendritic cells during T cell priming. Nevertheless, in our current studies, we cannot completely rule out a role for dendritic cells in the observed T cell priming, although the presence of Ag-specific B cells clearly enhances this priming by >3.5-fold.

Following the uptake of cognate Ag by IgR, B cells show a marked up-regulation of B7-2 molecules, an event that was independent of CD40:CD40L interactions (Fig. 7). Studies looking at the role of these interactions have provided evidence for their requirement to activate B cells to progress through the cell cycle (25), to up-regulate cytokine receptors (26) and costimulatory ligands such as B7 (27). While we have no data showing how the proliferation and cytokine receptors of B cells in our study were affected by the absence of CD40L, the initial up-regulation and maintenance of B7-2 molecules were completely unaffected, as has also been demonstrated indirectly in several in vitro studies in which B cells were provided with Ag in the complete absence of any T cells (15, 28). The critical component for this CD40L-independent up-regulation of B7 to occur is clearly the mechanism through which the Ag is being internalized by the B cell. In most of the studies looking at a role for CD40:CD40L interactions during B cell activation, Ag uptake was other than through IgR (reviewed in Ref. 8). Although the presence of CD40L was unnecessary for B7 expression on B cells in our model of immunization, it was certainly necessary for priming of the CD4+ T cells, since we were unable to recover the majority of labeled CD40L-/- T cells transferred into Ag-injected recipients (data not shown). This agrees with reports showing a role for CD40:CD40L interactions in the expansion phase of CD4+ T cells during priming (29). However, the main argument put forward to explain these data has usually been as a failure of the T cells to engage APC and induce up-regulation of costimulatory signals. Our current data would argue instead that, under some circumstances, the role of CD40:CD40L interactions is to provide signals that are more critical to the T cell, possibly by promoting T cell expansion.

Part of the goals of our studies was to establish the sequence and the kinetics of the molecular interactions taking place between Ag-specific T and B cells during an initial encounter with Ag. Based on our results, we suggest that an Ag-specific B cell takes up its cognate Ag via IgR, which in turn induces B7 molecules and antigenic complexes to be expressed within 2–4 h of uptake. Pierce and coworkers have previously shown that this is indeed a sufficient time period for an Ag to be internalized, processed, and presented efficiently (30). Under circumstances in which T cells of the appropriate specificity are not available, the expression of the two ligands will down-regulate, that of B7 being within 24 h of initial up-regulation (Fig. 4). MHC class II/peptide complexes remain longer on the B cell surface (see Fig. 5), but these too eventually become down-regulated by about 48 h (unpublished observations). It is possible that during this window of time, between 24 and 48 h, B cells expressing antigenic complexes in the absence of costimulatory molecules are most likely to induce T cell anergy. However, we have indirect evidence that argues against this. We found that using the 24 h in vivo pulsed Ag-specific B cells as APC to stimulate CD4+ T cells in vitro did not induce a state of anergy. Instead, several groups have reported that the induction of anergy, followed by death, in B cells is dependent on a prolonged occupancy of IgR by Ag, as demonstrated by the transfer of HEL-specific Ig transgenic B cells into HEL transgenic mice, in which there is a continuous exposure to the relevant Ag (31, 32). Interestingly, this very prolonged exposure to the Ag resulted in the maintenance of B7-2 expression for up to 20 h after Ag administration (31). The prolonged expression of B7-2 on the B cells may in itself have contributed to their anergy and subsequent death, since findings using B cells from B7-2 transgenic mice, in which B7 molecules are not able to be down-regulated, also showed elimination, specifically by an immune mechanism requiring the engagement of CD28 on T cells (33). Thus, under more physiologic situations, in which Ag is only available for a limited period and the expression of B7 molecules can be modulated, a fully activated Ag-presenting B cell is only available for up to 24 h after Ag uptake. After that, the B cell most likely reverts to a resting state, where it remains available for a subsequent encounter with cognate Ag.

In the event that a T cell of the correct specificity is available, Ag-specific B and T cells will interact. This results in a maintenance of the expression of B7 (Fig. 6), but, we would postulate, a fast down-regulation in the expression of antigenic complexes (Fig. 9). Studies looking at the rate of Ag internalization through IgR have shown that maximal internalization occurs within 20 min of binding (30), with Ag reported to appear in peptide-loading compartments as early as 15 min after binding (30). These data suggest that, despite antigenic complexes disappearing from the cell surface after 1–2 h, Ag will already have been processed and presented within this time frame. One of the net results of this down-regulation will be an inability of the B cell to interact with other Ag-specific T cells within the vicinity. Instead, the initial T:B cell interaction will continue through various other ligand pairs, including B7:CD28, CD40:CD40L, ICAM-1:LFA-1, and CD48:CD2. Based on the results with the transferred Ag-specific CD4+ T cells (Figs. 2 and 3), the T cells are clearly receiving signals to enter into the cell cycle. What we do not know at this stage in our
studies is whether these cells will go on to form a pool of long-lived memory cells, after which they can be reactivated into cytokine-producing effector cells, or whether they represent a short-lived population of Ag-specific effector cells. Interestingly, Townsend and Goodnow have reported that, following the transfer of Ag-specific B cells loaded with Ag in vitro, Ag-specific T cells undergo a burst of proliferation over a period of several days before almost completely disappearing from the lymphoid pool by 5 days after transfer (34). Unlike Townsend and Goodnow, we do not see any major decreases in the population of Ag-specific CD4+ T cells that have undergone proliferation either at 5 or 7 days after transfer (unpublished observations). We have found that using an in vitro approach for loading B lymphocytes with Ag, as was used by Townsend and Goodnow, induces artificially high levels of costimulatory molecules on the B cells (15), which could account for their ability to induce apoptotic signals to any interacting T cells. Our approach of allowing the B cells to become loaded with Ag in vivo may circumvent this outcome. In future studies, we intend to examine more closely the T cells that remain in the lymphoid pool after undergoing proliferation in vivo to establish their effector and memory status.

One further issue to be addressed is whether the precursor frequency of Ag-specific B cells in vivo is sufficient to initiate primary immune responses. Although the frequency of B lymphocytes of any one specificity is only in the order of 2.5–4 x 10^6 murine lymphoid organs (35), it should be pointed out that, as a result of Ag internalization followed by processing, the complexity of antigenic epitopes made available to the T cell pool by a particular B cell will be increased significantly. In addition, the studies of MacLennan and colleagues looking at the formation of germinal centers by Ag-specific B cells during primary immune responses in rats suggest a requirement of only two to three activated B cells for the initiation of these responses, with germinal center formation visible within 6–7 h after administration of soluble protein Ags (36). They concluded from these findings that cognate T:B cell interactions must be taking place within minutes to hours of Ag introduction, followed by an intense burst of proliferative activity. This is well within the 24-h time frame that Ag is being made available to an Ag-specific T cell by an Ag-loaded B cell. With these criteria in mind, the frequency of Ag-specific B lymphocytes may in fact not be a limiting factor during T cell priming, making a role for these B cells in the initiation of primary immune responses very feasible.

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References


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