Small Splenic B Cells That Bind to Antigen-specific T Helper (Th) Cells and Face the Site of Cytokine Production in the Th Cells Selectively Proliferate: Immunofluorescence Microscopic Studies of Th-B Antigen-presenting Cell Interactions

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Summary

Antigen (Ag)-specific T helper (Th) cells regulate the proliferation and differentiation of Ag-specific B cells by secreting cytokines and by expressing activating receptors like gp39. In vitro, the cytokines and the activating receptors function in an Ag-nonspecific manner. It is unclear, therefore, how Ag specificity is imposed on B cell responses in physiological Th–B cell interactions. Here we studied, at the single cell level, the interactions between cloned Th cells and small splenic B cells, which served as Ag-specific antigen-presenting cells (APCs) to the Th cells. Digital confocal immunofluorescence microscopy of Th–B cell conjugates revealed significant variability in the molecular and cellular properties of these interactions, in spite of the fact that all the interactions in this system were expected to be Ag specific. After 30 h of incubation B cells began to divide, and this process was entirely dependent on the presence of both Th cells and Ag. Immunofluorescence microscopic studies showed that essentially all the mitotic B cells were bound to Th cells and faced the microtubule organizing center (MTOC) in the Th cells where interleukin 4 was highly concentrated. Other B cells that were bound to the same Th cells but were not close to the Th-MTOC remained in interphase. These results provide the first direct structural and functional evidence that the site of interaction of B cells with Th cells affects their immune response. We propose that, during Ag-induced Th–B cell interactions, B cells that are bound facing the Th-MTOC proliferate preferentially because they are the recipients of locally secreted cytokines. In addition, these B cells may interact with newly expressed receptors, which may also be locally inserted into the Th membrane. The polarized delivery of activating molecules towards the Th-bound APCs may impose functional specificity on effector molecules that otherwise are not Ag specific.
B cell proliferation in an entirely Ag and MHC nonrestricted manner (12-14). On the other hand, it is unclear how specific B cell responses can be generated under physiological conditions unless there are additional mechanisms that can restrict the response.

Previous immunofluorescence microscopic observations of Th/APC conjugates revealed several rapid Ag-dependent membrane and intracellular rearrangements in the bound T cells (15). The TCR, CD4 and, most prominently, lymphocyte function-assocated antigen 1 (LFA-1)1, as well as the intracellular cytoskeletal protein talin, cluster at the cell–cell contact area (16, 17). It was proposed that the clustering of TCR and CD4 activates the T cell and is caused by their interaction with the Ag–MHC complex in the APCs (18). The clustering of talin and LFA-1 reflected the conversion of LFA-1 into its high avidity state and stabilizes the Th/ APC interaction (17). Inside the APC-bound T cells the microtubule organizing center (MTOC) was reoriented toward the T/B junction area (19). As the MTOC in other cell systems colocalizes with the Golgi apparatus it was suggested that the rapid and early MTOC reorientation may result in directional secretion of cytokines from the Th cells toward the bound B cells. If APC-activated Th cells can indeed restrict the induction of B cell proliferation by the directed secretion of cytokines, then it would be predicted that in a mixture of Ag-specific Th cells and B-APCs the B cells that would be the recipients of such locally secreted cytokines would preferentially divide. If, on the other hand, every Ag-specific B cell that bound the Th cell would proliferate, it would argue that the ligand of gp39 with CD40 primarily determines the specificity of the response and that either cytokines are not directly secreted or that the mode of secretion has little effect on the proliferation of B cells. To test these possibilities, the induction of B cell proliferation by Th cells was studied at the single cell level using immunofluorescence microscopy. In mixtures of cloned Th cells, specific for processed F(ab')2 fragments of rabbit IgG, and an excess of small splenic B cells that were pulsed with F(ab')2 of rabbit anti-mouse Ig, several B cells were often bound to each T cell. Digital confocal microscopy revealed that the newly synthesized IL-4 colocalized with the Th-MTOC and both were oriented towards only one of the bound B cells. Further microscopic observations indicated that after 30 h of coculture, mitotic B cells appeared. Essentially all of these mitotic B cells were bound to the Th cells, and most significantly, were facing the Th-MTOC. In the same cell conjugates, B cells that were bound to other areas of the Th membrane did not proliferate. These findings indicate that in physiological Th–B cell interactions the specificity of the response is determined by the contact-dependent signals and by the polarized and localized secretion of cytokines towards the same bound APCs that activated the Th cells.

1 Abbreviations used in this paper: LFA-1, lymphocyte function–associated antigen 1; MTOC, microtubule organizing center; RAMG, rabbit anti-mouse Ig.

Materials and Methods

Cells and Antibodies. The CDC35 Th2-cells were obtained from Dr. David Parker (University of Massachusetts Medical School, Worcester, MA) and were maintained as described (20). Small resting B cells (density ≈1.080) from spleens of (BALB/c × A/J)F1, (CAF1) mice were isolated as before (20). Affinity purified rabbit anti–chicken brain tubulin antibodies and rat mAb (1B11I) against IL-4 have been used before (20). Antitabin Abs were generated by injecting guinea pigs with a recombinant murine tain-fusion protein and were affinity purified with a different tain-fusion protein bound to Affigel 15. Tissue culture supernatants of the murine IgG2b mAbs 10-2.16 and H116.32, specific for I-A1, were obtained from Dr. Philippa Marrack (National Jewish Center for Immunology and Respiratory Medicine) and were used undiluted. All affinity-purified and cross-adsorbed secondary antibodies, except the anti–mouse IgG2b (Caltag Lab, S. San Francisco, CA) were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Conjugation and Immunofluorescence Labeling. CDC35 cells were used 2–3 wk after their last stimulation with Ag. The CDC35 cells and the splenic B cells were mixed at a 1:3 T/B cell ratio in the presence of 200 ng/ml F(ab')2 fragments of affinity purified rabbit anti–mouse Ig Abs [F(ab')2-RAMG] and placed in a CO2 incubator. Samples of cells (1–2 × 106 cells) were removed at the indicated times, placed on glass coverslips, which were pretreated with poly-D-lysin, and the cells were fixed with freshly prepared 3% paraformaldehyde. The cells were treated with 0.2% Triton X-100 for intracellular labeling. For digital confocal microscopy the cells were quadruply labeled with guinea pig antitulin (10 µg/ml), rabbit antitubulin (10 µg/ml), rat anti-IL-4, and murine anti-I-A1. The cells were further labeled with Cy-3 tagged donkey anti–guinea pig IgG, Cy-5 tagged donkey anti–rabbit IgG, FITC-donkey anti–rat IgG, and biotinylated-donkey anti–mouse IgG, each at a concentration of 5 µg/ml. The biotinylated Abs were visualized with AMCA-labeled streptavidin (5 µg/ml). In other experiments the cells were labeled with rabbit antitubulin and murine anti-I-A1 as described (20). All of the secondary Abs were passed over columns containing bound IgG from different species to eliminate cross-species reactivities. The cells were monitored with an Axiohoch microscope (Carl Zeiss, Inc., Thornwood, NY), which is equipped with the appropriate optical filter sets (Omega Optical Inc., and Chroma Technology Corp., Brattleboro, VT) that can selectively visualize each label. The Cy-5 dye, which emits in the near infrared, is invisible to the naked eye but is detectable by a peltier-cooled charge coupled device (CCD) (MCD1000; SpectraSource Inst., Westlake Village, CA) camera that is mounted on the microscope. The digital images obtained with the high resolution CCD camera were transferred to an accelerated Quadra 900 (Apple Comp., Cupertino, CA) computer that is linked to a high speed ComputeServer (Torque Systems, Palo Alto, CA), and the out-of-focus haze was analyzed and removed by software that we developed (Monks, C., and A. Kuper, unpublished software). At least 100 cell conjugates with enlarged or dividing B cells were analyzed in each experiment.

Results

Heterogeneous T-B Interactions Are Formed when Cloned Th Cells Are Mixed with Ag-specific APCs. The CDC35 Th2-cells recognize as specific Ag processed F(ab')2 fragments of rabbit IgG that are bound to I-A1 (21). When a polyclonal population of resting splenic B cells that express I-A1 is treated with F(ab')2-RAMG every B cell can serve as an Ag-specific APC to the CDC35 cells (21, 22), which produce IL-4 and...
IL-5 upon activation by APCs (23). Previously, we studied early events that occur within minutes in the Th cells immediately after Th-APC interaction. Now we were interested in later events (h) that lead to the proliferation of the APCs. CDC35 cells were mixed with a threefold excess of small resting B cells from spleens of CAF1 (I-A<sup>d</sup> x I-A<sup>k</sup>) mice in the presence of F(ab')<sub>2</sub>-RAMG. Samples of cells that were processed for immunofluorescence microscopy showed that almost all of the T cells were bound to B cells (not shown). In many of these cell conjugates more than one B cell was bound to a single CDC35 cell; a typical example is illustrated in Fig. 1. Morphologically (Fig. 1 F) the interactions between the CDC35 cells and any of the bound B cells appeared indistinguishable. However, analysis of the intracellular distribution of tubulin, talin, and IL-4 revealed major differences between the interacting cells (Fig. 1). In the example shown in Fig. 1, talin (Fig. 1 C) clustered in the contact area between the CDC35 cell and two of the three bound B cells, differentiating the interaction of these two B cells (labeled 1 and 2 in Fig. 1 F) from the third B cell (labeled 3 in Fig. 1 F). Even the interactions between CDC35 and the B cells labeled 1 and 2 were dissimilar since the Th-MTOC (Fig. 1 A) was oriented toward only one of the B cells (labeled 1). Accordingly, IL-4 (Fig. 1 B), which colocalized with the Th-MTOC (Fig. 1, A and F) was localized in the Th cell juxtaposed to cell contact with the same B cell (labeled 1). These findings demonstrate that even in a mixture of cloned Th cells and Ag-specific APCs the interactions between individual T and B cells vary significantly and may affect differently the responses of these B cells. The ability to identify such interactions at the single cell level was used to find if polarized secretion of cytokines influences the proliferation of B cells during physiological T-B interactions.

**Localization of Mitotic B Cells in Th2-B-APC Conjugates.** Small splenic B cells were mixed with CDC35 cells and Ag as described above. After 10 h of coincubation most of the cells formed large aggregates. To simplify the analysis of these cell clusters they were gently dispersed to dissociate the weaker

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**Figure 1.** Digital confocal immunolocalization of the MTOC, IL-4, talin, and I-A<sup>k</sup> in Ag-induced cell conjugates of CDC35 Th cells and small splenic B cells. CDC35 cells were mixed with a threefold excess of the B cells in the presence of 200 ng/ml F(ab')<sub>2</sub>-RAMG and 8 h later samples were removed and fixed. The cells were labeled with rabbit antitubulin Abs (A), to display the MTOC, rat anti-IL-4 (B), guinea pig antitalin Abs (C), and mouse anti-I-A<sup>k</sup> (D), to identify the B cells. The secondary Abs were donkey anti-rabbit, anti-rat, and anti-guinea pig Ig labeled with Cy5, FITC, and Cy3, respectively. The biotinylated goat antimurine IgG2b was visualized with AMCA-labeled streptavidin. The image in E is a computer generated overlay of the labeling for IL-4 (B), talin (C), and I-A<sup>k</sup> (D), and F shows the Nomarski images of the same cells. The bar in F represents 10 μm. Note the arrow in A pointing to the location of the Th-MTOC, which is facing the B cell that is marked 1 in F. Note also that IL-4 is localized in the Th close to the cell contact with B cell no. 1. Talin is clustered at the cell contacts between the Th cell and B cells no. 1 and no. 2.
Ag-nonspecific interactions while maintaining the stronger Ag-specific contacts. The cells were immunofluorescently labeled with mAbs specific for I-A^k, to identify the B cells, and with rabbit antibodies specific for tubulin. The labeling for tubulin served two purposes; it identified the location of the Th-MTOC, which is the site of cytokine production (Fig. 1) and it identified the mitotic spindle of dividing cells. During the first 20 h of culture essentially all of the bound

Figure 2. Microscopic observations of Th-B cell conjugates after 18 h of coculture. The cells were mixed and labeled with rabbit antitubulin (A and D) and mouse anti-I-A^k (B and E) as described in Materials and Methods. C and F show the Nomarski images of the same cells. The bar in C represents 10 μm. Note that the Th-MTOCs (wide arrows in A and D) face the bound B cells that are still very small. The MTOCs in the B cells (narrow arrows in A and D) are randomly oriented, facing in one B cell the T cell and in the other B cell facing away from the T cell.

Figure 3. Immunofluorescent microscopic analysis of CDC35: splenic B cell conjugates after 30 h of coculture. The cells were treated and labeled with antitubulin (A and D) and anti-I-A^k (B and E) as described in Fig. 2. Note the appearance of large B cells (B, C, E, and F) as well as small B cells. The large B cells were bound to the Th cell and were facing the Th-MTOC (wide arrows in A and D). The small B cells were either unbound (B and C) or bound to the Th cells but not facing the Th-MTOC (D-F). As in Fig. 2 the MTOCs in the B cells (narrow arrows in D and F) are randomly oriented, facing in one B cell the T cell and in the other B cell facing away from the T cell.
B cells remained small and viable (Fig. 2). By contrast, >80% of the B cells died when cultured for 20 h with CDC35 cells, but without Ag (not shown). Bigger B cells were first seen after 22 h of culture (as in Fig. 3). Essentially all (>90%) of the enlarged B cells were bound to CDC35 cells, whereas the unbound B cells remained small (as in Fig. 3, A–C). Binding to the CDC35 cells was not by itself sufficient to cause changes in cell size since not all bound B cells were enlarged even after 30 h (Fig. 3, D–F). Significantly, the enlarged B cells were bound facing the Th-MTOC, whereas the small B cells were bound at other sites (Fig. 3). Mitotic B cells were first observed after 32 h (Fig. 4) and essentially all (>90%) of them were in contact with CDC35 cells. Even in multicellular complexes, only one dividing B cell was seen in each cell conjugate (Fig. 5). Distinctly, the mitotic B cells were bound facing the CDC35 MTOC (Fig. 5). The induction of the CAF1 B cell proliferation was strictly Ag and MHC specific. It was not observed in the absence of either CDC35 cells or specific Ag. In addition, incubation of small B cells from spleens of A/J (I-Ak) mice with CDC35 cells and Ag failed to cause the proliferation of the B cells (not shown).

The interaction of the B cells with the CDC35 Th cells seemed to weaken when the B cells progressed through mitosis (Fig. 6, A–F). Excessive dispersion of the cells separated many dividing B cells from the CDC35 cells whereas interphase B cells tended to remain bound. When mitotic B cells dissociate from the Th cell the MTOC in the Th cell was oriented toward the other bound, but still nondividing, B cells (Fig. 6, G–I).

Discussion
The induction of proliferation of small splenic B cells by cloned Th2 cells was studied here at the single cell level. In this cell system the interactions between the Th cells and the B cells were Ag specific since the Th cells were specific to processed F(ab')2-rabbit Ig and the B cells were pulsed with F(ab')2-RAMG. It was surprising therefore that, at the single cell level, the interactions between individual Th and B cells varied greatly. Based on the immunofluorescence labeling, there were three types of cellular interaction. In the first, the cytoskeletal protein talin and the Th-MTOC were rearranged at the region of cell contact; in the second, talin, but not...
Figure 5. Immunolocalization of dividing B cells in multicellular complexes of T and B cells. The cells were labeled after 32 h of coculture with antitubulin (A, D, and G) and anti-I-Ak (B, E, and H). In each of the three conjugates shown here a single CDC35 cell is bound to two B cells. In each of these cell conjugates one of the bound B cells (the larger cell at the right side) is dividing while the other bound B cell is still in interphase. Note that the Th MTOCs (wide arrows in A, D, and G) are facing the mitotic B cells.

The Th-MTOC, was rearranged; and, in the third, neither talin nor the Th-MTOC were rearranged. The reasons for the formation of such heterogeneous cell conjugates in a mixture of Ag-specific Th and B cells remain uncertain. In previous studies, in which both the APCs and the Th cells were cloned cell lines, each of the above mentioned interactions could be selectively induced at the appropriate concentration of Ag (17). In the absence of Ag or in the presence of very low concentrations of Ag, the TCR was not engaged and it could not trigger the rearrangements of either talin or the Th-MTOC. Engagement of a small number of TCRs was sufficient to induce the clustering of talin, and an optimal activation of the Th cells by a larger number of TCRs was required to induce also the reorientation of the MTOC (17). In the present studies all the B cells were pulsed with the same optimal concentration of Ag and they still varied in their interactions with the CDC35 cells. One likely explanation is that this phenomenon was caused by the temporal order of interactions between the cells. According to this scheme, the first B cell that bound a Th cell engaged and clustered enough TCR, CD4, and LFA-1 (17) to cause the rearrangement of talin and of Th-MTOC towards that B cell. When a second B cell, which may express at least as much processed Ag as the first B cell, bound the same Th cell, it could only engage fewer available TCR molecules, which would suffice to cluster LFA-1 and talin but would not suffice to reorient the MTOC (17). Since most receptors in the Th cells would be already engaged with the earliest bound B cells, interactions with additional B cells might not engage enough receptors to cause even the clustering of talin and LFA-1. It is also possible that after the first B cell bound and activated the Th cell, resulting in the reorientation of the MTOC and clustering of talin, the activated T cell would bind additional B cells independent of the Ag that they present.

Regardless of the mechanisms responsible for the generation of such different cellular interactions between Ag-specific B and T cells, we have used this diversity to study the possible role of polarized secretion of Th-derived cytokines in
Figure 6. Immunofluorescence observations of Th/B cell conjugates after 36 h of incubation. The cells were labeled for tubulin (A, D, and G) and I-Ak (B, E, and H) as before. The three dividing B cells shown here appear to be in the final stages of mitosis (note the clustering of I-Ak at the cleavage furrow; arrows in B, E, and H), and seen to dissociate from the Th cells. Note that in G–I the dividing B cell is no longer bound to the Th cell and the Th MTOC (wide arrow in G) is facing the bound B cell that is still small.

restricting the induction of B cell proliferation. Secreted proteins, including cytokines, are processed and packaged into secretory vesicles in the Golgi apparatus, which colocalizes with the MTOC (24). The intracellular location of the Th-MTOC should accordingly determine the site of synthesis and packaging of Th-derived cytokines. Indeed, IL-4 colocalized with the MTOC in the CDC35 cells. The expression of IL-4 and IL-5 in the CDC35–B cell conjugates was spatially and temporally regulated and in all cases the cytokines colocalized with the Th-MTOC (Kupfer, A., unpublished results). A similar colocalization of IL-2 and the Th-MTOC was reported in Th1 clones (20). In CDC35–B cell conjugates the Th-MTOC was always oriented towards only one of the bound B cells, unlike talin, which was often clustered in the cell–cell contact sites of several B cells that were bound to the same Th cell. If such orientation of the Th-MTOC would result in polarized secretion of cytokines then one might expect that not all the Th-bound B cells would be similarly induced to proliferate. Instead, the Th-bound B cells that face the Th-MTOC should preferentially proliferate. Our immunofluorescence microscopic observations clearly detected such preferential proliferation. These results together with the findings that blocking anti-IL-4 and IL-5 mAbs failed to inhibit the proliferation of the bound B cells (Kupfer, A., and H. Kupfer, unpublished data) suggest that the polarized and directed secretion of cytokines may strongly influence the specificity of B cell proliferation. In interpreting these observations it is important to note that the reorientation of the MTOC may direct not only the secreted cytokines but also the insertion of new membrane proteins. In other cell systems it was shown that the intracellular location of the Golgi apparatus/MTOC determines the site of membrane insertion of newly synthesized membrane glycoproteins (24). In Th cells, proteins like gp39, which are transiently expressed after TCR activation, may also be locally inserted into the Th plasma membrane near the Th-MTOC. It is possible therefore that the selective proliferation of B cells that are bound facing the Th-MTOC may be caused by a combined effect...
of a high local number of receptor–ligand interactions, including CD40–gp39, and a higher local concentration of secreted cytokines.

The duration of the interaction between Th and B cells is another important issue that is still unsettled. The findings reported here are consistent with the notion that the Th and B cells remained stably bound since the early stages of the coculture, but such lengthy interaction was not directly proven here. Cellular interaction of T and B cells are mediated primarily by the ligation of TCR-activated LFA-1 and intercellular adhesion molecules (ICAMs) (25). When T cells are activated with anti-CD3 mAbs the high avidity state of LFA-1 is very transient, lasting <30 min (26). The half-life of conjugates between B cells and T cell hybridoma is reported to be 4 h (27). On the other hand, anti-CD2 (28) or anti-class II MHC mAbs (29) induce a persistent and stable activation of LFA-1-dependent cell adhesion. The interaction of gp39 with CD40 increases the expression of ICAM-1 and of B7 and triggers persistent LFA-1-dependent cell interactions (6, 30). In addition, soluble IL-4 also enhances the binding of T and B cells (31). Finally, it was reported that continued cell contact via adhesion molecules induces B cells to respond to lymphokines and to proliferate (3). Unlike the anti-CD3-induced cell interaction, physiological interactions are regulated by multiple receptors and the regulatory mechanisms mentioned above suggest that Ag-specific interactions of T and B cells may be stable and even strengthen after the initial contact. If this is indeed the case then it is likely that the B cells that were bound to the Th cells after 30 h of coculture were the same B cells that bound the Th cells at the beginning of the culture. Once the B cells become mitotic they seem to detach from the Th cells. Although we have not studied the avidity of LFA-1 in such cells it is possible that LFA-1 reverted into its resting low avidity state. Fibroblasts in interphase are stably bound to extracellular matrix proteins through their integrin adhesion receptors; but mitotic fibroblasts round up and easily detach from the substratum (Kupfer, A., unpublished data). It is possible, but untested, that during mitosis the interaction between adhesion receptors and the cytoskeleton are similarly affected in lymphocytes.

The high degree of specificity and selectively of B cell activation and proliferation by Th cells, reported here, seems to be at odds with published reports that, in mixtures of Ag-specific Th and B cells, bystander B cells also proliferate, albeit less efficiently than the specific APC (4, 32). In preliminary studies we have not detected the proliferation of bystander B cells during the first 32 h of culture but minor bystander proliferation was clearly observed after 40 h (Kupfer, A., unpublished data). The molecular basis for the apparent breakdown in the specificity of the B cell's response during the latter stages is now under investigation. It is interesting to note that the Ag-specific and the bystander B cells were bound to the CDC35 cells. In addition, even high concentrations of anti-IL-4 and anti-IL-5 mAbs failed to inhibit either the rate or the extent of proliferation of bystander and specific B cells, further supporting the notion of directed cytokine secretion (Kupfer, A., unpublished data).

In summary, these studies at the single level imply that in Ag-specific Th–B cell interactions the Th and B cells remain bound long enough for the Th cells to produce and directly secrete cytokines towards the bound B cells. By stably binding the B cells and by providing high local concentrations of cytokines and signaling receptors the Th cells can impose functional Ag specificity on the cytokines and on receptors that by themselves are not Ag specific.

We thank David Parker for providing us with the CDC35 cells.

This work was supported by National Institutes of Health grant AI-23764.

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Received for publication 19 October 1993 and in revised form 6 January 1994.

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