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Immunology

Polarized expression of cytokines in cell conjugates of helper T cells and splenic B cells

(cell differentiation/cell–cell interaction/directed secretion/double immunofluorescence microscopy)

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ABSTRACT **We describe the intracellular localization, by double immunofluorescence microscopy, of four cytokines that were produced during the prolonged interaction of cloned helper T cells with resting splenic B cells. When two rabbit immunoglobulin-specific helper-T-cell clones were mixed, either separately or together, with splenic B cells in the presence of the antigen rabbit anti-mouse immunoglobulin antibodies, stable T-cell–B-cell conjugates were seen up to 29 hr later. Microscopic observations of these cells revealed that interferon γ and interleukin 2, inside one of the T-cell clones, and interleukins 4 and 5, inside the other T-cell clone, were concentrated very close to the T-cell–B-cell contact area. The cytokines were not seen in the T cells prior to their interaction with the B cells and their production was strictly antigen-specific. These studies show, at the single-cell level, that helper-T-cell clones can remain bound to splenic B cells long enough for the T cells to produce cytokines, which are synthesized near the bound B cells. We propose that the polarized synthesis of the cytokines may result in their directed secretion toward the bound B cells. By locally secreting the cytokines, which are not antigen-specific, at the contacting T-cell–B-cell membranes, where T- and B-cell surface receptors are engaged and clustered, the helper T cells can induce selective and specific B-cell responses.**

The introduction of proteinaceous foreign antigens (Ags) into an animal generally results in the proliferation and differentiation of B cells that then secrete antibodies (Abs), which bind specifically to the foreign Ags. The B-cell response is largely controlled by soluble growth and differentiation factors, collectively termed lymphokines or cytokines (1, 2), which are produced by Ag-specific helper T cells (T_h cells). It is unclear yet how selective and regulated B-cell responses can be generated by the secretion of cytokines such as interferon γ (IFN-γ) and interleukins 2, 4, and 5 (IL-2, IL-4, and IL-5, respectively), since these cytokines are not Ag-specific or even cell-type-specific (2). In addition, each of these cytokines can have multiple effects on the cells and when present together they can either antagonize or synergize each other (3). However, the addition of crude or purified cytokines to resting B cells in the absence of T_h cells failed to cause B-cell proliferation and differentiation, implying that specific cell–cell interactions may be additionally required for effective B-cell responses (4–7). The molecular events that take place during such T_h-cell–B-cell (T_h–B) interactions are still largely unknown.

We have described an *in vitro* model system to study at the single-cell level events that occur within the first minutes of the interaction of cloned T_h cells and Ag-presenting B-cell (B-APC) lymphomas or hybridomas (8). By using immunofluorescence microscopy, we observed the clustering of the T-cell receptors (TCRs) for Ag, CD4, and lymphocyte function-associated antigen 1 (LFA-1) membrane receptors along with the cytoskeletal protein talin into the T_h–B contact area and the reorientation of the microtubule-organizing center (MTOC) inside the T_h cell to face the bound B-APC (9–12). It was also shown that the formation of Ag-specific cell–cell interactions and the reorientation of the MTOC were not tightly coupled since specific T_h–B-APC conjugates can form without causing this reorientation in the T_h cells (12). The MTOC is the nucleation site for the cytoplasmic microtubules and as such it controls the structural polarity of the cells. It was proposed (8) that the rapid MTOC reorientation, which is seen immediately after the T_h–B-APC interaction, may be responsible for directing the secretion of cytokines, which are expected to be produced by the T_h cells several hours later, toward the bound B cells to cause the selective activation of these cells. As a test of this hypothesis we have studied, at the single-cell level, the localized expression of four cytokines inside cloned T_h cells that interacted specifically and stably with resting splenic B cells. T_h–B couples were seen up to 29 hr after cell mixing. In these cells newly synthesized IFN-γ, IL-2, IL-4, and IL-5 were concentrated inside the T_h cells close to the contact area with the B cells. The implications of these findings on the mechanisms of B-cell proliferation and differentiation are discussed.

MATERIALS AND METHODS

Cells and Abs. The CDC35 (13) and D1.6 (14) cloned T_h lines were obtained from David Parker (University of Massachusetts Medical School, Worcester). The cells were maintained by stimulation with Ag and irradiated splenocytes from (BALB/c × A/J)F_1 (CAF1) mice every 2 weeks as described (13). Small resting B cells from spleens of CAF1 ($IA^d \times IA^k$) mice were purified essentially as described (15). Briefly, after the depletion of erythrocytes and T cells, the remaining cells with a density >1.080 were collected on a Percoll (Pharmacia) discontinuous gradient. Tissue culture supernatants of the murine monoclonal Abs (mAbs) 10-2.16 and 116.32, specific for IA^k, were obtained from Philippa Marrack (National Jewish Center for Immunology and Respiratory Medicine, Denver, CO). The following rat mAbs were used: XMG1.2 (16), specific for IFN-γ; S4B6 (17), specific for IL-2; 11B11 (18), specific for IL-4; and TRFK-4 and TRFK-5 (19), both specific for IL-5. The mAbs were purified on protein G-Sepharose columns (Pharmacia–LKB). In some experiments affinity-purified rabbit anti-murine IFN-γ Abs (16) were used. The biotinylated and rhodamine-conjugated

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Abbreviations: Ag, antigen; Ab, antibody; mAb, monoclonal Ab; T_h cell, helper T cell; IFN-γ, interferon γ; IL-2, -4, and -5, interleukin 2, 4, and 5; B-APC, antigen-presenting B cell; TCR, T-cell receptor; MTOC, microtubule-organizing center; $(Fab')_2$-RAM, $(Fab')_2$ fragments of rabbit anti-mouse IgG; GA, Golgi apparatus; LFA-1, lymphocyte function-associated antigen 1.

$F(ab')_2$ fragments of goat or donkey anti-rat, -mouse, or -rabbit IgG as well as the unconjugated $F(ab')_2$ fragments of rabbit anti-mouse IgG [$F(ab')_2$-RAM] and fluorescein-conjugated streptavidin were purchased from Jackson ImmunoResearch. All the Abs that were used as secondary reagents for immunofluorescence labeling were further purified by passage through the appropriate columns packaged with solid-phase-bound rat, mouse, or rabbit IgG to eliminate cross-species reactivities.

Cell Conjugation and Immunofluorescence Microscopy. The T_h cells were used at least 2 weeks after their last stimulation with Ag. The T_h cells were mixed with splenic B cells without centrifugation, at a ratio of 1:1.5 to 1:2, in the presence of $F(ab')_2$-RAM (200 ng/ml). At the indicated times, 2–3 × 10^5 cells were removed, plated on glass coverslips that were treated with poly(D-lysine), and fixed with 3% (wt/vol) paraformaldehyde (14 min at room temperature). The cells were doubly labeled first with the murine anti-IA^k mAbs (1 hr) to identify the B cells and then treated for 5 min with 0.2% Triton X-100 in isotonic phosphate-buffered saline (pH 7.5), followed with the rat anti-cytokine mAbs (1 hr) at the following concentrations: S4B6, 20 μg/ml; XMG1.2, 10 μg/ml; 11B11, 40 μg/ml; TRFK-4 or TRFK-5, 10 μg/ml. In some experiments the cells were doubly labeled with the rabbit anti-IFN-γ Abs and rat anti-cytokine mAbs. After 5 washes with phosphate-buffered saline, the cells were treated (1 hr) with mixtures of the above-listed secondary reagents, each at a concentration of 10 μg/ml. To prevent nonspecific labeling, all the Abs were diluted into Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum and normal donkey and goat γ-globulins (100 μg/ml). The fluorescent labeling was observed with a Zeiss Axiophot microscope. Kodak Tri-X film was used for photography. Each experiment was repeated three times and at least 200 cell couples were analyzed per coverslip.

RESULTS

The CDC35 and D1.6 cloned T_h lines were originally developed by David Parker and coworkers (13) and Abul Abbas and coworkers (14) to study the polyclonal activation of normal B cells by T_h cells. These T_h cells recognize processed

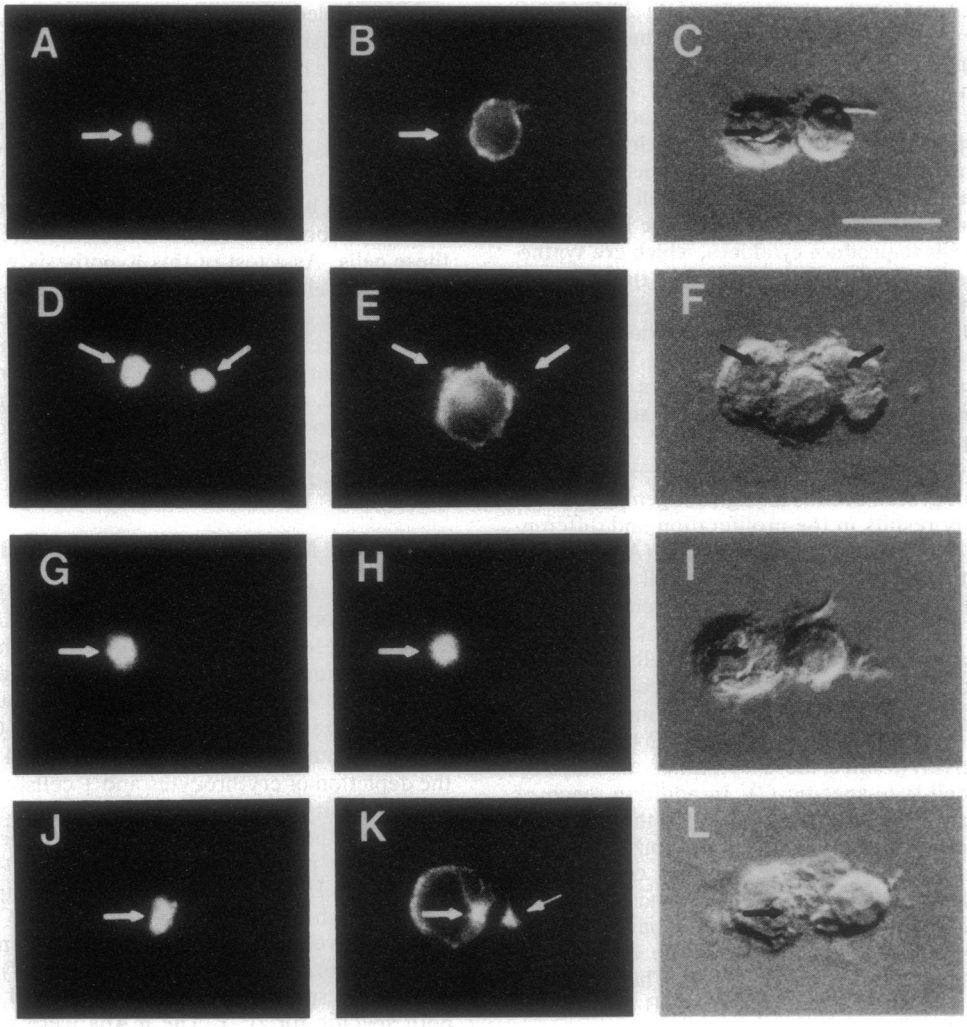

FIG. 1. Intracellular localization of the cytokines IL-2 (*A*, *G*, and *J*) and IFN-γ (*D* and *H*) by double immunofluorescence microscopy, in Ag-specific T_h–B conjugates of D1.6 cells and CAF1 splenic B cells, 18 hr after cell mixing. In Figs. 1, 3, and 4, each cell conjugate is depicted in a row of three micrographs. Micrographs to the right (*C*, *F*, *I*, and *L*) were made using Nomarski optics. (*A–F*) Cells were double labeled with murine mAbs specific for IA^k (*B* and *E*) to identify the B cells and with rat mAbs specific for IL-2 (*A*) or IFN-γ (*D*). (*G–I*) Cells were doubly labeled with the rat mAb specific for IL-2 (*G*) and with affinity-purified rabbit Abs specific for IFN-γ (*H*). (*J–L*) Cells were doubly labeled with the rat mAb specific for IL-2 (*J*) and with affinity-purified rabbit Abs specific for chicken brain tubulin (*K*) (9) to display the MTOC. Note that IL-2 colocalizes with the D1.6 MTOC (large arrow in *K*) but not the B-cell MTOC (small arrow in *K*). The arrows in each row point to an identical imaginary spot to assist in identifying the spatial correlations of the three consecutive pictures from the same cells. Note that IL-2 (*A* and *G*) is localized in the D1.6 cells (but not the B cells) by the T–B contact site and that IL-2 (*G*) colocalizes with IFN-γ (*H*) in the same cell. (*D–F*) Cell conjugate of two D1.6 cells bound to one B cell (*E*); note that IFN-γ (*D*) in both T cells is polarized toward the B cell. (*C*, bar = 10 μm.)

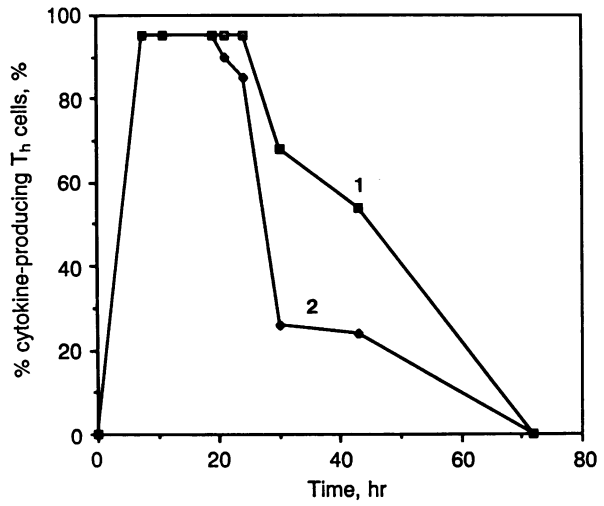

FIG. 2. Immunofluorescence microscopic studies of the effect of the duration of the coculture of D1.6 and B cells on the number of D1.6 cells in D1.6–B conjugates that produce IFN-γ (curve 1) or IL-2 (curve 2). Cell conjugation and labeling were as described in Fig. 1. This analysis at the single-cell level shows the fraction of B-cell-bound T_h cells that produce detectable amounts of cytokines and not the accumulating concentrations of the secreted cytokines in the medium.

$F(ab')_2$ fragments of rabbit IgG on APCs that express surface IA^d molecules. Treatment of normal B cells with low concentrations of $(Fab')_2$-RAM results in the polyclonal binding of the rabbit Abs to immunoglobulins on the surface of the B cells followed by internalization and processing of the rabbit Abs. Small splenic B cells, purified from CAF1 ($IA^d \times IA^k$) mice, when treated with $(Fab')_2$-RAM can serve as specific APCs to the CDC35 and the D1.6 T_h cells (13, 14). D1.6 and CDC35 cells were classified as T_h1 and T_h2 (20), respectively, since D1.6 cells secrete IL-2 and IFN-γ, whereas CDC35 secrete IL-4 and IL-5. When resting D1.6 or CDC35 cells were immunofluorescently labeled with any one of the anti-cytokine Abs, the cells did not display any specific labeling, indicating that these cytokines are not produced or stored inside resting T_h cells. Similarly, we could not detect any cytokine-labeling when the T_h cells were incubated for 18 hr with small B cells in the absence of Ag. When the specific Ag [$(Fab')_2$-RAM, 200 ng/ml] was added to mixtures of D1.6 cells and B cells, practically all (≥95% ± 5%) of the D1.6-cell–B-cell conjugates displayed, 18 hr later, bright intracellular labeling in the D1.6 cells (but not the B cells) for IFN-γ and IL-2 (Figs. 1 and 2) and, as expected, did not show any labeling for IL-4 or IL-5 (see Fig. 4). Inversely, in essentially all the CDC35-cell–B-cell couples the CDC35 cells were strongly labeled for IL-4 (96 ± 4%) and IL-5 (82 ± 5%) (Fig. 3) but were unlabeled for IFN-γ and IL-2 (see Fig. 4). Immunofluorescence labeling in the T cells was strictly Ag- and IA^d-specific, since changes or omissions of either one resulted in completely unlabeled cells (not shown). The specificity of the labeling is shown also in Fig. 4. When CDC35 and D1.6 cells were simultaneously mixed with B cells in the presence of Ag, the IL-4- or IL-5-producing cells (CDC35) were not labeled for IFN-γ and, inversely, the IFN-γ-producing cells (D1.6) were not labeled for IL-4 or IL-5. These results demonstrate also the potential usefulness of the immunofluorescence-labeling technique in detecting a single cytokine-producing cell even in the presence of cells that produce other cytokines. In addition, a control rat mAb against β-galactosidase (GL113) from *Escherichia coli* followed by the same secondary Ab reagents that were used for the labeling of the cytokines failed to stain the cells, further confirming the specificity of the labeling. The location of the cytokines inside the T_h–B complexes is particularly interesting. As seen in Figs. 1, 3, and 4, the cytokines were concentrated in the T_h cells next to the T_h–B contact area. This polarized localization was seen in essentially all (95 ± 5%) the cytokine-producing cell couples.

DISCUSSION

To understand how Ag-specific B-cell responses can be generated by the secretion of Ag-nonspecific cytokines, we localized at the single-cell level four cytokines, IFN-γ, IL-2, IL-4, and IL-5, inside cloned T_h cells during their specific and prolonged interactions with resting splenic B cells. In previous studies of the interactions of cloned T_h cells and B-APCs, we detected the rapid formation of T_h–B-APC couples and the reorientation of the MTOC inside the T_h cell toward the B-APC (9, 10). In other cell systems, the MTOC was colocalized with the Golgi apparatus (GA) (21). The GA is the organelle where the final processing of secretory proteins occurs and where these proteins are packaged into secretory

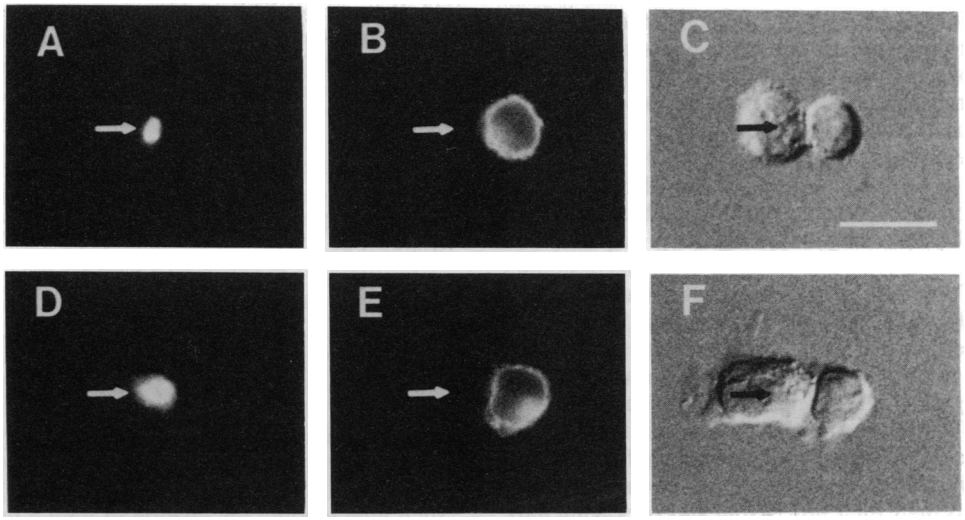

FIG. 3. Intracellular localization of the cytokines IL-4 (*A*) and IL-5 (*D*) in Ag-specific T_h–B couples of the CDC35 T_h cells and CAF1 splenic B cells, 18 hr after cell mixing. The cells were doubly labeled for IA^k (*B* and *E*) to identify the B cells and with rat mAbs specific for IL-4 (*A*) or for IL-5 (*D*). Note the concentration of IL-4 and IL-5 inside the T_h cells juxtaposed to the B cells. (*C* and *F*) Nomarski optics. (*C*, bar = 10 μm.)

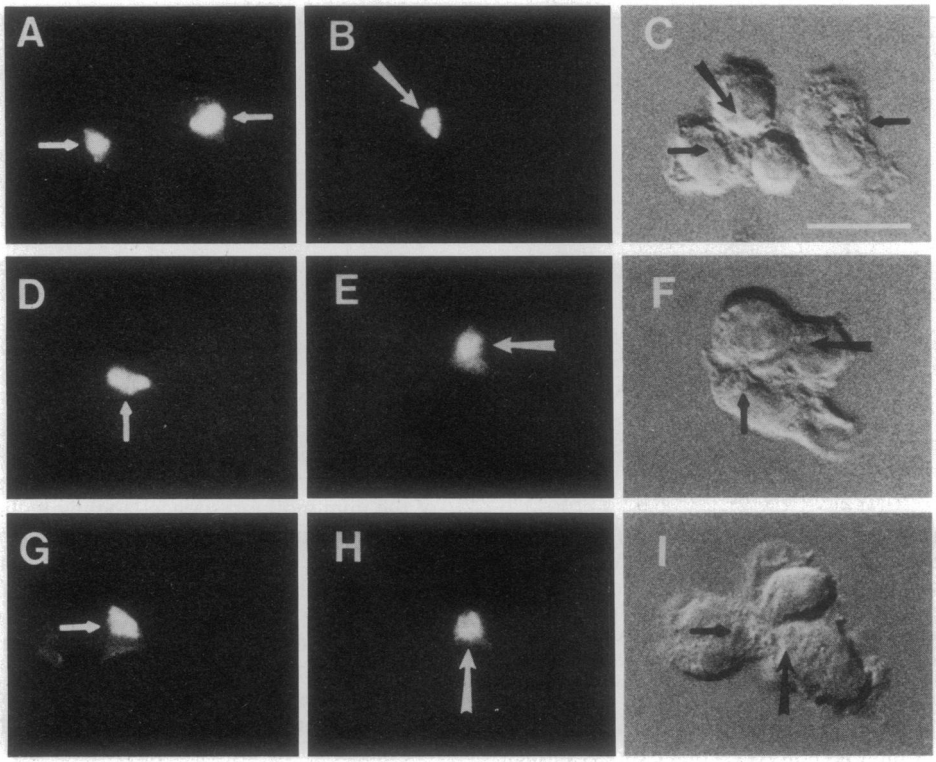

FIG. 4. Simultaneous immunolocalization of IFN-γ (*A*, *D*, and *G*) and IL-4 (*B*) or IL-5 (*E* and *H*) in cell conjugates of CAF1 splenic B cells bound to D1.6 and CDC35 T_h cells. The splenic B cells were mixed in the presence of Ag with D1.6 and CDC35 cells at a 2:1:1 cell ratio and samples were taken 16 hr (*A–C*), 22 hr (*D–F*), or 29 hr (*G–I*) later and doubly labeled with rabbit anti-IFN-γ Abs (*A*, *D*, and *G*) and rat mAbs against IL-4 (*B*) or IL-5 (*E* and *H*). The smaller arrows point to the location of IFN-γ and the larger arrows to the location of IL-4 or IL-5. (*D–I*) Each of the cell triplets contains one IFN-γ- and one IL-5-producing cell. The cells that were not labeled for cytokines were B cells. The identity of the B cells was confirmed by triple-immunofluorescence labeling with anti-IA^k Abs (not shown). Note that both cytokines face the T_h–B contact. (*A–C*) Cell complex contains three cytokine-producing cells. Note that the immunolabeling for IFN-γ in the cell to the right is facing away from the T_h–B contact but that IL-4 and IFN-γ in the two other T_h cells are polarized toward the central B cell (see *Discussion*). (*C*, *F*, and *I*) Nomarski optics. (*C*, bar = 10 μm.)

vesicles on their route to the plasma membrane (22); the location of the GA is thought to determine the site of secretion at the cell surface (23). We proposed (8) that the reorientation of the MTOC, and presumably its associated GA, that was seen at the initiation of the T_h–B-APC interaction may be responsible for the directed secretion of cytokines, which will occur hours later, from the T_h cells to the B cells. It was assumed that the T_h–B contact and the MTOC/GA reorientation would be maintained long enough for the T_h cells to produce sufficient amounts of the cytokines. It was, of course, possible that the early MTOC orientation was transient and would not determine the site of cytokine production. It was also possible that the rapid and early T_h–B conjugation, which resulted from the ligation of the TCRs as well as other receptors, would suffice to initiate the production of cytokines and further cell–cell contact would no longer be necessary, resulting in the dispersion of the T_h–B conjugates shortly thereafter (24). In this case too, the early MTOC reorientation would not affect the later secretion of cytokines. Herein we observed that most of the T_h and B cells were found in cell conjugates for up to 29 hr after cell mixing (Fig. 4). Most strikingly throughout this entire time IFN-γ, IL-2, IL-4, and IL-5 were found concentrated inside the T_h cell next to the T_h–B contact site (Figs. 1–4). This intracellular polarized localization reflects, most likely, the site in the GA where the newly synthesized cytokines undergo their final processing and packaging into secretory vesicles since the cytokines were colocalized with the T_h cell MTOC (Fig. 1 *J–L*). The finding that the cytokines could not be detected intracellularly, either before the T_h–B interaction or after 72 hr of cellular interactions (Fig. 2), indicates that the cytokines are synthesized and secreted upon activation and they are not stored in the T_h cells for later use.

The polarized production of cytokines as seen here is likely to result in their directed secretion toward the Ag-specific B cells. Recently splenic B cells, which were bound to the T_h cells next to the site of the production of IL-4/IL-5 in the CDC35 T_h cells, were found to be preferentially activated (A.K., unpublished results), strongly supporting the hypothesis of polarized cytokine secretion. It is interesting to compare these results with another study in which the polarized secretion of IL-4 was investigated in an entirely artificial system (25). There, instead of a physiological Ag, the cloned T_h cells were placed with an activating anti-TCR mAb on one side of a millipore filter and 16 hr later the media from both sides of the filter were collected separately and tested for IL-4 activity. Evidence for polarized secretion was found only when the T_h cells were activated suboptimally with very low concentrations of the mAb. But when the T_h cells were maximally activated with higher concentrations of the mAb this polarity was completely lost. In our studies the B cells maximally activated the two T_h clones (not shown), yet the cytokines were localized toward the T–B contact site (Figs. 1–3). This apparent difference may arise from the fact that the activation of the T_h cells by their physiological Ag, the B cells, involves the ligation of not only the TCRs but also additional receptors such as CD4 and LFA-1 as well (8). In such interactions, the ligation of only a fraction of the total number of TCRs and the concomitant ligation of other receptors, which can deliver additional signals, may be sufficient to fully activate the T_h cells and result in the polarized production and secretion of cytokines.

The *in vitro* mixing of Ag-specific T_h and B cells resulted in the formation of 1:1 T_h–B couples and also more complex cell conjugates (as in Fig. 4), the frequency of which was dependent on the initial T_h-cell/B-cell ratio (data not shown, but see ref. 26). When two T_h cells were bound to one B cell (Fig. 1 *D–F* and Fig. 4 *D–I*), the polarity of cytokine production in each T_h cell was maintained as in 1:1 T_h–B couples. But in about 60% of the more complex conjugates, which had three T_h cells bound simultaneously to one B cell (as in Fig. 4 *A–C*), IFN-γ, and presumably IL-2, in one of the T_h cells was not localized toward the bound B cell. What may be the cause for this lack of polarity?

When cloned IL-2 producing T_h cells were mixed with B-APCs in the presence of very low concentrations of Ag, they formed cell couples in which LFA-1 and talin were concentrated into the T_h–B contact area but the T_h-cell MTOC failed to reorient (12). Such interactions failed also to induce the proliferation of the T_h cells (12), but the production of cytokines was not determined. It was proposed that the engagement of a very small number of TCRs was sufficient to fully activate the LFA-1/talin cell–cell adhesion mechanisms but that additional signals, generated at higher levels of engagements of the TCR and possibly CD4, were needed to cause the MTOC reorientation and T-cell proliferation. It is possible that the signaling for the production of cytokines is more closely linked to the signaling pathways that activate the LFA-1/talin adhesion mechanisms and, at low Ag concentrations, cytokine production can be uncoupled from the MTOC/GA reorientation. In the absence of such a reorientation, the cytokines may be secreted randomly into the surrounding medium. In the multicellular complexes, the number of Ag ligands on the surface of a B cell may have been sufficient to induce T_h–B binding and cytokine production in all the T_h cells, but not sufficient to cause the MTOC/GA reorientation in all the bound T_h cells. Indeed, recent studies (A.K., unpublished results) indicate that D1.6–B couples formed in the presence of very low concentrations of Ag [$F(ab')_2$-RAM at 5 ng/ml] produced IL-2 and IFN-γ, but the cytokines were not localized at the T_h–B contact site in about 50% of the cell couples. It is of interest to determine whether *in vivo* immune responses involve the formation of 1:1 or more complex T_h–B conjugates.

In summary, the immunofluorescence studies reported here suggest that in response to an optimal antigenic stimulation, T_h cells direct the secretion of cytokines toward the Ag-specific B cells to which they are bound. Even if not all of the secreted cytokines reach the bound B cells and some of the secreted cytokines are released into the surrounding medium, the bound B cells have the advantage of encountering very high local concentrations of these cytokines. In addition, during the T_h–B interaction, receptors on the T-cell membrane are clustered into the T_h–B contact area (8, 11, 12) and it is likely that their ligands on the B cells are concomitantly clustered into the same contact area. Such clustering can deliver activating signals into both cells, resulting, for example, in the up-regulation of cytokine receptors that may then be enriched in the contact area, further favoring the activation of the bound B cells. The combined effects of the localized receptor engagements and the directed secretion of cytokines may be the mechanism by which T_h cells can generate specific B-cell responses.

Finally, the ability to detect and localize simultaneously multiple cytokines at the single-cell level can be applied also to study the regulation *in vivo* of cytokine production in freshly prepared normal T cells and frozen sections of lymphoid organs.

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