Cholera Toxin Discriminates Between T Helper 1 and 2 Cells in T Cell Receptor-Mediated Activation: Role of cAMP in T Cell Proliferation

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Summary

CD4+ T helper (Th) clones can be divided into interleukin 2 (IL2)-secreting Th1 and IL4-secreting Th2 cells. We show in the present report that these two Th subsets have different activation requirements for lymphokine production and proliferation: namely, cholera toxin (CT) as well as forskolin inhibit T cell receptor (TCR)-mediated IL2 production and proliferation in Th1 cells, while the same reagents fail to block IL4 production and proliferation in Th2 cells. In addition, CT and forskolin differentially influence the proto-oncogene mRNA expression in Th1 vs. Th2 cells after stimulation with Con A. Since both reagents lead to elevated levels of intracellular cAMP, it is likely that Th1 and Th2 cells differ in their sensitivity to an increase in cAMP. Our results indicate that the two Th subsets use different transmission signal pathways upon TCR-mediated activation.

Mature T cells are functionally specialized for recognition of antigen in the context of MHC gene products (1). They can be divided into class I-restricted CD8+ cytotoxic cells and class II-restricted CD4+ Th cells (2). Recently, in the murine system, two types of CD4+ Th cells have been described that differ in their autocrine growth factor and other lymphokine production. Namely, Th type I cells (Th1) make IL-2 and IFN-γ, and Th type II cells (Th2) secrete IL-4, IL-5, and IL-6 upon activation (for review, see reference 3). In addition, only Th2 cells are high expressors of the 80-kD IL-1-R, which has recently been cloned (4). While both types of cells are able to help B cells to secrete antibodies, the nature of this help is quite different. IL-4 induces selective isotype switching to IgG1, IgE, and IgA, while IFN-γ counteracts IL-4 and leads to IgG2a secretion (5). In addition, IL-4 increases class II expression on B cells, while IFN-γ blocks that induction (6).

Several lines of evidence indicate that the two types of cells use different pathways to transmit signals from the cell surface to the nucleus in response to mitogenic activation (7). We have described recently that Th2 cells are less dependent on protein kinase C (PKC) activation than Th1 cells (7a). We have continued our comparison of signal transmission pathways in activated Th1 vs. Th2 cells, and we have established a new distinction between the two types of helper cells. We report here that cholera toxin (CT), which ribosylates the α subunit of the G stimulatory protein, Gs, resulting in accumulation of intracellular cAMP (8), inhibits c-fos and c-myc mRNA expression, IL-2 production, and proliferation in Th1 cells in response to TCR-mediated triggering. On the other hand, the same doses of this toxin do not block IL-4 production and proliferation in Th2 cells. It is likely that the selective inhibitory effect of CT on Th1 vs. Th2 cells is due to their differential sensitivity to high levels of cAMP, since administration of forskolin, which is a direct activator of the adenylate cyclase, leading to accumulation of cAMP, produces a similar pattern of lymphokine secretion and proliferation in the two types of cells.

Materials and Methods

Mice. BALB/c (H-2d) and AKR/J (H-2k) mice were purchased from Taconic (Germantown, NY).

Media and Reagents. Complete culture medium was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 2 mM l-glutamine (Gibco Laboratories), 1 mM Hepes, 50 mM 2-ME antibiotics (Gibco Laboratories), and 10% FCS (HyClone Laboratories, Logan, UT). α-[32P]dCTP (800 Ci/mmol) was purchased from New England Nuclear (Boston, MA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. The generation and maintenance of the cloned Th2 cell lines D10.G4.1 (D10) (American Type Culture Collection, Rockville, MD) (specific for conalbumin in the context of I-Ak), CDC.25 (specific for rabbit IgG in the context of I-Ak), and the Th1 cell line D1.5 (identical specificity as CDC.25) have been described (9–11). Briefly, Th cell lines were stimulated every 2 wk with antigen and irradiated syngeneic splenocytes as APC. α-Methylmannoside-containing supernatant from rat spleen cells that had been stimulated with Con A for 48 h was added to the culture medium as a source of lymphokines. For the IL-2 and IL-4 bios-

[1] Abbreviations used in this paper: CT, cholera toxin; PKC, protein kinase C.
s says, the HT-2 indicator cell line was used, which proliferates in response to either lymphokine.

**Proliferation and Lymphokine Assays.** Proliferative responses of the T cell clones were assessed by purifying live cells on a Ficol-Paque gradient (Pharmacia Fine Chemicals, Piscataway, NJ) at least 2 wk after restimulation with antigen and irradiated spleen cells. Usually, 2.5 x 10^5/ml T cells were cultured in 200-μl aliquots in the presence of mAb 1452C11 (2C11, anti-CD3, used as 20% tissue culture supernatant) plus or minus IL-1 (1 ng/ml) in 96-well plates. PMA was used at 10 ng/ml, and ionomycin at 0.5 μg/ml. IL-2 was used at 5 U/ml. For the CT experiments, resting T cells (10^6/ml) were incubated in RPMI 1640 at 37°C for 2 h with the indicated doses of the toxin. The cells were then washed three times with complete medium and used for proliferation assays as described above. The cultures were incubated for 60 h at 37°C, and [3H]Tdr (0.5 μCi/well) was added for the final 12 h of culture. Radioactivity incorporated into DNA was measured by liquid scintillation counting.

To determine the lymphokine activity secreted by the stimulated T cell clones, supernatants were collected after a 24-h culture and assessed in a bioassay on the HT-2 indicator cell line. Briefly, 2 x 10^5 HT-2 cells/well were incubated with 25% supernatant for 24 h (final volume 200 liters/well). DNA synthesis was measured by the MTT method, as previously described (12).

**RNA Analysis.** Except for the time course experiment, resting T cells were stimulated with Con A (5 μg/ml) for 60 min for c-fos mRNA expression, and with Con A or IL-1 (1 ng/ml) for 150 min for c-myc and c-myb mRNA expression. Total RNA was then extracted as described elsewhere (15), with some modifications. Briefly, 10^6 cells were washed three times with cold PBS, pelleted, and resuspended in 3 ml of 3 M LiCl/6 M urea. The suspension was homogenized with a 60-s pulse using a polytron. The RNA was then precipitated at 4°C for 15 h and extracted twice with phenol/chloroform/isoamylalcohol. 20 μg of RNA was subjected to electrophoresis on a Northern gel (1% agarose/2% formaldehyde/1x MOPS) and the gel blotted onto a nylon membrane (Biodyne Nylon membrane; ICN, Irvine, CA). The RNA was cross-linked according to the protocol developed by Church and Gilbert (14). The blot was prehybridized in 5% SDS, 100 mM NaCl, 50 mM sodium phosphate, and 1 mM EDTA for 2 h at 65°C, and then hybridized for 12 h in a new aliquot of the same solution, containing an α-[32P] random primed probe. The hybridized membrane was washed three times for 5 min each at 65°C in 5% SDS, 1x SSC, and three times for 30 min each at 65°C in 0.1% SDS, 0.5x SSC. Autoradiographs were exposed at -70°C with enhancer screens.

**Quantitation of cAMP Levels.** Resting cells (10^6/ml) were incubated with CT for 1 h or forskolin for 30 min at 37°C. The reaction was stopped by adding 5 ml cold PBS. The cells were spun down and resuspended in 0.5 ml 10 mM Tris-HCl, pH 7.4, 1 mM MgCl2, and sonicated for 30 s. Ice-cold TCA was added to a final concentration of 6%, and the suspension was kept on ice for 30 min. After centrifugation at 13,000 rpm for 15 min, the supernatant was collected and extracted four times with 4 vol of H2O-saturated diethylether to remove the remaining TCA. The level of cAMP was determined using an RIA kit (Amersham Corp., Arlington Heights, IL).

**DNA Probes.** The following DNAs were used for the preparation of random primed probes (kit from Boehringer Mannheim Biochemicals, Mannheim, FRG). c-fos is an EcoRI/StuI cDNA fragment; c-myc is a genomic BamHI/Xba I fragment; c-myb is a genomic EcoRI fragment; and β-actin is a PstI cDNA fragment. All plasmids were obtained from American Type Culture Collection.

**Results**

Cholera Toxin Inhibits Th1 Cell Proliferation Induced by TCR-mediated Stimulation, but not by IL2. It has been reported that CT has an inhibitory effect on mitogen-induced proliferation in peripheral T cells, as well as in T cell clones (15). To test the effect of CT in a prototype Th1 clone, we pretreated D1.5 cells with various doses of toxin. The cells were then stimulated with IL-2, anti-CD3 (2C11), or PMA plus ionomycin. The two latter reagents mimic TCR-mediated stimulation (16). As can be seen in Fig. 1 A, the proliferation induced by 2C11 or PMA plus ionomycin is almost completely inhibited by 0.01 μg/ml CT, while the response to IL-2 is unaffected.

To test whether the inhibitory effect of CT on the proliferation of D1.5 cells is related to the accumulation of cAMP, we measured the proliferation of this clone in the presence of increasing doses of forskolin. Fig. 1 B shows a dose-dependent inhibition of T cell proliferation in response to CT.
PMA plus ionomycin, while, again, the response to exogenous IL-2 is unaffected. Similar results were obtained with the cAMP analogue 8-Br-cAMP (data not shown).

Cholera Toxin Does not Inhibit TCR-mediated Proliferation in Th2 Cells. It has been suggested that Th1 and Th2 cells use different transmission signaling pathways after TCR-mediated stimulation (7). Furthermore, the regulation of their respective autocrine growth factors is quite different (7a). To test the effect of CT on the proliferation of Th2 cells, we pretreated D10 cells with several doses of CT, before stimulation with anti-CD3. As shown in Fig. 2 A, no inhibition was detected, even with high doses (5 μg/ml) of the toxin. Similar results were obtained with the Th2 clone CDC25. A 50-fold higher dose of CT than was used in Th1 cells (0.01 compared with 0.5 μg/ml) was ineffective to block proliferation in these cells. In an additional experiment, even higher doses of CT (0.1–1 μg/ml) were tested and failed to inhibit TCR-mediated proliferation in this clone (data not shown). Again, to check the effect of cAMP on the proliferative response of these cells, forskolin was included in these experiments. As shown in Fig. 2 B, a dose of 25 μM forskolin was required to induce 40% inhibition of proliferation. This represents a 50-fold higher dose than the one used to induce an 85% inhibition in the proliferation of Th1 cells, mediated by PMA plus ionomycin. It is possible that 25 μM forskolin is slightly toxic for the cells, because this dose also inhibited the proliferative response of Th2 cells mediated by exogenous IL-2 (Fig. 2).

Cholera Toxin and Forskolin Block IL-2 Production in Th1 Cells, but not IL-4 Production in Th2 Cells. The inhibitory effect of CT and forskolin on the proliferative response of the Th1 clone D1.5 suggests that these agents block the secretion of the autocrine growth factor IL-2, because the addition of exogenous IL-2 restores proliferation. It is possible, however, that the autocrine growth factor IL-4 is released in stimulated Th2 cells in the presence of CT or forskolin. As can be seen in Fig. 3, this is the case. No inhibition of IL-4 production was seen in anti-CD3-stimulated CDC25 cells in the presence of CT or forskolin. Identical results were obtained in D10 cells (data not shown). In contrast, the Th1 clone D1.5 failed to secrete IL-2 when stimulated in the presence of CT or forskolin (Fig. 3, A and B).

Cholera Toxin and Forskolin Increase the Level of Intracellular cAMP in Th2 Cells. A previous report, as well as the results presented in this paper, indicates that the inhibition of proliferation mediated by CT in Th1 cells is likely due to an increase in the level of intracellular cAMP (15). One important step in this process is the ribosylation in the α subunit of a G protein (8, 17). The inability of CT to inhibit proliferation in activated Th2 cells could be explained by a missing G protein target of CT. Nevertheless, as we show in Fig. 4, both CT and forskolin were able to induce a significant increase in the level of intracellular cAMP in D10 (Fig. 4 A) as well as in CDC25 (Fig. 4 B) cells. Total values of cAMP fluctuated from experiment to experiment, but in all five tests, treatment with forskolin or CT lead to significant increases in the level of cAMP in comparison with untreated cells.

Cholera Toxin Induces c-myb Expression, but Inhibits c-fos and c-myc mRNA in Con A-activated Th1 Cells. As shown in Fig. 1, CT and forskolin block the proliferation of D1.5 cells in response to TCR-mediated activation. It was of interest, therefore, to analyze the effect of these agents on the proto-oncogene expression in these cells. Resting cells were incubated with CT for 2 h, washed, and recultured in the presence or absence of Con A (5 μg/ml). Total RNA was then extracted at different timepoints in order to evaluate steady state message levels of the c-myb, c-fos, and c-myc genes. As can be seen in Fig. 5 A, Con A and CT independently induced c-myb mRNA, and this expression was not affected when both agents were added together, or when CT was replaced by forskolin. In contrast, CT did not induce c-myc and c-fos mRNA on its own (Fig. 5, A and B), but blocked the expression of these proto-oncogenes induced by Con A. It is likely that this in-
Figure 3. Lymphokine production by TCR-stimulated Th1 and Th2 cells in the presence of CT or forskolin. D1.5 (Th1, IL-2 producer) and CDC25 (Th2, IL-4 producer) cells were either pretreated with CT (A) or cultured in the presence of forskolin (B). All cells were stimulated with soluble anti-CD3 mAb (1452C11) for 24 h. After this time, 100 μl of supernatant was removed and tested for lymphokine content on the HT-2 indicator cell line. The following values were obtained in control cells: unstimulated CDC25 cells, 0; CDC25 cells stimulated with anti-CD3 mAb 0.206 ± 0.017; unstimulated D1.5 cells, 0.004 ± 0.002; D1.5 cells stimulated with anti-CD3 mAb, 0.122 ± 0.007.

Figure 4. CT and forskolin induce accumulation of intracellular cAMP. (A) Resting D10 cells were treated with: unstimulated (a), 0.5 μg/ml CT (b), 1 μg/ml CT (c), 10 μM forskolin (d), and 25 μM forskolin (e). (B) Resting CDC25 cells were treated with: unstimulated (a), 0.1 μg/ml CT (b), 0.5 μg/ml CT (c), and 1 μg/ml CT (d). cAMP was measured as indicated in Materials and Methods.

Protein Ozoneenzyme Expression in Th2 Cells after Con A Stimulation. The expression of proto-oncogenes during T cell proliferation has been extensively studied (18, 19). It is thought that the induction of some of these genes is required for the progression of the cell cycle (20). c-fos, c-myc, and c-myb are transcribed after TCR-mediated stimulation or in response to other stimuli such as lymphokines (21, 22). It was, therefore, of interest to determine the expression of these proto-oncogenes in activated Th2 cells. For this purpose, resting D10 cells were stimulated with Con A (5 μg/ml), which has been shown to bind and precipitate TCR proteins (23) and to activate T cells through the TCR. Total RNA was extracted at different timepoints thereafter. As can be seen in Fig. 6, c-fos mRNA peaked at 1 h, while c-myb and c-myc mRNA were also detected in these cells and peaked at 2 and 3 h after activation, respectively. Thus, Th2 cells seem to have a similar profile of proto-oncogene expression as peripheral T cells (24).
Figure 5. CT inhibits c-fos and c-myc mRNA expression in Con A-activated D1.5 cells. (A) Resting cells were activated for 2.5 h under the following conditions: unstimulated (lane 1); Con A (lane 2); Con A plus 1 μg/ml CT (lane 3); Con A plus 10 μM forskolin (lane 4); 1 μg/ml CT (lane 5). (B) Resting cells were activated for 1 h under the following conditions: unstimulated (lane 1); Con A (lane 2); PMA (lane 3); Con A plus 1 μg/ml CT (lane 4); PMA plus 1 μg/ml CT (lane 5); 1 μg/ml CT (lane 6).

Discussion

The activation of T cells by specific antigen, mitogen, or mAb directed against TCR structures induces the hydrolysis of phosphatidylinositol 4'5' biphosphate, which is mediated by phospholipase C (26). This hydrolysis leads to the production of second messengers, such as diacylglycerol and inositol triphosphate. DAG can activate the enzyme PKC (27), while InsP3 increases the concentration of intracellular calcium (28). It is believed that these second messengers lead to increased levels of proto-oncogene mRNA, production of lymphokines, expression of lymphokine receptors, and cellular proliferation (29). Another system of second messengers is related to the adenylate cyclase pathway. Activation of this enzyme leads to accumulation of intracellular cAMP, which can activate a cAMP-dependent kinase (30). Increased levels of cAMP have been described in T cells in response to TCR-mediated stimulation (reviewed in reference 31) and in response to IL-1 (32). The PKC- and the cAMP-dependent kinase pathways interact in certain cell systems (33).
Figure 7. CT inhibits c-myc but not c-fos mRNA expression in Con A-activated D10 cells. (A) Resting D10 cells were stimulated for 2.5 h under the following conditions: unstimulated (lane 1); 1 μg/ml CT (lane 2); Con A (lane 3); Con A plus 1 μg/ml CT (lane 4); and Con A plus 25 μM forskolin (lane 5). (B) Resting D10 cells were stimulated for 1 h under the following conditions: unstimulated (lane 1); 1 μg/ml CT (lane 2); 25 μM forskolin (lane 3); 1 μg/ml CT plus Con A (lane 4); and Con A plus 25 μM forskolin (lane 5).

The role of cAMP as second messenger in T cells is controversial. There are many inducible genes that can be transcribed in response to cAMP. These include genes that contain a consensus sequence in the promoter that has been named cAMP-RE (cAMP response element) (34). On the other hand, elevated levels of cAMP are inhibitory for the proliferation of peripheral T cells, as well as for T cell clones (15, 35). Interestingly, as has been reported (35) and we show here, CT blocks the proliferation of Th1 cells induced with anti-TCR antibody or PMA plus ionomycin, but not with exogenous IL-2. This inhibition is probably mediated through increased levels of intracellular cAMP and is selective to stimuli that activate PKC. These results are in contrast to those reported by Farrar et al. (36), who found that in the CT6 cytotoxic T cell line, an increase in the level of intracellular cAMP blocked proliferation and c-myc expression in response to IL-2. It is important to note that in this cell line, the interaction of IL-2 with the IL-2-R leads to translocation of PKC from the cytosol to the membrane (37), which does not happen in other T cells (38–40). In the Th1 cells that we tested, CT was unable to block c-myc expression induced by exogenous IL-2 (E. Muñoz, A. Zubia, and B.T. Huber, unpublished results), but inhibited c-myc and c-fos mRNA expression induced either by Con A or PMA.

Figure 8. CT inhibits c-myc but not c-fos mRNA expression in Con A-activated CDC25 cells. (A) Resting cells were stimulated for 2.5 h under the following conditions: unstimulated (lane 1); Con A (lane 2); Con A plus 1 μg/ml CT (lane 3); Con A plus 25 μM forskolin (lane 4); and 1 μg/ml CT plus Con A (lane 5). (B) Resting cells were stimulated for 1 h under the following conditions: unstimulated (lane 1); Con A (lane 2); Con A plus 0.1 μg/ml CT (lane 3); Con A plus 0.5 μg/ml CT (lane 4); Con A plus 1 μg/ml CT (lane 5); Con A plus 1 μM forskolin (lane 6); Con A plus 10 μM forskolin (lane 7); and Con A plus 25 μM forskolin (lane 8).

Our present report shows that TCR-mediated proliferation in Th2 cells is not affected by CT, whereas Th1 cells are inhibited. This raises the possibility that Th2 cells do not use PKC as a major signal transmission pathway after TCR-mediated stimulation. In fact, we have previously shown that

Figure 9. IL-1-induced c-myc mRNA expression is not affected by CT, forskolin, or 8-Br cAMP. Resting D10 cells were stimulated for 2.5 h under the following conditions: unstimulated (lane 1); 1 μg/ml CT (lane 2); 25 μM forskolin (lane 3); 1 ng/ml IL-1 (lane 4); 1 ng/ml IL-1 plus 1 μg/ml CT (lane 5); 1 ng/ml IL-1 plus 25 μM forskolin (lane 6); and 1 ng/ml IL-1 plus 1 mM 8-Br cAMP (lane 8).
Th2 cells can respond to antigenic stimulation in the absence of functional PKC (7a). In addition, Th2 cells are dependent on IL-1 for proliferation (41), and we have shown recently that IL-1 induces high levels of intracellular cAMP in these cells (32). Therefore, while cAMP may be inhibitory in Th1 cells, it could be important for activation and proliferation in Th2 cells.

Some proto-oncogenes are known to encode proteins that act as transcription factors: e.g., c-fos and c-jun, which form an AP-1 binding heterodimer necessary for IL-2 gene transcription (42). Indeed, it has been confirmed that activation of the IL-2 gene requires de novo protein synthesis (43). We show here that CT and forskolin inhibit c-fos mRNA expression induced by Con A in Th1 cells. It is possible, therefore, that blocking of c-fos constitutes the mechanism of the CT-mediated inhibition of IL-2 production. In contrast, in Th2 cells that release IL-4 as an autocrine growth factor, c-fos mRNA induction is not affected by CT.

c-myc and c-myc have been shown to play a role in T cell proliferation induced by mitogens (44, 45). c-myc also seems to be important in T cell proliferation triggered by exogenous IL-2 (22), but probably requires the coexpression of c-myc for full T cell activation to occur. This is based on the results reported here, namely, that Th2 cells that accumulate c-myc, but not c-myc, mRNA after incubation with forskolin or CT, fail to proliferate in response to these agents. As shown in Fig. 9, exogenous IL-1 can provide the signal required for the induction of c-myc expression that is not affected by CT. Of interest is the fact that IL-1 can induce c-myc mRNA by a PKC-independent pathway (Muñoz, E., A. Zubiaga and BT. Huber, manuscript submitted for publication). This could explain why elevated levels of cAMP cannot block IL-1-induced c-myc mRNA expression. On this account, it has been reported recently that activated Th2 cells produce IL-1 (46), and we have observed that this lymphokine acts in an autocrine manner (Zubiaga, A., E. Muñoz and B.T. Huber, manuscript in preparation). Furthermore, IL-1 secretion is not affected by elevated levels of cAMP in other cell systems (47, 48). It is likely, therefore, that this growth factor is produced in CT-pretreated Con A-activated Th2 cells and leads to c-myc transcription, allowing proliferation of these cells. In fact, careful examination of the time course of proto-oncogene steady-state message in these cells suggests that CT pretreatment not only leads to prolonged c-fos and c-myc mRNA expression, but also, the observed inhibition of c-myc mRNA starts to be alleviated after 6 h (results not shown). A delay in c-myc transcription would be expected, if the activation of this gene depends on the previous production of IL-1.

The results presented in this paper are pertinent to long-term T cell lines of Th1 and Th2 types. There is growing evidence that the precursors of both types of cells are contained within primary CD4+ T cells (49). We are testing at the moment whether the findings in long-term in vitro propagated T cell clones can be extrapolated to these precursor cells.

In conclusion, from the results presented here, we hypothesize that Th1 and Th2 cells use different transmission signaling pathways after TCR-mediated stimulation. While the PKC pathway is the major system of activation in Th1 cells, different second messengers that are still unknown are generated in Th2 cells after activation.

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