Helper or Cytolytic Functions Can Be Selectively Induced in Bifunctional T Cell Clones

By Assia Eljaafari,* Catherine Vaquero,* J. L. Teillaud,† Georges Bismuth,$ Claire Hivroz,$ Isabelle Dorval,* Alain Bernard,$ and Ghislaine Sterkers*

From the *Laboratory of Immunology/Oncology, INSERM U152, Hôpital Cochin, 75014 Paris, France; the †Laboratory of Cellular and Clinical Immunology, INSERM U255, Institut Curie, Paris, France; the $Laboratory of Cellular Immunology, CHU Pitié Salpêtrière, Paris, France; the ‡Laboratory of Immunopathology, INSERM U108, Hôpital Saint-Louis, Paris, France; and the §Laboratory of Children’s Immunology, Institut Gustave Roussy, Paris, France

Summary

By using bifunctional T cell populations, we have shown in this report that elicitation of helper versus cytolytic function depends on the stimulatory signal at the membrane. Interestingly enough, the transduction of these signals is likely to be achieved via different metabolic pathways. Thus, helper function is associated with intracellular Ca2+ mobilization and PLC activation, while cytolsis can occur even in the absence of detectable levels of these second messengers. These results indicate that selective activation through the same membrane-transducing molecule may orientate T cell function through qualitatively or quantitatively different second messengers. This would be an important part of immune regulation.

Materials and Methods

T Cell Clones. T cell clones were produced as previously described (2-4). They are all helper and cytotoxic, specific for the influenza A/Texas virus, and restricted by HLA-DR molecule (2-4, and unpublished observations). They were maintained in long-term culture by weekly restimulation with PHA and irradiated feeder cells in the presence of rIL-2, as previously described (2-4). In all experiments described herein, T cells were used 7 d after their last restimulation.

Monoclonal Antibodies. The three anti-CD2 mAbs used were anti-TII, (OKT11A, IgG2a), anti-GT2 (IgG1), and anti-D66 (IgG2a), which have been previously characterized (5-7). The anti-CD3 mAb used was OKT3 (IgG2a). In dose-response experiments, anti-TII, anti-GT2, and anti-D66 were purified from ascitic fluids using protein A-Sepharose chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) and titrated by ELISA.

T Cell Proliferation Assays. Cells were cultured in triplicate in 96-well round-bottomed microtiter plates, with various stimulators, as indicated, in a total volume of 200 µl of culture medium for 3 d at 37°C, in 5% CO2 atmosphere. During the last 6 h of culture, [3H]thymidine (0.8 µCi) (Amersham, les Ulis, France) was added.

T Cell Cytotoxicity Assays. Cytotoxicity was assessed by the standard 51Cr-release assay described elsewhere (3). Briefly, effector cells (5 x 10⁴ cells per well) were seeded into round-bottomed microtiter plates, in RPMI 1640 medium supplemented with 10% FCS. mAbs were added 20 min before addition of target cells. 51Cr-labeled K562 target cells (5 x 10⁴ cells per well) were added in a total volume of 200 µl. After centrifugation at 100 g, cells were incubated for 4 h at 37°C in 5% CO2 atmosphere. Plates were then centrifuged again. 100 µl of supernatant was harvested from each well and the radioactivity was measured using a gamma counter.

Lymphokine mRNA Accumulation. Total RNA was isolated by the guanidinium isothiocyanate procedure (8). Equal amounts (10 µg) of RNA (this was confirmed by visualization of ribosomal RNA after staining of the gel with ethidium bromide) were resolved on
glyoxal-agarose gel, transferred to nylon filter, and sequentially hybridized at high stringency with specific probes as already described (9). These probes (listed on the left of the figure) were obtained after in vitro transcription of T3-T7 Bluescribe vector (Vector Cloning System, San Diego, CA) containing the 1.35-kb PstI-BamHI fragment from huIL-2 R3 cDNA (10), 0.3-kb XbaI-StuI from huIL-2 cDNA (9), 0.72-kb Khol-Hpal from huIL-3 cDNA (11), and 0.28-kb EcoRV-EcoRI from huIL-4 cDNA (12).

F: Rosettes and Inhibition of Binding by GT2, D66, and T11 Hybridoma Proteins. Rosette assays between IgG-coated SRBC and cells to be investigated have been previously described in detail (13). Briefly, SRBC were coated with two different BALB/c mouse mAbs directed to SRBC (U182.5, IgG1; UN2, IgG2a) kindly provided by Dr. M.D. Scharff (Albert Einstein College of Medicine, New York, NY), and tested for rosette formation with K562 cells. Cells associated with at least five SRBC were scored as FeR⁺. In another set of experiments, hybridoma proteins were purified from ascitic fluids by passing them over protein A-Sepharose columns (LKB-Pharmacia, Uppsala, Sweden). ELISAs were performed as previously described (14) to control the purity of these preparations. Hybridoma proteins were then heat-aggregated by incubation at 60°C for 20 min. Pellets of 2.5 × 10⁶ K562 cells were incubated for 45 min at room temperature with 100 μl of heat-aggregated proteins diluted in PBS. IgG1- or IgG2a-coated SRBC were then added in 0.4 ml of PBS and rosettes were counted after 10 min centrifugation at 900 rpm at 4°C.

Measurement of [Ca²⁺]i. T cells were washed and suspended at 5–7 × 10⁶/ml in culture medium containing 3 μM acetoxyethyl ester of indo 1, according to the method of Rabinovitch et al. (15). Briefly, after incubation with indo 1 for 30 min at 37°C to effect loading, cells were washed, resuspended in the same medium at 10⁶/ml, and analyzed on a cytofluorograph 50 HH cell sorter equipped with a 2150 computer (Ortho Pharmaceutical, Raritan, NJ). Analyses were conducted at 37°C in a flow rate of 500 cells.

P1 Turnover. Effector cells were washed twice in a phosphate-free buffer containing 20 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM MgCl₂, 5 mM KCl, and 0.1% Glc. The final pellet was resuspended in the same buffer and the cell suspension (10⁷/ml) was incubated with carrier-free ³²P orthophosphoric acid (Amersham, 20 μCi/ml) for 1.3 h at 37°C, until isotopic equilibrium. Cloned T cells as well as target cells were then washed twice; 100-μl aliquots of the effector cell suspension were transferred into polystyrene tubes and after adding or not adding the K562 cell suspension (10⁵/tube), effector cells were stimulated with anti-CD2 mAbs for different time periods. Then, reaction was stopped as previously described (16). Radiolabeled phospholipids were located by autoradiography and their nature was determined by parallel migration of standard phospholipids visualized by exposure to iodine vapors. Spots concerning PI-PA were subsequently scraped from the plates and evaluated by liquid scintillation counting.

Results and Discussion

Two bifunctional human T cell clones, i.e., TA4 and M3ap20 specific for the influenza virus associated with HLA class II molecule and isolated from two different donors, were selected for the present study. As previously demonstrated, these monoclonal T cell populations exhibit a CD2⁺/CD3⁺/CD4⁺/CD8⁻ phenotype and have the same specificity and restriction for both helper and cytolytic activities (2–4). Activation of T cell clones can be induced by mAbs to membrane proteins such as CD3 or CD2 (17–19). In the case of CD2, combination pairs of mAbs are required, such as D66 + T11, mAbs (1:200 final dilution for each) or anti-GT2 + T11 mAbs (12000 final dilution for each). To compare both assays, K562 cells were added in proliferation assay at the same E/T ratio than in cytotoxicity assay (10:1). Results of proliferation assays are expressed as mean counts per minute of triplicate. Results of cytotoxicity assay are expressed as percentage of specific cytotoxicity as calculated by the following formula: Percent cytotoxicity = (100 x ([³¹Cr release with effector cells]–[spontaneous ³¹Cr release])/(maximum ³¹Cr release)–[spontaneous ³¹Cr release]). (Asierik) In the absence of K562 cells, lysis could not be tested.
(IgG1) and D66 (IgG2a) to the FcγRII present on K562 cells. Indeed, these cells express FcγRII that bind immune-complexed IgG1 and IgG2a, as shown by their ability to form rosettes with both IgG1- and IgG2a-coated SRBC (Table 1). Furthermore, heat-aggregated GT2, D66, and T111 proteins were able to inhibit the rosette formation between K562 cells and IgG2a- or IgG1-coated SRBC (Table 1). In these functional assays, helper and cytotytic activities were assayed following different experimental conditions. Indeed, proliferation was tested after 3 d of culture in the absence of K562 cells, while cytolysis was analyzed in the presence of the labeled targets, and after a short 4-h period of stimulation. Therefore, these assays did not directly investigate whether both activities could be simultaneously expressed in a single T cell upon a strictly identical mode of stimulation. Thus, helper function was tested in short-term assay (5 vs. 4 h for cytolysis) by analyzing IL-2, IL-3, IL-4, and IL-2R mRNA accumulation. Moreover, K562 cells, which are required for cytolysis, were added at the same E/T cell ratio. As illustrated in Fig. 3 with clone M3ap20, when both functional assays were performed under strictly identical conditions of stimulation, GT2+T11 preferentially induced cytolysis; conversely, D66+T111 selectively induced helper function. These results strongly suggest that induction of either function depends upon different signals delivered through the same transducing CD2 molecule.

Concomitantly, we explore the second intracellular messengers induced by the two mAbs pairs. T cell helper function induction via CD2 molecule (20) or CD3/Ti antigen receptor complex (TCR) (21) has already been described to be associated with membrane phosphoinositide-derived second messengers: inositol 1, 4, 5 triphosphate (IP3) and diacylglycerol (DG). These two second messengers are the products of a rapid, transient hydrolysis of membrane phosphatidylinositol 4, 5 biphosphate (PIP2) by phospholipase C (PLC) and act synergistically to activate protein kinase C (PKC), leading to protein phosphorylation and modulation of gene expression. IP3 is responsible for Ca2+ mobilization from intracellular stores, whereas DG increases enzyme affinity for Ca2+ (22-24). Phosphatidylinositol (PI) turnover induced by PIP2 hydrolysis, resulting in phosphatidic acid (PA), and PI accumulation has been described to be associated with extracellular Ca2+ influx (25-27). In contrast, PKC activation, in the absence of free cytosolic [Ca2+], and PIP2 hydrolysis, has been reported to be absolutely required for cytotytic triggering. Indeed, PMA can trigger T cell-mediated cytolysis with no increase in free cytosolic [Ca2+]i (28). In our model of bifunctional T cells stimulated via CD2 molecule by anti-D66+T111 or anti-GT2+T111 mAbs, cytosolic free Ca2+ increment was investigated by measuring fluorescence with the indo-1 indicator, and phospholipid hydrolysis was determined by analyzing PI+PA accumulation. As shown in Fig. 4a, the helper function of M3ap20 clone triggered with anti-D66+T111 mAbs was accompanied by [Ca2+]i increase. Interestingly, and as also reported by Acover et al. (29) in other human T cell clones, a several minute latency in [Ca2+]i rise after mAb addition was observed. This contrasts with the rapid increase observed in a Jurkat T cell line stimulated through CD2 molecule, as reported by Pan-

---

**Table 1. Binding of GT2, D66, and T11; mAbs to FcyRII Expressed on K562 Cells**

| IgG added† | SRBC-γ1 | SRBC-γ2a |
|------------|---------|----------|
| None       | 47.1    | 42.0     |
| T11 (IgG2a)| 15.5    | 12.6     |
| D66 (IgG2a)| 15.1    | 20.5     |
| GT2 (IgG1) | 20.7    | 19.8     |

† Rosette assays between K562 cells and IgG1- or IgG2a-coated SRBC and inhibition of binding by hybridoma proteins were performed as described in Materials and Methods.

† Heat-aggregated hybridoma proteins were added at a final concentration of 20 μg/ml.
Expression of IL2R and lymphokine mRNA compared with cytotoxic activity in bifunctional T cell clones. (A) Cloned T cells were unstimulated (lane 1); stimulated for 5 h with anti-GT2+T11, anti-D66+T11, and K562 (lane 2), anti-GT2+T11, anti-D66+T11, and K562 (lane 3). K562 was cultured alone as control (lane 6). Culture conditions are identical as those described in Fig. 1. Then, total cellular RNA was extracted as detailed in Materials and Methods and analyzed by Northern Blotting. 10 μg of RNA were transferred on nylon membranes and hybridized with IL2R, IL4, IL2R riboprobes. (B) In the same experiment, the cloned T cells were tested for cytolytic activity under the same conditions of stimulation. Results are expressed as percentage of specific cytotoxicity calculated as described in Fig. 1. (Asterisk). In the absence of K562 cells, lysis could not be tested.

tateo et al. (20). In both clones, helper function was associated with PIP2 hydrolysis as determined by PI+PA accumulation and as illustrated with clone TA4 in Fig. 5. This is in good agreement with other reports, indicating that [Ca2+]i rise and phospholipid hydrolysis are associated with acquisition of T cell helper function through CD2 stimulation (20, 29). In contrast, cytotoxic activity triggered with anti-GT2+T11 mAb, even in the presence of K562 cells, was not associated with detectable changes in [Ca2+]i. (Fig. 4 b) nor with PI+PA accumulation (Fig. 5). It must be noticed that chronic treatment of our bifunctional T cell clones with phorbol ester abolished their ability to express helper and cytotoxic functions when subsequently stimulated by anti-CD2 mAbs (data not shown). Since this treatment is known to inactivate PKC (30, 31), one may assume that PKC activation is involved in the induction of both functions.

Altogether, these data suggest that different second messenger metabolic pathways are triggered, depending on the combination pairs of anti-CD2 used for stimulation. This clearly correlates with subsequent expression of selective functions. Nevertheless, one cannot rule out that the GT2+T11 combination might be less effective at increasing steady-state levels of phosphoinositide turnover and Ca2+ influx, both detected by relatively low sensitive methods. In that case, the same metabolic pathways would be involved but the threshold required for cytolytic function induction would be lower than that for helper function. Consistent with this hypothesis, we recently demonstrated that a secondary signal provided by IL-2 in addition to GT2+T11, can lead to helper function (16, 32) and PA+PI accumulation (16). In addition, it must be noticed that in the present study, K562 cells in association with anti-GT2+T11 provide a secondary signal leading to some proliferation (Fig. 1) associated with low IL-2, IL-4, IL-2R mRNA accumulation (Fig. 3, lane 3).

Finally, the induction of helper but not cytotoxic function after D66+T11 activation strengthens the assumption that GT2+T11 or D66+T11 mAbs trigger different functions through two distinct metabolic pathways. Indeed, if the low levels of GT2+T11-activated second messengers are
Cloned T cells were tested for lysis of K562 cells in different conditions of stimulation. 30 min after triggering with anti-1366+ TⅠ or CD3 mAbs, target cells alone (lanes 1 and 2, respectively) or in association with anti-1366+ TⅠ (lane 3) or, anti-CD3 (lane 4) were added. Then cytolysis was performed during 4 h. In Exp. 2, the same clone was tested in other conditions of stimulation: anti-1366+ TⅠ was added 30 min after triggering of cloned T cells with anti-CD3 plus target cells (lane 5). Results are expressed as percentage of specific cytotoxicity, as calculated in Fig. 1.

Even if the exact biochemical mechanisms by which CD2 can differently orientate T cell functions are not fully understood, one may speculate that this phenomenon could be an important part in the human in vivo immune regulation, by directing the response towards cellular and/or humoral immunity. Although experiments are more difficult to carry out, further studies are now in progress to determine whether a similar dichotomy exist in TCR/CD3-mediated T cell activation.

We thank Michel Seman for helpful comments on the manuscript, Sylvie Benslama and Ms. J. Moncuit for excellent technical assistance, Isabelle Rivenex for typing the manuscript.

This work was partly supported by grants from the Ligue Nationale Contre Le Cancer and La Fondation de France.

Address correspondence to Dr. Ghislaine Sterkers, INSERM U152, Hôpital Cochin, 27 rue du Faubourg Saint Jacques, 75014 Paris, Cedex 14, France.

Received for publication 21 November 1989 and in revised form 22 March 1990.

References
1. Fleisher, B. 1984. Acquisition of specific cytotoxic activity by human T lymphocytes in culture. Nature (Lond). 308:365.
2. Henin, Y, G. Sterkers, E. Gomard, L. Gebuhrer, and J.P. Levy. 1985. Functional subdivision of HLA DRw8 with influenza specific cloned cell lines. Immunogenetics. 22:407.
3. Sterkers, G., J. Michon, Y. Henin, E. Gomard, C. Hannoun, and J.P. Levy. 1985. Fine specificity analysis of human influenza specific cloned cell lines. Cell. Immunol. 94:394.
4. Zeliszewski, D., J.M. Tiercy, I. Dorval, C. Kaplan, B. Mach, and G. Sterkers. 1990. DR-restricted T cell reactivities associated with the Dw19 specificity can be directed against the products of either DRB3 (DRw52) or locus DRB1. Hum. Immunol. In press.
5. Verbi, W., M.J. Greaves, C. Shneider, K. Koubeck, and G.
Goldstein. 1982. Monoclonal antibodies OKT11 and OKT11A have pan T reactivity and block sheep erythrocyte receptors. 
*J. Immunol.* 129:81.

6. Bernard, A., C. Gelin, B. Raynal, P. Phan, C. Gousse, and L. Boumsell. 1982. Phenomenon on human T cells reswimming with sheep erythrocytes analyzed with monoclonal antibodies. Modulation of a partially hidden epitope determining the conditions of interaction between T cells and erythrocytes. *J. Exp. Med.* 155:1317.

7. Huet, S., H. Wakasugi, G. Sterkers, J. Gilnour, T. Tursz, L. Boumsell, and A. Bernard. 1986. T cell activation via CD2, the role of accessory cells in activating resting T cells via CD2. *J. Immunol.* 137:1420.

8. Vaquero, C., J. Sanceau, P. Sondermeyer, and R. Falcoff. 1984. Kinetics of messenger accumulation coding for IFN gamma related to modifications in the poly(A) RNA population of activated human lymphocytes. *R. Nucleic Acids Res.* 12:2629.

9. Paillard, F., G. Sterkers, G. Bismuth, E. Gomard, and C. Vaquero. 1988. Lymphokine mRNA and T cell multipurpose mRNA of the Ig supergene family are reciprocally modulated during human T cell activation. *Eur. J. Immunol.* 18:3643.

10. Bernard, W.J., J.M. Depper, G.R. Crabtree, S. Rudikoff, J. Pumphrey, R.J. Robb, M. Konuke, P.B. Suetlik, N.J. Peffer, T.A. Waldmann, and W.C. Greene. 1984. Molecular cloning and expression of cDNAs for the human Interleukin 2 receptor. *Nature (Lond.)* 311:626.

11. Yang, Y.C., A.B. Ciarietta, P.A. Temple, M.P. Chung, S. Kovacic, J.S. Witek-Giannoti, A.C. Leary, R. Kiz, R.E. Donahue, G.G. Wong, and S.C. Clark. 1985. Human IL3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL3. *Cell.* 47:3.

12. Yokota, T., T. Otsuka, J. Mowmann, J. Banchereau, T. De France, D. Blanchard, J.E. Vries, F. Lee, and K.I. Arai. 1986. Isolation and characterization of a human interleukin CDNA clone, homologous to mouse B cell stimulatory factor 1 that expresses B cells and T cells activities. *Proc. Natl. Acad. Sci. USA.* 83:5894.

13. Teillaud, J.L., B. Diamond, R.R. Pollock, V. Fajtova, and M.D. Scharff. 1985. Fc receptors on cultured myeloma and hybridoma cells. *J. Immunol.* 134:1774.

14. Thibaut, E., S. Amigorena, J. Moncuit, W.H. Fridman, and J.L. Teillaud. 1987. Software for the quantitative evaluation of in vitro monoclonal antibody production from ELISA data. *J. Immunol. Methods.* 104:15.

15. Rabinovitch, P.S., J.H. June, C.H. Grossman, and J.A. Ledbetter. 1986. Heterogeneity among T cells in intracellular free calcium responses after mitogen stimulation with PHA or anti-CD3, simultaneous use of indo-1 and immunofluorescence with flow cytometry. *J. Immunol.* 137:952.

16. Hu, J., C. Vaquero, G. Bismuth, A. Eljasafar, A. Bernard, J.P. Levy, and G. Sterkers. 1988. Activation signals via CD2 molecule and IL2 receptor act in synergy for helper function induction. *Eur. J. Immunol.* 18:1123.

17. Reinherz, E.L. 1985. A molecular basis for thymic selection: regulation of T11 induced thymocyte expansion by the T3-ti antigen/MHC receptor pathway. *Immunol. Today.* 3:75.

18. Spitz, H., H. Ysel, J. Leeuwenberg, and J.E. De Vries. 1985. Antigen specific cytotoxic T cell clones and antigen specific proliferation T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. *Eur. J. Immunol.* 15:88.

19. Siliciano, R.F., J.C. Pratt, R.E. Schmidt, J. Ritz, and E.L. Reinherz. 1985. Activation of cytolytic T lymphocyte and natural killer cell function through the T11 sheep erythrocyte binding protein. *Nature (Lond.)* 317:428.

20. Pantaleo, G., D. Olive, A. Poggi, W.J. Kozombo, L. Moretta, and A. Moretta. 1987. Transmembranin signalling via the T11 dependent pathway of human T cell activation. Evidence for the involvement of 1,2 diacylglycerol and inositol phosphate. *Eur. J. Immunol.* 17:55.

21. Imboden, J.B., and J.D. Stobo. 1985. Transmembrane signalling by the T cell antigen receptor. Perturbation of the T3-antigen receptor complex generates inositol trisphosphate and releases Ca**+** ions from intracellular stores. *J. Exp. Med.* 161:446.

22. Berridge, M. 1984. Inositol trisphosphate and diacylglycerol as second messengers. *J. Biochem.* 220:345.

23. Nishizuka, Y. 1984. Turnover of inositol phospholipids and signal transduction. *Science.* 225:1365.

24. Majerus, P.W., E.J. Neufeld, and D.B. Wilson. 1984. Production of phosphoinositides-derived second messengers. *Cell.* 37:701.

25. Gelfand, E.W., G.B. Mills, R.K. Cheung, J.W.W. Lee, and S. Grinstein. 1987. Transmembrane ion fluxes during activation of human T lymphocytes: role of Ca**+**, Na**, H**+** exchange and phospholipid turnover. *Immunol. Rev.* 95:59.

26. Nisbet-Brown, E., R.K. Cheung, J.W.W. Lee, and E.W. Gelfand. 1985. Antigen dependent increase in cytosolic free Ca**+** in specific human T lymphocyte clones. *Nature (Lond.)* 316:543.

27. Shapiro, D.N., B.S. Adams, and J.E. Niederhuber. 1985. Antigen specific T cell activation results in an increase in cytoplasmic free Ca**+**. *J. Immunol.* 135:2256.

28. Sitkovsky, M.V. 1986. Mechanistic, functional, and immuno- pharmacological implications of biochemical studies. *Immunol. Rev.* 103:127.

29. Alcover, A., D. Ramarli, N.E. Richardson, H.C. Chang, and E.L. Reinherz. 1987. Functional and molecular aspects of human T lymphocyte activation via T3-Ti and Ti-Ti pathways. *Immunol. Rev.* 95:5.

30. Russel, J.H. 1984. Phorbol esters inactivate the lytic apparatus of cytotoxic T lymphocytes. *J. Immunol.* 133:907.

31. Pasti, G., J.C. Laval, B.S. Warren, S.A. Aaronson, and P.M. Blumberg. 1986. Loss of mouse fibroblast cellular response to phorbol esters restored by microinjected protein kinase C. *Nature (Land.)* 325:161.

32. Hu, J., C. Vaquero, S. Huet, A. Bernard, and G. Sterkers. 1987. Interleukine 2 upregulates its own receptor. *J. Immunol.* 139:4109.

33. Grynkiewicz, G., M. Poenie, and R.Y. Tsien. 1985. A new generation of Ca**+** indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440.