Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4611–4615, July 1987
Immunology

Induction of cytotoxicity in resting human T lymphocytes bound to tumor cells by antibody heteroconjugates

(monocyte-independent T-cell activation/melanoma/anti-CD3 monoclonal antibody/anti-CD28 monoclonal antibody/anti-tumor antibody)

GUNDRAM JUNG*, JEFFREY A. LEDBETTER†, AND HANS J. MÜLLER-EBERHARD*

*Division of Molecular Immunology, Research Institute of Scripps Clinic, La Jolla, CA 92037; and †Oncogen Corporation, Seattle, WA 98121

Contributed by Hans J. Müller-Eberhard, March 25, 1987

ABSTRACT **An *in vitro* model for peripheral human T-cell activation and resultant tumor cell killing is described. Cytotoxic T lymphocytes may be generated from resting lymphocytes by incubation of human peripheral blood mononuclear cells for 3 days with the anti-CD3 monoclonal antibody OKT3. Cytotoxicity in peripheral blood mononuclear cells can also be induced by adding an anti-target-OKT3 antibody conjugate and 10% (vol/vol) fetal calf serum to the culture medium. Conjugate activation of T cells was almost completely blocked, however, when 20% (vol/vol) human serum was added to the medium. Conjugate-mediated peripheral blood mononuclear cells activation was restored to some extent by the addition of melanoma target cells to the culture and was markedly enhanced by a second conjugate containing anti-target cell and anti-CD28 antibody. Monoclonal antibody 9.3 (anti-CD28) provides a progression signal in T-lymphocyte activation when used in combination with anti-CD3. Thus, presentation by the tumor target cells of anti-CD3 and anti-CD28 to resting human lymphocytes causes T-cell activation, which is independent of monocytes, proceeds in the presence of human serum, and results in tumor cell killing.**

Monoclonal antibodies, directed to the murine T-cell antigen receptor (1) or to the receptor-associated CD3 structure on human T cells (2, 3) and chemically linked to anti-target cell antibodies, can overcome the major histocompatibility complex restriction of cytotoxic T lymphocytes (CTL) and trigger cloned CTL to lyse the respective target cells. Thus such antibody heteroconjugates may be capable of directing CTL to target tumor cells *in vivo*. Since T cells in peripheral human blood are resting cells that are virtually noncytolytic, the generation of active CTL from resting T cells is required if anti-CD3-containing heteroconjugates are to be used in any therapeutic strategy. Our purpose in this study was to devise a method for the monocyte-independent activation of resting human T lymphocytes at the surface of tumor cells that results in tumor cell killing. It is pertinent that certain anti-CD3 antibodies can induce proliferation of resting T cells in a monocyte-dependent system (4, 5).

Human peripheral blood mononuclear cells (PBMC) become potent CTL after 4 days of culture with the anti-CD3 monoclonal antibody OKT3. After the 4-day activation period, the cells could kill tumor target cells in presence of the anti-target-OKT3 heteroconjugates, as determined by a 4-hr cytotoxicity assay (6). The time-dependent acquisition of cytotoxic activity correlated with *de novo* synthesis of the cytolytic C9-related protein (C9RP) (7, 8) by the OKT3-stimulated lymphocytes (9).

Monocytes are required for the activation of lymphocytes by OKT3 for the solid-phase presentation of OKT3 by Fc receptors and for supplying second signals (4, 10, 11). Serum IgG interferes with OKT3 binding to monocyte Fc receptors and thus with T-cell activation (12, 13). Given these facts, we have determined conditions that allow T-cell activation in human serum (HS) without the participation of monocytes. Considering the potential utility of antibody heteroconjugates for tumor immunotherapy, the ability of such conjugates to activate resting T cells was investigated. It was found that: (*i*) Anti-target-OKT3 conjugates activate human PBMC held in medium containing fetal calf serum (FCS), but that in medium containing 20% (vol/vol) HS activation was minimal. (*ii*) Inhibition by HS could be partly overcome by utilizing the surface of the tumor cells for solid-phase presentation of OKT3 within the anti-target-OKT3 conjugate. (*iii*) Employing monocyte-depleted PBMC, the second signals ordinarily contributed by monocytes could be provided effectively by an additional anti-target conjugate containing monoclonal antibody (mAb) 9.3 that is known to transmit progression signals in T-lymphocyte activation.

MATERIALS AND METHODS

Cells and Antibodies. M21 melanoma cells, originally provided by D. L. Morton (University of California, Los Angeles), were grown in complete medium [RPMI 1640 supplemented with 10% (vol/vol) FCS, 2 mM glutamine, and gentamicin at 50 μg/ml]. mAb 9.2.27, which is directed to a melanoma-associated glycoprotein–proteoglycan complex (14), was supplied by Ralph Reisfeld (Research Institute of Scripps Clinic). mAb 9.3 recognizes the T-cell differentiation antigen Tp44 (CD28) found on most T cells (15–17) and acts as a progression signal in T-cell activation (18–20). OKT3 was purified from hybridoma (American Type Culture Collection) supernatants. mAb 9.2.27 and mAb 9.3 were purified from ascites fluid using protein A-Sepharose chromatography (21).

Conjugation of Antibodies. The mAb 9.2.27 was cross-linked to OKT3 or mAb 9.3 using the heterobifunctional reagent SPDP [*N*-succinimidyl 3-(2-pyridyldithio)propionate, Sigma] as described (6, 22).

Isolation of PBMC. Human PBMC were isolated from heparinized blood of healthy donors (General Clinical Research Center of Scripps Clinic and Research Foundation) by Ficoll-Hypaque density gradient centrifugation (Pharmacia). Depletion of monocytes from PBMC was achieved by replicate adherence to plastic tissue culture flasks, followed by treatment with 5 mM leucine methyl ester for 40 min at room temperature (23). Treated cells no longer responded to mitogenic concentrations of OKT3 or OKT3 and mAb 9.3.

Induction of PBMC Activation. PBMC and monocyte-depleted PBMC were cultured for 3 days under various

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: CTL, cytotoxic T lymphocytes; PBMC, peripheral blood mononuclear cells; HS, human serum; FCS, fetal calf serum; mAb, monoclonal antibody; IL-2, interleukin 2.

conditions as described in the figure legends. DNA synthesis was measured by pulse-labeling 10^6 cells per ml with [^{3}H]thymidine at 5 μCi/ml (1 Ci = 37 GBq; Amersham) for 6 hr. The cells were also analyzed with fluorescent mAb IL-2 R1 directed against the interleukin 2 (IL-2) receptor (Coulter) and fluorescence cytometry using a FACS IV (Becton Dickinson). In all figures mean values of at least three different experiments with PBMC from different donors are given.

Cytotoxicity Assay. To measure the cytotoxic activity of PBMC after 3 days in culture, M21 melanoma cells labeled with sodium [^{51}Cr]chromate were incubated with mAb 9.2.27-OKT3 conjugate at 3 μg/ml for 30 min at 37°C and washed. PBMC activated under various conditions were then added to 10^4 tumor cells per well at an effector/target cell ratio of 40:1, and cytotoxicity was determined in a 4-hr ^{51}Cr-release assay as described (6).

RESULTS

Inhibition by Human Serum of PBMC Proliferation Induced by the mAb Conjugate 9.2.27-OKT3. Fig. 1*A* shows that during 3 days of culture in medium containing 10% (vol/vol) FCS unconjugated OKT3 stimulated PBMC proliferation over a wide concentration range, as determined by [^{3}H]thymidine incorporation. In 20% (vol/vol) HS more antibody was required than in 10% (vol/vol) FCS to achieve maximal activation, and the inhibitory effect of high antibody concentrations was more pronounced. If an anti-target cell-OKT3 conjugate (mAb 9.2.27-OKT3) (Fig. 1*B*) was used for activation of PBMC, the dose required for comparable activation was higher than that needed of OKT3 alone; activation proceeded in FCS, but was minimal in HS occurring only in a narrow anti-target-OKT3 antibody concentration range. Also, IL-2 receptor expression and induction of cytotoxicity were almost completely inhibited by HS (Fig. 2).

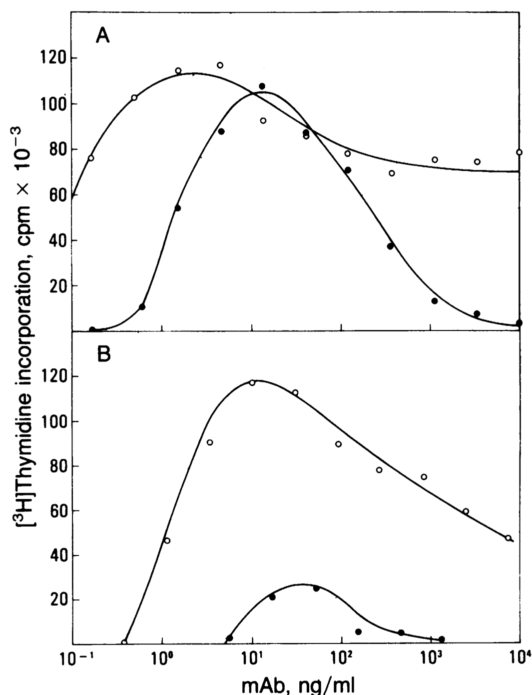

FIG. 1. Inhibition by HS of proliferation of PBMC induced by the mAb 9.2.27-OKT3 conjugate. PBMC (8 × 10^5 cells per ml) were cultured for 3 days in medium containing 10% (vol/vol) FCS (○) or 20% (vol/vol) HS (●) with the indicated amounts of OKT3 (*A*) or mAb 9.2.27-OKT3 conjugate (*B*).

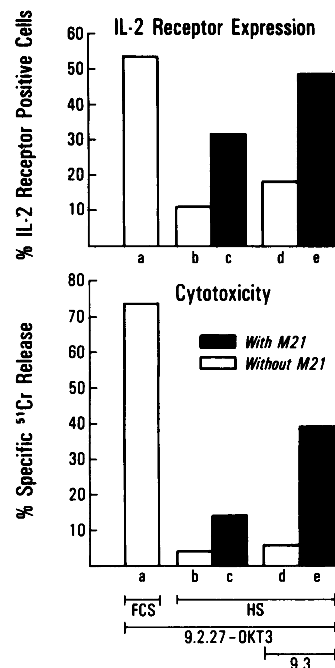

FIG. 2. Positive effect of melanoma target cells on PBMC activation by the mAb 9.2.27-OKT3 conjugate in the presence of HS. PBMC (8 × 10^5 cells per ml) were cultured in 10% (vol/vol) FCS (bar a), 20% (vol/vol) HS (bars b–e), mAb 9.2.27-OKT3 conjugate (150 ng/ml) (bars a–e), mAb 9.3 (600 ng/ml) (bars d and e), and M21 melanoma cells (initial PBMC/M21 cell ratio, 15:1) (bars c and e). After 3 days the lymphocytes were removed, washed, and subjected to analysis for IL-2 receptor expression and cytotoxic activity toward M21 cells.

Effects of Target Cells on mAb 9.2.27-OKT3 Conjugate-Mediated PBMC Activation. In view of the HS inhibition of conjugate-mediated PBMC activation, we explored whether target cells with conjugate bound to their surface could substitute for the OKT3-presenting function of monocytes. The addition of target cells at an initial concentration comparable to that of monocytes in PBMC had a positive effect on conjugate-mediated PBMC activation as assessed by IL-2 receptor expression and also on the cytotoxic activity of the cells, although to a lesser extent (Fig. 2). That these effects were not due to alloreactivity of PBMC toward the target cells is supported by the following findings: (*i*) PBMC cultured in 20% (vol/vol) HS for 3 days with or without M21 cells showed no signs of activation. (*ii*) When unconjugated OKT3 was used for activation in HS (Fig. 1*A*), addition of target cells resulted in variable inhibition rather than stimulation of activation. (*iii*) Excess mAb 9.2.27 specifically inhibited the target cell effects in conjugate-induced activation.

Effects of mAb 9.3 on PBMC Activation by mAb 9.2.27-OKT3 Conjugate and Target Cells. Target cells that present anti-CD3 in the form of anti-target-anti-CD3 conjugate can only stimulate the receptor–CD3 complex; they cannot supply second signals provided by monocytes that are also necessary for T-cell activation to proceed (18–20, 24). We attempted, therefore, to supply second-signal functions by using mAb 9.3, an agonistic antibody directed to Tp44 (CD28) furnishing progression signals (18–20). Fig. 2 shows that this antibody markedly increased the positive target-cell effect in mAb 9.2.27-OKT3-mediated PBMC activation. The effect of mAb 9.3 was similarly pronounced when monocyte-depleted PBMC were used (Fig. 3). No activation was seen if unconjugated OKT3 was used under identical experimental conditions (data not shown).

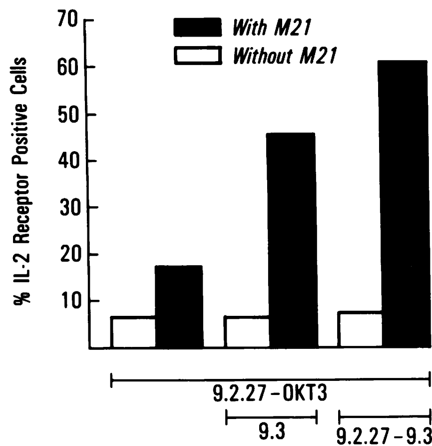

FIG. 3. Activation of monocyte-depleted PBMC by mAb 9.2.27-OKT3 and mAb 9.2.27-9.3 conjugates in the presence of melanoma target cells assessed by IL-2 receptor expression. Monocyte-depleted PBMC (8×10^5 cells per ml) were cultured for 3 days in 20% (vol/vol) HS in the presence of M21 cells (initial PBMC/M21 cell ratio, 10:1), mAb 9.2.27-OKT3 conjugate (150 ng/ml), mAb 9.3 (600 ng/ml), and the mAb 9.2.27-9.3 conjugate (150 ng/ml) as indicated.

Activation of Monocyte-Depleted PBMC by Target Cells Presenting OKT3 and mAb 9.3. A mixture of mAb 9.2.27-OKT3 and mAb 9.2.27-9.3 conjugates in the presence of melanoma target cells activated monocyte-depleted PBMC as judged by IL-2 receptor expression (Fig. 3). When the target cells were precoated with saturating amounts of the respective conjugates and washed before addition to monocyte-depleted PBMC, again a pronounced synergistic response to the two conjugates was observed (Fig. 4). The impact of the mAb 9.3-containing conjugate on the 3-day activation phase was particularly apparent when the cytotoxic activity of the pretreated cells was determined in a 4-hr ^{51}Cr-release assay (Fig. 5). In contrast, monocyte-depleted PBMC incubated with target cells and the mAb 9.2.27-OKT3 conjugate alone had no cytotoxic activity. Effective PBMC activation could be observed at concentrations of each of the two conjugates as low as 8 ng/ml.

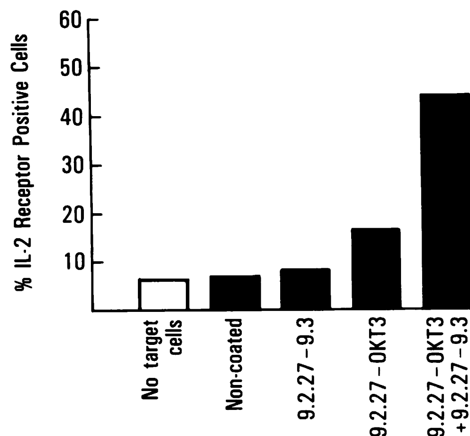

FIG. 4. Activation of monocyte-depleted PBMC by melanoma cells precoated with mAb 9.2.27-OKT3 and mAb 9.2.27-9.3 conjugates assessed by IL-2 receptor expression. M21 melanoma cells (10^6 cells per ml) were incubated with medium only, mAb 9.2.27-OKT3 conjugate (5 μg/ml), mAb 9.2.27-9.3 (5 μg/ml), or a mixture of mAb 9.2.27-OKT3 (5 μg/ml) and mAb 9.2.27-9.3 (5 μg/ml). After incubation at 37°C for 30 min, cells were washed twice and added to monocyte-depleted PBMC (8×10^5 cells per ml) at a PBMC/M21 cell ratio of 10:1 and cultured in 20% (vol/vol) HS for 3 days. The lymphocytes were then separated from the adhering tumor cells and analyzed for IL-2 receptor expression.

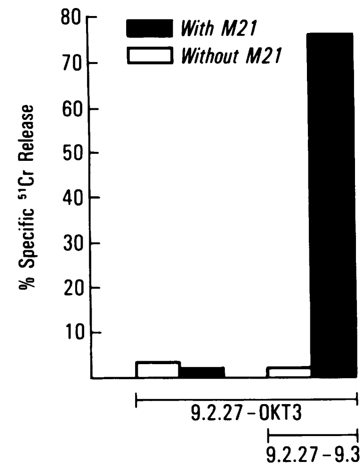

FIG. 5. Cytotoxic activity of monocyte-depleted PBMC activated by mAb 9.2.27-OKT3 and mAb 9.2.27-9.3 conjugates in presence of melanoma target cells. Monocyte-depleted PBMC were incubated in 20% (vol/vol) HS with or without M21 cells (initial PBMC/M21 cell ratio, 10:1) in the presence of mAb 9.2.27-OKT3 alone (150 ng/ml) or in combination with mAb 9.2.27-9.3 (150 ng/ml). Cytotoxicity was determined after 3 days in culture.

Fig. 6 shows photomicrographs of M21 cells cultured with monocyte-depleted PBMC for 6 days. With mAb 9.2.27-OKT3 (150 ng/ml) alone, the tumor cells proliferated forming a dense layer of adhering cells. With mAb 9.2.27-OKT3 (150 ng/ml) and mAb 9.2.27-9.3 (150 ng/ml), the tumor cells died, and only lymphocytes are seen. Three days after initiation of these cultures, samples of lymphocytes were removed from both flasks and tested for IL-2 receptor expression and cytotoxicity. With mAb 9.2.27-OKT3 alone, receptor expression was only slightly increased (Fig. 3), and cytotoxicity was low (Fig. 5). When both conjugates were present, the cells were IL-2 receptor positive (50–60%) (Fig. 3) and strongly cytotoxic toward melanoma cells as determined in a 4-hr ^{51}Cr-release assay (Fig. 5).

DISCUSSION

An *in vitro* model for T-cell activation and tumor cell killing has been designed to fulfill several key requirements pertinent to possible *in vivo* application. (*i*) The cells used are uncloned resting T lymphocytes as they normally occur in human blood. These cells lack the cytolytic, pore-forming protein C9RP (8, 9) and need to be activated to acquire cytotoxic potential. (*ii*) The resting cells are rendered cytotoxic by stimulation of the T-cell antigen receptor structure CD3 with monoclonal antibody that, during the ensuing 3-day period, results in critical changes of cellular functions including *de novo* synthesis of C9RP (8, 9). (*iii*) The solid-phase presentation of anti-CD3, obligatory for T-cell activation by this antibody, has been assigned to the tumor target cells rather than to the Fc receptor-bearing cells. Presentation of anti-CD3 by the tumor cells is accomplished by means of an anti-target-anti-CD3 heteroconjugate. This strategy obviates Fc receptor involvement and, therefore, circumvents the problem of inhibition of Fc receptor–anti-CD3 interaction by free IgG in serum. (*iv*) The heteroconjugates employed show little interaction with monocytes in presence of human serum. The T-cell activating function of these conjugates is, therefore, confined to the tumor cell surface. (*v*) The second signal needed for T-cell activation by anti-CD3 and normally provided by monocytes is supplied in the monocyte-independent system by mAb 9.3 (18–20). (*vi*) To direct the second-signal effect of mAb 9.3 toward the tumor cell surface, this

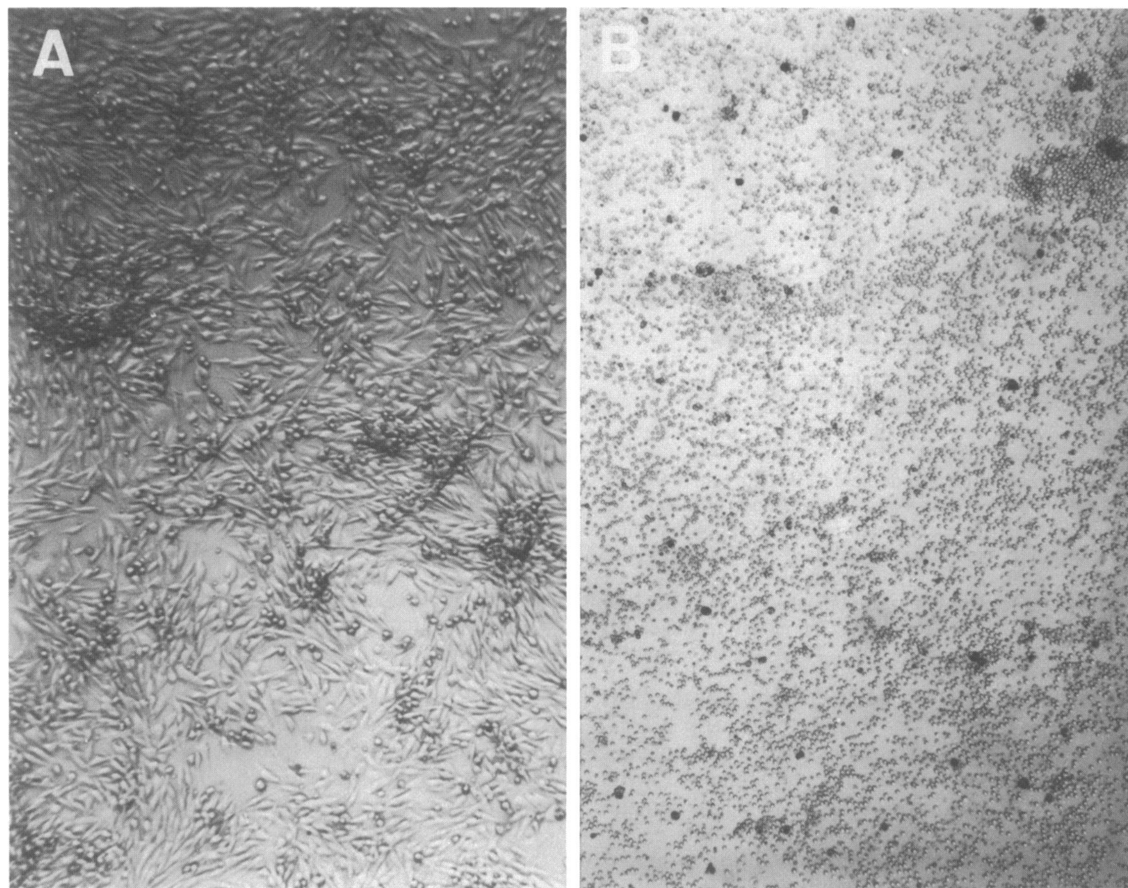

FIG. 6. Photomicrographs of M21 cells cultured with monocyte-depleted PBMC in 20% (vol/vol) HS for 6 days. The initial PBMC/M21 cell ratio was 10:1. Cells were incubated at initiation of culture with mAb 9.2.27-OKT3 conjugate (150 ng/ml) (*A*) or a mixture of mAb 9.2.27-OKT3 (150 ng/ml) and mAb 9.2.27-9.3 conjugates (150 ng/ml) (*B*). (×32.) Whereas the melanoma cells in *A* have proliferated to form a dense monolayer, in *B* primarily lymphocytes are seen without evidence for surviving tumor cells.

antibody is furnished also in the form of a conjugate with anti-target antibody. Thus, two distinct antibody heteroconjugates that alone or in combination have no apparent effects on T lymphocytes induce T-cell activation in presence of target cells to which they specifically bind (Figs. 3 and 5). Activation was efficient in that minute amounts of the conjugates sufficed; it was monocyte independent and, therefore, not inhibited by 20% (vol/vol) HS.

Tp44 is a T-cell differentiation antigen, and mAb 9.3, which reacts with Tp44, constitutes one of the second signals for T-cell proliferation (18–20). Interleukin 1, IL-2 (10, 24), and anti-Tp67 (CD5) (18, 19) are other second signals that are capable of replacing monocyte requirement. mAb 9.3 by itself has no detectable effect on T cells. However, in combination with the tumor promoter phorbol 12-myristate 13-acetate (15) or in conjunction with immobilized anti-CD3 (18, 20), mAb 9.3 provides a powerful stimulus for T-cell activation. In the experiments described above, mAb 9.3 markedly enhanced T-cell activation by the anti-CD3 presenting tumor target cells. The biochemical events that are directly triggered by the mAb 9.3-Tp44 interaction and that might also be elicited by an as yet unidentified physiological ligand are currently unknown, except for the reported increase in the concentration of free cytoplasmic calcium ions (20, 25, 26).

Whereas the described strategy of human T-cell activation and resultant tumor cell killing is exceedingly efficient *in vitro*, its feasibility and efficacy *in vivo* remain to be investigated.

The authors thank Dr. Ralph A. Reisfeld for supplying mAb 9.2.27. This is publication 4775-IMM from the Department of Immunology, Research Institute of Scripps Clinic. This investigation was supported by U.S. Public Health Service Grants AI 17354, CA 27489, HL 07195, HL 16411, and RR 00833. G.J. was supported by Fellowship 300-402-516-5 from the Stiftung Deutsche Krebshilfe (Mildred Scheel Stipendium).

1. Staerz, U. D., Kanagawa, O. & Bevan, M. J. (1985) *Nature (London)* **314**, 628–631.
2. Perez, P., Hoffman, R. W., Shaw, S., Bluestone, J. A. & Segal, D. M. (1985) *Nature (London)* **316**, 354–356.
3. Liu, M. A., Kranz, D. M., Kurnick, J. T., Boyle, L. A., Levy, R. & Eisen, H. N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 8648–8652.
4. Chang, T. W. (1985) *Immunol. Today* **6**, 245–249.
5. Wauwe, J. V. & Goossens, J. (1981) *Int. J. Immunopharmacol.* **3**, 203–208.
6. Jung, G., Honsik, C. J., Reisfeld, R. A. & Müller-Eberhard, H. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 4479–4483.
7. Zalman, L. S., Brothers, M. A., Chiu, F. J. & Müller-Eberhard, H. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 5262–5266.
8. Zalman, L. S., Martin, D. E., Jung, G. & Müller-Eberhard, H. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2426–2429.
9. Martin, D. E., Zalman, L. S., Jung, G. & Müller-Eberhard, H. J. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2946–2950.
10. Manger, B., Weiss, A., Weyand, C., Goronzy, J. & Stobo, J. D. (1985) *J. Immunol.* **135**, 3669–3673.
11. Williams, J. M., Deloria, D., Hansen, J. A., Dinarello, C. A., Loertscher, R., Shapiro, H. M. & Strom, T. B. (1985) *J. Immunol.* **135**, 2249–2255.
12. Landegren, U., Andersson, J. & Wigzell, H. (1984) *Eur. J. Immunol.* **14**, 325–328.
13. Looney, R. J. & Abraham, G. N. (1984) *J. Immunol.* **133**, 154–156.
14. Bumol, T. F. & Reisfeld, R. A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1245–1249.
15. Hara, T., Fu, S. M. & Hansen, J. A. (1985) *J. Exp. Med.* **161**, 1513–1524.

16. Gmünder, H. & Lesslauer, W. (1984) *Eur. J. Biochem.* **142,** 153–160.
17. Lesslauer, W., Koning, F., Ottenhoff, T., Giphart, M., Goulmy, E. & van Rood, J. J. (1986) *Eur. J. Immunol.* **16,** 1289–1296.
18. Ledbetter, J. A., June, C. H., Martin, P. J., Spooner, C. E., Hansen, J. A. & Meier, K. E. (1986) *J. Immunol.* **136,** 3945–3952.
19. Clark, E. A. & Ledbetter, J. A. (1986) *Immunol. Today* **7,** 267–270.
20. Weiss, A., Manger, B. & Imboden, J. (1986) *J. Immunol.* **137,** 819–825.
21. Ey, P. L., Prowse, S. J. & Jenkin, C. R. (1978) *Immunochemistry* **15,** 429–436.
22. Carlsson, J., Drevin, H. & Axen, R. (1978) *Biochem. J.* **173,** 723–737.
23. Thiele, D. L., Kurosaka, M. & Lipsky, P. E. (1983) *J. Immunol.* **131,** 2282–2290.
24. Meuer, S. C. & Meyer zum Büschenfelde, K.-H. (1986) *J. Immunol.* **136,** 4106–4112.
25. Pantaleo, G., Olive, D., Harris, D., Poggi, A., Moretta, L. & Moretta, A. (1986) *Eur. J. Immunol.* **16,** 1639–1642.
26. Ledbetter, J. A., June, C. H., Grosmaire, L. S. & Rabinovitch, P. S. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1384–1388.