25°C, followed by two washes for 30 min in 1× SSC, 0.1% SDS at 50°C. After exposure to film, the blot was washed and rehybridized with an actin probe, which indicated that the lanes (in Fig. 2) contained similar quantities of nondegraded RNA.

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11. We thank D. Page for providing pDP1007. Supported by the University of Texas William and Gladys Reeder Fellowship (J.B.) and by NSF grants DEB 8415745 (J.B.) and BSR 8657640 (D.H.). D. K. Hews, G. A. Mengden, and M. Dixon provided tissues of some the species used; live specimens collected in Texas were authorized under Texas Parks and Wildlife Scientific Permit #329 to J.B. D. Crews and P. Harvey commented on the manuscript.

8 June 1988; accepted 23 August 1988

In Vivo Administration of Anti-CD3 Prevents Malignant Progressor Tumor Growth

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Malignant progressor tumors are only weakly immunogenic and can evade host recognition and rejection. One approach to therapy involves activation of the host antitumor cellular effector mechanisms. Since monoclonal antibodies to CD3 (anti-CD3) can activate T cells in vitro, an attempt was made to determine if tumor immunity could be achieved by the administration of anti-CD3 in vivo. T lymphocytes from mice injected with anti-CD3 showed increased interleukin-2 receptor (IL-2R) expression, increased proliferation to recombinant IL-2 (rIL-2), and enhanced reactivity in both an allogeneic mixed lymphocyte reaction and a mixed lymphocyte tumor culture. Malignant tumor growth in treated mice was also examined. The anti-CD3 treatment prevented tumor outgrowth that would have killed untreated animals and also stimulated an in vivo response against a malignant progressor tumor providing lasting tumor immunity.

MONOCLONAL ANTIBODIES (MABS) to T lymphocyte antigens have been used to suppress immune responses in vivo and in vitro by blocking T cell receptor-mediated antigen recognition (*1*). This property has been exploited in the clinical setting to prevent and reverse organ transplant rejection (*2*). In addition to their immunosuppressive properties, these MAbs can activate resting T cells in vitro. This suggests that they might be efficacious in augmenting immune responsiveness in a clinical setting. We initially observed that T cells become activated in vivo within hours after treatment with anti-CD3. However, under the conditions used, the stimulatory effects were transient and ultimately led to long-term immune dysfunction. We have therefore determined the optimal conditions to facilitate specific beneficial biologic responses with the use of anti-CD3 as an immunostimulatory reagent.

Intravenous administration of 400 μg of the anti-CD3 in vivo has previously been shown to activate T cells (*3*). This high dose, however, ultimately results in suppression of T cell function as demonstrated by inhibition of mixed lymphocyte reaction (MLR), cell-mediated lympholysis (CML), and transplantation responses (*1*). In an attempt to separate these two seemingly contradictory effects of anti-CD3, we examined the effects of lower MAb doses on T cell activation. Mice were given different doses of anti-CD3, and their lymph node and spleen cells were examined for IL-2R expression 18 hours later by flow cytometry (FCM) (Fig. 1). The IL-2R expression was enhanced at the three doses tested (4 μg, 40 μg, and 400 μg) and plateaued at the 40-μg dose. In other experiments maximal IL-2R expression was found at the 400-μg dose. When the same lymphoid cells were incubated in media containing human rIL-2, their proliferation was enhanced in proportion to their IL-2R expression (Fig. 2). This proliferation was not the result of the carry-over of injected anti-CD3 from the mouse to the culture media, since the addition of MAb 2.4G2 [anti-Fc receptor (FcR)], to the culture media did not affect the proliferative response (*4*). By comparison, the addition of 2.4G2 to control cells incubated with IL-2 and anti-CD3 eliminated the FcR-dependent proliferative response.

The immunosuppression which results from a dose of 400 μg of anti-CD3 is the result of T cell depletion, T cell receptor (TCR) blockade, and modulation of the TCR complex (*1*). The net result is that the amount of cell surface CD3 available to react with antigen is decreased, rendering the T cells unable to respond to antigenic stimuli because efficient antigen-specific activation depends on the presence of intact TCR. Therefore, the quantity of available CD3 on lymph node cells from control mice and those treated with various doses of anti-CD3 was examined 18 hours after treatment. Cells were stained with fluorescein

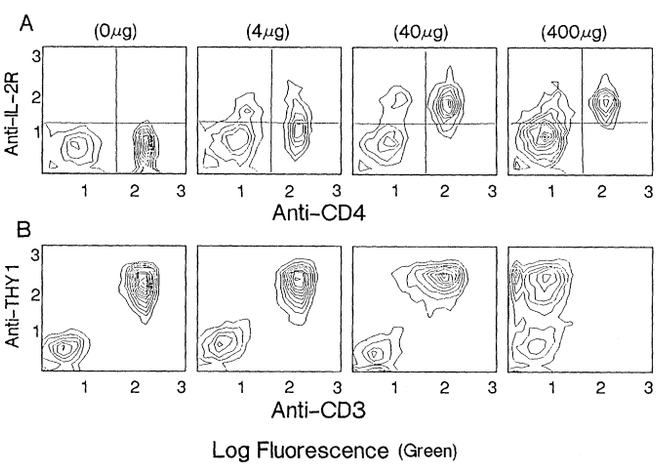

Fig. 1. Flow cytometry of peripheral lymph node cells from anti-CD3–treated C3H mice. Two-color FCM from control, 4, 40, and 400 μg of anti-CD3 treated animals are displayed as contour plots on a logarithmic scale. Intensity of green FITC fluorescence is plotted along the *x*-axis and red (B-phycoerythrin) fluorescence is plotted along the *y*-axis. (**A**) Anti-CD4 staining on the *x*-axis and anti-IL2R staining on the *y*-axis. (**B**) Anti-CD3 staining on the *x*-axis and anti-Thy-1 staining on the *y*-axis. C3H/HeN MTV⁻ mice (Frederick Cancer Research Foundation, Frederick, Maryland) were housed and cared for in accordance with the guidelines of the University of Chicago Animal Research Committee. The animals were killed 18 hours after intravenous injection of purified anti-CD3 (MAb 145-2C11) that was grown and purified as described (*1*). Femoral, axillary, and mesenteric lymph nodes were removed and dissociated into a single-cell suspension and FCM analysis was performed (*9*). Cells were stained with FITC–anti-CD3 or FITC–anti-CD4 (MAb GK1.5) (Becton Dickinson), and biotin-conjugated anti–IL-2R (MAb 3C7) or biotin-conjugated MAb to Thy-1.2 (Becton Dickinson), then counterstained with B-phycoerythrin–conjugated egg white avidin (Jackson Immuno Research Laboratories).

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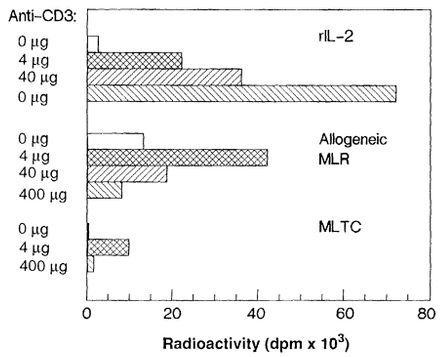

Fig. 2. In vitro proliferation of lymph node cells from treated C3H mice. Eighteen hours after intravenous anti-CD3 administration lymph nodes were removed from animals and the cells (1×10^5 cells for rIL-2 assays, 2×10^5 cells for MLR and MLTC) were incubated in medium that contained irradiated syngeneic spleen cells (2×10^5) plus rIL-2 or irradiated allogeneic (C57BL/10) spleen cells, or mitomycin C–treated Pro-4L tumor cells (5×10^3). Proliferation was measured by [^{3}H]thymidine uptake at 48 hours (rIL-2) or 72 hours (MLR and MLTC). Background (generally less than 5×10^3 cpm) was determined from lymph node cells stimulated with syngeneic irradiated accessory cells, or from mitomycin C–treated tumor cells cultured alone, and was subtracted. All assays were performed in triplicate; standard deviations were less than 5%.

isothiocyanate (FITC)–conjugated anti-CD3 and Thy-1$^+$ T cells were examined (Fig. 1B). Although T cells from the 400-μg treated animals expressed the highest level of IL-2R, they were negative for CD3 because of the modulation of cell surface CD3/TCR and the coating of cell surface CD3 by MAbs (*1*). This coating was seen as a positive shift of the cells when stained with an FITC-conjugated antibody to hamster immunoglobulin (*4*). Thus, while activated T cells from 400-μg treated animals showed the most IL-2R expression and proliferation to IL-2, this treatment led to immunosuppression. IL-2R expression on cells from 40-μg treated animals was also elevated, but there was a decrease in CD3 expression by these cells (Fig. 1) and an increase in the amount of anti-CD3 bound to the cells (*4*). In contrast, animals treated with 4 μg of anti-CD3 demonstrated normal CD3 expression. Therefore, optimal conditions for anti-CD3 mediated T cell activation might be those in which the concentration of anti-CD3 is low enough to permit activation without adversely affecting CD3/TCR expression.

Additional evidence that the low dose anti-CD3 treatment not only nonspecifically activated T cells, but increased immune responsiveness, was obtained from allogeneic mixed lymphocyte reaction (MLR) and mixed lymphocyte tumor culture (MLTC) studies. Lymphocytes harvested from mouse peripheral lymph nodes 18 hours after anti-CD3 treatment were stimulated with syngeneic or allogeneic lymphocytes, or with the syngeneic C3H fibrosarcoma 1591-Pro-4L (Pro-4L) tumor cells. As predicted by the FCM analysis of TCR expression, treatment with 400 μg of anti-CD3 resulted in a diminished ability to proliferate in an MLR and minimal proliferation in the MLTC (Fig. 2). In contrast, treatment with 4 μg of anti-CD3 enhanced the proliferative response against both allogeneic spleen and syngeneic tumor cells. Thus, low doses of anti-CD3 stimulated T cells without interfering with CD3/TCR function. This activation can be measured as the enhancement of a specific physiologic response in vitro. Furthermore, cells from mice treated with 4 μg of anti-CD3 had enhanced proliferation when incubated in an MLTC along with Pro-4L cells.

Since in vivo treatment with anti-CD3 could enhance specific MLR and MLTC responses, we tested the effects of anti-CD3 on malignant tumor growth in vivo. Many malignant tumors do elicit a specific immune response, but this may not be sufficient to effect a reversal of tumor growth (*5*), so we attempted to augment the already existent antitumor immune response to effect tumor regression. To examine this potential in a murine system, we used the C3H fibrosarcoma 1591-Pro-4L (*6*). Pro-4L is a weakly immunogenic ultraviolet light–induced murine tumor that lacks cell surface CD3 and FcR, and therefore does not react directly with the anti-CD3 used for treatment. This malignant tumor grows progressively in about 95% of normal C3H mice and eventually kills all of the mice by infiltrative growth without macroscopic evidence of metastasis (*6*). None of the mice treated with 4 μg of anti-CD3 developed tumors in this experiment (Fig. 3). Animals treated with 4 μg of anti-CD3 also developed tumor immunity since they failed to develop tumors following a second inoculation of Pro-4L 60 days later, with no additional MAb therapy. All naïve animals (11 of 11) challenged with tumor fragments at that time developed tumors. Treatment of animals with F(ab′)$_2$ fragments of the anti-CD3 (equivalent in moles to 4 μg of intact anti-CD3) had no effect on tumor growth (Table 1). The F(ab′)$_2$ is immunosuppressive, yet does not stimulate T cells (*3*). Therefore, the effect of the anti-CD3 on tumor growth was probably due to the immunopotentiating effects of the treatment MAbs. These immunopotentiating effects might be manifested in increased tumor-specific T cells, lymphokine-activated killer cell activity, or the induction of antitumor cytokines. In this regard, tumor necrosis factor (TNF) was detected (*7*) in the serum of anti-CD3–treated, but not control, animals (*4*), and has been suggested as a possible mediator of antitumor effects (*8*).

Overall, after treatment with 4-μg of anti-CD3, 65% of the animals in accumulated

Table 1. Summary of tumor incidence at day 28 in anti-CD3–treated mice (from three separate experiments). There was no recurrence, late outgrowth, or tumor regression after 28 days. The *P* value (χ^2 method) for the difference between the control animals and those treated with 4 μg of anti-CD3 was determined (*). Five control animals and five of those treated with 4 μg of anti-CD3 also received two intraperitoneal doses of human rIL-2 (10,000 U). This additional treatment had no detectable effect on tumor growth.

Treatment group	Tumor incidence (day 28) no. with tumor/ total no. (%)
Control	42/44 (95)
Anti-CD3 (4 μg)	11/31 (35)
F(ab′)$_2$ anti-CD3 (2.6 μg)	9/10 (90)

*$P < 0.001$.

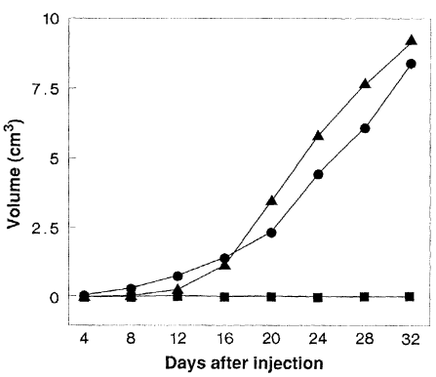

Fig. 3. Inhibition of Pro-4L growth by intravenous anti-CD3. The growth curves of tumors inoculated in one experiment. Tumor volume is a group average (four animals per treatment group). Groups received 4 μg of anti-CD3 (■), 40 μg of anti-CD3 (▲), or phosphate-buffered saline (●) when the tumor fragments (2 mm^3) were subcutaneously injected. Animals that received 4 μg of anti-CD3 developed small tumors which regressed during the first 3 weeks following treatment; all of the control animals and those treated with 40 μg of anti-CD3 developed progressor tumors. The animals treated with 40 μg of anti-CD3 seemed to develop more rapidly growing tumors; however, the difference in tumor volume between the control animals and those treated with 40 μg was not significant. One of the animals treated with 40 μg of anti-CD3 died at day 29 with a large tumor burden. The mean tumor size of control groups and those treated with 40 μg of anti-CD3 differed from the mean of the 4-μg treated group by greater than 2 standard deviations at all measurements done after day 20. After 45 days, three of four animals treated with 40 μg of anti-CD3 and two of four controls had died with large tumor burdens, but none of the 4-μg treated animals had palpable tumors.

experiments did not develop tumors (Table 1). Thus, we have demonstrated that the in vivo activation of T cells by anti-CD3 can result in the enhancement of an antitumor response, the prevention of malignant tumor growth, and lasting tumor-associated immunity. In fact, it is now clear that the immunity is tumor-specific (*4*).

Although we did not observe immunosuppression after administration of low doses of anti-CD3, the potential for inadvertent immunosuppression is always present. The 40-μg dose of anti-CD3 caused enhanced tumor outgrowth (Fig. 3) with earlier animal death. This enhanced growth may be due to the immunosuppression that correlates with the reduction of T cell surface CD3 expression following treatment with 40 μg of anti-CD3. The use of several different activating MAbs that are not immunosuppressive when administered in the appropriate regimen may ultimately be the best approach to optimal immunoenhancement without immunosuppression.

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10. We thank G. Perdriget for his assistance in developing the tumor transplant model, J. Rothstein for performing the TNF assay, P. Hartley for his expertise with flow cytometry, and F. Fitch for review of this manuscript. J.D.I.E. is the recipient of an NIH training grant, and J.A.B. is a Gould Foundation faculty scholar with support from the Lucille P. Markey Charitable Trust.

6 June 1988; accepted 3 August 1988

T Cell CD3-ζη Heterodimer Expression and Coupling to Phosphoinositide Hydrolysis

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The T cell antigen receptor consists of an antigen-binding heterodimer that is noncovalently associated with at least five CD3 subunits (γ, δ, ε, ζ, and η). The CD3-ζ chains are either disulfide-linked homodimers (CD3-ζ_2) or disulfide-linked heterodimers with η (CD3-ζη). Variants of a murine antigen-specific T cell hybridoma that express normal amounts of CD3-ζ_2 but decreased amounts of CD3-ζη were isolated. When activated, the parental cell line increased both phosphatidylinositol hydrolysis and serine-specific protein kinase activity to a much greater extent than the variants. In contrast, the activation of a tyrosine-specific kinase after stimulation with a cross-linking antibody to CD3 was similar among these cells. There was a positive linear relation between the expression of CD3-ζη and phosphoinositide hydrolysis stimulated by the TCR, suggesting a differential coupling of the T cell αβ heterodimer to signal transduction mechanisms due to αβ association with either CD3-ζ_2 or CD3-ζη.

THE T CELL ANTIGEN RECEPTOR (TCR) consists of an antigen-binding heterodimer noncovalently associated with at least five nonpolymorphic subunits of CD3: γ, δ, ε, ζ, and η (*1, 2*). The large majority (~90%) of the CD3-ζ chains exists as disulfide-linked homodimers, CD3-ζ_2, with the remainder being disulfide-linked heterodimers with η, CD3-ζη (*2*). The gene encoding the CD3-ζ chain has been cloned (*3*); its product is predicted to have a single cysteine residue available for covalent interactions. Therefore, a CD3-ζ chain is either disulfide-linked to a second CD3-ζ chain or a CD3-η chain, but not both. This has suggested a model in which two structurally distinct TCR complexes exist, the predominant one containing CD3-ζ_2 and the remainder containing CD3-ζη (*2*).

T cells bearing variant TCR complexes were produced by the subcutaneous injection of syngeneic mice with 2B4.11 T hybridoma cells and then intraperitoneal injections of monoclonal antibody (MAb) 2C11 (anti–CD3-ε) (*4*). Activation of 2B4.11 T cells in vitro with this MAb leads to a cell cycle block and autolysis (*5*). Despite treatment with anti–CD3-ε in vivo, tumors appeared at the site of inoculation in two different mice. The tumors were excised, and the cells were cultured and subcloned by limiting dilution (*6*). Since 2B4.11 cells frequently lose expression of CD3-ζ (*7, 8*), the subclones were analyzed by immunoblotting with an antiserum to CD3-ζ (anti–CD3-ζ). All five subclones obtained from one tumor line expressed little or no CD3-ζ chain and had low amounts of cell surface TCR, consistent with a previous analysis of a CD3-ζ–deficient 2B4.11 variant (*7*). The second tumor line was heterogeneous in its expression of cell surface TCR, and many subclones from this line expressed normal amounts of CD3-ζ. Two cells, EV.3 and 2M.44, from separate subclonings of this tumor were further analyzed.

Both anti–CD3-ε and a MAb to the 2B4 TCR-α chain, A2B4-2 (*9*), were used to evaluate cell surface expression of TCR (Fig. 1). The 2B4.11 cell line and both variants had single peaks of fluorescence, with 2B4.11 > 2M.44 > EV.3 (EV.3 cells had ~60 to 75% the mean fluorescence intensity of 2B4.11 cells on multiple analyses). Solu-

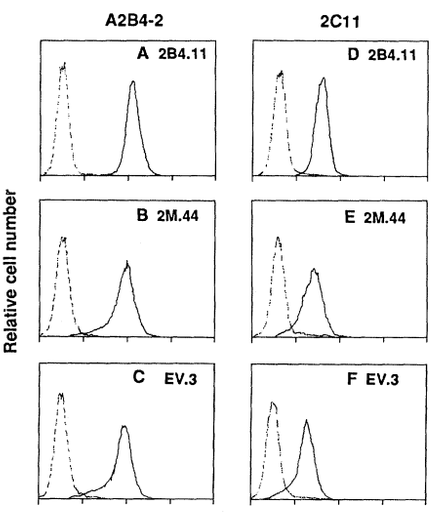

Fig. 1. Cell surface expression of TCR by 2B4.11 and two variants. 2B4.11, 2M.44, and EV.3 were analyzed by indirect immunofluorescence flow cytometry for cell surface expression of the TCR-α chain (**A** to **C**) or the CD3-ε chain (**D** to **F**) (solid lines). Background immunofluorescence was determined by staining with the fluoresceinated F(ab')$_2$ second antibody alone, either goat antibody to mouse immunoglobulin or goat antibody to hamster immunoglobulin, respectively (dotted lines). The 2B4.11 and EV.3 cells stained weakly or not at all with anti-L3T4, and both cells stained brightly with antibodies to Thy-1 and Ly-6 (*10*).

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