Anti-CD2 Antibodies Induce T Cell Unresponsiveness In Vivo

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Summary

The CD2 receptor functions as an adhesion and signal molecule in T cell recognition. Multimeric binding of CD2 on T cells to its physiologic ligand LFA-3 on cognate partner cells in vitro efficiently augments the antigen-specific T cell signal delivered by the T cell receptor/CD3 complex. The precise contribution of the antigen-nonspecific CD2-LFA-3 interactions to T cell immune responses in vivo, however, has been difficult to assess. Here we analyzed the role of CD2 in the murine immune response using a nondepleting anti-CD2 monoclonal antibody that induces a marked, reversible modulation of CD2 expression on murine T and B cells in situ. This modulation is dose and time dependent, specific for CD2, and does not require the Fc portion of the antibody. Anti-CD2 antibodies [rat IgG1 or $F(ab')_2$] significantly inhibit the $CD4^+$ T cell-mediated response to hen egg lysozyme and the cytotoxic $CD8^+$ T cell response to a syngeneic tumor cell line. In both cases, anti-CD2 antibodies are only effective when administered before or within 24 h after antigen priming. The suppression of the antitumor response corresponds to a sixfold reduction of specific cytotoxic T lymphocyte precursor cells and results in the abrogation of protective antitumor immunity. Anti-CD2 antibodies also affect the humoral immune response to oxazolone: the isotype switch from specific IgM to IgG1 antibodies is delayed, whereas the IgM response is unaltered. In addition, a single antibody injection results in sustained polyclonal unresponsiveness of T cells irrespective of antigen priming and CD2 modulation. These results document that CD2-mediated signals induce a state of T cell unresponsiveness in vivo.

The differentiation, activation, and effector phase of T cells is determined and regulated by interactions of an array of receptors on T cells with their specific ligands on cognate partner cells. Interactions of the T cell receptor with the MHC/antigen complex determine the specificity of T cell recognition. Interactions between the surface receptor pairs CD8 and MHC class I, CD4 and MHC class II, lymphokine function-associated antigen 1 (LFA-1)[1] and ICAM-1/2, or CD2 and LFA-3 provide cosignals which augment the specific signal and regulate the adhesion process (1–8). This multiplicity of receptor pairs allows for a subtle regulation of T cell recognition and underlines the importance of this process for the immune system. Among the "auxillary" recognition molecules CD2 has been most intensely studied. CD2 is a 50–55 kD membrane glycoprotein which is expressed on 95% of thymocytes early on during intrathymic T cell maturation and on most peripheral T cells and natural killer cells, although species differ with respect to CD2 expression on T cell subsets (9–11). So far mice provide an exception in that murine CD2 is also expressed on B cells (12–14). The physiological ligand for human CD2 has been unequivocally identified as LFA-3, a widely distributed cell surface glycoprotein (15). Numerous studies revealed a dual function for CD2 as a signal and adhesion molecule. The signal function is most clearly documented by the activation of mature T cells by a combination of certain anti-CD2 antibodies (Abs) (16, 17) and the ligand of CD2 on SRBCs (18) and additionally the potentiation of T cell activation by crosslinking CD2 and the TCR with bispecific Abs in vitro (19). The adhesion function has been originally demonstrated by blocking T cell-target cell binding with anti-CD2 and anti-LFA-3 antibodies. Although the affinity between soluble CD2 and LFA-3 molecules is low, the adhesion provided during cell-cell contact is the sum of multimeric interactions between both molecules on apposing membranes (20). The signal function has been assigned to the membrane proximal part of the cyto-

[1] *Abbreviations used in this paper:* CTL-p, cytotoxic T lymphocyte precursor; HEL, hen egg lysozyme; ICAM, intercellular adhesion molecule; LFA, lymphocyte function-associated antigen; PEC, peritoneal exudate cells; PPD, protein purified derivative; SEB, staphylococcal enterotoxin B.

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Volume 174 November 1991 957–967

plasmic tail, amino acids 253–287 being essential for human CD2. The adhesion function resides in the outer extracellular domain encoded by exon 2 (3, 6, 21). Both functions can be dissected experimentally, whereby a signal-defective, adhesive CD2 molecule is far less effective in augmenting T cell activation than the intact, signal competent molecule (22). Whether CD2 can serve as an antigen-independent T cell activation receptor ("alternative pathway") or merely as a coreceptor for the TCR in vivo remains unclear (23).

The apparent redundancy in T cell interaction molecules raises the question whether CD2-LFA-3 interactions are indispensable for T cell recognition in vivo, or can be compensated for by other receptor pairs. Earlier studies have shown that anti-CD2 Abs can block T cell activation in vitro suggesting an important role for CD2 in this event (24). The contribution of CD2 to the immune response in vivo has been difficult to assess given the lack of appropriate studies in animal models. Here we report on experiments using a mAb against murine CD2, which efficiently modulates CD2 expression on lymphocytes in vivo. This modulation of CD2 expression has profound effects on T cell-mediated immune responses in vivo.

Materials and Methods

Mice. DBA/2 and C3H/He mice, 8–10 wk of age, were obtained from the Zentrale Versuchstierzuchtanstalt, Hannover, FRG; Balb/c mice were bred at the Institute of Genetics, Cologne. Animals were kept under specific pathogen-free conditions in isolators during the whole course of experimentation.

Reagents. The rat IgG1 anti-murine CD2 mAb 12.15A (25) and the control Abs anti-CD5 (rat IgG2a, 53-7.3, reference 31) and anti-tenascin (rat IgG1, 576, reference 26, kindly provided by Dr. Faissner, Institute of Neurobiology, University of Heidelberg) were semipurified from ascites fluid by Protein G affinity chromatography. Purified mAbs were administered i.p. in 200 μl sterile PBS. $F(ab')_2$ fragments of mAb 12.15A were prepared according to Rousseaux et al. (27). Briefly, Abs (10 mg/ml) were extensively dialyzed first against pH 2.8 (0.1 M sodium formate) and then against pH 4.5, (0.1 M sodium acetate), subsequently 1% pepsin (wt/wt) (Boehringer-Mannheim) was added for 4 h at 37°C and the reaction was stopped by neutralizing the pH with 1 M Tris buffer. $F(ab')_2$ fragments were purified from undigested Abs and smaller fragments by Mono Q ion-exchange FPLC chromatography.

The following mAbs were used for staining either as purified reagents or directly coupled to FITC, PE, or biotinylated: anti-CD3 (500 A3, reference 28), anti-$\alpha\,\beta$ TCR (H57.597, reference 29), anti-CD4 (GK 1.5, reference 30), anti-CD8 (53-6.7, reference 31) anti-Thy 1.2 (30H12, reference 31), anti-LFA-1 (FD 18.5, reference 32), and anti-B220 (RA3-3A1, reference 33).

Hen egg lysozyme (HEL) and staphylococcal enterotoxin B (SEB) were purchased from Sigma Chemical Co. (Munich, FRG), protein purified derivative (PPD) from Behringwerke AG (Marburg, FRG), Con A and myoglobin (Myo) from Serva (Heidelberg, FRG). Recombinant IL-2 was a gift of Cetus Immune (Emeryville, CA) and rIL-4 a gift from Dr. W. Müller, Cologne.

The DBA/2 mouse derived tumor cell line ESb is a spontaneous, highly metastatic variant of the methylcholanthrene-induced T cell lymphoma L5178Ye (Eb) (34). Tumor cell lines were passaged in RPMI 1640 (Gibco, Paisley, UK) containing 5×10^{-5} M 2-ME, 10 mM Hepes, pH 7.3, 2 mM glutamine, and 10% FCS. The cells were routinely tested to be free of mycoplasma and virus infections.

Quantitative Flow Cytometry. Staining of thymocytes, mesenteric lymph node cells, splenocytes, and peritoneal exudate cells was performed as described previously (35). Goat anti-rat IgG-PE, rabbit anti-hamster Ig-FITC, or streptavidin-PE (Jackson Lab., Avondale, PA) were used as second stage reagents. Analysis was performed on a FACScan® cytometer (Becton & Dickinson, Heidelberg, FRG) recording 10,000 events for single color and 20,000 events for dual color analysis. Dead cells and nonlymphoid cells were excluded from analysis by a combination of forward and side scatter gating. Data were processed with FACScan® Research software.

Immunization with HEL. C3H/He mice were immunized subcutaneously at the base of the tail with 100 μg HEL emulsified in 100 μl PBS/CFA (1/1; vol/vol). 7 d after immunization, the draining lymph nodes were removed and cells were cultured in 96-well microtiter plates (2×10^5 cells/well) with various concentrations of HEL and control antigens. Cultures were set up in triplicates from pooled lymph node cells of two to three mice, incubated for 4 d under standard conditions, and pulsed for the last 12 h before harvesting with 1 μCi [^{3}H]TdR/well (2 Ci/mmol, Amersham, Braunschweig, FRG). Incorporation of [^{3}H]TdR was measured according to standard procedures.

Immunization with Syngeneic Tumor Cells. DBA/2 mice were injected with 5×10^4 live ESb tumor cells in 50 μl PBS into the external ear under anaesthesia. Injection at this site proved to be most effective in inducing antitumor immunity and thus preventing local tumor growth and metastasis (36). 7 d after priming the mice received a second injection with 10^7 inactivated ESb cells i.p. 3 d after the second immunization peritoneal exudate cells (PEC) and splenocytes were removed. PECs were tested directly in a 4 h ^{51}Cr release assay for antitumor cytotoxicity ("secondary response"), whereas splenocytes were restimulated with syngeneic tumor cells for 4 d in vitro and then assayed for ESb-specific cytotoxicity ("tertiary response") as described previously (37). The parental tumor cell line Eb, which does not express the ESb-specific tumor antigen, was always included as a negative target cell control. Although Eb cells are sensitive to NK cells, lysis of Eb cells did not exceed 10%. We showed previously that ESb-specific cytotoxicity is exerted by $CD8^+$ T cells which recognize a class I MHC (K^d) associated ESb-specific antigen (34).

Limiting dilution analysis of PECs and splenocytes was performed according to a protocol described previously (37).

T Cell Activation Assays. Flat-bottomed 96-well microtiter plates were coated with various concentrations of purified anti-CD3 or anti-α/β TCR mAbs by preincubating the plates with the titered Abs in 100 μl of serum free medium for 20 h at 37°C followed by extensive washing. Unseparated lymph node cells (2×10^5/well) or thymocytes (5×10^5/well) were added in complete medium and cultured for 2–3 d. Alternatively, T cells were stimulated with Con A (2,5 μg/ml) or graded doses of SEB in flat-bottomed 96-well plates. Allogeneic MLR responses were measured after cocultivation of 2×10^5 DBA/2 lymph node cells with 2×10^5 irradiated C57Bl/6 splenocytes/well for 3 d in flat-bottomed 96-well plates. T cell proliferation was measured as described above.

Immunization with Oxazolone. BALB/c mice received 100 μg of phOXCSA (2-phenyl oxazolone coupled to chicken serum albumin, referred to as oxazolone) i.p. which was precipitated by alum (38). 1 d before and 7, 14, and 28 d after priming the mice were tail-bled and their serum IgM and IgG1 titers of anti-oxazolone Abs were determined in an ELISA assay using isotype-specific mAbs. Mice pretreated with 0.5–1 mg anti-CD2 Ab 1 d before antigen priming were compared to PBS-injected controls.

Results

Modulation of CD2 Expression In Vivo. The previously described mAb 12.15A (25) against mouse CD2 was used to study the role of CD2 during the immune response in vivo. A single injection of purified anti-CD2 mAb i.p. led to a significant modulation of CD2 expression on both T and B cells in peripheral and central lymphoid organs, the mean fluorescence being reduced by 80% (T cells) and 73% (B cells) (Fig. 1). Modulation was defined by highly reduced binding of anti-rat IgG-PE and biotinylated anti-CD2 Abs to lymphoid cells. The mAb-mediated modulation was dose-dependent, 100 μg intact IgG per animal being sufficient to induce maximal modulation. The same degree of CD2 modulation was achieved with a single injection of purified $F(ab')_2$ fragments, albeit at an $\sim$ 10-fold higher dose (Fig. 2). CD2 modulation did not result in comodulation of CD3, B220 (Figs. 1 and 2 *B*), TCR α/β, CD4, CD8, LFA-1, and Thy-1 surface molecules (data not shown).

Maximal modulation was effected 20 h after Ab injection and persisted for $\sim$3 d, thereafter CD2 expression was gradually regained and control CD2 levels were attained by day 14 (Fig. 3). The anti-CD2 mAb [intact IgG and $F(ab')_2$] led to a transient increase in the frequency of T cells by 5–20%

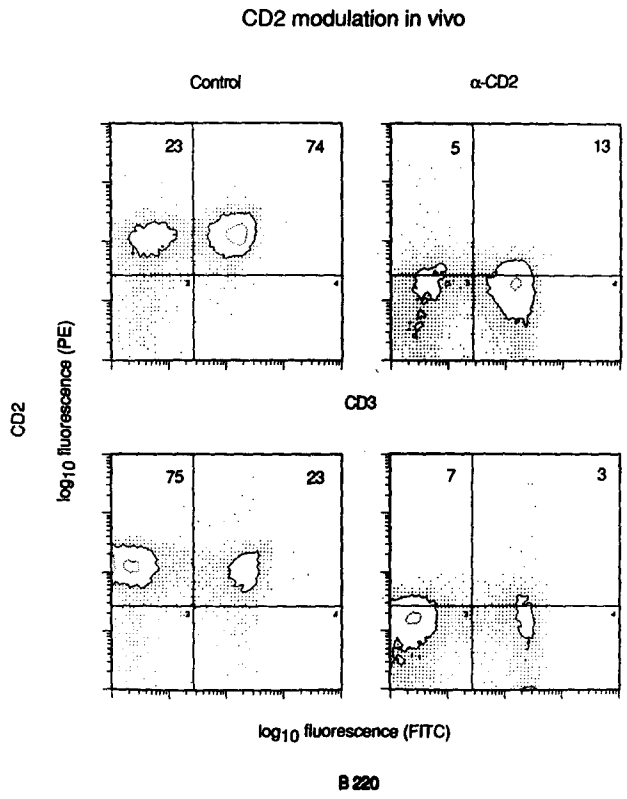

Figure 1. Modulation of CD2 on peripheral T and B cells in vivo. DBA/2 mice were injected with 1 mg anti-CD2 Ab i.p. and 20 h later mesenteric lymph node cells were double-labeled with CD2-biotin/streptavidin-PE and CD3-FITC or B220-FITC antibodies. Note the reduced expression of CD2 (reduction of mean fluorescence value by 75%) and the unaltered expression of CD3 and B220. CD2 positive cells are indicated in percent.

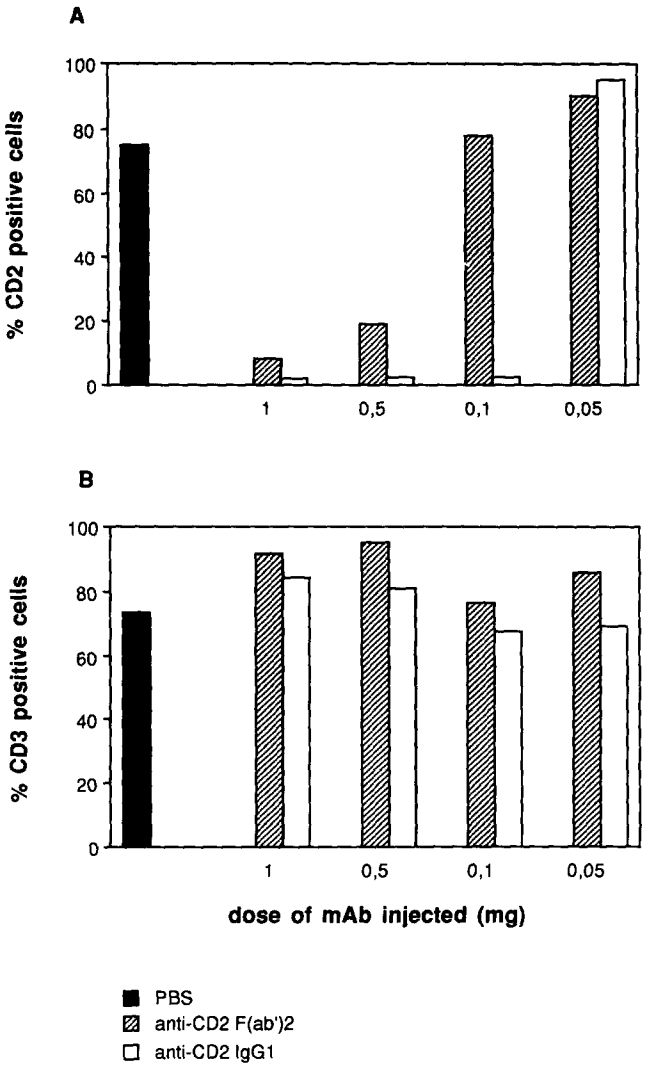

Figure 2. Modulation of CD2 is dose-dependent. DBA/2 mice were injected with different doses of intact or $F(ab')_2$ fragments of anti-CD2 Abs. 48 h later lymph node cells were labeled with CD2-biotin/streptavidin-PE (*A*) or CD3-FITC (*B*). $F(ab')_2$ fragments were $\sim$ 10 times less efficient than intact IgG Abs in inducing modulation in vivo. Cells with surface fluorescence above control staining with streptavidin-PE alone scored as positive. Each value represents one animal. In all experiments control mice received an equal volume of PBS.

(as assessed by four independent markers, data not shown) in spleen and lymph nodes and by day 8–14 to a relative decrease in numbers of T cells by 10–30% accompanied by a proportional increase in B cells. By day 30–40 post-injection the frequency of $CD4^+$ and $CD8^+$ T cells ranged between 85 and 100% of control levels. (Fig. 3). There was no consistent change in the absolute frequency of peripheral lymphocytes following Ab treatment.

Thus anti-CD2 Abs induced a marked modulation of CD2 receptors on T and B cells, which was specific for CD2, reversible, independent of the Fc-portion of the Ab, and site-independent.

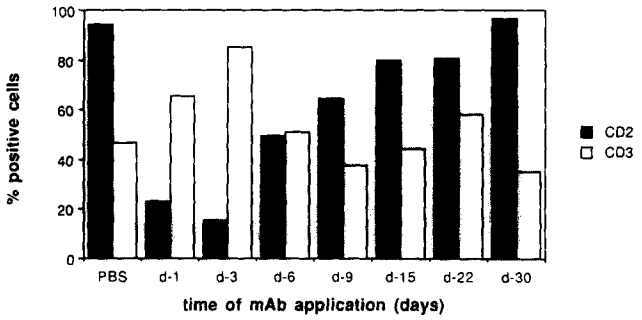

Figure 3. Time course of CD2 modulation in vivo. DBA/2 mice received 0.5 mg anti-CD2 Ab and at the time intervals indicated mesenteric lymph node cells were labeled as described in Fig. 2. Note the time-dependent reversion of CD2 modulation and the transient increase of $CD3^+$ cells. Each value represents one animal.

Anti-CD2 Abs Inhibit the Immune Response of $CD4^+$ T Cells to Hen Egg Lysozyme In Vivo. The efficient modulation of CD2 expression on murine T and B cells in vivo allowed us to assess the role of the CD2 receptor in the immune response in vivo. Does the highly reduced expression

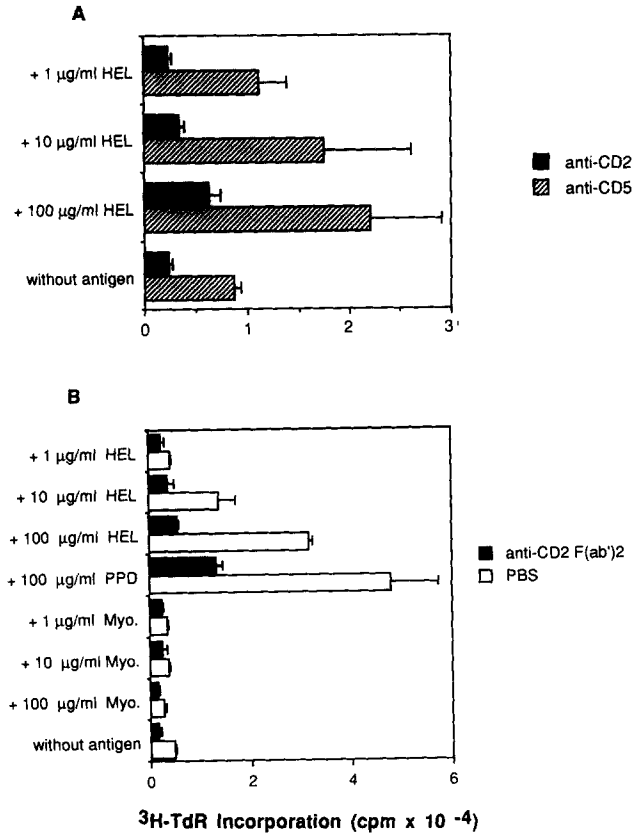

Figure 4. Anti-CD2 mAbs suppress the $CD4^+$ T cell-mediated response to HEL. C3H/He mice received 0.5 mg intact anti-CD2 or anti-CD5 Ab (*A*) or 1 mg $F(ab')_2$ fragments of the anti-CD2 Ab (*B*) 1 d before immunization with HEL. 7 d later the proliferative response of cells from draining lymph nodes to the antigens indicated was measured. Both protocols resulted in marked suppression of the HEL-specific response.

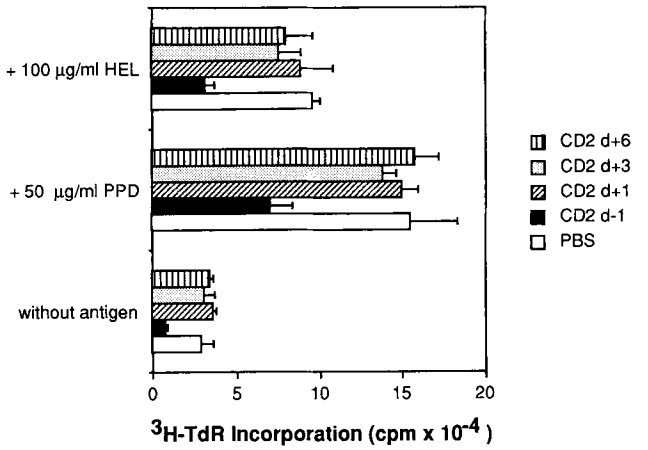

Figure 5. Anti-CD2 Abs only affect the early phase of HEL priming. C3H/He mice received 1 mg of anti-CD2 Ab 1 d before or various days after priming with HEL and the specific proliferative response of draining lymph node cells was analyzed in vitro. Anti-CD2 Abs were no longer effective, when administered 24 h after priming or later.

of CD2 molecules perturb the interactions of effector precursor cells with accessory cells and thus influence the recruitment of antigen-specific lymphocytes in situ? The in vivo priming of $CD4^+$ T helper cells was studied by immunizing C3H/He mice with HEL in CFA. Anti-CD2 Abs were administered 1 d before immunization. They reduced the antigen-specific proliferation of primed lymph node cells by more than 50% as compared to control animals, likewise the proliferative response of T cells against PPD, and an irrelevant antigen (myoglobin) was markedly inhibited. Anti-CD2 $F(ab')_2$ fragments reduced the antigen-specific T cell response to a similar extent (Fig. 4). This effect was specific for anti-CD2 Abs and not seen with an anti-CD5 (Fig. 4 *A*) or a rat control IgG1 Ab (data not shown).

To delineate the critical phase during priming at which anti-CD2 Abs interfere, anti-CD2 Abs were administered before and at different intervals after antigen injection. Anti-CD2 Abs were effective when given simultaneously (data not shown) or 1 d before antigen priming. When administration of anti-CD2 Abs, however, was delayed by 1 or more d with respect to antigen injection, they proved ineffective (Fig. 5).

These results demonstrate that modulation of CD2 expression on murine lymphocytes interferes with the earliest phase of priming of $CD4^+$ T helper cells in vivo.

Anti-CD2 Abs Inhibit the $CD8^+$ T Cell-mediated In Vivo Immune Response to Syngeneic Tumor Cells. The immune response of DBA/2 mice against the syngeneic tumor ESb has been well characterized (34). These tumor cells express tumor-associated transplantation antigen(s) which can induce a long lasting antitumor response. Priming with these tumor cells recruits MHC-restricted, tumor antigen-specific $CD8^+$ T effector cells which confer protective immunity against tumor cells upon adoptive transfer (39). The in vivo induction of these $CD8^+$ T effector cells is dependent on antigen-specific $CD4^+$ T helper cells (36). Mice received a primary and 7 d later a secondary immunization with syngeneic tumor cells,

3 d thereafter spleen cells were removed and restimulated with inactivated tumor cells in vitro to reveal tumor-specific cytotoxic T cells. When mice were pretreated with anti-CD2 Abs one day before or simultaneously with first tumor cell priming, the in vivo induction of cytotoxic antitumor T cells was significantly reduced (Fig. 6). The reduction of the tumor-specific CTL response could not be overcome by addition

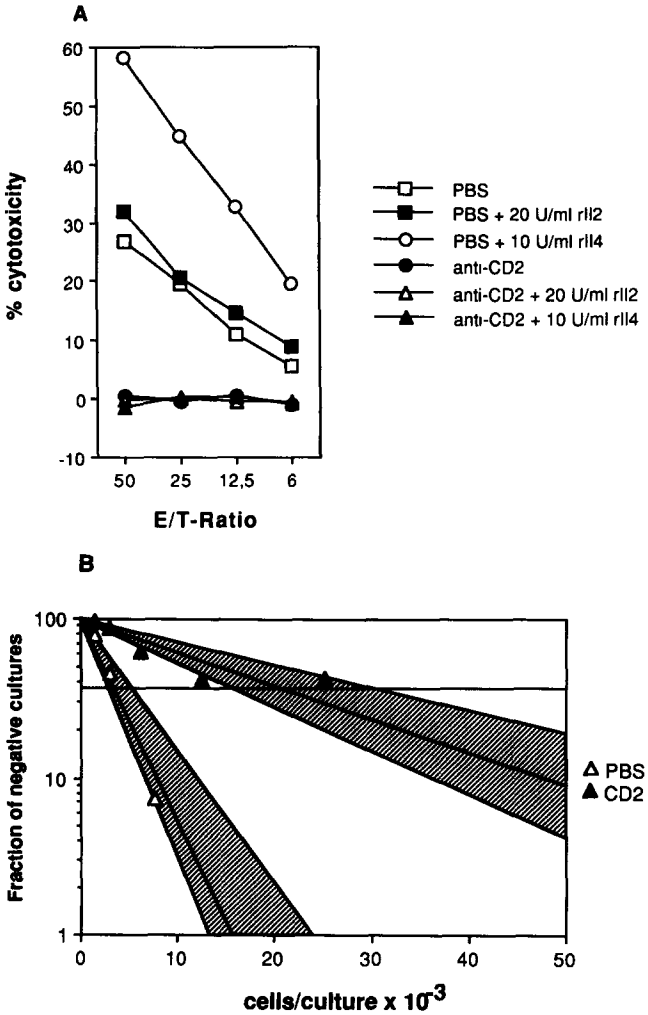

Figure 6. (*A*) Anti-CD2 Abs suppress the $CD8^+$ T cell mediated immune response to a syngeneic tumor. DBA/2 mice were immunized twice with ESb tumor cells (day 0 and 7), splenocytes were removed at day 10 and restimulated in vitro with the same tumor cells for 4 d without or with addition of recombinant IL-2 or -4. Specific antitumor cytotoxicity was measured in a standard ^{51}Cr release assay, nonspecific cytotoxicity as tested against Eb tumor cells was <5%. Experimental mice received 1 mg anti-CD2 Ab 1 d before the first immunization. Anti-CD2 Abs suppressed the specific CTL response irrespective of addition of interleukins. For each group cells from three animals were pooled. (*B*) Anti-CD2 Abs reduce the frequency of antitumor specific CTL-p sixfold. DBA/2 were immunized twice with the syngeneic tumor cell line ESb (day 0 and 7) and at day 10 peritoneal exudate cells were removed and directly assayed for the frequency of tumor-specific CTL-p. Control group: f = 1/3,606 (shaded areas indicate the 95% confidence limits: 2,709–5,394), anti-CD2 Ab treated group: f = 1/20,928 (15,883–30,669). For details see Materials and Methods and reference 37.

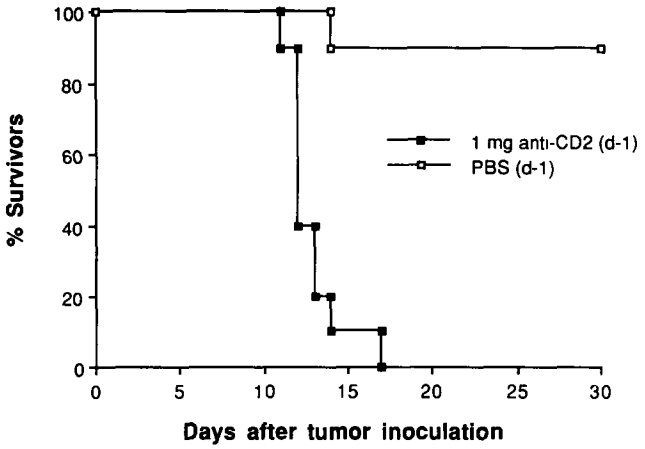

Figure 7. Anti-CD2 Abs abrogate protective antitumor immunity in vivo. DBA/2 mice received 1 mg anti-CD2 Ab 1 d before immunization with ESb tumor cells. Whereas control mice developed protective immunity and rejected the tumor cells, all treated mice died of tumor spread within 17 d. Each group comprised 10 mice.

of low doses of IL-2 or -4 to the culture in vitro, although IL-4 markedly enhanced the control response (Fig. 6 *A*).

Suppression of the antitumor CTL response by anti-CD2 Abs corresponded to a reduction in the frequency of specific CTL-precursors (CTL-p) as determined by limiting dilution analysis. Splenic CTL-p frequencies were reduced from 1/18,901 to 1/46,439 and peritoneal CTL-p frequencies from 1/3,606 to 1/20,928 in mice pretreated with 500 μg Ab/mouse. (Fig. 6 *B*). This reduction in tumor cell-specific T cell immunity was sufficient to abrogate protection against lethal tumor cell spread. All mice treated with anti-CD2 Abs (10/10) succumbed to the tumor load within 17 days, whereas 90% of

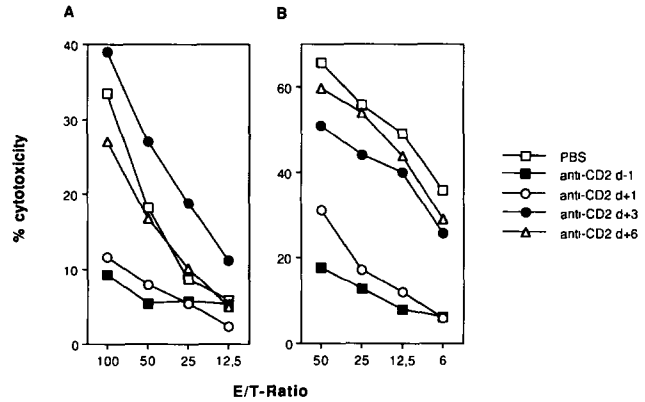

Figure 8. Anti-CD2 Abs affect the early phase of tumor cell priming in vivo. DBA/2 mice were immunized with ESb tumor cells as described in legend to Fig. 6. The specific antitumor cell cytotoxicity of peritoneal exudate cells was assessed directly ("secondary response") (*A*) whereas splenocytes were restimulated in vitro ("tertiary response") (*B*). Mice received 0.5 mg anti-CD2 Ab before or various intervals after the first priming. Specific cytotoxicity was only reduced, when mice were treated within 24 h after priming.

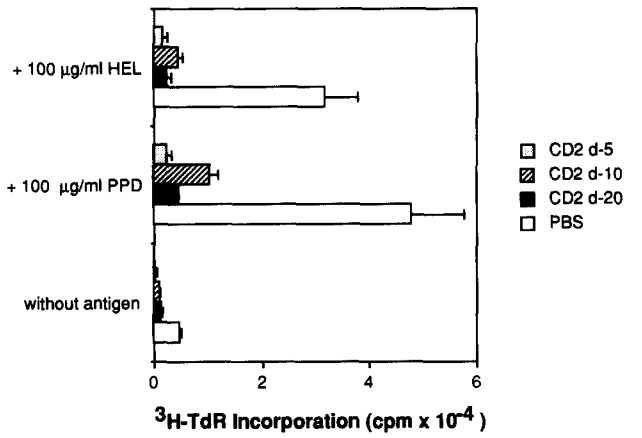

Figure 9. Anti-CD2 Abs induce sustained T cell unresponsiveness against HEL. DBA/2 mice received 1 mg of anti-CD2 Abs at different time intervals before immunization with HEL. 7 d after antigen priming draining lymph node cells were removed and their proliferative response was measured. Even 20 d after Ab administration mice did not develop a T cell-specific response.

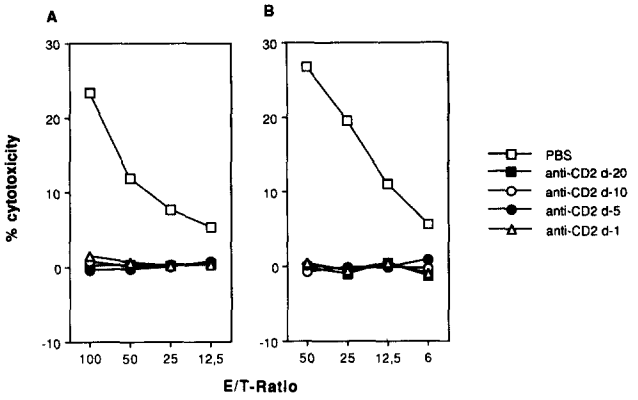

Figure 10. Anti-CD2 Abs induce sustained T cell unresponsiveness against syngeneic tumor cells. DBA/2 mice received 1 mg of anti-CD2 Ab at different time intervals before the first immunization with ESb tumor cells. The "secondary" (*A*) and "tertiary" (*B*) cytotoxic antitumor response was assessed according to the protocol in legend to Fig. 6. Even 20 d after Ab injection mice remained unresponsive towards the tumor cells.

the mice in the control group (9/10) were still alive 40 d after tumor cell inoculation (Fig. 7).

To define the window of susceptibility to anti-CD2 Ab treatment in this immune response, Abs were administered before (day −1) and after (day +1, +3, +6) the first priming with tumor cells. Peritoneal cells were tested for specific CTLs ex vivo without further restimulation ("secondary response", Fig. 8 *A*), whereas spleen cells were restimulated with inactivated tumor cells in vitro ("tertiary response", Fig. 8 *B*). A significant reduction in the cytotoxic response was observed in both protocols, when Abs were administered within 24 h after initiation of the immune response. 3 d after priming anti-CD2 Abs did no longer interfere with the development of antigen-specific T cells in vivo (Fig. 8).

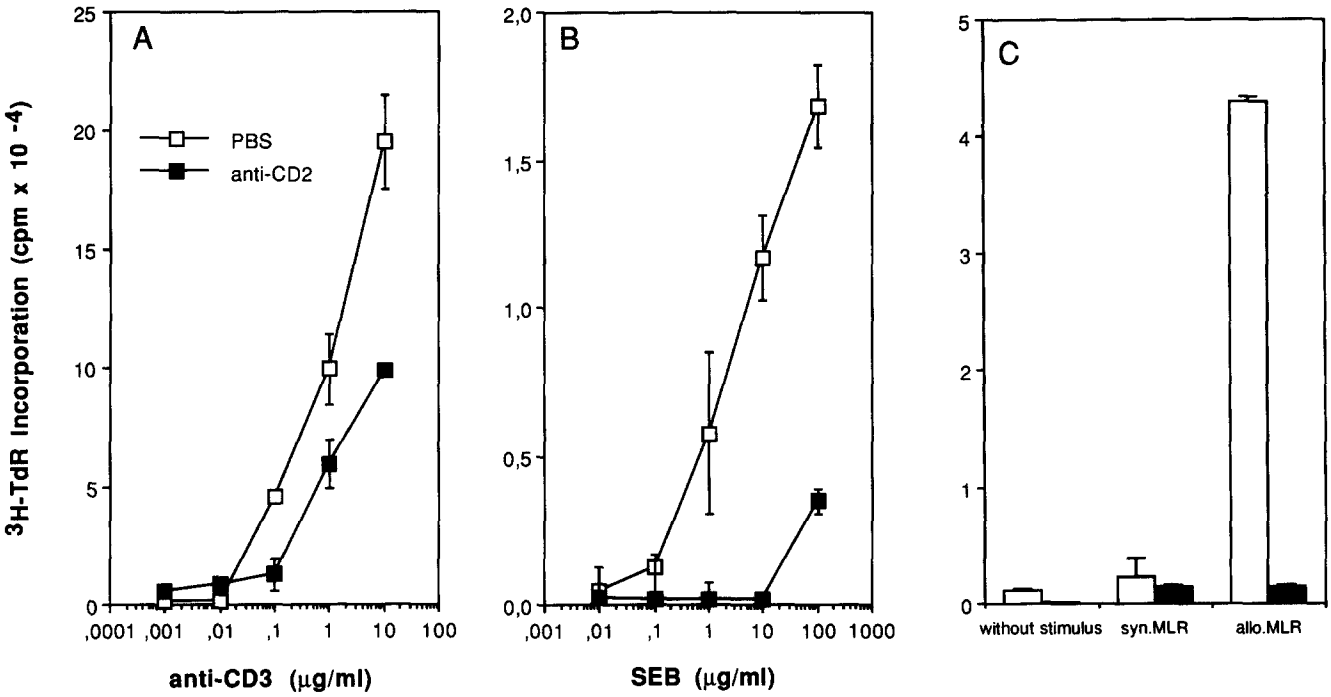

Figure 11. Anti-CD2 Abs induce a sustained reduction in polyclonal T cell responsiveness. DBA/2 mice were pretreated with 1 mg anti-CD2 Ab 28 d before removal of mesenteric lymph node cells. T cells were polyclonally activated by graded doses of matrix-bound anti-CD3 Abs (*A*), the superantigen SEB (*B*), and allogeneic stimulator cells (*C*). Even 28 d after treatment with anti-CD2 Abs peripheral T cells remained less responsive than those of control mice.

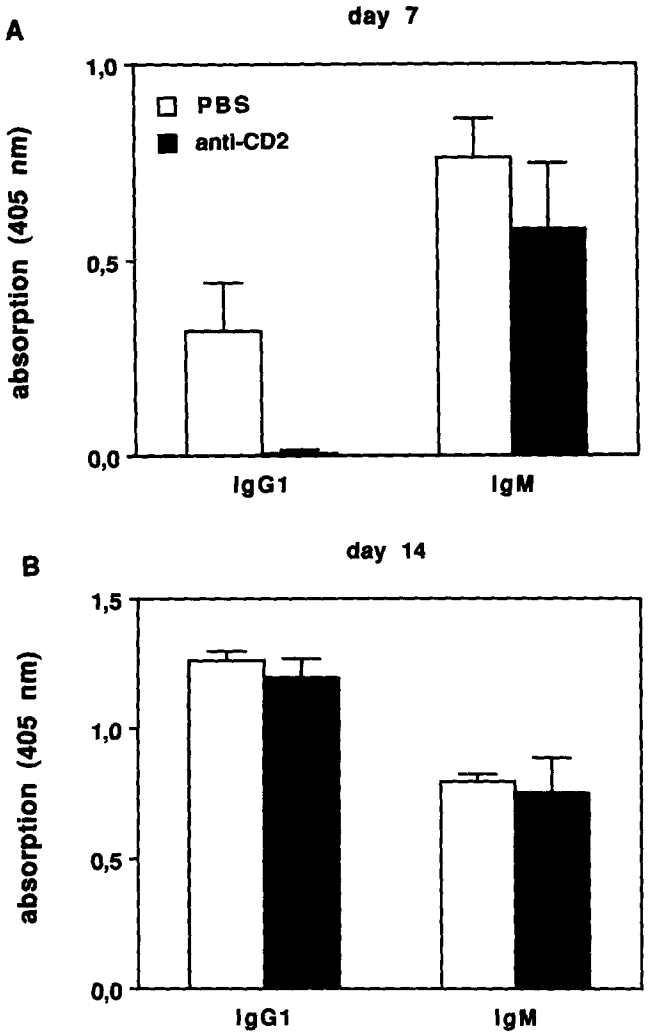

Figure 12. Anti-CD2 Abs delay the IgG1-specific Ab response to oxazolone: BALB/c mice received 1 mg anti-CD2 Ab 1 d before immunization with oxazolone. 7 and 14 d after priming the specific IgM and IgG1 Ab titers against oxazolone were determined. Treatment led to highly reduced IgG1 titers at day 7 (*A*) which regained control levels by day 14 (*B*). In contrast, specific IgM titers were unaffected by anti-CD2 Abs at day 7 and 14. Serum antibody titers as measured by ELISA are shown (dilution 1:10). Values represent the $\bar{x} \pm$ SD of four animals.

Thus, anti-CD2 Abs suppress the clonal development of specific $CD8^+$ T effector cells against syngeneic tumor cells by interfering at an early stage of the immune response in vivo.

Anti-CD2 Abs Induce Sustained T Cell Unresponsiveness In Vivo Independent of CD2 Modulation. The stage at which anti-CD2 Abs suppressed the HEL-specific and antitumor cell response coincided with maximal modulation of CD2 expression (see Fig. 3) and thus the effect may be explained by blocking CD2-LFA-3 interactions resulting in reduced adhesion between T cells and cognate partner cells. Given the dual function of CD2 as an adhesion and signal molecule, we tested whether the immuno-suppressive effects of anti-CD2 Abs do require CD2 modulation. Mice were pretreated with anti-CD2 Abs 5, 10, or 20 d before priming with HEL or ESb tumor cells, whereby CD2 modulation was reversed 10–14 d post-injection (Fig. 3). Pretreatment with anti-CD2 Abs even 20 d before priming resulted in a nearly complete inhibition of the anti-HEL, -PPD (Fig. 9), and secondary and "tertiary" antitumor cell response (Fig. 10, *A* and *B*). When mice were killed 28 d after application of anti-CD2 Abs, CD2 expression was restored with regard to the frequency of $CD2^+$ cells/organ and the density of CD2 receptors/cell and lymphocytes were phenotypically indistinguishable from those of control mice and no residual rat IgG was detectable in vivo (data not shown). In addition to antigen-specific T cell responses peripheral T cells of treated mice were less responsive to polyclonal activation by TCR crosslinking, activation by SEB, and allogeneic stimulator cells (Fig. 11, *A–C*). This latter result indicates that anti-CD2 Abs but not control Abs induced reduced polyclonal responsiveness of T cells in vivo which was independent of CD2 surface expression.

Effects of Anti-CD2 Abs on the Humoral Immune Response. In distinction to other species (rat, sheep, human) in mice CD2 is expressed both on T and B cells (12–14). Anti-CD2 Abs modulate CD2 expression on B and T cells to a similar extent (Fig. 1) and may thus affect T-B cell recognition and/or B effector cells directly. Production of Ab subclasses of the IgG isotypes is strictly T helper cell-dependent while IgM Ab production is less T cell-dependent. We analyzed the effect of anti-CD2 Abs on the well characterized humoral response of BALB/c mice to oxazolone (38). A single injection of antigen in adjuvant results in production of specific IgM Abs (day 7) followed by IgG1 Abs (day 7–14). Pretreatment of mice with 0.5 or 1 mg anti-CD2 Abs did not influence the production of antigen-specific IgM Abs, whereas the IgG1 response was significantly reduced at day 7 (Fig. 12, *A* and *B*). The reduction of IgG1 titers, however, was transient and by day 14 the IgG1 titers of treated mice did not differ from those of controls (Fig. 12 *B*). These observations have been reproduced in three independent experiments. Thus, anti-CD2 Abs obviously caused a delay in the isotype switch from IgM to IgG1 Abs. This result is consistent with an indirect role of CD2 in the humoral response probably affecting T helper cell function.

Discussion

The human CD2 receptor has been extensively characterized with regard to its structure and function primarily based on in vitro studies (1–8). Thus detailed knowledge has accumulated on delineating the function of the extracellular and cytoplasmic domains of CD2 which are involved in transmembrane signaling and adhesion to LFA-3. In contrast, little is known about the contribution of the CD2 receptor to activation, clonal expansion, and distribution of antigen-specific lymphocytes during an immune response in situ. Using a mAb against murine CD2 we report experiments which support a significant role of CD2 in T cell immunity in vivo. Coinjection of anti-CD2 Abs with a soluble or cellular antigen results in marked inhibition of $CD4^+$ and $CD8^+$ T cell-mediated, antigen-specific immune responses. Moreover,

injection of anti-CD2 Abs induces a prolonged downregulation of T cell responsiveness independent of antigen exposure.

The immunosuppressive effects are specific for anti-CD2 Abs and cannot be explained by ablation of $CD2^+$ lymphocytes. A single injection in adult or multiple injections of anti-CD2 Abs into newborn mice did not lead to numerical depletion of thymocytes or peripheral lymphocytes, more than 98% of which express CD2. The unaltered presence of CD2-synthesizing cells in Ab-treated animals was easily revealed when lymphocytes were cultured in vitro in the absence of Abs. Reexpression of modulated CD2 occurred within 24 h (9). Moreover, nondepleting $F(ab')_2$ fragments of anti-CD2 Abs induce CD2 modulation and immunosuppression in vivo.

CD2 modulation was dose and time dependent, whereby a single dose of 0.1–5 mg purified Ab resulted in maximal modulation within 24 h gradually reverting to normal CD2 expression levels within 10–14 d. Biodistribution of ^{125}I-labeled anti-CD2 Abs revealed a rapid clearance from blood and a quantitative accumulation in spleen, lymph nodes and bone marrow. Within 32 h splenic radioactivity fell to <5% of initial levels indicating a rapid internalization, degradation and excretion of iodinated degradation products (40). This fate of labeled 12.15A Ab confirms the cytofluorometric analysis on the modulation kinetics of CD2 surface expression.

Given the dual function of CD2 as an adhesion and signal molecule the immunosuppressive effects of anti-CD2 Abs could be due to interference with either of these functions. Injection of 0.5–1 mg of anti-CD2 Abs results in marked modulation of CD2 surface expression for ~1 wk (Fig. 3). This lowered expression of CD2 would diminish the contribution to cell-cell adhesion by CD2/LFA-3 interactions and the cooperation between CD2 and TCR/CD3 in T cell activation and may thus impede the recruitment of antigen-specific T cell precursors. Alternatively, crosslinking of CD2 by bivalent Abs [IgG or $F(ab')_2$] could deliver a signal which induces T cell unresponsiveness irrespective of CD2 surface expression and antigen recognition. Either of these mechanisms, "adhesion/signal deficiency" or "unresponsiveness" of T cell precursors in vivo may thus explain the immunosuppression which is observed when antigen is injected at the time of CD2 modulation. Since a reduction in polyclonal T cell responsiveness (Fig. 11) was only observed 8–21 but not 2 d after Ab injection (data not shown), we favor "adhesion/signal deficiency" by CD2 modulation to account for the reduced response when antigen and anti-CD2 Abs are coinjected. It this context it is worth mentioning that the mouse homologue of human LFA-3 has not yet been identified and the natural ligand for murine CD2 remains to be defined.

Anti-CD2 Abs are only effective when administered within 24 h after antigen priming. This narrow window of susceptibility indicates a role for CD2 in the early recruitment phase of antigen-specific precursor cells. Once primed, differentiation and expansion of antigen-specific cells obviously proceeds independent of CD2-mediated signals. Similar results have been observed for the development of TNP-specific, $CD8^+$ T cells (41). This time-dependency of CD2 immunomodulation was stricter for the $CD4^+$ T cell–mediated response to HEL than for the antitumor response (compare Figs. 5 and 8). Whether this is due to different kinetics of these particular immune responses or due to the effector subsets involved ($CD4^+$ versus $CD4^+$ plus $CD8^+$ T cells) remains to be clarified.

The immune response to HEL clearly identifies $CD4^+$ T cells as target cells for CD2-mediated suppression. Likewise the specific albeit transient effect of anti-CD2 Abs on the IgG1-specific immune response against oxazolone is likely to be due to insufficient help by $CD4^+$ T cells. The unaltered IgM response argues against a direct effect on B cells. At present it is open whether CD2 modulation on B cells directly affects B cell-specific functions. In contrast to the T cell response, the effect on the humoral immune response, however, is short-lived.

The antitumor response is mediated by $CD8^+$ T effector cells. Protective immunity against ESb tumor cells can be induced after injection of live tumor cells in the ear within 2–3 d and it involves multicellular interactions including intradermal antigen presenting cells (Langerhans cells), $CD4^+$ helper cells, and $CD8^+$ CTL-p. These critical interactions take place within 24–48 h after priming (34, 36). Frequency analysis revealed a significant reduction of specific CTL-p in treated mice. The deficient cytotoxic response could not be overcome by supplying exogenous IL-2 or -4 during restimulation in vitro indicating an insufficient recruitment of interleukin-responsive CTL-p in vivo. This defect could be due to abrogation of help by $CD4^+$ T cells and/or direct impairment of $CD8^+$ T cell function At present the effects on all three antigen-specific responses are compatible with lack of $CD4^+$ T helper cell function and it remains open whether $CD8^+$ T cells can be direct targets of CD2-mediated immune suppression. Interestingly, the $CD4^+$ T cell subset has been shown to be selectively susceptible to anergy induction by SEB (42).

It is of interest to note that the anti-CD2 mAb 12.15A did not affect T cell-mediated immune responses in vitro (e.g., syngeneic and allogeneic MLR, Con A, TCR/CD3 or SEB-mediated T cell activation, secondary responses to HEL and tumor cells, data not shown) when added to cultures. Likewise, the anti-CD2 Ab on its own did not activate mature T cells (as measured by cell proliferation, induction of IL-2 receptor expression or raise in intracellular Ca^{2+}) even when coupled to sepharose. The reason for this difference may relate to the rapid modulation of CD2 receptors in vivo after Ab injection (Figs. 1 and 3), whereas the Ab does not modulate CD2 on resting T cells in vitro (43). Additionally, different levels of adhesion receptors on precursor T cells and memory cells (2, 8), the different microenvironments in situ and in tissue culture and the different modes and kinetics of T cell activation involved my contribute to this difference. Alternatively, the different in vitro and in vivo effects may be a characteristic of the mAb used in this study (44). In particular, for rat and human CD2 it has been shown that Abs directed against different epitopes may exert different biological effects in vitro (16, 17) and possibly in vivo.

Beyond inhibition of antigen-specific immune responses,

a single injection of anti-CD2 Abs induced a sustained reduction in polyclonal T cell responsiveness (Figs. 9–11), which did not correlate with CD2 modulation, altered expression of TCR/CD3, CD4, CD8, and LFA-1 or reduction of T cells. While the in vivo response to HEL or tumor cells was nearly completely suppressed even 20 d after Ab injection, T cells were still responsive to activation by anti-TCR/CD3 Abs, SEB and allogeneic stimulator cells in vitro (Fig. 11) but less so than control cells. Thus, functional unresponsiveness in vivo corresponds to a mere quantitative reduction in T cell responsiveness in vitro. A similar dose shift in polyclonal T cell activation has been observed in mice which are transgenic for TCR $V_{\beta 8.1}$ and express the corresponding autoantigen Mls^a. These mice did not display autoreactivity but developed a state of functional tolerance by anergy (45).

The CD2-mediated state of T cell unresponsiveness is reminiscent of peripheral T cell unresponsiveness induced by inappropriate antigen presentation in vitro or in vivo (46–49), recognition of superantigens in vivo (42, 50), and administration of nondepleting anti-CD4 or anti-CD8 Abs at the time of antigen priming (51). In contrast to these protocols of peripheral tolerance induction, CD2-mediated unresponsiveness is not confined to antigen-specific or subset-defined T cells. The mechanism and significance of this CD2-mediated "negative" signal remains a matter of speculation. In the context of the two signal hypothesis (46) crosslinking of CD2 could provide signal one (either directly via an CD2-specific or indirectly via a CD3-dependent signal pathway [52]) which in the absence of signal two would render T cells unresponsive. In addition to induction of anergy among $V_{\beta 8}^+$ T cells, the superantigen SEB has recently been shown to induce partial deletion of this T cell subset in vivo (50). In two out of three experiments we observed that the frequency of peripheral $CD4^+$ and $CD8^+$ T cells was reduced by 10–15% 2–4 wk after anti-CD2 Ab injection possibly indicating heterogeneity among T cells with regard to deletion by CD2-signaling or redistribution of T cells in vivo.

Preliminary data indicate that the CD2-mediated unresponsiveness is readily induced in peripheral lymph node and spleen T cells but not in mature thymocytes, an intriguing difference between both compartments which was already observed in transgenic mice (45). Likewise, different anti-CD2 Abs lack as yet any demonstrable in vivo effect on intrathymic T cell differentiation and selection (e.g. deletion of $V_{\beta 6}^+$ T cells in Mls^a-positive mouse strains [9, 53, 54]) which is in contrast to the marked effect on the immunocompetence of postthymic mature T cells.

The immunosuppressive effects of anti-CD2 Abs are potentially applicable for therapeutic intervention, e.g., prolongation of allo-transplants or the treatment of autoimmune syndromes.

We are indebted to Dr. T. Hünig for critical comments on the manuscript and Sonja Höflinger and Karin Rensch for competent technical assistance.

These studies have been supported by grants from the German Cancer Research Center and the Deutsche Forschungsgemeinschaft.

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Received for publication 6 February 1991 and in revised form 10 June 1991.

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