Heat-stable Antigen Is a Costimulatory Molecule for CD4 T Cell Growth

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Summary

Optimal induction of clonal expansion by normal CD4 T cells requires a ligand that can engage the T cell receptor as well as functionally defined costimulatory activity on the same antigen-presenting cell surface. While the presence of effective costimulation induces proliferation, T cell receptor ligation in its absence renders T cells inactive or anergic. The molecular basis of this costimulatory activity remains to be defined. Here we describe a monoclonal antibody that can block the costimulatory activity of splenic accessory cells. Treatment with this antibody not only blocks the proliferation of CD4 T cells to a T cell receptor ligand, but also induces T cell nonresponsiveness to subsequent stimulation. Sequence analysis of the antigen recognized by this antibody indicates that it recognizes a protein that is identical to heat-stable antigen. Gene transfer experiments directly demonstrate that this protein has costimulatory activity. Thus, heat-stable antigen meets the criteria for a costimulator of T cell clonal expansion.

One of the most prominent features of the adaptive immune system is the generation of diversity by random somatic gene rearrangement (1). The generation of diversity provides the receptors needed to recognize novel pathogens. However, this diversity creates two problems for the immune system. The first problem is self-nonself discrimination. Because the repertoire of receptors is somatically generated, each clone has to be tested for self-reactivity, and autoreactive clones have to be inactivated or eliminated. The second problem is the low frequency of specific lymphocytes. As the frequency of any specific clone is the reciprocal of the diversity of receptors, the adaptive immune response requires clonal expansion of specific lymphocytes. This requirement for clonal expansion in adaptive immunity (including autoimmune responses) provides the immune system with a crucial control point at which these two problems may be solved.

Many recent experiments have demonstrated that the clonal expansion of all lymphocytes requires both receptor ligation and the receipt of a poorly defined costimulatory signal(s). In the case of T cells, clonal expansion requires both a specific peptide/MHC ligand and costimulatory activity on APCs. Early work from Lafferty et al. (2, 3) indicted that treatment of APCs with UV light or heat inactivation could destroy the immunogenicity of the APC without affecting its antigenicity. They referred to the activity being destroyed as "costimulatory activity." Most importantly, they showed that this activity is intrinsic to cells of hemopoietic origin, as depletion of hemopoietic cells from allografts leads to acceptance of the graft and a gradual induction of immune tolerance. More recently, Schwartz and coworkers (4, 5) have used a well-defined tissue culture model to demonstrate that fixation of APCs leads to clonal anergy in the presence of specific peptides. Such treatment does not affect the generation of the TCR ligand, so it is postulated that clonal anergy is induced by engaging the TCR in the absence of costimulation. More recently, we have demonstrated that engaging the TCR of cloned Th1 cells in the absence of a costimulatory signal leads to the death of Th1 effector cells (6). Finally, the finding that stimulation of T cells with the TCR ligand alone leads to clonal anergy has been confirmed in transgenic mice that express MHC antigens on nonhemopoietic tissues such as β cells of the islets of Langerhans (7, 8). Together, these studies demonstrate that costimulatory activity on APCs plays a key role in determining the consequences of the interaction of the TCR with its ligand. It is therefore of great interest to define molecules on APC that are involved in costimulation of T cells.

Clonal expansion of T cells involves the binding of the TCR and its coreceptors to a peptide/MHC ligand. mAbs to a variety of other T cell and APC molecules have been shown to inhibit T cell proliferation, including lymphocyte function-associated molecule 1/intracellular adhesion molecule 1 (LFA-1/ICAM-1) (9), CD2/LEA3 (10), and CD28/B7BB1 (11-13). Although all of these receptor/ligand pairs would be defined as costimulatory, we prefer to divide these interactions into two distinguishable sets. mAbs to one set...
of molecules disrupts T cell responses even in situations where clonal expansion is not critical, as in target cell recognition by killer T cells. Molecules of this class are involved in antigen recognition by the T cell. mAbs to the other set affect only clonal expansion. These recognize what we would call costimulators, as they regulate T cell behavior after an antigen is recognized. In the presence of mAbs to such costimulatory molecules, not only would clonal expansion be blocked, but also one would expect a state of T cell inactivation orergy to be induced. By contrast, mAbs that prevent TCR signaling should prevent the induction of anergy. This provides a test for distinguishing between the two sets of mAbs that prevent T cell clonal expansion.

In this report, we describe a mAb, 20C9, that blocks clonal expansion of normal CD4 T cells. Engagement of the TCR in the presence of this mAb leads to functional inactivation of T cells. Thus, this mAb meets the criteria for inhibition of the delivery of costimulatory activity by APCs. Expression cloning and cDNA sequencing reveal that this mAb recognizes the heat-stable antigen. Gene transfer experiments confirm that heat-stable antigen has a direct costimulatory activity for clonal expansion of normal CD4 T cells.

### Materials and Methods

**Production of mAbs.** BALB/c ByJ and CBA/CaJ mice, used at 8–10 wk old, were purchased from The Jackson Laboratory (Bar Harbor, ME). Armenian hamsters (Cytogen Research and Development, West Roxbury, MA) were immunized by four consecutive intraperitoneal injections each of 10⁸ LPS-activated B cells from CBA/CaJ mice. The spleen cells were fused to Ag8.653 and hybrids selected in HAT medium. The supernatants were screened for their ability to inhibit the proliferation of CD4 T cells purified from BALB/cByJ mice to the anti-CD3 mAb YCD3-1 (14) and irradiated LPS-activated syngeneic B cells described (15). Positive clones were subcloned three times. mAbs were purified from hybridoma supernatants on a protein G–Sepharose column.

**Expression Cloning of 20C9.** cDNA and Generation of CHO Transfectants. 20C9 cDNA was cloned as previously described (16). Briefly, a cDNA library was prepared from mRNA isolated from LPS- and IFN-activated BALB/c spleen cells and inserted into the pCDM8 expression vector. The cDNA library was transfected into COS cells by the DEAE-dextran method (16). COS cells were incubated with 20C9 mAb, and the cells expressing 20C9 protein were isolated by panning onto plates coated with goat anti-hamster IgG (Caltag Laboratories). Anti-Thy-1 mAb HO 13.4.9; reference 19) and complement lysis, B cells were prepared from spleen nonadherent cells after two rounds of anti-Thy-1 mAb (HO 13.4.9; reference 19) and complement lysis, and were activated with 10 μg/ml of LPS (15). These B cells were either irradiated (2,000 rad) or fixed with 1% paraformaldehyde (15) before being used as accessory cells. Unless specified in the text, CD4 T cells were purified as previously described and incubated with accessory cells and a 1:40 dilution of YCD3-1. Proliferation was determined after 42 h by pulsing the culture for 6 h with 1 μCi/well of [³H]THBR. Results of duplicate/triplicate cultures in which the variation was <20% are reported.

**Cleavage of Glycosyl-Phosphatidylinositol (GPI) Linkage by Phosphinositol-specific Phospholipase C (PI-PLC) and Flow Cytometry.** A20 cells (10⁶/ml) in serum-free Click's EHAA medium were incubated with PI-PLC (152354; ICN Biochemicals, Cleveland, OH), at a 1:200 dilution at 37°C in a water bath for 1 h. The enzyme was washed away with PBS, and the treated and untreated cells were stained with 20C9 mAb undiluted hybridoma supernatant followed by FITC-labeled goat anti–hamster IgG (mouse and rat Ig adsorbed; Caltag Laboratories). Anti-Mac-1 mAb M1/70 (20), anti-FcR mAb 2.4G2 (21), and anti–heat-stable antigen mAbs J11d (22) and M1/69 (23) were also used in flow cytometry and/or proliferation assays, which was performed as described previously (15).

### Results

**A mAb that Inhibits T Cell Clonal Expansion by Blocking the Delivery of Costimulatory Activity by APC.** To study the clonal expansion of normal CD4 T cells, we have used anti-CD3 mAb–induced proliferation of CD4 T cells as a model system. Our previous work has demonstrated that B cells are the major APC for this response in murine spleen (15). Activation with LPS induces costimulatory activity in B cells that is resistant to aldehyde fixation. To generate mAbs that can block the delivery of this costimulatory signal for T cell activation, we immunized hamsters with LPS-activated B cells and made hybridomas. Supernatants were screened for inhibitory effects on the proliferation of normal CD4 T cells to anti-CD3 mAb and irradiated, LPS-activated B cells as accessory cells. The hybridomas that secreted inhibitory mAbs were subcloned three times. One of these inhibitory mAbs, 20C9, is characterized here. 20C9 significantly inhibits the proliferation of CD4 T cells induced by anti-CD3 (Fig. 1 a) or by allogeneic LPS-activated B cells (Fig. 1 b). When a cloned T cell is used as a responder, 20C9 blocks proliferation of this cloned line to its antigen, OVA (Fig. 1 c). 20C9 also inhibits proliferation of total spleen cells to anti-CD3 (see Fig. 3 a), indicating that the antibody has a broad spectrum.

1 **Abbreviations used in this paper:** GPI, glycosyl-phosphatidylinositol; PI-PLC, phosphinositol-specific phospholipase C.
of inhibitory effects on the clonal expansion of T cells induced via the TCR.

As the assay system contains both T cells and APCs, 20C9 could have inhibited T cell proliferation by binding to either T cells or LPS-activated B cells. To resolve this issue, we incubated 20C9 with LPS-activated B cells. After washing away unbound 20C9, LPS-activated B cells were fixed with paraformaldehyde. As shown in Fig. 2a, preincubation of 20C9 with LPS-activated B cells profoundly inhibits the proliferation of CD4 T cells induced by anti-CD3. Control experiments indicate that the functional antibody cannot be eluted from the fixed B cells because the antibody-coated cells do not inhibit if fixed LPS-activating B cells are added to the same well (data not shown). The activity of 20C9 on the B cells is consistent with two-color FACS® data, which indicate that 20C9 binds only to non-T spleen cells (Fig. 2b). The protein

Figure 1. mAb 20C9 inhibits proliferation of T cells. (a) Proliferation of CD4 T cells to anti-CD3. BALB/c CD4 T cells (5 × 10^4/well) were cultured with 2 × 10^4 irradiated, LPS-activated B cells in the presence of anti-CD3 mAb (YCD3-1, hybridoma supernatant; 1:40). Varying concentrations of 20C9 supernatant were added at the beginning of the culture. (b) Proliferation of BALB/ByJ CD4 T cells to allogenic LPS-activated B cells from CBA/CaJ mice. 20C9 mAb (100 μg/ml) was incubated with LPS-activated B cells at 4°C for 1 h, unbound antibody was washed away, and the LPS-blasts were fixed with paraformaldehyde before being used as accessory cells. Data shown are the change in cpm, with the proliferation of the CD4 T cells in the absence of allogeneic cells subtracted. (c) Proliferation of the Th1 clone 5.9 (2 × 10^4/well) to OVA (100 μg/ml) presented by irradiated adherent cells (10^5/well) from BALB/c mice. 20C9 mAb was added at a 1:2 dilution of hybridoma supernatant.

Figure 2. 20C9 mAb inhibits proliferation of CD4 T cells by blocking a component on LPS-activated B cells. (a) Preincubating 20C9 mAb with LPS-activated B cells is sufficient to inhibit the proliferation of the CD4 T cells. LPS-activated B cells were incubated with 20C9 mAb at 4°C for 1 h, unbound antibodies were washed away, and the cells were fixed with paraformaldehyde and used as accessory cells in anti-CD3-induced proliferation of CD4 T cells. Preincubation with normal hamster Ig or anti-CD45 mAb TIB122 has no effect on the function of the LPS-activated B cells in this assay (data not shown). (b) Analysis of the cellular distribution of the 20C9 epitope on BALB/c ByJ spleen cells double stained with the anti-Thy-1 mAb Y19 and with 20C9. (Top) No 20C9 added; (bottom) 20C9 present. (c) Cleavage with PI-PLC removes the 20C9 epitope from A20 cells as determined by FACSscan®. Similar cleavage was found in normal spleen cells (not shown). (d) 20C9 mAb inhibits the APC function of fixed LPS-activated B cells. LPS-activated B cells were fixed with paraformaldehyde and used as accessory cells in the proliferative response of CD4 T cells from normal CBA/CaJ mice to anti-CD3 (YCD3-1; 1:40). 20C9 mAb supernatants were added at the onset of the culture.
that this antibody recognizes is anchored to the cell membrane by a GPI linkage, as PI-PLC can specifically cleave the 20C9 epitope from B cells (Fig. 2 c). Furthermore, this antibody strongly inhibits the accessory cell function of fixed B cells (Fig. 2 d). As paraformaldehyde-fixed B cells are metabolically inert, it is most likely that 20C9 mAb binds to and inhibits the action of a component expressed on LPS-activated B cells, which is necessary for proliferation of CD4 T cells. 20C9 mAb does not bind to the FeR, as it binds to FeR+ A20 cells and an FeR− variant equally well (data not shown). In addition, 20C9 mAb does not affect the function of the FeR on LPS-activated B cells in anti-CD3-induced activation of cloned T cells (Liu, Y., and C. A. Janeway, manuscript submitted for publication). Thus, 20C9 mAb inhibits T cell proliferation by a mechanism other than affecting the function of the FeR.

Many mAbs specific for accessory molecules required for T cell activation inhibit the formation of T cell APC clusters. 20C9 inhibits anti-CD3-induced proliferation of spleen cells without inhibiting cluster formation induced by anti-CD3 mAb. This contrasts with the effects of anti-LFA-1 mAb M17/5.2 (20) and anti-FeR mAb 2.4G2 (21), which inhibit cluster formation as well as proliferation. Anti-LFA-1 mAb M17.5.2 and anti-FeR mAb 2.4G2 inhibit spleen prolifera-
Figure 4. The effect of preculture of CBA/CaJ spleen cells on subsequent responses to anti-CD3 plus spleen accessory cells. (a) Spleen cells were precultured in the presence or absence of anti-CD3 (1:40 dilution of hybridoma supernatant) and/or 20C9 mAb (0.5 μg/ml) for 48 h. (b) Spleen cells were treated with anti-CD3 together with 1 μg/ml of either anti-LFA-1, anti-FcR, or 20C9 mAb. (c) Spleen cells were treated with either no antibody or anti-LFA-1, anti-FcR, or 20C9 mAbs at 1 μg/ml in the absence of anti-CD3 mAb. In all cases, viable cells were isolated on lymphocyte separation medium 48 h after initial stimulation and washed three times. These cells were then stimulated with fresh anti-CD3 mAb and fresh irradiated CBA/CaJ spleen cells (10^5/well) as APC. Proliferation of the pretreated spleen cells was determined at 72 h (a) or 48 h (b and c) of stimulation.

Inhibition of Costimulation by 20C9 mAb Leads to CD4 T Cell Unresponsiveness to Subsequent Stimulation with Anti-CD3 mAb and APCs. As stated in the introduction, stimulating T cells with anti-CD3 in the presence of antibodies that block costimulatory activity should lead to functional inactivation of T cells. By contrast, T cells treated with anti-CD3 in the presence of mAbs that block TCR recognition should behave like untreated cells upon subsequent stimulation. This distinction can be used to divide inhibitory antibodies into two groups. To examine whether 20C9 blocked TCR signals or costimulatory signals, we restimulated spleen cells recovered from cultures stimulated with anti-CD3 mAb in the presence or absence of mAbs that inhibit the proliferation of T cells to anti-CD3 (see Fig. 3 a). As shown in Fig. 4 a, cells that had been previously stimulated with anti-CD3 and APC mount what appears to be a secondary proliferative response, a substantial proliferative response being seen with very low cell numbers. The low total response may reflect the kinetics of this response as the experiment was harvested at the time of peak primary proliferation (day 3 of restimulation). Cells treated with medium alone proliferate well, while
cells that were pretreated with anti-CD3 in the presence of 20C9 mAb make only a marginal proliferative response requiring very high cell numbers, indicating that the pretreatment induces functional inactivation of the T cells. The reduction in response observed is ~20-fold relative to untreated cells. This inactivation requires exposure to anti-CD3 in the first culture and was not due to the carry-over of the 20C9 mAb, because treatment of the cells with 20C9 mAb in the absence of anti-CD3 mAb allows a proliferative response in the second culture similar to that of untreated cells. These data suggest that engaging the TCR in the absence of 20C9 protein leads to functional inactivation of the T cells.

As seen in Fig. 3 a, mAbs directed at several different cell surface molecules can prevent clonal expansion of CD4 T cells induced by anti-CD3 and APCs. To test whether treatment with mAbs that interfere with cell adhesion (anti-LFA-1) or TCR ligation (anti-FcR) also results in functional inactivation of T cells exposed to anti-CD3, we treated spleen cells with anti-LFA-1, anti-FcR, and 20C9 mAbs in the presence or absence of anti-CD3 mAb. 2 d later, the antibodies were washed away and the viable cells were restimulated with anti-CD3 in the presence of competent APC. As shown in Fig. 4 b, cells treated with anti-LFA-1 and anti-FcR mAbs plus anti-CD3 generate a vigorous proliferative response upon restimulation, comparable with that of untreated spleen cells (Fig. 4 c), while those pretreated with 20C9 plus anti-CD3 give a significantly reduced response. As the dose of all the mAbs used (1 μg/ml) can almost totally inhibit the primary proliferative response of T cells to anti-CD3 (Fig. 3 a), these results indicate that anti-CD3 combined with anti-LFA-1 or anti-FcR mAb does not induce functional inactivation of the T cells, in contrast to anti-CD3 plus 20C9 mAb, nor do we observe the augmented response induced by anti-CD3 alone, as seen in Fig. 4 a, in any of these cultures. The inhibition observed with 20C9 mAb was not due to carry-over of the mAb in the culture, as spleen cells treated with 20C9 in the absence of the anti-CD3 mAb mount a normal proliferative response in secondary stimulation (Fig. 4 c). Thus, the inactivation observed requires both anti-CD3 and the presence of blocking concentrations of 20C9.

Taken together, the above data indicate that 20C9 antibody blocks clonal expansion of T cells induced by anti-CD3, allogeneic LPS-activated B cells, and antigen presented by syngeneic spleen cells. As 20C9 does not affect ligand recognition by the TCR or cell adhesion, it is likely to block a costimulatory signal. This conclusion is supported by the finding that exposure of T cells to anti-CD3 in the presence of 20C9 leads to functional inactivation of T cells.

Expression Cloning of 20C9 Antigen Reveals Its Identity with Heat-stable Antigen. To clone the gene encoding the 20C9 antigen, COS cells were transfected with a pCDM8-cDNA library prepared from LPS-activated spleen B cells. Positive

**Figure 6.** 20C9, J11d, and M1/69 all bind related epitopes. (a) 20C9 and M1/69 block the binding of J11d to spleen cells. (b) M1/69 blocks the binding of 20C9 mAb to spleen cells. (c) Expression of 20C9 protein on CHO-FcR cells transfected with 20C9C7 cloned cDNA. (d) J11d and M1/69 bind to CHO-FcR cells transfected with the 20C9/C7 cDNA.
clones were enriched by four cycles of transfection followed by panning for 20C9-positive COS cells. Plasmid DNA prepared from the fourth cycle of panning was found to transfer 20C9 reactivity to COS cells. The plasmid DNA was cloned, and of 55 clones tested, four transferred 20C9 reactivity to COS cells. The plasmid DNA was cloned, and of 55 clones tested, four transferred 20C9 reactivity to COS cells. The plasmid DNA was cloned, and of 55 clones tested, four transferred 20C9 reactivity to COS cells. The plasmid DNA was cloned, and of 55 clones tested, four transferred 20C9 reactivity to COS cells. The plasmid DNA was cloned, and of 55 clones tested, four transferred 20C9 reactivity to COS cells.

**Discussion**

The role of costimulators in the clonal expansion of T cells has been defined primarily by the effect of their absence. The lack of costimulation in T cell responses to ligand leads to two distinct phenomena, the lack of clonal expansion and the inactivation of T cells in forms such as clonal anergy and induced cell death (4-6). The inactivation of T cells in the absence of costimulation suggests that expression of costimulatory molecules on hematopoietic cells but not on most cells of other tissues could be critical for self-nonself discrimination by mature T cells in the periphery (2, 4, 7, 8). Although many molecules have been demonstrated to play a role in T cell clonal expansion, it remains to be tested whether inactivation of T cells can be achieved by stimulation in the presence of antibodies that block the activity of any of these molecules. As most of the treatments used to destroy costimulatory activity have been nonspecific, it is possible that these two effects are caused by the functional elimination of different molecules. Here we report that treatment with the mAb 20C9 can inhibit clonal expansion of T cells induced by a variety of stimuli, indicating that the protein recognized by 20C9 plays an important role in clonal expansion of T cells. Furthermore, adding 20C9 to cultures of T cells stimulated by anti-CD3 and APCs induces nonresponsiveness of T cells to further stimulation. These results demonstrate that a single molecule is responsible both for clonal expansion and for prevention of clonal inactivation of T cells. Thus, the 20C9 protein fulfills the criteria for a costimulatory molecule on APCs.

The molecules that mediate cell-cell adhesion have also been
regarded as costimulatory molecules because antibodies that block their function can block T cell proliferation, while transfection of these molecules into fibroblasts can enhance the T cell response to ligand. As T cells normally respond to peptide/MHC on the surface of APCs, cell-cell contact is a prerequisite for TCR engagement. We suggest that it is appropriate to differentiate those molecules that are required for ligand recognition, that is, delivery of signal one, from costimulatory molecules that deliver signal two. Despite their similar effects on T cell proliferation, the distinctive effects of anti-LFA-1 and 20C9 on cluster formation and on the induction of unresponsiveness clearly discriminates these two classes of molecules.

Molecular cloning and partial DNA sequencing reveals that the gene encoding the 20C9 protein is identical to the previously cloned gene encoding heat-stable antigen. Transfection with cDNA encoding heat-stable antigen is sufficient to transfer costimulatory activity to CHO cells, demonstrating directly that heat-stable antigen costimulates T cells. It has been documented that expression of the heat-stable antigen is tightly controlled in immature T cells and in B cells at different functional stages (25). The function of this molecule has remained elusive. We show here that heat-stable antigen cDNA from activated B cells encodes a costimulatory molecule that can participate in clonal expansion of CD4 T cells.

The heat-stable antigen contains a very small peptide core with a large number of potential N-linked and O-linked glycosylation sites. The structural basis of its costimulatory activity is at present not understood. It is worth noting that the level of 20C9 binding activity does not correlate directly with the costimulatory activity of a cell. Normal B cells do not have constitutive costimulatory activity (15), yet they bind 20C9 significantly. This discrepancy can be explained at least in two ways. First, normal B cells may express an inhibitor for the costimulatory activity of the heat-stable antigen, and this inhibitor is inactivated during B cell activation. Second, the heat-stable antigen may be modified during B cell activation. Since different cell types seem to have different glycosylation patterns of the heat-stable antigen (24), one possibility would be that glycosylation regulates the costimulatory activity of the heat-stable antigen. This notion is consistent with the earlier findings by Frohman and Cowing (26), who showed that treatment with neuraminidase can enhance T cell stimulation by B cells.

Adaptive immune responses by cells having clonally distributed receptors must discriminate self from nonself. Many experiments have demonstrated that clonal deletion mediated by the interaction of immature thymocytes with antigen on APCs is a major mechanism for removing from the mature repertoire those T cells that recognize ligands borne by APCs (27, 28). This mechanism, however, does not apply to antigens that are expressed in tissues but are not present on APCs in the thymus (7, 8). Thus, there must be mechanisms to ensure self-nonself discrimination by mature T cells in the periphery. TCR engagement on mature T cells leads to a number of different consequences depending on the costimulatory activity of the cells that present antigens (4–6). Self antigens presented by tissue cells induce immune tolerance by inactivating specific T cells (7, 8), whereas antigens borne by microbes that induce costimulatory activity on APCs induce potent adaptive immune responses (29, 30). Although much data are consistent with the two-signal theory (30–32), this hypothesis has not been subjected to a stringent experimental test because the nature of signal two on APCs has been obscure. Hopefully, with the identification of costimulatory molecules such as B7 (11–13) and heat-stable antigen, the two-signal theory itself can now be directly tested.

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