Induction of Autoreactive B Cells Allows Priming of Autoreactive T Cells

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Summary

A novel mechanism for breaking T cell self tolerance is described. B cells induced to make autoantibody by immunization of mice with the non-self protein human cytochrome *c* can present the self protein mouse cytochrome *c* to autoreactive T cells in immunogenic form. This mechanism of breaking T cell self tolerance could account for the role of foreign antigens in breaking not only B cell but also T cell self tolerance, leading to sustained autoantibody production in the absence of the foreign antigen.

Systemic lupus erythematosus (SLE) is a severe autoimmune disease in which antibody production against selected cellular antigens (1) leads to the generation of soluble immune complexes that damage the microvasculature (2). Many of the autoantibodies in SLE are directed at nucleoprotein complexes, and studies of antibody production in individual patients have suggested that antibodies directed at different components of the same particle are found together in the same patient (3). Moreover, in animal models, certain ribonucleoproteins elicit autoantibodies that resemble those found in patient sera (4–6), supporting a role for the autoantigens themselves in inducing the autoantibody responses. In addition to antibodies directed at components of nucleosomes, ribosomes, and spliceosomes, antibodies to a number of other intracellular proteins have been observed (7). Recently, one of us (M. J. Mamula) has detected production of autoantibodies to the well-characterized protein human cytochrome *c* (cyt *c*)[1] in SLE patient sera (8). Cyt *c* is a highly conserved protein and shares several features common to other nucleoprotein autoantigens (5). Anti–cyt *c* autoantibodies from SLE patient sera and animal sera bind selected sites of evolutionary divergence on the cyt *c* molecule.

The clustering of autoantibody specificities in SLE suggests that the central defect in the disease could be the presence of helper T cells directed at a peptide component of one of the proteins in the complex, allowing B cells that bind any component of the complex to present this peptide to the autoreactive T cell. The autoreactive CD4 T cell recognizing the autoantigenic complex of self peptide/self class II MHC protein on the antigen-specific B cell would then induce synthesis of autoantibodies. Sequence analysis of anti-DNA antibodies in mice has suggested that both antigen and antigen-specific helper T cells are driving this autoimmune response (9, 10). If this synthesis is correct, then the analysis of autoreactivity in helper T cells could provide a key to understanding autoantibody production in SLE.

Early studies on breaking tolerance used foreign molecules both to induce and to break tolerance. Weigle's laboratory (11) showed that tolerance to foreign serum albumins could be broken by immunization with a related serum albumin, leading to the production of antibody reacting with both the immunogen and the tolerogen. Loss of tolerance probably reflects the ability of foreign (non-self) peptides to stimulate helper T cells that in turn induce B cells to make antibodies to epitopes shared by both the immunizing and the tolerated protein. Indeed, Chiller et al. (12) showed that acquired tolerance in helper T cells is much more profound and sustained than is tolerance in B cells. While this may serve as a model for how B cell self tolerance could be broken by infectious agents through "molecular mimicry" (13), this mechanism does not explain the generation of autoimmune diseases such as SLE, since the T cell epitope recognized in these experimental models is foreign. Thus, this process cannot lead to the sustained loss of self tolerance required for the pathogenesis of autoimmune disease. Similar considerations apply to models leading to production of autoantibodies by immunization with foreign erythrocytes (14–16).

T cell tolerance is established in one of three ways. First, developing T cells that recognize ubiquitous self antigens are clonally deleted in the thymus (17, 18). T cells specific for antigens found only on certain tissue cells and not available in the thymus appear to be clonally inactivated or anergized by encountering ligand in the absence of required co-stim-

[1] *Abbreviations used in this paper:* cyt *c*, cytochrome *c*; PPD, purified protein derivative.

ulatory signals on tissue cells (19–22). Finally, some evidence exists for dominant suppression of self-reactivity in the periphery (23). Clearly, clonally deleted T cells cannot be immunized by any antigen; however, T cells that are not anergized might be inducible to autoreactivity if antigen is presented by a "professional" APC capable of delivering a potent co-stimulatory signal (19, 20, 24). In the present studies, we have obtained evidence for a mechanism by which immunization with a crossreacting antigen might break not only B cell but also T cell self tolerance. When B cells are simulated with a foreign antigen to make antibody that also binds a related self protein, these activated autoreactive B cells can present the self protein in immunogenic form to T cells, leading to T cell autoreactivity. By this means, not only are autoantibodies produced, but also, autoreactive T cells may be generated, potentially leading to a sustained autoimmune response.

Materials and Methods

Antigens. Horse heart cyt *c* (type VI) and rat heart cyt *c* (type XX), which is sequence-identical to mouse cyt *c*, were purchased from Sigma Chemical Co. (St. Louis, MO). Human cyt *c* was a generous gift of Dr. Morris Reichlin (Oklahoma City, OK).

Animals. MRL/mpJ +/+, A/J, CBA/J, C57BL/10J, B10.BR, and BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME). BALB.K and B10.A(4R) mice were produced in our breeding colony. In general, 4–8-wk-old mice were used in the experiments.

Immunization. Mice were immunized subcutaneously with a total of 50 or 100 μg cyt *c* emulsified 1:1 in CFA (Difco Laboratories, Detroit, MI) in the base of the tail.

Anti-cytochrome c ELISA. Solid phase immunoassays (ELISA) were used to detect the presence of cyt *c* binding antibodies from mouse sera (5). Rat cyt *c* and human cyt *c* were repurified by cation exchange chromatography and adsorbed to microtiter plates at a concentration of 20 μg/ml. Plates were blocked with 1% BSA followed by the addition of 10^{-2} dilutions of mouse serum. Rabbit anti-mouse IgG-alkaline phosphatase (Southern Biotechnology Associates Inc., Birmingham, AL) followed by *p*-nitrophenylphosphate substrate (Sigma Chemical Co.) was used to determine the binding of antibody. In some assays, serum dilutions were first preincubated with human or rat cyt *c* (20 μg/ml) before their addition to the ELISA as a measure of specificity to the two species of cyt *c*. As determined previously, the inhibition assays were performed in the range of solid phase and solution phase antigen excess.

Proliferation Assays. 10 d after immunization with cyt *c* in CFA in the base of the tail, paraortic and inguinal lymph nodes were collected aseptically and single cell suspensions were prepared by pressing the nodes between the ends of frosted glass slides. Lymph node cells (LNCs) were washed and resuspended in Click's EHAA medium containing 5% FCS. LNCs (5×10^5/well) were cultured with and without antigen in flat-bottomed 96-well microculture plates. The plates were incubated at 37°C in a 5% CO_2, 95% air humidified incubator for 3 d. 18 h before harvesting, 1 μCi of [^{3}H]thymidine was added to each well in 20 μl of Click's medium. The cultures were harvested using a semiautomatic cell harvester (Pharmacia LKB Nuclear Inc., Gaithersburg, MD) and counted in a liquid scintillation counter (Betaplate; Pharmacia LKB Nuclear Inc.). For assessment of proliferation of T cell clones, cells were tested at a concentration of 2×10^4 cells per well together with varying numbers of mitomycin C–treated syngeneic spleen cells as APC. The cultures were incubated for 48 h and tested as described above. In some assays, cells were incubated with mAbs anti-CD4 (GK 1.5), anti-CD8 (53-6.72), anti-I-A^k (10-3.62), or anti-I-E^k (Y-17) before proliferation assays.

Generation of Mouse cyt c–specific T Cell Clones. Mouse cyt *c*-specific T cell clones were derived from LNCs of MRL+/+ mice immunized with 50 μg mouse cyt *c* and 50 μg human cyt *c*. The LNCs from primed mice were stimulated with 100 μg/ml mouse cyt *c* and cloned by limiting dilution (0.2 cells/well) after 48 h in culture. Culture medium contained 2 U/ml of IL-2. Clones were restimulated for 48 h with 20 μg mouse cyt *c* in the presence of 3,000 rad irradiated syngeneic spleen cells (3×10^5/well) and further expanded with 1 U/ml of IL-2 and 2% rat Con A supernatant–containing medium. The clones were restimulated and expanded weekly in 24-well plates by the same protocol.

Isolation and Transfer of Immune Cells. MRL+/+ mice were immunized in the base of the tail and intraperitoneally with human cyt *c* in CFA, boosted at 2 wk with antigen in IFA, and at 3 wk with antigen in PBS. At 4 wk, the mice possessed high titer serum antibodies that bound both human and mouse cyt *c* in ELISA. Spleen and lymph node cells were then removed 3 d after the last boost. B cell populations were enriched by two treatments of spleen cells with anti-Thy-1 antibody (Y-19) (25) and complement. The adherent cells were depleted by panning on tissue culture plates at 37°C for 1 h. T cells were enriched by anti-Ig columns to remove B cells as described previously (26). 1.5×10^7 purified B or T cells from either human cyt *c* immunized or normal control mice were transferred by subcutaneous injection in the thighs as previously described (27). One group of mice received 100 μl immune serum from human cyt *c*–immunized mice. The mice were injected in the base of the tail with 100 μl CFA containing 100 μg mouse cyt *c*. 8 d later, inguinal lymph nodes were collected and T cell proliferation was measured. In a second experiment, two groups of mice were primed with CFA alone or CFA containing 50 μg human cyt *c* in the hind foot pad. 7 d later, the draining lymph nodes were removed, treated with anti-Thy-1 +/C, followed by adherence to plastic for 1 h. Nonadherent cells were transferred to recipients that were then primed with mouse cyt *c* as in the previous experiments.

Results

Murine T and B Cells Are Tolerant to Murine Cytochrome c. Cyt *c* is found in all cells, and mice are tolerant to their own cyt *c*. We demonstrated tolerance of T cells by immunizing mice with the sequence-identical rat cyt *c* and measuring the ability of immunized lymph node T cells to proliferate to rat cyt *c* (Fig. 1 *A*). No response was observed, but immunization did elicit a response to the purified protein derivative (PPD) component of the *Mycobacteria* found in CFA. Furthermore, no autoantibodies were detected in the serum of such immunized mice (Fig. 1 *B*), showing that both humoral and cell-mediated immunity to mouse cyt *c* is absent in normal mice.

Mice Immunized with Human Cyt c Make Antibody but not T Cell Responses to Mouse cyt c. The failure of mice to make antibody upon immunization with mouse cyt *c* could reflect tolerance of B cells, helper T cells, or both. To test whether the B cells of mice are tolerant to mouse cyt *c*, mice were immunized with human cyt *c*, and sera were tested for antibodies specific for human or mouse cyt *c* (Fig. 1 *D*). Specific

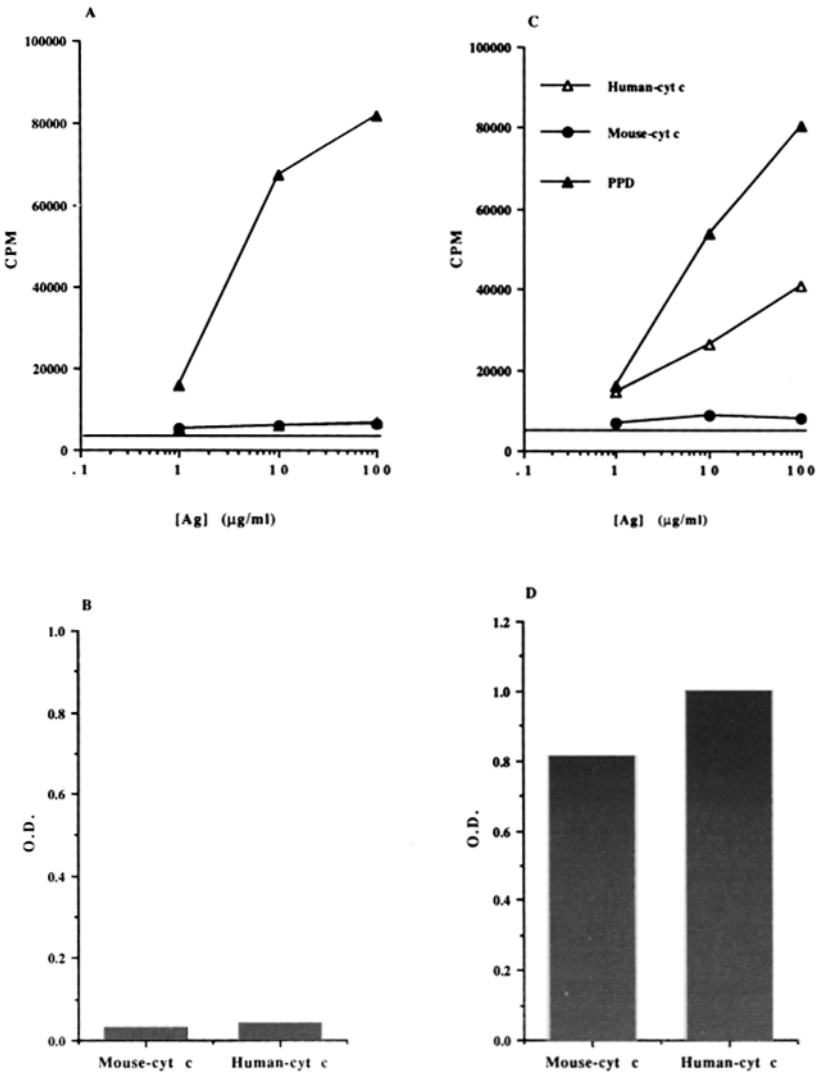

Figure 1. The cellular and humoral immune responses of MRL+/+ mice to cyt *c*. The proliferative response of LNCs (4×10^5 cells/well) from (*A*) 100 μg mouse cyt *c*– and (*C*) 50 μg human cyt *c*–immunized mice to in vitro stimulation by human cyt *c* (△), mouse cyt *c* (●), PPD (▲), or no antigen (–). Humoral IgG immune response of (*B*) mouse cyt *c*– and (*D*) human cyt *c*–immunized mice to human or mouse cyt *c*.

antibodies that bind mouse cyt *c* were produced in response to immunization with human cyt *c*. This autoantibody is fully crossreactive with human cyt *c*, implying that T cell help could be delivered entirely by cells specific for peptides unique to human cyt *c* (Fig. 2). Furthermore, mice immunized with human cyt *c* have potent T cell responses to human cyt *c*, but fail to respond to mouse cyt *c*, confirming the tolerance of mouse T cells to mouse cyt *c* (Fig. 1 *C*). Thus, these experiments are analogues of the earlier studies of Weigle (11) on tolerance to foreign antigens; humoral responses to tolerated self proteins can be induced by a related foreign protein that can activate effective helper T cells. However, the autoantibody response cannot be expected to be sustained by helper T cells once the foreign stimulus is removed, since autoreactive helper T cells are not induced by this immunization.

Immunization with both Mouse and Human cyt c Breaks Tolerance to Mouse Cytochrome c. When mice are immunized with human cyt *c*, the absence of a T cell response to mouse cyt *c* could reflect true self-tolerance. Alternatively, there could be an insufficient amount of mouse cyt *c* available to initiate a T cell response. To test this, mouse and human cyt *c* were mixed in CFA and used to immunize mice. Such mice produced antibody to human and mouse cyt *c* (Fig. 2). However, in this case, primed T cells responded to both human and mouse cyt *c* (Fig. 3). To examine the specificity of such cells, lines and clones were prepared by culturing these cells with mouse cyt *c* and syngeneic APC. The proliferative responses of these cells show strict specificity for mouse cyt *c* (Fig. 4 *A*). This species specificity was found in all five individual clones (data not shown). The response is inhibitable by antibodies specific for CD4 and I-E^k (Fig. 4 *B*), suggesting that the responding cells are CD4 T cells that recognize a peptide of murine cyt *c* presented by the syngeneic I-E molecule. This is the exact phenotype of the most common mouse T cells that respond to non-self cyt *c* (28). This response is observed using four different H-2^k strains of mice, but not congenic H-2^d or H-2^b mice (data not shown). Thus, the response does not require MRL background genes, although it may require H-2^k. The priming of T cells to re-

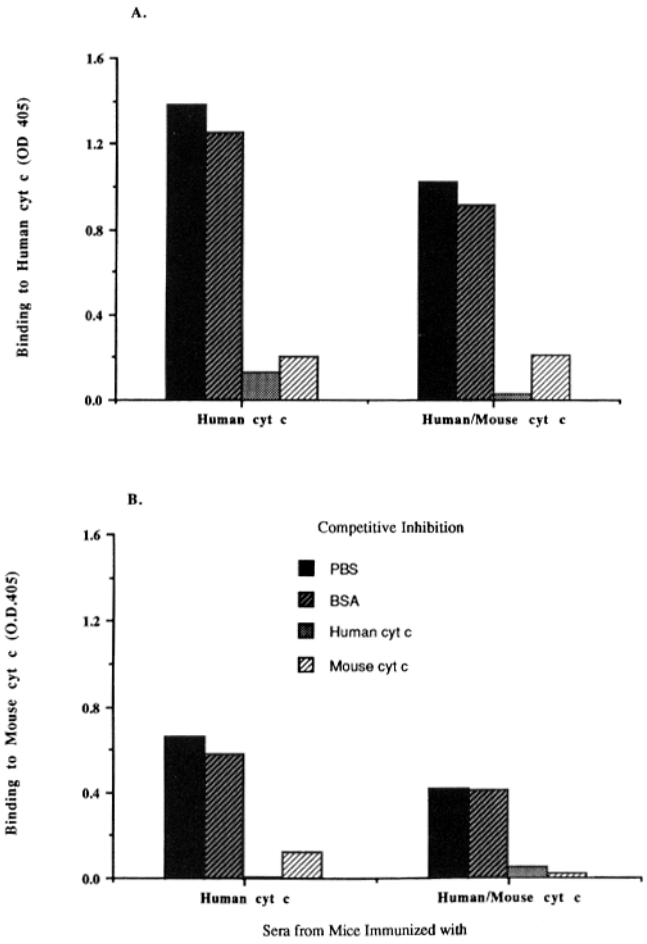

Figure 2. The specificity of anti-cyt *c* antibodies from immunized mice. Sera from MRL+/+ mice immunized with 50 μg human cyt *c* with or without 100 μg mouse cyt *c* in CFA were assayed by ELISA for binding to human cyt *c* (*A*) or to mouse cyt *c* (*B*). As a measure of specificity, sera were preincubated with 20 μg/ml of mouse or human cyt *c*, or BSA as a control protein before the ELISA.

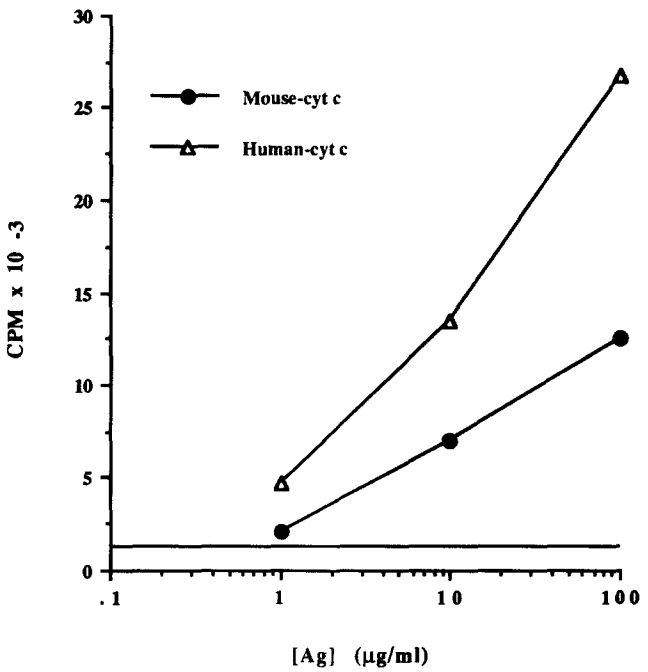

Figure 3. The proliferative response of LNCs from MRL+/+ mice immunized with both human cyt *c* and mouse cyt *c*. LNCs from primed mice were stimulated in vitro with human (△) or mouse (●) cyt *c* or no antigen (–). Data presented are means of triplicate wells.

spond to mouse cyt *c* requires the presence of a crossreacting foreign cyt *c*. Both human and horse cyt *c* have this activity, while OVA, although highly immunogenic for OVA-reactive T cells, does not allow priming to mouse cyt *c* when these two antigens are mixed in CFA (data not shown).

B Cells from Mice Immune to Human cyt c Allow Priming of Autoreactive T Cells with Mouse cyt c. Because priming with human cyt *c* breaks B cell but not T cell self tolerance, we thought that crossreactive B cells might be involved in the loss of T cell tolerance to mouse cyt *c* when both are used for priming. Furthermore, the T cell response to mouse cyt *c* lags behind that to human cyt *c* by ~5 d in these dual-immunized mice, consistent with a requirement for prior B cell activation (data not shown). Antigen-specific, activated B cells have been shown to be effective APC in a number of experimental systems (24, 27, 29–32). To determine whether B cells, their secreted antibody, or T cells primed with human cyt *c* can adoptively transfer the ability to induce T cell responses to mouse cyt *c*, mice were primed with human cyt *c* and then killed after bleeding. Recipient mice were injected with purified immune B or T cells, with B cells from unimmunized mice, or with immune serum, and primed with mouse cyt *c*. As can be seen in Figs. 5, *A* and *B* and 6, lymph node T cells from the primed recipient mice responded to mouse cyt *c* only if the recipient had previously received B cells from mice immunized with human cyt *c*. Immune serum, immune T cells, and B cells from nonimmune donors would not transfer the ability to prime T cells with mouse cyt *c*. Furthermore, the response of the recipient mice was entirely specific for mouse cyt *c*, as no proliferative response of recipient mice to human cyt *c* was observed. Thus, the critical cell involved in breaking tolerance to self cyt *c* is the antigen-specific B cell.

Discussion

In these experiments, we describe a new mechanism by which self tolerance in T cells may be broken by crossreacting immunogens. These studies demonstrate the presence of T cells specific for mouse cyt *c* in normal H-2^k mice. When self cyt *c* is presented on its own, no T or B cell response is observed. However, when the same antigen is presented by antigen-specific B cells elicited with a serologically crossreactive antigen, T cell priming is achieved. These studies may explain how sustained, T cell–dependent autoantibody production can be initiated by crossreactive foreign antigens, such as molecular mimics on infectious agents.

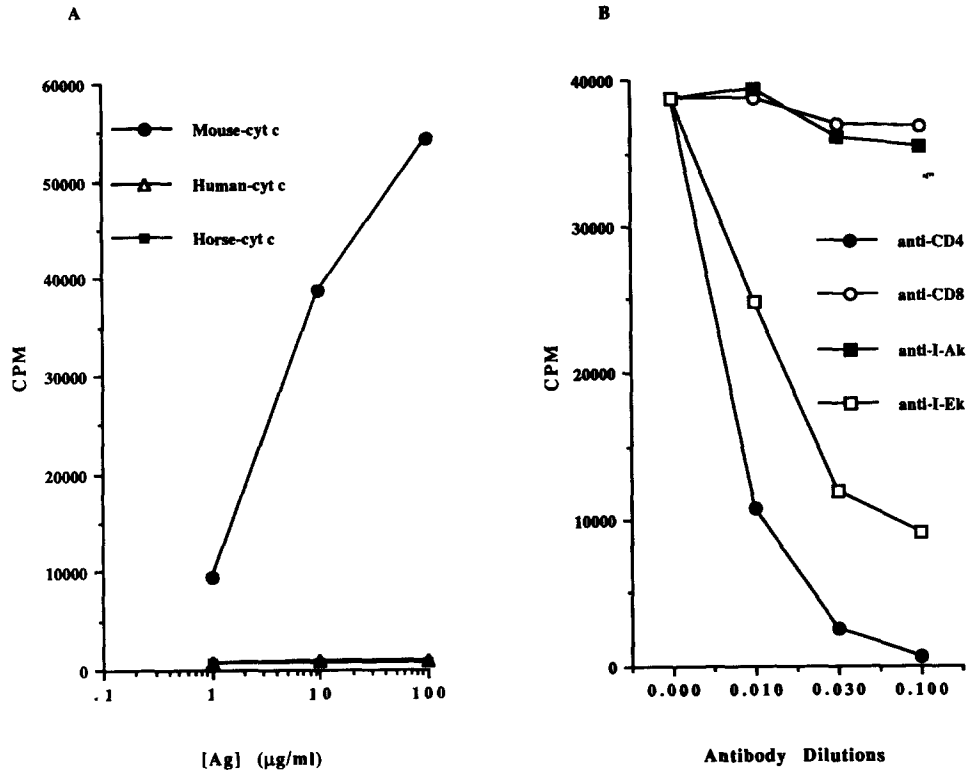

Figure 4. The specificity and MHC class II restriction of mouse cyt *c*–specific cloned T cells. (*A*) The proliferative response of a representative mouse cyt *c*–specific T cell clone stimulated by mouse (●), human (△), and horse (■) cyt *c*. (*B*) The proliferative response of a mouse cyt *c*–specific T cell line to mouse cyt *c* (50 μg/ml) in the presence of blocking antibody culture supernatants: (●) anti-CD4, (○) anti-CD8, (■) anti-I-A^k, (□) anti-I-E^k.

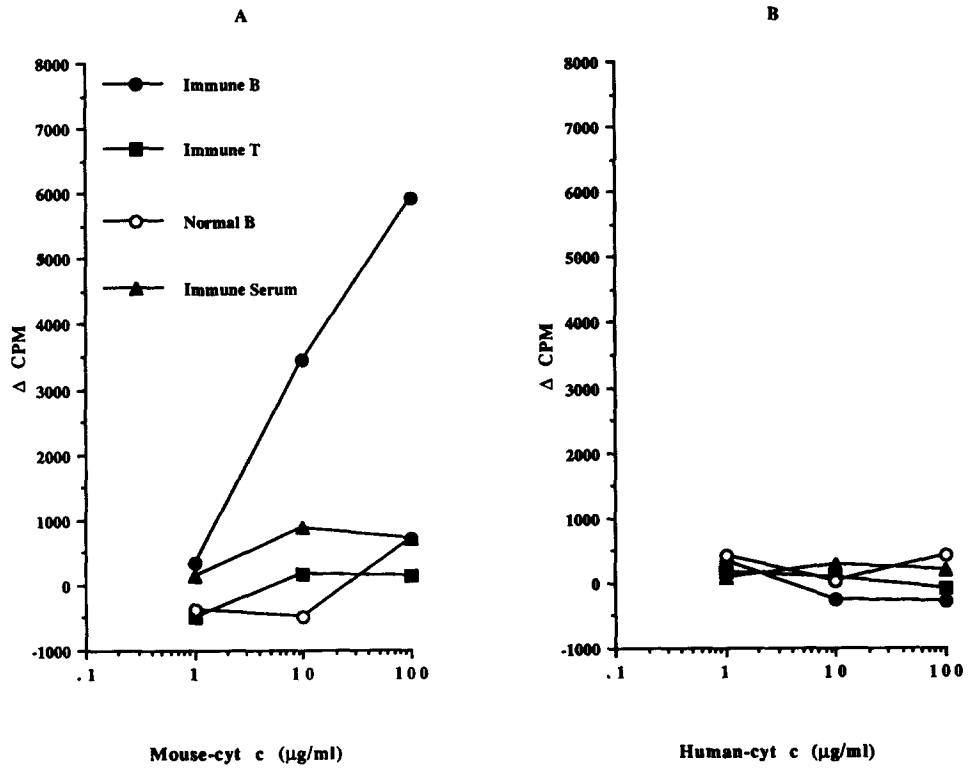

Figure 5. Purified B cells (●) and T cells (■) from mice immunized with human cyt *c*, or B cells from normal mice (○) were adoptively transferred into naive mice (see Materials and Methods). Mouse anti–human cyt *c* serum (▲) was also transferred into recipient mice. The mice were then immunized with 100 μg mouse cyt *c* in CFA. After 8 d, the LNCs (5×10^5 cells/well) from primed mice were stimulated with various concentrations of mouse cyt *c* (*A*) or human cyt *c* (*B*). Background cpm for each group ranged from 2,308 to 3,940 and are subtracted to yield (△) cpm.

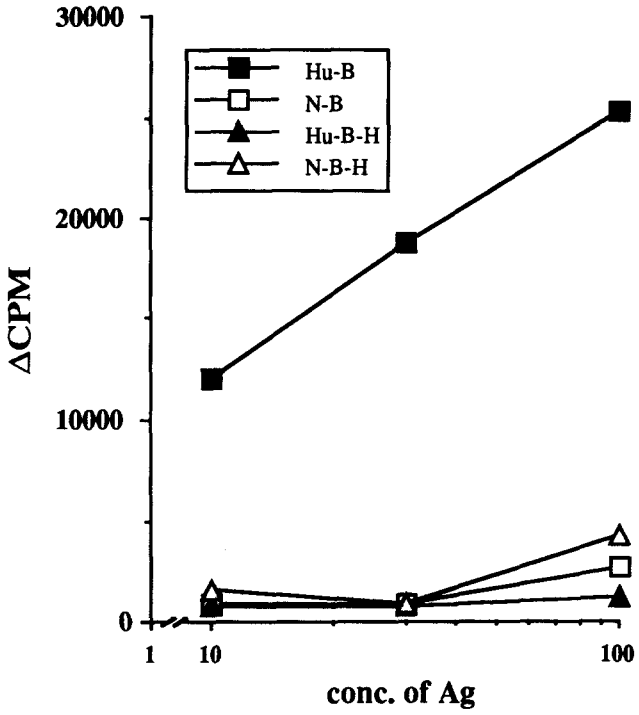

Figure 6. Purified B cells from mice primed with CFA containing 50 μg human cyt *c* (*filled symbols*) or CFA alone (*open symbols*) were isolated 7 d after immunization and used for adoptive transfer experiments (see Materials and Methods). The mice were then immunized and lymph node proliferation responses were measured in the presence of various concentrations of mouse cyt *c* (*squares*) or human cyt *c* (*triangles*).

Antigen-specific B cells are known to be highly effective at concentrating protein antigens for presentation to CD4 T cells (33, 34). In vivo, B cells have been implicated in T cell responses by the failure of B cell–deficient mice to become immunized for a T cell–proliferative response to protein antigens (30, 31). Furthermore, Kurt-Jones et al. (27) have reconstituted such mice with antigen-specific B cells and have shown that these cells are required for the induction of T cell responses. However, it should also be noted that these statements apply to the T cell–proliferative response, whereas the priming of helper T cells may have different requirements, since B cell–deficient mice can be primed to yield competent helper T cells (35–37). Whether the autoreactive T cells we have identified can function as helper T cells in an antibody response remains to be determined.

The activation of CD4 T cells requires presentation of an antigen/self MHC class II ligand and also the provision of a suitable co-stimulatory signal (20, 24). Resting B cells apparently lack the ability to co-stimulate T cells, but activated B cells are excellent co-stimulators of T cell clonal expansion in many systems (24, 29). Furthermore, existing data suggest that both signals for T cell activation must be delivered by the same APC (Liu, Y., and C.A. Janeway, manuscript submitted for publication). Thus, the activation of antigen-specific B cells may aid in breaking T cell self tolerance by providing both a high level of stimulatory ligand and a high level of costimulator activity on the same APC. Human cyt *c*, being a non-self protein, may have less stringent requirements for priming, as it can presumably stimulate a more extensive repertoire of T cells.

Thus, our data provide a potential mechanism for the breaking of T cell self tolerance. Clearly, this mechanism cannot operate if autoreactive T cells are not present in the mouse, as may occur if effective clonal deletion or complete anergy exist. Nevertheless, this mechanism may explain how an infectious agent that carries epitopes that induce autoantibody production but not an autoreactive T cell response can lead to autoreactive T cell priming and the sustained autoantibody production typical of SLE and other antibody-mediated autoimmune diseases.

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