*Brief Definitive Report*

# The Inability to Process a Self-Peptide Allows Autoreactive T Cells to Escape Tolerance

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## Summary

It is now clear that antigen presenting cells (APCs) do not present all the possible peptides of self-proteins to the immune system. What then, is the fate of T cells specific for those self-peptides that escape processing? In this study, the COOH-terminal peptide (residues 81-104) of self cytochrome *c* (cyt *c*) elicited strong autoimmune T cells, as well as autoantibodies specific for this immunogen. These T cells did not respond to stimulation with the whole self cyt *c* molecule, demonstrating that APCs cannot process and present the self 81-104 peptide. Whereas mice were unresponsive to immunization with the whole mouse cyt *c* molecule, the mouse 81-104 fragment together with the whole self-molecule induced and amplified the autoimmune T cell response to sites within the 1-80 peptide. T cells that never contact the relevant self-peptide are functionally ignorant. They do not become tolerized or deleted, nor do they normally participate in immune responses to the native whole self-protein, since APCs cannot present the 81-104 peptide.

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Successful immune responses rely on the intelligence of B and T cells to differentiate self from foreign components. Self-reactive T cells are controlled either by their elimination in the thymus or by the induction of tolerance in the periphery (1, 2). Both mechanisms depend on the ability of APCs to process and present self-peptides in the context of MHC molecules, although it is now apparent that not all possible peptides from a self-antigen are presented (3, 4). These limitations in processing suggest that T cells specific for some determinants on self-proteins may escape tolerance and therefore be a part of the normal repertoire of T cells. The importance of such autoreactive T cells in pathological conditions may become apparent only under conditions that cause the presentation of normally hidden self-peptides or when the immune system is confronted with foreign molecular mimics.

Recent studies of autoantigens in SLE have shown that B cells elicited with foreign cross-reactive antigen can present self-peptides resulting in the priming of autoreactive T cells (5–7; Mamula, M., S. Fatehnejad, and J. Craft, manuscript submitted for publication). The present study has identified a peptide from a self-protein, cytochrome *c* (cyt *c*), capable of inducing strong autoreactive T cell responses. This cryptic self-peptide, in the presence of the whole self-protein, breaks T cell tolerance to other sites on the self-protein. These observations may have important implications in the induction and expansion of the autoimmune T cell responses in human disease.

## Materials and Methods

*Antigens.* Rat heart cyt *c* (type XX) was purchased from Sigma Chemical Co. (St. Louis, MO). Rat cyt *c* is identical to mouse cyt *c* in amino acid sequence, and is hereafter known as mouse cyt *c*. Human cyt *c* was the generous gift of Dr. Morris Reichlin (University of Oklahoma, Oklahoma City, OK). Both mouse and human cytochromes *c* were repurified by cation exchange chromatography as previously described (8).

Polypeptides encompassing amino acids 81-104 of human cyt *c* or amino acids 37-51, 54-68, 81-95, or 81-104 of mouse cyt *c* were made on a polypeptide synthesizer (RAMPS; DuPont Corp., Wilmington, DE) or by the Yale University School of Medicine Protein and Nucleic Acid Chemistry Facility (New Haven, CT). The synthetic polypeptides were analyzed for purity by HPLC and by mass spectroscopy.

Cyanogen bromide (CNBr)-derived polypeptides of mouse cyt *c* were prepared by a modification of a previously described method (8). Briefly, repurified whole mouse cyt *c* was resuspended in 70% formic acid and digested for 24 h with CNBr (10:1, CNBr/cyt *c* molar ratio). The digested cyt *c* was resuspended in sterile PBS for use in lymph node cell (LNC) proliferation assays. A fraction of the digest was resuspended in 7% formic acid and analyzed by fast protein liquid chromatography (FPLC; Pharmacia LKB Biotechnology, Piscataway, NJ) on a gel filtration column (Superose 12; Pharmacia LKB Biotechnology). CNBr-derived peptides of mouse cyt *c* encompassing 1-80 and 1-65 were purified by FPLC as previously described (6).

*Animals.* Immune responses among different murine haplotypes were analyzed in mice purchased from The Jackson Laboratory (Bar Harbor, ME) or in mice kindly provided by Dr. Charles A. Janeway, Jr., (Yale University School of Medicine). The mouse strains used included B10.A (5R), B10.A (4R), B10.BR, B10.F, B10/Sn, B10.R III, B10.S, DBA 1, Sm/J, BALB/c, and AKR. Detailed analysis of T cell responses to individual polypeptides were performed in B10.BR mice.

*Immunization and Proliferation Assay.* B10.BR mice and the other haplotypes examined were immunized with 50 μg of antigen

emulsified 1:1 in CFA in the base of the tail and in one hind footpad. After 10 d, inguinal, periaortic, and popliteal LNCs were aseptically collected and washed in Click's EHAA medium supplemented with 5% FCS, antibiotics and L-glutamine. The LNCs ($5 \times 10^5$) were incubated in flat-bottomed 96-well microtiter wells with and without antigen for 3 d. The culture wells were pulsed with 1 $\mu$Ci [$^3$H]thymidine 18 h before the end of the assay. Experimental wells were harvested onto paper with a semiautomatic cell harvester (Skatron Instruments, Inc., Sterling, VA), and radioactive counts were measured in scintillation fluid (Beckman Instruments, Inc., Fullerton, CA). Averages of duplicate wells were plotted and deviations from the mean were <10% for all experiments. Purified protein derivative (PPD, 20 $\mu$g/ml) and negative (no antigen) control values are noted in each figure legend.

*Anti-cyt c ELISA.* Serum from B10.BR mice immunized and twice boosted with mouse cyt *c* peptide 81-104 were examined by ELISA for binding to whole mouse cyt *c* and the COOH-terminal fragments 81-104 of mouse and human cyt *c* according to previous methods (9). The peptides or whole mouse cyt *c* was adsorbed to the solid phase of microtiter plates followed by a blocking step with 1% BSA in PBS. Serum dilutions were applied to the plates and detected with anti-mouse IgG alkaline phosphatase conjugate (Southern Biotechnology Associates, Birmingham, AL). In some assays, dilutions of sera were first incubated for 2 h at 4°C with whole cyt *c* or peptides before their application to ELISA plates. Inhibition studies were always performed in conditions of antigen excess. Percent inhibition was calculated as $100 \times [1-(\text{inhibited OD}/\text{uninhibited OD})]$.

## Results and Discussion

The immune system has evolved to ignore self-proteins as demonstrated in Fig. 1. T cells from mice immunized with native whole mouse cyt *c* failed to proliferate in response to stimulation with the whole molecule, to a peptide from the COOH terminus (81-104), or to CNBr-derived peptides of

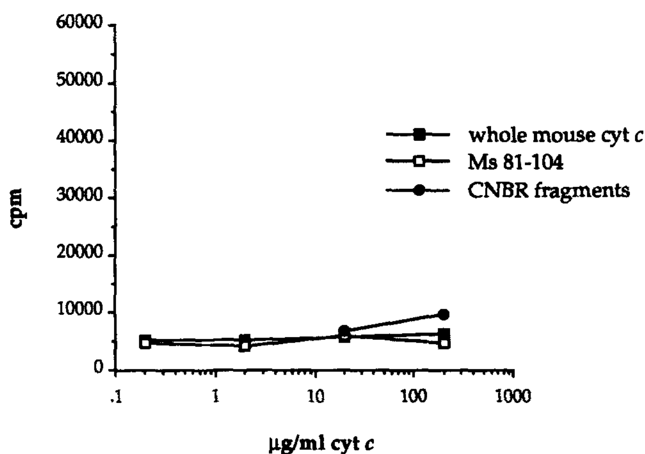


**Figure 1.** Mice are unresponsive to immunization with whole mouse cyt *c*. B10.BR mice were immunized with 50 $\mu$g of whole mouse cyt *c* in CFA and standard LN proliferation assays were performed at day 10. T cells were stimulated with titrations of either whole mouse cyt *c*, synthetic fragment 81-104 of mouse cyt *c*, or total CNBr digests of mouse cyt *c*. The data points represent the average of duplicate wells with a deviation of <1,000 cpm for all experiments. PPD and negative control wells averaged 86,563 and 3,430 cpm, respectively.

the whole molecule. In addition, no T lymphocyte or antibody responses (as measured by ELISA) were observed in any haplotype (p, q, r, s, f, v, b, and d) of mouse immunized with its own cyt *c* (data not shown).

Mice immunized with human cyt *c* peptide 81-104 elicited T cells that responded to stimulation with the 81-104 peptide (Fig. 2 *A*). Additionally, these T cells responded to stimulation with the whole human cyt *c* molecule, suggesting that intracellular processing of the native whole human cyt *c* molecule resulted in the expression of a COOH-terminal fragment on the APC surface, a response that is inhibited by antibodies to I-E$^k$ (data not shown). These T cells were not cross-reactive with whole mouse cyt *c* or its 81-104 fragment. In contrast, immunization with fragment 81-104 of mouse cyt *c* elicited T cells that responded only to the mouse 81-104 fragment (Fig. 2 *B*) and not to stimulation with whole mouse cyt *c* or the human 81-104 fragment. As a control, T cells could be stimulated with CNBr digests of the whole mouse cyt *c*, a cleavage that generates the 81-104 peptide from the whole molecule. This important observation demonstrated that the response to the synthetic self-peptide 81-104 was not a result of contamination with foreign peptides. Therefore, these T cells could be stimulated by whole mouse cyt *c* if the processing of this molecule is performed in vitro for the APC. It is also apparent that T cells specific or the mouse cyt *c* 81-104 fragment have not been deleted or tolerized in the periphery presumably from the inability of mouse APCs to express this fragment.

The contrasting responses between self and foreign cyt *c* are due to differences in primary amino acid sequences. Mouse and human cyt *c* differ by a total of nine amino acids among their 104 total residues, with two differences existing within the COOH-terminal peptide (residues 83 and 89). Preliminary studies have demonstrated that changes made in the recombinant mouse cyt *c* protein to the corresponding human amino acids at either residues 83 or 89 partially restore the ability of APCs to process and present the COOH-terminal peptide of the self-antigen (Mamula, M., B. Crump, and E. Margoliash, manuscript in preparation). It is not yet clear whether amino acids outside the COOH-terminal region are critical in processing this peptide of mouse cyt *c*.

A previous study has demonstrated that immunization of mice with whole mouse cyt *c* together with various peptides along the entire length of foreign (human) cyt *c* elicited autoreactive T cells (6). These autoimmune T cell responses were dependent on the presence of a foreign peptide immunogen or molecular mimic. After demonstrating that mouse fragment 81-104 could induce strong T cell responses, the ability of this self-determinant to elicit T cell responses to other sites on the self-protein was examined (Fig. 3). Mice were immunized with a mixture of whole mouse cyt *c* and the COOH-terminal mouse peptide 81-104. As expected from earlier results, T cells from these mice responded to stimulation with the the 81-104 peptide and CNBr fragments of mouse cyt *c*. In contrast to earlier results using mouse 81-104 alone as an immunogen, these T cells now also responded to the whole mouse cyt *c* protein and to purified mouse peptide 1-80 (Fig. 3). These observations demonstrated that the



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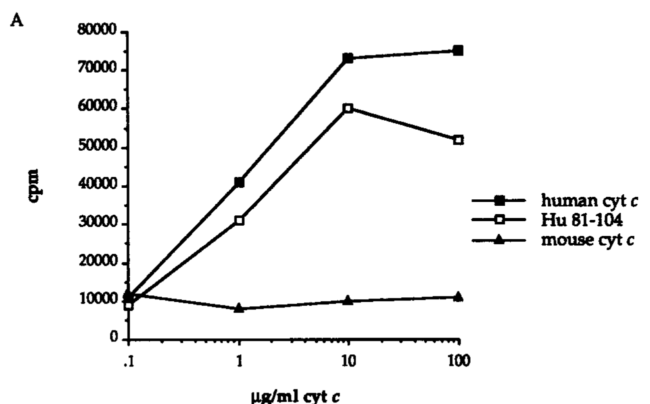


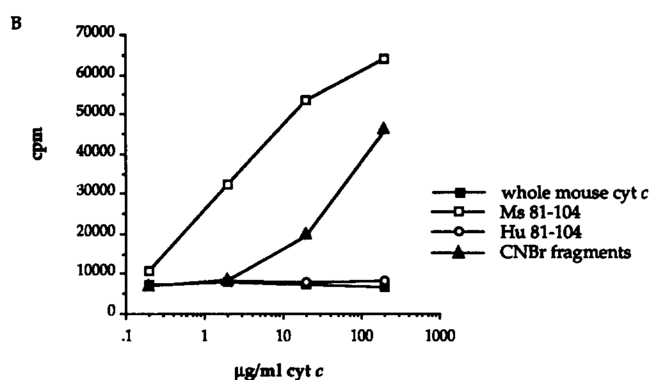


**Figure 2.** Distinct T cell responses of mice immunized with foreign (*human*) or self-(*mouse*) peptide 81-104 of cyt *c*. B10.BR mice were immunized with 50 $\mu$g of human cyt *c* peptide 81-104 (*A*) or mouse peptide 81-104 (*B*) in CFA at the base of the tail. LN cells were stimulated at day 10 with titrations of either whole mouse or human cyt *c*, synthesized peptides 81-104 of mouse or human cyt *c*, or CNBr digests of whole mouse cyt *c*. PPD controls averaged >100,000 cpm and negative controls were <5,878 cpm for all experiments.

81-104 self-peptide of cyt *c* can break T cell tolerance to other sites on the self-protein.

One likely possibility to explain these results is that B cells specific for mouse 81-104 peptide also bind and process the whole mouse cyt *c* molecule. Peptides within the first 80 amino acids of the protein are then presented in the priming of this expanded autoimmune T cell response. This mechanism is supported by previous studies with cyt *c* (5, 6) and by studies of the antibody responses from mice immunized with the mouse 81-104 fragment (Table 1). Sera from B10.BR mice chronically immunized with mouse cyt *c* 81-104 bound both human and mouse 81-104 cyt *c* fragments in ELISAs, as well as binding the whole molecule adsorbed to the solid phase of microtiter plates. However, the binding to whole mouse cyt *c* was dependent on partial denaturation of this protein since the native protein did not significantly inhibit binding in solution phase. Therefore, the release of intracellular cyt *c* by tissue pathology or perhaps the apoptosis of LNCs may allow sufficient denaturation of the whole protein by extracellular proteases in order to be bound, processed, and presented by B cells specific for the mouse 81-104 peptide.

The observations of this study may help explain the diver-

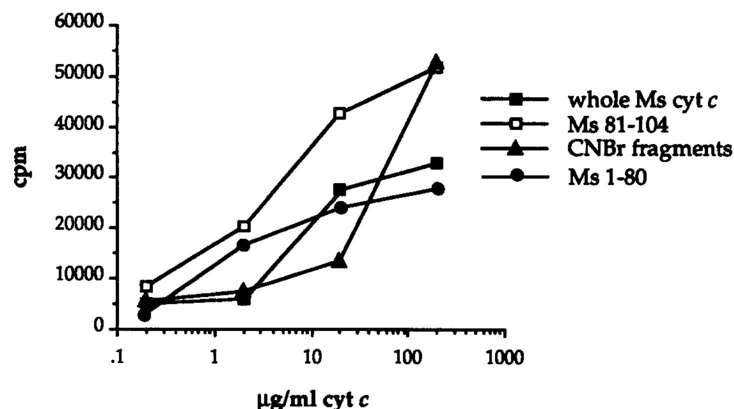


**Figure 3.** Coimmunization of whole mouse cyt *c* with peptide 81-104 elicits T cells specific for the whole mouse cyt *c* protein. B10.BR mice were immunized with whole mouse cyt *c* (50 $\mu$g) together with mouse cyt *c* peptide 81-104 (50 $\mu$g) in CFA at the base of the tail. LN proliferation assays were performed at day 10. PPD and negative control wells averaged 84,899 and 3,544 cpm, respectively.



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**Table 1.** *Binding Properties of Anti–Mouse cyt c 81-104 Antisera*

| Binding to: | OD (405) | Percent inhibition with: | | |
|---|---|---|---|---|
| | | Ms 81-104 | Hu 81-104 | Whole Ms cyt *c* |
| Mouse 81-104 | 0.97 | 82 | 66 | 3 |
| Human 81-104 | 1.31 | 88 | 92 | ND |
| Whole Ms cyt *c* | 0.76 | 72 | ND | 18 |

Serum dilutions (1/100) from mice immunized with mouse cyt *c* peptide 81-104 were examined by ELISA for binding to mouse or human peptide 81-104 or to whole mouse cyt *c*. Serum dilutions were then incubated with 8 $\mu$M of antigen before their application to the ELISA plates. Percent inhibition = 100 × [1 − inhibited OD/uninhibited OD)].

sity of autoimmune T cell responses in murine models of experimental allergic encephalomyelitis (EAE; 10). Immunization with a peptide from foreign (guinea pig) myelin basic protein will initiate EAE in mice and elicit T cell responses to the immunogen. Over the course of disease, the specificity of autoimmune T cells diversifies to several other sites on the mouse myelin basic protein (10). This observation demonstrates that the self-protein has entered into the expanding autoimmune T cell response in the mouse. It is likely that the initial immunization with foreign peptide had elicited B cells that bound the self-myelin basic protein. The B cells then processed and presented a variety of other self-peptides in the priming of autoreactive T cells that were observed over time. In similar studies, MRL lpr/lpr mice (the murine model of human SLE) immunized with mouse cyt *c* 81-104 exhibited spontaneous expansion of autoreactive T cell specificity to sites within the 1-80 peptide over the course of several months (M. Mamula, unpublished observations). This diversified T cell response occurred only after clinical symptoms of SLE appeared in the mouse. This response also suggested that self cyt *c* released by tissue pathology had contributed to the autoimmune response. In either of these examples, it is not yet clear how the diversity of the autoreactive T cell response may correlate with the severity of disease.

This study has identified a subset of autoreactive T cells that are functionally ignorant in vivo. Such cells are allowed to exist in the host because of the processing properties of self-proteins rather than secondary defects in mechanisms of tolerance or deletion. The consequences of activation of these T cells may not be completely known until mechanisms that may interfere with normal processing functions of self proteins are understood. Dissection of these processes may help decipher the early events in the initiation of autoimmune disease.

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