T CELL TOLERANCE STUDIED AT THE LEVEL OF ANTIGENIC DETERMINANTS

I. Latent Reactivity to Lysozyme Peptides that Lack Suppressogenic Epitopes Can Be Revealed in Lysozyme-tolerant Mice

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A prevailing and persistent subject of controversy in immunology is whether the mechanism of immune tolerance to self antigens involves active, cell-mediated regulatory processes or is effected centrally and directly via interactions between antigen and effector cell precursors (e.g., 1–7). The debate continues to be fueled by evidence for both regulatory (7) and direct mechanisms (8, 9) in similar systems. Many investigators now believe that multiple pathways lead to self-tolerance, forming a web of failsafe mechanisms against autoimmunity (4, 5).

The mechanism of T cell tolerance has been especially difficult to approach because of our limited understanding of (*a*) antigen recognition by T cells, (*b*) the complex T cell regulatory circuitry, and (*c*) the molecular and physiological basis for antigen-specific, T cell–mediated suppression (i.e., the nature of the off signal, half-life of clonal inactivation, reversibility, target cell death). Conventional approaches to the question of whether tolerance is mediated by suppressor T lymphocytes (Ts)[1] or through direct clonal inactivation by antigen have relied upon attempts to demonstrate Ts by in vitro cell mixing experiments or in vivo adoptive transfers. However, recent work by several groups has demonstrated many intricate cellular requirements of T cell–mediated suppression (e.g., multiple interacting Ts subsets, distinct levels of suppression) (10, 11), and the several cellular activities that may mask T cell suppression (e.g., augmenting T cells, multiple subsets of contrasuppressor T cells) (11, 12). The simple inability to demonstrate active suppression is not evidence for clonal deletion, and, conversely, the mere demonstration of active suppression does not rule out the role of clonal deletion mechanisms in tolerance.

Recent work in several laboratories has demonstrated that cytotoxic T lymphocyte (CTL) precursor frequency, determined by limiting dilution analysis, is

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[1] *Abbreviations used in this paper:* AP-HEL, aminopeptidase-treated HEL; A-TOL(2 mg) and A-TOL(20 mg), tolerance induced in adult mice by injection of 2 or 20 mg HEL, respectively; CFA, complete Freund's adjuvant; CTL, cytotoxic T lymphocyte; HEL, hen (chicken) eggwhite lysozyme; HGG, human gamma globulin; IFA, incomplete Freund's adjuvant; MHC, major histocompatibility complex; N-TOL, neonatally induced tolerance; RCM-HEL, reduced, carboxymethylated HEL; SD, Ts-inducing determinant; TdR, thymidine; Th, Tp, and Ts cells, T helper, proliferative, and suppressor cells.

dramatically lowered in mice neonatally tolerized for either major histocompatibility complex (MHC) (13, 14), virus (15), or hapten-conjugated self-responses (16). Precursor frequencies of allo-MHC–specific, interleukin 2–producing cells are also dramatically lowered in neonatally tolerized mice (14). These findings are an important demonstration that CTL clonal nonresponsiveness can be manifest in the short-term absence of Ts cells. However, lower CTL precursor frequencies may occur through clonal paralysis or death, reflecting the mechanism of Ts action. Recent work by Huerer et al. (17) has demonstrated that a cloned Ts cell can lyse antigen-specific target T cells.

Even less is known about tolerance of protein antigen-specific T helper (Th) or proliferating (Tp) cells. The lack of a primary in vitro single-cell assay prevents the quantitation of Th/p, relatively free of Ts regulation, by limiting dilution assay. The recent demonstration, by Lamb et al. (18), of in vitro inactivation of human T cell clones, specific for influenza hemagglutinin, by high doses of hemagglutinin peptides, indicates that deletion/anergy mechanisms are possible for proliferating Th cell clones. However, the difficulty of tolerizing human gamma globulin (HGG)-primed T cells in vivo with doses of deaggregated HGG that inactivate HGG-specific naive T cells, as well as primed or unprimed B cells (19), suggests that these findings should not yet be generalized to in vivo T cell tolerance.

The approach outlined in this paper is based on previous work in this laboratory and others showing that the specificities of Th and Ts cells are nonoverlapping in the protein antigen systems that have been examined (20). In the C57BL/6 stain, which is nonresponsive to hen (chicken) eggwhite lysozyme (HEL), Ts cells are restricted to the recognition of an amino-terminal epitope, while antigen-specific Ts and Tp cells are restricted to "internal" epitopes. "Amputation" of the Ts-inducing epitope from the rest of the molecule reveals the latent capacity to induce Tp or Th cell response by freeing these cells from Ts regulation (21–23). It is thereby possible to determine whether antigen-specific Tp remain responsive in a tolerant animal without precursor frequency analysis, by using peptide probes that lack Ts-inducing determinants (SD).

In this report, the amputation approach clearly indicates that in acute, low dose tolerance of adult responder strain B10.A mice, proliferative HEL-specific T cells are regulated by an active mechanism, presumably mediated by Ts cells. Two other states of tolerance were examined: neonatal tolerance, after which HEL-specific T cells were not responsive to the available peptide probes; and high dose adult tolerance, in which T cells of discrete specificities were affected differentially. Whether functional clonal deletion had taken place, and/or whether the activation of usually quiescent Ts with specificity for SD within the peptide probes used for challenge had occurred, awaits critical analysis with minimal peptide determinants that are small enough to exclude SD.

Materials and Methods

Animals. B10.A mice were bred from mating pairs purchased from The Jackson Laboratory, Bar Harbor, ME, and raised in the vivarium facility maintained by this laboratory. Mice of both sexes were used at 8–24 wk of age for all experiments.

Antigens. HEL was obtained from Societa Prodotti Antibiotici (Milan, Italy) and was chromatographed before use on Biorex 70 (Bio-Rad Laboratories, Richmond, CA) as previously described (22). Detailed preparation and characterization of NC (22), aminopeptidase-treated HEL (AP-HEL) (23), L2, and L3 (24), are given elsewhere: a summary appears in Fig. 1.

Acute Adult Tolerance (A-TOL) Induction. 8–24-wk-old B10.A mice were administered 2 mg of HEL [A-TOL(2 mg)] dissolved in 0.2 ml of normal saline, or 20 mg of HEL [A-TOL(20 mg)] in 0.2 ml aqueous solution by retroorbital injection. Control mice received normal saline. Mice were rested 10–14 d before in vivo immunization.

Neonatal Tolerance (N-TOL) Induction. Newborn B10.A mice (24–48 h old) were administered a single dose of 0.1 mg of HEL in 0.05 ml of a saline emulsion with incomplete Freund's adjuvant (IFA) (Gibco Laboratories, Grand Island, NY), intraperitoneally. Initially, control mice received equivalent volumes of saline-IFA emulsion. No difference was observed between control mice and untreated mice in response to HEL or its derivative peptide fragments in the T-dependent, lymph node cell proliferation assay. Subsequent experiments used untreated age- and sex-matched control mice. All neonatally tolerized mice were immunized at 8–16 wk of age; however, such mice remained tolerant to HEL challenge at >26 wk of age (data not shown).

In Vivo Immunizations. Mice were injected subcutaneously in each rear footpad with 50 μg of lysozyme (3.5 nmol), or the molar equivalent for peptide fragments (used as antigens for in vivo immunization), in saline emulsion with complete Freund's adjuvant (CFA), containing 1 mg/ml *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories, Inc., Detroit, MI). Immunizations with L2 and AP-HEL were performed with either 3.5 or 0.35 nmol per footpad. Either of these doses will induce vigorous proliferative lymph node cell responses in the B10.A mouse.

Antigen-dependent Lymph Node T Cell Proliferation Assay. The in vitro culture system used in this study was adapted with slight modification (26) from that of Corradin et al. (25). Inguinal and popliteal lymph nodes were removed and single-cell suspensions were made 9–11 d after subcutaneous immunization in each rear footpad. Lymph node cells were washed three times and adjusted to 4×10^6 viable cells/ml in Click's medium, modified by the addition of Hepes (15 mM), gentamycin sulfate (0.010 mg/ml), and the substitution of normal mouse serum (0.5%) for fetal calf serum. 4×10^5 lymph node cells in 0.1 ml Click's medium was added to an equal volume of soluble antigen (for in vitro challenge) in Click's medium to yield a final antigen concentration of 6.8 mM. Control cultures for unstimulated background proliferation received medium alone. These cultures, in flat-bottom, 96-well microtiter plates (3040; Falcon Labware, Oxnard, CA), were incubated at 37°C in a humidified atmosphere of 2% CO_2 in air for 5 d. DNA synthesis was assessed by incorporation of tritiated thymidine ([^{3}H]TdR) (6.7 Ci/mmol; New England Nuclear, Boston, MA); 1 μCi [^{3}H]TdR was added to each culture for the last 20–22 h of culture. Cultures were harvested by a multiple automated sample harvester onto glass fiber filter strips (grade 934AH; Whatman, Inc., Clifton, NJ), and discs for each culture were immersed in 1 ml Aquasol (New England Nuclear) for scintillation counting.

Statistical Treatment of Data. The following experimental design, in which the responses of individual mice were evaluated, was selected to avoid the problem of false positives, which may result if a small number of break-through responses occur in a group of tolerant mice whose cells are pooled for assay. The representation of individual responses as a fraction of the mean of control responses was used to normalize responses, which can be variable in magnitude between experiments. This normalization was necessary to present the combined results of multiple experiments. Each experiment involved three to six individuals in control and experimental groups, and experiments were repeated one or more times.

Each experimental and control animal was individually assayed in the in vitro, antigen-specific, T cell–dependent, lymph node cell proliferation assay. Arithmetic means were calculated for [^{3}H]TdR incorporation of triplicate cultures of each individual. Antigen-specific incorporation was determined by subtracting the mean incorporation of cultures that received medium alone from the mean incorporation of antigen-stimulated cultures. The antigen-specific incorporation for each experimental and control individual was evaluated as a percentage of the mean response of the control group in each experiment. The histograms in Figs. 2–5 represent the arithmetic mean value of the experimental group expressed as a percentage of the mean of the untreated control responses. The scattered points within the histograms represent the spread of individual responses,

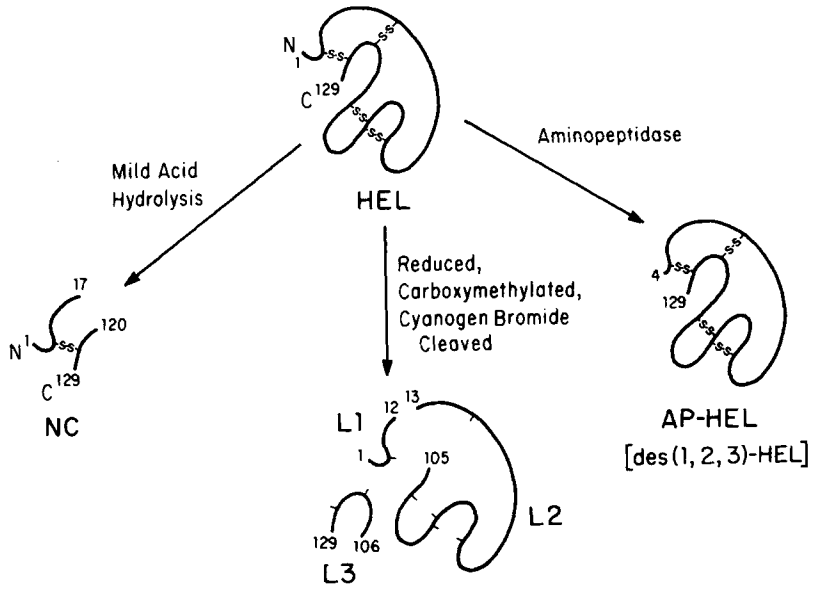

FIGURE 1. HEL peptide probes used in this study. See text for details.

expressed as a percentage of the mean control response, and are representative of the variation between individual mice in this assay system.

Results

Peptide Probes of HEL Used in this Study. Previous studies in this laboratory with the B10 mouse, a genetic nonresponder to HEL, demonstrated that Ts cell–inducing determinants (SD) in one region of HEL prevent the response of Th cells directed against other determinants within the molecule. A mild acid hydrolysis fragment of HEL, the NC peptide (amino acid residues 1–17:cys 6-cys 127:120–129), induces HEL-specific Ts cells that can suppress the entire subsequent response to HEL (22). Despite this lack of response, peptide fragments derived from HEL (Fig. 1) could be used to demonstrate "latent" T cell responsiveness to determinants within HEL in B10 mice. We obtained latent T cell proliferative responses, through removal of the dominant SD, to the largest product of cyanogen bromide cleavage, L2 (residues 13–105), and even to the aminopeptidase product, AP-HEL [des-(1,2,3)-HEL]. This localized the B10 HEL-induced Ts activity to the "TIP" determinant, which contains, or is structurally influenced by, the amino-terminal tripeptide (23).

The L2 (26) and AP-HEL (23) derivatives can also be used as immuonogenic probes for HEL responder B10.A mice. The NC peptide is normally immunogenic in B10.A mice (26) by virtue of determinants at residues 13–17 and 120–129.[2] Another cyanogen bromide cleavage fragment, L3 (residues 106–129), primes for a vigorous proliferative response in the B10.A mice that can be stimulated with either L3 or HEL in vitro. However, HEL immunization results

[2] Oki, A., G. Gammon, N. Shastri, A. Miller, and E. E. Sercarz. T cell tolerance studied at the level of antigenic determinants. II. Neonatal tolerance to lysozyme can be induced in genetic responder strain B10.A mice with small fragments of lysozyme which contain an amino-terminal T suppressor cell-inducing determinant. Manuscript in preparation.

in a response that hierarchically favors determinants within L2, with little or no activation of L3-specific clones (26). Thus, L3 should represent a qualitatively distinct probe for latent responses in the HEL-tolerant B10.A.

Experimental Scheme. We used these peptide fragments to examine B10.A T cell responses under three conditions of HEL tolerance: (*a*) low dose (2 mg HEL) intravenous administration into adult mice followed by immunization in 10–14 d [A-TOL(2 mg)], (*b*) neonatal (24–48 h old) intraperitoneal administration of 100 μg HEL emulsified in IFA, followed by immunization in 8–12 wk (N-TOL), (*c*) high dose (20 mg HEL) intravenous injection into adult mice followed by immunization in 10–14 d [A-TOL(20 mg)]. The experimental sequence, consisting of exposure of the putatively tolerant mice to immunogen in vivo followed by restimulation of activated lymphocytes with antigen or antigen peptide fragments in vitro, tests the efficacy of the immunogen for eliciting proliferative responses in tolerized vs. untreated mice. The in vitro restimulation only assesses responses that have been activated during the in vivo immunization phase.

Acute HEL Tolerance in Adult B10.A Mice: Latent T Cell Responses Can Be Revealed by Immunization With Peptide Fragments of HEL. A single intravenous injection of 2 mg of HEL in saline induced a profound T cell unresponsiveness to HEL in adult B10.A genetic responder strain mice, as assessed by a T cell–dependent lymph node proliferation assay (Fig. 2). Such A-TOL(2 mg) mice also

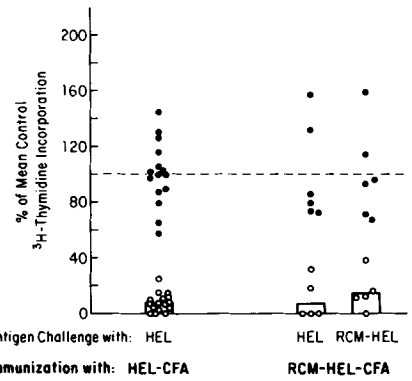

FIGURE 2. T cell tolerance to HEL induced in adult mice. B10.A mice were tolerized with 2 mg HEL in saline [A-TOL(2 mg)], or sham-tolerized with saline alone at 8–24 wk of age. These mice were immunized with HEL or reduced, carboxymethylated HEL (RCM-HEL). Inguinal and popliteal lymph nodes were removed 9–11 d after immunization and cultured as described in Materials and Methods (see text for details). Triplicate 0.2-ml suspension cultures were prepared with either medium alone (for unstimulated background [^{3}H]TdR incorporation), or the antigens HEL or RCM-HEL at 7 μM concentration for 5 d (in vitro challenge). Incorporation of [^{3}H]TdR was assayed during the last 18 h of culture. The arithmetic mean [^{3}H]TdR incorporation was calculated for triplicate cultures, and the mean [^{3}H]TdR incorporation of unstimulated cultures was subtracted to obtain the antigen-specific response for each treated and sham-tolerized mouse. The arithmetic mean antigen-specific response was calculated for the sham-tolerized control group, and is represented by the dashed horizontal line as the 100 value. The arithmetic mean antigen-specific response was calculated for the A-TOL(2 mg) group. This value was determined as a percentage of the mean antigen-specific response of the sham-tolerized control group, and is represented as a vertical bar. The antigen-specific response of each untreated control individual (●) was evaluated as a percentage of the mean response of the untreated control group. Similarly, the antigen-specific response of each A-TOL(2 mg) individual was evaluated as a percentage of the mean response of the untreated control group (○). The rationale for this representation is discussed in Materials and Methods.

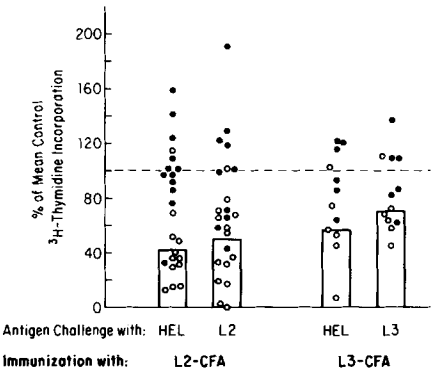

FIGURE 3. Latent responsiveness to HEL determinants can be revealed in acute, A-TOL(2 mg) mice by immunization with L2 and L3 peptide fragments. A-TOL(2 mg) B10.A mice were immunized with 3.5 nmol (equivalent to 50 μg of HEL) of L3 emulsified in CFA, or either 3.5 or 0.35 nmol of L2 (see Materials and Methods), and assayed as described in the legend for Fig. 2. The antigen-specific responses of sham-tolerized control individuals (●) and A-TOL(2 mg) individuals (○) are represented as a percent of the mean control response (---). A vertical bar represents the mean antigen-specific control responses of the A-TOL(2 mg) group, as a percentage of the mean control response.

were unresponsive to reduced and carboxymethylated HEL, demonstrating that the lack of response was evident for both native and denatured forms of HEL. To determine whether underlying T cell responsiveness to epitopes within HEL remains in such acutely tolerized B10.A mice, A-TOL(2 mg) mice were challenged with L2 or L3 (Fig. 3). These peptides can induce a vigorous lymph node proliferation in untreated B10.A mice (26) that is completely crossreactive with the intact HEL molecule. The L2 and L3 fragments appear to span all linear proliferation-inducing determinants of HEL, since we were unable to induce T cell proliferation with L1 (residues 1–12) in the B10.A strain (unpublished data). Fig. 3 illustrates that latent HEL responsiveness can be revealed in HEL A-TOL(2 mg) mice by immunization with either L2 or L3 peptides. The responses to L2 and L3 were directed at epitopes that are shared with HEL and are not unique to these peptides, as evidenced by proliferative responses to HEL in vitro after peptide immunizations. Although these latent responses were clearly significant when compared with the lack of responses to HEL and reduced, carboxymethylated HEL (RCM-HEL) in Fig. 3, it is noteworthy that the mean responses of acutely HEL-tolerized B10.A mice to L2 and L3 represented only 42 and 57%, respectively, of untreated mean control responses with HEL in vitro, and 50 and 71%, respectively, with the peptide fragments in vitro (see Results below, and Discussion).

Therefore, in summary, the A-TOL(2 mg) treatment induces an active regulatory mechanism, presumably mediated by Ts cells, which does not permanently inactivate all T proliferative cells directed against other portions of the HEL molecule. Accordingly, immunization of these HEL-tolerant mice can evoke reactivity to peptides lacking SD. These results have led to experiments that demonstrated the presence of Ts cells in A-TOL(2 mg) B10.A mice.[3]

[3] Oki, A., G. Gammon, and E. E. Sercarz. T suppressor and suppressor-inducer activity is masked in lysozyme tolerant mice by the presence of an augmenting Lyt-1$^+$,2$^+$ T population. Manuscript in preparation.

Acute HEL Tolerance in Adult B10.A Mice: An amino-terminal Ts cell–inducing determinant that is used by nonresponder strain H-2^b mice is also used by HEL-tolerized responder strain B10.A mice. The strong suggestion from the previous experiment was that a Ts cell, involving an L1 determinant, was responsible for A-TOL(2 mg) HEL unresponsiveness. In the following experiments, two HEL derivatives were used for the attempted immunization of HEL-tolerant animals. These derivatives were chosen to specifically test the possibility that the major SD for the B10.A strain might lie within the same region as had been shown for B10 mice. Fig. 4 shows the result of immunization to NC and AP-HEL in A-TOL(2 mg). Despite the presence of immunogenic epitopes on the NC disulfide peptide, immunization of A-TOL(2 mg) mice with this peptide did not result in a proliferative response. This indicated that an SD exists on the NC peptide that does not permit expression of reactivities to any other attached epitopes. Thus, as in the B10 mouse, the reactivity to L2 and L3, but not NC, localizes the dominant SD in HEL-tolerant B10.A mice to that region of NC which is not overlapped by L2 or L3, i.e., residues 1–12 (L1 fragment).

To further investigate the Ts-inducing antigenic requirements in the N-terminal region of HEL, A-TOL(2 mg) B10.A mice were challenged with AP-HEL (residues 4–129). AP-HEL retains secondary structure through four disulfide linkages (see Fig. 1), and possesses the tertiary structure of the intact HEL molecule, as evidenced by its undiminished enzymatic activity and its crossreactivity with almost all of the 50 anti-HEL monoclonal antibodies we have tested. This minimal alteration of HEL was sufficient to preclude the activation of B10 Ts cells (23). Remarkably, in this tolerance context, AP-HEL induced a proliferative response in a large proportion of acutely HEL-tolerant B10.A mice and this response could be recalled by HEL or AP-HEL in vitro (Fig. 4). Thus, the

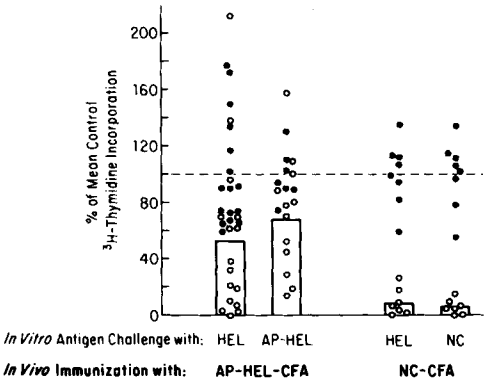

FIGURE 4. Low dose acute tolerance in adult B10.A mice: an amino-terminal Ts cell–inducing determinant is used by A-TOL(2 mg) B10.A mice. Latent responsiveness to HEL determinants could be revealed by immunization with AP-HEL but not with NC. This indicated that the amino-terminal tripeptide, present in NC and absent in AP-HEL, is important for the expression of HEL-induced suppression. A-TOL(2 mg) B10.A mice were immunized with either 3.4 or 0.34 nmol of AP-HEL (see Materials and Methods), or with 3.4 nmol of NC, and assayed as described in the legend for Fig. 2. The antigen-specific responses of sham-tolerized control individuals (●) and A-TOL(2 mg) individuals (○) are represented as a percent of the mean control response (---). The graphic representation is described in the legends to Figs. 2 and 3.

TIP determinant is the major Ts-inducing HEL element in the A-TOL(2 mg) B10.A mouse as well as in the B10 nonresponder strain.

Neonatal HEL Tolerance: No Latent T Cell Response Could Be Revealed by Immunization with HEL Fragments. A single intraperitoneal injection, at 24–48 h of age, of 0.1 mg HEL emulsified in IFA induced unresponsiveness at the level of T cell proliferation to immunization with intact HEL at 8–24 wk of age (Fig. 5). No proliferative reactivity was revealed in response to either L2 or L3, whether intact HEL or the relevant peptide fragments were used for the in vitro stimulation (Fig. 5). The inability of these mice to respond to the L2 or L3 peptide is consistent with a model of functional clonal deletion of all lysozyme-specific clones in the neonatal environment. If Ts were involved, they would have to be specific for subdominant SD within L2 and L3, which may gain special access to Ts precursors within the neonatal milieu.

High Dose Acute HEL Tolerance: Loss of L2 Reactivity, But Retention of L3 Reactivity. Administration of a single intravenous injection of 20 mg of HEL in saline negated subsequent B10.A T cell proliferative responses to HEL (Fig. 6). Interestingly, as in the neonatal tolerance situation, and in contrast to A-TOL(2 mg)-induced tolerance, no latent T cell responses could be revealed when high dose–tolerized mice were challenged with L2. This suggests that clonal deletion or anergy was induced in the L2-reactive T cell population that is exposed to high doses of HEL. However, unlike the results in neonatal tolerance, significant L3-induced responses remained after high dose tolerance. This may reflect the inefficient presentation of L3 determinants for clonal deletion with HEL as toleragen, just as L3 epitopes are ineffectively presented as immunogens with HEL in the B10.A (26; see Discussion).

Alternatively, the differential responsiveness to L2 in A-TOL(2 mg) and A-TOL(20 mg) mice may reflect a hierarchy in the efficiency of Ts cell–inducing determinants in HEL, with a dominant SD at the amino terminus and one or more subdominant SD within L2. The TIP SD could effectively induce the dominant Ts in A-TOL(2 mg) mice. Subdominant SD within L2, which do not efficiently induce Ts in the A-TOL(2 mg) condition, may be engaged at higher

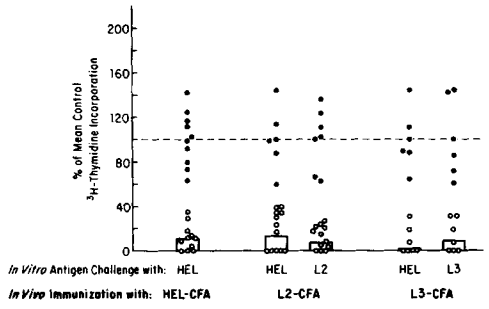

FIGURE 5. Neonatal HEL tolerance: no latent T cell responses can be revealed by immunization with HEL fragments. Neonatal B10.A mice (24–48 h old) were injected intraperitoneally with 100 μg of HEL, emulsified in IFA. Control mice received saline emulsified in IFA, or were untreated: no difference was detected between these protocols. Mice were immunized at 12–24 wk of age with HEL, L2, or L3, and assayed as described in the legend to Fig. 2. The antigen-specific responses of sham-tolerized control individuals (●) and N-TOL individuals (○) are represented as a percent of the mean control response (---). The graphic representation is described in the legends to Figs. 2 and 3.

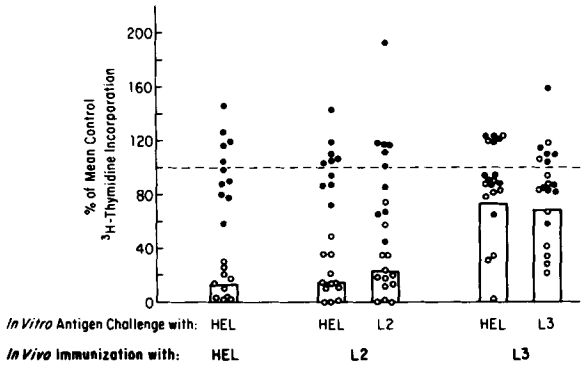

FIGURE 6. High dose acute HEL tolerance: loss of L2 reactivity, but retention of L3 reactivity. Adult B10.A mice were injected retroorbitally with 20 mg HEL in saline [A-TOL(20 mg)] or saline alone (controls). Mice were immunized 10–14 d later with HEL, L2, or L3, and assayed as described in legend to Fig. 2. The antigen-specific responses of sham-tolerized control individuals (●) and A-TOL(20 mg) individuals (○) are represented as a percent of the mean control response (– – –). The graphic representation is described in the legends to Figs. 2 and 3.

toleragen concentrations, thus accounting for the loss of responsiveness to L2 in A-TOL(20 mg) mice. Furthermore, a suboptimal activation of L2-specific Ts in A-TOL(2 mg) mice may account for the partial suppression of L2-induced responses seen in Fig. 3. In this case, the resistance of L3-specific responses to A-TOL(2 mg) and A-TOL(20 mg) treatment suggests the absence of an SD within this fragment, or it may reflect a very inefficient L3 SD that gains optimal expression only in the neonatal milieu.

Discussion

The rationale for our using peptide fragments of protein toleragens as probes for latent T cell responsiveness in tolerant mice derives from the finding that antigenic determinants that are recognized by Ts and Th/p are distinct and nonoverlapping in several protein antigen systems (20). The amputation of a Ts-inducing determinant (SD) away from Th/p cell–inducing determinants releases the Th/p clones from Ts regulation, thus revealing latent responses to those derivatives lacking the SD. In the B10 nonresponder mouse, the prototype amputation experiment had been performed earlier (21, 23), giving rise to the notion that a *single* Ts cell–inducing determinant existed at the amino terminus of HEL for this haplotype. Study of HEL-induced tolerance in the B10.A mouse promised to be especially interesting, owing to a number of distinct T cell proliferation–inducing determinants within the molecule (26), coupled with the availability of several immunogenic peptide probes (L2, L3, NC, and AP-HEL), which allowed the differential analysis of the effects of three toleragenic regimens on these responses. Another advantage of this experimental design is the ability to study the induction of tolerance in the in vivo setting, at the determinant level.

This approach has disclosed three distinct states of unresponsiveness in responder B10.A mice that have been tolerized to HEL. Superficially, these tolerant states are identically manifested by Tp cell unresponsiveness to the intact HEL molecule. However, each condition can be distinguished by differential

latent responsiveness to two fragments of HEL: (*a*) low dose, acutely tolerized mice can respond to L2 and L3, (*b*) neonatally tolerized mice fail to respond to either L2 or L3, (*c*) high dose, acutely tolerized mice can respond to L3 but have lost the potential to respond to L2. These findings suggest that HEL unresponsiveness results through distinct mechanisms in each tolerant state. As several investigators have recently noted (3–6), multiple pathways of tolerance induction must exist to insure the integrity of the organism, considering the heterogeneity, both qualitatively and quantitatively, of self molecules.

Low Dose Acute Tolerance: Suppression Induced by a Dominant Determinant at the Amino Terminus of Lysozyme. The analysis of acute low dose tolerance illustrates the efficacy of the SD amputation approach. The A-TOL(2 mg) B10.A mouse did not respond to HEL or NC. The existence of latent responsiveness to L2 and L3 establishes that an active suppression mechanism occurs in the B10.A strain to lysozyme: previously, Ts had not been examined in this strain. The latent responses also rule out clonal deletion mechanisms in the nonresponsiveness to HEL in A-TOL(2 mg) mice, except possibly to explain the lack of maximal L2, L3, and AP-HEL responses. The fact that A-TOL(2 mg) mice responded to AP-HEL suggests that a single dominant SD exists at the amino terminus of HEL in the B10.A as well as in the B10 nonresponder. This is the first report of apparently identical SDs in nonresponder and responder strain H-2-congenic mice. This surprising finding implies a common mechanism for restricted SD specificity, and one which possibly involves a non-H-2 genetic component. Strain survey experiments are currently under way to test the interesting possibility that the TIP epitope may play an important role in suppressor induction in a wide range of haplotypes, as a possible "universal" and dominant SD in the species which may be linked to naturally occurring immune tolerance to a self antigen, such as murine lysozyme or lactalbumin.

The normal B10.A proliferative response to HEL is largely directed at determinants within L2, one of which crossreacts with NC (26) because of the overlap of residues 13–17. Other dominant HEL T proliferation–inducing determinants may exist within L2, but remain to be identified in the B10.A. The inability of A-TOL(2 mg) mice to respond to the residues 13–17 and 120–129 determinants after NC immunization, or to all L2 determinants after HEL immunization, presumably reflects antigen-bridging mechanisms operative between the Ts-directed towards TIP and the Tp cells directed towards the immunogenic determinants.

The presence of Ts in A-TOL(2 mg) B10.A lymph nodes has been demonstrated by classical in vitro mixing experiments, but these experiments require manipulations to remove augmenting T cells that coexist in these populations and mask the Ts activity in vitro.[3] Study of the cell types that exist in the A-TOL(2 mg) B10.A mouse indicate the presence of both Lyt-2^+ Ts effector cells and Lyt-1^+ Ts inducer cells as well as Lyt-1^+,2^+ T augmenting cells. Therefore, the presence of active Ts that can ablate normal T cell help for an antibody response can be directly demonstrated in the B10.A mouse. This work strengthens and broadens the scope of the tolerance experiments presented in this paper at the level of the Tp cell.

Neonatal Tolerance: Evidence for Clonal Deletion. When tolerance is acutely induced in an adult animal, the toleragenic impulse acts upon a mature immune

system with existing capacity for complex effector and regulatory functions in response to antigenic challenge. T cell unresponsiveness may result from amplification of the Ts circuitry, which is a regulatory component of the normal response to antigen (10, 11, 27). Alternatively, it may result from inactivation of mature effector precursor or inducer cells by a tolerogenic signal, either in the form of nominal antigen or, more likely, antigen in the context of MHC gene products. In contrast, neonatally induced tolerance initially acts upon a developmentally immature immune system that is deficient in peripheral Ia expression and poor in accessory function (28–30). However, the toleragenic stimuli must continue to impinge upon the maturing system as effector precursors emerge from the stem cell pool.

We investigated the HEL peptide-induced proliferative response potential of neonatally tolerized mice in an attempt to compare the neonatally induced unresponsive state with both acutely induced tolerance and the prototype case of the genetic nonresponder. Interestingly, our initial attempts to induce neonatal tolerance with soluble antigen at high doses failed, but a single 24–48 h postnatal, intraperitoneal injection of 0.1 mg of HEL in emulsion with IFA effectively induced T cell proliferative unresponsiveness, which remained evident for at least 180 d (unpublished data). This contrasts with the relatively short-lived unresponsiveness of acute adult tolerance, induced by intravenous injection of HEL in solution, which begins to break at ~35 d. It also demonstrates the toleragenic efficacy of small quantities of antigen when released slowly from an oil emulsion reservoir during postnatal maturation. Continued low level presence of antigen may be required for chronic stimulation of Ts cells or may be required to functionally delete emerging effector precursor populations as they differentiate from stem cells and express receptors for recognition of antigen plus Ia.

Further differences between neonatal and acute adult tolerance are evident when responses to the HEL peptides are compared. Unlike either low dose or high dose acute tolerance, no responsiveness was observed in neonatally HEL-tolerized mice challenged with L2 or L3 peptides. Two facts, that only a single Ts determinant is evident in the acute low dose–tolerant mouse, and that L3 responsiveness is resistant to even a 20 mg acute toleragenic challenge with HEL (Fig. 6), indicate that neonatally induced unresponsiveness to HEL determinants, induced and presumably maintained by slow release of HEL, is mechanistically distinct from the acute tolerance states of adults. The loss of responses to all HEL determinants is consistent with a model of functional deletion of specific T cell clones in neonatal tolerance and contrasts with the Ts-mediated unresponsiveness of acute low dose tolerance in the adult, or the situation in the genetic nonresponder B10 mouse.

High Dose Acute Tolerance: A Hierarchy of Tolerance Induction. Analysis of latent responsiveness in the high dose (20 mg HEL) acute tolerance model led to a surprising "split tolerance" result. Tolerance to L3 was more difficult to induce than to L2 determinants because, even at this higher dose, L3 was able to elicit a response in the HEL-tolerant mouse. This becomes more understandable in the context of the dominance of L2 determinants over L3 determinants when HEL is presented as immunogen (26); L3 essentially is silent (0–20% of the maximal proliferation with HEL or L2). In contrast, L3 immunization induces a vigorous proliferative response that crossreacts well with HEL in vitro.

These results suggest that processing of intact HEL by antigen-presenting cells favors presentation of L2, thus skewing the response towards L2-specific reactivity at a critical inductive phase of the response. After L3 immunization, the lower level of L3 presentation with HEL in vitro may still be sufficient to drive the in vitro proliferative response. Thus, if it is assumed that antigen recognition by T cells does not fundamentally differ, whether the recognition leads to T cell activation or deletion, the favored susceptibility of L2-specific clones to clonal deletion over L3-specific clones would follow from favored presentation of L2 over L3 determinants at higher doses of HEL-induced acute tolerance. This assumption is supported by the recent demonstration that T cell tolerance is MHC restricted (31, 32) and the fact that acute functional clonal deletion in vitro is inhibitable by anti-MHC class II antibody (33). A prediction of the clonal deletion mechanism is that L3-reactive clones would be tolerized under conditions in which L3 could be presented efficiently to delete developing precursor T cells. Possibly, the L3-induced responses would be negated if even higher doses of HEL, or high doses of L3, were used for acute tolerance induction.

Potential Subsidiary Ts-inducing Determinants in L2 and L3. Up until now, we have assumed that the only SD on HEL occurs at the TIP epitope in the B10.A mouse, as in the nonresponder B10. However, it is possible that other SDs may exist within the L2 and L3 fragments. The differential responsiveness to HEL, L2, and L3 in the three tolerant states examined may result from differences in efficiency of Ts induction by distinct SDs within HEL. The TIP SD is evidently dominant to putative SDs within L2, which, in turn, may be dominant to SDs within L3, similar to the "hierarchical" dominance of L2 determinants over L3 determinants for induction of proliferative responses. A possible mechanism may be a differential processing of distinct regions in lysozyme, or differential association of these determinants with antigen presentation structures that are important in T cell recognition events.

Thus, the TIP SD is the only SD that optimally induces Ts in A-TOL(2 mg) mice. The putative, subdominant SDs within L2 may only be engaged at higher toleragen concentrations, and might account for the loss of L2 responsiveness in A-TOL(20 mg) mice. Furthermore, a low level of activation of L2-specific Ts in A-TOL(2 mg) mice may account for the submaximal L2-induced responses depicted in Fig. 3. Similar arguments can be made for the submaximal responses induced by L3 shown in Figs. 3 and 6. However, the resistance of L3 responses to A-TOL(2 mg) and A-TOL(20 mg) treatments suggests the absence of an SD within this fragment. Nevertheless, an alternative explanation to clonal deletion for the loss of L3 responses in N-TOL mice is that a subdominant SD within L3 may gain expression in the neonatal milieu.

"Minimal Peptide" Approach Towards Clarifying the Suppression/Deletion Controversy. Previous experience in the lysozyme and β-galactosidase (GZ) systems indicates that determinants which address suppressor cells and helper cells are nonoverlapping (21–23, 34). This implies a sequence-dependent chemical or structural basis for the distinction of suppressor and helper determinants. A minimal Th/Tp-inducing determinant can be defined that requires the inclusion of both an agretope (35) (a site for association with an MHC molecule) and an epitope (a site for interaction with the T cell receptor). It is unlikely that within a stretch of about 10 amino acids, regarded as a minimal size for a complete

determinant as defined above, both a Th- and a Ts-inducing determinant would be included. Accordingly, the immune status of responsiveness to minimal determinants such as that centralized at residues 13–17, or within T11 (residues 74–96) could be used to distinguish whether suppression or clonal deletion, or conceivably a combination of these mechanisms, is responsible for loss of responses to L2 in neonatal or high dose, HEL-tolerized, B10.A mice. Studies with such minimal determinants are in progress in our laboratory.

Summary

Whether T cell tolerance represents direct inactivation of antigen-specific T cells via recognition of antigen plus major histocompatibility complex, or via T suppressor (Ts) cells, or a combination of these mechanisms, remains to be clarified. This problem was investigated using a novel approach based on the finding in several systems that T helper/proliferative (Th/Tp) cell–inducing antigenic determinants are dissociable from Ts cell–inducing determinants. Thus, peptide probes containing known sites that stimulate T proliferative activity, as well as peptides from distinct sites assumed to bear Ts-inducing determinants, were used in studying hen (chicken) eggwhite lysozyme (HEL)-tolerant mice. The clear prediction from clonal deletion models is that Th/Tp response potential to short peptides in the tolerant mouse would not exist, while regulatory suppression models predict the coexistence of antigen-reactive cells and antigen-specific regulatory cells that prevent their expression.

Adult mice, treated with 2 mg HEL in saline, were tolerant to HEL in complete Freund's adjuvant (CFA). Latent T cell proliferative responses could be revealed to determinants within two HEL peptide probes, which lacked the amino-terminal region of the molecule. This responsiveness suggested two conclusions: first, Ts cells directed against the amino terminus of lysozyme exist in the tolerant genetic responder B10.A; second, these Ts regulate the activity of functional antigen-reactive T cells directed against epitopes elsewhere on the molecule, but only in the presence of the complete molecule, HEL.

Examination of neonatally induced tolerance did not reveal any latent responsiveness, supporting the hypothesis that clonal deletion or anergy is the relevant mechanism in this situation. Possible reservations in these explanations of the two tolerant states, plus analysis of the more complex "split tolerance" resulting from 20 mg HEL in saline treatment in adults, are discussed. The approach of dissociation of proliferation-inducing determinants from suppression-inducing determinants clarifies our understanding of the tolerant state and holds promise for more definitive exploration of mechanisms of T cell tolerance.

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