

T Cell Receptor Complementarity Determining Region 3 Length Analysis Reveals the Absence of a Characteristic Public T Cell Repertoire in Neonatal Tolerance: The Response in the “Tolerant” Mouse within the Residual Repertoire Is Quantitatively Similar but Qualitatively Different

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Abstract

All adult BALB/c mice immunized with hen egg white lysozyme (HEL) or its dominant determinant, peptide (p)106–116, mount a T cell response using a “public” V β 8.2J β 1.5 T cell clone. Neonatal exposure to tolerance-inducing doses of antigen can drastically diminish responsiveness in the draining lymph nodes but not in the spleens of animals challenged as adults with the cognate antigen. To determine the role of T cell deletion or anergy within the mechanisms of observed neonatal “tolerance,” we treated neonatal BALB/c mice with HEL and directly followed the characteristic public clone using complementarity determining region 3 length T cell repertoire analysis. Our results confirm that despite intraperitoneal injection of neonates with a high dose of HEL emulsified in incomplete Freund’s adjuvant, a strong splenic proliferative response to HEL was observed upon recall. However, the adult splenic T cell response of these neonatally treated mice lacked the usual V β 8.2J β 1.5 public clone characteristic of HEL-primed BALB/c mice. After challenge with HEL–complete Freund’s adjuvant as adults, immunoglobulin (Ig)G2a isotype antibody was drastically reduced, and IgG1 was found to be the predominant anti-HEL IgG isotype expressed, indicating a deviation of cytokine response toward T helper type 2. 5-wk-old mice, nasally instilled with tolerogenic doses of HEL p106–116, also showed significant inhibition of this public T cell expansion. These results demonstrate that during neonatal and adult nasal tolerance induction, deletion/anergy removes the public clone, exposing a response of similar specificity but that is characterized by the T helper type 2 phenotype and a splenic residence.

Key words: neonatal tolerance • deletion/anergy • nasal instillation • HEL • T cell repertoire

Introduction

Early discussions of transplantation tolerance and high dose tolerance (1, 2) revolved around the debate as to whether they represented a “central” or a “peripheral” response deficit. Recently, this discussion has centered on assessing roles of deletion or anergy versus cytokine deviation in Ag-induced tolerance (3, 4). Additionally, regulation via Ag- and receptor-centered devices has also been considered as a plausible component of response tolerance (5–10).

Several experiments have supported the hypothesis that tolerance to a minimal determinant results in clonal inactivation via deletion or anergy (11), whereas direct thymic studies demonstrate a loss of T cells by negative selection in the thymus (12). Recently, however, additional studies have suggested that Th1/Th2 deviation may be the sole consequence of neonatal Ag administration (3, 4). In those studies, neonatal BALB/c mice were given an intraperitoneal injection of the model Ag, hen egg white lysozyme (HEL)¹ emulsified in IFA, and then were analyzed as adults

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¹Abbreviations used in this paper: HEL, hen egg white lysozyme; RIS, relative intensity of signal.

for T cell–proliferative and cytokine recall responses to cognate Ag. The results showed that although the draining LN proliferative response was dramatically reduced, the splenic proliferative response to Ag remained significant and Th2 in nature (3, 4). A similar result was obtained in a different H-2 haplotype using a high-level neonatal exposure to a murine leukemia virus (13). These findings would appear to contradict our previous results indicating that neonatal tolerance to a minimal determinant results in clonal inactivation via deletion or anergy (11). However, the current results demonstrate that clonal inactivation of Th1 cells can proceed concomitantly with the appearance of different Th2 cells of the same antigenic specificity. In this study, we have addressed the question of whether the residual responsive splenic T cell repertoire seen in neonatally treated (“tolerized”) mice utilizes a qualitatively different responder T cell population compared with untreated mice. Splenic T cells were specifically targeted because historically, LN cells display a classic “tolerant” phenotype after neonatal treatment (11, 14, 15), whereas splenic T cells remain responsive (3, 4).

In contrast to other studies that focus on the cytokine profile of a single clone in response to in vitro stimulation with altered peptide ligands (16) or differing Ag concentrations (17, 18), we investigated the T cell repertoire after tolerance induction at the clonal level within the polyclonal context of the spleen. This distinction allowed us to directly follow the fate of the cell(s) as opposed to the fate of the response.

Despite the existence of many other potential determinants, BALB/c mice (H-2^d) immunized with whole HEL mount a response directed almost completely to a single determinant of the molecule with a core of peptide (p)108–116 (19). Most importantly, repertoire analysis has identified a public TCR gene rearrangement that is used by all BALB/c mice when mounting an anti-HEL T cell response (20). This T cell expansion, originally described by Cibotti et al. (20), has also held true for all unmanipulated, HEL-primed BALB/c mice tested to date in our laboratory. It is characterized by a TCR gene rearrangement containing a V β 8.2 variable region and a J β 1.5 joining region that together encode for a CDR3 length of eight amino acids, GTGNNQAP (20). The technique of CDR3 length repertoire analysis (immunoscope) allows such public expansions to be followed in the presence of a polyclonal repertoire. Here we show that BALB/c mice tolerized as neonates with HEL in IFA can generate a significant splenic proliferative response to HEL as adults; however, this response excludes the public clone upon subsequent challenge with HEL–CFA. Similar results were obtained when animals were nasally instilled with p106–116 and then challenged with HEL–CFA. Thus, these methods of tolerance induction can induce both clonal deletion (or anergy) of the dominant T cell clone as well as immune deviation of other Ag-specific T cells. These findings demonstrate that previous observations concerning clonal deletion and cytokine deviation of the T cell re-

sponse after neonatal exposure to Ag were describing the fates of different populations.

Materials and Methods

Mice. BALB/cJ mice were purchased from The Jackson Laboratory and bred in our specific pathogen-free colony at the La Jolla Institute for Allergy and Immunology. Neonatal mice used in all experiments were age matched, housed in filter top cages, and fed autoclaved chow. For the nasal instillation experiments, the mice were also sex matched.

Neonatal Tolerance Induction. Three groups of neonatal mice, receiving a total of 0.01, 100, or 500 μ g of HEL, were tolerized as described (11). In brief, the two groups of neonates were given intraperitoneal injections of 50 μ l containing 0.005, 50, or 250 μ g of HEL, respectively. Injections were given within the first 18 h of life and then again at 72 h. HEL was dissolved in PBS and emulsified 1:1 in IFA (Difco Labs., Inc.). A control group of neonates was given intraperitoneal injections of IFA without Ag, also administered at 18 and 72 h.

Adult Nasal Tolerance Induction. Nasal instillation treatment was initiated in female mice at 5 wk of age. Mice were separated into two groups, one receiving 10 and the other 100 μ g of HEL p106–116 dissolved in 20 μ l of PBS and delivered to the tip of the nose after light anesthesia (halothane). 7 d later, animals were given a second identical instillation. A control group was given two 20- μ l instillations of PBS, also 1 wk apart.

Adult Antigenic Challenge. In neonatal tolerance experiments, mice were immunized at 6 wk of age in the hind foot pads (\sim 25 μ l per foot pad) with HEL emulsified 1:1 in CFA, such that each mouse received a total of 100 μ g of HEL–CFA. In the nasal instillation experiments, mice were immunized with 100 μ g of HEL emulsified 1:1 in CFA 10 d after the final nasal instillation of p106–116. 14 d after the HEL–CFA immunization, tissues were harvested.

Tissue Culture. Individual spleens and LNs were aseptically removed, and single-cell suspensions were prepared in petri dishes containing DME (GIBCO BRL Life Technologies, Inc.). Large debris was removed by decanting, followed by two washes in DME. Splenocytes and LN cells were adjusted to 6×10^6 and 4×10^6 cells/ml, respectively, for subsequent culture. The medium employed in all tissue culture was serum-free HL-1 (Bio-Whittaker) supplemented with 5×10^{-5} M 2-ME (Sigma Chemical Co.), 4 mM of l-glutamine, 100 U/ml of benzyl penicillin, and 100 μ g/ml of streptomycin sulfate (all three from GIBCO BRL). All cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂.

Protein and Peptide Ags. HEL was purchased from Sigma Chemical Co. and then further purified on a Bio-Rex 70 column (Bio-Rad Labs.). HEL p106–116 (NAWVAWRNRCK) was purchased from Macromolecular Resources and found to be 95% pure by mass spectrometry.

T Cell Proliferation Assay. Splenocytes and LN cells were cultured in 96-well plates at 6×10^5 and 4×10^5 cells/well, respectively, in the presence or absence of Ag. Proliferation was measured by incorporation of 1 μ Ci of [³H]thymidine (ICN, Inc.) for the last 18 h of a 4-d culture. The cells were harvested onto glass fiber filter mats (LKB-Wallac Ltd.) using a Mach III Harvester 96 (Tomtec), and [³H]thymidine incorporation was measured using a scintillation counter (Microbeta +; LKB-Wallac).

ELISPOT Cellular Assays for Cytokine Production. After 36 h of culture in the presence or absence of Ag, splenocytes and LN

cells were washed once and adjusted to 5×10^6 cells/ml in fresh, supplemented HL-1 serum-free medium. IFN- γ , IL-4, and IL-5 production was then determined using standard ELISPOT assays, as described in detail elsewhere (21). In brief, cells were incubated on nitrocellulose plates previously coated with anticytokine mAb and blocked with 1% FBS/PBS for 24 h at multiple dilutions. After washing and lysing of cells in chilled PBS/0.5% Tween 20, bound cytokines were detected by adding biotinylated anticytokine mAb directed to a nonoverlapping epitope on the cytokine. After incubation of plates with streptavidin peroxidase (Vector Labs.), plate-bound enzyme was visualized using 3-amino-9-ethyl carbazole (Sigma Chemical Co.) as a substrate. Plates were washed in distilled water and dried before subsequent visual spot enumeration under a dissecting microscope.

Assays for Serum Abs. ELISA with anti-mouse Ig was used to determine serum levels of Abs specific for HEL. Affinity-purified rabbit Ab specific for IgG1 or IgG2a H chains were obtained from Zymed Labs. In brief, Maxisorp Immunoplates were coated for 1 h at 37°C with 50 μ l per well of HEL diluted to 10 μ g/ml in carbonate/bicarbonate buffer, pH 9.6. After washing twice in PBS/0.05% Tween 20, plates were blocked at 37°C for 1 h with 200 μ l per well of a PBS/1% BSA solution. After two washes, sera to be tested were then added at 50 μ l per well in dilutions ranging from 1/10 to 1/500 and incubated overnight at 4°C. Pooled sera from HEL-immunized mice were used as a positive standard. After three further washes in PBS/Tween 20, antiisotype conjugate was added at an optimal (1/500) dilution in PBS/BSA and incubated at 37°C for 1 h. After eight additional washes, the amount of enzyme bound to the wells was assessed using *p*-nitrophenyl phosphate as the substrate for 20–30 min at 37°C. The plates were then assayed at OD 405 nm on a multiscan plate reader.

T Cell Receptor Repertoire Analysis. Repertoire analyses were performed using a modified protocol similar that described by Pannetier et al. (22). Total RNA was isolated from cell suspensions of individual samples using Trizol reagent according to product instructions (GIBCO BRL Life Technologies). cDNA syntheses were then performed using an oligo-dT primer (dT)₁₅ according to the manufacturer's instructions (GIBCO BRL Life Technologies). From each cDNA, PCR reactions were then performed using a V β 8.2 primer (CATTATTCATATGGTGCTGGC) and a common C β primer (CACTGATGTTCTGTGTGACA). Using 2 μ l of this product as a template, run-off reactions were performed with a single internal fluorescent primer for each J β tested, a 1-min 94°C denature and then five cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s, followed by a 1-min extension at 72°C. These products were then denatured in formamide and analyzed on an Applied Biosystems 310 Prism using GeneScan 2.0 software (Perkin-Elmer Corp.). Labeled products were analyzed separately or duplexed as four-color electrofluorographs. These data determine peak areas and confirm appropriate product specificity based upon size and color (primer specificity). The relative intensity of signal (RIS) value was calculated as the area under the experimental peak divided by the area under the control peak found within a Gaussian distribution. Peaks were normalized before division. Control peaks obtained from either IFA/CFA-immunized animals or naive animals gave equivalent RIS values. RIS values >4 are considered significant (22).

Results and Discussion

Neonatal Administration of HEL Inhibits the Clonal Expansion of the Public Repertoire. As all HEL-primed BALB/c

mice utilize an identical "public" clone that could be readily followed, we measured its expansion in tolerized and intolerized animals to determine whether the mechanism of neonatal tolerance involves T cell deletion or anergy. BALB/c mice were treated neonatally with HEL in IFA (as described in Materials and Methods). Upon reaching adulthood, spleens from neonatally treated animals that had not been challenged in vivo were examined for T cell response after in vitro stimulation with HEL. Fig. 1 shows that adult BALB/c mice treated neonatally with HEL continue to mount a strong in vitro splenic proliferative response to whole HEL as well as to its dominant determinant, p106–116. Clearly, not all HEL-specific T cells are deleted in this form of tolerance induction.

Because it was possible that these responses represented an expansion of the public clone or a variety of other clones of similar specificity, immunoscope analysis was conducted on these samples. Fig. 2 A presents RIS values (an expression of the relative expansion of the public clone; see Materials and Methods) for the public clonotype. Notably, the data in the first column (from left) of Fig. 2 A illustrate that although the T cell response shown in Fig. 1 was robust, it failed to show the characteristic V β 8.2J β 1.5 public T cell expansion seen in the control HEL-immunized animals (fifth column). Fig. 2 B illustrates representative "immunoscope profiles" showing the different CDR3 chain lengths within the V β 8.2J β 1.5 repertoire. A Gaus-

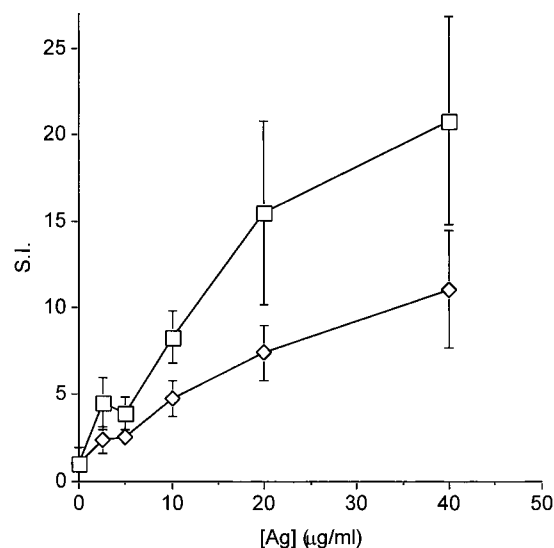


Figure 1. Strong splenic proliferative responses persist after neonatal administration of HEL. BALB/c mice received intraperitoneal injections of 50 μ l containing 50 μ g of HEL emulsified in IFA within the first 18 h of life and again at 72 h. Spleens were harvested at 8 wk of age, and the splenic recall responses to HEL (□) or its dominant determinant, p106–116 (◇), were measured using a [3 H]thymidine incorporation assay. Results are expressed as stimulation indices (S.I.; CPM in sample/CPM in control). In vitro recall responses were significantly elevated (S.I. > 3) for both HEL and p106–116. These data represent the average of three groups of two to three mice.

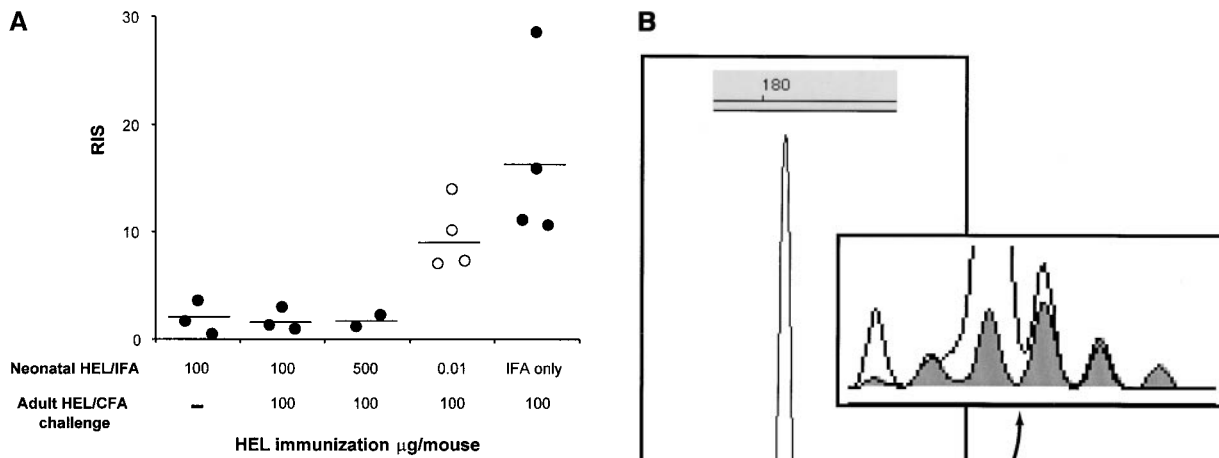


Figure 2. V β 8.2J β 1.5 CDR3 length analysis of spleen cells reveals that neonatal administration of HEL inhibits the clonal expansion of the public repertoire. Groups of BALB/c mice were treated neonatally with IFA-HEL (0.01, 100, or 500 μg) as described in Materials and Methods. As shown in A, groups were then challenged at 6 wk of age in the hind foot pads ($\sim 25 \mu\text{l}$ per foot pad) with 100 μg of HEL emulsified 1:1 in CFA (Difco Labs.). In A, each circle represents two to three animals whose cells were pooled for analysis (open circles represent individual animals, 0.01 μg group only). An additional group that was treated with 100 μg of HEL-IFA as neonates was left unchallenged as adults. 14 d after challenge, spleens were harvested and cultured in vitro with HEL (10 $\mu\text{g}/\text{ml}$). Immunoscope analysis was conducted after 4 d of culture (see Materials and Methods). Results are displayed as RIS values of histogram peaks from J β 1.5 run-off reactions of V β 8.2 amplicons, corresponding to a CDR3 length of eight amino acids. The RIS value plotted here equals the area of the experimental peak divided by the area of the naive control peak (shown in B). B shows representative histogram comparisons of the J β 1.5 run-off reaction of the V β 8.2 amplicons. Normally, unimmunized animals display peaks in a Gaussian distribution centered on a CDR3 length of nine. Only the low dose and IFA control groups, which were immunized with HEL-CFA at 6 wk of age, showed the highly significant expansion (RIS > 4) of the HEL-specific public clone. B shows representative distributions of V β 8.2J β 1.5 for tolerized (normally distributed) and intolerized animals immunized with HEL-CFA. An inset is provided that shows an expanded view of the Gaussian distribution of the V β 8.2J β 1.5 products from a tolerized individual. Note that when scaled to match, the signal detected from the expanded V β 8.2J β 1.5 products is largely reduced in comparison with the tolerized (normally distributed) electrofluorograph. The peak that corresponds to an amino acid length of eight is indicated by the vertical arrow.

sian distribution is seen in the tolerized mouse (Fig. 2 B, inset) in which no clear expansion is measured. Fig. 2 B shows the characteristic marked expansion of the public clone with its CDR3 length of eight amino acids. Thus, although many responsive clones can be measured that respond to HEL and p106-116 (Fig. 1), there is no visible expansion of the public clone in tolerized mice.

To determine whether the absence of the public expansion to HEL or p106-116 could be attributed to immunization with an inappropriate adjuvant, animals neonatally treated with HEL-IFA were immunized with HEL-CFA as adults. As with neonatal treatment alone, these animals responded with a strong splenic proliferative T cell response; however, this response again lacked a significant expansion of the dominant public clone (Fig. 2, second and third columns). The absence of the expanded public clone in the neonatally pretreated animals is strong evidence supporting deletion or anergy as contributory mechanisms to neonatal tolerance induction. Included in these data is a third group of mice that were neonatally treated with an extremely low dose (0.01 μg) of HEL-IFA. Notably, these mice did not lack the characteristic expansion when subsequently challenged with HEL-CFA.

To further characterize the residual HEL-specific T cells in mice that were both neonatally treated and challenged in

adulthood with HEL, the Ig isotypes and cytokines produced in response to HEL were identified. When compared with intolerized animals, the IgG2a anti-HEL responses (Th1) were significantly reduced in the neonatally treated group. In fact, IgG2a responses were essentially undetectable in five out of seven animals in the tolerized group and were marginal in the remaining two animals. IgG1 responses (Th2), however, were not affected in six out of seven tolerized animals (Fig. 3). In addition, ELISPOT cytokine analysis revealed that IL-5 responses were significantly increased in the neonatally treated animals, whereas IFN- γ responses were lower or remained unchanged (data not shown).

Nasal Instillation Results in a Reduced Expansion of the Dominant Clone. In addition to neonatal tolerance, there is now overwhelming evidence that peptide Ag provided via nasal instillation has a pronounced effect on the animal's immune response to that particular determinant and in some cases to other determinants on the same or different molecules (23). The consequences of nasal administration of Ag are not easily predictable from strain to strain; in many instances, nasal instillation clearly results in a radical Th1 to Th2 shift in the cytokine profile of the Ag-specific T cells (24, 25). In other systems, there is a simple down-regulation of T cell proliferation and cytokine secretion

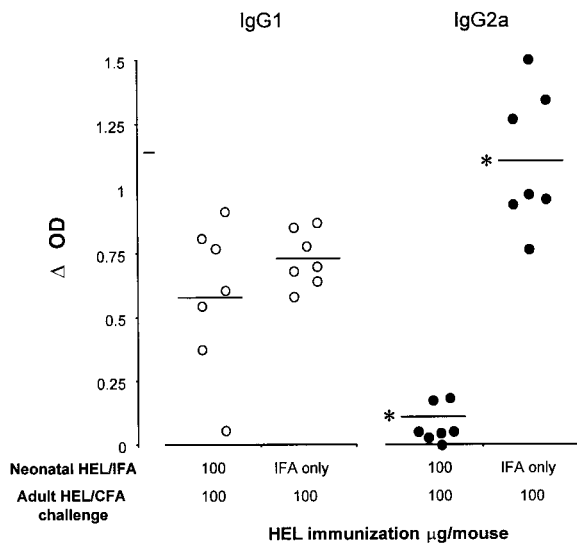


Figure 3. Neonatally tolerized animals mount a predominant IgG1 anti-HEL response when challenged with Ag as adults. BALB/c mice were neonatally tolerized with HEL emulsified in IFA or IFA alone, as described in Fig. 2. As adults, they were challenged with HEL-CFA. Blood samples were collected before organs were harvested, and the sera separated from these samples were used in an ELISA to measure levels of anti-HEL Ab isotypes. Only two of seven mice in the tolerized group mounted an anti-HEL IgG2a (Th1-associated) response upon HEL in vivo challenge, and then only marginally. In contrast, six of seven of the mice showed strong IgG1 anti-HEL responses (associated with Th2 responses). Thus, when compared with untolerized control animals, neonatal tolerance resulted in a significant reduction in IgG2a ($P < 0.005$, Student's *t* test, indicated by *) and no significant change in IgG1 ($P > 0.15$). Each circle represents the serum level of anti-HEL Abs of the indicated isotype from a single animal. Results are expressed as OD. Immunoscope analyses on T lymphocytes from pools of these animals are shown in Fig. 2.

without a noticeable Th1 to Th2 shift (23). It is likely that several factors, such as the peptide's MHC binding affinity as well as the array of TCR affinities for the peptide-bound MHC complex, would influence the effect of nasal instillation on the Ag-specific immune response. Experiments in our laboratory have shown that BALB/c mice nasally instilled with HEL or its dominant determinant, p106-116, mount a predominant Th2 T cell response upon subsequent in vivo challenge with HEL-CFA (25). Therefore, to determine whether the residual HEL-specific T cell repertoire, remaining after neonatal treatment, was unique to such pretreatment or indistinguishable from other tolerizing regimes, we nasally instilled BALB/c mice with HEL p106-116 and performed immunoscope analysis on splenic T cells 14 d after subsequent HEL-CFA challenge. Similar to the findings of a previous report (23), nasal instillation resulted in a significant reduction of LN proliferative responses (Fig. 4 A). Splenic proliferative responses, however, were similar or higher between groups of animals nasally instilled with p106-116 (known to be Th2; reference 25; Fig. 4 B) and those nasally instilled with PBS alone (Th1), indicating that cytokine deviation was not reflected in the proliferative response. Interestingly, the most striking difference seen in the nasally instilled group was revealed by immunoscope analysis. As was the case in the neonatal treatment experiments, BALB/c mice treated by nasal instillation with p106-116 showed a dose-dependent decrease in the expansion of the HEL-specific public clone (Fig. 5). Although not as dramatic as in the neonatally pretreated animals, there was a sixfold reduction seen in the p106-116 group nasally instilled with 200 μg , which was significant at the $P < 0.005$ level (Fig. 5). In mice treated similarly with HEL, a robust IL-5 response to p106-116 appears, whereas responses to subdominant and cryptic determinants were unaffected (25).

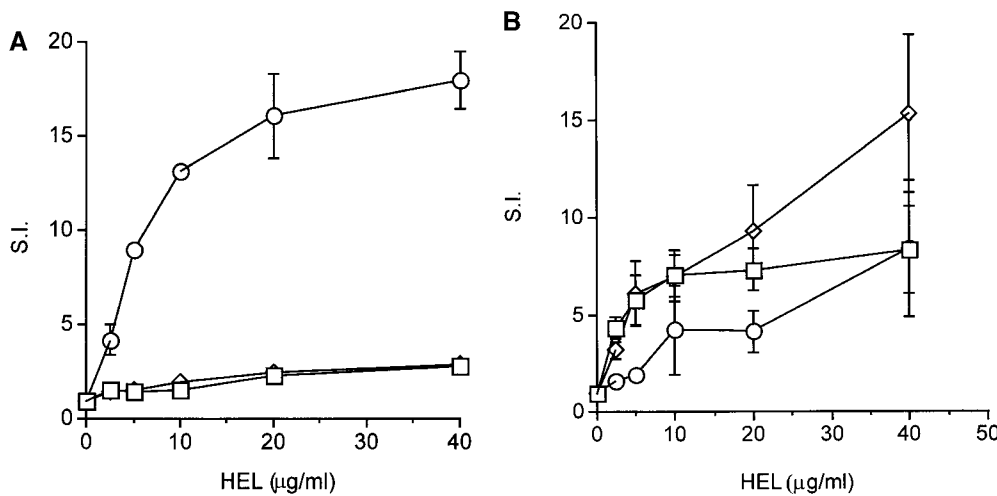


Figure 4. Nasal administration of HEL p106-116 results in a reduced LN but a significant splenic proliferative response. Animals were nasally instilled twice with a total of 0 (\circ), 20 (\diamond), or 200 μg (\square) of HEL p106-116 dissolved in 20 μl of PBS; half of the dose was administered at each delivery, 7 d apart. 10 d after the second instillation, animals were immunized in the hind foot pads with 100 μg of HEL emulsified in CFA. 14 d thereafter, splenic and LN restimulation cultures were established, and responses to HEL were measured in a [^3H]thymidine incorporation assay as described in Fig. 1. Results are presented as stimulation

indices. Pretreatment by nasal instillation resulted in a significant reduction of LN proliferative responses (A). However, as shown in B, splenic proliferative responses were similar or higher in groups of animals nasally instilled with p106-116 compared to those pretreated with PBS alone. Parallel spectratype analyses of splenic lymphocytes from these nasally instilled animals are shown as RIS values in Fig. 5.

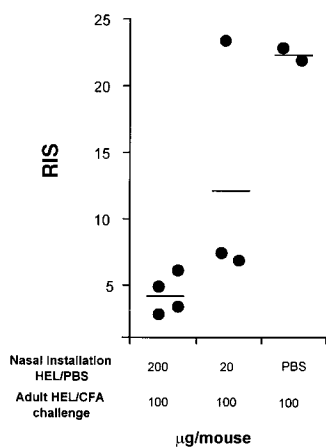


Figure 5. Nasal instillation of p106-116 results in a significant reduction of the V β 8.2J β 1.5 public expansion. BALB/c mice were nasally instilled twice with 0, 10, or 100 μ g of HEL p106-116 as described in Fig. 4. TCR spectratype analysis was conducted after 4 d of in vitro culture with Ag (10 μ g/ml). Results are displayed as RIS values as described in Materials and Methods. Each circle represents two animals whose cells were pooled for analysis. As was the case in the neonatally pretreated animals, animals nasally instilled with p106-116 showed a marked decrease in the expansion of the

HEL-specific public clone. In comparison to the control group, mice nasally instilled with 200 μ g did not exhibit a significantly expanded public repertoire ($P < 0.005$, Student's t test).

The Nature of the T Cell Repertoire Remaining after Neonatal Tolerance. The data presented here indicate that clonal deletion or anergy can occur even though a significant T cell response persists. The seemingly paradoxical result of a quantitatively similar but qualitatively different response suggests that in some situations, among T cells with similar specificity, some can escape tolerance induction, whereas others do not. These data can be explained by considering the diversity of the HEL-specific T cell repertoire. Studies of HEL-specific T cells in the BALB/c mouse have shown that individual mice use from 2 to 20 different clones in response to an HEL immunization (20). Each clone with a unique TCR will exhibit an interaction with its ligand along the continuum of affinities. The individuality of T cell recognition is also evident in that T cells have unique patterns of degeneracy (26, 27) and thereby cross-recognize different ligands (our unpublished results). We may assume that a given ligand can therefore signal for tolerance induction of only a subpopulation of Ag-specific T cells. Indeed, we have observed that the V β 8.2J β 1.5, HEL-specific public clone is more sensitive to tolerance induction than other clones recognizing determinants within the p103-120 determinant envelope. A simple explanation is that these T cells bear T cell receptors with avidities that are sufficiently sensitive to signals delivered by the HEL-pulsed APCs, resulting in clonal silencing through elimination or anergy induction. In addition, in these studies, many clones with avidity similar to the public clone could have been affected by the tolerance-inducing regimen without our knowledge.

Furthermore, there is evidence that IL-2-producing Th1 cells are more susceptible to activation-induced cell death than Th2 cells (28). In our study, there is a clear deficit of at least one major TCR expansion in the tolerized animals that are immunologically challenged. Therefore, we postulate that the Th2 nature of the residual splenic T cells arises from the relatively weak interaction of their TCRs with p106-116-bound MHC class II complexes. This notion is

supported by the finding that low concentrations of Ag tend to promote the development of Th2 cells (17, 18, 29). Therefore, a low-affinity TCR might signal for a similar response. In accord with this concept, it has been shown in the I-A^u system that Th2 responses to the NH₂-terminal region of myelin basic protein result from sparse ligand display, low-affinity TCR, or both. However, T cells with a higher avidity receptor may adopt either a Th1 or Th2 phenotype depending on the density of the ligand (16). In short, in the spectrum of p103-120-specific T cells, there exist some low-avidity T cells that are stimulated by HEL-derived peptides from this region to respond in a Th2 fashion just as there exist altered peptide ligands capable of inducing Th2 responses (30).

Determinant Display and the Induction of Tolerance. It has been shown that tolerance is induced best to well-processed and -presented determinants and that subdominant and cryptic determinants are less efficient at tolerance induction using whole protein Ags (31, 32). Conceivably, the recall response to HEL seen in Fig. 1 might be augmented by responses to previously subdominant and latent cryptic determinants. This could explain why the response to p106-116 is slightly lower than that to HEL (Fig. 1). In addition, it has been postulated that the same peptide can bind in multiple overlapping registers (our unpublished results) or possibly in multiple conformations to the class II MHC molecule (33). Thus, a portion of the residual response measured after tolerance induction might be specific for a particular conformation or register that is disfavored when processed from the intact protein. In either case, the Th2 phenotype can be explained in terms of a low density of determinant display in the appropriate conformation/register, which, as in the case of a low Ag concentration, would favor the development of Th2 cells (17, 18). Perhaps several of these possibilities simultaneously contribute to our observed results.

A third consideration, which is not addressed experimentally at present, is the cytokine milieu surrounding the differentiating T cell. The amount of IL-12 and IL-4 in this milieu has been shown to have profound effects on the development of Th1 and Th2 cells, respectively (34). We cannot dismiss the importance of these effects. In our experiments, however, both tolerized and control animals were immunized with HEL emulsified in CFA. Because of this, the cytokine milieu should have remained largely constant between tolerized and untolerized groups, except for cytokines produced by HEL-specific cells, which could have helped to drive the response one way or the other. In fact, adult immunization with HEL-IFA, presumably a Th2-inducing stimulus (3), does expand the public clone (data not shown). Thus, the probability of a T cell responding to an antigenic stimulus in a Th1 or Th2 fashion depends on multiple factors, one of which is the particular TCR borne by the T cell.

Tolerance Redefined. In earlier experiments when Ab-producing B cell activity was sought after neonatal or adult tolerance to high-level Ag (BSA), none was found (35). Furthermore, early T cell tolerance experiments were in-

terpreted to indicate that central T cell activity had been inhibited. Therefore, in light of the finding that considerable residual responsiveness remains or is revealed in the spleen when the appropriate T cell population is studied directly despite neonatal or nasal "tolerance regimens," the term "tolerance" needs to be carefully defined. Classic experiments by Billingham et al. (2) and Owen et al. (36) showed that fetal or neonatal exposure to transplant Ags resulted in an immune system that would tolerate and not react against these same alloantigens. Although it is possible that all alloreactive cells were simply converted to a nonaggressive phenotype (Th2), it is likely that as shown here with HEL, many of the highly reactive cells could have been deleted or anergized. Accordingly, the residual T cell repertoire directed to any self-Ag would largely remain as a contingent of cells capable of responding to many of its subdominant/cryptic determinants, regardless of the extent of previous tolerogenic exposure. Within the body of T cells directed against the dominant determinant(s), the high-avidity members would become tolerized, whereas the lower avidity group should remain available to respond to sufficient levels of appropriate versions of the dominant determinant. It appears that complete silencing of the whole contingent of immune cells directed against the whole Ag cannot be achieved. Nevertheless, the term "tolerance," implying a qualitative variety of ways of safely dealing with self-Ags, might usefully be retained as an envelope term, providing that the context of its usage does not overstep its limitations.

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