Brief Definitive Report

INDUCTION OF TOLERANCE TO ONE DETERMINANT ON A SYNTHETIC PEPTIDE DOES NOT AFFECT THE RESPONSE TO A SECOND LINKED DETERMINANT

Implications for the Mechanism of Neonatal Tolerance Induction

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Neonatal T lymphocyte tolerance to proteins has been extensively studied, but the underlying mechanisms are still unknown (1–3). One hypothesis is that tolerance is due to the inactivation of T cells on contact with antigen at an early stage of development. Alternatively, tolerance may be the result of the stimulation of other T cells, which actively suppress subsequent responses. We have developed a novel experimental system using peptides to analyze the tolerant state at the level of individual peptide determinants, and to detect functional suppression. This approach makes use of the finding that different T cell subpopulations appear to recognize different determinants (4, 5). Thus, Ts specific for a distinct determinant(s) on a protein molecule suppress the response of T cells specific for other regions of the molecule. Although the mechanism of this antigen-bridging suppression is poorly understood, the ability of Ts to act across an antigen bridge has been confirmed experimentally by the coupling of Ts-inducing determinants (SD) to those recognized by the responding T cells (6, 7). Further, proliferative T cells (Tp) can be stimulated, despite the presence of Ts, by fragments of the whole protein that bear the Tp-inducing determinant but lack the Ts-inducing determinant (8). Peptides lacking a suppressor determinant can be used as probes to reveal latent responses in tolerant mice, i.e., responses not induced by challenge with the whole molecule. Previously (9), we showed that neonatal tolerance can be induced by small synthetic cytochrome c peptides. In that system, the specificity of tolerance matched that of the response to the peptide, providing evidence for direct clonal inactivation. In this report, we show that the induction of tolerance to one determinant on a 23–amino acid peptide does not affect the response to a second determinant on the same peptide, as would be predicted in a suppressor model. Furthermore, the congruence of minimal immunogenic and tolerogenic peptides is demonstrated.

Materials and Methods

Mice. B10.A mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and bred at our facility. Mice of either sex were used at 8–12 wk of age.

Antigens. The synthetic peptides shown in Table I were prepared by solid-phase peptide synthesis, as described (10).

Neonatal Tolerance Induction. Neonatal B10.A mice received a single intraperitoneal injection of 14 nmoles of peptide in 0.05 ml of a saline emulsion with IFA (Gibco

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TABLE I

Synthetic HEL Peptides Used to Study Tolerance

Synthetic peptide	Amino acid residues of HEL																						
	75						80					85					90					95	
T11(74-96)	N	L	C	N	I	P	C	S	A	L	L	S	S	D	I	T	A	S	V	N	C	A	K
85-96												—	—	—	—	—	—	—	—	—	—	—	—
74-86	—	—	—	—	—	—	—	—	—	—	—	—	—										
74-82	—	—	—	—	—	—	—	—	—														
77-86				—	—	—	—	—	—	—	—	—	—										

Laboratories, Grand Island, NY) at 24–48 h after birth. Tolerance was induced to the peptides 74-82 and 77-86 by two intraperitoneal injections of 7 nmoles of peptide in IFA, the first between 24–48 h and the second between 72–96 h after birth. Control mice were normal age- and sex-matched B10.A mice. We had found earlier (our unpublished data) that injection of saline/IFA emulsion into neonatal mice does not affect subsequent proliferative responses to peptides in adult mice.

Immunizations. 8–12-wk-old mice were injected in each rear footpad with 3.5 nmoles of peptide in a saline emulsion with CFA containing 1 mg/ml *Mycobacterium tuberculosis* strain H37Ra (Difco Laboratories, Detroit, MI).

In Vitro Proliferation Assay. The in vitro culture system has been described previously (11). Inguinal and popliteal lymph nodes were removed 9–11 d after immunization, and single-cell suspensions were prepared. 4×10^5 cells were cultured in 96-well microtiter plates in 200 μl of supplemented Click's medium. Tuberculin PPD (Connaught Laboratories, Ltd., Willowdale, Canada) was used as a positive control for each mouse, at a final concentration of 5 μg/ml. The cultures were incubated at 37°C in 2% CO_2 and 98% air for 5 d. Proliferation was assayed by addition of 1 μCi [^{3}H]thymidine (sp act 33 Ci/mmol; International Chemical and Nuclear, Irvine, CA) for the last 18 h of the culture, and incorporation of label was measured by liquid scintillation counting.

Results

B10.A Response to T11. Immunization of adult B10.A mice with the synthetic peptide T11, which corresponds to amino acid residues 74-96 of hen eggwhite lysozyme (HEL), induces a strong in vitro proliferative response. Analysis of T cell clones derived from three T11-specific lines has revealed the presence of distinct determinants contained within the smaller peptides 74-86 and 85-96 (10 and our unpublished results). All the clones obtained responded either to peptide 74-86 or 85-96, as well as to T11. Thus, there are at least two determinants on T11, and both peptides 74-86 and 85-96 recall in vitro proliferation in T11-primed lymph node cells (Table II). Interestingly, T11 is much more efficient in stimulating an in vitro proliferative response than the smaller peptides, (i.e., the sum of the responses to the two smaller peptides does not equal the T11 response). Although this could be due to the presence of T cells specific for a third determinant on T11, the evidence from the analysis of the T cell lines argues against this. Changes in the relative ability of peptides of varying lengths to recall a response in vitro has been observed previously (12), and may reflect the ability of the different peptides to maintain a stable secondary structure under these culture conditions.

Induction of Tolerance with T11 (peptide 74–96). To investigate the underlying mechanism of neonatal tolerance to HEL peptides, neonatal B10.A mice were injected with T11 in an IFA emulsion at 24–48 h after birth. Challenge with

TABLE II

Responses of T11- and 74-86-tolerant Mice

Line	Tolerogen	Immunization	[^{3}H]Thymidine incorporation (Δcpm × 10^{-3})				
			Medium alone	T11(74-96)	74-86	85-96	PPD
a	—	T11	4.9	61.9 ± 6.7	9.6 ± 2.2	17.5 ± 4.1	83.8 ± 3.9
b	T11	T11	6.3	3.9 ± 1.0	1.8 ± 0.6	3.7 ± 0.8	90.6 ± 7.2
c	—	74-86	3.7	13.7 ± 1.6	13.9 ± 1.2	—	46.5 ± 2.6
d	T11	74-86	3.4	2.2 ± 0.5	1.2 ± 0.7	—	51.3 ± 2.6
e	74-86	74-86	3.5	1.8 ± 0.5	1.7 ± 0.6	—	48.7 ± 3.9
f	74-86	T11	7.5	39.5 ± 7.3	1.5 ± 0.7	16.5 ± 4.8	94.9 ± 5.1

Normal, and T11- and 74-86-tolerant mice were challenged with peptide in CFA at 8–10 wk of age. The in vitro proliferative response was measured by [^{3}H]thymidine incorporation, and the values shown are the average of the responses of six or seven individual mice ± SE. Background (medium alone) has been subtracted.

T11 in CFA at 8–10 wk of age showed that these mice were profoundly tolerant (Table II, line *b*). There was no significant response in vitro to T11, 74-86, or 85-96 after in vivo T11 challenge. To test for latent responsiveness to the peptide 74-86, T11-tolerant mice were challenged with 74-86 in CFA. No response was observed (Table II, lines *c* and *d*). The failure of T11-tolerant mice to respond to challenge with 74-86 could only be due to suppression if 74-86 fortuitously contained a Ts-inducing determinant (SD) in addition to a Tp-inducing determinant (PD). This seems unlikely given the small size (13 amino acids) of the peptide. Nevertheless, this possibility can be tested by examining the effect of 74-86-induced tolerance on the response to the neighboring determinant within the sequence 85-96 on T11.

Specificity of Tolerance to 74–86. The possibility that Ts specific for 74-86 exist in mice tolerant to 74-86 was tested by inducing tolerance to this peptide and then challenging with T11-CFA. As discussed earlier, these are many examples of Ts acting via an antigen bridge to suppress the response to linked determinants. Thus, 74-86-specific Ts should also act on Tp responding to the *C*-terminal determinant on T11 and suppress the response to both determinants on T11. On the other hand, if tolerance were due to clonal inactivation, it would be restricted to epitopes on the tolerogen, and the response to linked determinants would be unaffected. Therefore, in this example, 74-86-tolerant mice should respond to the determinant on peptide 85-96 after challenge with T11.

Mice injected with 74-86 between 24 and 48 h after birth were tolerant to later challenge with 74-86 (Table II, lines *c* and *e*). Challenge with T11-CFA stimulated a response that was limited to 85-96, and which was of approximately equal strength to the control response (Table II, compare lines *a* and *f*). Thus, 74-86 induces tolerance to itself only, and the response to the *C*-terminal determinant on T11 was unaltered. This result is further evidence against suppression and hence support for clonal inactivation. It is conceivable that 74-86 induces Ts that are functionally restricted and can only suppress the 74-86 response and not the response to the other determinant within T11. Although we considered this unlikely, we could not discount it, so we sought further evidence against active suppression by using the smaller constituent peptides 74-82 and 77-86.

Tolerance Induction Using Minimal Peptides. If tolerance were the result of

TABLE III
Specificity of Response to 74-86

Exp.	[^{3}H]Thymidine incorporation (Δcpm $\times 10^{-3}$)					
	Medium alone	T11	74-86	74-82	77-86	PPD
1	2.7	36.9 ± 1.3	20.6 ± 3.9	22.4 ± 3.1	1.2 ± 0.2	96.2 ± 3.0
2	4.3	41.3 ± 2.6	29.5 ± 4.4	17.2 ± 3.2	0 ± 0.2	98.2 ± 5.2

Popliteal and inguinal lymph nodes from three normal B10.A mice immunized with 74-86 were pooled, and the in vitro proliferative response to medium alone, to 5 μg/ml PPD, and to 7 μM T11, 74-86, 74-82, and 77-86, was measured. The values shown are the average of triplicate wells ± SE.

TABLE IV
Response to HEL Peptide 74-86 after Neonatal Injection with 74-82 or 77-86

Tolerogen	[^{3}H]Thymidine incorporation per culture (Δcpm $\times 10^{-3}$)		
	Medium alone	74-86	PPD
—	3.7	20.2 ± 6.1	137.4 ± 11.9
74-82	2.5	4.7 ± 2.0	157.3 ± 10.4
77-86	2.0	31.3 ± 6.7	142.8 ± 8.6

The in vitro proliferative response of peptide 74-86-primed lymph node cells to medium alone, PPD (5 μg/ml), and 74-86 (14 μM) was assayed. The values shown are the mean of the responses of seven or eight individual mice ± SE.

clonal inactivation, exactly the same peptides that stimulate a response should be able to induce tolerance. However, if tolerance were due to active suppression, the critical requirement for the induction of tolerance by a peptide would be that it contained a Ts-inducing determinant; any peptide lacking an SD would not be tolerogenic, but could be immunogenic. Alternatively, a peptide could be tolerogenic, containing an SD, but could lack a Tp-inducing determinant (PD), and be nonimmunogenic. The use of peptides smaller than the 13–amino acid 74-86 might fortuitously separate the PD and the putatively overlapping SD. To test this hypothesis, we used the T11 amino-end peptides, 74-82 and 77-86. 74-82 can stimulate in vitro proliferation in 74-86-immunized lymph node cells (Table III), while 77-86 does not (but might conceivably contain an SD). Neonatal mice were injected with 74-82 or 77-86, and challenged at 8 wk with 74-86. 74-82 induced tolerance to 74-86, while 77-86 had no effect on the subsequent response (Table IV). Thus, the ability to induce tolerance correlated precisely with the ability to stimulate a response; even a nine–amino acid peptide, near the minimal size reported for an immunogenic peptide (12), could induce neonatal tolerance.

Discussion

The crux of this approach to the study of tolerance induction is the separate analysis of clonal inactivation and suppression, taking advantage of two general findings: (*a*) that T suppressor cell–inducing and T-helper/proliferative cell–inducing determinants are distinct and nonoverlapping, and (*b*) that Ts effects can be expressed both on nearby and distinct determinants, while clonal inactivation can only affect T cells directed at the determinants on the tolerogen.

Furthermore, the possibility of a peptide containing both an SD and a PD becomes increasingly remote as smaller peptides are used, so that at the level of minimal immunogenic peptides, the only remaining mechanisms to be considered are those involving clonal inactivation.

Administration of the peptide 74-86 to neonatal mice induces tolerance to itself. However, this tolerance does not affect the independent response to 85-96 when the tolerant mouse is injected with 74-96, a situation in which putative Ts within 74-86 should have been able to influence the response to the neighboring peptide. B10.A mice can respond to many different peptides from lysozyme, but Ts directed against the *N*-terminal determinant of HEL can turn off the antibody response or the proliferative response to the whole molecule (15), including that to a nearby determinant.

These experiments indicated that no SD was contained within 74-86, but to test the possibility that an overlapping SD might exist within the tridecapeptide, we tried, and were able to use the smallest immunogenic peptide 74-82 to induce tolerance. To see whether the unnecessary 83-86 stretch might have included useful Ts-inducing residues, we used 77-86 to try to induce tolerance for 74-86; it did not. In conclusion, the smallest immunogenic peptide also could induce tolerance, virtually excluding any other situation than one in which the minimal immunogenic peptide and the putative minimal suppressogenic peptide were exactly congruent. Further, the suppressive effect in this presumptive case must be very circumscribed, as the response to the nearby determinant contained within 85-96 was unaffected. Such congruent suppression has yet to be demonstrated directly.

As well as antigen-specific Ts, Ts recognizing the idiotypy of the antigen-reactive T cell must be considered a possible mechanism of tolerance. These Ts would act directly on the Tp, recognizing idiotopes on the T cell receptor and suppressing activation. However, there is little evidence for such Ts in neonatal tolerance to proteins, and we were unable to show any Ts in cell mixing experiments using an in vitro suppressor assay in cytochrome c–tolerant mice (13). To assess a possible role for Ts, we have commenced studies in neonatal tolerance using anti–suppressor cell or antifactor mAbs to see to what extent, if any, the pattern of tolerance induction is affected.

Summary

To investigate the mechanism underlying neonatal T cell tolerance, we used synthetic peptides to induce tolerance. We found that induction of tolerance to one determinant on a 23–amino acid peptide did not affect the response to an adjacent determinant on the same peptide. There was no evidence of suppression of the response to the second determinant. Furthermore, even small peptides near the minimal size for a determinant, which would be very unlikely to possess a suppressor T cell–inducing determinant as well as a proliferative T cell–inducing determinant, could induce tolerance. These studies provide in vivo experiments supporting clonal inactivation as the mechanism of neonatal tolerance to immunogenic peptides.

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