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Research Article

Extended blood half-life of monomethoxypolyethylene glycol-conjugated hen lysozyme is a key parameter controlling immunological tolerogenicity

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Abstract. The blood half-life of a protein is prolonged by conjugating a protein with a linear amphiphilic polymer, monomethoxypolyethylene glycol (mPEG). The conjugation gives a protein immunotolerogenicity; hence, it is likely that the long half-life is crucial for the tolerogenicity. We prepared a tolerogenic mPEG conjugate of hen egg lysozyme ($mPEG_{1.5}$-HEL), which is conjugated 1.5-fold the molecular weight of mPEG against that of HEL, and evaluated the relationship between in vivo stability and the tolerogenicity. $mPEG_{1.5}$-HEL retained immunogenicity to prime HEL-specific T cell and antibody responses and had a long blood half-life, more than 27 times that of native HEL. The tolerant state was maintained as long as $mPEG_{1.5}$-HEL was detected in sera. With a decrease in the blood $mPEG_{1.5}$-HEL level, the tolerant state returned gradually to the responsive state; however, reinjection of $mPEG_{1.5}$-HEL again restored the tolerance. Thus, the extended blood half-life of HEL by mPEG conjugation is probably vital for establishing and maintaining the tolerant states.

Key words. Immunological tolerance; monomethoxypolyethylene glycol; extended blood half-life; hen egg lysozyme; immunochemistry.

To enhance in vivo resistance time of proteins, residues on the surface of a protein are conjugated with monomethoxypolyethylene glycol (mPEG), which is a synthetic straight-chain amphiphilic polymer [1]. In general, the pegylation increases the blood half-life of proteins by a factor of 3- to 486-fold, which derives from reduced intracellular uptake, proteolysis and renal filtration [2]. In this regard, adenosine deaminase [3], L-asparaginase [4], and interferon α-2a [5], have been pegylated.

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On the other hand, introduction of an appropriate amount of mPEG into a protein renders the protein most tolerogenic [6, 7], i.e. preinjection of adult mice with the mPEG protein effectively tolerizes immune responses against subsequent injection of the unconjugated counterpart.

We have proposed that mPEG-type II collagen and mPEG-hen egg lysozyme (HEL) are effective in tolerizing both T helper type 1 (Th1) and T helper type 2 (Th2) immune responses, whereas the native counterpart suppresses Th1-type responses but tends to spare Th2-type responses [8, 9]. This means that mPEG

proteins are effective in suppressing both cell-mediated and humoral immune responses against the proteins, while the conventional protocol for tolerance induction is not effective in suppressing the latter response.

It has been proposed that $CD8^+$ suppressor T cells play a major role in suppression of antibody production in mice treated with mPEG proteins [6, 10]. However, it is not clear how $CD8^+$ suppressor T cells are activated by mPEG proteins. We demonstrated that the suppressor activities were not detected in lymph node and spleen cells of mPEG-HEL-injected BALB/c mice, i.e. lymph node cells from mPEG-HEL-preinjected and native HEL-immunized mice did not suppress HEL-specific T cell proliferative responses in vitro, and transfer of spleen cells of mPEG-HEL-injected mice into naive mice did not suppress anti-HEL immunoglobulin G (IgG) and T cell responses [9]. Hence, it was necessary to reexamine the mechanism from another aspect.

Despite numerous reports on the advantageous features of mPEG proteins, no study has ever evaluated the meaning of the in vivo stability of mPEG proteins for immunotolerogenicity. We therefore used HEL as a model protein antigen and examined the relationship between the blood half-life of mPEG-HEL and the tolerogenicity. This report clarifies the critical role of the prolonged blood half-life of mPEG proteins in tolerogenicity.

Materials and methods

Animals. BALB/c mice were obtained from the Center of Biomedical Research, Kyushu University. The mice were immunized at 8 to 12 weeks of age.

Antigens. Five times recrystallized HEL was kindly donated by QP (Tokyo, Japan). A purified protein derivative of *Mycobacterium tuberculosis* H37Ra (PPD) was purchased from Kainosu (Tokyo, Japan).

Preparation of mPEG-HEL. mPEGs (average MW 5 kDa) were purchased from Aldrich (Milwaukee, WI, USA). Activated $mPEG_2$, 2,4-bis(*O*-methoxypolyethylene glycol)-6-chloro-*s*-triazine, was synthesized according to Ono et al. [11]. HEL (20 mg/ml, 1.4 mM) was incubated with an approximately fourfold molar excess of activated $mPEG_2$ (6 mM), in 0.1 M borate buffer, pH 10, for 8 h at 20 °C. Unreacted mPEG was removed by Sephacryl S-100 (Pharmacia, column size: 2.6×60 cm) equilibrated with 0.1 M NH_4CO_3, and the fraction of mPEG-HEL was exhaustively dialyzed against saline. The degree of mPEG introduction was determined by measuring the amount of free amino groups in the HEL molecule, which are unreacted with activated $mPEG_2$, using trinitrobenzenesulfonate. A tolerogenic mPEG-HEL ($mPEG_{1.5}$-HEL), in which the available amino groups of HEL are conjugated with 1.5-fold molecular weight of mPEG against HEL in average, was used in this experiment.

Tolerance induction and immunization. Prior to challenge immunization, mice were given i.p. (intraperitoneal) injection of native HEL or $mPEG_{1.5}$-HEL dissolved in saline. Control mice were injected with saline. The mice were challenge-immunized intracutaneously in both hind foot pads with native HEL in 0.1 ml of emulsion with complete Freund's adjuvant (CFA; Difco Laboratories, Detroit, MI, USA).

Measurement of T cell and antibody responses. Mice were killed 9 days after immunization, and draining lymph node cells and sera were obtained. Cultures for a lymph node T cell proliferation assay were set up according to Adorini et al. [12], and the proliferation was measured after 96 h using a colorimetric assay based on tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), as described [13]. Antigen-specific serum IgG titers were measured using enzyme-linked immunosorbent assay (ELISA), as described elsewhere [8, 9].

Measurement of protein concentrations in blood circulation. Groups of five mice were injected i.p. with 0.5 ml of saline containing native HEL or $mPEG_{1.5}$-HEL. At various times after injection, blood samples were drawn from the orbital sinus into capillary tubes. Concentrations of native HEL and $mPEG_{1.5}$-HEL in serum samples were determined using sandwich ELISA. ELISA plates were coated with 50 μl of rabbit anti-HEL polyclonal antibody in 0.1 M sodium carbonate buffer (pH 9.6) at 5 μg/ml overnight at 4 °C, and blocking was done with 2% nonfat dry milk in phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). After washing with PBST, 50 μl of serum samples serially diluted with blocking buffer were incubated overnight at 4 °C. After washing with PBST, 50 μl of biotinylated mouse anti-HEL monoclonal antibody (1 μg/ml) in the blocking buffer was added to each well followed by incubation for 2 h at room temperature. The reacted biotinylated monoclonal antibody was subsequently probed with an alkaline phosphatase-biotin/avidin complex (Vector Laboratories, Burlingame, CA, USA). To determine the serum concentration, known concentrations of native HEL and $mPEG_{1.5}$-HEL were included in each plate, as an internal standard. Data were expressed as mean concentrations with standard errors.

Results

Relationship between blood concentration of HEL and tolerogenic activity. To evaluate the correlation between tolerogenicity of HEL and in vivo stability, native HEL or $mPEG_{1.5}$-HEL was intraperitoneally injected into mice, and their tolerogenic activities and blood clearance profiles were measured. $mPEG_{1.5}$-HEL tolerized both T cell and antibody responses in an antigen-specific manner, whereas native HEL only suppressed the

T cell response (fig. 1A–C), as we previously demonstrated [9]. We examined the effect of the injection route, i.e. i.p., i.v. (intravenous) and s.c. (subcutaneous), on tolerance induction and found that the administration of $mPEG_{1.5}$-HEL through the three routes could effectively establish similar tolerance states, indicating that the tolerance induction in this experimental system may not depend on routes of administration.

Native HEL disappeared from the blood circulation to a detection limit (3×10^{-9} M) within 1 day following injection, whereas $mPEG_{1.5}$-HEL was cleared more slowly and we could detect $mPEG_{1.5}$-HEL even 28 days later (fig. 1D). From the clearance profiles, the half-life of native HEL was calculated to be less than 0.1 days, whereas that of $mPEG_{1.5}$-HEL was about 2.7 days. Thus, the long half-life of $mPEG_{1.5}$-HEL was suggested to play an important role in tolerizing imune responses.

$mPEG_{1.5}$-HEL primes anti-HEL T cell and antibody responses. Pegylation of a protein antigen inhibits both antigen-antibody interaction [14, 15] and generation of antigenic peptides for T cell stimulation [13]. Thus, with an increase in the extent of pegylation, the heavier pegylated molecule may be neglected by the specific T and B cells, and effective tolerance states seem not to be established. Indeed, heavy pegylation attenuated the tolerogenicity of mPEG-HEL [7]. To test the immunogenicity of the $mPEG_{1.5}$-HEL used in this study, we administrated $mPEG_{1.5}$-HEL with CFA, and T cell and antibody responses to HEL were measured. A decrease in HEL-specific T cell-dependent proliferative response was observed in $mPEG_{1.5}$-HEL-primed mice; however,

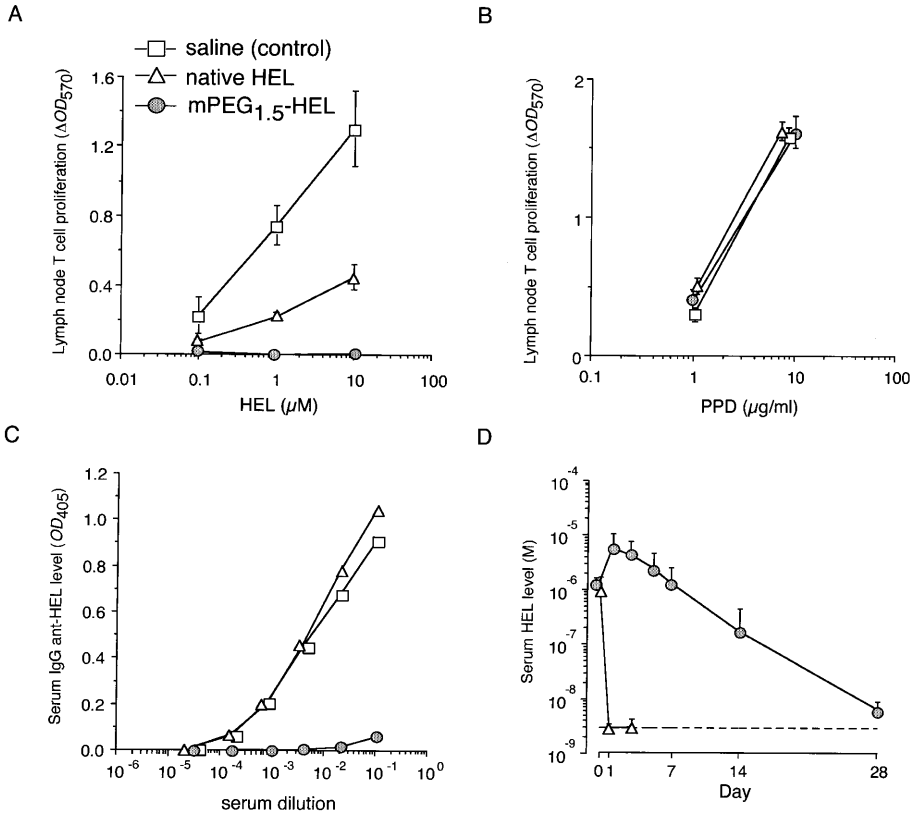

Figure 1. Immunotolerogenicities and blood clearance profiles of native HEL and $mPEG_{1.5}$-HEL. Groups of two BALB/c mice were administrated native HEL, $mPEG_{1.5}$-HEL (each contained 1 mg protein in 0.5 ml of saline) or saline i.p. on day −7 relative to the challenge immunization of native HEL (50 μg/mouse) with CFA on day 0. Nine days later, draining lymph node cells and peripheral blood samples were harvested. The cells were cultured in the presence of indicated doses of native HEL (*A*) and PPD (*B*). After 4 days of incubation, the number of live cells in each well was evaluated, using the MTT assay. Cultures were set up in triplicate from pooled lymph node cells of two mice per group. Data are expressed as mean OD_{570} and standard deviation of triplicate cultures with background values (cultured with no antigen) subtracted (ΔOD_{570}). Serum IgG antibody levels to HEL were evaluated, using ELISA (*C*). Serially diluted pooled sera from two mice per group were measured. Data are expressed by plotting OD_{405} against log10 of dilution factors. Blood concentrations of native HEL and $mPEG_{1.5}$-HEL were evaluated, using sandwich ELISA (*D*). Sera were obtained at different day points from BALB/c mice given a single administration of native HEL or $mPEG_{1.5}$-HEL (each contained 1 mg of protein in 0.5 ml of saline). Concentrations of native HEL and $mPEG_{1.5}$-HEL in serum samples were determined as described in 'Materials and methods'. Each point represents the mean concentration (M) and the standard error of 5 mice. Dotted line is the detection limit (3×10^{-9} M).

the T cell response was not completely inhibited and was about 1/100 of native HEL (fig. 2A). On the other hand, anti-HEL IgG titer of $mPEG_{1.5}$-HEL-primed mice was similar to that of native HEL (fig. 2C). From these experiments, we concluded that tolerogenic $mPEG_{1.5}$-HEL possesses weak but substantial immunogenicity to induce HEL-specific immune responses.

Tolerizing both T cell and antibody responses by consecutive administration of native HEL. One milligram of native HEL suppressed T cell response but spared antibody response (fig. 1). If the blood antigen level is the critical determinant for tolerance, native HEL should induce a tolerance state similar to $mPEG_{1.5}$-HEL by maintaining the blood HEL level. Thus, to keep the serum concentration, we injected 10 mg of native HEL daily. This protocol maintained the serum level over 5×10^{-8} M, as shown in figure 3D. The consecutive injection of native HEL significantly suppressed T cell and antibody responses (fig. 3A–C). Thus retention of a certain blood antigen concentration is critical for establishment and maintenance of immunological tolerant state for the antigen.

Elimination of $mPEG_{1.5}$-HEL from blood circulation breaks the tolerance state. $mPEG_{1.5}$-HEL was sustained in vivo for an extended period (fig. 1D). If the longer half-life of $mPEG_{1.5}$-HEL is important for tolerance, immune responses may recover when $mPEG_{1.5}$-HEL is eliminated from blood circulation. To test this possibility, mice preinjected with $mPEG_{1.5}$-HEL were immunized with native HEL at different day points, 2–84 days after the $mPEG_{1.5}$-HEL injection, and the duration of tolerance was evaluated. At days 7 and 28, the level of suppression in T cell and antibody responses nearly peaked, but after that, both responses reverted to the responsive state, and the tolerant state was almost lost at day 84 (fig. 4). The tolerance established with $mPEG_{1.5}$-HEL was found to persist for at least 28 days, which suggests an important association between the magnitude of tolerance and the blood concentration of $mPEG_{1.5}$-HEL.

Restoration of tolerance in mice recovered from the previous tolerant state. The recovery of T cell and antibody responses after day 28 in $mPEG_{1.5}$-HEL-treated mice is supposed to be the cause of elimination of $mPEG_{1.5}$-HEL from the body. We found this to be true (fig. 5). HEL-specific T cell proliferative response and antibody responses were both suppressed with the second injection of $mPEG_{1.5}$-HEL given on day 84, whereas mice injected only at day 0 recovered completely from tolerance. Thus, this result also supports the importance of the increased blood half-life of mPEG-HEL in maintaining the immunological tolerance state.

Sustained antigen concentration in blood circulation is likely to be the key factor for maintaining the tolerance state in which both T and B cell responses are sup-

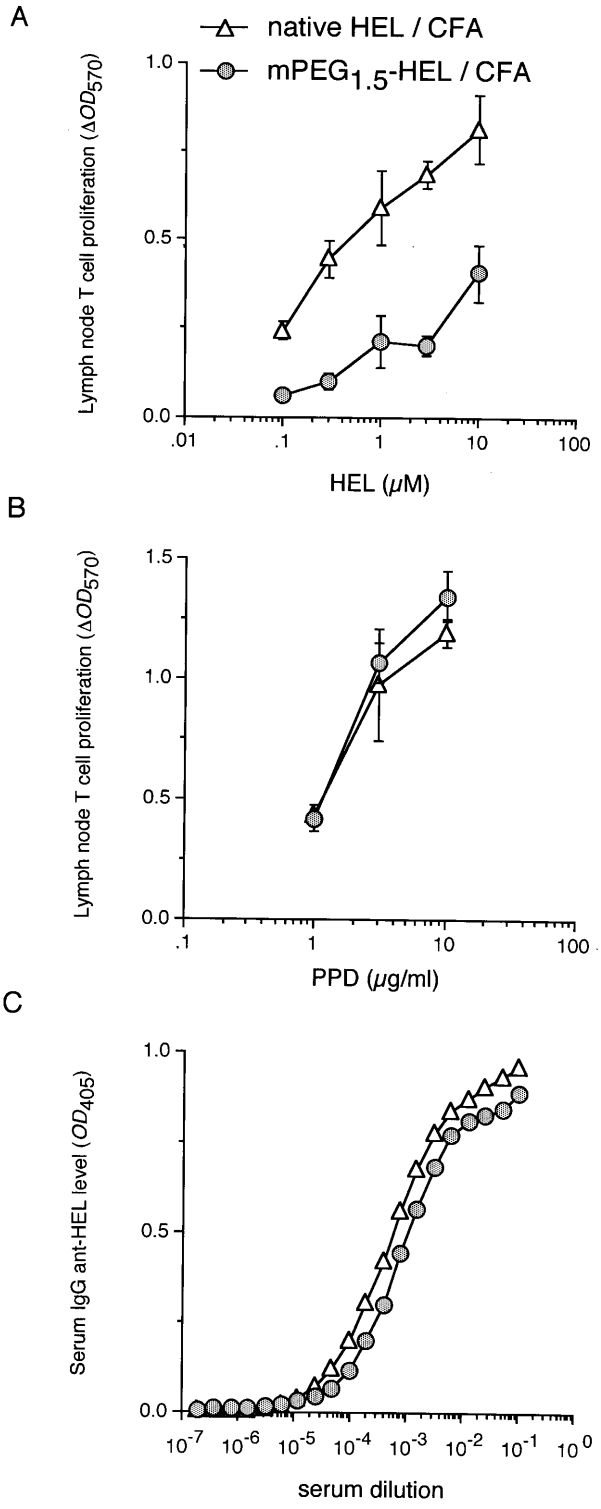

Figure 2. Immunogenicity of $mPEG_{1.5}$-HEL. Groups of two BALB/c mice were immunized with native HEL or $mPEG_{1.5}$-HEL (50 μg protein/mouse) emulsified in CFA. Nine days later, draining lymph node cells and peripheral blood samples were harvested, and T cell proliferative response to HEL (*A*), PPD (*B*) and anti-HEL serum IgG titer (*C*) were measured as described in legend to Figure 1.

pressed. Tolerogenicity of mPEG-HEL can be explained by their in vivo long life acquired by pegylation.

Discussion

Weigle proposed that the soluble fraction of xenogenic gamma globulins possess immunotolerogenicity, which derives from their long half-life in vivo [16]. From his observation, it was supposed that the soluble and the stable nature of mPEG proteins played important roles in tolerogenicity. As we previously demonstrated, however, deaggregation of native HEL by ultracentrifugation did not significantly alter the immunogenicity, and the tolerogenic activity was different from that of mPEG-HEL, concluding that the tolerogenicity of mPEG-HEL is unlikely to be due to increased solubility by pegylation [9].

In this study, we concentrated on another question, i.e., whether the in vivo stability of mPEG proteins plays a critical role in the tolerogenicity. Our results are in agreement with the findings of Weigle. However, we propose that pegylation techniques are an effective, general method of preparing tolerogenic molecules regardless of original character of the proteins. Gamma globulin is a high molecular weight ($\sim$ 150 kDa) protein and relatively stable itself in physiological conditions [16]; thus, the tolerogenicity should be an inherent property. On the other hand, HEL is a stable but small protein (14 kDa) and is easily removed at kidney [17]. This may be the reason why the repeated injection of native HEL was required to establish the similar tolerant state to $mPEG_{1.5}$-HEL (fig. 3). In addition, many proteins have been converted into tolerogenic molecules by pegylation [6–10, 18–20], and the tolerogenicity did not depend on the genetic background of mice [unpublished observation]. Hence, the protein-pegylation technique may be generally applicable to tolerization of undesired immune responses against proteins.

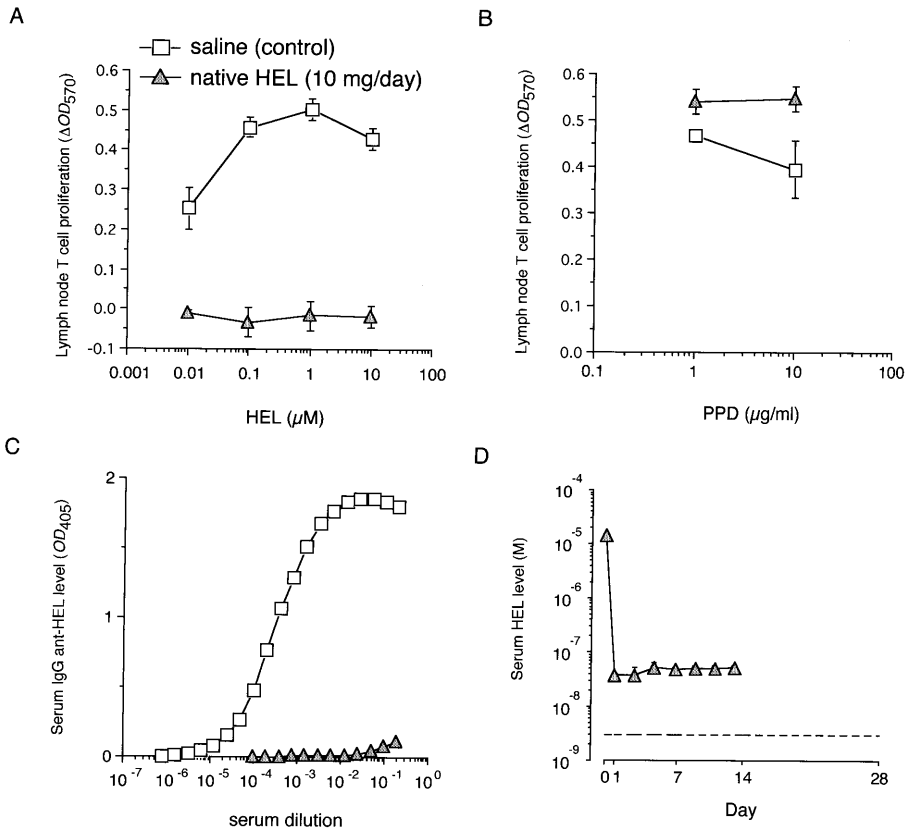

Figure 3. Maintenance of the serum HEL level is effective in tolerizing both anti-HEL T cell and antibody responses. Groups of two BALB/c mice were injected daily with 10 mg of native HEL or saline from days 0 to 23. Mice were immunized with 50 μg of native HEL in CFA at day 14. Nine days later, the T cell proliferative response to HEL (*A*) PPD (*B*) and anti-HEL serum IgG titer (*C*) were measured as described in the legend to figure 1. The blood concentration of native HEL was evaluated, using sandwich ELISA (*D*). Sera were obtained from BALB/c mice given a daily injection of 10 mg of native HEL on odd days before the daily injection. Concentrations of native HEL in serum samples were determined as described in 'Materials and methods'. Each point represents the mean concentration (M) and the standard error of five mice. The dotted line is the detection limit (3×10^{-9} M).

In HEL transgenic models, the serum concentration of HEL is demonstrated to be a critical factor in deleting and inactivating autoreactive lymphocytes. Goodnow et al., using HEL- and anti-HEL antibody-double transgenic mice, reported that mature B cells were inactivated when the blood concentration of HEL exceeded a critical threshold, i.e. more than 10 ng/ml (0.7 nM) [21]. Similarly, using T cell receptor transgenic mice, Cibotti et al. indicated that induction of T cell tolerance for an im-

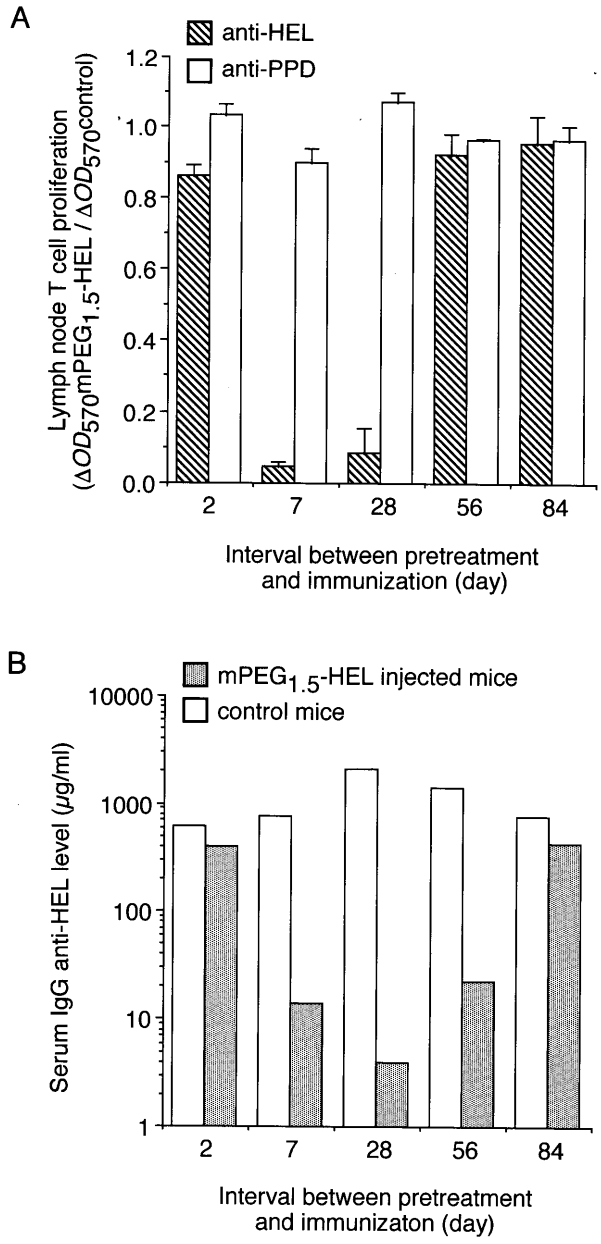

Figure 4. The duration of tolerance induced by mPEG$_{1.5}$-HEL. Groups of two BALB/c mice were injected with 1 mg of mPEG$_{1.5}$-HEL or saline at day 0 and immunized with 50 μg of native HEL in CFA at days of 2, 7, 28, 56 or 84. Nine days after immunization, draining lymph node cells and peripheral blood samples were harvested, and lymph node T cell proliferation in response to 10 μM HEL and 10 μg/ml PPD (*A*), and serum IgG response to HEL (*B*) were measured. T cell proliferative response was measured, as shown in the legend to figure 1, and data are expressed as the ratio of proliferative responses (ΔOD$_{570}$ mPEG$_{1.5}$-HEL/ ΔOD$_{570}$ control) and standard deviations. Serum anti-HEL IgG concentration was measured using ELISA, and data are expressed in arbitrary ELISA units, determined using an anti-HEL monoclonal antibody.

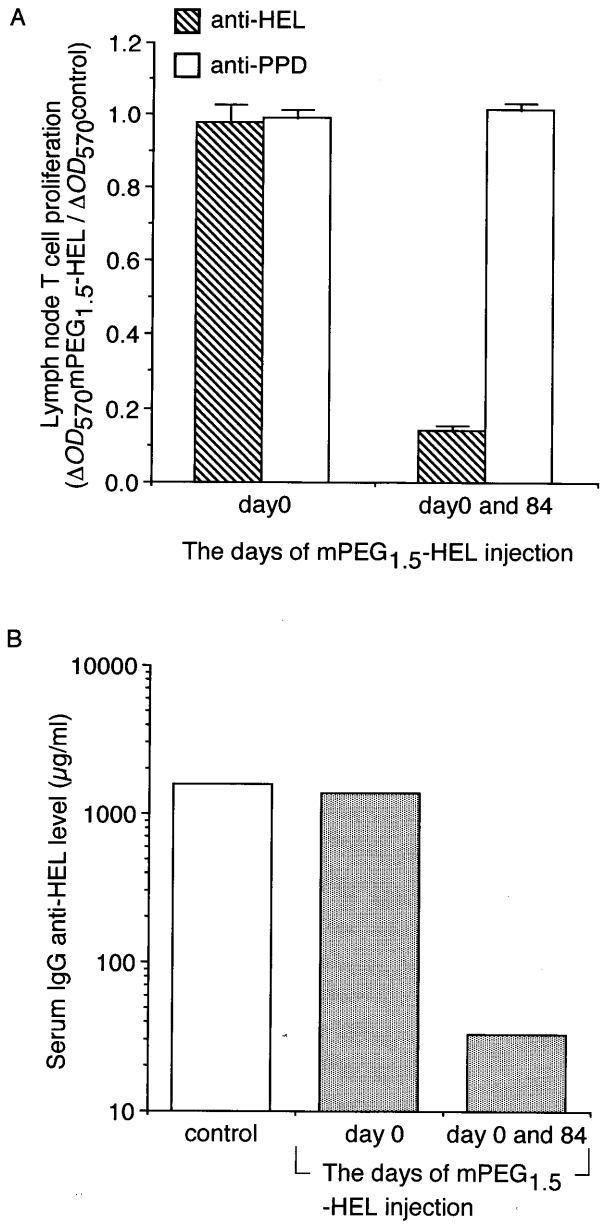

Figure 5. Secondary injection of mPEG$_{1.5}$-HEL retolerizes T cell and antibody responses. Groups of two BALB/c mice were injected with 1 mg of mPEG$_{1.5}$-HEL at day 0, or day 0 and 84; control mice were injected with saline only. On day 91, mice were immunized with 50 μg of native HEL in CFA. Then 9 days later, lymph node T cell proliferation in response to 10 μM HEL and 10 μg/ml PPD (*A*) and serum IgG response to HEL (*B*) were measured, as described in the legend to figure 4.

munodominant peptide determinant of HEL also required a blood level over 10 ng/ml [22]. In our experiment, the blood concentration in mice injected with 1 mg of $mPEG_{1.5}$-HEL was over 10 ng/ml for 36 days (fig. 1D). This explains the recovery from the immune suppression after day 28, using normal mice (fig. 4).

The recovery from the tolerant state after day 28, however, could be retolerized by the second injection of $mPEG_{1.5}$-HEL (fig. 5). This means that the presence or reexposure of antigens is necessary to maintain the tolerant state. A similar conclusion is indicated by Scully et al. using the transplantation model. They induced a tolerance state in skin grafts with an anti-CD4 monoclonal antibody and demonstrated that tolerant T cells reverted to the responsive state when these cells were not stimulated by transplantation antigens and also that reexposure of the T cells to the antigens was needed to maintain the tolerant state [23]. Thus, continuous exposure of T and B cells to their specific antigens is a critical factor in establishing and maintaining the tolerant state.

In vivo encounters of lymphocytes with the soluble form of antigens results in a deletion of the majority of antigen-specific T and B cells in the periphery by a process termed activation-induced apoptosis. However, the deletion has never been complete. There is always a small percentage of the specific lymphocytes that are able to escape from activation-induced apoptosis and persist in the periphery of the host in an unresponsive state [24, 25]. Administrated mPEG-proteins persist in vivo; thus, the frequency of association of mPEG-HEL with HEL-specific lymphocytes increases, and clonal deletion or inactivation of the lymphocytes may occur. This may be a possible mechanism of tolerance by mPEG-proteins. Recovery from the tolerant state in $mPEG_{1.5}$-HEL-treated mice might be attributable to restoration of the anergic lymphocytes to the reactive state against HEL or to the newly generated repertoires of HEL-specific lymphocytes from bone marrow.

This report clarifies the important correlation between sustained blood half-life and tolerogenicity in mPEG proteins. The result provides practical information in tolerizing undesired immune responses to protein antigens and may enhance the usefulness of mPEG proteins in therapeutics.

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