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Tolerogenic activity of polyethylene glycol-conjugated lysozyme distinct from that of the native counterpart

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SUMMARY

Conjugation of proteins with polyethylene glycol (PEG) has been reported to make the proteins tolerogenic. Native proteins are also potentially tolerogenic when given without adjuvants. We compared immunotolerogenic activities between PEG-conjugated and native hen egg-white lysozyme (HEL). BALB/c mice were given consecutive weekly intraperitoneal administrations of PEG-conjugated HEL, unmodified HEL or phosphate-buffered saline (PBS), for 3 weeks, then challenged with HEL in Freund's complete adjuvant. The pretreatment with PEG–HEL tolerized both T-cell and humoral responses, while native HEL tolerized only the T-cell response. Immunoglobulin G1 (IgG1) antibody was already elevated in HEL-pretreated mice prior to challenge immunization, followed by suppressed IgG2a and IgG2b, but spared IgG1 production after the antigen challenge, whereas PEG–HEL-pretreated mice produced no antibody in all IgG subclasses prior and subsequent to the antigen-challenge. Production of interleukin-2 (IL-2) and interferon-γ (IFN-γ) by lymphoid cells in response to HEL *in vitro* was markedly suppressed in both the antigen-pretreated groups, while suppression of IL-4 production was evident only in PEG–HEL-, not in HEL-pretreated animals. Involvement of suppressor cells in these tolerance states was found to be unlikely, and the immunological property of PEG–HEL differed from deaggregated HEL that was similar to the original HEL. These results suggest a unique immunotolerogenic activity of PEG-conjugated proteins to suppress both T-helper type-1 (Th1)- and Th2-type responses, the result being extensive inhibition of all IgG subclass responses, while tolerance induction by unconjugated soluble proteins tends to be targeted on Th1-, but spares Th2-type responses.

INTRODUCTION

The most desired strategy to control unwanted immune responses, such as allergy, autoimmune diseases and graft rejection, would be achieved by induction of antigen-specific immunological tolerance, rather than antigen-non-specific immunosuppression as currently being used. Therefore, searching for procedures to effectively induce immunological tolerance in adult animals is the subject of interest. The recent discovery of essential roles of co-stimulatory signals in full activation of T and B lymphocytes[1,2] has provided important implications in this regard. Interruptions of CD28/B7 and CD40/CD40L pathways, either alone or both in combination, are promising regimens to suppress immune responses, in an antigen-specific manner, in animal models.[3–8] Immunological tolerance is also achieved by feeding antigens, thus was named oral tolerance.[9] This approach has been applied not only to diseases in experimental animals but also to a clinical trial for human rheumatoid arthritis, and encouraging results are reported.[10] Despite the attention to those specific procedures, tolerance states can also be induced in adult animals by simple parenteral administration of soluble antigens[11–13] (for a review see ref. 14), but the underlying mechanisms are poorly understood.

Polyethylene glycol (PEG) is an amphipathic polymer and is poorly immunogenic itself. This substance is introduced to xenogenic proteins to be administered as therapeutic agents,[15,16] because the chemical modification prolongs survival of proteins in body fluids, and in addition, lowers the antigenicity and/or immunogenicity intrinsic to the proteins.[17–20] We have shown that PEG-conjugation abrogates the T-cell stimulating capacity of hen egg-white lysozyme (HEL), probably because the conjugate resisted proteolytic

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Abbreviations: PEG, polyethylene glycol; HEL, hen egg-white lysozyme; CII, type II collagen; PPD, purified protein derivative of *Mycobacterium tuberculosis* H37Ra; HGG, human gamma globulin; FCA, Freund's complete adjuvant; i.p., intraperitoneal; IL, interleukin; IFN, interferon; Th1, T helper type 1; Th2, T helper type 2; Ts, suppressor T cells; ELISA, enzyme-linked immunosorbent assay.

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degradation, an essential step for antigen processing.[20] In addition to the immunologically inert nature of PEG-conjugated proteins, an immunologically active, beneficial property of the conjugates has been proposed; proteins become strongly tolerogenic with the attachment of PEG.[21,22] We[23] previously compared tolerogenic activities between native type II collagen (CII) and the PEG-conjugate, using DBA/1 mice, which are susceptible to collagen-induced arthritis, an animal model for rheumatoid arthritis. In the experiment, we observed that antibody response of immunoglobulin G1 (IgG1) subclass was markedly suppressed in mice pre-injected intraperitoneally (i.p.) with the PEG-conjugate, and less effectively in mice pretreated with the native antigen, whereas three other IgG subclasses and T-cell responses were equally suppressed either by native or by PEG-conjugated CII.

The present study was done to determine whether what we have observed with CII in DBA/1 mice was a general phenomenon associated with PEG-conjugation of proteins, and to clarify the differences in detail. Using HEL and BALB/c mice, we again observed that the i.p. injection of both PEG-conjugated HEL and unconjugated HEL equally tolerized the antigen-specific T-cell proliferative responses, while antibody production, especially in the IgG1 subclass, was suppressed in the mice pretreated with the PEG-conjugate, but not with native HEL. Profiles of cytokine production in response to HEL were in agreement with the IgG subclass distributions. Thus, the tolerance states induced by PEG-conjugates and the native counterparts differed. We propose that PEG-conjugated proteins may be generally effective in tolerizing both T helper type 1 (Th1)- and Th2-type immune responses, while native proteins do suppress Th1-type responses but tend to spare Th2-type responses.

MATERIALS AND METHODS

Animals

Female BALB/c mice were obtained from Seac Yoshitomi Ltd. (Fukuoka, Japan) and were immunized at age 8–12 weeks.

Antigens

Five times recrystallized HEL was kindly donated by QP Co. (Tokyo, Japan). Purified protein derivative (PPD) of *Mycobacterium tuberculosis* H37Ra was purchased from Kainosu Inc. (Tokyo, Japan). Coupling of HEL with PEG was performed as previously described[20] using cyanuric chloride-activated monomethoxyPEG (average MW 5000, Sigma Chemical Co., St. Louis, MO). The degree of modification of HEL with PEG was determined by measuring the amount of free amino groups in the HEL molecule, using trinitrobenzenesulphonate,[24] and more than 50% reduction in primary amino groups of HEL was used. Deaggregated HEL was prepared just prior to being injected, according to the protocol described by Romball and Weigle[13] to prepare deaggregated human gammaglobulin (HGG). The solution of native HEL was centrifuged at 150 000 ***g*** for 180 min and the upper quarter of the supernatant was collected and designated as deaggregated HEL.

Tolerance induction and immunization

Schedule for tolerance induction was essentially the same as previously described for DBA/1 mice and CII.[23] BALB/c mice were i.p. administered native HEL or PEG–HEL (each contained 0·2 mg HEL in 1 ml phosphate-buffered saline (PBS)) on days −21, −14, and −7 relative to the challenge-immunization on day 0. Control mice were injected with PBS. The mice were challenge immunized intracutaneously in both hind foot pads with HEL (50 μg/mouse) in 0·1 ml emulsion with Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI).

Cell cultures, T-cell proliferation assay and cytokine assays

Mice were killed 9 days after the immunization and draining lymph node cells and spleen cells were obtained. Cultures for lymph node T-cell proliferation assay were set up according to Adorini *et al.*[25] and the proliferation was measured after 96 hr using a colorimetric assay based on tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), as previously described.[20] To evaluate cytokine production, cells were cultured at 10^6 cells/well for 36 hr in the presence or absence of HEL (50 μg/ml). Levels of interleukin-2 (IL-2) in the culture supernatants were measured by a bioassay using a CTLL-2 cell line,[26] as previously described.[20] Interferon-γ (IFN-γ), IL-4 and IL-10 were measured using murine cytokine enzyme-linked immunosorbent assay (ELISA) kits (PerSeptive, Cambridge, MA). For IFN-γ and IL-10, amounts of cytokines in the medium were determined according to the manufacturer's instructions. For IL-4, antibody-coated plates provided in the kit were first treated with sterile PBS containing penicillin (100 U/ml), streptomycin (100 μg/ml) and 2% human albumin at 37° for 24 hr, washed three times with sterile PBS, then cells were directly cultured in the plates. The cultures were terminated by extensive washing with PBS containing 1% Triton-X-100 and 0·5% Tween 20. After complete removal of cells and the debris, IL-4 associated with the solid phase was measured following the ordinal ELISA protocol described in the manufacturer's instructions. Other conditions for cell cultures were the same as previously described,[20,23] except that cultures were performed under a serum-free condition using HL-1 medium (Hycor, Irvine, CA).

Measurement of serum antibody level

Sera were obtained at various time points, as indicated in the figure legends, and the antibody levels to HEL were determined by ELISA, as described elsewhere.[23] Materials used in the present study are all described in our previous reports.

Adoptive transfer of splenocytes

Spleen cells were obtained from mice in which tolerance was induced using the protocol described above. Control cells were obtained from mice injected with PBS instead of HEL or PEG–HEL. Cells from two mice per group were washed and resuspended in 1 ml PBS ($\approx 2 \times 10^8$ nucleated cells/ml) and the 0·5 ml was transfused to each of two naive mice per group, through the lateral tail vein. The next day (day 0), recipients were immunized with HEL in FCA, and serum antibody levels and lymph node T-cell proliferation in response to HEL were evaluated on day 9, as described above.

RESULTS

Pretreatment with PEG–HEL tolerized both T-cell and humoral responses, while native HEL tolerized only the T-cell response

To determine whether an antigen-specific tolerance was established, mice preinjected with native HEL or PEG–HEL were

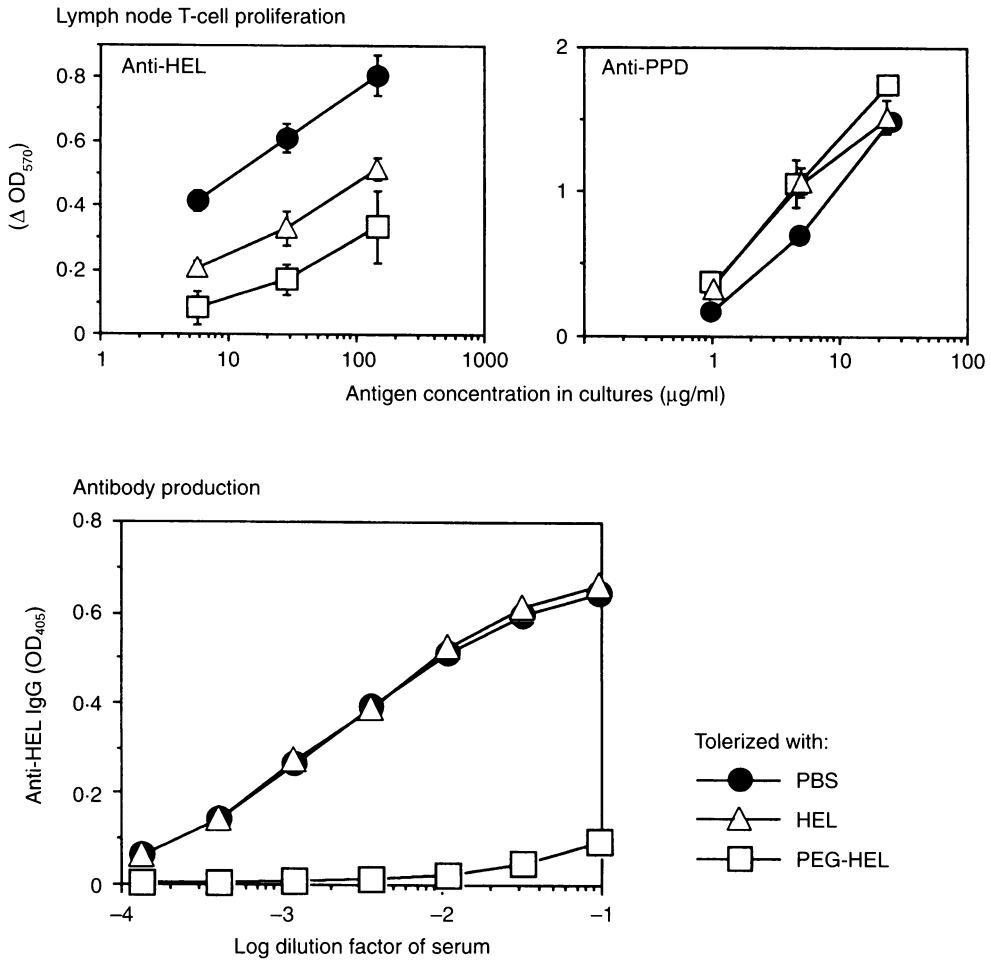

Figure 1. Proliferative responses of lymph node T cells and serum antibody levels to HEL in mice pretreated with HEL or PEG–HEL and challenged with HEL in FCA. Mice pretreated with HEL (△), PEG–HEL (□), or PBS (●) were immunized with HEL (50 μg/mouse) in FCA. Nine days later, draining lymph node cells and sera were obtained. The cells were cultured in the presence of indicated amounts of HEL (upper left), PPD (right), or no antigens at 5×10^5 cells in 0·2 ml per well. Four days later, the number of live cells in each well was evaluated by 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 (Δ OD_{570}). Serum IgG antibody levels to HEL (lower panel) were evaluated using ELISA. Serially diluted pooled sera from two mice per group were measured. Data are expressed by plotting OD_{405} against $\log_{10}$ of dilution factors.

challenge-immunized with HEL in FCA. Lymph node cells were harvested on day 9 and stimulated *in vitro* with HEL. As shown in Fig. 1, mice pretreated with PEG–HEL or with native HEL showed a marked reduction in responses to HEL, while responses to PPD and concanavalin A (data not shown) were unaffected. Thus, antigen-specific tolerance was successfully induced.

In contrast to the T-cell response, the level of serum anti-HEL antibody in mice pretreated with native HEL was not significantly lower than in control mice 9 days after immunization, while the antibody production in PEG–HEL-pretreated mice was suppressed (Fig. 1, lower).

Native HEL triggers IgG1 and suppresses other IgG subclasses, while PEG–HEL suppresses all IgG subclasses of anti-HEL antibody production

Sera were obtained from mice given three consecutive weekly injections of PEG–HEL, native HEL or PBS, before and 9 days after challenge-immunization with HEL in FCA. The antibody levels were examined in an isotype-specific manner. As shown in Fig. 2, native HEL-pretreated mice had elevated levels of IgG1 antibody to HEL on day 0, while no elevation was observed in mice treated with PEG–HEL. On day 9, native HEL-pretreated mice had lower levels of IgG2a, IgG2b and IgG3 antibodies than in control mice preinjected with PBS. Levels of IgG1 antibodies of the PBS-pretreated and HEL-pretreated mice were almost the same on day 9. On the other hand, responses of serum antibodies of all IgG subclasses were strongly suppressed in PEG–HEL-pretreated mice, although some elevation of IgM and IgG3 antibody was noted both on day 0 and day 9 in compared with native HEL-pretreated mice.

Cytokine production of cells in peripheral lymphoid organs

From the IgG subclass distributions of anti-HEL antibodies (Fig. 2), as well as from our previous results in DBA/1 mice

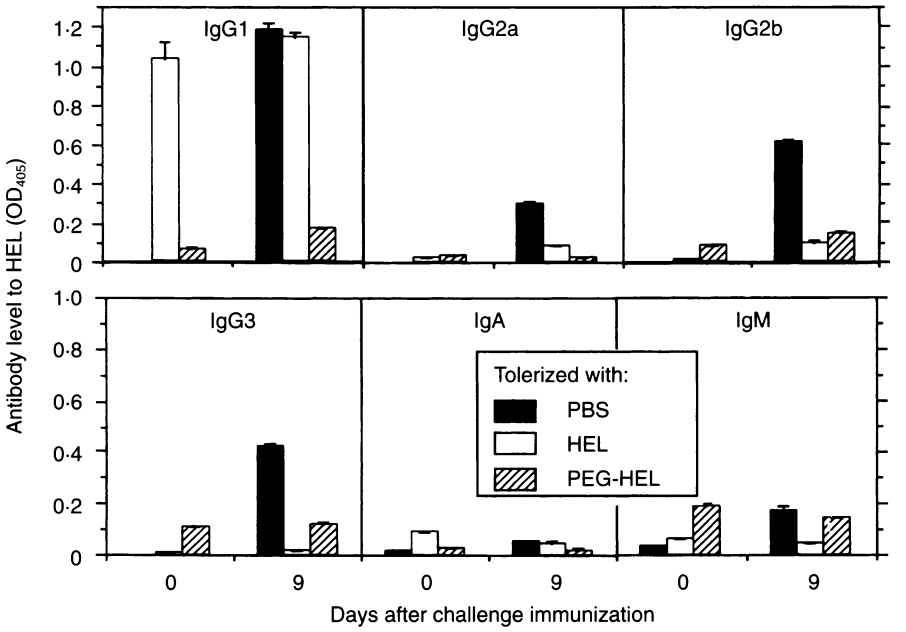

Figure 2. Isotype distribution profiles of serum anti-HEL antibodies. Mice were weekly injected with HEL, PEG–HEL or PBS three times. Their sera were obtained on day 0 just prior to challenge-immunization with HEL in FCA, and on day 9 after the immunization. Sera from two mice per group at each point were pooled and used in triplicate for ELISA at 1/100 dilution. Anti-HEL antibody levels of four IgG subclasses, IgA and IgM were evaluated using rabbit antibodies specific for each isotype followed by alkaline phosphatase-conjugated goat anti-rabbit IgG. Data are expressed as mean $OD_{405} \pm SD$ of the triplicate wells.

using CII, we considered that PEG-conjugated proteins could tolerize both Th1- and Th2-type immune responses, whereas native soluble antigens tolerized only Th1-, but spared or enhanced Th2-type reactions. To verify this hypothesis, we determined representative Th1- and Th2-cytokines produced by lymphoid cells from the tolerized and challenge-immunized mice in response to HEL *in vitro*.

The two Th1-cytokines, namely IL-2 and IFN-γ, were suppressed in both HEL- and PEG–HEL-pretreated mice (Fig. 3, upper two rows), in accordance with the similar suppression of IgG2a antibody production (Fig. 2) and T-cell proliferative response in the two treated groups (Fig. 1). Compared with the Th1 cytokines, difference in production of two representative Th2 cytokine, IL-4 and IL-10, was less clear cut. However, it was noticeable in spleen cells that pretreatment with PEG–HEL suppressed the ability to produce IL-4, and IL-10 to a lesser extent. In contrast, native HEL tended to spare them both in spleens and lymph nodes (Fig. 3, lower two rows). The less clear-cut data obtained as to IL-10 may be because of the different methods used to detect the two cytokines. IL-10 was readily detectable in culture supernatant of lymph node cells, while IL-4 levels in the culture medium under the standard condition of lymph node cell cultures were always below the detection limit of the ELISA system (10 pg/ml). However, as IL-4 is the essential cytokine for IgG1 antibody production,[27] the isotype possessed the most conspicuous difference (see Fig. 2), we tested a modified condition to detect this cytokine, as described in Materials and Methods. We detected IL-4 production of lymph node cells from PBS- and HEL-treated mice, and a lower level in PEG–HEL-treated mice; however, the difference between mice given the differential pretreatments was still not clear. When IL-4 was measured in spleen cell cultures using the modified condition, we found a marked suppression of its production in PEG–HEL-treated mice, as compared with findings in the other two groups. Recently, for the same purpose of improving the sensitivity of ELISA for IL-4, a similar modification has been also reported.[28]

Tolerance induced by PEG–HEL is not mediated by active suppression

It has been reported that PEG-conjugated proteins are potent inducers of antigen-specific $CD8^+$ suppressor T cells (Ts).[21,22] To determine if the unique tolerance state induced by PEG–HEL in our experiments was caused by specific induction of Ts by the conjugate, we carried out two kinds of experiments. First, lymph node cells were obtained from mice tolerized either with PEG–HEL or with native HEL on day 9 following challenge-immunization. These cells were mixed with HEL-responsive T cells obtained from untolerized and HEL-primed mice (Fig. 4a). The cells from tolerant mice were unable to suppress the proliferation of HEL-responsive T cells, a finding which suggests that the unresponsiveness in T-cell proliferation induced by either PEG–HEL or HEL was not mediated by regulatory cells which actively suppress the reaction. We next prepared spleen cells from tolerant mice i.p. injected with HEL or PEG–HEL, or from control mice injected with PBS. These cells were transferred into naive syngeneic mice and the mice were immunized with HEL in FCA. All the transfused mice possessed a similar potential to mount antigen-specific T-cell responses and antibody production following the subsequent challenge with HEL in FCA (Fig. 4b and c). Thus, we obtained no evidence implying that the tolerance

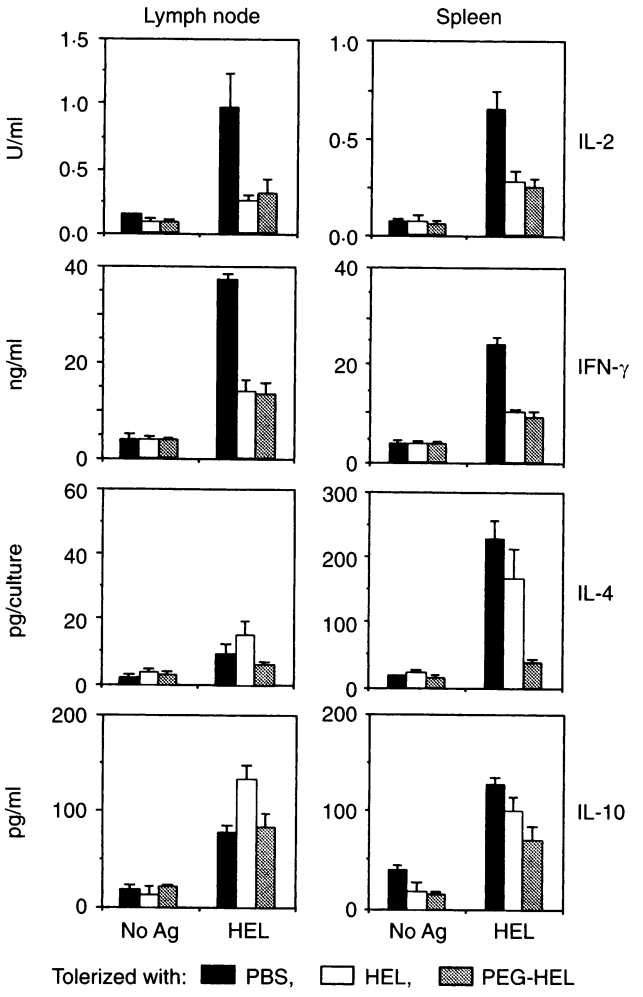

Figure 3. Production of Th1- and Th2-cytokines. Lymph node and spleen cells were prepared from mice preinjected with HEL (□), PEG–HEL (▨), or PBS (■) and subsequently immunized with HEL in FCA on day 9 post immunization, and cultured in the presence or absence of 50 μg/ml HEL at 10^6 cells/well in 96-well culture plates (for IL-2, IL-10 and IFN-γ) or in anti-IL-4 antibody-precoated plates (for IL-4) for 36 hr. Data are expressed as mean ± SD of triplicate cultures from pooled lymph node cells of two mice per group. Detection limit of each assay was ≈0·2 U/ml for IL-2, 10 pg/ml for IFN-γ, 10 pg/ml for IL-4, and 20 pg/ml for IL-10.

state induced by i.p. injection of PEG–HEL was mediated by regulatory cells with a capacity of active suppression.

Distinct tolerogenic property of PEG–HEL from deaggregated HEL

Water-solubility of proteins can be increased by coupling with PEG which has hydrophilic properties.[29] In addition, soluble proteins are reported to be tolerogens, whereas aggregated proteins are strong immunogen.[14] Thus, it was possible that conjugation of HEL with PEG increased its water-solubility, and this in turn augmented its intrinsic tolerogenicity. We next treated mice with consecutive, weekly injections of deaggregated HEL or PEG–HEL and anti-HEL IgG levels in their sera were monitored. Native HEL stimulated antibody production when given without adjuvants (Fig. 5a).

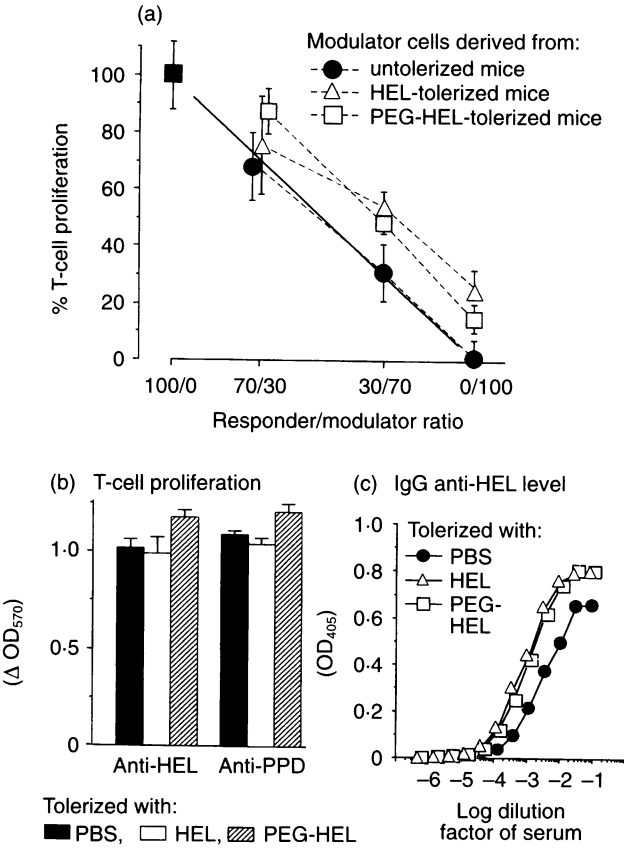

Figure 4. Lack of suppressor cell activity in spleen and lymph node cells from mice tolerized with PEG–HEL and native HEL. (a) Untolerized, HEL-primed lymph node cells (responder cells) were mixed with HEL-unresponsive cells which possibly had suppressor activity (modulator cells), i.e. lymph node cells from HEL-tolerized and HEL-immunized mice (△), PEG–HEL-tolerized and HEL-immunized mice (□), or untolerized mice immunized only with FCA (●). Each well contained total of 5×10^5 cells, and the ratios of responders to modulators were varied, as indicated by the abscissa. Percentage T-cell proliferation was calculated as [Δ OD_{570} of test wells: containing responders and modulators/mean Δ OD_{570} of wells composed of 100% responder cells (■)]. The solid line indicates the anticipated result when no modulating potential is present in the cell preparations. (b) and (c) Groups of two mice were i.p. injected with PEG–HEL, HEL, or PBS on days −22, −15 and −8. On day 1, spleen cells were obtained and transfused i.v. into groups of two naive mice, at ≈10^8 cells per mouse. Twenty-four hours after adoptive transfer, the mice were immunized with HEL in FCA. Lymph node T cell proliferation (b) and serum anti-HEL IgG activity (c) were evaluated on day 9, as described for Fig. 1.

Deaggregation of HEL by ultracentrifugation did not significantly alter the immunostimulatory activity of native HEL (Fig. 5b). In contrast, injection of PEG–HEL did not stimulate production of the antibody (Fig. 5c). Furthermore, these mice injected with PEG–HEL became unable to produce anti-HEL antibody when they were subsequently given consecutive i.p. injections of the immunogenic native HEL without adjuvants. This observation also confirmed the unique tolerogenic property of PEG–HEL regarding the humoral immune response, and this property of PEG–HEL was unlikely to be due to increased water-solubility acquired by PEG-conjugation.

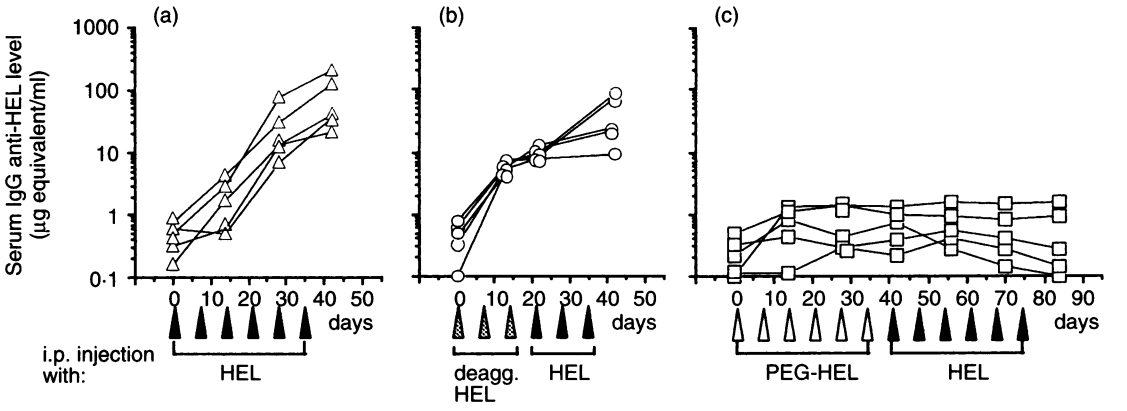

Figure 5. Serum IgG antibody levels to HEL in mice given consecutive, weekly injections of native HEL, deaggregated HEL, or PEG–HEL, without adjuvants. (a) Five mice were given six consecutive i.p. injections with native HEL (closed arrows) every week. (b) Five mice were injected with freshly deaggregated HEL (dotted arrows) three times, followed by injections with native HEL (closed arrows). (c) Five mice were given six consecutive weekly injections with PEG–HEL (open arrows), followed by injection with native HEL (closed arrows). Each preparation was adjusted to contain 0·2 mg/ml of HEL in PBS, and 1 ml of the solution was i.p. injected into each mouse at each injection without adjuvants. Sera were obtained at various time points and the levels of IgG antibodies to HEL were determined in ELISA. A standard curve was generated using an anti-HEL mAb and the data are expressed in arbitrary ELISA units.

DISCUSSION

The most important difference between PEG-conjugated and unconjugated HEL found in this study may be that the consecutive administration of native antigen without adjuvant, which is tolerogenic for T-cell responses, indeed triggered a systemic antibody response, while PEG–HEL administration suppressed antibody production upon subsequent challenge with the immunogenic native protein. The unresponsiveness in humoral immune response induced by PEG-conjugates was specific for the antigen, since mice pretreated with a PEG-conjugate produce specific IgG antibody as well as T-cell proliferation to another antigen irrelevant to the tolerogen; this is the same as naive mice, when the mice were challenged with the second antigen in FCA. PEG needed to be covalently attached to the proteins for suppression of the antibody production, since a mixture of free PEG and a protein suppressed only the T-cell proliferative response but not the antibody production, as was seen with the protein alone. These findings were common for PEG-conjugated CII[23] and HEL (unpublished observations).

The administration of soluble proteins prior to challenge immunization, parenterally or orally, suppresses subsequent Th1-type responses, including IgG2a and IgG2b antibodies that depend on cytokines produced by Th1, but spares Th2-type responses, including IgG1 antibodies.[11,12,30] In our present experiments, the capacity of mice pretreated with native proteins to mount the T-cell proliferative response as well as IgG2a and IgG2b antibody production following subsequent challenge immunization was suppressed, while IgG1 antibody production was little affected. These observations were much the same as previously noted in association with acquired tolerance, using different native soluble proteins.[11,12,30–32] Other investigators[13,33,34] found that both Th1- and Th2-type responses can be tolerized. However, most researchers are in agreement that the Th2-response is more resistant to tolerization than is Th1.[11,13,34]

Using PEG-conjugated proteins, different states of acquired tolerance have been suggested by Sehon and colleagues.[21,22,35] The IgE antibody response, which is dependent on IL-4, a Th2-cytokine,[27] was suppressed by PEG-conjugated proteins. In our present and previous[23] studies, production of specific antibodies of IgG1 isotype, also requiring IL-4,[27] was suppressed in mice pretreated with PEG-conjugated proteins, but not in mice treated with native proteins. It is therefore suggested that chemical modification with PEG alters the tolerogenic property of proteins. Parallel results obtained using both a globular protein (HEL) and a fibrous protein (CII) conjugated with PEG in different strains of mice suggest that the unique tolerogenicity is the general property of PEG-conjugated proteins. The IgG subclass profiles are in accordance with the cytokine profiles examined in the present study. Production of IFN-γ by lymph node and spleen cells in response to HEL *in vitro* was markedly suppressed in both pretreated groups, while suppression of IL-4 was noticeable in spleen cell cultures from PEG–HEL-, but not from HEL-pretreated mice. Although increase in Th2-cytokine production was not evident in HEL-pretreated mice, the elevated IgG1 antibody in these mice indicates that the repeated i.p. injections of HEL without adjuvant induce a Th2-type immune response prior to challenge-immunization with the antigen in FCA. Therefore, we propose that administration of native soluble proteins causes an immune deviation toward Th2-dominance, as has been previously suggested,[11,12] and that this may play an important role in the subsequent suppression of Th1-type responses. On the other hand, treatment with PEG–protein conjugates may simultaneously tolerize both Th1- and Th2-type responses.

Sehon and colleagues[22,35] emphasized the role of regulatory T cells, namely $CD8^+$ Ts, in establishment and maintenance of the tolerance induced by PEG-conjugated proteins, but why PEG-conjugates activate Ts has not been discussed. In our experiments, lymph node cells obtained from PEG–HEL-pretreated and HEL-challenged mice did not actively suppress responses of HEL-responsive T cells obtained from non-

pretreated, HEL-challenged mice, and the tolerant state was not passively transferred by spleen cells from the PEG–HEL treated mice to naive mice, as well as from HEL-treated mice. We have also found that suppressor activity was not observed in lymph nodes on day 9 when mice were challenged with PEG–HEL in FCA.[20] Thus, so far, we have no evidence indicating that PEG–HEL activates suppressor cells. The possibility still remains that Ts may be involved in maintenance of the tolerance state, or in the induction of tolerance by other treatment regimens. Cellular and molecular mechanisms involved in the unique tolerance state induced by PEG-conjugated proteins are the subject of ongoing study.

The distinct tolerogenic properties of PEG-conjugated proteins observed in our study may be better explained by changes in chemical properties rather than Ts involvement. PEG-conjugation increases resistance to proteolytic degradation and decreases plasma clearance rates,[17,18,20,36] properties considered to be important for tolerogenicity of proteins, as described by Weigle *et al.*[13,14] Therefore, one might assume that PEG-conjugation enhances the intrinsic tolerogenicity of proteins, then the PEG-conjugates exhibit tolerogenicity similar to the deaggregated HGG that suppressed both Th1- and Th2-responses in their study.[13] However, the immunostimulatory property of native HEL was not altered when using the same centrifugation procedure for deaggregating HGG. Thus the immunogenic property of HEL and tolerogenic property of PEG–HEL were, at least, not caused by their differential water solubility. These authors[13] also argue against a different tolerance state reported by other investigators induced by injection with native proteins (i.e. the suppressed Th1- and spared or increased Th2-type responses)[11,12] would be caused by contamination of endotoxin in protein preparations used as tolerogens). Endotoxin promotes production of inflammatory cytokines, such as IL-1, that cancels tolerance induction.[37] When we measured endotoxin levels in our various samples, the level in the HEL preparation (less than 1 endotoxin unit/mg HEL) was much lower than other commercially available preparations of purified proteins (range from 10 to 50 U/mg protein), and the level in our previous CII preparation (27 U/mg protein) was similar to that of other proteins. In addition, PEG-conjugation did not alter the contents of endotoxin in our protein preparations. The possibility of endotoxin-involvement in the distinct tolerogenicity of PEG-conjugated HEL and CII from the unmodified counterparts can be excluded.

Despite numerous reports describing acquired tolerance induction with native proteins, related mechanisms *in vivo* remain to be elucidated. In addition, there is limited information on the chemical properties required for tolerogenicity. PEG-conjugated proteins may offer a tool useful for elucidation of these factors. It remains to be determined whether the PEG-conjugation enhances the tolerogenicity of native proteins, or if it creates a novel immunological property on the proteins. It is of particular interest as to how PEG-conjugated proteins suppress Th2-responses. Other strategies to induce tolerance using native antigens and by blockade of the B7/CD28 co-stimulatory signalling,[4,6] both of which preferentially suppress Th1-type immune responses, are unlikely to have implications for some allergic diseases mediated by IgE that depend on a Th2-cytokine (IL-4).[27] A recent report, however, has shown that blockade of the CD40/CD40 L pathway, in addition to the CD28/B7 pathway, in combination, successfully attenuates Th2-type responses.[8] In conclusion, our present study suggests a possible therapeutic potential of a PEG-conjugated protein which may be superior to the native protein as a tolerogen for manipulating immunological disorders mediated by Th2-type responses that are considered to be more refractory than Th1.

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