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Prevention of collagen-induced arthritis (CIA) by treatment with polyethylene glycol-conjugated type II collagen; distinct tolerogenic property of the conjugated collagen from the native one

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SUMMARY

Administration of a soluble protein into animals prior to challenge immunization induces immunological tolerance which is specific for the protein. In addition, chemical modification of proteins with polyethylene glycol (PEG) has been reported to convert the immunogenic proteins to become tolerogenic. However, differences in tolerogenic properties between PEG-modified proteins and the native counterparts have never been analysed. The ability of PEG-conjugated type II collagen (PEG-CII) to attenuate CIA, an animal model for rheumatoid arthritis, was compared with the native unconjugated CII. Groups of DBA/1 J mice were treated weekly with i.p. injections with PEG-CII, native CII, or vehicle alone for 3 weeks, before they were challenged with CII in adjuvants. The induction of tolerance was confirmed in both PEG-CII- and CII-pretreated mice when suppression of lymph node T cell proliferation in response to CII was noted. The degrees of suppression of T cell proliferation were comparable between the two pretreated groups. However, induction of arthritis and production of IgG anti-CII antibody were more markedly suppressed in PEG-CII-pretreated mice than in native CII-pretreated mice, although the severity of arthritis and antibody levels in the latter group were also lower than in control mice. IgG2a and IgG2b antibody levels were equally suppressed in the two pretreated groups, whereas the IgG1 level was significantly lower in the PEG-CII-pretreated group than in the native CII-pretreated group. The results provide the first evidence that attachment of PEG to CII renders the protein more tolerogenic.

Keywords polyethylene glycol type II collagen collagen-induced arthritis tolerance

INTRODUCTION

The desired therapeutic strategy to control autoimmune diseases is to induce antigen-specific tolerance, rather than antigen-non-specific immunosuppressive treatments that are currently being used. It is well known that tolerance states can be induced in adult animals by either simple parenteral [1,2] or oral [3] administration of soluble antigens. However, the underlying mechanisms are still not fully understood and are a subject of much current interest.

CIA, an animal model of human rheumatoid arthritis (RA), is induced in susceptible strains of mice and rats by immunizing them with native type II collagen (CII), and is considered to be a useful model for evaluating biological-based therapies intended for human RA (see [4] for a review). CII-specific tolerance has been shown to occur in adult animals either by parenteral [5–8] or oral [9,10] administration of the protein, and to be effective in suppressing the induction of CIA induced by subsequent immunization with CII.

Polyethylene glycol (PEG), a straight amphiphilic polymer, is attached to several xenogeneic proteins and the conjugates are administered as therapeutic agents, since the chemical modification prolongs survival of the proteins in the body fluids and lowers the antigenicity and/or immunogenicity [11–17]. We have previously shown that PEG modification abrogates the immunogenicity of hen egg-white lysozyme (HEL), since the PEG conjugate fails to be presented to T helper cells [17]. Other investigators have reported an additional immunological change in proteins due to the PEG modification; proteins could be rendered not only non-immunogenic but also tolerogenic [18,19]. However, the necessity or benefit of the PEG conjugation of antigens to induce tolerance has been left undetermined, since, as mentioned above, unconjugated native proteins are also capable of inducing tolerance when the treatments are properly scheduled [1,2,20,21]**.** It is therefore important to compare the tolerogenic activity of PEG–protein conjugates with that of the native counterparts. In addition, most of the studies of PEG conjugates were intended for prophylaxis of allergic diseases or for therapeutic use of xenogeneic enzymes in patients suffering from deficiency in the enzymes, and only a few [22,23] have been intended for autoimmune disorders.

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In the present study, the ability of PEG-conjugated CII to prevent CIA was compared with the native one. We found that induction of arthritis and anti-CII antibody production following challenge immunization with native CII were more markedly suppressed in mice pretreated with PEG-CII than in mice pretreated with native CII.

MATERIALS AND METHODS

Animals

Male DBA/1 J mice were obtained from Seiwa Experimental Animals (Fukuoka, Japan) and immunized at 10 weeks of age.

Antigens

Bovine CII (3 mg/ml in 10 mM acetic acid) was purchased from Collagen Research Centre (Tokyo, Japan) and used without further purification. Coupling of CII with PEG was performed as previously described for HEL [17]. The solution of CII was diluted six-fold in 0·1 M sodium tetraborate buffer to give a final concentration of 0·5 mg/ml at pH 9·2. Cyanuric chloride-activated monomethoxyPEG (mol. wt 5000 on average; Sigma Chemical Co., St Louis, MO) was dissolved at 20 mg/ml in the tetraborate buffer and mixed with the CII solution at a volume ratio of 1:5 (PEG to CII). The mixture was incubated overnight at 4°C with gentle agitation, then extensively dialysed against PBS to remove unconjugated free PEG molecules and stored at −20°C. Coupling was monitored by SDS–PAGE, where PEG-CII showed a mol. wt > 200 kD, while unmodified CII was about 100 kD. The average number of PEG molecules attached to one CII molecule was determined by measuring the amount of free amino groups in the CII molecule before and after the coupling, using trinitrobenzenesulfonate [24]. Conformation of CII, before and following chemical modification, was assessed by circular dichroic (CD) spectroscopy, as described for type I collagen [25].

Tolerance induction

Mice were injected intraperitoneally with native CII or PEG-CII (each contained 0·2 mg CII in 1 ml PBS) on days −21, −14, and −7 relative to the challenge immunization with CII in Freund's complete adjuvant (FCA) on day 0. Control mice were injected with PBS.

Assessment of T cell proliferation in vitro

Lymph node T cell proliferation assay was performed as described [17,26]. Mice pretreated as above were immunized intracutaneously in both hind footpads with 100 μg CII in 0·1 ml emulsion with FCA (Difco Labs, Detroit, MI) on day 0 (7 days after the third i.p. injection with PEG-conjugated or unconjugated CII). In some experiments, mice were challenged with a mixture of CII and HEL in FCA (100 μg and 50 μg, respectively). Five-times recrystallized HEL was used as a control antigen which was irrelevant to the tolerogen CII. Mice were killed on day 9 and draining lymph node cells were obtained. The cells (5×10^5 cells/well) were cultured in 96-well microtitre plates with or without CII, HEL, or purified protein derivatives (PPD; of *Mycobacterium tuberculosis* H37Ra; Kainosu Inc., Tokyo, Japan) in Dulbecco's modified Eagle's medium (DME; Sigma) containing 1% heat-inactivated pooled mouse serum. Cultures were set up in triplicate from pooled lymph node cells consisting of two mice per group. Proliferation was measured after 96 h using a colourimetric assay based on tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) [17,27]. Data are presented as mean optical density $(OD)_{570}$ and s.d. of triplicate cultures with background values (cultured without antigens) subtracted (Δ OD_{570}).

Induction of CIA and clinical evaluation of arthritis

CIA was induced in DBA/1 J mice as previously described [28]. One week after the third i.p. injection for tolerance induction, mice were challenge-immunized intracutaneously with native CII (150 μg/mouse) emulsified in FCA into the left footpad (day 0). After 3 weeks (on day 21), mice were subcutaneously injected at the base of the tail with the same dose of native CII emulsified in Freund's incomplete adjuvant (FIA). Observations were carried out three times a week for the presence of distal joint swelling and erythema. Mice were graded for severity of arthritis as previously described [28] according to the method originally developed for scoring adjuvant arthritis in rats [29]. Lesions of extremities distal to the elbow or knee were graded on a scale of 0–4. Since the left foot that was used for immunization was excluded from the evaluation, the maximal score per mouse was 12. A mouse was considered arthritic when the symptom was observed on consecutive measurement dates in, at least, one paw.

Measurement of serum antibody levels

Serum antibody levels to CII were assessed by ELISA. Wells of microtitre ELISA plates (Nunc, Roskilde, Denmark) were coated with 50 μl CII (5 μg/ml in sodium carbonate buffer, 0·1 M, pH 9·5). Blocking was done with 2% non-fat dry milk in PBS. Diluent for sera and other antibody reagents was PBS containing 0·05% Tween-20 (PBS–T) and 2% non-fat dry milk, and washing buffer was PBS–T. Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG (γ-chain specific) was used for evaluating total IgG antibody levels. Colouring was obtained using *p*-nitrophenyl phosphate (Wako Pure Chemicals, Osaka, Japan) as an enzyme substrate, and absorbance at 405 nm (OD_{405}) of each well was

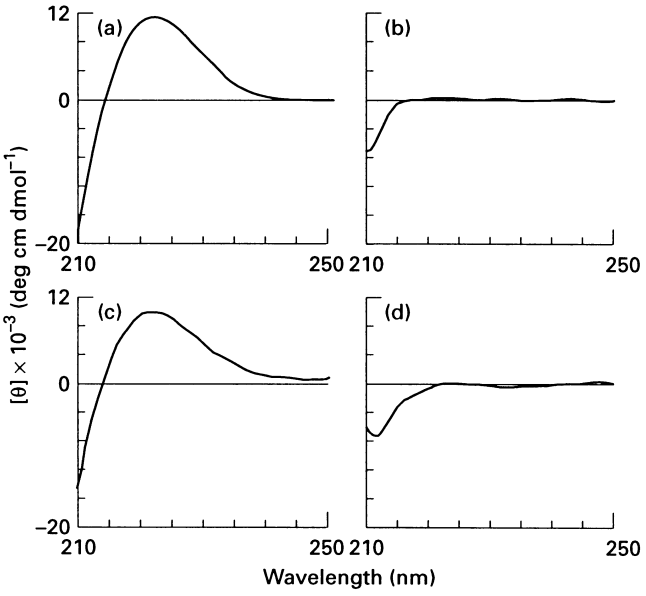

Fig. 1. Circular dichroic (CD) spectra of type II collagen (CII) and its derivatives. CD spectra of unmodified native CII (a), unmodified CII denatured in the presence of 6 M guanidium-HCl (b), polyethylene glycol (PEG)-CII (c), PEG-CII in the presence of 6 M guanidium-HCl (d), were measured at 20°C using 0·6 μM protein in 0·05 M glycine-HCl buffer (pH 3).

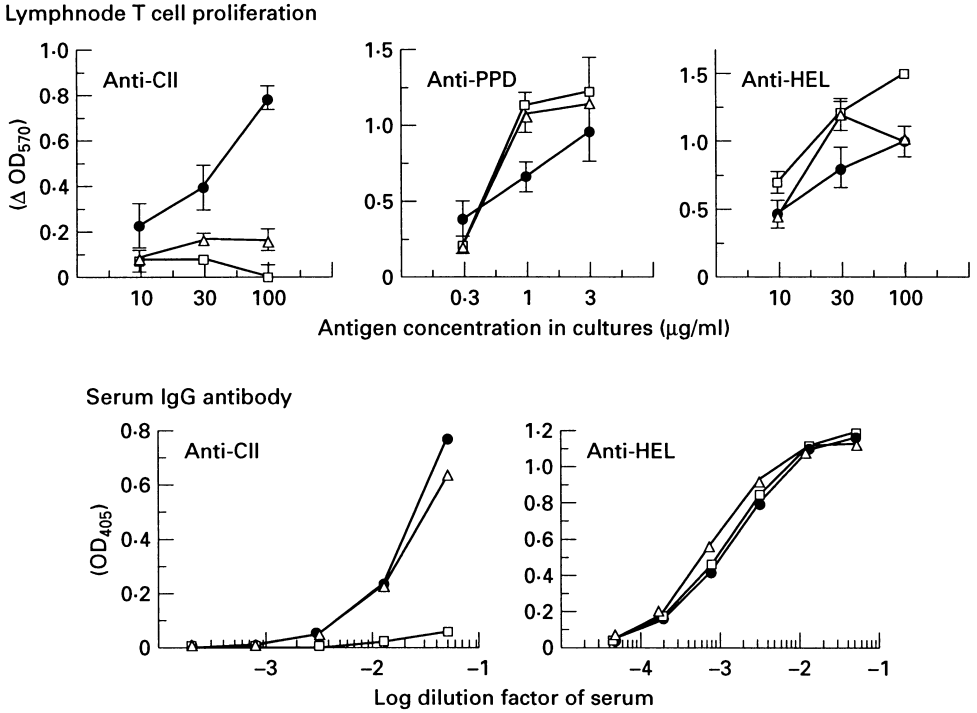

Fig. 2. Proliferative responses of lymph node T cells and serum antibody levels to type II collagen (CII) in mice pretreated with polyethylene glycol (PEG)-CII or CII and challenged with CII plus hen egg-white lysozyme (HEL) in Freund's complete adjuvant (FCA). Mice pretreated with native CII (Δ), PEG-CII (□), or PBS (●) were immunized with a mixture of CII (100 μg) and HEL (50 μg) in FCA. Nine days later, draining lymph node cells and peripheral blood were harvested. The cells were cultured in the presence of CII (upper left), purified protein derivative (PPD) (upper middle), HEL (upper right), or no antigen in Dulbecco's modified Eagle's medium (DME) supplemented with 1% mouse serum. Cultures were set up in triplicate from pooled lymph node cells of two mice per group. Number of live cells per well was evaluated by MTT assay 4 days later. Data are expressed as mean OD_{570} and s.d. of triplicate cultures with background values (cultured with no antigen) subtracted (Δ OD_{570}). Serum IgG antibody levels to CII (lower left) and to HEL (lower right) 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. It was confirmed, in preliminary experiments, that immunization with FCA alone (with no antigen) primed the lymph node cells to proliferate *in vitro* in response only to PPD, but not to CII or to HEL. The FCA-immunized mice did not produce detectable levels of serum antibodies to all these antigens.

recorded. Total IgG antibody levels of the individual mouse at days 0, 21, and 36 were normalized by relating the OD values to a standard titration curve obtained from serially diluted standard serum, which is pooled serum derived from CII-hyperimmunized DBA/1 J mice; the titration curve was obtained by plotting the OD values against the $\log_{10}$ of the dilution, and values along the linear portion were used to fit a linear equation of the regression. Mean OD value of the triplicate ELISA of each mouse serum (1 : 100 or 1 : 500 diluted) was related to the standard curve, and the result was expressed as ELISA units (EU), which means antibody activity relative to the reference standard serum that was regarded as 1 EU. Antibody levels of the four IgG subclasses were evaluated using rabbit antibodies specific for each immunoglobulin isotype and AP-conjugated goat anti-rabbit IgG, and data were expressed by OD_{405} values. All immunological reagents were purchased from Zymed Labs (South San Francisco, CA).

Statistical analysis

Arthritis indices were expressed as means ± s.e.m., and the comparison was performed by unpaired Student's *t*-test. Incidence of arthritic mice or that of arthritic limbs was compared with the χ^2 test. Comparison of antibody levels was performed using the Mann–Whitney *U*-test. $P < 0{\cdot}05$ was considered significant.

RESULTS

Chemical characterization of PEG-CII

The conjugate used in this study was found to contain 15·7 PEG molecules per α_1 chain of CII, thus about 47 PEG molecules were attached to one trimeric native CII molecule consisting of three identical α_1 chains. Among the 38 possible modification sites (37 ϵ-amino groups derived from lysine residues and one α-amino group per α_1 chain), 41·3% were modified. Since the higher order structure of native CII is critical for its ability to induce CIA [30,31], and is probably required for inducing immunological tolerance [9], we evaluated the conformation of CII before and after the conjugation with PEG (Fig. 1). The CD spectrum obtained from the conjugate was indistinguishable from that of unmodified native CII. This observation suggests that conjugation with PEG does not alter the native CII conformation. The spectra obtained from unmodified and PEG-conjugated CII in the native or in the denatured states were in agreement with those previously reported for type I collagen [25].

States of immunological tolerance induced by PEG-CII and native CII

To determine whether an antigen-specific tolerance was established, mice preinjected intraperitoneally with native CII or with

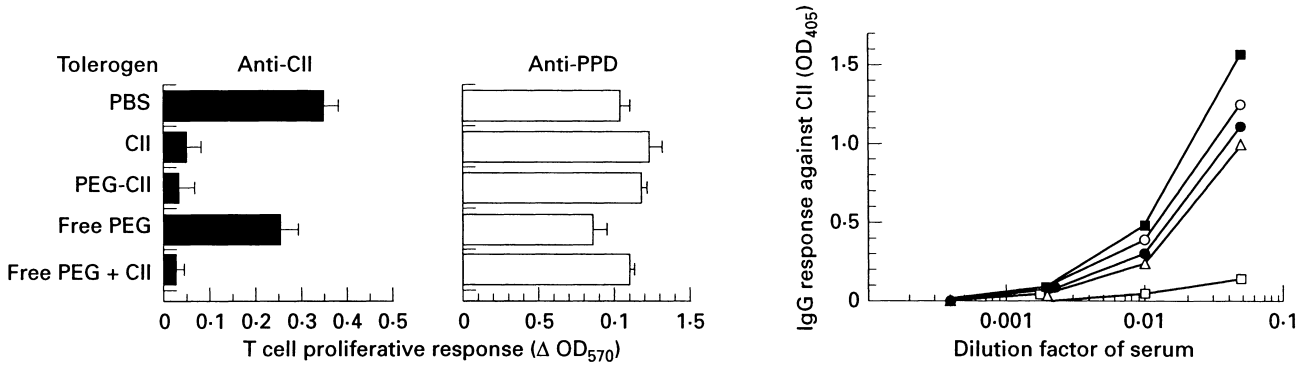

Fig. 3. Comparison of tolerogenic properties between type II collagen (CII) coupled with polyethylene glycol (PEG) and CII mixed with free PEG. Mice pretreated with native CII, PEG-CII (both contained 200 μg CII), unconjugated free PEG (2 mg), or a mixture of CII and free PEG (200 μg and 2 mg, respectively) were challenge-immunized with CII in Freund's complete adjuvant (FCA). T cell proliferation in response to CII (left, 100 μg/ml CII in cultures) or to purified protein derivative (PPD) (middle, 10 μg/ml in cultures), and serum IgG anti-CII levels (right) were evaluated as described in the legend to Fig. 2. ●, PBS; △, CII; □, PEG-CII; ○, free PEG; ■, free PEG + CII.

PEG-CII were immunized with native CII plus HEL in FCA. Nine days after the challenge-immunization, lymph node cells were harvested and cultured in the presence of CII, PPD, or HEL (Fig. 2, upper). Proliferation of lymph node T cells in response to CII was suppressed in mice pretreated either with PEG-CII or with native CII, while their responsiveness to PPD or to HEL was unaffected. Thus, antigen-specific tolerance was induced both in native CII- and in PEG-CII-pretreated mice. In contrast to the T cell responsiveness, suppression of serum IgG antibody production was apparent only in PEG-CII-pretreated mice (Fig. 2, lower). Native CII-pretreated mice had an equivalent level of the antibody compared with control mice pretreated with PBS on day 9. Anti-HEL antibody levels were equally elevated in all groups of mice. These results suggest that the suppression of antibody response to CII in PEG-CII-pretreated mice was also antigen-specific. Antibodies to *M. tuberculosis* cells or to PPD were undetectable in sera of any mice. When mice were pretreated with a mixture of free PEG and CII, responsiveness to CII was much the same as that observed in unconjugated CII-pretreated mice; the two groups of mice showed a significantly reduced T cell response and a conserved anti-CII antibody response on day 9 (Fig. 3). Pretreatment with free PEG alone had a marginal effect on the immune responses. The results suggest that CII conjugated with PEG, but not CII mixed with PEG, has different tolerogenic capacity from CII alone.

Induction of CIA in PEG-CII- and CII-pretreated mice

DBA/1 J mice pretreated with PEG-CII or native CII, and control mice pretreated with PBS were tested for their susceptibility to CIA. The disease could be induced in all eight control mice following the booster immunization, within 2 weeks. PEG-CII-pretreated mice exhibited a marked resistance to induction of CIA. As shown in Fig. 4, suppression of both severity and incidence of CIA were more marked in this group than in the group pretreated with native CII (e.g. PEG-CII-treated *versus* CII-treated on day 43, mean arthritis index: $0{\cdot}25 \pm 0{\cdot}71$ *versus* $1{\cdot}7 \pm 1{\cdot}7$, $P = 0{\cdot}039$; incidence of arthritic mice: 1/8 *versus* 8/10, $P = 0{\cdot}0044$; incidence of arthritic limbs: 2/24 *versus* 12/30, $P = 0{\cdot}0083$), although significant suppression of CIA was also observed in the latter group compared with the control group. The native CII-treated group possessed less joint inflammation than the control group throughout the experimental period (Fig. 4a; mean arthritis index $1{\cdot}7 \pm 1{\cdot}7$ *versus* $4{\cdot}13 \pm 1{\cdot}35$, $P = 0{\cdot}0048$ on day 43). However, the incidence of neither arthritic mice nor arthritic limbs of the native CII-treated group was significantly different from that of the control group after day 36 (Fig. 4b,c). The resistance to CIA in mice pretreated

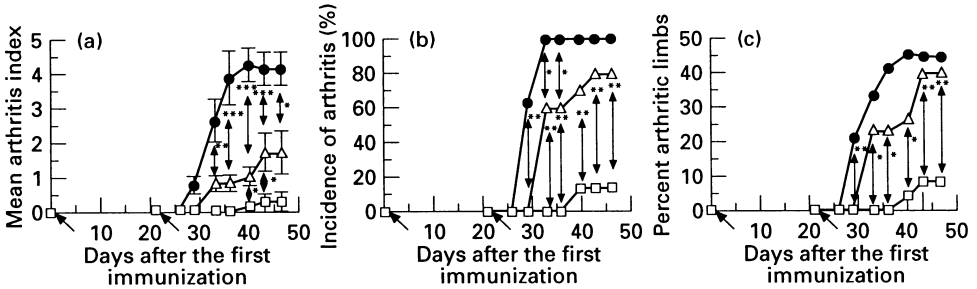

Fig. 4. Clinical symptoms of arthritis in mice injected with polyethylene glycol (PEG)-type II collagen (CII), native CII, or PBS following subsequent challenges with CII/Freund's complete adjuvant (FCA) and CII/Freund's incomplete adjuvant (FIA). DBA/1 J male mice were injected intraperitoneally with CII ($n = 10$, Δ), PEG-CII ($n = 8$, □) on days −21, −14 and −7. Control mice were injected with 1 ml PBS ($n = 8$, ●). These mice were immunized on day 0 with CII in FCA and boosted on day 21 with CII in FIA (arrows), and the presence of arthritis and its severity were monitored. Data are expressed by mean arthritis index (a), incidence of arthritic mice (b), and incidence of arthritic limbs (c). Comparison of means were performed by Student's *t*-test (a), and differences in incidence were analysed using χ^2 test (b,c). *P* values: * < 0·05; ** < 0·01; *** < 0·002.

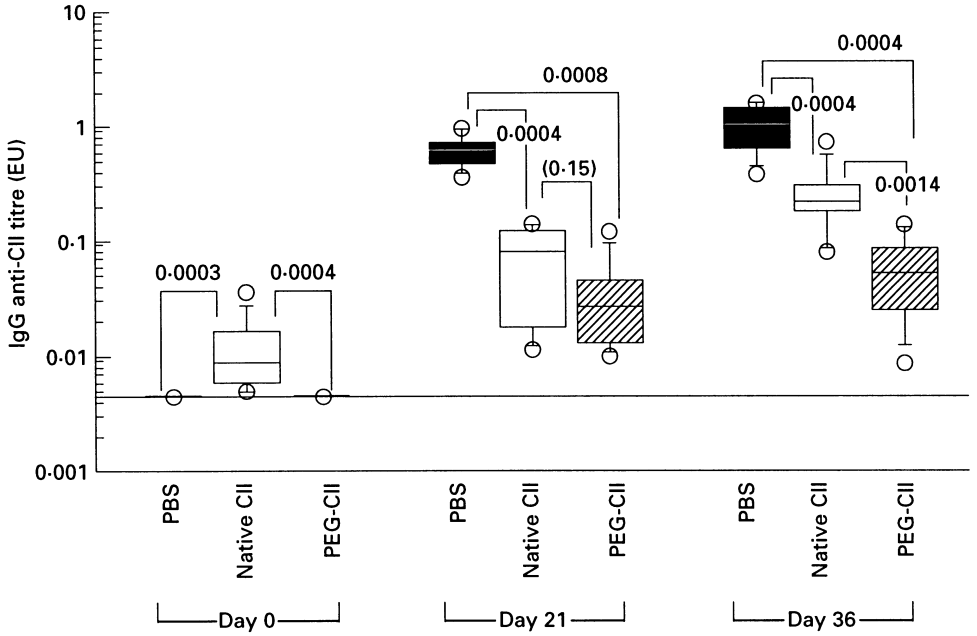

Fig. 5. Levels of IgG antibody to type II collagen (CII) during CIA induction. Sera obtained on the indicated days were analysed by ELISA. Antibody titres of individual mice was calculated and expressed by ELISA units (EU), as described in Materials and Methods. Box plots of EU indicate the 10th, 25th, 50th, 75th, and 90th percentiles (■). ○, Maximal and minimal values of each group. Comparison between different groups was performed by the Mann–Whitney *U*-test. *P* values are indicated in the figure and statistically insignificant values are parenthesized. A long horizontal line at EU 0·0042 indicates the detection limit of this ELISA.

with PEG-CII appeared to be stable, since an additional booster injection given to the mice on day 45 was incapable of increasing the severity or incidence of arthritis by the end of observation on day 67 (data not shown).

Response of anti-CII antibody in PEG-CII- and native CII-pretreated mice

Serum IgG antibody titre to CII was positively correlated with arthritis progression (Fig. 5). Native CII-pretreated mice possessed significantly lower antibody levels than control mice on day 21 and day 36 (Mann–Whitney *U*-test, $P = 0{\cdot}0004$), while the level of this antibody was higher than in controls on day 0 before immunization ($P = 0{\cdot}0003$). PEG-CII-pretreated group possessed the lowest antibody levels among the three groups throughout the observation period. Although the difference between the PEG-CII- and native CII-pretreated groups was statistically insignificant on day 21, just before booster immunization, the antibody titre in PEG-CII-pretreated mice was significantly lower than in CII-pretreated mice on

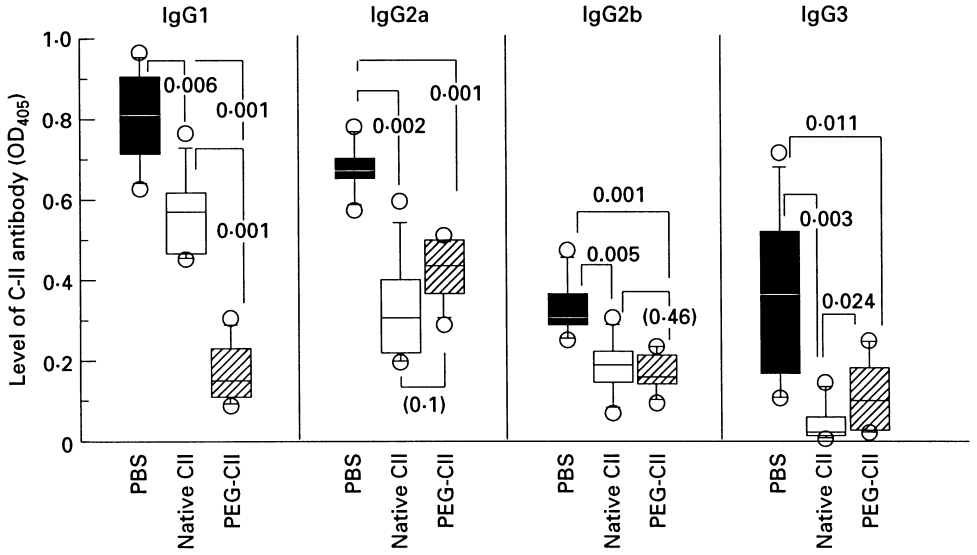

Fig. 6. Levels of IgG subclass antibodies to type II collagen (CII). Antibody levels in individual mouse sera obtained on day 36 were measured by ELISA in an IgG subclass-specific manner. Results are expressed by optical density $(OD)_{405}$ values, and shown by box plots as described in the legend to Fig. 5. Comparison between different groups was performed by the Mann–Whitney *U*-test. *P* values are indicated in the figure and statistically insignificant values are parenthesized.

day 36 ($P = 0{\cdot}0014$), when signs of arthritis became apparent in the latter group. IgG subclass antibody levels were also examined. Figure 6 shows the results obtained from sera on day 36. Suppression of all four IgG subclass antibodies was apparent in the two pretreated groups compared with the control group ($P < 0{\cdot}01$, except PBS *versus* PEG-CII in IgG3: $P = 0{\cdot}011$). However, the PEG-CII-pretreated group exhibited significantly lower IgG1 levels ($P < 0{\cdot}0008$) and slightly higher IgG3 levels ($P < 0{\cdot}024$) than the native CII-pretreated group, while IgG2a and IgG2b antibody levels were equally suppressed in the two pretreated groups.

DISCUSSION

CIA is a useful model for evaluating biological-based therapies intended for human RA [4]. Both cellular and humoral immune responses towards CII, which is cross-reactive with autologous CII in the joints, play important roles in the pathogenesis of CIA [30]. Various strategies have been exploited to manipulate this disease (see [4] for a review). Antigen-specific tolerance is induced in adult animals by administration of CII before challenge immunization with the antigen, and it effectively prevents the induction of CIA [5–10].

The present study demonstrates that pretreatment with PEG-CII is more effective than native CII in preventing CIA. Suppression of IgG1 anti-CII antibody production was also more marked in PEG-CII-pretreated mice than in native CII-pretreated ones. In contrast, suppression of IgG2a and IgG2b anti-CII antibody production and that of T cell response, determined in a conventional lymph node T cell proliferation assay, occurred equally in PEG-CII- and native CII-pretreated mice. This means that antigen-specific tolerance can be induced either by unconjugated native CII or by PEG-CII, but the consequent immunological states are different.

It was previously reported that the IgE antibody response was successfully tolerized by PEG-conjugated proteins [18,32]. Production of IgE antibodies depends on IL-4 [33], a cytokine chiefly produced by type 2-helper (Th2) T cells [34]. In our present study, production of IgG1 antibody, also known to be an IL-4-dependent isotype [33], was more markedly suppressed in mice pretreated with PEG-CII proteins than in mice pretreated with the native one. In contrast, administration of native proteins before challenge immunization, either parenterally or orally, is shown to preferentially suppress subsequent Th1-type responses, i.e. IL-2, interferon-gamma (IFN-γ) production and proliferation of lymph node T cells in response to the proteins, and IgG2a antibody production, but tends to spare Th2-type responses [2,20,35]. Our finding of preferential suppression of IgG2a and IgG2b antibody production in native CII-pretreated mice was consistent with the earlier observations. Authors of those reports proposed that the administration of native soluble proteins may cause an immune deviation towards Th2 dominance. Although some researchers contradicted this idea [21,36], there is agreement that Th2 is more resistant to being tolerized than Th1 [2,21,36]. Thus, tolerance induced by native proteins may tend to be targeted on Th1, while treatment with PEG–protein conjugates may tolerize both Th1 and Th2 equally.

CIA is considered to be a disease dominated by Th1-type responses, since IgG2a is shown to be the dominant isotype among the pathogenic anti-CII antibodies [9,37], and IFN-γ accelerates the disease [38]. Blockade of the CD28/B7 interaction by CTLA4-Ig, that suppresses the Th1-, but not Th2-type responses [39,40], successfully prevents the disease [41]. However, we observed better prevention of CIA in animals tolerized with PEG-CII than in those with native CII. Thus, it appears possible that Th2 and/or IgG1 subclass antibody may have some roles in the pathogenesis of CIA, although other unknown mechanisms might be involved in the tolerant state induced by PEG-CII.

Sehon and colleagues suggest that $CD8^+$ suppressor T cells play an important role in the tolerance induced by PEG-conjugated antigens [19,32], although the mechanism by which PEG conjugates activate this type of regulatory T cells is still unknown. Alternatively, the distinct tolerogenic properties of PEG-conjugated proteins may be explained as a consequence of changes in chemical properties; PEG modification is shown to increase resistance to proteolytic degradation and decrease plasma clearance rates [11,12,42], the properties considered to be important for the tolerogenic capacity of proteins [21]. However, the precise mechanism for the different tolerogenic property of PEG-conjugated proteins remains to be elucidated, and investigation is in progress in our laboratories.

In a previous study [17] we have shown that conjugation of HEL with PEG resulted in annulment of the immunogenicity of the protein when emulsified with FCA and injected into mice as an immunogen, implying the immunologically inert property of the conjugate. However, the results of the present study clearly demonstrate an immunologically active nature of PEG-CII conjugate, which induces an antigen-specific tolerance state. This paradox is not due to the difference of proteins used in the two independent studies, but to a difference in the administration schedules, since PEG-HEL also induces tolerance, which was similar to what we report here with PEG-CII, when assessed using the same protocol (data not shown).

CII retained the native helical structure after PEG conjugation (Fig. 1). It might be of interest to address whether denatured CII coupled with PEG possesses as strong a tolerogenicity as PEG-CII used in this study. Denatured CII is theoretically equivalent to the native one, as it can be recognized by antigen-specific Th cells. Administration of denatured CII in fact renders the animals resistant to CIA by suppressing the Th1 response [7]. Therefore, CIA is presumably prevented by denatured PEG-CII, too. However, Nagler-Anderson *et al.* [9] reported that the native conformation was required to suppress CIA when CII was orally administered as a tolerogen. In a different report [43], these authors also demonstrated that chick CII in the native form, but not the denatured one, was able to protect B10.RIII mice from CIA elicited by CII from bovine. These findings indicate some additional roles of B cell epitopes on native CII in its tolerogenicity. It is therefore plausible that PEG-CII with conserved native CII conformation may have some advantages over the denatured one, especially when an application to human RA is considered.

In conclusion, the results provide evidence that the attachment of PEG to CII alters the immunological property of the protein to become more tolerogenic. The data also provide a possible therapeutic potential of PEG-conjugated antigens for manipulating autoimmune diseases.

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