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Therapeutic effects of estradiol benzoate on development of collagen-induced arthritis (CIA) in the Lewis rat are mediated via suppression of the humoral response against denatured collagen type II (CII)

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SUMMARY

The effects of estradiol benzoate (EB) on the development of anti-CII antibodies and their pathogenic potential were studied during the progress of established CIA in the rat. CIA was induced in mature female Lewis rats by two subcutaneous inoculations containing bovine native CII (BCIIn), emulsified in Freund's incomplete adjuvant. Clinical arthritis fully developed by day 18 and then EB (1 mg/kg body wt per day, diluted in corn oil (CO)) was administered intramuscularly every second day thereafter. Antibodies binding four different CIIs (bovine or rat, either native or heat-denatured) were detected in sera and joint tissue extracts by means of solid-phase ELISA. Pharmacological doses of EB (>0·2 mg/kg body wt per day) caused significant remission of established CIA 5–7 days after treatment, and selectively suppressed the production of antibodies specific for denatured CII. To evaluate the arthritogenic potential of circulating anti-CIId IgG, transfer experiments were performed. IgG anti-CIIn, purified from EB-treated CIA rats, was not arthritogenic, whereas IgG anti-denatured (CIId), purified from CO-treated CIA rats, caused severe passive arthritis. Furthermore, pretreatment with rat CIId protected against subsequent induction of CIA, and this protection was associated with suppressed antibody production against CIId. Collectively, our results indicate that antibodies specific for CIId are involved in the pathogenesis of CIA, and that oestrogen-related remission of clinical arthritis may be caused by a selective suppression of antibodies produced against degraded/denatured CII.

Keywords collagen-induced arthritis sex hormones anti-collagen antibodies

INTRODUCTION

Rheumatoid arthritis (RA), an inflammatory joint disease, is one of a number of autoimmune diseases with marked female excess in incidence and severity [1–3]. The importance of sex and sex hormones in the pathology and etiology of RA is supported by evidence of a striking clinical remission during pregnancy [3,4], exacerbation post-partum, and ameliorating effects by oral contraceptives and hormone replacement therapy in pre- and post-menopausal women [5–8]. The mechanisms responsible for the regulation of RA by female sex hormones are unclear, and may involve long-term alterations of oestrogen levels or metabolism as well as oestrogen receptor functions. CIA in mice [9] and rats [10] has many features similar to those of RA and serves as a useful model for studying the mechanisms involved in the pathogenesis of the disease in humans [11–13]. Increased severity and frequency in females resembles the human state, and oestrogen-mediated protection from subsequent CIA induction is well documented [14–17], though little is known of the effects of oestrogen treatment on fully developed rat CIA.

Immunologically, CIA is characterized by development of both cellular [18] and humoral [11,19,20] immune responses directed against heterologous and autologous CII. Thus, CIA can be passively transferred to syngeneic naive recipients by serum antibodies specific for CII [11,21–23] as well as by adoptive transfer of lymphoid cells [13], CII-specific T cell lines and clones or CII-sensitized spleen cells [18]. Oestrogen pretreatment has been shown in murine CIA to reduce the humoral response towards CII, which was positively correlated with observed reduction in disease severity [14]. This observation indicates a possible link between CIA pathogenesis, anti-CII antibody responses and oestrogen therapy. Although many studies have focused on evaluating the transition from antibodies specific for heterologous CII to those specific for autologous CII as a cause for pathogenesis in CIA and RA [24], less is known of the concomitant shift from antibodies specific for native CII (CIIn) to those specific for denatured CII (CIId).

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Rats challenged with bovine CII (BCII) emulsified in Freund's incomplete adjuvant (FIA) produce antibodies which bind to both CIIn and CIId [21–23]. Sera of RA patients at advanced stages of disease contain anti-CIIn and CIId antibodies as well [24–28]. In mice, a synergistic activity of both humoral and cellular immune responses towards CIIn and CIId was found to be crucial for CIA development [20]. Moreover, histochemical studies show that hyaline cartilage obtained from joints of RA patients contains high amounts of unwound CII (i.e. CIId), whereas tissues from healthy controls contain only CIIn [29,30]. While the production of anti-CIId antibodies may be secondary to the release of CII fragments in diseased and damaged joints, the possible involvement of these antibodies in the pathogenesis of arthritis should also be considered in light of recent studies demonstrating partial protection against CIA in rodents and monkeys by pretreatment with BCIId [31]. To investigate these issues we evaluated antibody production during the course of CIA towards four different CIIs (autologous or heterologous, either native or denatured), and studied the relationship between these responses and pathogenesis of CIA in the presence or absence of oestrogen therapy. Our findings indicate a direct relationship between antibodies directed toward autologous CIId and CIA severity, and that both disease severity and anti-CIId antibodies are dramatically reduced by oestrogen therapy.

MATERIALS AND METHODS

Animals and surgical procedures

Female Lewis rats were bred in our animal unit, and maintained in a light- and temperature-controlled facility with free access to feed (standard rodent chow) and water. Rats were used when sexually mature (about 10 weeks of age, mean body weight $192 \cdot 1 \pm 12 \cdot 4$ g). Implantation of Silastic tubes was done under chloral hydrate (0·4 mg/kg body wt) anaesthesia. Tubes (20 mm long; internal diameter 1·6 mm; Dow Corning, Valbonne, France) were filled with crystalline 17-β estradiol 3-benzoate (EB; Sigma Chemical Co., St Louis, MO), sealed with Silastic adhesive (type B; Dow Corning), and placed subcutaneously at various locations.

Collagens

BCIIn from nasal septum or articular cartilage, and native rat CII (RCIIn) from processus xiphoideus of sternalis were prepared by partial pepsin (Boehringer, Mannheim, Germany) digestion (1 : 50 enzyme:substrate ratio) in 0·5 M glacial acetic acid pH 2·5, after two consecutive pretreatments with Tris-buffered (pH 7·4) 2 M $MgCl_2$ and 4 M guanidine hydrochloride, at 4°C as previously described [10,11,13,32,33]. Further purification of extracted CIIn was achieved by DEAE-cellulose ion exchange chromatography after equilibration in neutral salt solution (0·2 M NaCl/0·05 M Tris–HCl pH 7·4), followed by a series of precipitation steps at 4°C in which CIIn was precipitated from neutral (pH 7·4) or acidic (pH 2·5) solvents by differential NaCl (3 M and 1 M, respectively) precipitation. CIIn was collected by centrifugation (20 000 *g*), dissolved in 0·5 M acetic aicd, precipitated by dialysis against 10 mM Na_2HPO_4, washed, lyophilized and stored at −20°C. The chemical purity of each CII preparation was verified by SDS–PAGE analysis [32,33]. For experiments, CIIn was dissolved in 0·05 M acetic acid (pH 3·0; 4 mg/ml) or in neutral salt solution (pH 7·4; 2 mg/ml) by stirring overnight at 4°C. CIIn was denatured by heating a CIIn solution in a water bath at 56°C for 30 min immediately before use; the gentle heating causes cleavage of interchain hydrogen bonds, so that the individual α-chains dissociate, thus revealing putative cryptic antigenic determinants.

Induction and evaluation of arthritis

Active induction. CIA was induced in Lewis rats by two identical injections, given a week apart, containing BCIIn emulsified in FIA as previously described [10–13]. Each injection was administered intradermally around the tail root (100 μl emulsion containing 200 μg BCIIn). Using this protocol, 97 ± 2% rats developed severe arthritis 5 days after the booster injection (12 days after the first antigenic challenge), which peaked about 1 week after the onset of disease (16–18 days after the first antigenic challenge). No spontaneous recovery occurred within 60 days, a time at which all rats were killed. In several experiments, rats received injections containing BCIId, RCIIn or RCIId emulsified in FIA. Dosage and regimen were identical to that used for injection of BCIIn.

Passive induction. CIA was passively induced by transfer of IgG isolated from rats inflicted with active CIA. IgG from donor rats was injected intravenously via the lateral tail vein into syngeneic recipients (1·6 mg/rat, divided into two identical injections, 24 h apart).

Severity of CIA was measured in all four paws, each of which was graded 0–4 according to the extent of periarticular erythema, oedema, joint deformity and ankylosis as described [10–13]. The sum of scores of all paws was recorded as the arthritis score (AS) for each individual rat. Results are group (at least five rats per group) averages ± s.e.m.

EB administration

Two different routes of EB administration were used. In the first, EB (1 mg/kg body wt per day, unless otherwise stated) was dissolved in 0·2 ml corn oil (CO) and injected intramuscularly every second day, into the upper hind paw muscle (left and right intermittently), while control rats were injected with the same volume of CO (vehicle) alone. In the second, EB (4 mg/rat) was implanted in Silastic tubes; control rats were implanted with empty tubes.

Antibody purification and ELISA

Anaesthetized rats were bled from the tail, and collected sera were aliquoted and stored at −20°C until use. Antibodies were extracted from hind paw interphalangeal joint homogenates in cold PBS by 0·1 M citric acid pH 3·0, followed by immediate neutralization and dialysis as previously described [34]. IgG was purified by ammonium sulphate precipitation as described [35]. Aliquots of purified antibodies were stored at −20°C, and their purity was verified by SDS–PAGE analysis [35]. Affinity purification of anti-CII antibodies from IgG was performed essentially as previously described [34]. In brief, CIIn and CIId from bovine and rat were coupled to cyanogen bromide (CNBr)-activated Sepharose 4B beads (Pharmacia, Uppsala, Sweden). After equilibration with PBS, pooled IgG samples (3 ml per 1 g of dry beads) were added and incubated overnight at 4°C with the respective CII Sepharose conjugates. Bound antibodies were eluted by 0·2 M glycine pH 2·5, collected, dialysed against PBS pH 7·4, and concentrated by Amicon

filtration (cellulose-ester molecular filtration membrane; Spectrum, CA). Aliquots of the purified antibodies were stored at −20°C. The ELISA used in the present study for measurement of anti-CII antibodies in serum and joint extracts was essentially as previously described [10,11,13,14]. Measurements of antibody levels were performed in duplicate for individual rats, and results are group averages expressed in absorption units at 405 nm ± s.e.m. Test results were normalized to standard positive and negative pooled sera that were run in duplicate for each antigen in every ELISA plate.

Statistical analysis

Continuous variables were analysed by analysis of variance with two-tailed *t*-tests between each pair of treatments.

RESULTS

EB causes remission of full blown CIA

Prompted by observations that had demonstrated marked suppression of CIA following EB pre-treatment [36,37], we evaluated the therapeutic effects of EB on female Lewis rats inflicted with full blown CIA. EB was administered 18 days after CIA was induced, either by direct i.m. injection, or via subcutaneously implanted Silastic tubes (Fig. 1a). Both routes significantly reduced CIA severity compared with control CIA rats (i.m. delivery of EB, $P < 0.01$ after 5 days and $P < 0.001$ after 7 days of treatment; s.c. Silastic tube delivery of EB, $P < 0.01$ after 9 days and $P < 0.001$ after 15 days of treatment). The effect of the s.c. route was slightly slower compared with that of the i.m. route, probably due to the time required for optimal hormone release from the implanted Silastic tubes.

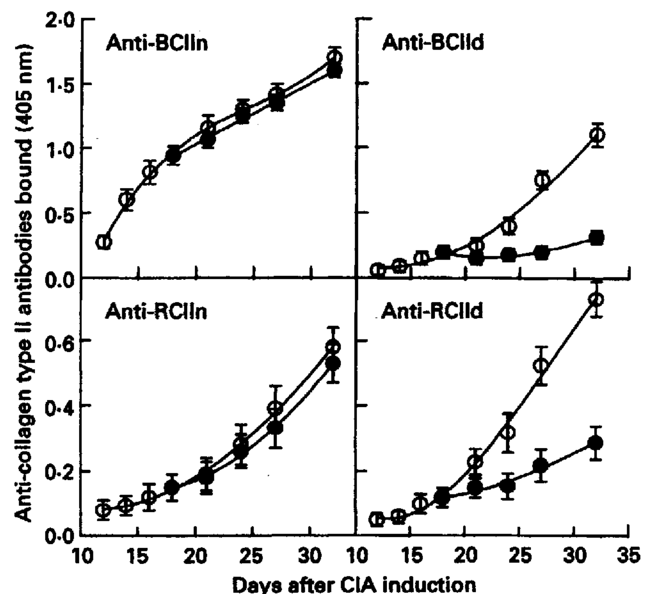

Fig. 2. Effect of 17-β estradiol 3-benzoate (EB) treatment in fully developed CIA on the humoral responses towards different CIIs. Circulating anti-CII antibodies were assayed in individual rats by means of solid-phase ELISA and plotted as a function of time. The upper panels depict anti-heterologous (bovine) CII, and the lower panels anti-autologous (rat) CII antibodies. The left panels depict anti-native CII, and the right panels anti-denatured CII antibodies. ○, Corn oil alone; ●, 1·0 mg EB/kg per day, intramuscularly. Each point is the average ± s.e.m. of at least five individual rats. Similar results were obtained in three identical experiments.

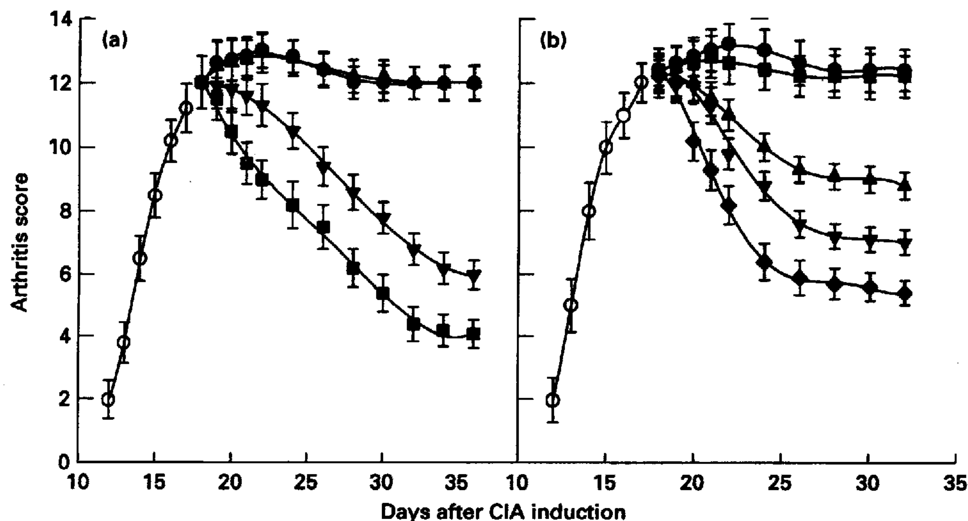

Fig. 1. Therapeutic effects of 17-β estradiol 3-benzoate (EB) treatment on the development of established CIA in female rats. (a) Routes of hormone administration. CIA was induced in mature female rats by two intradermal inoculations of bovine native CII (BCIIn; 200 μg/rat each) emulsified in Freund's incomplete adjuvant (FIA) a week apart. Eighteen days after CIA induction, only rats that had developed severe arthritis (○; 97 ± 2%) were randomly divided into four experimental groups (at least five rats per group) and treated as follows: ●, corn oil (CO) alone; ▲, empty Silastic tubes; ▼, EB administered subcutaneously in Silastic tubes; ■, EB administered intramuscularly in CO. Similar results were obtained in four identical experiments. (b) Dose response to EB *in vivo*. CIA-inflicted rats (○; at least five per group) were treated with EB as follows: ●, CO alone; ■, 0·05 mg/kg per day; ▲, 0·2 mg/kg per day; ▼, 0·8 mg/kg per day; ◆, 1·6 mg/kg per day. Similar results were obtained in three identical experiments.

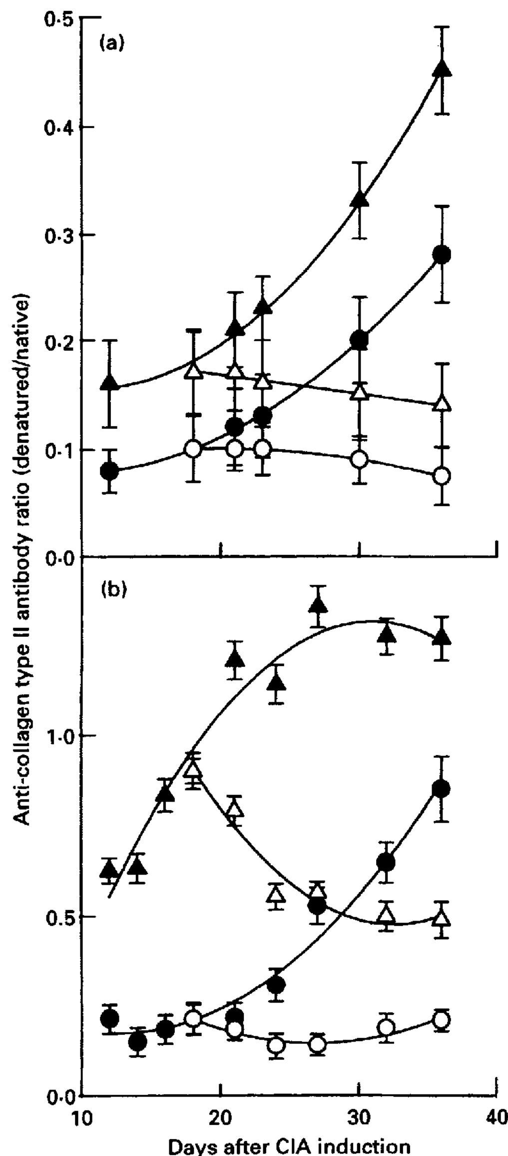

Fig. 3. The effect of 17-β estradiol 3-benzoate (EB) treatment on anti-CII antibody ratio (anti-denatured CII (CIId)/anti-native CII (CIIn)) in serum and joints. Rats inflicted with full blown CIA were divided randomly into two experimental groups (at least six per group): CIA-corn oil (CO) (full symbols) and CIA-EB (1·0 mg/kg per day, intramuscularly; empty symbols). Anti-CII antibodies from serum (b) and joint extracts (a) were assayed by means of ELISA, and two ratios were calculated and plotted as a function of time: anti-rat (R)CIId/anti-RCIIn (triangles) and anti-bovine (B)CIId/anti-BCIIn (circles). Ratios were calculated from absorbance units measured at several serum and joint extract dilutions; the results presented are from a serum dilution of 1 : 800, and from a joint extract dilution of 1 : 100. Similar results were obtained in three identical experiments.

The therapeutic effects of EB treatment on CIA severity were dose-dependent (Fig. 1b). Doses within the physiological range had negligible effects on CIA severity, whereas pharmacological dosages (0·8–1·6 mg/kg per day) induced rapid amelioration of CIA symptoms within 3–5 days of treatment. The effects of pharmacological doses of EB on CIA severity were significant (for 0·8 mg/kg body wt per day, $P < 0{\cdot}001$ after 9 days; and for 1·6 mg, $P < 0{\cdot}001$ after 5 days).

EB therapy suppresses antibodies specific for degraded collagen

Since CIA has been reported to be mediated by anti-CII antibodies [21–23], we investigated the effects of EB therapy on serum antibody titres specific for bovine (heterologous) or rat (autologous) CII, in either their native or denatured form (Fig. 2). EB therapy significantly reduced antibody titres specific for both types of denatured CIIs (BCIId, $P < 0{\cdot}01$ after 9 days and $P < 0{\cdot}001$ after 15 days; RCIId, $P < 0{\cdot}01$ after 15 days), whereas antibody levels specific for CIIn (autologous and heterologous) were not altered. A similar pattern was observed in antibody titres directly extracted from hind paw interphalangeal joints (see Fig. 3). The temporal increase in antibody levels specific for denatured rat and bovine CIIs relative to that of native CIIs is described in Fig. 3. In the absence of EB therapy, levels of antibodies specific for denatured CIIs increased exponentially relative to those specific for native CIIs. However, this increase was completely arrested by EB therapy (in joints, $P < 0{\cdot}01$ for both bovine and rat collagens 15 days after treatment; in serum, $P < 0{\cdot}001$ for both collagens 5 days after treatment). These observations suggest that the humoral response directed against denatured CII might be involved in the pathogenesis of CIA.

EB therapy reduces severity of passively induced CIA

To address specifically the relationship between EB therapy and serum antibodies, CIA was passively induced by transfer of purified IgG derived from rats inflicted with active CIA and undergoing treatment with EB (CIA-EB) or CO alone (CIA-CO). IgG from CIA-EB rats induced light and transient CIA (maximal score 1·8 by day 10, and 0 by day 15), whereas that induced by IgG from CIA-CO rats was more severe (maximal score 5·5) and incessant (score 4·2 by day 25). To investigate the direct role of IgG specific for either native or denatured CII in passively induced CIA, IgG anti-CIId and IgG anti-CIIn were purified from IgG from either CIA-CO or CIA-EB female rats, respectively, 7 days after initiation of hormonal treatment (25 days after CIA induction), and then injected into naive, age- and sex-matched syngeneic recipients (Fig. 4). IgG anti-CIIn induced light and transient symptoms, which were significantly lower than those induced by IgG anti-CIId ($P \leqslant 0{\cdot}01$ 8–23 days after the first IgG injection).

Pretreatment with denatured rat CII protects against CIA

The severity of CIA was associated with increased antibody levels specific for denatured CII, and reduction of these antibodies following EB treatment was associated with disease amelioration. These observations raised the possibility that CIId and antibodies specific for CIId were important for CIA development and could be important mediators or regulators of CIA. To address this issue, rats were challenged (two injections in FIA, a week apart) by denatured rat, denatured bovine or by native rat CIIs. These treatments did not cause CIA (not shown). To determine whether these presensitizations protected against subsequent CIA induction, all rats were exposed to BCIIn emulsified in FIA 1 week after the second presensitization, and then once again 1 week later (Fig. 5). Pre-exposure to RCIId fully protected against CIA. BCIId and RCIIn were not protective, though disease severity was lower and transient in rats presensitized to BCIId; in both cases a single exposure to BCIIn was sufficient to induce CIA, which became more severe after the second inoculation.

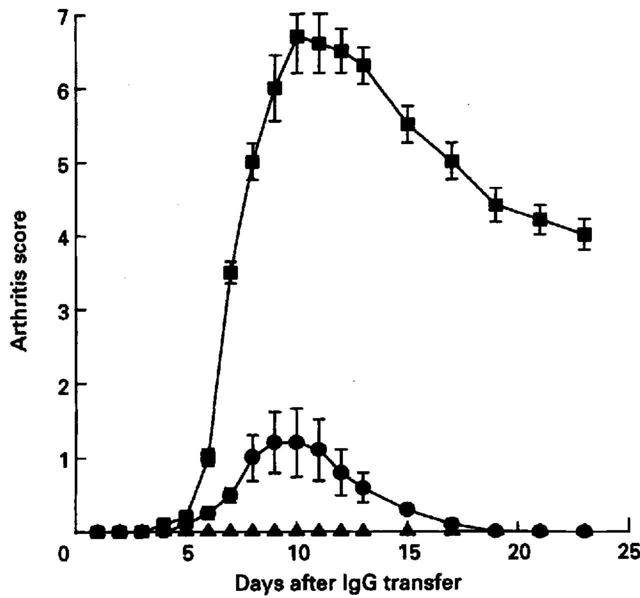

Fig. 4. Effect of IgG anti-denatured CII (CIId) and IgG anti-native CII (CIIn) transfer on the development of passive arthritis. Naive, syngeneic recipients (at least six rats/group) received i.v. injections containing affinity-purified IgG anti-CIId (■) or IgG anti-CIIn (●) (two injections, 24 h apart, containing 1·6 mg IgG each). A control group recieved IgG purified from naive rats (▲). Similar results were obtained in three identical experiments.

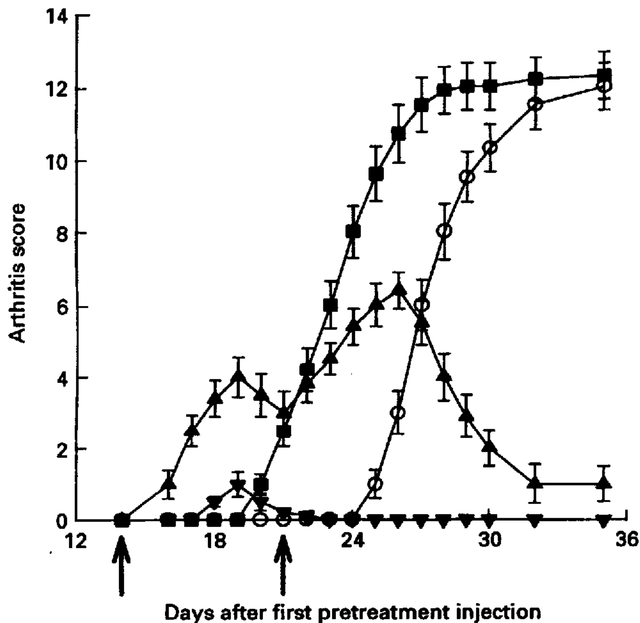

Fig. 5. Effects of pretreatment with various CIIs on the severity of subsequent CIA induced by bovine native CII (BCIIn)/Freund's incomplete adjuvant (FIA). Rats were presensitized twice with different CIIs (200 μg/rat) emulsified in FIA and 1 week after the second injection, CIA was induced by two inoculations of BCIIn/FIA (200 μg/rat). Presensitization groups (at least six rats per group): no presensitization (○); bovine denatured CII (BCIId)/FIA (▲); rat (R)CIId/FIA (▼); and RCIIn/FIA (■). Similar results were obtained in six identical experiments.

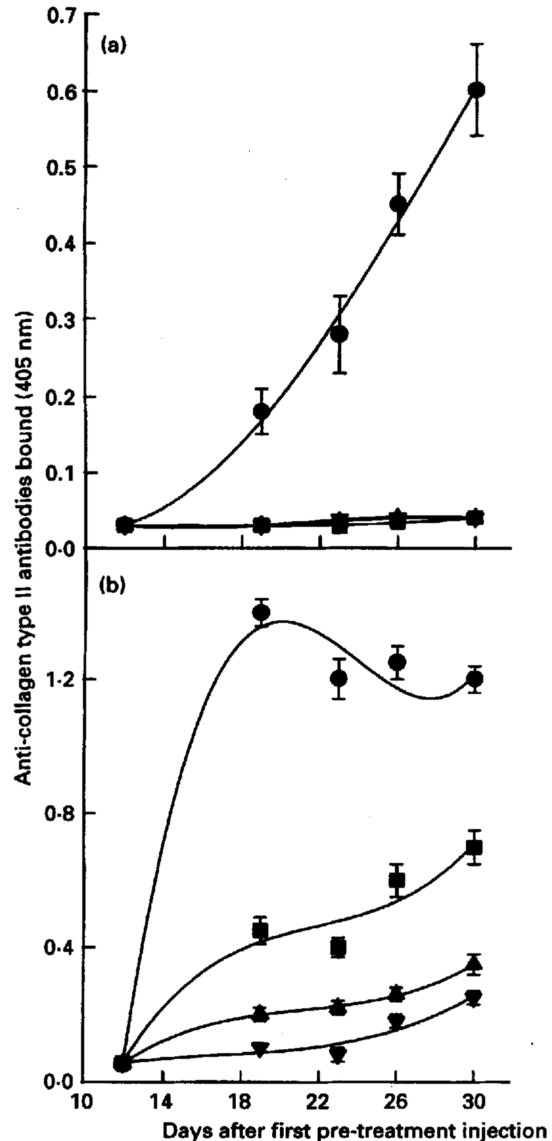

Fig. 6. Anti-CII antibody production in denatured CII (CIId)/Freund's incomplete adjuvant (FIA)-presensitized rats. Experimental conditions are as described in legend of Fig. 5. Circulating anti-CII antibodies in rats presensitized by rat (R)CIId (a) or by bovine (B)CIId (b) were assayed by means of solid-phase ELISA. CII antibodies evaluated: anti-bovine native CII (BCIIn; ●); anti-BCIId (▲); anti-rat (R)CIIn (■); and anti-RCIId (▼). Similar results were obtained in six identical experiments.

Anti-CII antibodies were determined in sera of rats presensitized by CIId and after challenge by BCIIn/FIA (Fig. 6). Presensitization by RCIId or BCIId did not lead to production of antibodies specific for CIIs (Fig. 6a,b; compare with Fig. 2). Furthermore, exposure of RCIId-presensitized rats to an arthritogenic challenge of BCIIn did not induce production of antibodies specific for RCIId, BCIId or RCIIn; the only antibodies produced in these rats bound unique epitopes on BCIIn (Fig. 6a,b; compare Fig. 2). In contrast, all antibody specificities were produced in BCIId-presensitized rats receiving an arthritogenic challenge of BCIIn, though their level was lower than that found in rats challenged by BCIIn alone

(Fig. 6b; compare Fig. 2). Thus, it appears that CIA is associated with the appearance of antibodies specific for denatured CII, and particularly with antibodies specific for rat denatured CII, and that pre-exposing rats to autologous denatured CII prevents the production of these antibodies following an arthritogenic challenge with BCIIn.

DISCUSSION

This study investigated the relationship between development of CIA in the female Lewis rat, therapeutic effects of EB on full blown CIA, and CII specificity of antibodies produced during the course of the disease. Previous studies had indicated the involvement of anti-CII autoantibodies in the pathogenesis of CIA, and in murine CIA where CIA was suppressed by estradiol pretreatment, anti-CII antibody levels were suppressed too [38]. These studies, however, did not address therapeutic effects of EB on full blown CIA, which is more germane to the human condition, nor did they study the possible involvement of antibodies specific for denatured CII in the pathogenesis of CIA.

Pharmacological dosages of EB, administered intramuscularly in an oil vehicle or subcutaneously in crystalline form encapsulated by Silastic tubes, caused significant suppression of full blown CIA, as determined by diminishing clinical symptoms. Furthermore, the same dosages suppressed anti-CIId antibody production. Dosages within the physiological range were less effective. Similar suppression of clinical symptoms by pharmacological dosages of estradiol were demonstrated in several experimental models of autoimmune diseases, such as autoimmune thyroiditis [39], experimental allergic neuritis [40], sialadenitis and vasculitis in MLR/*lpr-lpr* mice [41,42] and murine lupus [43], which together imply general anti-inflammatory effects of EB. The mechanism for this anti-inflammatory effect is presently obscure, but the presence of high-affinity, low-capacity cytosolic receptors for oestrogen in mammalian lymphoid cells might indicate a direct modulating effect on activated immune cells [2,3].

The humoral immune response towards CII in RA and CIA is a complex phenomenon, as multiple arthritogenic and tolerogenic T and B lymphocyte epitopes were found to be distributed along the CB11 fragment of the CII molecule, and various (polyclonal) anti-CII autoantibodies were found to be produced at different stages of arthritis development, even in the same individual [44–46]. This gradual production of antibody specificities could result from gradual exposure of antigenic epitopes in CII concomitant with its inflammatory degradation. In addition to the development of the antibody repertoire during arthritis, susceptibility to CIA in monkeys was found to be directly related to the production of antibodies cross-reactive with heterologous and autologous CIIn [24], which was attributed to a repertoire shift from antibodies specific for heterologous CIIn to an antibody population specific for autologous CIIn. This observation was confirmed by the present investigation, and is extended to demonstrate an additional shift from the production of antibodies specific for CIIn to the production of antibodies specific for CIId during the course of CIA. The shift is well demonstrated by the anti-CIId/anti-CIIn antibody ratio in both serum and joints, which increased gradually with intensifying disease severity. Furthermore, the shift was completely suppressed by EB treatment, which was directly related to the amelioration of clinical symptoms. Thus, it is obvious that a high anti-CIId/anti-CIIn antibody ratio is typical of advanced stages of arthritis development and the decrease in this ratio indicates the therapeutic effect of EB treatment. No such relationship was found with antibodies specific for CIIn, which confirms previous reports that failed to demonstrate a positive correlation between protective effects of estradiol pre-treatment in rats and the humoral response towards CIIn [15]. Our results indicate that decreased circulating anti-CIId antibodies in CIA-EB rats was preceded by a parallel decrease in the accumulation of similar antibodies in joints, and confirm earlier data that showed local production of anti-CIId antibodies in the joints of RA patients [26].

The direct relevance of antibodies specific for CIId in the pathogenesis of CIA was determined by transfer of affinity-purified IgG to syngeneic recipients. IgG anti-CIId, purified from CIA rats at advanced stages of disease (high anti-CIId titres) induced passive arthritis, whereas IgG anti-CIIn, purified from CIA-EB rats (devoid of anti-CIId activity) were unable to induce the passive disease. To investigate further the involvement of anti-CIId humoral response in the pathogenesis of CIA in rats, presensitization with BCIId/FIA or RCIId/FIA was performed. Interestingly, complete protection from subsequent CIA induction was observed in rats pretreated twice with autologous CIId, whereas rats pretreated with heterologous CIId were not protected. Protection against subsequent CIA induction in RCIId/FIA-pretreated rats was characterized by totally suppressed humoral responses towards RCIIn and CIId. These results extend earlier data showing partial protection from subsequent CIA induction as a result of a single pre-treatment with heterologous CIId/FIA in rats and non-human primates [31]. Resistance to CIA induction and inhibition of autologous CII autoantibody production may be regulated by peripheral mechanisms responsible for the generation of tolerance [47]. Thus, the presentation of self epitopes in the absence of inflammatory co-stimulatory signals could down-regulate the response to autologous CII by either deletion or anergy, which would explain the absence of antibody shifts (native to degraded) following exposure of these rats to an arthritogenic challenge [47].

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