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Tolerogenic activity of polymerized type II collagen in preventing collagen-induced arthritis in rats

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SUMMARY

Rats were exposed parenterally or pergastrically to polymerized type II collagen (POLCII) and became resistant to the subsequent induction of disease with arthritogenic type II collagen (CII) administered intradermally in Freund's incomplete adjuvant (FIA). POLCII was prepared by cross-linking native soluble arthritogenic CII, from bovine nasal septal cartilage, with glutaraldehyde. POLCII injected intradermally in FIA did not induce arthritis. Animals treated in this manner were resistant for a period of at least 100 days to induced disease. The change in the properties of the CII from an arthritogen to a tolerogen was related to the amount of glutaraldehyde (used to polymerize the CII) which was assumed to control the extent of cross-linking of the CII. Highly cross-linked POLCII administered pergastrically, like soluble CII, was not arthritogenic but was tolerogenic, inducing a state of unresponsiveness to a challenge with arthritogenic CII. In general serum anti-CII antibody levels were higher in arthritic than in tolerized non-arthritic rats. It is concluded that the breaking of self-tolerance to CII depends upon its physical state. When polymerized and insoluble, a form analogous to that in which it exists naturally, it is tolerogenic.

Keywords type II collagen arthritis polymerization gut

INTRODUCTION

Collagens of articular cartilage become powerful arthritogens, when they have been solubilized by enzymic proteolysis, capable of inducing experimental systemic polyarthritides in rats (Trentham, Townes & Kang, 1977), mice (Courtenay *et al.*, 1980) and monkeys (Cathcart *et al.*, 1986). The disease induced actively with antigen involves complex interactions between B cells and T cells reactive with type II collagen (CII). Arthritic symptoms, which do not necessarily reflect all those of the active disease, have been induced passively with serum antibodies against CII (Stuart *et al.*, 1982), monoclonal antibodies against CII (Holmdahl *et al.*, 1986), CII-specific L3T4$^+$ T cells (Holmdahl *et al.*, 1985) and a 65 kD lymphokine (Helfgott *et al.*, 1985). However, resistance to subsequent disease induction has also been induced passively with serum antibodies against CII (Staines *et al.*, 1981) and CII-specific W3/25$^+$ T cells (Brahn & Trentham, 1987).

CII is not only arthritogenic and immunogenic but it is also protective and tolerogenic when administered appropriately. Resistance to CII-induced arthritis in rats has been demonstrated following intravenous injection of either soluble native CII (Staines *et al.*, 1981) or a constituent peptide (Englert *et al.*, 1984), or CII coupled to spleen cells (Schoen, Greene & Trentham, 1982). Recently, we have found that soluble CII administered pergastrically also induces immunologically specific resistance to disease induction (Thompson & Staines, 1986a, b).

Collagens exist normally in cartilage as cross-linked fibrils and are not immunogenic or arthritogenic in this form; intact cartilage is immunologically inert and may behave as an immunologically privileged site (Elves, 1974). To investigate the factors that influence the immunogenicity, arthritogenicity and tolerogenicity of CII we have chemically polymerized soluble CII.

MATERIALS AND METHODS

Animals

Inbred Wistar WA/KIR strain rats were either bred at King's College or obtained from the Kennedy Institute, Hammersmith,

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London. Young adults were housed in groups of 4–8 in solid-bottomed plastic cages with wood shavings as litter. The numbers of animals used are indicated in the text.

Preparation of type II collagen

Soluble native CII was prepared from bovine nasal septa by pepsin digestion (Staines *et al.*, 1981). Its purity was confirmed by polyacrylamide gel electrophoresis and by amino acid analysis.

Polymerized CII (POLCII)

CII was dissolved in 0·1 M ethanoic acid at 3 mg/ml and was dialysed for 3 days at 4°C against 0·1 M $NaHCO_3$ + 0·2 M NaCl pH 7·8. Volumes (7 ml) of dialysed collagen were mixed with equal volumes of glutaraldehyde solution (British Drug House, Poole, Dorset), in 0·1 M $NaHCO_3$ + 0·2 M NaCl at final concentrations ranging from 10% to 0·001%. The glutaraldehyde: CII mixtures were mixed gently by orbital oscillation for 1 h at room temperature and were then dialysed against three changes (5 litres each) of 0·1 M $NaHCO_3$ + 0·2 M NaCl for 3 days at 4°C to remove unconjugated glutaraldehyde. The amount of glutaraldehyde used in each preparation is indicated in the text in parentheses; thus, CII polymerized with 10% glutaraldehyde is referred to as POLCII(10%).

Immunization of animals

Solutions of native soluble CII (1·5 mg/ml) or suspensions of POLCII were emulsified with equal volumes of Freund's incomplete adjuvant (FIA) (Gibco, Paisley). Each animal received 0·75 mg collagen intradermally, either in 1 ml of emulsion or in 0·5 ml of solution or suspension, divided between five injection sites in the suprascapular region. Some animals subsequently received a secondary immunization with 0·5 mg CII in 0·1 M ethanoic acid intraperitoneally.

CII or POLCII were administered pergastrically by gavage on five consecutive days (up to and including the day of intradermal challenge with CII emulsified in FIA) at 2·5 μg collagen/g body weight daily. Control animals were gavaged with 0·1 M ethanoic acid.

Assessment of disease

Clinical symptoms were recorded daily during the first 3 weeks and twice weekly thereafter, assessed as described previously (Burrai *et al.*, 1985) and expressed here as numbers of animals in each group showing unequivocal signs of disease.

Serological procedures

Sera prepared from blood collected under ether anaesthesia from the tail artery were stored in fractions at −20°C and their levels of antibodies against soluble CII determined by enzyme-linked immunosorbent assay (ELISA) (Staines *et al.*, 1981; Thompson & Staines, 1986a). Titres are given as the reciprocal of the interpolated dilution giving a mid point reading (A450 = approximately 0·8) in the titration curve.

The immunoglobulin subclasses of serum anti-CII antibodies and biliary anti-CII immunoglobulin A (IgA) levels were determined in a modified ELISA using rabbit anti-rat isotype reagents (Rose, Peppard & Hobbs, 1984) detected with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin reagent (ICN Biochemicals, High Wycombe, Bucks.).

Immunohistochemical analysis of antigen uptake in the small intestine

POLCII(10%) or CII or 0·1 M ethanoic acid were either administered pergastrically by the gavage regime described above or in a single bolus of 35 mg (equivalent to 10 times the total amount given in five gavages of 2·5 μg/g body weight) administered in the same way or by direct injection into the lumen of the ileum of anaesthetized normal rats. The common bile duct was cannulated and bile samples were collected at intervals throughout the experiment. Individual animals were killed either 48 h after the last gavage or 1·5, 5, 24 or 48 h after the single bolus. Their intestines were flushed with phosphate buffered saline and Peyer's patches, ileum, mesenteric lymph nodes, spleen and brachial lymph nodes were removed and snap frozen. Absorbed POLCII and CII were detected immunohistochemically on cryostat sections (air-dried and acetone fixed) with a mouse anti-CII monoclonal antibody, 1083-16 (prepared by S. Omar, King's College London), and an anti-mouse IgG immunoperoxidase reagent (Dako, High Wycombe, Bucks.) diluted in 10% normal rat serum in Tris-buffered saline.

RESULTS

Effects of polymerization with glutaraldehyde on the arthritogenicity of CII

In control animals receiving pepsin-solubilized unpolymerized CII, intradermally in FIA, 72/91 animals (80%) developed disease. The appearance of symptoms in most arthritic animals between 11 and 14 days coincided with a sharp rise in serum anti-CII antibody activity and antibody levels were higher on average in arthritic animals than in those that did not develop clinical disease, confirming previous studies (Staines *et al.*, 1981; Henderson *et al.*, 1984; Burrai *et al.*, 1985).

Preparations of CII polymerized with 1·0% or 10% glutaraldehyde were no longer arthritogenic whereas POLCII(0·1%) and POLCII(0·01%) still induced disease, but in fewer animals. Treatment, of CII with 0·001% glutaraldehyde did not change its arthritogenicity. Non-arthritic animals remained free of clinical disease throughout the 100 day observation period. These results are summarized in Table 1.

POLCII induced lower antibody responses than CII (Fig. 1). The depression of anti-CII serum antibody levels was parallel to

Table 1. Incidence of arthritis in animals immunized parenterally with POLCII in FIA

Antigen preparation used for immunization	Animals in group	Days after immunization				
		4	10	17	42	100
CII	6	0	0	3	4	4
POLCII(0·001%)	6	0	0	2	5	5
POLCII(0·01%)	6	0	0	0	2	2
POLCII(0·1%)	7	0	0	2	4	4
POLCII(1·0%)	7	0	0	0	0	0
POLCII(10·0%)	7	0	0	0	1*	0

* Transient and mild swelling of one digit in one foot probably due to traumatic injury.

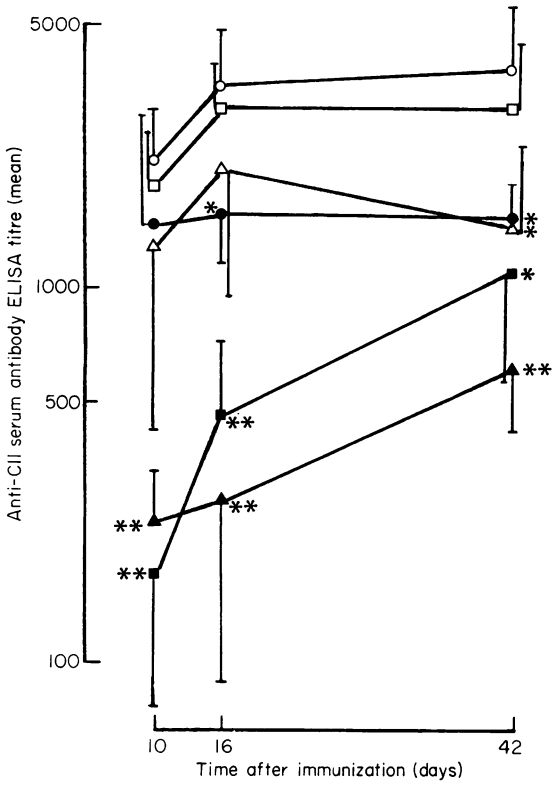

Fig. 1. Serum anti-CII antibody titres of rats immunized parenterally with POLCII in FIA. Responses of control animals immunized with soluble CII (○) compared with responses of others to POLCII(0·001%) (□), POLCII(0·01%) (△), POLCII(0·1%) (●), POLCII(1·0%) (■) and POLCII(10%) (▲). Values plotted are means with vertical bars representing one standard deviation. (*$P < 0{\cdot}01$; **$P < 0{\cdot}001$, from control values, Student's *t*-test). Animal numbers shown in Table 1.

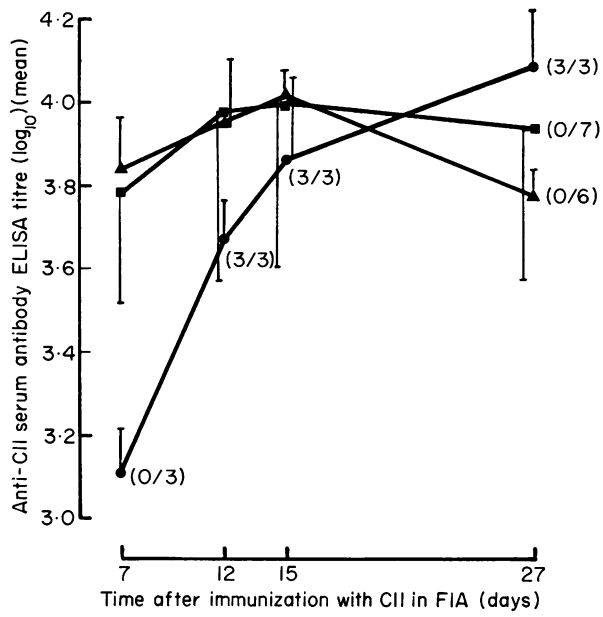

Fig. 2. Anti-CII antibody responses in rats preimmunized parenterally with POLCII. 100 days after intradermal immunization with POLCII(1%) (■) ($n = 7$) or POLCII(10%) (▲) ($n = 6$) administered in FIA, or POLCII(10%) in saline without FIA (●) ($n = 3$), rats were injected with soluble CII in FIA; numbers of arthritic rats/group given in brackets. Antibody responses were measured at times indicated and are mean values for groups. Vertical bars represent one standard deviation.

the reduction in disease incidence and was directly related to the amount of glutaraldehyde used to polymerize the collagen.

Animals immunized intradermally with POLCII preparations in the absence of FIA had only very low titres (average 30) of anti-CII antibody. Injection of FIA alone did not induce antibody formation nor did it induce arthritis (results not shown).

Effects of parenteral exposure to POLCII on the subsequent induction of disease

One hundred days after exposure to POLCII intradermally, selected animals received an arthritogenic dose of CII intradermally in FIA. Of three arthritic and three non-arthritic animals, which had been injected previously with POLCII(0·1%) in FIA, two arthritic animals showed exacerbated symptoms (within 7 days) and one healthy animal developed disease for the first time (by 12 days), after being challenged intradermally with arthritogenic CII in FIA (results not shown). By contrast, none of the animals that had previously received (non-arthritogenic) POLCII(10%) or POLCII(1·0%) in FIA developed disease upon an arthritogenic challenge (Fig. 2). Three rats previously injected intradermally with POLCII(10%) in saline without FIA first showed symptoms of disease between 12 and 15 days, that is they responded normally to the arthritogenic challenge.

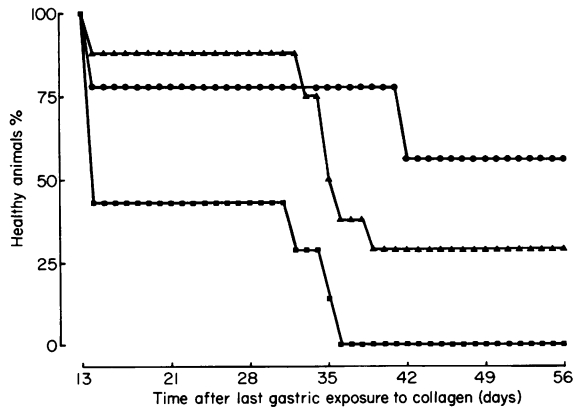

Fig. 3. Suppressive effects of pergastrically administered collagen on subsequent induction of arthritis. Animals were gavaged daily with either 2·5 μg/g POLCII(10%) (●) ($n = 9$) or 2·5 μg/g soluble CII (▲) ($n = 8$) or 0·1 M ethanoic acid (■) ($n = 7$) for 5 days, then immunized with arthritogen (0·5 mg CII in FIA) intradermally on the last day and with 0·5 mg CII in 0·1 M ethanoic acid intraperitoneally 21 days later. These represent three distinct populations with respect to times of disease onset ($P < 0{\cdot}05$, Kruskal–Wallis test).

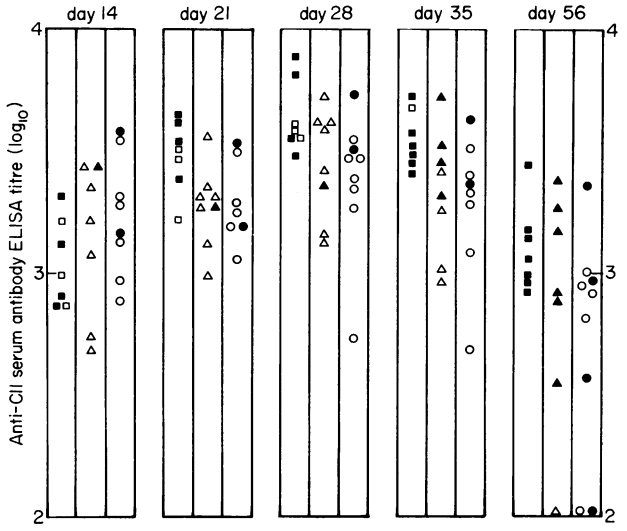

Fig. 4. Effects of pergastrically administered collagen on serum antibody levels induced by parenteral immunization. Experimental details as in Fig. 3. Open symbols represent healthy animals, and closed symbols those with clinical disease gavaged with POLCII(10%) (○,●), CII (△,▲) or ethanoic acid (□,■). There is a significant difference ($P<0.05$, Student's t-test) between each experimental group and the control on days 21, 28 and 35 but not between the experimental groups.

The serum anti-CII antibody titres were high in all groups, falling within the normal range at some point after immunization (Fig. 2). The elevation of antibody levels in rats 7 days after the arthritogenic challenge was presumably a result, in part, of persisting preformed antibody. The injection of FIA alone did not modify subsequent disease induction in control animals in any way (results not shown).

Effects of pergastric exposure to POLCII and CII on the subsequent induction of disease

POLCII and CII administered pergastrically before intradermally administered CII were found to modify subsequent disease induction (Fig. 3). CII, administered pergastrically, delayed the time of disease onset as described previously (Thompson & Staines, 1986a), whereas POLCII, administered pergastrically in the same way, both lowered the incidence of CII-induced arthritis and also delayed the time of disease onset in those animals which became diseased. Neither POLCII nor CII, administered pergastrically, modified the severity of the CII-induced arthritis in those animals that developed clinical disease (results not shown).

The serum anti-CII antibody titres were high in all groups although animals gavaged with either POLCII or CII had depressed ($P<0.05$) anti-CII antibody titres on days 21, 28 and 35 after parenteral challenge with arthritogenic CII (Fig. 4). The serum anti-CII antibodies were predominantly of the IgG isotype (IgM, IgA and IgE antibodies were not detected) and almost exclusively of the IgG2a and IgG2b subclasses (Fig. 5), (antibodies of the IgG2c subclass were not tested for). Antibody

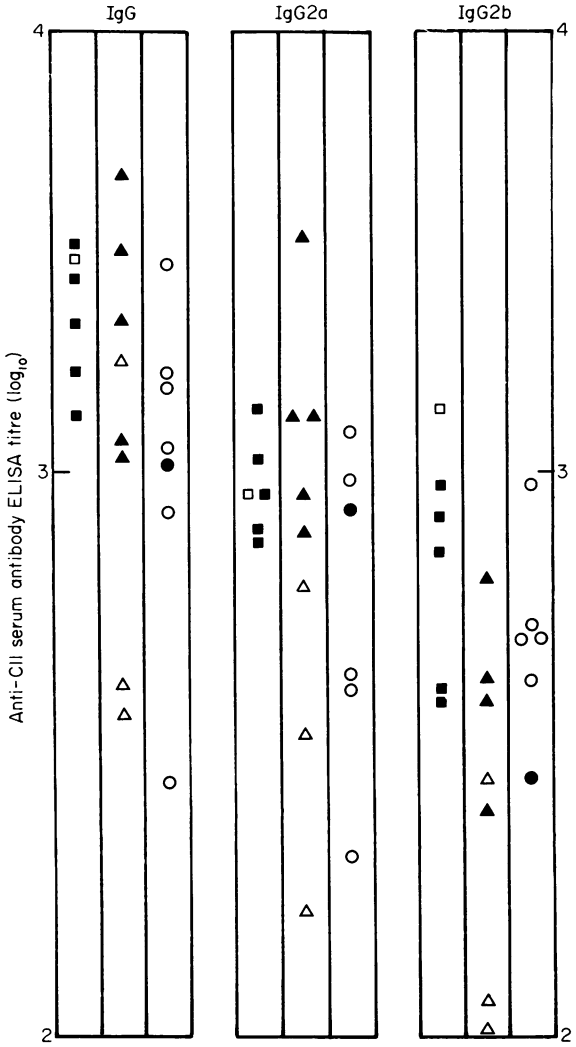

Fig. 5. Effects of pergastrically administered collagen on the isotype of serum anti-CII antibodies 35 days after parenteral immunization. Experimental details as Figs 3 and 4. The only significant change ($P<0.01$, Student's t-test) was in antibodies of the IgG2b isotype in animals gavaged with CII. At this time (35 days) antibodies of the IgG1 subclass and the IgM, IgA and IgE classes were not detected.

levels of both IgG2 subclasses tended to be lowered but the changes achieved significance only with IgG2b antibodies in rats gavaged with CII.

Uptake of POLCII and CII from the lumen of the intestine

Both POLCII and soluble CII were detected in the lamina propria of the villi in the ileum within 5 h of their introduction as a bolus directly into the lumen. However, neither form of collagen was still detectable in the lamina propria at 24 or 48 h. Neither POLCII nor CII could be detected in the Peyer's patches, mesenteric lymph nodes, spleen or brachial lymph nodes at any of the times tested, up to 48 h.

In contrast, tissues, including the lamina propria, taken from animals 48 h after they had received the last of five standard daily consecutive gavages of either POLCII or CII contained no persisting absorbed collagen. Treatment with

POLCII by this gavage regime elicited a greater biliary IgA anti-CII antibody response (mid point titre 41) than CII (mid point titre 4) or 0·1 M ethanoic acid (mid point titre 2).

DISCUSSION

The polymerization of CII with glutaraldehyde reduced its arthritogenicity. The most extensively cross-linked CII, POLCII(10%), was still, however, immunogenic as parenteral administration in FIA induced low levels of anti-CII antibodies and pergastric administration elicited a biliary IgA anti-CII response. In this form, on a weight-for-weight basis it was also more tolerogenic than soluble CII; animals exposed to it either pergastrically or parenterally in FIA became refractory to challenge with an otherwise immunogenic and arthritogenic soluble CII preparation administered intradermally in FIA. For this tolerogenic effect to be seen with parenterally administered POLCII it had to be administered in FIA, without which it was not tolerogenic and did not significantly alter the subsequent arthritic or immune response of the rats to soluble CII.

The abrogation of arthritis with only a slight depression of serum anti-CII antibody levels in animals gavaged with either POLCII or soluble CII is consistent with our previous observations that pergastrically administered CII had differential suppressive effects on subsequent antibody levels and arthritis (Thompson & Staines, 1986a, b). The only significant change in the anti-CII antibody isotype profile of gavaged rats was associated with the IgG2b subclass— which activates both the classical and the alternative complement pathways—in animals gavaged with soluble CII but not those gavaged with POLCII, though there was a lower incidence of disease following treatment with the latter. A tendency towards lowered IgG2 antibody responses has also been shown in mice suppressed for arthritis by pergastrically administered CII (Nagler-Anderson *et al.*, 1986). In all experiments antibodies were quantified by ELISA using soluble CII as the antigen. Because of purely practical considerations no attempts were made to detect antibodies reacting with POLCII since this antigen, by virtue of its particulate nature, is not suitable for use in ELISA.

Polymerized, and to a lesser extent soluble CII, induced a biliary IgA anti-CII antibody response, detected 48 h after the last of five daily gavages, which was not accompanied by the appearance of anti-CII antibodies of any immunoglobulin class in the serum. This biliary antibody may play no direct role in the abrogation of arthritis, but IgA antibody secretion across mucosal surfaces has been shown to correlate with the establishment of systemic unresponsiveness following pergastric exposure to other antigens (Challacombe & Tomasi, 1980). When administered pergastrically, particulate antigens are known to be more potent initiators of secretory immune responses than soluble antigens (Cox & Taubman, 1984). This may be due to the greater ability of some particulate antigens to gain access to Peyer's patches (Jeurissen, 1986). In this study, however, both particulate POLCII and soluble CII appear to be absorbed into the lamina propria, and not into the Peyer's patches. It is expected that the extent to which the collagens are differentially degraded, the route by which they reach antigen reactive cells, the form in which they are presented and the way they interact with antigen processing/presenting cells, will all influence the subsequent immune response.

The refractory condition of animals exposed to POLCII (by either route of administration studied) is associated with a change in their immune status although the cellular basis for this is not known. One change in immune activity, associated with this antigen-specific pergastrically-induced resistance to disease, can be detected in lymphoid tissues not primarily associated with mucosal surfaces: resistance can be adoptively transferred from gavaged rats to naive rats by spleen cells, but not by mesenteric lymph node cells or by serum (our observations, to be published).

In animals gavaged with either POLCII or CII there appears to be a state of split tolerance; classically defined as 'the phenomenon in which animals are immunologically tolerant to a portion of the total antigenic moieties of the tolerance-inducing inoculum' (Weissman, 1966). Furthermore, it has been shown that T cells are more easily tolerized than are B cells, the latter requiring higher doses and longer exposure to the tolerogen (Chiller, Habicht & Weigle, 1971). It is known that T cells proliferate in response to CII (Rosenwasser, Bhatnager & Stobo, 1980), CII cross-linked with glutaraldehyde (Hom *et al.*, 1986) and that the antibody response to collagen and synthetic collagen-like polypeptides has both T-dependent and T-independent components (Fuchs *et al.*, 1974). Pergastrically administered T-dependent antigens have also been shown to induce systemic tolerance whereas pergastrically administered T-independent antigens do not (Chiller, Titus & Etlinger, 1979). We propose that glutaraldehyde polymerization of CII converts this partially T-dependent immunogenic and arthritogenic molecule into a potent T cell but not B cell tolerogen; its tolerogenic properties are particularly evident when it is administered pergastrically.

It is not known why polymerization of CII changes its immunogenic properties but exposure of collagen to glutaraldehyde is known to increase its mechanical and thermal stability as well as causing some axial contraction leading to a change in its tertiary structure (Meek & Chapman, 1985) (an analysis of the structural and immunochemical properties of POLCII is in progress). The effects of polymerization, which converts the autoantigenic CII from a soluble immunogen to an insoluble tolerogen, are in contrast to the changes to the properties of both globular and fibrous foreign proteins which are not autoantigens or cross-reactive with autoantigens. Their polymerization or aggregation increases immunogenicity and destroys tolerogenicity (reviewed for example by Arnon & Geiger, 1977).

However, if, by analogy to the antigenic structure of hen egg lysozyme (Goodman & Sercarz, 1983), CII has different epitopes associated with its immunogenic, arthritogenic and tolerogenic properties, polymerization would be expected to change the relative expression of these. In this context, and in general support of this hypothesis, it is known that heat-denatured CII is not arthritogenic and expresses epitopes different from those on native CII (Thompson & Staines, 1986b).

In CII-induced arthritis there may be some parallel to the situation in spontaneous inflammatory joint disease in which the form of the collagen and its naturally slow rate of catabolism and release from cartilage may be altered so as to break the maintained tolerant state of the immune system. It is proposed that collagens in their natural cross-linked states in cartilage maintain self-tolerance actively in the same way as artificially

polymerized CII. POLCII may have a therapeutic role in the active enhancement of self-tolerance to articular cartilage collagens.

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