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Oxidative modification of type II collagen differentially affects its arthritogenic and tolerogenic capacity in experimental arthritis

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Summary

Introduction: Oxidative modification of proteins affects their biological properties. Previously we have shown that hypochlorite (HOCl), the product of activated neutrophils, enhances protein immunogenecity. Collagen type II, a primary component of cartilage, is commonly used in the induction of arthritis in animals (CIA). The aim of this study was to examine whether HOCl may affect immunogenic, tolerogenic, and arthritogenic properties of collagen.

Materials and Methods: DBA/J mice were injected with either native (C_{NAT}) or chlorinated collagen (C_{HOCl}) to induce arthritis. The effect of chlorination on collagen properties was measured by evaluation of incidence and severity of CIA. Moreover, the concentration of serum anti-collagen IgG antibodies and myeloperoxidase (MPO) activity in inflamed joints was determined.

Results: Mice immunized with C_{NAT} in adjuvant developed arthritis (CIA) with an incidence of 69%. C_{NAT} also exerted tolerogenic properties when injected intravenously either before or shortly after primary immunization, resulting in decreased incidence and severity of CIA, reduced MPO activity in inflamed joints, and lowered serum levels of anti-C_{NAT} IgG antibodies. Chlorination of collagen significantly diminished its ability to induce CIA and to trigger generation of anti-C_{NAT} IgG antibodies. Interestingly, chlorination did not affect tolerogenic properties of collagen administered prior to primary immunization with C_{NAT}.

Conclusions: These results suggest that chlorination of collagen may selectively affect functional epitopes of collagen. It is likely that in inflamed joints, neutrophil-derived HOCl, in some circumstances, will destroy arthritogenic and immunogenic B cell epitopes, while regulatory T cell epitopes will be preserved.

Key words: **collagen-induced arthritis • DBA/1 mice • protein oxidation • neutrophils • myeloperoxidase • hypochlorous acid**

Abbreviations: **CIA** – collagen-induced arthritis, **CII** – collagen type II, **C_{NAT}** – native collagen, **C_{HOCl}** – chlorinated collagen, **DTH** – delayed type hypersensitivity, **MPO** – myeloperoxidase, **SAA** – serum amyloid A, **CFA** – complete Freund's adjuvant, **OVA** – ovalbumin, **HOCl** – hypochlorite

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INTRODUCTION

Collagen-induced arthritis (CIA) is an experimental model for autoimmune arthritis that resembles human arthritis in a number of aspects[24, 25]. CIA can be induced in genetically susceptible animals by intradermal injection of homologous or heterologous type II collagen (CII), a primary component of cartilage, in adjuvant[3, 16]. Although the incidence and severity of arthritis is highest after immunization with intact, native collagen (C_{NAT}), denaturated collagen and even its fragments are capable of causing CIA provided that arthritogenic epitopes are not destroyed[3, 8].

It is commonly accepted that oxidative modification of proteins may play a role in the pathogenesis of disorders ranging from atherosclerosis to autoimmune diseases[19]. Activated neutrophils, the primary cell population in the synovial fluids of arthritic joints, release hypochlorite (HOCl), the major oxidant produced by the myeloperoxidase (MPO)-halide system[6, 28]. HOCl contributes to the destruction of cartilage in inflamed joints by direct fragmentation of collagen and also by activation of proteolytic enzymes, including collagenase[4, 26].

Oxidation via HOCl may also contribute to disease pathogenesis by altering the immune response. CII is known to contain different sets of epitopes, including arthritogenic (both B and T cell), non-arthritogenic, and regulatory (suppressor) epitopes[3, 16, 18]. Oxidative modifications may therefore include destruction of any of these epitopes or, conversely, enhancement of immunogenicity, as has been demonstrated for other proteins, through mechanisms including increased susceptibility to degradation by enzymes involved in antigen processing[11, 20]. Some studies have shown that heat-denaturation may increase susceptibility to gelatinase B while maintaining arthritogenic epitopes intact[26]. Heat denaturation, however, is a non-physiological process, while, in contrast, oxidation is a sequence-selective modification known to occur during inflammation[19]. In particular, protein thiols are highly susceptible to oxidation and HOCl induces deamination, leading to the formation of carbonyl groups and transformation of tyrosine residues to dichlorotyrosine[11]. Selective modification of collagen may also provide a useful tool for therapy, since the ability to destroy potential arthritogenic epitopes may be used to enhance tolerogenic strategies, such as intravenous (i.v.) delivery of CII.

In this study we utilize the murine CIA model to investigate the immunological properties of two forms of chicken CII, i.e. C_{NAT} and chlorinated (C_{HOCl}), both in their ability to induce arthritis and to induce tolerance and suppress CIA. This model mimics the effects of the oxidation of cartilage protein in rheumatoid arthritis (RA) as a result of synovial neutrophil activation[7, 10, 17] and suggests that such oxidation may differentially affect distinct sets of epitopes.

MATERIALS AND METHODS

Mice

Male DBA/J mice between 8–12 weeks of age from the breeding unit of the Department of Immunology, Jagiellonian University Medical College, Kraków, Poland, were used. All mice were housed 3 per cage in the laboratory room with water and standard diet provided *ad libitum*. The authors were granted permission by the Local Ethics Committee to use mice in this study.

Collagen chlorination with HOCl/OCl⁻

Samples of chicken CII (2 mg/ml) in 0.2 M phosphate buffer were incubated for 2 h at different concentrations of HOCl. To stop the reaction, the samples were treated with the stoichiometrical amount of thiosulfate.

Induction of CIA and evaluation of arthritis

Immunization. Male DBA/1 mice were immunized with 200 μg of chicken CII (C_{NAT} or C_{HOCl}) emulsified in complete Freund's adjuvant (CFA; Sigma, Steinham, Germany) by intradermal injection at the base of the tail (primary immunization). On day 21 after the first immunization the mice were immunized subcutaneously with 100 μg of collagen II in CFA (booster immunization)[24]. In some experiments, the mice received only the primary immunization.

Tolerization. CII (C_{NAT} or C_{HOCl}) was dialyzed against phosphate buffer saline (PBS). Mice were tolerized by i.v. injection of 100 μg of CII on three consecutive days, either before or after primary immunization. Control animals were injected with ovalbumin (OVA) using the same protocol.

Measurement of the incidence and severity of arthritis. The mice were examined visually for the appearance of arthritis in the peripheral joints 3 times a week for 42 days after the primary immunization. There were two separate examiners (utilizing a visual scoring system) "blind" to the experimental groups. Mice were considered to have arthritis when swelling and erythema in at least 1 digit or paw were observed on 2 consecutive observation dates (Fig. 1).

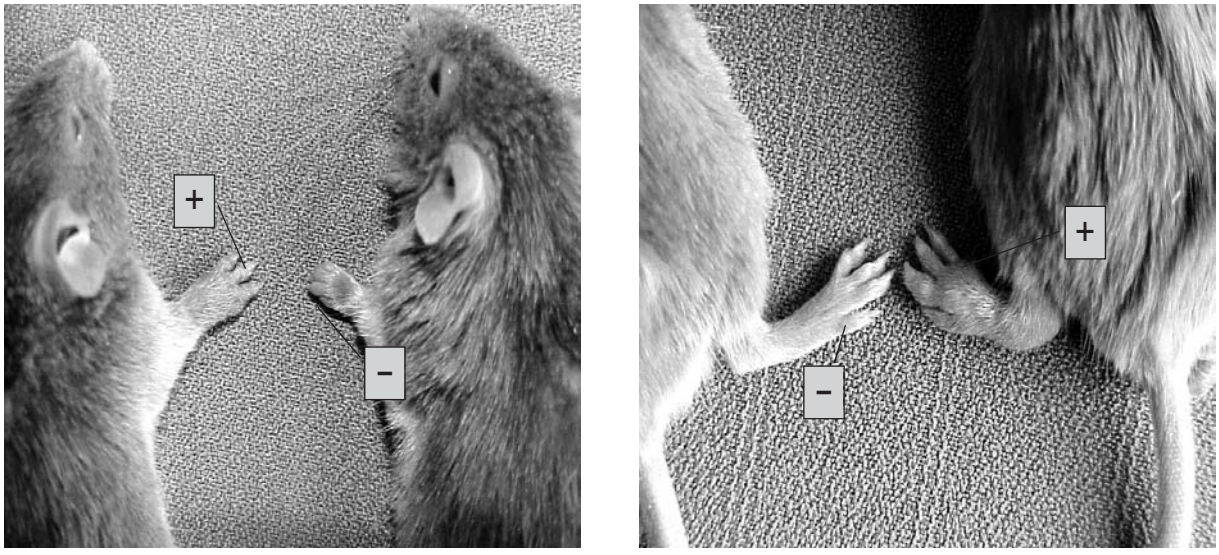

Figure 1. Pictures showing normal (–) and arthritic (+) paws in male DBA/1 mice.

The severity of arthritis in each affected paw was graded according to an established scoring system as follows: 0 – normal, 1 – visible inflammation of interphalengeal joints or mild footpad swelling, 2 – pronounced footpad swelling associated with erythema and edema, 3 – joint deformation, and 4 – ankylosis. The cumulative score of all 4 paws of each mouse was used as the "arthritis index" (maximum score of 16 per mouse) to represent overall disease severity and progression in an animal[9, 25]. The incidence of CIA is presented as percentage values in the tables. The onset of CIA is not shown in this paper since we did not find statistically significant differences between the experimental groups.

Measurement of serum anti-collagen antibody titers

Mice were anesthetized and bled on days 21 and 42 after primary immunization. Serum level of antibody against CII was measured using a standard ELISA assay. Briefly, serum samples were stored at –80°C until they were used for the ELISA.

Microtiter plates (Corning, NY, USA) were coated overnight with 5 μg/ml of CII (acid soluble; Sigma, Steinham, Germany) in PBS at 4°C. Non-specific binding was blocked with 4% bovine serum albumin (BSA) in PBS at room temperature for 1 h. Diluted serum samples (in 1% BSA in PBS) were added and incubated for 1 h at room temperature. The plates were then incubated with biotinylated goat anti-mouse IgG antibody (Sigma) for 45 min at room temperature. Horseradish peroxidase-conjugated streptavidin diluted 1:1000 in 1% BSA/PBS was added and the plates were incubated for 45 min at room temperature. Then o-phenylenediamine dihydrochloride (OPD; Sigma, Steinham, Germany) was used as a substrate (5 mg of OPD in 10 ml of phosphate-citrate buffer, pH 5.0) and incubated with 40 μl of 30% H_2O_2 for 30 min at room temperature. The reaction was stopped with 3 M H_2SO_4. Optical density was measured at 492 nm.

Measurement of MPO activity

On day 42 of the experiment, MPO activity was measured in the periarticular tissue as described before[9]. Briefly, periarticular tissue was homogenized in ice-cold 0.5% hexadecyltrimethylammonium (Sigma, Steinham, Germany) in 50 mM potassium phosphate buffer, pH 6.0. The tissue was freeze-thawed 3 times and dispersed by vortexing. Suspensions were centrifuged at 4000 × g for 15 min at 4°C. Aliquots (0.1 ml) of supernatant were mixed with 2.9 ml of 50 mM phosphate buffer, pH 6.0, containing 0.167 mg/ml o--dianisidine dihydrochloride (Sigma, Steinham, Germany) and 0.0005% hydrogen peroxide. 200 μl of the mixture were placed in a 96-well flat-bottom plate and incubated for 20 min in a room temperature. The absorbance was measured at 460 nm using a Sumal-PE2 spectrophotometer. The activity of MPO was calculated from MPO (Calbiochem, San Diego, USA) standard curve and expressed in units in which one unit of MPO activity was defined as that degrading 1 μmol of hydrogen peroxide per minute at room temperature. Each sample was measured in duplicate.

Measurement of serum amyloid A

Mice were bled on days 7, 21, and 42 after immunization. In each experiment, serum samples from at least

6 mice per group were analyzed for serum amyloid A (SAA) using the Mouse Serum Amyloid A ELISA Kit (BioSource International, Inc., Camarillo, USA).

Statistical analysis

The non-parametric Mann-Whitney U test was applied to examine differences in the amounts of antibodies and cytokines. Differences in the cumulative incidence of arthritis were analyzed by χ^2 analysis.

Results are expressed as mean ±SE if not otherwise stated. Probability values less than 0.05 were considered to be statistically significant.

RESULTS

Serum levels of SAA and IgG specific to CII during the development of CIA

In order to confirm the role of collagen-specific immune reactions in the development of CIA, serum levels of IgG specific to native CII (IgG) and SAA were tested at the time of induction and after the onset of arthritis. The levels of SAA after 7 days, which represent the acute-phase inflammatory response of innate immunity, were increased to the same degree in mice which had received either CFA alone or CFA plus collagen. At this time, IgG anti-C_{NAT} antibodies, which represent the humoral response, were not detected in the sera of either group of mice. Fourteen days later (21 days after immunization) SAA decreased to the level of SAA in the serum of naive mice. On day 42, approximately 1 week after the onset of arthritis, SAA was detected again in the serum, but only in the arthritic mice, reaching concentrations 2–4 times higher than those reached during the induction stage of CIA. Elevated SAA at day 42, but not during initial priming, therefore reflected chronic inflammation and always correlated to the development of CIA.

Monitoring of the collagen-specific humoral response during the development of CIA shows that on day 21 the high level of IgG anti-C_{NAT} was detected only in the serum of mice immunized with collagen in CFA. After an additional 21 days, mice with CIA (arthritic mice) showed 2–4 times higher titers of IgG anti-C_{NAT} than immunized, non-arthritic mice. This higher concentration of IgG anti-C_{NAT} correlated with the increased marker of inflammatory response, SAA (see Table 1).

Induction of CIA by immunization with C_{NAT} and C_{HOCl}

To determine how chlorination alters the arthritogenic capacity of collagen, DBA/1J mice were treated with either C_{NAT} or C_{HOCl}. In preliminary experiments we used collagen modified with HOCl at concentrations ranging 0.5–5.0 mM. Such concentrations of HOCl are used in experimental models investigating the biological properties of oxidized proteins[21, 22]. Quantitative analyses demonstrated that 10^6 maximally triggered neutrophils produced approximately 200 nmol of HOCl. Given the fact that a large interstitial inflammatory site contains approximately 25×10^6 neutrophils in 0.1 ml, enormous quantities of HOCl, up to 5 mM, may be achieved *in vivo* in close proximity to the activated neutrophils[27]. Moreover, such a concentration of H_2O_2 and HOCl may be achieved within the neutrophil vacuoles during phagocytosis[23]. Since neutrophils are major cells of the synovial fluid of inflamed joints, one may speculate that similar collagen chlorination may occur during the development of arthritis.

In our study, HOCl at concentrations below 1.0 mM affected neither the capacity of collagen to induce CIA nor its ability to react with standard antibodies specific to epitopes expressed on C_{NAT} (not shown). In further experiments, the effect of 3 mM HOCl on properties of collagen was investigated. At this concentration, HOCl caused deamination, transformation of tyrosine residues to dichlorotyrosine and, in

Table 1. Serum levels of SAA and IgG α C_{NAT} during the development of CIA

Immunization[a]		SAA (μg/ml)[b]			IgG α C_{NAT}[c]			Arthritis
primary (0)	boost (21)	(7)	(21)	(42)	(7)	(21)	(42)	CIA
C_{NAT} + CFA	C_{NAT} + CFA	150–300	< 10	200–600	< 4	256–512	1024–4000	(+)
		150–300	< 10	< 10	< 4	256–512	256–1024	(–)
CFA	CFA	> 150	< 10	< 10	< 4	< 4	< 4	(–)

[a] Mice were immunized on day 0 followed by a booster injection (day 21) with C_{NAT} + CFA. (n) – day of experiment after primary immunization. Control mice received CFA alone (as described in Materials and Methods). [b] SAA and IgG α C_{NAT} were measured in sera obtained 7, 21, and 42 days after immunization. Sera from arthritic (+) and non-arthritic (–) mice were pooled separately. Results represent the data taken from 3 independent experiments (SAA was tested only in 3 experiments out of 25 successfully evoked CIA). Each experimental group consists of 6–10 mice. [c] IgG titer expressed in arbitrary units, 1 U – IgG α C_{NAT} titer 1/100. Statistical analysis was not performed for the data presented in this table.

Table 2. Induction of CIA by immunization with native (C_{NAT}) and chlorinated (C_{HOCl}) collagen

Immunization with antigen in CFA[a]		Incidence of CIA[b]	Severity of CIA[b] (arthritic index)	MPO[c] (U × 10^{-3}/mg protein)	
primary	boost				
C_{NAT}	–	13/26 (50%)	6.92 ± 3.55	339 ± 33	(+)
				< 10	(–)
C_{NAT}	C_{NAT}	44/64 (69%)	6.82 ± 3.16	364 ± 28	(+)
				< 10	(–)
C_{NAT}	C_{HOCl}	20/30 (67%)	6.02 ± 2.83	394 ± 12	(+)
				< 10	(–)
C_{HOCl}	C_{NAT}	0/24	0	< 10	
C_{HOCl}	C_{HOCl}	0/18	0	< 10	
–	–	non-tested	non-tested	< 10	

[a]Mice were immunized on day 0 (priming) and day 21 (booster) as described in Materials and Methods. [b]The presence of arthritis (CIA) and severity was evaluated 42 days after immunization. [c]MPO activity was measured in periarticular tissue of the hind limb separately in arthritic mice (+) and non-arthritic mice (–) as described in Materials and Methods.

Table 3. Suppression of CIA development following intravenous (i.v.) administration of native (C_{NAT}) and chlorinated (C_{HOCl}) collagen

Tolerization (i.v. administration)	Incidence of CIA[a]	Severity of CIA (arthritic index)	MPO (U × 10^{-3}/mg protein)
None (+ve)	44/64 (69%)	6.82 ± 3.16	364 ± 284
Before primary immunization			
C_{NAT}	0/12	0	< 10
C_{HOCl}	1/12 (8%)	3.0	184
OVA	6/10 (60%)	6.22 ± 1.11	non-tested
After primary immunization			
C_{NAT}	0/42	0	< 10
C_{HOCl}	11/25 (44%)*	5.94 ± 3.75	237 ± 232*
OVA	7/10 (70%)	6.55 ± 2.02	non-tested

Mice were immunized as described in Materials and Methods. Tolerization was done either before or after the primary immunization (for details see Materials and Methods). [a]The incidence, severity and MPO activity in inflamed joints is reported on day 42 after immunization. *$p < 0.05$ C_{HOCl} tolerized mice vs. control mice (+ve).

addition, collagen fragmentation[21]. As shown in Table 2, mice immunized (primed) with C_{NAT} in CFA developed a high incidence of severe arthritis. Periarticular tissue taken from the arthritic mice showed a high activity of MPO, which indicates that neutrophils are present in the inflamed joints. The effects of chlorination were different if tested at priming or boost. Priming with C_{HOCl} failed to induce either disease or neutrophil infiltration, even after a subsequent boost with C_{NAT}. In contrast, after priming with C_{NAT} there were no statistically significant differences in CIA incidence, severity, or MPO activity between mice boosted with either C_{NAT} or C_{HOCl}. Chlorinated collagen therefore retains the ability to boost, but not to prime for an arthritogenic response.

Suppression of development of CIA following i.v. administration of C_{NAT} and C_{HOCl}

In order to determine the tolerogenic capacity of C_{NAT} and C_{HOCl}, mice were injected i.v. with these two forms of antigen (C_{NAT}, C_{HOCl}) either prior to or after intradermal immunization with C_{NAT} in adjuvant (CFA). C_{NAT} administered prior to immunization completely abolished the development of CIA (Table 3), confirming previous studies[7, 10]. C_{HOCl} also almost completely abolished the development of CIA when injected i.v. one week prior to immunization. Injecting (tolerizing) control mice with OVA did not affect the induction and development of arthritis.

However, while intravenous administration of C_{NAT} shortly after immunization also completely prevented the development of CIA, C_{HOCl} only slightly diminished the incidence of CIA under these conditions.

Suppression of IgG anti-C_{NAT} antibody production following i.v. administration of C_{NAT} and C_{HOCl}

In order to determine the capacity of C_{HOCl} to affect the production of IgG antibodies specific to collagen used for the induction of arthritis, the levels of serum

IgG anti-C_{NAT} were measured. As shown in Table 1, primary immunization with C_{NAT} in adjuvant (CFA) resulted in a massive production of IgG anti-C_{NAT}. Booster immunization with the same antigen increased the serum levels of specific IgG more than 4 times in arthritic mice, and less than 2 times in non-arthritic mice. The same primary immunization with C_{HOCl} in CFA did not induce production of IgG specific to C_{NAT}. An enhanced production of anti-C_{NAT} IgG was observed when primary immunization with C_{HOCl} in CFA was followed by booster immunization with C_{NAT} (Table 4), suggesting that C_{HOCl} generates memory T helper cells specific to carrier epitopes displayed by both forms of collagen (C_{NAT} and C_{HOCl}). Moreover, as shown in Table 5, C_{HOCl} injected i.v. suppressed the production of IgG specific to C_{NAT}. Nevertheless, the inhibition of C_{NAT}-specific IgG production evoked by C_{HOCl} was much weaker than that evoked by C_{NAT}. Tolerization with the control, irrelevant antigen (OVA) did not affect the production of collagen-specific antibodies. Experiments performed on CBA mice, a CIA-resistant mice strain, showed similar effects of C_{NAT} and C_{HOCl} on the production of IgG specific to the native form of collagen (data not presented).

Table 4. Antibody responses to native collagen (C_{NAT}) after immunization with native (C_{NAT}) or chlorinated (C_{HOCl}) collagen

Immunization[a]		Anti-C_{NAT} IgG (units)[b]	
primary	boost	day 21	day 42
–	C_{NAT} (12)[c]	≤ 4	24 ± 9
C_{NAT} + CFA	C_{NAT} (64)	384 ± 70	1390 ± 594
C_{HOCl} + CFA	C_{NAT} (24)	9 ± 8*	438 ± 263**
–	– (10)	≤ 4	non-tested

[a]Mice were immunized either with C_{NAT} or C_{HOCl} in CFA. 21 days after priming immunization all mice were injected subcutaneously with C_{NAT} (boost). Serum level of IgG anti-C_{NAT} was analyzed on days 21 and 42. [b]Results are reported using arbitrarily defined units (1 U – anti-C_{NAT} IgG titer 1/100). [c](n) – number of mice. *$p < 0.0001$, **$p < 0.05$, C_{NAT} vs. C_{HOCl} (primary immunization).

DISCUSSION

An animal model of CIA, relevant to RA, can be induced in genetically susceptible mice by immunization with CII[25]. Intradermal immunization with native collagen in CFA results in the development of CIA and stimulation of antigen-specific humoral and cell-mediated (DTH) immune response. On the other hand, i.v., oral, or nasal application of C_{NAT} has been used to induce collagen-specific tolerance and to block the development of CIA[3, 14, 15], although protection was not complete and was relatively short lasting[7, 10].

It is commonly accepted that distinct collagen epitopes are recognized by B and T cells. Moreover, it has been shown that intact native CII displays distinct arthritogenic and suppressive epitopes[3, 16, 18]. It has also been documented that heat denaturation of CII destroys its arthritogenic capacity and heat-sensitive B cell epitopes, but leaves most T cell epitopes intact[26]. Therefore, immunization of susceptible animals with heat-denaturated CII fails to induce arthritis and the production of antibodies, but these animals develop a positive DTH reaction[8]. Moreover, tolerization is effective and can prevent CIA development if the collagen expresses intact suppressive epitopes. Although the incidence and severity of arthritis is highest after immunization with native CII, there are some reports that denaturated CII and even fragments of collagen are capable of causing disease[18, 25]. The presence of intact functional epitopes is essential for disease induction[14, 15, 18]. Antigen modification that selectively destroys arthritogenic epitopes and leaves suppressive epitopes intact would be of value for clinical

Table 5. Suppression of IgG α C_{NAT} production following intravenous (i.v.) administration of native (C_{NAT}) and chlorinated (C_{HOCl}) collagen

Tolerization (i.v. administration of Ag)	IgG α C_{NAT} (units)			
	day 21		day 42	
None (+ve)	387 ± 70	100%	1390 ± 594	100%
Before primary immunization				
C_{NAT}	45 ± 16*	12%	120 ± 59*	9%
C_{HOCl}	256 ± 140	66%	682 ± 241*	49%
OVA	384 ± 64	100%	1024 ± 102	74%
After primary immunization				
C_{NAT}	52 ± 21*	14%	296 ± 218*	21%
C_{HOCl}	256 ± 140	66%	1408 ± 608	101%
OVA	341 ± 160	88%	1256 ± 631	90%

Mice were bled on days 21 and 42 and sera from each group of mice were pooled and analyzed by ELISA to measure IgG specific to C_{NAT}. Data represent cumulative results from 5 independent experiments. Each experimental group consists of 6–10 mice. *$p < 0.05$ in comparison with the non-tolerized control mice (+ve).

application to diminish the danger of inducing adverse arthritic symptoms. Some data suggest distinct sensitivity of functionally different epitopes to treatment with different agents (heat denaturation, oxidative stress)[1, 4, 21]. In this study we addressed the issue whether chlorination of collagen may selectively modify its biological functions related to the induction and regulation of arthritis.

Previous studies have shown that protein modification by chlorination may enhance immunogenecity[12]. OVA treated with HOCl and incubated with antigen-presenting cells stimulated OVA-specific T cells more effectively than did native albumin[11]. The effect of enhanced immunogenecity after chlorination of proteins was dose dependent. Chlorination with HOCl used at concentrations over 5 mM abolished OVA immunogenecity due to protein fragmentation into non-immunogenic peptides[22]. In the context of RA, collagen epitopes may be destroyed either by direct attack of neutrophil-derived HOCl on collagen and protein fragmentation, or indirectly by enhancement of collagenase activity by HOCl[4, 26]. Collagen may be cleaved further by gelatinase B, while leaving some remaining immunodominant epitopes intact[25]. In addition, collagen fragments produced by reaction with HOCl may have a direct inhibitory effect on the ability of collagenase to further degrade the intact collagen substrate[5]. The overall effect of chlorination on disease progression *in vivo* is therefore complex.

In this study we have shown that C_{HOCl} with HOCl lost its capacity to induce CIA. In parallel, immunization of DBA/1J mice with C_{HOCl} in adjuvant did not produce IgG antibodies specific to C_{NAT}. These results suggest that both arthritogenic and immunodominant B cell epitopes of native protein were destroyed. We did not test the formation of immunodominant neoepitopes by chlorination. Thus, it is not clear if C_{HOCl} changes its antigenicity and can stimulate B cells specific to epitopes formed by chlorination. Further studies will be done to evaluate this problem.

Although C_{HOCl} was shown to lose its arthritogenic and immunogenic capacity (humoral response), the tolerogenic capacity of C_{HOCl} was surprisingly similar to that of $C_{NAT.}$ When antigens were injected intravenously prior to immunization, both forms of collagen equally and completely reduced the incidence of CIA. Taken in conjunction with the results discussed above, these data therefore imply that chlorination destroys type II collagen's arthritogenic potential while retaining its tolerogenic activity. Anti-collagen IgG levels were not correlated with protection in these experiments, since only C_{NAT} suppressed the antigen-specific humoral response. This form of tolerance appears to be mediated primarily by the induction of immunoregulatory T cells with altered cytokine profiles and reduced cellular immunity, even in the presence of high serum titers of IgG antibody[2, 8, 9, 13]. In addition, levels of specific IgM, which were not measured in our study, may also be crucial determinants of CIA[8].

Differences in the tolerogenic capacities of C_{NAT} and C_{HOCl} were observed in experiments in which mice were tolerized after the induction of CIA. In this model of tolerance, only native collagen was able to prevent the development of arthritis. Although the mechanism for these differences has not yet been explored further, the present study suggests that different mechanisms underlie the CIA suppression induced by the i.v. administration of collagen before or after primary immunization.

In conclusion, our results suggest that C_{HOCl} may be used as a tolerogen to prevent the development of CIA. Strong tolerogenic activity along with loss of arthritogenic properties by C_{HOCl} suggest that its administration *in vivo* may decrease the risk of the induction of immune reaction against native, intact collagen. The ability to selectively induce pathogenic epitope loss suggests that chlorination may offer specific advantages in preparing tolerogenic preparations for therapeutic tolerance induction and thus reduce joint degeneration in RA.

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