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Modulation of antigen-specific T-cell activation *in vitro* by taurine chloramine

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SUMMARY

Taurine chloramine (TauCl) is produced during inflammation by reaction of hypochlorous acid (HOCl) with taurine, the most abundant free amino acid in neutrophils. We previously reported that TauCl inhibits the generation of macrophage inflammatory mediators such as nitric oxide, prostaglandin E_2 (PGE_2), tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6). In this study, the activity of TauCl in modulating T-cell activation was investigated. Treatment of T cells with TauCl (0·1–0·3 mM), prior to activation, was found to inhibit interleukin-2 (IL-2) release in response to both mitogen and antigen stimulation. Similarly, pretreatment of A-20 antigen presenting cells (APCs), at low cell numbers, was found to inhibit their ability to process and present ovalbumin (OVA) to a specific T-cell hybridoma. In contrast, pretreatment of higher numbers of A-20 cells with TauCl in the presence of OVA enhanced subsequent presentation of OVA. Finally, OVA modified with TauCl was processed and presented more efficiently than native OVA. Thus, TauCl is able to modulate induction of a specific adaptive immune response at several independent points of the overall antigen-presenting pathway.

INTRODUCTION

Invasion by pathogenic micro-organisms induces a complex sequence of events known as the inflammatory response. Professional phagocytes, neutrophils (PMNs) and macrophages (Ms), are the major cells of acute and chronic inflammation, respectively. During phagocytosis a variety of microbicidal and proinflammatory agents are generated, for example, reactive oxygen species such as hypochlorous acid (HOCl), hydroxyl radicles (OH^-) and hydrogen peroxide (H_2O_2), eicosanoids such as prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4), and cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6).[1–3] These agents are responsible for the intracellular killing of pathogens but, paradoxically, they are also involved in damage of host tissue.[4] At the same time, sentinel dendritic cells (DC) may engulf antigen and, after migration out of an inflammatory site and into the T-cell area of lymphoid organs, initiate an adaptive immune response against the invading pathogen.[5] It is now apparent that there is a close interplay between components of the 'innate' inflammatory response and the initiation of specific immunity.[6,7] For example, many functions of DCs, such as final maturation and migration, are also under the control of inflammatory cytokines.[5] Thus, complex reactions, both beneficial and pathological, and both innate and adaptive, may be stimulated by the same proinflammatory agents.[4,6] To maintain the balance between mounting an effective immune response and minimizing the destructive effect of the inflammatory cells, and to link the innate and adaptive responses, many regulatory mediators are also produced at a site of inflammation.[4,7] Taurine chloramine (TauCl), a product of activated neutrophils, appears to play a special role in this context.[8,9]

Taurine (Tau), the most abundant free amino acid in the cytosol of leucocytes, acts as a scavenger for HOCl, a microbicidal agent produced by the myeloperoxidase–hydrogen peroxide–halide ($MPO–H_2O_2–Cl$) system of activated neutrophils and monocytes.[3,10] The reaction of Tau and HOCl forms taurine chloramine (TauCl), a long-lived oxidant, which is much less toxic than HOCl.[11,12] Therefore, Tau present in the cytosol of neutrophils may protect these cells against attack by chlorinated oxidants that escape from phagolysosomes. In addition, Tau released extracellularly may protect the tissue at a site of inflammation.[13–15] TauCl itself, however, may exert prolonged oxidative and chlorinating effects long after the initiation of the respiratory burst.[8]

Recently, TauCl has been shown to play roles other than simply as an end product of HOCl detoxification. TauCl was postulated to be a modulator of inflammation, on the basis of its ability to inhibit production of macrophage proinflammatory mediators, such as nitric oxide, IL-1, TNF-α and

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Abbreviations: APCs, antigen-presenting cells; IL-2, interleukin 2; Ms, macrophages; OVA, ovalbumin; PMNs, polymorphonuclear cells; Tau, taurine; TauCl, taurine chloramine.

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PGE_2.[16–19] Nothing, however, is known about the ability of TauCl to modulate the initiation of the adaptive immune response via direct contact with antigen-presenting cells (APCs) and T cells.

The aim of this study was to determine whether taurine chloramine was able to modulate the induction phase of the immune response by altering the activation of antigen-stimulated T cells.

MATERIALS AND METHODS

Synthesis of taurine chloramine

TauCl was prepared by dropwise addition of 5 ml of 20 mM NaOCl (Sigma, St Louis, MO) solution in 0·05 M phosphate buffer (pH 7·4–7·5), with vigorous stirring, to 5 ml of 29 mM taurine (Tau) (Sigma).[17] Each preparation of TauCl was monitored by ultraviolet (UV) absorption spectra (200–400 nm) to confirm the presence of monochloramine (TauCl) and the absence of dichloramine ($TauCl_2$), NH_2Cl, and unreacted $HOCl/OCl^-$. The concentration of taurine monochloramine was determined by absorption at 252 nm ($\varepsilon_M = 415$).[20] Stock solutions of Tau and TauCl (10 mM) were kept at 4° for a maximum period of 5 days before use.

Mice

Inbred CBA/J male mice from the breeding unit, Department of Immunology, Jagiellonian University Medical College, Cracow, Poland, were used between 6 and 8 weeks of age.

Cells

The cloned, antigen-specific ovalbumin (OVA), H-2^d restricted DO-11-10 T-hybridoma cells were used as responder cells. A-20 B-lymphoma line (H-2^d positive) cells were used as APCs.[21]

Trinitrophenyl (TNP)-specific CD4 ± T cells were prepared as follows. CBA mice were sensitized by topical application of 150 µl of 5% trinitrochlorobenzene (Sigma) in ethanol/acetone (4:1 ratio). Draining lymph nodes from four animals were collected 4 days later, and CD4 cells were purified as follows: inguinal lymph node cells ($1–2 \times 10^7$/ml) were incubated for 60 min at 4° with anti-CD8 monoclonal antibody (mAb) 3.168 (rat anti-CD8 culture supernatant diluted 1:5). After two washes, cells were incubated on petri dishes (Falcon, New Haven, CT) ($2·5 \times 10^7$ cells/plate) precoated with rabbit anti-mouse immunoglobulin [DAKO (Glostrup, Denmark), 1:300], and rabbit anti-rat immunoglobulin (DAKO, 1:200) for 90 min at 4°. Non-adherent cells were removed by gentle washing with RPMI medium. The non-adherent population, which constituted ≈30–35% of the total lymph node population was 96% positive for $CD4^+$ T cells, as judged by flow cytometry.

Measurement of cell viability

Viability of the cells incubated with different concentrations of TauCl was routinely monitored by cellular exclusion of Trypan blue. In some experiments cell respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenylotetrazolium bromide (MTT) (Sigma) to formazan.[22] Cells were incubated with MTT (500 µg/ml) for 3 hr at 37°. Culture medium was removed, by aspiration, and the cells were solubilized in an isopropanol solution (PoCh, Gliwice, Poland). The extent of reduction of MTT to formazan within cells was quantified by measurement of optical density at 570 nm.

Cell culture and induction of IL-2 production

OVA system: APCs (A-20) were cultured (at 37° in an atmosphere of 5% CO_2) in 96-well U-bottom plates at a density of 3×10^3–10^5 cells per well, in RPMI-1640 medium (Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (FCS) (Gibco) and 2×10^{-5} M 2-mercaptoethanol (2-ME). Antigens – ovalbumin or $OVA_{329–338}$ peptide (ICRF Peptide Facility, London, UK) – were added to the culture at a concentration of 1·0 mg/ml or 5 µg/ml, respectively, unless stated otherwise. After incubation for 24 hr, supernatants were removed, cells were washed with RPMI-1640 medium, and the OVA-specific T-hybridoma (DO-11-10) cells (5×10^4/well) were added to the culture. After a further 24 hr incubation, supernatants were collected and frozen at −20° prior to assay for interleukin-2 (IL-2).

TNP system: $CD4^+$ T cells (2×10^5/well) and TNP-coupled spleen cells (5×10^4/well) were cultured in RPMI-1640 medium as described previously.[23] After 24 hr supernatants were collected and frozen prior to assay for IL-2.

Concanavalin A (Con A): spleen cells from the naive animals were cultured in 96-well U-bottom plates (1×10^6/well) in the presence of Con A (1–10 µg/ml). After 24 hr supernatants were collected and frozen prior to assay for IL-2.

Ovalbumin chlorination with TauCl

Ovalbumin dissolved in 0·05 M phosphate buffer, pH 7·5 was mixed, by stirring, with taurine chloramine solutions (prepared in the same buffer) of 0·1–0·3 mM TauCl/mg protein (equivalent to a 2–6 molar excess of TauCl). The mixture was incubated at 37° for 24 hr and then dialysed against RPMI-1640 medium for an additional 24 hr. Under these conditions, three or four sulphydryl groups per molecule of OVA were modified.[24] In some experiments, OVA was treated with TauCl in the presence of equimolar concentrations of 2-ME (Sigma).

IL-2 assay

IL-2 was measured using a capture enzyme-linked immunosorbent assay (ELISA), with microtitre plates (Corning, NY) coated with the rat monoclonal antibody against murine IL-2 (Pharmingen, San Diego, CA), and a biotinylated rat monoclonal antibody against murine IL-2 (Pharmingen), horseradish peroxidase streptavidin (Pharmingen) and ophenylenediamine and H_2O_2 (both Sigma) as substrate. The plates were read at 492 nm using a 96-well plate reader. Recombinant murine IL-2 (Pharmingen) was used as a standard.

Statistical analysis

Results are expressed as mean ± SEM. Statistical significance was determined by the Student's *t*-test and the differences were regarded as significant at $P < 0·05$.

RESULTS

The inhibition of IL-2 production by T cells in the presence of TauCl

The activity of TauCl (and unmodified Tau) was tested in three different models of *in vitro* T-cell activation. In the first

model, unfractionated spleen cells were stimulated with Con A mitogen; in the second, TNP-primed $CD4^+$-enriched lymph node T cells were stimulated with TNP–spleen cells as antigen; and in the third, the OVA-specific T-cell hybridoma DO-11-10 was stimulated by OVA and the A-20 antigen-presenting B-cell line.

Tau did not influence the production of IL-2 by stimulated T cells. In contrast, as shown in Table 1, TauCl inhibited the release of IL-2 in a dose-dependent manner in all three experimental models. At the concentrations tested (30–300 μM) TauCl did not affect cell viability in any model with the exception that 300 μM TauCl was cytotoxic for DO-11-10 cells (data not shown). In the Con A system we also tested the influence of TauCl on the release of IL-6, the cytokine generated by a different subset of T cells. TauCl inhibited the release of IL-6 in the same dose-dependent manner (data not shown).

The effect of TauCl on A-20-J cells

A-20 cells were preincubated with either TauCl or Tau, and then the cells were washed to remove excess free amino acids before addition of T cells (DO-11-10) and antigen. In this way, the influence of TauCl on the A-20 APCs could be studied independently of any effect on T cells or antigen. The antigen was added either as whole native OVA protein or as a synthetic peptide representing the epitope recognized by DO-11-10 cells (amino acids 323–339 of the OVA sequence). Both forms of antigen are recognized by OVA-specific T-hybridoma cells (DO-11-10). However, to stimulate IL-2 production, OVA protein requires both processing and presentation, while the OVA peptide requires only presentation by APCs.[21]

As shown in Table 2, TauCl inhibited the ability of A-20 cells to present either intact OVA or OVA peptide, although stronger inhibition was observed for whole OVA. The inhibition of antigen presentation by TauCl is dose dependent, and the inhibition depends on the number of APCs. When A-20 cells were used at a concentration of 10^5/well, we did not observe any effect of TauCl. The same concentration of TauCl (300 μM) completely blocked the production of IL-2 when 3×10^3 A-20 cells were used to stimulate T cells. Taurine alone did not affect the production of IL-2.

TauCl enhances the production of IL-2 when incubated with OVA in the presence of excess of A-20-J cells

A-20 cells (10^5/well) were cultured with OVA and TauCl prior to the addition of DO-11-10 T cells. At these concentrations, the previous set of experiments showed that TauCl does not inhibit antigen presentation by A-20-J cells. As shown in

Table 1. Inhibition of interleukin-2 (IL-2) production by T cells in the presence of taurine chloramine (TauCl)

				IL-2	
Antigen + APCs	T cells	Tau (μM)	TauCL (μM)	pg/ml	%
Concanavalin A*	Spleen cell	–	–	5713 ± 150	100
		300	–	5569 ± 248	101 ± 6
		100	–	5575 ± 112	99 ± 3
		30	–	5691 ± 192	100 ± 5
		–	300	869 ± 37**	8 ± 4**
		–	100	3634 ± 62	73 ± 14
		–	30	5655 ± 144	96 ± 5
Trinitrophyenyl spleen† cells	$CD4^+$ T cells	–	–	769 ± 26	100
		300	–	690 ± 18	82 ± 8
		100	–	757 ± 48	90 ± 7
		30	–	728 ± 44	98 ± 4
		–	300	155 ± 19**	17 ± 5**
		–	100	399 ± 24*	39 ± 11*
		–	30	617 ± 61	90 ± 4
Ovalbumin‡ + A-20 cells	DO-11-10 cells	–	–	1297 ± 72	100
		300	–	1410 ± 230	114 ± 9
		100	–	1365 ± 54	107 ± 6
		30	–	1269 ± 44	99 ± 3
		–	300	< 64	< 5***
		–	100	499 ± 48	43 ± 6*
		–	30	1035 ± 110	105 ± 28

*Spleen cells (10^6/well), stimulated with concanvalin A (Con A) (1 μg/ml), were cultured either with taurine or taurine chloramine for 24 hr.

†$CD4^+$ T cells 2×10^5/well, stimulated with trinitrophyenyl (TNP) spleen cells 5×10^4/well (see Materials and methods), were cultured for 24 hr in the presence of Tau or TauCl.

‡A-20 APC, 1×10^5/well, were cultured for 24 hr with OVA 1·0 mg/ml. After 24 hr the medium was replaced and the ovalbumin (OVA)-specific DO-11-10 T cells, 5×10^4/well were added. The cells were cultured for a further 24 hr in the presence of Tau or TauCl. Results are shown as a mean + SEM of triplicate cultures from one experiment. Each experimental condition was repeated in four or more separate experiments. Unstimulated *in vitro*, TNP-specific $CD4^+$ T cells released approximately 5% of the stimulated cell production of IL-2, whereas unstimulated T cells from naive animals and unstimulated T-cell hybridoma DO-11-10 did not release IL-2.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 2. The effect of taurine chloramine (TauCl) on A-20 cells

A-20 cells		IL-2%	
Number/well	Incubated with μM	OVA	$OVA_{323-339}$
10^5	–	100	ND
	Tau 300	95±4	
	TauCl 300	99±3	
	TauCl 100	96±7	
	TauCl 30	102±5	
3×10^4	–	100	100
	Tau 300	90±9	102±4
	TauCl 300	64±7*	82±6
	TauCl 100	92±4	99±5
	TauCl 30	100±4	101±4
10^4	–	100	100
	Tau 300	94±7	97±5
	TauCl 300	31±5**	56±11*
	TauCl 100	63±9*	76±7
	TauCl 30	103±4	96±12
3×10^3	–	100	100
	Tau 300	96±9	83±9
	TauCl 300	5±3**	22±7**
	TauCl 100	21±6**	49±10*
	TauCl 30	62±14*	71±12

APC A-20, at different numbers per well were incubated with Tau or TauCl. After 24 hr, the medium was replaced, DO-11-10 cells (5×10^4/well) were added and cultured for a further 24 hr in the presence of either whole ovalbumin (OVA) protein (1·0 mg/ml), or $OVA_{323-339}$ peptide (5 μg/ml). Supernatants were collected and tested for interleukin-2 (IL-2) content. IL-2 release is expressed as percentage of the release from control group, without Tau/TauCl. (100% = approximately 800–1500 pg/ml, depending on the number of APCs). Results are shown as mean ± SEM from three independent experiments.

*$P<0.05$; **$P<0.01$.

Fig. 1, under conditions when TauCl, OVA and A-20-J cells were all present at the same time, but T cells were added later and had no contact with TauCl, an increase in IL-2 release by DO-11-10 cells was observed. The maximum effect was observed at 200–300 μM TauCl (mean enhancement of 227%, range 132–305%). As shown in Fig. 1, TauCl did not influence the presentation of the OVA 323–339 peptide. Taurine alone had no effect on either antigen (data not shown).

IL-2 production stimulated by native and TauCl-modified OVA

To determine if the enhancement of IL-2 production, observed in Fig. 1, could be attributed to a change in the immunogenicity of OVA by reaction with TauCl, OVA was pretreated with TauCl before addition to the bioassay. As shown in Fig. 2, OVA modified by chlorination stimulates the production of IL-2 more effectively than does native protein. This effect was dependent on the extent of the chlorination and was observed in the range 0·1–0·3 mM TauCl. The enhanced immunogenic properties of TauCl-treated OVA were observed most clearly with suboptimal doses of antigen (<0·5 mg OVA/ml) (>250% of the release of IL-2 stimulated by native OVA). However, TauCl did not alter the immunogenic properties of OVA when incubated with antigen in the presence of an equimolar concentration of 2-ME (data not shown).

DISCUSSION

During the inflammatory response, activated neutrophils release myeloperoxidase (MPO) into phagosomal vacuoles and also extracellularly into inflamed tissue. MPO, together with a product of the respiratory burst – H_2O_2 – and ubiquitous chloride ions, forms a chlorinating system.[2,3,12] HOCl, the major product of this system, is highly bactericidal and is also harmful to neighbouring tissue. Owing to its extremely high oxidant activity, HOCl is short-lived and reacts with many biomolecules.[3,24] It has been described previously that proteins modified by chlorination show enhanced susceptibility to degradation by endoproteinases, a phenomenon that may have both pathological and immunological significance.[25,26] For example, proteins modified by HOCl may lose their biological properties, but also may become more immunogenic than the native ones.[14,27,28]

Taurine is the major scavenger of HOCl that produces the less toxic TauCl.[10,15] Recent studies, however, have shown that TauCl, in addition to its role of mopping up excess HOCl, has strong anti-inflammatory activity, inhibiting the production of NO, PGE_2 and TNF-α.[16–18] In the present study, we tested whether TauCl might also modulate the induction of an antigen-specific T-cell response by affecting antigen, APCs or activated T cells.

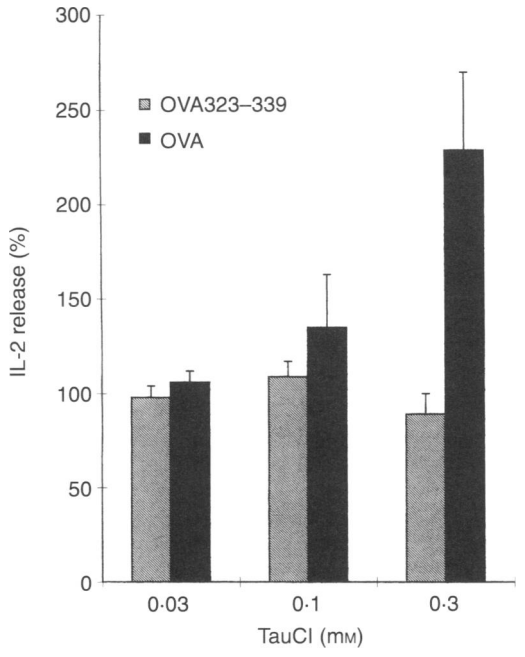

Figure 1. The effect of TauCl on antigen processing and/or presentation by A-20 cells. A-20 cells (1×10^5/well) were cultured in the presence of either OVA (1 mg/ml) or $OVA_{323-339}$ peptide (5 μg/ml) and Tau or TauCl. After 24 hr the medium was replaced, DO-11-10 cells (5×10^4/well) were added and cultured for a further 24 hr. IL-2 release is expressed as percentage of the release from the control group (incubated minus Tau/TauCl). Results represent four (OVA) and three ($OVA_{323-339}$) experiments, respectively.

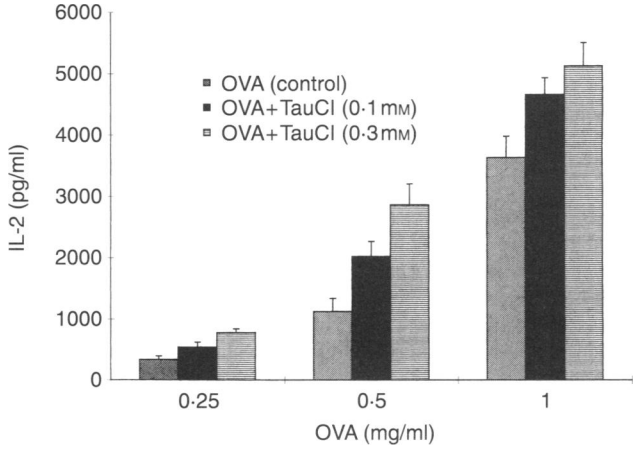

Figure 2. Production of IL-2 induced by native OVA and TauCl-modified OVA. OVA was treated with TauCl as described in the Materials and methods. A-20 APC (3×10^4/well) and DO-11-10 cells (5×10^4/well) were cultured together with different doses of either native or TauCl-modified OVA. After 24 hr supernatants were collected and IL-2 release was tested. Results represent one of five experiments.

We have shown in three *in vitro* models that TauCl, when added at the time of antigen stimulation and incubated with T cells, inhibited the generation of IL-2, with an IC_{50} value below 0·1 mM. It is difficult to estimate *in vivo* concentrations of TauCl. However, the intracellular concentration of Tau in neutrophils is very high (15–20 mM). As neutrophils can produce up to 2 μmol HOCl/hr per 10^7 cells, it is possible to estimate that the concentration of TauCl generated within the phagolysosome can reach 2 mM or greater.[29] TauCl also down-regulates (IC_{50} value 0·3–0·5 mM) the production of cytokines by activated phagocytes, as described previously.[16–18] This suppressor activity of TauCl seems to be common for various agents (nitric oxide, eicosanoids, cytokines) generated *de novo* in stimulated cells, such as macrophages, neutrophils and T cells. However, the mechanisms of inhibition may be different. For example, TauCl inhibits transcription of the iNOS (inducible nitric oxide synthase) gene, or some earlier event in the signal transduction pathway.[16,18] In contrast, TauCl appears to suppress the translation of TNF-α mRNA and affects the post-transcriptional regulation of COX-2 (inducible form of cyclooxygenase) protein expression, which results in decreased release of TNF-α and PGE_2 molecules, respectively.[18,30] Which mechanism is responsible for the inhibition of IL-2 production still needs to be determined. *In vivo*, the generation of TauCl usually occurs at a site of inflammation, at a distance from the lymphoid tissue in which T cells are initially activated by antigen.[8] Thus, it is unlikely that the inhibitory action of TauCl on T cells affects T-cell priming. However, TauCl may act to moderate the development of a local effector T-cell response, at least early in an inflammatory response.

A second target for TauCl action might be APCs (macrophages, sentinel DCs) or antigen, in this way modulating the induction phase of the antigen-specific immune response. To address these questions we looked, in the first instance, at the action of TauCl on a highly simplified model system, the activation of the OVA-specific DO-11-10 hybridoma by A-20 B-lymphoma cells and antigen. TauCl, when preincubated with A-20 cells, diminished their ability to stimulate IL-2 production by both forms of the antigen (native ovalbumin and the $OVA_{323-339}$ peptide). The inhibition could be masked by excess A-20 cells (10^5 cells/well). Surprisingly, when TauCl was incubated simultaneously with native OVA and with the higher concentration of A-20 cells, an enhancement of IL-2 production was observed. As the release of IL-2 was not affected when peptide was used instead of whole OVA, we could conclude that this phenomenon was modified by an effect of TauCl on the processing of OVA.[31] Further experiments confirmed this suggestion, as OVA modified by TauCl was more immunogenic then native OVA. In this model system the maximum enhancement was observed when OVA was incubated with 200–300 μM TauCl/mg protein. The effect could be abolished by addition of 2-ME, whose sulphydryl groups preferentially react with TauCl.[24,25] A similar enhancement had been observed previously when OVA was chlorinated by HOCl, and OVA modified by HOCl was more susceptible to proteolytic degradation by trypsin and cathepsin D.[26,27] HOCl reacts with several functional groups of proteins. Reaction with sulphydryl chains results in formation of additional inter- or intra- S–S bonds, followed by a decrease in total sulphydryl group content. Amino groups are oxidized to carbonyl moieties with a concomitant acidic shift of pI.[24,25] Thus, the increased susceptibility of chlorinated protein to proteolysis is caused by partial unfolding of the protein and/or by a change in the surface charge.[25,26] A similar series of reactions may occur between OVA and TauCl,[24] although further experiments will be required to determine if the increase in immunogenicity of

TauCl-treated OVA results from increased denaturation, and enhanced susceptibility to degradation.

In conclusion, it is apparent that TauCl has pleiotropic effects on the inductive phase of an immune response. In the microenvironment of the localized inflammatory response, our *in vitro* data would suggest that TauCl will exert predominantly a negative regulatory influence, inhibiting both T-cell and APC function. However, interaction between antigen and TauCl or HOCl will result in enhanced immunogenicity of protein antigens, thus enhancing the initiation of a subsequent adaptive specific response. Although HOCl is active at 10-fold lower concentrations than TauCl,[27] the much greater stability of TauCl over HOCl suggests that TauCl will play the major role in these processes *in vivo*, especially during the later phases of inflammation. More generally, our study provides further evidence of a role for neutrophils in modulation of adaptive immunity. As neutrophils are frequently the first cells to arrive at the site of infection, they, or the products they release, have the capacity to influence both APC function and the subsequent function of effector T cells. The activity of TauCl documented in this paper therefore provides another molecular link between innate and adaptive immunity.

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