

Immunological specificity of chloramine-T-induced IgE antibodies in serum from a sensitized worker

U. WASS*, L. BELIN*, and N. E. ERIKSSON†

*The Occupational Allergy Unit, Asthma and Allergy Research Centre, Sahlgrenska Hospital, Göteborg, and

†Department of Medicine, Länssjukhuset, Halmstad, Sweden

Summary

A procedure for the preparation of chloramine-T (CT) conjugates used to assay IgE antibodies was developed using response surface methodology and serum from a subject occupationally exposed to the substance. The conjugates, synthesized by reacting CT with human serum albumin (HSA) and other protein carriers, were used as antigens in a radio-allergosorbent test (RAST). Human serum albumin was found to be a suitable carrier, although other protein carriers also gave specific IgE-binding of a similar extent. The CT-HSA conjugates used in the RAST were characterized by high performance liquid chromatography, electrophoresis, immunodiffusion and ammonium sulphate precipitation. However, no strong correlation was seen between the ability of the conjugates to bind IgE and their physical or immuno-chemical properties. The hapten and carrier specificity of CT-induced IgE antibodies in the subject's serum were studied by direct RAST and RAST inhibition. No existence of new antigenic determinants related to the carrier could be demonstrated. Although HSA as a carrier was altered immunochemically by CT, the IgE antibodies were found to be specific to hapten only. Chloramine-T-specific IgG antibodies could not be demonstrated in the subject's serum.

Clinical and Experimental Allergy, Vol. 19, pp. 463–471. Submitted 14 October 1988; revised 16 February 1989; accepted 22 February 1989.

Introduction

Chloramine-T (CT), $\text{CH}_3\text{-C}_6\text{H}_4\text{-SO}_2\text{-N(Na)-Cl}$, is the sodium salt of *N*-chloro-*p*-toluene sulphonamide. It is an oxidative agent with antiseptic and disinfectant properties and has been used in cleaning of floors and other surfaces in dairies, breweries, the food industry, kitchens and operating theatres as well as in the treatment of infected wounds. The substance has bactericidal, fungicidal and virucidal properties. Furthermore, it is well known among biochemists because it has been used frequently for the radio-iodination of proteins for *in-vitro* procedures. The chemistry of CT has been reviewed by Campbell and Johnson [1].

Hypersensitivity reactions to CT have been known for a long time. Such reactions were discussed in a Swedish medical journal as early as 1935 [2], and in 1945 Feinberg and Watrous [3] observed 14 cases of occupational

asthma and rhinitis among workers exposed to the substance. They also noticed weal-and-flare reactions to CT. In 1968, Hård and Bengtsson [4] reported six cases among 40 workers in the factory hall of a paper pulp mill, where CT was sprayed on the pulp in order to suppress the growth of moulds and other microorganisms. Five of the workers had experienced both asthma and rhinitis, while rhinitis was the only symptom in the sixth worker. All six workers were found to have positive skin 'scratch' tests to CT. In 1979, Bourne *et al.* [5] described seven brewery workers with CT-induced asthma and positive weal-and-flare reactions to the substance. A further case was presented by Charles the same year [6]. Dijkman *et al.* described, in 1981, five cases with respiratory symptoms due to CT exposure [7]. They found that skin-prick tests, performed in four patients, as well as inhalation tests, performed in three patients, were positive. In a separate paper, the presence of IgE antibodies specifically directed against CT was demonstrated by this group, using both a polystyrene tube radioimmunoassay (PTRIA) and a radio-allergosorbent test (RAST) based on CNBr-

Correspondence: Dr Urban Wass, The Asthma and Allergy Research Centre, Department of Medicine I, Sahlgrenska Hospital, S-413 45 Göteborg, Sweden.

activated Sepharose 4B [8]. A further case with CT-specific IgE antibodies demonstrated by RAST was reported by Romeo *et al.* [9]. Allergic contact urticaria due to chloramine has also been reported [10,11]. One of the patients with allergic contact urticaria also suffered from dyspnoea and rhinitis, and was shown to have CT-specific IgE antibodies in serum [11].

The aim of this study was: to optimize the preparation of CT-conjugates with respect to their ability to bind specific IgE antibodies; to characterize the conjugates and investigate whether there was any correlation between their physical and immunochemical properties and the IgE-binding ability; and to study the specificity of the antibodies detected in this serum, with regard to both the CT hapten and the carrier.

Subject and methods

Subject

The subject, a 42-year-old non-smoking man, working part-time as a farmer (keeping cows and pigs), employed for 6 yr as a driver at a dairy. Three days a week he collects milk with his lorry at 60 farms. He checks the milk using a scoop which he disinfects with a CT solution. On these days, he mixes the CT solution twice by adding 8 l of hot water to about 0.05 l of a stock CT solution.

For 2 yr the subject has suffered from sneezing, nasal discharge, nasal blockage and eye irritation. The symptoms appear occasionally but seem to occur more often on days when he uses CT solution. A few times he has noticed more severe symptoms, with headache in addition to sneezing, when mixing the solution. On the other hand he had been free from symptoms for many days in spite of working with CT. No exposure data were available, however, and the CT exposure might have been low on the days when he was free from symptoms. Total IgE in the patient's serum was 35 kU/l.

Physical examination revealed only a slightly swollen nasal mucosa. The subject was successfully treated with prophylactic nasal corticosteroid aerosol (beclomethasone) and disodium cromoglycate eye drops.

Skin-prick and provocation tests

Skin-prick tests were performed on the patient's arms with CT (0.01–10 mg/ml) as well as with extracts of the common inhalant allergens, *Dermatophagoides pteronyssinus*, *Cladosporium*, *Alternaria*, *Aspergillus*, *Candida albicans*, horse, dog, cat, timothy, mugwort, birch and chironomid extracts. Skin-prick tests with native human serum albumin (HSA) (1 mg/ml) and CT-HSA conjugates (0.01, 0.1 and 1 mg/ml) were performed on the back of the subject. In these tests, the test solution was

applied in duplicate, either on both the left and the right arm, or on both the left and the right side of the subject's back. In all tests, saline-glycerol was the negative, and histamine dihydrochloride (10 mg/ml) the positive control. Five unexposed control individuals from our staff were also skin-prick tested with CT and CT-HSA.

Nasal provocation and conjunctival provocation tests were performed with CT solutions in the concentration ranges of 0.01–1.0 mg/ml and 1.0–10 mg/ml respectively.

Control sera used in the RAST

The non-specific IgE-binding to RAST discs was investigated using sera from individuals without any known CT exposure. Firstly, RAST discs with various CT-HSA conjugates were studied using a pool of sera from 20 unexposed individuals with the same level of total IgE (35 kU/l) in the pool as in the subject's serum as well as a pool of 20 sera from unexposed individuals with high levels of total IgE (2000 kU/l in the pool). Secondly, discs with the conjugate shown to give optimal RAST results were used together with sera from 18 randomly selected blood donors and 13 sera from other unexposed individuals with various serum levels of total IgE (from 8 to 5600 kU/l).

Reagents

Chloramine-T trihydrate, pro analys, was purchased from E. Merck (Darmstadt, FRG). Throughout this work, all amounts of chloramine-T refer to the trihydrate (molecular weight 281.69). Methanol, pro analys, was supplied by May & Baker Ltd (Dagenham, U.K.). Human serum albumin, fraction V, ovalbumin (OA), bovine serum albumin (BSA), porcine serum albumin (PSA), immunoglobulin A (IgA) from human colostrum, immunoglobulin G (IgG), human and *o*-phenylenediamine dihydrochloride were obtained from Sigma (St Louis, U.S.A.). Peroxidase-conjugated rabbit immunoglobulins to human IgG and anti-human albumin anti-serum produced in rabbits, were supplied by Dako-Immunoglobulins A/S (Copenhagen, Denmark). Polystyrene microplates (Nunc-Immuno plate I) for the enzyme-linked immunosorbent assay (ELISA), were purchased from A/S Nunc (Roskilde, Denmark). ¹²⁵I-labelled anti-IgE was purchased from Pharmacia Diagnostic AB (Uppsala, Sweden). Cyanogen bromide-activated paper discs were supplied by MIAB (Uppsala, Sweden).

In all analytical tests, duplicate samples were used.

Apparatus and equipment

For high performance liquid chromatography (HPLC) analysis, an LDC Constametric III pump (Laboratory

Data Control, Division of Milton Roy Co., Riviera Beach, U.S.A.) equipped with a Rheodyne 7125 injection valve, an LDC Spectromonitor III variable wavelength spectrophotometric detector, a Vitatron 2001 recorder, and a Hewlett-Packard 3390A integrator were used. Organic-free water was produced using a Millipore water purification system. For the separation of proteins by HPLC, a 30 cm $\times$ 7.5 mm i.d. TSK G3000 SW column (Toyo Soda Manufacturing Co., Tonda, Japan) was used.

For electrophoresis, an electrophoresis constant power supply (ECPS 2000/300) and a flat bed apparatus (FBE 3000, Pharmacia Fine Chemicals, Uppsala, Sweden) were used. Radioactivity bound to RAST discs was determined using a gamma-counter from LKB (Bromma, Sweden).

For dialysis purposes, Minicon-B15 sample concentrators were used (Amicon Corporation, Lexington, U.S.A.), with each chamber holding 5 ml of sample. Water and salt molecules passed through a membrane while proteins were retained and concentrated. RAST discs were washed using a DA 300 dispenser-aspirator from Pharmacia.

The light absorbance of samples on microtitre plates was recorded at 490 nm by means of a MR 700 microplate reader (Dynatech Laboratories Inc., Laboratorie Design AB, Lidingö, Sweden). The reader was run in dual wavelength mode with a 570 nm filter used as reference to reduce the effects of interfering factors such as dust and scratches. Microplates were washed by emptying and filling the wells using a Dynatech Autowash 2000 washer/aspirator (Dynatech Laboratories Inc., Lidingö, Sweden).

Preparation of conjugates and RAST procedure

One volume of a protein carrier solution was mixed with four volumes of a CT solution and left at room temperature. After a pre-defined time, 5-ml portions of the mixture were transferred to the chambers of a Minicon-B15 sample concentrator. The mixture was concentrated to a volume of 0.2 ml (concentration ratio=25) and 4.8 ml of 0.1 mol/l NaHCO_3 was then added to the chamber. This concentration and addition procedure was repeated once more before the solution was used in the RAST. The sample concentrator was used in order to separate the CT molecules not bound to the carrier from the conjugate. By this procedure, the phosphate buffer was also changed to an NaHCO_3 solution suitable for coupling to RAST discs.

The freshly prepared 0.1 mol/l NaHCO_3 solution containing the CT conjugate, was then added to the activated paper discs (100 discs per 20 ml). The discs were incubated at room temperature overnight and washed twice (10 min each time) in 0.1 mol/l NaHCO_3 , twice (60 min) in 0.1 mol/l NaHCO_3 solution containing

0.1 mol/l ethanolamine, twice (10 min) in 0.1 mol/l sodium acetate pH 4.0 and twice (10 min) in a phosphate-buffered saline solution (PBS) containing Tween and sodium azide. Furthermore, some activated paper discs were directly exposed to either 1 or 10 mg/ml of CT at pH 6.3 for 1 hr and then washed as described above.

In the RAST, 50 μ l of serum were added to the disc and the disc was incubated for 3 hr at room temperature. The disc was then washed three times, 10 min each time, with 2.5 ml of saline, containing Tween as a surfactant. Fifty microlitres of ^{125}I -labelled anti-IgE with an activity of approximately 40 000–65 000 c.p.m. was added and the disc was incubated overnight at room temperature. The next morning the samples were washed three times, as before, and counted for 60 sec on a gamma-counter. Results were expressed as a percentage of the total activity added, or for some experiments with CT-HSA discs, as the ratio between the uptake on a CT-HSA disc and on an HSA disc.

The relative standard deviation in RAST was calculated within the run by the performance of duplicate measurements [12].

Optimization of specific IgE-binding in the RAST

Various conjugates were prepared from HSA and CT in order to optimize the specific IgE-binding to RAST discs. A priori, it was assumed that for the reaction between CT and HSA there were four possible and significant variables:

- (1) pH of the reaction medium;
- (2) time for which CT was allowed to react;
- (3) concentration of CT; and
- (4) concentration of HSA.

In order to reduce the number of variables, the concentration of HSA was chosen to be 3 mg/ml in the final solution (after mixture with the CT solution). This concentration had been used successfully in other studies where low molecular weight haptens had been coupled to HSA and used in RAST [13,14].

Thus, there remained three variables to study: pH, time and CT concentration. The first step was to find out whether all of them were significant and needed further consideration. This was achieved by an initial screening procedure, using 8 (2^3) experiments in a factorial design [15]. Each of these three variables was tested on one low and one high level. The concentration of CT (after mixture with the HSA solution) was either 4 or 40 mg/ml, the time 1 or 3 hr and the pH of the 0.025 mol/l phosphate buffer was 5.7 or 8.

The time-dependence of the reaction was studied further in a separate experiment where CT (10 mg/ml) was allowed to react with HSA (3 mg/ml) at pH 6.25. The

reaction was monitored over time by HPLC and results were expressed as moles CT bound per mole of HSA.

Later, another 14 experiments, based on the results from the initial factorial design experiment, were conducted in order to find the optimal reaction conditions. This optimum was determined theoretically by response surface methodology [15,16]. Calculations were performed with the aid of the RSREG procedure within the Statistical Analysis System (SAS Institute Inc., Cary, NC, U.S.A.). The procedure was used to fit the parameters of a complete quadratic response surface and determine critical values to optimize the RAST response [17].

Preparation of conjugates using carriers other than HSA

When optimal conditions were established for the preparation of conjugates between CT and HSA, conjugates from other carriers were synthesized using the same procedure. Chloramine-T was reacted with BSA, PSA, OA, IgA, and IgG.

RAST inhibition

The capacity of a conjugate to inhibit a positive RAST reaction was studied by standard inhibition techniques. Thus, 50 μ l of human RAST-positive serum was mixed with 10 μ l of the different conjugates in 0.1 mol/l NaHCO₃ buffer and left at 6°C overnight. Discs were incubated with the mixture the next morning at room temperature for 3 hr and treated according to the standard RAST procedure as described above.

The percentage inhibition was calculated as follows:

$$\text{percentage inhibition} = 100 \times \frac{(\text{c.p.m.}_b - \text{c.p.m.}_i)}{(\text{c.p.m.}_b - \text{c.p.m.}_n)}$$

Where c.p.m._b = c.p.m. with buffer but without inhibitor, c.p.m._i = c.p.m. with inhibitor, and c.p.m._n = c.p.m. for a normal, negative serum.

ELISA

The CT-HSA conjugate was diluted with 3 mmol/l PBS solution, pH 7.2, to a final concentration of 0.1 mg/ml and added in 100- μ l amounts to the polystyrene plate. A standard ELISA procedure using peroxidase-conjugated anti-IgG was adopted. Each serum tested was used in eight fivefold dilutions, starting at 1/5.

Characterization of CT-HSA conjugates

High performance liquid chromatography was used to determine the average number of CT molecules bound per molecule of HSA. The calculation was based on the peak areas recorded at 254 nm. Peak areas for conjugates

were compared with areas for pure HSA as well as areas for free CT. It was assumed that CT bound to HSA showed the same ultraviolet spectrum as the free molecules. The conjugates were chromatographed using a 0.1 mol/l sodium phosphate buffer, pH 7.0, containing 0.3 mol/l NaCl as mobile phase.

The solubility of the different CT-HSA conjugates was studied by an ammonium sulphate precipitation experiment. To 300 μ l of a 3 mg/ml CT-HSA solution in pH 7.2 PBS, 300 μ l of saturated ammonium sulphate solution was added, giving 50% saturation of the mixture. The mixture was left at room temperature for 1 hr and then centrifuged at 6000 g for 15 min. The precipitated amount was calculated by comparing the absorbance at 254 nm of the supernatant with the absorbance of a CT-HSA sample that had not been treated with ammonium sulphate.

The electrophoretic mobility of the CT-HSA conjugates was studied using agarose gel electrophoresis with a pH 8.6 barbitone buffer run at 100 V for 75 min. The distance of anodal migration from the starting point was measured for both CT-HSA conjugates and native HSA. The change in mobility (%) was calculated as follows:

$$\text{change} = 100 \times \frac{(D_C - D_H)}{D_H}$$

Where D_C = distance of migration of the conjugate and D_H = distance of migration of native HSA.

The change in antigenicity of HSA, when reacted with various amounts of CT, was estimated by immunodiffusion in agarose gels as well as 'rocket' immunoelectrophoresis using rabbit antibodies to HSA. Conjugates prepared from carriers other than HSA were used in RAST without any characterization.

Results

Subject

Skin-prick tests were negative to common inhalant allergens. Skin-prick tests, nasal provocation and conjunctival provocation were positive using CT (Table 1). Nasal and conjunctival reactions were elicited at a CT concentration of 1.0 mg/ml, while positive skin tests already occurred at 0.1 mg/ml.

In the skin-prick test with CT-HSA conjugates, a significant weal reaction was already noted at 0.01 mg/ml. The weal size increased with increasing CT-HSA concentration. The results for 1 mg/ml solutions of conjugates with approximately 8, 40 and 100 CT haptens per HSA molecules were 5, 7 and 7 mm respectively. The mean weal diameter for the positive histamine reference was 5 mm, while no weal was elicited by the saline control or by native HSA. The five control individuals that were skin-prick tested with CT and CT-HSA were all negative.

Table 1. Results of skin-prick tests and nasal/conjunctival provocation tests with chloramine-T. The mean weal diameter elicited by the positive histamine reference was 5 mm

Chloramine-T concentration (mg/ml)	Skin-prick test, mean weal diameter (mm)	Nasal provocation test	Conjunctival provocation test
0.01	1	Negative	n.d.
0.1	4	Negative	n.d.
1.0	5	Moderate discharge, severe blockage	Slight itching
10	8	n.d.	Redness, itching, tears

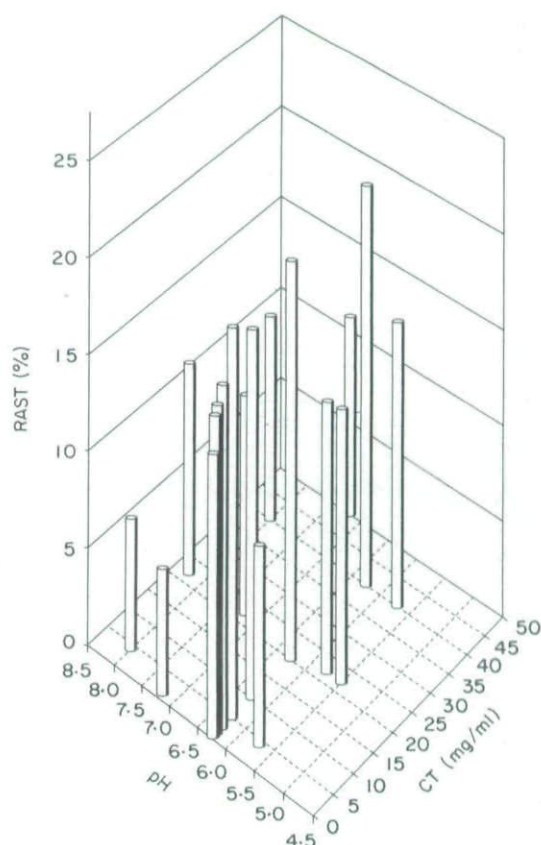
Table 2. Result of the factorial design experiment

Chloramine-T concentration (mg/ml)	Time (hr)	pH	Result of RAST (percentage of total activity)
4	1	5.7	5.2
40	1	5.7	13.1
4	3	5.7	10.0
40	3	5.7	15.0
4	1	8.0	7.1
40	1	8.0	11.2
4	3	8.0	6.8
40	3	8.0	11.1

Synthesis of HSA-conjugates and their use in the RAST

The initial factorial design experiment showed that the CT concentration was an important variable in the preparation of conjugates (Table 2). At pH 5.7 or 8, a concentration of 40 mg/ml elicited higher binding in the RAST than a concentration of 4 mg/ml. Time was found to be less important, especially at high CT concentrations. The reaction between CT and HSA was found, by an additional experiment, to continue for at least 7 hr. The rate of reaction was fast during the first 30 min and approximately 40 mol of CT was bound per mol of HSA during that time. Then, two periods of lower reaction rates followed; from 30 min to 2 hr, the rate of reaction was approximately one-sixth, and after 2 hr approximately one-twentieth of its initial value. A reaction time of 3 hr would thus cover the time periods characterized by fast and moderate reaction rates, while a time of 1 hr would not.

An additional 14 experiments were carried out with varying concentrations and pH values while the time of reaction was kept constant at 3 hr. The results, together with results from the screening procedure obtained using a 3-hr reaction time, are shown in Fig. 1. The outcome of the RAST is illustrated by the height of the bars. As shown, various combinations of CT concentration and

**Fig. 1.** The result of RAST (percentage of the total activity added) as a function of pH and CT concentration (mg/ml) during the preparation of the CT-HSA conjugates.

pH produced widely varying results. The range of IgE binding was from 6 to 21% of the total activity added.

The optimal conditions were found theoretically by calculating a response surface based on the experimentally determined points. The response surface, based on the experimental data in Fig. 1, is shown in Fig. 2. The pH had a significant influence on the RAST result ($P=0.03$). The CT concentration, on the other hand, was of minor importance above approximately 5 mg/ml when con-

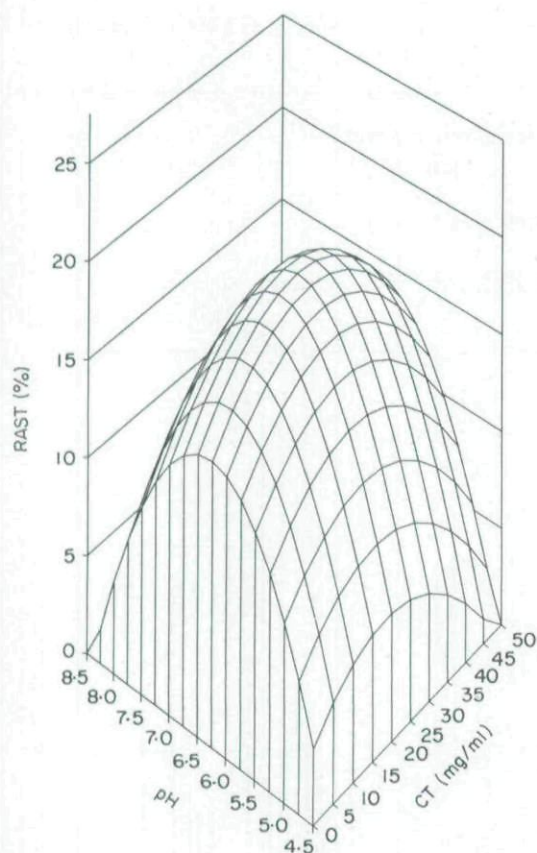


Fig. 2. A response surface fitted to the experimental data shown in Fig. 1.

jugates were prepared at pH 6.25. The predicted optimum conditions corresponded to pH 6.5 and a CT concentration of 25 mg/ml, and the predicted RAST value at that point was 19% of the total activity. However, some of the experiments shown in Fig. 1 had been conducted at pH 6.25 and with CT concentrations ranging from 5 to 40 mg/ml. As these experiments had produced results between 19% and 21%, we considered these experimental conditions to be sufficiently close to the real optimum and no further optimization was performed. The relative standard deviation in RAST for the subject's serum using the discs prepared at pH 6.25 was found to be 9% when calculated within the run by the performance of duplicate measurements.

The non-specific binding to discs, due to high levels of total IgE, was low for conjugates prepared with ≤ 20 mg/ml CT but increased slightly when the amount of CT was 40 mg/ml. Significant binding, 10% of the total activity, was noted when the subject's serum was added to RAST discs that had been exposed directly to CT (without the use of any carrier). However, the same result

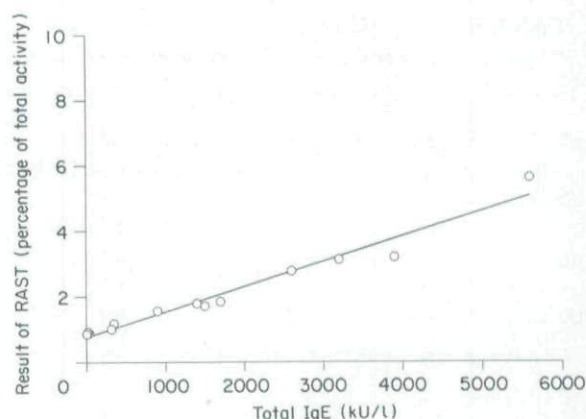


Fig. 3. Correlation between non-specific binding to optimal RAST discs and total IgE levels in sera from non-exposed controls.

was achieved when the two serum pools were used, revealing that the discs bound IgE non-specifically and thus produced unreliable results.

Optimal discs

Based on the above results, the following conditions of preparation of CT-HSA conjugates for RAST were chosen: HSA 3 mg/ml, CT 20 mg/ml, pH 6.25 and time 3 hr. Such discs were named 'optimal discs'. The optimal discs were studied using sera from blood donors and sera with various levels of total IgE. The 18 blood donors' results were in the range 0.8 to 1.1% of the total activity compared with the borderline of a positive RAST, according to the Phadebas reference system (Pharmacia Diagnostics, Uppsala, Sweden), which was approximately 3%. The correlation between the non-specific binding to optimal RAST discs and total IgE in serum is shown in Fig. 3. A total IgE level of approximately 3000 kU/l was required to obtain a false positive result. Sera with high total IgE concentrations, however, also show a high degree of binding to a pure HSA disc. The ratio between the uptake on a CT-HSA disc and on an HSA disc was 13 for the patient, 2.3 for the serum with 5600 kU/l of total IgE and < 2 for the other sera illustrated in Fig. 3.

RAST with carriers other than HSA

The binding of specific IgE from the subject's serum to CT-OA, CT-IgA, CT-IgG and CT-BSA discs was not significantly different from that obtained with CT-HSA discs, while CT-PSA discs elicited 30% lower binding.

RAST inhibition

Native HSA was completely unable to inhibit the binding of specific IgE to CT-HSA discs. Conjugates of CT and

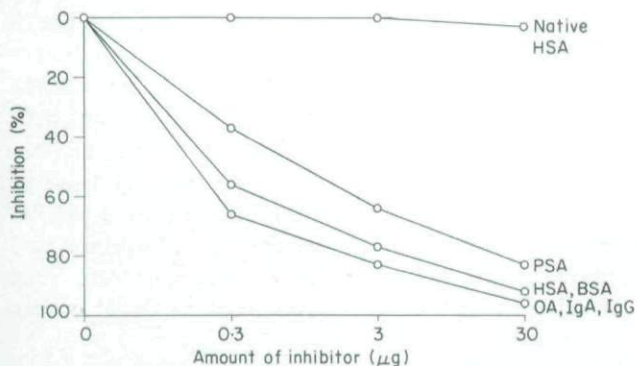


Fig. 4. RAST inhibition using CT-HSA discs, serum from the subject and various CT-carrier conjugates (or native HSA) as inhibitors. Carriers which gave very close inhibition curves have been represented by one curve only. Note that the scale of the x axis is logarithmic and the point 0 is thus not mathematically defined but has been added for reasons of clarity.

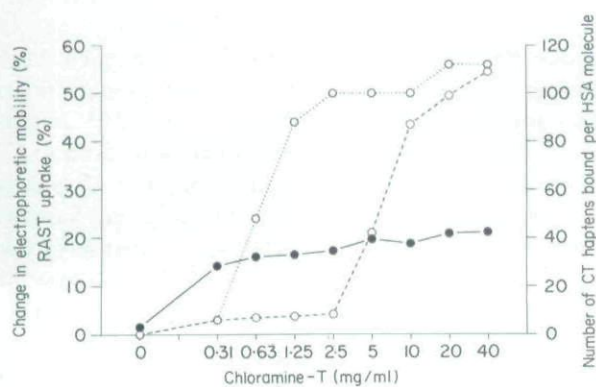


Fig. 5. Characteristics of CT-HSA conjugates as a function of the CT concentration used for preparation. (○.....○) Shows the increase in electrophoretic mobility at pH 8.6 relative to HSA, expressed as a percentage. (●—●) Shows the outcome of RAST for the respective conjugate. The number of CT haptens bound per HSA molecule is shown by (○---○). This line corresponds to the y axis on the right side. Note that the scale of the x axis is logarithmic and the point 0, which represents native HSA, is thus not mathematically defined but has been added for reasons of clarity.

the other carriers, on the other hand, gave a significant dose-related inhibition. The PSA conjugate was a somewhat less efficient inhibitor than the other conjugates (Fig. 4).

ELISA

No CT-specific IgG-antibodies could be demonstrated in the subject's serum by ELISA. Serum from the subject,

sera from blood donors and the two serum pools were analysed. The absorbances were generally low at a serum dilution of 1:125 or more and the subject's serum did not give significantly higher absorbance values than did the other sera.

Characterization of the HSA-conjugates

The conjugates were studied regarding their physical and immunochemical properties. The results from these analyses were compared with the respective conjugate's ability to bind IgE specifically in RAST. The specific IgE-binding in RAST did not, however, correlate closely with either the number of CT haptens or electrophoretic mobility (Fig. 5). The conjugate prepared with 5 mg/ml of CT showed a detectable but weaker antigenicity in immunodiffusion, using anti-HSA antibodies, than did pure HSA. Conjugates prepared with less CT were not different from pure HSA in this respect, while higher concentrations of CT completely eliminated the antigenicity. Conjugates prepared with ≥ 2.5 mg/ml of CT precipitated at 50% ammonium sulphate saturation. The IgE-binding ability of the conjugates did not change significantly when the antigenicity, using anti-HSA antibodies, disappeared or when the conjugates became less soluble.

Discussion

The subject's history in combination with the results of skin tests, provocation tests and RAST, indicated that CT exposure was the cause of his symptoms. The subject left work, for reasons other than his allergy, and since then has been free from symptoms. As sensitization by haptens is increasingly recognized, it was considered interesting to study some aspects of CT-induced IgE antibodies in detail and to optimize the RAST system.

It was desirable that conjugates used in the RAST gave the highest possible specific binding of CT-induced IgE-antibodies, while the non-specific binding due to high levels of total IgE was minimal. Several variables, such as concentration, time, and pH, might influence the characteristics of the synthesized conjugates. It was desirable to allow for a simultaneous variation of all experimental variables when searching for optimal conditions for preparation, since a joint influence by two or more variables may exist. However, the number of experiments required grows rapidly when the number of variables increases. For example, each of five variables tested on four levels requires 1024 (4^5) experiments. However, methods are available that deal with such problems. We chose to use factorial design as a first screening and then response surface analysis, an approach based on papers published by Carlson and co-workers [15,16].

The need to optimize RAST discs was obvious. During this work, the specific binding of CT-specific IgE antibodies to CT-HSA discs ranged from 5 to 21%. Furthermore, the need for negative control sera was illustrated by the discs prepared by direct exposure of CT without any protein carrier; IgE was bound non-specifically and any serum will be 'positive' when analysed with such discs.

The CT-HSA conjugates were characterized by HPLC with regard to the number of CT haptens per HSA molecule. This procedure eliminated interferences from molecules not bound to the protein, since low molecular weight substances and HSA conjugates eluted separately. When we estimated this number from ultraviolet absorption data, we assumed that the ultraviolet spectrum of CT was not shifted when it was bound to the macromolecule carrier. However, as the spectrum of CT might change when the substance is bound, and as CT is likely to oxidize HSA and to change the ultraviolet spectrum of HSA itself, the results obtained cannot be considered absolute values. However, our results are of the same order of magnitude as those reported by Evans *et al.* [18] who used radio-labelled CT to study the covalent binding of CT to HSA. They found 24 CT molecules per molecule of HSA when the concentrations of both CT and HSA were 10 mg/ml. The experiment in our work closest to theirs employed 5 mg/ml of CT and 3 mg/ml of HSA, i.e. 67% more CT was added per HSA molecule in our experiment, and we found approximately 42 molecules of CT per HSA molecule under these conditions.

Our study shows that the carrier protein is not of definite importance for the binding of CT-induced IgE antibodies in the RAST, and thus confirms the findings of Kramps *et al.* [8]. We found that several carriers could be used successfully in RAST as well as in RAST inhibition. Porcine serum albumin was somewhat less efficient than HSA and the other carriers. However, it is not possible to draw any definite conclusions from this fact, as the conjugation procedure was not optimized for this protein, as it was for HSA. The antibodies were hapten-specific only, indicating that CT did not induce new antigenic determinants of the protein. Kramps *et al.* have suggested that the antigenic determinant could be formed by the *p*-toluenesulfon group of CT, as low molecular weight compounds structurally related to CT could block the specific IgE-antibodies [8].

Conjugates with < 10 CT molecules per HSA molecule were almost as efficient in the RAST as conjugates with approximately 100 haptens or more per carrier. This is probably due to the exclusive hapten specificity of the subject's IgE and the fact that the number of binding sites on a RAST disc was already in excess with conjugates of the lower epitope density. In a study of isocyanate-specific IgE antibodies, the antibodies were directed

against both hapten and carrier, and a large number of haptens per carrier was found to have a negative influence on the outcome of RAST [14]. In this study, however, the modification of the carrier seemed to be of very little importance. Conjugates with greatly varying electrophoretic mobility, solubility and antigenicity towards anti-HSA antiserum bound specific IgE to a similar extent. We could not demonstrate any CT-specific IgG antibodies and other attempts to demonstrate such antibodies among sensitized individuals have also been negative [8].

Hapten sensitization implies *in-vivo* conjugation to an accessible body protein. Furthermore, according to present knowledge, IgE-mediated mast cell response requires bridging of adjacent IgE molecules by at least bivalent allergens. Nevertheless, pure (monovalent) CT solutions elicited immediate prick-test reactions in this subject. However, the molar concentration of monovalent CT had to be about 100 times higher than carrier-bound CT in order to elicit weal reactions of comparable size. This would indicate that, during conditions of excess, rapid conjugation occurs *in vivo* at the test site.

Since the outcome of the RAST was dependent upon the conjugate used, the conditions during preparation of such conjugates should be carefully controlled. *In-vitro* studies of IgE antibodies from workers sensitized to CT may be of diagnostic help and may increase our general understanding of allergic reactions to chemical haptens. Furthermore, the factorial design and response surface methods illustrated by this study might be generally useful in studies concerning reactive chemical haptens.

Acknowledgment

This study was supported by grant 84-1240 from the Swedish Work Environment Fund.

References

- 1 Campbell MM, Johnson G. Chloramine-T and related *N*-halogeno-*N*-metallo reagents. *Chem Rev* 1978; 78:65-79.
- 2 Salén EB. Några hittills föga beaktade allergiformer. *Nord Med Tidskr* 1935; 9:241-6.
- 3 Feinberg SM, Watrous RM. Atopy to simple chemical compounds—sulfone-chloramides. *J Allergy* 1945; 209: 16.
- 4 Hård S, Bengtson K. Hypersensitivity to chloramine at a paper pulp mill. *Proc 18 Meeting Scand Dermatol Ass, Turku*, 1968:166-171.
- 5 Bourne MS, Flindt MLH, Miles Walker J. Asthma due to industrial use of chloramine. *Br Med J* 1979; 2 (6181): 10-12.

- 6 Charles TJ. Asthma due to industrial use of chloramine (letter). *Br Med J* 1979; 2 (6185):334.
- 7 Dijkman JH, Vooren PH, Kramps JA. Occupational asthma due to inhalation of chloramine-T. I. Clinical observations and inhalation-provocation studies. *Int Arch Allergy Appl Immunol* 1981; 64:422-7.
- 8 Kramps JA, van Toorenenbergen AW, Vooren PH, Dijkman JH. Occupational asthma due to inhalation of chloramine-T. II. Demonstration of specific IgE antibodies. *Int Arch Allergy Appl Immunol* 1981; 64:428-38.
- 9 Romeo L, Gobbi M, Pezzini A, Caruso B, Costa G. Asma da tosilcloramide sodica: descrizione di un caso. *Med Lav* 1988; 79:237-40.
- 10 Beck HI. Type I reaction to chloramine. *Contact Dermatitis* 1983; 9:155-6.
- 11 Dooms-Goossens A, Gevers D, Mertens A, Vanderheyden D. Allergic contact urticaria due to chloramine. *Contact Dermatitis* 1983; 9:319-20.
- 12 RN Barnett. *Clinical Laboratory Statistics*, 2nd Ed. Boston: Little, Brown and Company, 1979:105.
- 13 Wass U, Belin L. Longitudinal study of specific IgE and IgG antibodies in a patient sensitized to ethylene oxide through dialysis. *J Allergy Clin Immunol* 1988; 82:679-86.
- 14 Wass U, Belin L. Immunological specificity of isocyanate-induced IgE-antibodies in serum from ten sensitized workers. *J Allergy Clin Immunol* 1989; 83:126-35.
- 15 Carlson R, Lundstedt T, Phan-Tan-Luu R, Mathieu D. On the necessity of using multivariate methods for optimization in synthetic chemistry. An instructive example with the Willgerodt reaction. *Nouveau Journal de Chimie* 1983; 7:315-19.
- 16 Carlson R, Hansson L, Lundstedt T. Optimization in organic synthesis. Strategies when the desired reaction is accompanied by parasitic side reactions. An example with enamine synthesis. *Acta Chem Scand* 1986; B40:444-52.
- 17 SAS users guide: statistics, Version 5 Edn. SAS Institute Inc., Cary, 1985:725-34.
- 18 Evans JC, Jackson SK, Rowlands CC, Baratt MD. Covalent binding of human serum albumin and ovalbumin by chloramine-T and chemical modification of the proteins. *Anal Chim Acta* 1986; 186:319-23.

Copyright of Clinical & Experimental Allergy is the property of Blackwell Publishing Limited and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.