Immunology 1993 **79** 667–672

Characterization of a monoclonal antibody to *cis*-urocanic acid: detection of *cis*-urocanic acid in the serum of irradiated mice by immunoassay

A. M. MOODYCLIFFE, M. NORVAL, I. KIMBER* & T. J. SIMPSON† *Department of Medical Microbiology, University of Edinburgh Medical School, Edinburgh, *Zeneca, Central Toxicology Laboratory, Macclesfield and †School of Chemistry, University of Bristol, Bristol*

Accepted for publication 28 March 1993

SUMMARY

Cis-urocanic acid (*cis*-UCA), which is formed from the naturally occurring *trans*-isomer on ultraviolet (UV) irradiation, has been suggested as a photoreceptor for and mediator of the suppressive effects of UV irradiation on systemic immune responses. *Trans*-UCA is located predominantly in the stratum corneum, and the extent of isomerization to *cis*-UCA may be analysed by high-performance liquid chromatography (HPLC) of skin extracts. Such an analysis is not suitable for other tissues. In this study a murine monoclonal antibody to *cis*-UCA was prepared and tested by ELISA using UCA isomers conjugated to protein as antigens. The interaction of the antibody with structural analogues of UCA was assessed by competitive inhibition ELISA which indicated that the antibody had a high specificity for *cis*-UCA. Screening of sera at various times after UVB irradiation of mice by competitive inhibition ELISA using the monoclonal antibody showed that *cis*-UCA was present, probably in an unbound form, for at least 2 days after the exposure. Thus, *cis*-UCA produced in the epidermis following UVB irradiation reaches the serum a few hours later. The implications of this finding for the generation of suppressed immune responses are discussed.

INTRODUCTION

Irradiation with ultraviolet-B light (UVB) results in suppression of some T-cell-mediated immune responses to antigens encountered within a short period after exposure. This has been shown in a variety of species including man (reviewed in ref. 1) and mice (reviewed in ref. 2). Several hypotheses have been suggested to explain the immunomodulation, one of which involves initiation by a specific photoreceptor, urocanic acid (UCA), in the skin which then mediates immunosuppression.[3] UCA is produced in the stratum corneum during keratinization by the action of the enzyme histidine ammonia-lyase.[4] It occurs naturally as the *trans*-isomer and represents a major UV absorbing component of skin. On UV irradiation *in vitro* or *in vivo*, *trans*-UCA converts to *cis*-UCA in a dose-dependent manner until a photostationary state is reached.[5–7] Evidence from a variety of experimental systems, most in mice, indicates that *cis*-UCA mimics a number of the effects of UV irradiation on immune responses. These include modification of antigen-presenting cell function *in vivo*,[8] suppression of contact hypersensitivity[9,10] and delayed hypersensitivity[11] responses, delay in rejection of transplant allografts[12] and enhancement of UV-induced tumour yield and malignancy.[13]

Correspondence: Dr M. Norval, Dept. of Medical Microbiology, University of Edinburgh Medical School, Teviot Place, Edinburgh EH8 9AG, U.K.

It is not known exactly how *cis*-UCA alters immune function or, indeed, if it is confined solely to the epidermis after irradiation. High-performance liquid chromatography (HPLC) has been used successfully to quantify UCA isomers in skin extracts.[14–16] However, it has not proved possible to analyse other tissues easily by HPLC because of the complexity of substances present and the fact that UCA represents a major UV absorbing constituent only in the skin. One approach has been to label UCA with ^{14}C and to follow its distribution after topical application in mice.[17] The novel approach, used in the present study, was to prepare a monoclonal antibody with specificity for *cis*-UCA and to use this to detect *cis*-UCA in serum following UVB irradiation of mice.

MATERIALS AND METHODS

UCA isomers and analogues

Trans-UCA was purchased from Sigma (Poole, U.K.) and *cis*-UCA prepared from it as outlined in Norval *et al.*[18] The following analogues of UCA were synthesized as described previously:[18] *cis*- and *trans*-2-methyl UCA, *cis*- and *trans*-3-furanacrylic acid, *cis*- and *trans*-2-thiopheneacrylic acid, *cis*- and *trans*-3-thiopheneacrylic acid and dihydrourocanic acid. Histidine and histamine were purchased from Sigma. The *trans*-isomer of 2-pyridineacrylic acid was prepared by Knoevenagel condensation of 2-pyridinecarboxaldehyde with malonic acid in the presence of pyridine. *Trans*-3-pyridineacrylic acid was

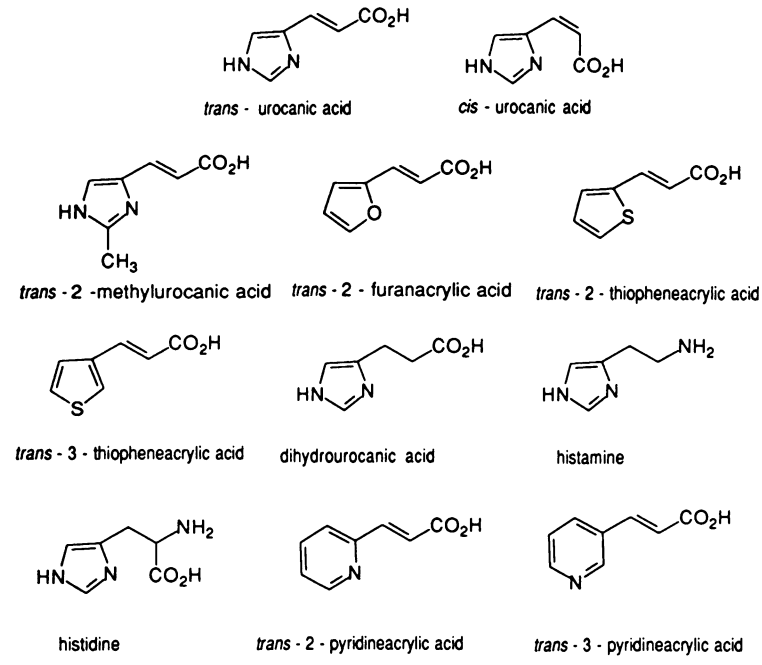

Figure 1. Structure of urocanic acid isomers and analogues.

prepared similarly from 3-pyridinecarboxaldehyde. *Cis*-2-pyridineacrylic acid was prepared from the corresponding *trans*-isomer by irradiation followed by thin-layer chromatography using a solvent system of 90% ethyl acetate, 8% methanol and 2% formic acid. The solvent was removed under vacuum and the *cis*-isomer purified on a dry-flash column eluted with ethyl acetate followed by sublimation after removal of the solvent. The *cis*-isomer of 3-pyridineacrylic acid was prepared similarly from the corresponding *trans*-isomer except it was recrystallized from ethanol in the final step. The identity and purity of the compounds were established as outlined in Norval *et al.*[18] The structures of these molecules are shown in Fig. 1.

Conjugates

Cis- and *trans*-UCA were coupled to keyhole limpet haemocyanin (KLH; Sigma) and bovine serum albumin (BSA; Sigma) using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide as a condensing agent[19] followed by purification by fast protein liquid chromatography (FPLC) using a Sephadex G25 column eluted with sodium borate buffer (200 mM, pH 9). The hapten–carrier conjugate was obtained by freeze drying of the protein-containing fractions. Histamine was coupled to BSA similarly.

Trans-[2,5-^{3}H] UCA was prepared from L-[2,5-^{3}H] histidine using the enzyme histidine ammonia-lyase (Sigma), then irradiated and the labelled isomers separated by HPLC[14] and counted. Each was reacted with KLH and BSA in the presence of the relevant cold UCA isomer, and the final conjugate counted to give an estimate of the number of hapten molecules per molecule of protein. The epitope density was found to be 62 *trans*-UCA molecules and 29 *cis*-UCA molecules/KLH molecule, and 4·5 *trans*-UCA molecules and 3·7 *cis*-UCA molecules/BSA molecule.

Preparation of monoclonal antibody

Female BALB/c mice, aged 6 weeks and bred in the Department of Medical Microbiology Animal House, were immunized by subcutaneous injection of 200 μg *cis*-UCA–KLH conjugate in 0·1 ml incomplete Freund's adjuvant. A first booster injection consisting of 200 μg *cis*-UCA–KLH conjugate in 0·2 ml sterile 0·01 M phosphate-buffered saline pH 7·2 (PBS) given intraperitoneally was administered after 36 days, followed by a second after 61 days and a third after 166 days. Mice were bled 7 days after the second booster and sera screened for anti-*cis*-UCA antibody by ELISA (see below). Mice showing antibody titres to *cis*-UCA were killed 3 days after the third booster and spleen cells fused with NS-0 mouse myeloma cells (a gift from Dr M. McCann, Department of Surgery, Edinburgh University, Edinburgh, U.K.) using a standard fusion technique. Culture supernatants were screened 14 days after fusion for antibodies to *cis*-UCA. The single positive culture was cloned and recloned by limiting dilution. The immunoglobulin subclass of the monoclonal antibody was determined by an isotyping kit (Amersham International, Amersham, U.K.).

ELISA

ELISA plates (Gibco BRL, Paisley, U.K.) were coated overnight at 4° with *cis*-UCA–BSA conjugate diluted in 0·1 M carbonate–bicarbonate buffer pH 9·6 (5 μg in 100 μl/well). After extensive washing with PBS–Tween pH 7·2, 100 μl hybridoma culture supernatant or serum diluted as appropriate in PBS–Tween containing 1% BSA was added and incubation continued for 3 hr at room temperature, followed by washing, incubation with anti-mouse IgG alkaline phosphatase conjugate (Sigma) and development with *p*-nitrophenyl phosphate. Absorbance was measured at 405 nm. Tissue culture medium or pre-bleed serum, as appropriate, were used as negative controls. The test was considered positive if the absorbance was more than the mean of the negative control + 3 SD of the mean.

In other assays *trans*-UCA–BSA conjugate, histamine–BSA

conjugate, KLH or BSA were used as antigen, all at 5 μg in 100 μl/well.

Competitive inhibition ELISA to test the specificity of the monoclonal antibody

Wells of an ELISA plate were coated with *cis*-UCA–BSA conjugate at 0·08 μg/well and incubated overnight at 4°. After extensive washing with PBS–Tween, 50 μl of the appropriate inhibitor was added to triplicate wells or more, followed by 50 μl of a 1/400 dilution of the hybridoma culture supernatant. The inhibitors were dissolved in dimethyl sulphoxide (DMSO) at 37° for 5 min at concentrations of 20, 5 or 1 mg/ml and then diluted appropriately in PBS–Tween containing 1% BSA. The plates were incubated for 3 hr at room temperature, washed and developed as above.

The negative control contained 100 μl PBS–Tween with 1% BSA. The positive control contained 50 μl of the diluted hybridoma culture supernatant and 50 μl of a dilution of DMSO in PBS–Tween containing 1% BSA, prepared as for the inhibitors above. The inhibition of binding of the monoclonal antibody was calculated as:

$$\% \text{ inhibition} = 100 \times 1 - \left[\frac{(\text{mean absorbance test} - \text{mean absorbance} - \text{ve control})}{(\text{mean absorbance} + \text{ve control} - \text{mean absorbance} - \text{ve control})} \right]$$

The SD from the mean absorbances of test and positive samples was less than 10%.

Screening of murine sera for cis-*UCA*

In a preliminary experiment, *cis*- and *trans*-UCA at various concentrations were added to murine serum diluted 1:3 in PBS–Tween with 1% BSA. Fifty microlitres of this was added to each of six wells coated with *cis*-UCA–BSA conjugate at 0·08 μg/well as already described; 50 μl hybridoma at a dilution of 1:400 was also added. One control contained serum without monoclonal antibody or UCA isomer and another contained serum with monoclonal antibody but no UCA isomer. The plates were incubated at room temperature for 3 hr, washed and developed as above.

C3Hf Bu/Kam mice were bred and maintained in the Department of Medical Microbiology Animal House. The dorsal sides of female mice, aged 7–8 weeks, were shaved and they were irradiated once with 216 mJ/cm^2 UVB or 144 mJ/m^2 UVB twice with a 24-hr interval between exposures. The conditions of irradiation and the lamp used have already been described.[20] Five hours before irradiation the mice were eye bled and the sera pooled to give the pre-bleed sample. At various times following irradiation, the mice were bled from the vena cava, serum samples from two mice being pooled for each time-point.

ELISA plates were coated with *cis*-UCA–BSA conjugate at 0·08 μg/well as already described. After washing, pre-bleed serum or sera from irradiated mice were diluted 1:3 in PBS–Tween with 1% BSA and 50 μl added to each of six wells or more followed by 50 μl hybridoma culture supernatant diluted appropriately. The plates were incubated for 3 hr at room temperature, washed and developed as above. One control contained 50 μl pre-bleed serum diluted 1:3 in PBS–Tween with 1% BSA and 50 μl PBS–Tween with 1% BSA. This value was subtracted from the test value. Another control consisted of hybridoma culture supernatant and pre-bleed serum diluted 1:3 and containing various concentrations of *cis*- or *trans*-UCA.

In a further experiment a group of 10 mice were eye bled and the serum pooled. After 5 hr the shaved mice were UVB irradiated with 216 mJ/cm^2 and 25 hr later they were bled from the vena cava and the serum pooled. Control mice were treated similarly but without irradiation. Half of each serum sample was dialysed against PBS at 4° for 14 hr using Visking tubing with molecular weight cut off in the range 12,000–14,000. The volume of the dialysed samples was measured and they were diluted appropriately in PBS–Tween with 1% BSA to be equivalent to 1:3 of the original volume. The undialysed samples were also diluted 1:3 in the same diluent. A competitive inhibition ELISA was carried out as above.

RESULTS

Preparation of monoclonal antibody against *cis*-UCA

Mice were immunized with *cis*-UCA–KLH and spleen cells from mice showing antibody titres to *cis*-UCA–BSA by ELISA were fused with NS-0 cells. One hybridoma was found which produced an antibody recognizing *cis*-UCA–BSA conjugate on ELISA. After cloning by limiting dilution twice, dilutions of the hybridoma culture supernatant were tested by ELISA, using 5 μg *cis*-UCA–BSA/well as antigen. The highest dilution which gave a mean absorbance more than the mean of an equivalent dilution of culture medium + 3 SD of the mean was 256,000. Using an isotyping kit, the monoclonal antibody was found to be $IgG_1\kappa$.

This culture supernatant was tested for activity against other conjugates and proteins as antigens as shown in Table 1. *Trans*-UCA–BSA and histamine–BSA were not recognized, nor was BSA or KLH.

Specificity of the monoclonal antibody

A checkerboard titration was performed with doubling dilutions of both *cis*-UCA–BSA starting at 1·25 μg/well, and the monoclonal antibody starting at 1:100 (Fig. 2). Antigen at 0·08 μg/well and antibody at a dilution of 1:400 gave an absorbance of 0·30. These conditions were chosen for the following competitive inhibition ELISA to demonstrate the specificity of the monoclonal antibody. In these tests UCA analogues or conjugates were added to the well together with the monoclonal antibody and the inhibition of binding of the monoclonal antibody to *cis*-UCA–BSA calculated. The results

Table 1. Binding of monoclonal antibody (1:1600 dilution) to various antigens (5 μg/well) measured by ELISA

	Absorbance ± SD
cis-UCA–BSA	0·408 ± 0·015
trans-UCA–BSA	0·044 ± 0·005
histamine–BSA	0·049 ± 0·011
BSA	0·037 ± 0·005
KLH	0·060 ± 0·005

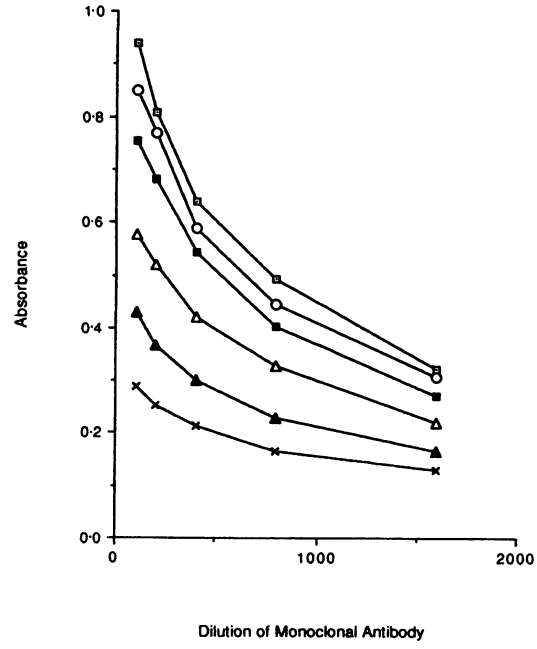

Figure 2. Binding of monoclonal antibody to *cis*-UCA–BSA conjugate at concentrations of 1·26 (□), 0·63 (○), 0·32 (■), 0·16 (△), 0·08 (▲) and 0·04 (×) μg/well by ELISA.

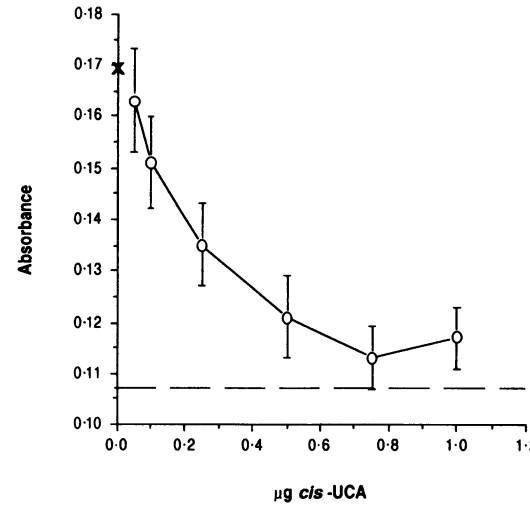

Figure 3. Standard curve showing the effect of varying concentrations of *cis*-UCA in serum in the competitive inhibition ELISA. The monoclonal antibody was used at a dilution of 1:400 and the *cis*-UCA–BSA conjugate at 0·08 μg/well. Absorbances are shown as mean ± standard deviation. *Trans*-UCA, added at 1 μg and 0·5 μg/well, showed absorbances of 0·176 ± 0·010 and 0·188 ± 0·007 respectively. The mean absorbance of wells with monoclonal antibody but no UCA isomer (X); mean absorbance of wells with neither monoclonal antibody nor UCA isomer (- - -).

Table 2. Competitive inhibition ELISA to test effects of UCA analogues and conjugates on binding of monoclonal antibody (1:400 dilution) to *cis*-UCA–BSA conjugate (0·08 μg/well)

	% inhibition of binding			
μg analogue or conjugate per well	5	1	0·1	0·03
cis-UCA	100	80	16	0
trans-UCA	27	0	0	0
histamine	ND	0	0	0
histamine–BSA	ND	0	0	ND
cis-UCA–KLH	ND	96	60	21
trans-UCA–KLH	ND	83	6	2
cis-2-methyl-UCA	59	6	0	0
trans-2-methyl-UCA	0	0	0	0

Cis- and *trans*-2-pyridineacrylic acid, *cis*- and *trans*-3-pyridineacrylic acid, *cis*- and *trans*-2-furanacrylic acid, *cis*- and *trans*-2-thiopheneacrylic acid, *cis*- and *trans*-3-thiopheneacrylic acid, dihydrourocanic acid and histidine (all at 1 μg and 0·1 μg/well) did not inhibit the binding of the monoclonal antibody.

ND, not done.

are shown in Table 2 where it can be seen that, while *cis*-UCA inhibited the binding of the monoclonal antibody to the antigen down to a level of 0·1 μg/well, *trans*-UCA had little effect in comparison; inhibition was only apparent if *trans*-UCA was present at 5 μg/well. The only UCA analogue to show any inhibition in binding was *cis*-2-methyl-UCA, again at the high concentration of 5 μg/well. *Trans*-2-methyl-UCA had no effect. *Cis*- and *trans*-UCA–KLH conjugate both had inhibitory activity.

Screening of murine sera for cis-*UCA*

Initially various amounts of *cis*- and *trans*-UCA were added to murine serum to obtain a standard curve for the quantification of UCA isomers using the competitive inhibition ELISA. The result of one such experiment is shown in Fig. 3. It may be seen that amounts of *cis*-UCA between 0·75 μg and 0·1 μg may be detected quantitatively by this method, while *trans*-UCA is not detected at all.

The assay was then applied to the measurement of *cis*-UCA in serum at various times after UVB irradiation of mice (Table 3). In the first experiment, after a single UVB exposure, the inhibition in binding of the monoclonal antibody indicated a maximum amount of *cis*-UCA in the serum 25 hr after irradiation, equivalent to approximately 0·1 μg *cis*-UCA in each well of the assay, i.e. about 6 μg *cis*-UCA/ml serum. In the second experiment after two exposures to UVB, *cis*-UCA was detected in the serum 1 hr after the second irradiation and reached a maximum at 5 hr of approximately 30 μg/ml serum.

It was not known whether the *cis*-UCA in the serum was present in a free form or conjugated to another molecule. To test this, the serum from irradiated mice was dialysed and used in a competitive inhibition ELISA (Table 4). It can be seen that there was inhibition of binding of the monoclonal antibody to the antigen in the presence of serum from irradiated animals but this inhibition was removed by dialysis of the serum. Therefore *cis*-UCA in the serum of irradiated animals was in an unbound form or conjugated to a molecule with molecular weight below 12,000.

DISCUSSION

To define the role of *cis*-UCA in UV-induced immunosuppression, it is necessary to identify its site of action and cellular

Table 3. Competitive inhibition ELISA to assay *cis*-UCA in serum at various times after irradiation of mice. The monoclonal antibody was used at a dilution of 1:400, and the *cis*-UCA–BSA conjugate at 0·08 μg/well

Experiment 1				*Experiment 2*			
Time after irradiation (hr) (one exposure of 216 mJ/cm²)	Absorbance	Significance (*P*)	% inhibition of binding	Time after second irradiation (hr) (two exposures of 144 mJ/cm² with 24 hr between)	Absorbance	Significance* (*P*)	% inhibition of binding
Pre-bleed serum	0·157			Pre-bleed serum	0·355		
14	0·124	NS	21	1	0·294	<0·01	17
25	0·116	<0·001	26	5	0·225	<0·01	35
39	0·139	NS	11	11	0·256	<0·001	28
48	0·142	<0·01	10	24	0·291	<0·01	18
114	0·161	NS	0	29	0·283	<0·01	20
				47	0·341	NS	4
				114	0·338	NS	5
Pre-bleed + 0·5 μg *cis*-UCA	0·050	<0·001	68	Pre-bleed + 0·5 μg *cis*-UCA	0·224	<0·001	37
Pre-bleed + 0·5 μg *trans*-UCA	0·163	NS	0	Pre-bleed + 0·5 μg *trans*-UCA	0·340	NS	4
Pre-bleed + 0·1 μg *cis*-UCA	0·112	<0·001	29	Pre-bleed + 0·1 μg *cis*-UCA	0·291	<0·001	18
Pre-bleed + 0·1 μg *trans*-UCA	0·171	NS	0	Pre-bleed + 0·1 μg *trans*-UCA	0·335	NS	6

* Significance of difference from pre-bleed serum (Student's *t*-test).
NS, not significant.

Table 4. Competitive inhibition ELISA (conditions as in Table 3) to assay *cis*-UCA in dialysed and undialysed serum 25 hr after irradiation of mice with 216 mJ/cm²

Serum sample	Absorbance	% inhibition of binding
Pre-bleed: undialysed	0·149[1]	
Pre-bleed: dialysed	0·109[2]	
Post-irradiation: undialysed	0·085[3]	43
Post-irradiation: dialysed	0·108[4]	0

[3] significantly different from [1] ($P < 0·001$; Student's *t*-test).
[4] not significantly different from [2] ($P < 0·01$; Student's *t*-test).

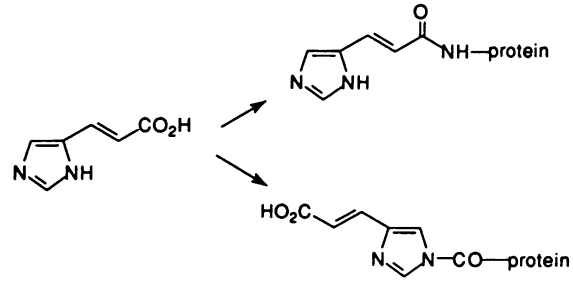

Figure 4. Possible modes of coupling of *trans*-UCA to a protein carrier.

target. In the present study, a monoclonal antibody with specificity for *cis*-UCA was developed and applied to the detection of UCA in serum of irradiated mice.

UCA isomers were conjugated to KLH and BSA by the carbodiimide method which was chosen for its convenience and simplicity. One problem which can occur is the precipitation of protein which has become denatured by cross-linking but very little precipitation was, in fact, observed. As UCA is amphoteric with both a basic imidazole ring and an acidic carboxylic acid group, there is the possibility of coupling through either group to the carrier protein to form two different structures (Fig. 4). It is not known which of these forms was synthesized or which the monoclonal antibody recognized. The epitope density of the conjugates was fairly light, as measured by radioactive labelling, but the *cis*-UCA–KLH used for immunization elicited a good antibody response and the conjugates used as antigens in ELISA proved sensitive and specific. A minimum number of haptens/protein of 15–30 is recommended for the induction of antibodies,[19] yet as few as five have been reported to give good IgG responses.[21]

From Table 1 it may be seen that the monoclonal antibody recognized only *cis*-UCA–BSA conjugate as antigen and this was corroborated in the competitive inhibition ELISA (Table 2) where *cis*-UCA competed, even at low concentrations, with the antigen bound to the well for binding of the antibody. *Trans*-UCA only competed when it was present at high concentration. The only UCA analogue out of the 15 tested which showed any inhibitory activity was *cis*-2-methyl UCA, although it was not so active as *cis*-UCA itself. When the UCA–KLH conjugates were used as competitors, both *cis*-UCA–KLH and *trans*-UCA–KLH demonstrated inhibitory activity. From the inhibition data in Table 2, the UCA–KLH conjugates were recognized approximately 1000-fold better in molar terms than *cis*-UCA itself. As KLH itself does not interact with the monoclonal antibody (Table 1), this result implies that the antibody is specific for the combined structure of UCA and the region on

the carrier to which it is bound. In this state it cannot distinguish *trans*- or *cis*-UCA–KLH conjugates. However, the isomers alone or conjugated to another protein were distinguished and we conclude that the monoclonal antibody is specific for *cis*-UCA unless *trans*-UCA is present in large quantities or *trans*-UCA is conjugated to KLH.

Analysis of UCA isomers in skin extracts by HPLC is straightforward and quantitative; in our hands the sensitivity of detection in homogenized ears is about 0·14 μM, representing 1 ng/mg wet weight tissue.[14] The identification of such isomers in serum by this method proved impossible due to the complexity of UV absorbing substances present. Use of the monoclonal antibody gave us the opportunity to assay *cis*-UCA in serum of irradiated mice by competitive inhibition ELISA. The sensitivity of detection was about 40 μM. However, despite the low sensitivity in comparison with HPLC, *cis*-UCA was detected in serum soon after irradiation, its concentration diminishing over the next 2 days. By dialysis of the serum, *cis*-UCA was demonstrated to be in a dialysable form and therefore unbound or conjugated to a small molecule, and unlikely to be conjugated to serum proteins or membranes. It has been reported that radiolabelled *cis*-UCA can be detected in urine of mice 24 hr after topical application (D. Hug and J. K. Hunter, quoted in ref. 22). In addition Reeve *et al.*[17] have found low but significant levels of ^{14}C in serum of mice after painting them with ^{14}C-UCA synthesized from [2-^{14}C]malonic acid. The counts were highest in animals that were UVB irradiated after painting, and were present 2 hr after exposure and not 6 days later. Electrophoresis of serum proteins suggested that ^{14}C was associated with specific proteins.

Thus there is evidence that a proportion of *cis*-UCA formed in the epidermis on UV irradiation reaches the serum either through capillaries or lymph, and may also be excreted in the urine. The cellular target for *cis*-UCA may be in the skin but, as a result of the systemic spread of *cis*-UCA after irradiation, it may act in lymph nodes or spleen, there to influence interactions of antigen-presenting cells and T cells. We are in the process of analysing murine tissues from these sites for *cis*-UCA and also have the opportunity to use the monoclonal antibody in experiments to block the effects of UV irradiation on the immune system.

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