Acta Derm Venereol 2001; 81: 87–91

INVESTIGATIVE REPORT

Urocanic Acid Isomers do not Modulate Intracellular Calcium and Cyclic AMP in Human Natural Killer Cells

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Ultraviolet irradiation influences natural killer cell function both *in vitro* and *in vivo*. The postulated ultraviolet photoreceptor in the epidermis, urocanic acid, has been reported to depress the cytotoxic activity of human natural killer cells. Therefore, this study investigated whether this would occur through specific second messengers, using a radioimmunoassay for intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) and Fluo-3 staining plus flow cytometry for free calcium. Both isolated lymphocytes and enriched $CD16^+$ cells were used. A combination of the *trans*- and *cis*-isomers of urocanic acid (200 μg/ml) induced cAMP in both $CD16^+$ and $CD16^-$ cells, but individual, stereospecific effects were not demonstrable. Urocanic acid did not induce significant changes in calcium levels in lymphocytes, or natural killer cells alone or conjugated to K562 target cells. Evidently, the biochemistry of urocanic acid-mediated natural killer-cell modulation is complex, and the cellular receptor(s) and specific signal transduction pathway(s) mediating the biological effects of urocanic acid remain elusive. *Key words: flow cytometry; immunomagnetic separation; CD16; K562 cells.*

(Accepted January 17, 2001.)

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Ultraviolet (UV) light exposure on the mammalian skin induces immunosuppression and immunological tolerance. A major UV chromophore component in the stratum corneum, urocanic acid (UCA), has materialized as a candidate photoreceptor initiating the photoimmunological processes (1). *Trans*-UCA absorbs UV radiation and photoisomerizes to *cis*-UCA. Various studies have demonstrated that *cis*-UCA contributes to UV-induced suppression of cell-mediated immunity, although the mechanisms have remained uncertain (for review see Ref. 2). *Cis*-UCA is substantially more water soluble (2, 3), and it has been detected in the circulation of mice (4) and in the urine of human subjects (5) following UV irradiation.

Natural killer (NK) cells mediate the spontaneous lysis of certain malignant cells. The classical experimental target for human NK cells is the erythroleukaemic cell line K562, but NK cells are also capable of lysing a variety of neoplastic cells, and there is evidence for a role of NK and lymphokine-activated killer cells in immunosurveillance of cutaneous tumours *in vivo*, including malignant melanoma (6). UV light regulates NK cell cytotoxicity both *in vitro* (7, 8) and *in vivo* (9). Experimental evidence also suggests that UCA isomers affect the NK activity of human peripheral blood lymphocytes (10, 11). Second messengers are involved in the cytotoxic activity of NK cells, but their roles in the function of UCA *in vitro* are unknown. The cellular receptors for UCA have not been characterized, and the present study was undertaken to delineate the regulatory pathways at the intracellular level. Data are presented on 2 second messengers, intracellular calcium (Ca_i^{2+}) and cyclic AMP (cAMP), in human peripheral blood lymphocytes and enriched NK cells, which suggest that *trans*- and *cis*-UCA do not stereospecifically modulate the levels of these mediators.

MATERIAL AND METHODS

Urocanic acid

Trans-UCA [3-(1*H*-imidazol-4-yl)-propenoic acid] was purchased from Sigma (St Louis, MO, USA). *Cis*-UCA was prepared from *trans*-UCA as described previously (12). The purity of both isomers was more than 98%, as detected by high-performance liquid chromatography (HPLC) (12). The isomers were dissolved in dimethyl sulphoxide (DMSO) at 5 mg/ml, diluted in test buffers and added to cells. Corresponding amounts of DMSO were added to control tubes.

Antibodies

For the identification and isolation of peripheral blood cells, the following primary anti-human monoclonal antibodies were used: anti-CD16 (Leu-11a; Becton-Dickinson, Mountain View, CA, USA), anti-human T-cell (OKT3 IgG2a; ATCC CRL-8001) and anti-human monocyte (3C10 IgG2b; ATCC TIB228) supernatants. Antibody labelling was performed at 4°C in phosphate-buffered saline (PBS) containing 2% fetal calf serum (Biological Industries, Kibbutz Beth Haemek, Israel).

Enrichment of lymphocytes and natural killer cells

Mononuclear cells were isolated from venous blood of apparently healthy volunteers by Ficoll-Isopaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. In assays for intracellular cAMP, the isolated cells were depleted of monocytes by adherence to plastic tissue culture flasks (Costar, Cambridge, MA, USA) after incubation in RPMI 1640 medium (Gibco, Paisley, UK) supplemented with gentamycin (50 μg/ml; Orion, Espoo, Finland) and 10% fetal calf serum, referred to as complete medium, at 37°C for 1 h. The non-adherent peripheral blood lymphocytes were collected, washed and resuspended in complete medium. The proportion of $CD16^+$ cells within peripheral blood lymphocytes ranged between 9.7 and 11.9%.

Magnetic enrichment of cells was performed using 2 separate methods. Peripheral blood lymphocytes were depleted of T-cells by reaction with the OKT3 antibody against the CD3 epitope at 4°C for 30 min. The $CD3^+$ cells were removed with magnetic beads (Dynabeads; Dynal, Oslo, Norway) coated with sheep anti-mouse immunoglobulin G (IgG) antibodies. The yield of $CD3^-$ cells was 29.1% of the total number of peripheral blood lymphocytes. Alternatively, rat anti-mouse IgG2a/2b-conjugated superparamagnetic microbeads (MACS; Miltenyi, Bergisch Gladbach, Germany) were applied to cells labelled with both mouse anti-human T-cell and mouse anti-human monocyte antibodies. After negative selection, 23.5% of the cells were recovered, representing $CD3^-/CD14^-$ double-negative cells. The enriched NK population was identified with phycoerythrin-conjugated anti-CD16 antibody. Relative fluorescence intensities were

 ISSN 0001-5555

monitored with flow cytometry (FACScan; Becton Dickinson). For selected cAMP analyses, the cells were also sorted using a FACStar PLUS cytometer (Becton Dickinson).

Assay for intracellular cyclic AMP

Peripheral blood lymphocytes or CD3-depleted peripheral blood lymphocytes were incubated with or without UCA for various periods. The concentrations of the UCA isomers did not affect the viability of the lymphocytes. No spontaneous lysis was observed. DMSO was used as a diluent control for UCA. In some experiments, 3-isobutyl-1-methylxanthine (MIX) and forskolin were used. The stock solution of MIX was heated in a boiling water bath and diluted 1:100 in complete medium to keep the agent in solution. Forskolin was dissolved in ethanol and corresponding volumes of ethanol were added to test tubes without forskolin. The incubation was stopped either in a block heater (about 95°C) for 5 min or by adding ethanol to a final concentration of 60% to lyse the cells. Total intracellular cAMP in the lymphocytes cultured with or without UCA was measured by radioimmunoassay using the acetylation method (13). Radio-iodinated 2′-*O*-monosuccinyladenosine 3′,5′-cyclic monophosphate tyrosyl methyl ester was used as a tracer, and it was labelled according to Brooker *et al.* (14). With lymphocyte incubations stopped with ethanol, the same portion of ethanol was added to the standards.

Analysis of intracellular free calcium

The isolated cells were loaded with the fluorescent calcium indicator as follows. Fluo-3/AM (50 μg corresponding to 44 nmol of F-1242; Molecular Probes, Leiden, The Netherlands) was reconstituted in DMSO to yield a stock solution of 4 mM. Then, 3–10 × 10^6 cells/ml in 10 mM HEPES-buffered Hank's balanced salt solution (HBSS, without phenol red), pH 7.4, were incubated with 1 or 2 μM Fluo-3/AM in polypropylene tubes at 37°C for approximately 30 min. Corresponding concentrations of DMSO served as unloaded controls. Ionomycin at 2 μM was added as a positive control for maximal Ca_i^{2+} response.

Conjugation of lymphocytes or enriched natural killer cells with K562 targets

Conjugation studies were constructed according to the guidelines provided by Van Graft *et al.* (15) and Callewaert *et al.* (16). K562 cells were maintained in exponential growth phase in complete medium. After enrichment, antibody labelling or calcium indicator loading, the effector cells were washed with HBSS/HEPES and resuspended to 2.5–10 × 10^6 cells/ml. UCA isomers, freshly dissolved in the same medium, were added. Keeping all cells at 0°C, the effector lymphocytes and K562 targets (ratio 1:1) were brought together and briefly centrifuged at 4°C. The supernatant was aspirated, and the cell suspension was gently shaken without vortexing and kept on ice until analysis with flow cytometry. PBS containing the appropriate concentrations of UCA was added, and the cells were incubated with occasional shaking in a water bath at about 37°C for various periods and analysed immediately with a FACScan flow cytometer.

Statistics

Measured concentrations of cAMP and Ca_i^{2+} were compared between the UCA-treated and control cells using a 2-tailed Student's *t*-test after preanalysis with the *F*-test to determine whether the samples had different variances.

RESULTS

Cyclic AMP

Immunostaining and flow-cytometric cell sorting were performed to enrich $CD16^+$ cells. $CD16^-$ cells served as a reference. The cAMP levels measured after incubation of triplicate samples with high concentrations (200 μg/ml) of *cis*- and *trans*-UCA for 2 h were always above those of control cells (Fig. 1). This was true especially for *trans*-UCA in the $CD16^+$ cells and for *cis*-UCA in $CD16^-$ lymphocytes. In addition, *cis*-UCA inhibited cAMP in $CD16^+$ cells at 10 μg/ml, although these effects lacked statistical significance. The isomers additively stimulated both cell populations at 200 μg/ml (total UCA concentrations 400 μg/ml). The elevation in cAMP was proportionally larger in $CD16^+$ cells, where the basal concentrations were lower than in $CD16^-$ cells ($p = 0.014$ and $p = 0.015$, respectively) (Fig. 1).

Attempts were made to elevate the basal steady-state levels of cAMP by inhibiting its further metabolism in peripheral blood lymphocytes by adding the phosphodiesterase inhibitor MIX (0.1 mM). The *cis*- and *trans*-UCA-incubated peripheral blood lymphocytes showed equally slight increases in cAMP levels after 20 min, but the levels were reduced close to control values after 2 h (data not shown). No increase could be observed without MIX. The isomers acted additively and stimulated cAMP during the 20-min incubation, decreasing after 2 h (Table I).

When NK cells were enriched by depletion of $CD3^+$ (T) cells and incubated with UCA and MIX for 20 min, no effect of either the UCA isomers or MIX could be seen. The cAMP-generating adenylyl cyclase stimulator forskolin (50 μM) was then combined with MIX to induce the synthesis of cAMP in peripheral blood lymphocytes. Cyclic AMP levels increased up to a plateau of 3–4 times the initial concentration with no effect of UCA (data not shown).

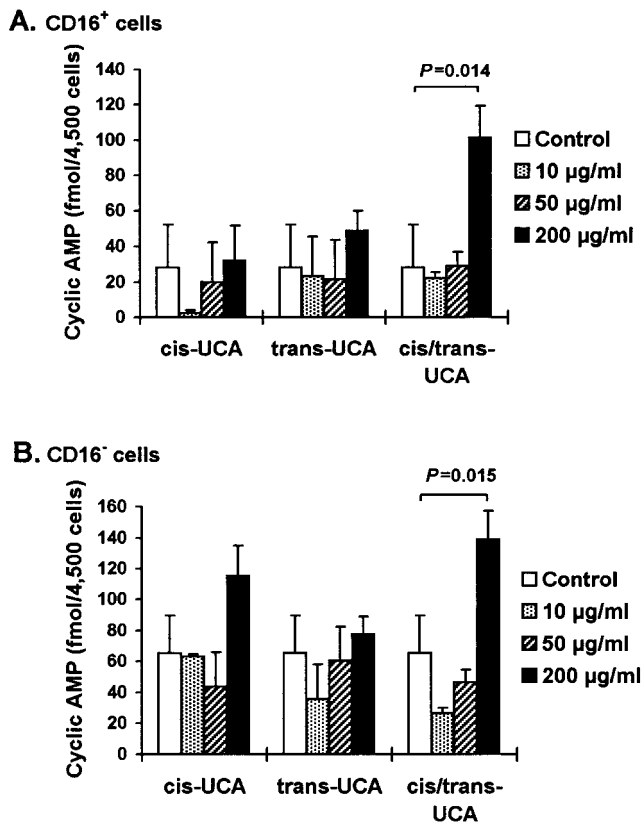

Fig. 1. Concentrations of intracellular cyclic AMP (mean, SEM) in (*A*) CD16-positive and (*B*) CD16-negative cells after incubation with urocanic acid (UCA) isomers. The sorted cells (90,000/tube) were incubated in triplicate tubes for 2 h.

Table I. *Levels of cyclic AMP in peripheral blood lymphocytes, 250,000 cells/0.5 ml, incubated in duplicate tubes with equal amounts (1:1) of the urocanic acid (UCA) isomers and 0.1 mM 3-isobutyl-1-methylxanthine (MIX)*

Incubation time (min)	MIX	Concentration of total UCA (μg/ml) 0	20	400
20	–	42.9 ± 2.7	50.7 ± 5.0	
	–	73.5 ± 21.1		56.8 ± 0.3
	+	81.0 ± 4.8	94.5 ± 11.2	
	+	62.8 ± 7.8		115.0 ± 0.3
120	–	17.4 ± 3.3	21.0 ± 4.5	
	–	22.7 ± 6.4		37.5 ± 1.0
	+	19.3 ± 7.8	25.1 ± 0.2	
	+	41.2 ± 6.5		23.6 ± 5.9

The figures are in fmol/25,000 cells (mean ± SEM). Dimethyl sulphoxide was added into control tubes in place of UCA.

Intracellular calcium

Freshly isolated peripheral blood lymphocytes were labelled with anti-CD16, loaded with Fluo-3 and incubated with UCA isomers (200 μg/ml). After 45 min, Ca_i^{2+} levels were somewhat higher in both *cis*- and *trans*-UCA-treated cells. This could be seen in $CD16^+$ and $CD16^-$ cells as well as in lymphocytes gated by size (forward scatter) and granularity (side scatter) in flow cytometry (Fig. 2). However, none of these changes reached statistical significance. Anti-CD16 antibody alone did not change the fluorescence in Fluo-3-loaded control cells at 1, 15 or 45 min (Fig. 2 and additional data not shown). Ca_i^{2+} fluorescence levels were on average 90% of those measured in the ionomycin-treated control cells.

Conjugate formation

In order to study how NK cells bind to their target cells and how they become activated in the presence of UCA isomers, magnetically (MACS) enriched NK cells ($CD3^-/CD14^-$) were allowed to form conjugates with K562 targets and were identified by analysing the cells in Fluo-3 versus forward scatter gates by FACS (Fig. 3A). The number of conjugates

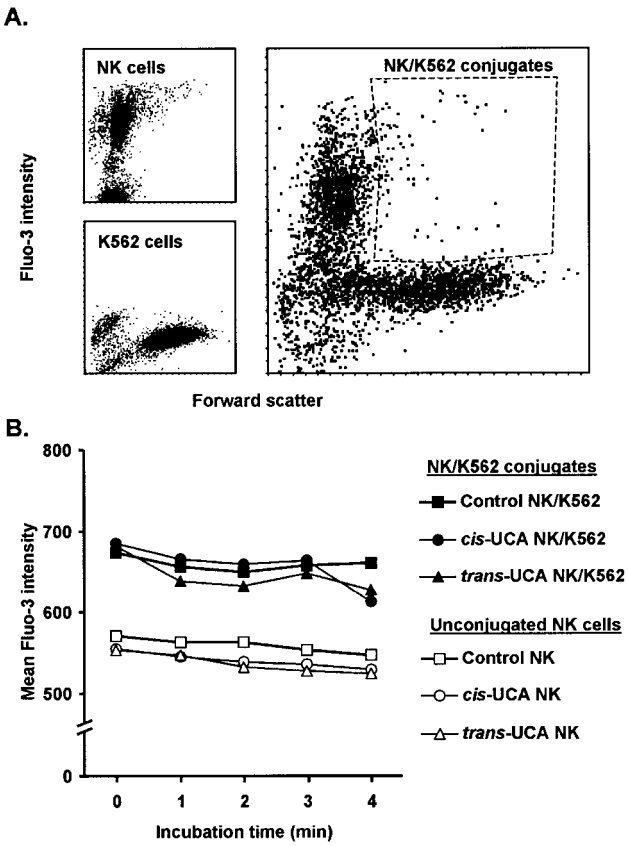

Fig. 3. Ca_i^{2+} response of natural killer (NK)/K562 target cell conjugates treated with urocanic acid (UCA) isomers. Human peripheral blood lymphocytes were magnetically (MACS) depleted of T cells ($CD3^+$) and monocytes ($CD14^+$), and they were allowed to conjugate with K562 cells (cell ratio 1:1) at 0°C in the presence of UCA isomers (200 μg/ml). The cells were then incubated at 37°C. The conjugates of NK effector lymphocytes and K562 targets were electronically localized in flow cytometry (*A*), and Ca_i^{2+} levels were measured by Fluo-3 fluorescence (*B*). The Fluo-3 intensity of the gated unconjugated lymphocytes in the same samples is shown for reference (*open symbols*). A total of 10,000 cells was analysed at the indicated incubation time points within 29–31 s.

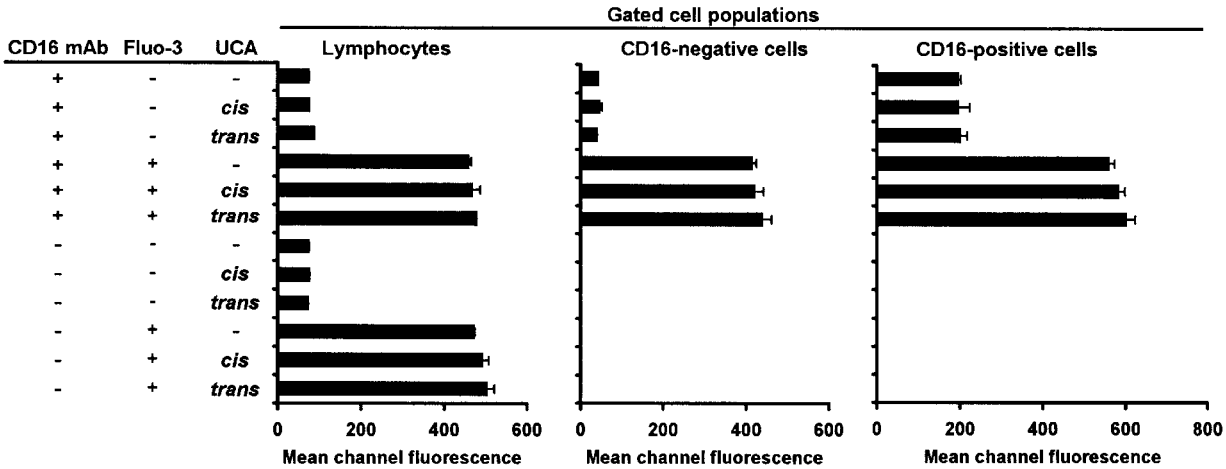

Fig. 2. Effect of urocanic acid (UCA) on Ca_i^{2+} in lymphocyte populations. The cells were labelled with anti-CD16 antibody, loaded with Fluo-3, and incubated with UCA isomers (200 μg/ml) in duplicate for 45 min, with untreated cells as controls. The portion of $CD16^+$ cells in the lymphocyte gate was 10.4 ± 0.56%, which was unaffected by Fluo-3 loading.

remained the same in the presence of *trans*-UCA (200 μg/ml; mean number of conjugates 6.7%, range 5.7–9.8%) and *cis*-UCA (mean 5.9%, range 5.3–7.3%) in comparison with control cells (5.7%, range 5.4–5.9%). In peripheral blood lymphocytes without enrichment, the proportion of conjugated cells ranged between 4.1 and 4.4%. The total number of analysed cells in FACS was 10,000, including effector cells, K562 target cells and their conjugates. The Fluo-3-loaded enriched NK cells had higher fluorescence in the conjugate gate with K562 targets than the NK cells alone. UCA had no effect on Ca_i^{2+} in the NK cells conjugated to targets (Fig. 3B).

DISCUSSION

Although the immunological functions of UCA have been widely investigated, little is known about the cellular receptor(s) or signal transduction system(s) mediating these effects (2). Based on both the close structural relationship with histamine and some indirect functional evidence, it has been postulated that histamine-like receptors would mediate the biological signal of *cis*-UCA at the cellular level. Recent reports have suggested that *cis*-UCA attenuates cAMP signalling induced by histamine in cultured fibroblasts (17, 18) and keratinocytes from the human skin (19), and epidermal cells from the pig skin (20), but not in human monocytes (21), hepatocytes isolated from the hamsters or an adenocarcinoma cell line (18). In all but one (17) of these reports UCA isomers alone failed to stimulate cAMP synthesis. It is presently widely accepted that although histaminergic signalling may take place in the whole process of UV-induced immunosuppression, histamine receptors are not the actual receptors for *cis*-UCA (3, 21–23). Other candidate receptors such as the imidazol(in)e (24) and α_2-adrenergic receptors (18, 24) have also proven non-reactive to UCA.

The cytotoxic activity of NK cells is regulated by both activating and inhibitory signals. Two groups of receptors transmitting signals that inhibit human NK cell function have recently been identified (for review see Ref. 25). Both of these receptor classes, the Ig superfamily-like and the lectin-like receptors, carry the immune tyrosine-based inhibitory motifs and, when bound to major histocompatibility complex class I molecules on the target cell, abrogate the signal transduction cascade initiated by the activating receptors. Measurement of specific second messengers provides indirect information on the receptor types involved in cell signalling. For example, adenylyl cyclase-coupled receptors produce cAMP as a second messenger, whereas phospholipase C-mediated effects typically involve a Ca_i^{2+} increase in the cell. Taking advantage of the effect of UCA isomers on human NK cell function (10, 11) and the fact that second messengers, such as Ca_i^{2+} (26) and cAMP (27, 28), are critically involved in NK cell activation, human NK cells were used in this study as the model system for investigating the signal transduction pathways in UCA action. Most biological effects of UCA have been attributed to the *cis* isomer, but Palaszynski *et al.* showed that *trans*-UCA induces the formation of cAMP in a human dermal fibroblast lineage and that *cis*-UCA reverses this effect (17). The cytotoxic activity of human NK cells is strongly suppressed in the presence of *trans*-UCA *in vitro* (10). In the present signal-transduction experiments neither *cis*- nor *trans*-UCA showed activity alone, but a combination of the isomers elicited a significant stimulation of cAMP production in both NK cells and non-NK cells (Fig. 1, Table I). Findings indicating inhibition of human polymorphonuclear leukocyte function *in vitro* without stereospecific effects by UCA isomers have been published (29). In corroboration with the present results, UCA did not affect cAMP signalling in those cells (29).

In the experiments on target cell binding of NK cells, the number of NK–K562 conjugates was only slightly increased by both *trans*- and *cis*-UCA. The Ca_i^{2+} levels in NK cells conjugated to targets were clearly higher than in unconjugated NK cells, but the values were not affected by UCA isomers either (Fig. 3B). Thus, the isomers do not regulate NK cell activity at a signal-transduction point characteristic of target cell recognition (26). This does not exclude the possibility that the lytic process may be inhibited at the post-binding level (30). According to recent data, conjugation of NK effectors and K562 targets initializes a process of lipid raft polarization to the NK-cell membrane site contacting with the target cell (31). The functional and temporal correlation of raft formation and intracellular signalling is not fully clear (31), and it may be that exogenous substances capable of directly modifying membrane raft polarization are not distinguishable at the second messenger level such as cAMP and Ca_i^{2+} signalling. If it were hypothesized that UCA would affect NK cell function through lipid raft formation, the observed stereospecific effects (10, 11) could partly reflect the differential solubility (lipophilicity) of *cis*- and *trans*-UCA.

Evidently, the biochemistry of UCA-mediated effects is complex. The present data do not clarify the general nature of the putative cellular UCA receptor(s) or the more specific question of the cellular signalling pathways in UCA-mediated NK modulation. There may be different receptors and signal-transduction pathways in different cell types. In addition, accessory effector cells may be involved; for example, the presence of monocytes has been found to regulate NK cell activity *in vitro*. The contradictory results of two recent investigations (10, 11) may be derived from the influence of monocytes and dendritic cells on NK cells in the latter study, as pointed out by Norval *et al.* (32). Elucidation of the cell-binding and signal-transduction mechanisms of UCA will need further research.

ACKNOWLEDGEMENTS

This work was supported by The Academy of Finland and by EC grant ENV4-CT96-0192.

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