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A new sensitive assay for measurement of cell-mediated cytotoxicity to intact layers of cultured human keratinocytes

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A cytotoxicity assay for sensitive measurement of cell-mediated lympholysis (CML) of human cultured keratinocytes (cK) is described The usage of ^{51}Cr-labeled keratinocytes in intact layers as target cells in this assay allows objective and accurate determination of lysis of keratinocytes which have not undergone trypsin- and suspension-induced membrane changes Furthermore, the problem of high spontaneous ^{51}Cr release values encountered with suspended keratinocytes is overcome The assay was applied to study antigen-specific CML of cK by cloned cytotoxic T cells (CTL) and to determine the effect of IFN-γ on the susceptibility of cK to lysis The results showed that HLA-A_2 specific CTLs could reproducibly lyse cK of HLA-A_2 positive healthy skin donors both with and *without* incubation of cK with IFN-γ.

Applications of this keratinocyte cytotoxicity assay lie in determining the antigenic expression of human cK, in analysis of effector cell/keratinocyte interactions in CML and of the modulatory effects of cytokines on these mechanisms The assay thus may provide a helpful tool in gaining insight into the role of CML of keratinocytes in the destruction of inflamed skin

Key words Cell-mediated lympholysis assay, Cultured keratinocyte, Cytotoxic T lymphocyte

Introduction

Cell-mediated lympholysis (CML) of keratinocytes (skin epidermal cells) may be one of the causes of skin destruction observed in various immunologic cutaneous diseases Immunofluorescence studies of infiltrated tissue, for example in graft versus host disease (Lampert et al, 1982, Guyotat et al, 1986), revealed $CD8^+$ T cells in close association to degenerating keratinocytes, suggesting a possible role for cytotoxic T cells (CTL) in the lysis of keratinocytes

In vitro, CML of human keratinocytes by sensitized CTL has been studied by only a small number of investigators (Bagot et al, 1985, Faure et al, 1985, Niederwieser et al, 1988, Kalish, 1989) In all these studies single cell suspensions of freshly isolated skin epidermal cells or of cultured keratinocytes (cK) obtained by trypsinisation were used as target cells in the conventional ^{51}Cr release assay, as originally described by

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Abbreviations CML, cell-mediated lympholysis, CTL cytotoxic T lymphocyte, K, human keratinocyte, cK, cultured human keratinocyte KM, keratinocyte culture medium, E T, effector-to-target ratio, rIFN-γ, recombinant interferon-γ ADCC antibody dependent cellular cytotoxicity, NK natural killer cell

Brunner (1968) There are several reasons however, why this assay may not to be optimal for measurement of CML of cK We found that suspended cK exhibited high values of spontaneous chromium leakage, which prevented accurate measurement of specific lysis In addition to this technical complication, cell suspensions of trypsinized cK may form an in vitro target cell model with only limited value for assessing CML of keratinocytes in vivo Firstly, exposure of cK to trypsin is thought to induce rigorous membrane changes (Norris et al, 1985) Secondly, suspended cK, being deprived of anchorage, rapidly undergo terminal differentiation, marked by transformation of the extracellular matrix glycoproteins, which may affect the susceptibility of cK to lysis (Rheinwald et al, 1980) Norris (1985) systematically compared the susceptibility of human cK in suspension versus those in intact layers to ADCC, NK activity and antibody-mediated lysis, using either ^{51}Cr release or fluorescein diacetate (FD) uptake as measure of lysis Exposure to trypsin rendered cK susceptible to NK activity, thereby indicating that the use of intact layers of cK is preferable for obtaining qualitative information on target cell determinants expressed on cK in vitro The FD uptake assay is a sensitive assay for assessment of lysis of cK in intact layers, however, this method is subjective and does not allow quantification as does, for example, a ^{51}Cr-release assay (Norris et al, 1985) In our study we attempted to develop an assay in which intact layers of cK are used and which provides an objective and quantitative system to study effector cell/keratinocyte interactions

For that end, a modified ^{51}Cr release assay was devised in which ^{51}Cr-labeled human cK as target cells are adherent to flat-bottomed 96-well microtiter plates Our data on the lysis of cK by allo-HLA antigen-specific CTL clones indicate that this assay allows a reproducible read-out of CML of keratinocytes in vitro

Materials and methods

Human keratinocyte culture

Skin biopsies (1 cm^2) were taken from the upper arms of healthy HLA-typed individuals Epidermal cells were isolated according to the method originally described by Liu and Karasek (1978) The resulting suspensions (97% keratinocytes, 2–3% Langerhans cells and melanocytes) were seeded together with irradiated 3T3 in culture flasks and cultured according to Rheinwald and Green (1975) in a humidified incubator at 37 °C, 10% CO_2 Keratinocyte culture medium (KM) consisted of a 3 1 mixture of Dulbecco Vogt and Ham's F12 (Flow Laboratories), supplemented with 5% FCS (Flow), 10^{-6} M isoproterenol (Sigma), and 0 4 μg/ml hydrocortisone At day 4, epidermal growth factor (10 ng/ml, Sigma) was added to the medium Cultures reached confluence after 7–10 days and, after trypsinization, cells were stored in liquid nitrogen until use in KM containing 6 6% DMSO 8–9 days before use as target cells in the CML assay, the cryopreserved keratinocytes were thawed and subcultured as described above Confluence was reached after 6–7 days

Preparation of keratinocyte layers

2 days prior usage, confluent layers of cK, cultured as described above, were washed two times with PBS of 37 °C and harvested by trypsinization in 0 25% trypsin (Difco) in Ca/Mg-free PBS, supplemented with 0 05 M EDTA, 0 1% glucose, pH 7 5 After incubation for 20–30 min at 37 °C, detached cells were suspended in KM, counted and diluted to a concentration of 5×10^4 cK/ml (unless stated otherwise) 200 μl of the homogenous suspension (containing 10^4 cK) was transferred to the wells of 96 well flat-bottomed microtiter plates (Greiner no 655160, unless stated otherwise) using a multichannel pipettor Cells were incubated for 36 h in a humified incubator (37 °C, 5% CO_2) In those cases where the effect of IFN-γ was studied, rIFN-γ (Genentech, San Francisco, CA) was added to the wells during adhesion, ranging from 250 to 1000 U/ml, 16–48 h Routinely the effect of a 20 h incubation of 250 U/ml IFN-γ was assesed Prior to isotope labeling, non-adherent cK were removed by decanting the KM and washing the cells with PBS at 37 °C To each well, 100 μl of KM containing 20 μCi/ml ^{51}Cr (sodium chromate, 1 mCi/ml, New England Nuclear, Boston, MA) was added After incubation for 1 5 h at 37 °C, the labeling fluid was removed and the cells were washed three times with PBS

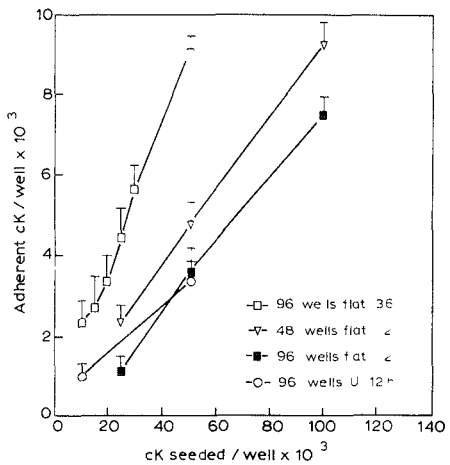

Fig 1 Effect of plate type and adhesion period on plating efficiency of keratinocytes seeded at various densities cK were seeded from 10 to 100×10^3 cells/well and incubated for 12 h in Costar (cat no 3548) 48 well flat-bottomed (▽), Costar (cat no 3799) 96-well round-bottomed plates (○) and for 12 h (■) and 36 h (□) in Greiner (cat no 655160) 96-well flat-bottomed plates Numbers of adherent cK/well were measured as described in the materials and methods section, mean values ± SE of the 5-plicate measurements are plotted

Measurement of the target cell number

Measurement of the number of adherent cK/well was performed first, to determine the efficacy of adhesion under the various seeding conditions studied to optimize the assay (Figs 1 and 2), and subsequently to routinely determine the exact target cell number present per well for each different cK culture used. In short, the following procedure was used The wells containing the adherent cK to be counted, were washed three times with PBS, incubated for 20–30 min in 50 μl of 0.25% trypsin (Difco), and after adding 150 μl PBS + 1% BSA, the wells' contents were collected using a multichannel pipettor The wells were rinsed using another 100 μl of PBS + 1% BSA per well. To obtain an accurate estimate of the adherent cell number per well, contents of 20–40 identical wells were pooled, centrifuged (8 min, 1000 rpm), adjusted to 0 5 ml and counted undiluted

Under standard conditions, i.e., 10^4 cK seeded per well in Greiner 96-well flat-bottomed plates, incubated for 36 h, 16–29% of the seeded cK adhered depending on the keratinocyte culture used (data not shown) For the keratinocyte cell line used in all experiments shown here, adhesion was 23%, corresponding to $2.3 \pm 0.5 \times 10^3$ adherent cK/well

Effector cells

Human alloimmune CTL, sensitized selectively against the HLA-A_2 antigen (Horai et al., 1982) were cloned by limiting dilution. The HLA-A_2-specific CTL clone 1E2 was used in the experiments after restimulation using allogenic feeder cells (Van de Griend et al., 1987). CTLs were cultured and tested in RPMI 1640 (Gibco) supplemented with gentamicin and 15% heat-inactivated pooled human serum (referred to as medium).

^{51}Cr release assay

Wells containing adherent ^{51}Cr-labeled cK were incubated in a volume of 200 μl with effector cells (ranging from 1.5×10^4 to 15×10^4 CTLs/well), with medium alone, or with medium containing a 5% Triton X-100 solution, for measurement of experimental (ER), spontaneous (SR) and maximal release (MR) respectively. Incubation with 5% Triton X-100 freed 90–95% of the total amount of ^{51}Cr incorporated in adherent cK (data not shown),

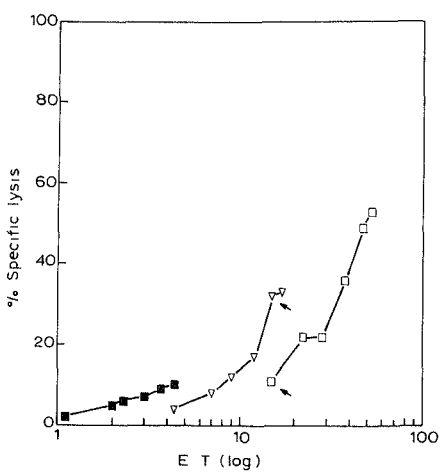

Fig 2 Effect of effector-to-target ratio (E T) and the absolute number of adherent cK/well on antigen specific lysis of adherent cK HLA-A_2-specific CTL clones were added at three different concentrations (■ 1×10^4, ▽ 4×10^4 and □ 12.5×10^4 CTL/well) to wells containing adherent cK of an HLA-A_2 +ve donor Densities of cK ranged from 2 3 to 9.0×10^3 adherent cK/well and had been generated by seeding $1–5 \times 10^4$ cK/well Mean values of % lysis of 5-plicates (SE < 15%) at resulting E T are presented per CTL concentration, ◄ indicate different % lysis at E T = 15

and therefore was considered appropriate for measurement of MR All measurements were carried out in 5-plicate The plates were centrifuged shortly to enhance cell-cell interaction (10 s, 2000 rpm) and incubated at 37°C for 4 h Thereafter, the plates were centrifuged for 5 min at 2000 rpm, supernatants were harvested using a collector system (Skatron SCS harvesting frames no 15772, Norway) and radioactivity was counted in a Packard gamma counter Mean values and standard errors were calculated for all 5-plicate measurements of cpm released in experimental wells (ER), in wells for spontaneous release (SR) and for maximal release (MR) Relative spontaneous release (SR/MR × 100%) never exceeded 20% Percentage of specific lysis was determined using the following formula

$$\% \text{ specific lysis} = \frac{ER - SR}{MR - SR} \times 100\%$$

Results

Optimization of the keratinocyte layers

Prior to using adherent cK as target cells in the ^{51}Cr release assay it was important to establish optimum conditions for (a) cell adhesion, (b) ^{51}Cr labeling, and (c) washing of the layers of cK

Adhesion We assessed the effects on adhesion of the type of tissue-culture wells used, the duration of the incubation period and the cell density seeded As is shown in Fig 1, after 12 h incubation the plating efficiency (cK adhered/cK seeded ×100%) was very similar for all types of wells tested (7 5–9 0%) We selected Greiner 96 wells flat-bottomed microtitre for further use Prolonging the incubation time up to 36 h caused a two-fold increase in the number of adherent cK in these wells Under these seeding conditions relative variation of the 5-plicate samples in the number of adhered cK/well did not exceed 20%, irrespective of the seeding density (Fig 1)

^{51}Cr-labeling For the subsequent ^{51}Cr labeling of the cK adhered to the microtiter wells it was pursued to minimize the amount of radioactive material needed The use of 2 μCi/well was considered acceptable and represented a 5-fold reduction in the amount of μCi used per well as compared to similar labeling procedures used for adherent fibroblasts (Russel et al , 1988) and endothelial cells (Miltenburg et al , 1988)

Washing procedure To optimize the washing procedure of the labeled cK (i e , to minimize the variance in adherent cells/well), two ways of removing the washing fluid were compared, namely the usage of a multichannel hand pipettor or decanting the fluid The variance in number of adherent cells in the 5-plicate wells did not differ significantly between the two methods, the latter method was chosen as standard procedure for reasons of efficiency (data not shown)

Optimization of the effector-target ratio and the absolute target cell number

To determine the effector-to-target ratio (E T) and the number of target cells optimal for lysis of cK in the adherent assay, both the cK seeding concentration and the effector cell concentration were varied Since cK have been shown to express the serologically defined HLA class I antigens (Mauduit et al , 1987), we studied HLA-A_2-specific lysis using the A_2-specific CTL clone $1E_2$ as effector cells cK were seeded at densities ranging from 10^4 to 5×10^4 per well of which ±20% was found to be present as target cells after 36 h of incubation (see Fig 1) CTLs were added at concentrations ranging from 1×10^4 to $12\,5 \times 10^4$ CTL/well As can be seen from Fig 2, the percentage of specific lysis increased with increasing E T, but was also affected by the absolute numbers of effector cells and adherent cK per well For example, at E T = 15, specific lysis was 33% at 4×10^4 CTL and $2\,6 \times 10^3$ adhered cK/well, as compared to 11% when $12\,5 \times 10^4$ CTL and $8\,5 \times 10^3$ cK/well were used This apparent unfavorable effect of high numbers of keratinocytes and effector cells on lysis could either be due to crowding of the effectors (which is not very likely at $12\,5 \times 10^4$ CTL/well) or to a less efficient exposure of cK to CTLs when cK cultures reach confluency Layers containing low numbers of adherent cK thus seemed to provide a more sensitive system for measurement of specific lysis

For purposes of standardisation of the assay, the seeding density was set at 10^4 cK/well which corresponded to numbers of adherent cK ranging

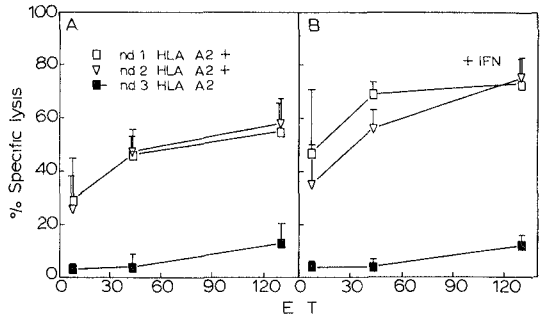

Fig 3 Antigen specific lysis of untreated (Fig 3A) and rIFN treated (Fig 3B 250 U/ml, 20 hrs) cK of 3 HLA typed donors 2 HLA A_2 positive (ind 1, ind 2) and 1 HLA A_2 negative (ind 3) Layers of cK were standardly generated (10^4 cK seeded per well 36 hrs adhesion in 96 wells Greiner no 655160 flatbottomed plates), clone 1E2 was used as effector (1 5-15 $\times 10^4$ CTL/well) Values of % specific lysis represent mean $\pm$ SE of 3 serial experiments using sequential subcultures (passage 1, 2 and 3) of cK of the same donors, SE of % specific lysis of the 5-plicate samples did not exceed 20% (not shown)

from $1\,6 \times 10^3$ to $2\,9 \times 10^3$ per well for distinct cK cultures used (not shown)

Antigen-specific lysis of cK with and without IFN γ pretreatment

The assay was applied to study the susceptibility of human cK to lysis by CTLs directed to HLA class I antigens In addition, the effect of IFN-γ on CTL/keratinocyte interaction was studied As can be seen in Fig 3*A*, HLA-A_2 specific CTLs showed dose-dependent lysis of cK of two HLA-A_2 positive individuals, whereas no lysis of HLA-A_2 negative cK was observed unless very high numbers of CTLs were used (< 5% lysis at E T < 100) Preincubation of cK with rIFN-γ increased the susceptibility of the HLA-A_2 cK to lysis from 45 to 70% for donor 1, from 45 to 55% for donor 2, at E T = 42 Aspecific lysis of cK of the HLA-A_2 negative individual was not induced by IFN-γ pretreatment (Fig 3*B*)

Discussion

Cultured human keratinocytes are frequently used as an in vitro target cell model to analyse mechanisms of CML of skin epithelial tissue Most investigators so far used suspensions of trypsinized cultured keratinocytes as target cells in the standard ^{51}Cr release assay However, the susceptibility of cK in this setting may differ significantly from that in situ due to changes in expression of cell surface molecules as a result of the trypsin treatment and the induction of differentiation of cK in suspension Additionally, high values of spontaneous ^{51}Cr release were commonly found to complicate the use of suspended ^{51}Cr-labeled keratinocytes as targets for CML As reported here, we developed a cytotoxicity assay in which intact layers of cK in 96 well flat-bottomed microtiter plates are used as target cells Spontaneous ^{51}Cr release values of adherent cK in the 4 h assay are reduced to 15% of the Triton X-100 values Applying this assay for the analysis of antigen specific CML of cK using cloned CTL specific for the HLA-A_2 antigen and cK derived from three HLA-typed donors, we demonstrated that the assay was sensitive (45% specific lysis at E T = 40), reproducible and provided no significant aspecific lysis (4% at E T = 40, see Fig 3) We found that pretreatment with IFN-γ increased the susceptibility of cK to A_2-specific lysis (Fig 3*B*) This presumably can be attributed to a IFN-γ-modulated induction of (a) a higher expression of HLA class I molecules (as was demonstrated by flow cytometric analysis, data not shown) and (b) de novo expression of ICAM-1 on cK (Dustin et al , 1988)

The finding that IFN-γ treatment rendered cK more sensitive to CML was compatible with findings of other investigators who used suspended cK to assess antigen specific (Niederwieser et al , 1988, Kalish, 1989) or non-specific lysis (Kalish, 1989) In contrast, these groups did not find significant antigen specific lysis of basal, IFN-γ untreated cK, whereas we found lysis up to 50% of cK in this assay when using CTLs directed to HLA-A_2 (Fig 3*A*) or other HLA and non-HLA antigens (Van Els et al , submitted) We assume this discrepancy is due to differences in the cytolytic capicity and specificity of the antigen-specific T cell lines and clones used

Our observations that cK were highly susceptible to antigen-specific CTLs in the absence of IFN-γ pretreatment may put CTL/keratinocyte interactions in a new light Binding of CTLs to keratinocytes via the adhesion molecules LFA-1 and its ligand ICAM-1 has been suggested to be crucial in cell-mediated lympholysis of keratinocytes (Dustin et al , 1988, Niederwieser et al ,

1988; Kalish, 1989). However, since expression of ICAM-1 on basal, unstimulated cK has been demonstrated to be extremely low or absent (Dustin et al., 1988), our results may indicate that LFA-1/ICAM-1 interaction is not an absolute requirement for CTL to lyse keratinocytes. If extrapolation to in vivo is allowed, CTL could target keratinocytes in inflamed skin, irrespective of ICAM-1 expression brought about by lymphokine production of infiltrated mononuclear cells. Whether in vivo the effector cells present in inflamed skin have sufficient cytolytic capacity to target keratinocytes without the additional 'help' of antigen-independent adhesion via ICAM-1, as demonstrated by our allo-CTL lines and clones in vitro, remains as yet unanswered.

We conclude that the cytotoxicity assay reported here represents a reproducible method to measure CML of intact layers of cultured keratinocytes The assay can serve to determine which cell-defined antigens are expressed by cK and what the modulatory effects of lymphokines are on effector cell/keratinocyte interactions. In this way it may provide a useful tool to understand the role of CML of keratinocytes in various T cell-mediated cutaneous disorders.

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