Human keratinocytes activate primed major and minor Histocompatibility antigen specific Th cells in vitro.

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Transplant Immunology 1993, **1:** 52–59

Human keratinocytes activate primed major and minor histocompatibility antigen specific Th cells *in vitro*

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Abstract: Keratinocytes are activated to express MHC class II and ICAM-1 molecules during cutaneous inflammatory reactions It is controversial how the interaction between these 'nonprofessional' antigen presenting cells (APC) and infiltrating T cells affects the local inflammatory response To address this issue we analyzed whether IFNγ-treated cultured human keratinocytes would activate established Th cell clones *in vitro* Three allo DR specific T cell clones were induced to proliferate in a HLA DR and LFA-1/ICAM 1 dependent fashion upon coculture with intact layers of IFNγ stimulated keratinocytes Likewise, keratinocytes also could activate two out of four minor histocompatibility (mH) antigen specific Th cell clones obtained from peripheral blood leukocytes (PBL) of graft versus host disease patients The T cell activating potential of MHC class II+ keratinocytes was shown to be relatively low compared to specialized APC as PMNC and EBV BLCL Most strikingly, measurable allo MHC and mH antigen specific Th cell proliferation was only induced by using *adherent* keratinocytes at low cell densities, but not by keratinocytes in suspension The results presented here indicate that *in vitro* conditions may crucially influence observations regarding the T cell activating potential of MHC class II expressing keratinocytes Furthermore, our results indicate that, in addition to a tolerizing effect as suggested by previous reports, interaction of primed antigen specific T cells with activated keratinocytes may also result in enhancement of a cutaneous immune response *in vivo*

Introduction

Epidermal keratinocytes are activated to express class II MHC and ICAM-1 molecules in a variety of lymphocyte-mediated skin diseases, such as graft versus host disease (GvHD) after allogeneic bone marrow transplantation (BMT) [1 3] Activation of keratinocytes is thought to be induced by IFNγ and TNFα locally secreted by infiltrating mononuclear cells [4–6] The biological consequences of activated class II+ ICAM-1+ keratinocytes on a cutaneous inflammatory response are not fully understood In response to IFNγ, keratinocytes efficiently bind T lymphocytes via interaction of ICAM-1 with its ligand LFA-1 [4 7] Furthermore, activated keratinocytes are rendered susceptible to lysis by MHC class I and class II restricted CD8+ and CD4+ cytotoxic T cells *in vitro*,[8 12] and thus may serve as targets for antigen-specific effector cells in inflamed skin With respect to the events initiating cutaneous T cell inflammation, MHC class II+ keratinocytes are thought not to be involved in the initial antigen-specific triggering of resting T cells [13–15] This process appears to be strictly mediated by the bone marrow-derived population of class II+ epidermal Langerhans' cells (LC) which are highly efficient in presenting exogenous as well as modified self antigens to T cells [13 16] By contrast, activated keratinocytes are considered to be responsible for persistence of inflammatory reactions via amplification of the local T cell response [17–19] Keratinocytes are assumed to do so via secretion of numerous T cell stimulatory lymphokines including IL-1, IL-6 and IL-8 [18 20 21] A second mechanism by which keratinocytes might cause progression of ongoing inflammation, would be to directly activate adhering T cells by triggering their antigen-specific receptor To what extent this second mechanism could occur and which antigenic epitopes are involved in the distinct inflammatory diseases is as yet unclear In *ex vivo* models developed to define the role of class II+ keratinocytes in psoriasis,[22] tuberculin-reactivity[23] and GvHD,[24] MHC II+ keratinocytes isolated from affected skin were unable to stimulate primed PPD or allo MHC-specific Th cells Furthermore, controversial results were obtained in *in vitro* studies aiming to elucidate the intrinsic potential of suspensions of cultured or uncultured class II+ keratinocytes to present allo MHC[8 25] hapten-modified self,[26] exogenous[8] or peptidic antigens[8 25] to primed Th cells Not only differences in T cell activation[8 25] but also induction of antigen specific T cell unresponsiveness[25 26] were observed.

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In the present study an *in vitro* model employing intact layers instead of suspensions of cultured IFNγ treated keratinocytes was developed and used to assess the capacity of MHC class II+human keratinocytes to activate allo major and minor histocompatibility (H) antigen specific Th cell clones We report that under restricted *in vitro* conditions adherent keratinocytes can induce proliferation of primed allo DR antigen specific Th cells Furthermore, preliminary results are reported suggesting that allo mH antigen specific Th cells detected in the blood of GvHD patients might be susceptible to stimulation by keratinocytes in GvHD-affected skin

Objective

The objective of this study is to address the controversial issue of whether IFNγ activated epidermal keratinocytes have the capacity to activate skin infiltrating primed allo-antigen specific Th cells This, in order to gain insight into the contribution of epidermal cells to the cascade of inflammatory events occurring in cutaneous allo-graft rejection, DTH, and graft versus host reactions

Materials and methods

T cell clones and lines

The DR3 specific Th cell clone 1603, a DR2 specific Th clone 2610, a DRw11 specific T cell line RT222 and a DPw4 1 specific Th line were all kindly provided by Dr A Termijtelen mH antigen specific Th cell lines were all established in our laboratory from primed PBL of patients after HLA genotypically identical BMT as previously described in detail [27] The CD4+ Th cell lines DAx and FAx display DR5 respectively *DR3 + DR5 restricted recognition of as yet unidentified* autosomal mH antigens, each expressed on PBL and EBV-LCL of the BM recipient and on PBL and EBV-BLCL of some of a panel of restriction antigen positive unrelated individuals A CD8+ Th cell clone R26 and a CD4+ Th cell clone R416[28] recognize the male specific mH antigen H-Y presented by the MHC class I molecules HLA-B60 and -A2 respectively All Th cell lines and clones were expanded by adding PMNC of the original stimulator (10^7, 3000 rad) or EBV-LCL of the original stimulator (2×10^6, 5000 rad) and autologous PMNC (10^7, 3000 rad) as feeder cells to 1×10^6 T cells in 10 ml of medium (RPMI 1640 supplemented with 15% human serum) with 20 U rIl-2/ml At day 9–10 after restimulation, the T cells were frozen and after thawing directly using as responder cells for proliferation

Human keratinocyte cultures

Keratinocytes (KC) were standardly isolated from skin biopsies of a selected panel of 9 HLA and mH antigen typed donors Cells were cultured in culture flasks coated with irradiated 3T3 feeder cells in culture medium (KM) composed of a 3 1 mixture of DMEM and Ham's F12 (Gibco), supplemented with 5% FCS, 10^{-6} M isoproterenol (Sigma), 0 4 μg/ml hydrocortisone and 10 ng/ml EGF (Sigma) as previously described[9] KC cell suspensions to be used for proliferation assays (direct or after adhesion) or FACS analysis were obtained by detaching KC from subconfluent 3–4th passage cultures using 0 25% trypsin (Difco) supplemented with 0 05 M EDTA and 0 1% glucose (pH = 7 5)

Immunofluorescence analysis

Single cell suspensions obtained via trypsinization of subconfluent KC cultures were analyzed on a FACScan flow cytometer (Becton Dickinson, Palo Alto, CA) Cells were incubated with mAb, washed and stained with FITC-goat anti-mouse IgG for measurement of indirect fluorescence

Proliferation assays

In the conventional primed lymphocyte test (PLT) $1–2 \times 10^4$ T cells were cocultured with 10^5 PMNC (2000 rad), or $0\,25 \times 10^5$ EBV LCL (7500 rad), or KC (3000 rad, see below) in a volume of 150 μl RPMI + 15% HS in 96 wells flat-bottomed plates (Greiner 655160) for 48 hours Triplicate cultures were labelled with 1 μCi [^{3}H] thymidine and after 16 hours assayed for isotype incorporation in a liquid scintilation counter Layers of KC to be used as stimulator cells (SC), were generated (unless stated otherwise) by adding a suspension of 200 μl KM containing 10^4 undifferentiated KC in 96 wells flat bottomed plates, allowed to adhere for 24 hours, followed by the addition of rIFNγ (Genentech, San Francisco, CA) to a final concentration of 200 U/ml After six days of IFNγ incubation, subconfluent KC layers were washed three times with 37°C PBS, irradiated 3000 rad and cocultured with $1–2 \times 10^4$ T cells/w for 64 hours as described above When KC in suspension were used as SC, IFNγ was added in the flasks to subconfluent cultures Single cell suspensions generated by trypsinization were washed, irradiated 3000 rad and directly used as SC in PLT Where the effect of mAb against cell surface molecules on antigen-specific proliferation was studied, either responder T cells of KC were preincubated in 100 μl of the indicated mAb dilution for 30 minutes at 20°C mAb were left in the culture medium for the duration of the assay

Assay for T cell unresponsiveness

KC were plated out in 96 wells plates (10 000 and 40 000 KC/w), allowed to adhere overnight and cultured for 72 hours in the presence of IFNγ at 200 U/ml Generated subconfluent (10 000) and confluent (40 000) KC layers were washed using PBS (3×), irradiated 3000 rad and incubated with 3×10^4 T cells/w in 15% HS in RPMI for 24 or 48 hours T cells were carefully removed, extensively washed and tested for induced unresponsiveness in a conventional PLT assay

Monoclonal antibodies (mAb)

HLA-reactive mAb were W6/32 and B9 12 1 (anticlass I framework), PvD5 2 (anticlass II framework), B8 11 2 (anti HLA DR) and B7 21 (anti HLA-DP) The anti LFA-1 mAb 8 3 14 1 was produced in this laboratory ICAM 1 reactive mAb were RR1/1 (kindly provided by Dr T Springer) and 15 2 (kindly provided by Dr N Hogg)

Results

IFNγ-treated keratinocytes induce proliferation of allo DR specific Th cell clones

We set out to address the controversial issue of whether activated DR + ICAM + KC might serve to perpetuate ongoing T cell responses in inflamed skin *in vivo* To mimick inflamed epidermis, an *in vitro* model was chosen which utilizes intact layers of IFNγ-treated cultured KC instead of cell suspensions In this culture system, which was initially developed to study cell mediated cytotoxicity of KC,[9] single cell suspensions obtained by trypsin treatment of subconfluent KC cultures are seeded at 10 000 KC/well in 96 flat-bottom plates, allowed to adhere overnight and incubated with rIFNγ at 200 U/ml for three days [9] In a first series of experiments, intact KC layers were generated from nine

selected HLA-typed individuals, washed, irradiated (3000 rad) and used as stimulator cells (SC) for 3 allo DR and 1 allo DP specific Th cell clones in a PLT assay Table 1 gives the results of a representative experiment, where IFNγ treated and untreated KC layers of four donors were cocultured for 64 hours with 10^4 RC/w of a DR3 specific Th cell clone (1603) or a DR2 specific Th cell clone (2610) KC were observed to induce significant (4 2–25 7 × 10^3 cpm) [^{3}H] thymidine incorporation of DR2 and DR3 specific Th cells provided KC expressed the right DR allele and had been rIFNγ pretreated (Table 1) To determine which accessory molecules were involved in the observed KC-mediated T cell proliferation, experiments were performed in the presence of antibodies to several cell surface antigens (Figure 1) Anti-DR (B 8 11 2) and αMHC class II (PvD5 2) mAbs almost completely prevented proliferation, in contrast to irrelevant mAbs specific for MHC class I (W6/32) or HLA-DP (B7 21) which did not significantly inhibit proliferation The LFA 1/ICAM-1 interaction was indicated to contribute to allo DR specific proliferation, even though both αICAM-1 (RR1/1) and αLFA-1 (8 3 14 1) mAbs did not completely abrogate T cell activation (Figure 1)

Table 1 IFNγ treated keratinocytes induce DR specific T cell proliferation

Keratinocytes[a]		Proliferation (cpm × 10^3)	
Donor	HLA DR	1603 (αDR3)	2610 (αDR2)
AT	3	25 7[b] (0 4)[c]	0 6 (0 4)
EG	3,7	16 8 (0 3)	0 6 (0 3)
VD	2,5	0 6 (0 5)	7 0 (0 3)
DH	2,3	10 9 (0 5)	4 2 (0 4)

[a] KC layers generated by seeding and allowing to adhere 10 000 K/w, followed by a 72 hour period with 200 U/ml IFNγ were used as SC after washing and 3000 rad irraddiation

[b] Values represent mean [^{3}H] thymidine incorporation in 10^3 cpm of triplicate wells containing 10^4 T cells and IFNγ treated KC in a 64 hours PLT assay

[c] Values between brackets stand for proliferation induced by IFNγ untreated KC, background values of T cells or KC alone were always below 0 5 × 10^3 cpm

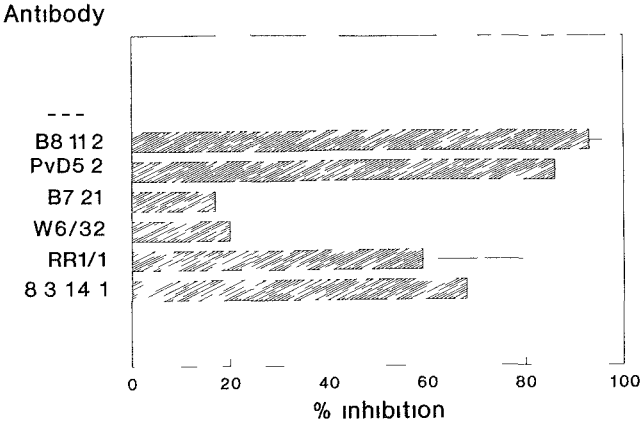

Figure 1 Effect of mAbs on KC induced proliferation of anti DR3 Th cell clone 1603 KC layers, prepared as described in Table 1, were incubated for 30 minutes at 20°C in 100 μl of a 1 200 dilution of W6/32 (anti MHC class I), PvD5 2 (anti class II), B8 11 2 (anti HLA DR), B7 21 (anti HLA DP) and RR1/1 (anti ICAM-1) The anti LFA 1 mAb 8 3 14 1 was preincubated with RC instead of SC Mean values of [^{3}H] thymidine incorporation of three experiments are presented as percentages ±SD of the unblocked response (always >10 × 10^3 cpm)

In vitro parameters influence T cell activation by IFNγ-treated keratinocytes

Effect of ICAM 1 and HLA-DR expression

IFNγ pretreatment of KC layers was varied with respect to time (zero to six days) and concentration (50–500 U/ml) to determine optimal conditions for *in vitro* T cell activation IFNγ was added at distinct time points to wells simultaneously seeded with 10 000 KC/w As Table 2 indicates, concentrations exceeding 50 U/ml did not further enhance KC-induced T cell proliferation Prolonged IFNγ incubation of KC for five or six days appeared to optimize the resulting proliferative T cell response (Table 2) This result was unexpected since both ICAM-1 and HLA-DR expression are known to be rapidly induced by IFNγ [29 30] To confirm the kinetics of IFNγ-induced ICAM 1 and HLA DR cell surface expression in our KC culture system, FACS analysis was performed with single cell suspensions obtained via trypsinization of KC cultures preincubated with 200 U/ml rIFNγ for zero, one, two, four and seven days Maximal densities of HLA DR (B8 11 1) and ICAM 1 (RR1/1) were observed after two and one day respectively (Figure 2) This rapid induction and the declining ICAM 1 expression after four days (Figure 2B) indicates that cell surface expression of HLA-DR and ICAM-1 are not the only factors determining T cell proliferation induced by IFNγ treated KC (Table 2) The fact that IFNγ exerts a strong inhibitory effect on KC growth (data not shown and [31]), might contribute to the finding that longer pretreated KC layers (containing less adherent KC) induce higher values of T cell proliferation (see KC density)

Table 2 Effect of IFNγ pretreatment of keratinocytes on DR specific Th cell proliferation

time[a] (days)	rIFNγ (U/ml) 0	50	100	200	500
2	0 3[b]	6 0	6 3	6 2	6 5
3	0 1	14 9	16 2	14 9	13 0
4	0 4	14 8	18 3	18 5	15 4
5	0 3	22 3	22 3	27 6	22 5
6	0 2	20 0	24 9	27 2	23 0

[a] Keratinocytes of a DR3+ donor were seeded at 10^4 cells/well seven days before usage as SC layers in a PLT assay IFNγ was added at the indicated concentrations on consecutive days starting 12 hours after KC seeding corresponding to six days IFNγ

[b] Mean [^{3}H]thymidine incorporation (cpm × 10^3) of triplicate wells containing 10^4 αDR3 T cells in a 64 hours PLT assay Results of one of two experiments are shown

Effect of KC density

To determine the effect of KC density, cell suspensions of four DR3+ KC lines ranging from 5–400 × 10^3 KC/ml (i e 1–80 × 10^3 KC/well) were seeded in 96 wells plates, allowed to adhere overnight, incubated with 200 U/ml IFNγ for six days and used as SC in PLT Proliferation induction of the αDR3 T cell clone strongly varied (2–11 × 10^3 cpm) with the number of seeded KC/w, with an optimal stimulation at 5–10 × 10^3 KC/well (Figure 3) A standard protocol for most sensitive (within our test ranges) measurement of T cell proliferation induction by KC was used in all future experiments and consisted of (washed and 3000 rad irradiated) subconfluent KC layers generated by seeding 5000 as well as 10 000 KC/w, followed by a six-day incubation period with 200 U/ml rIFNγ

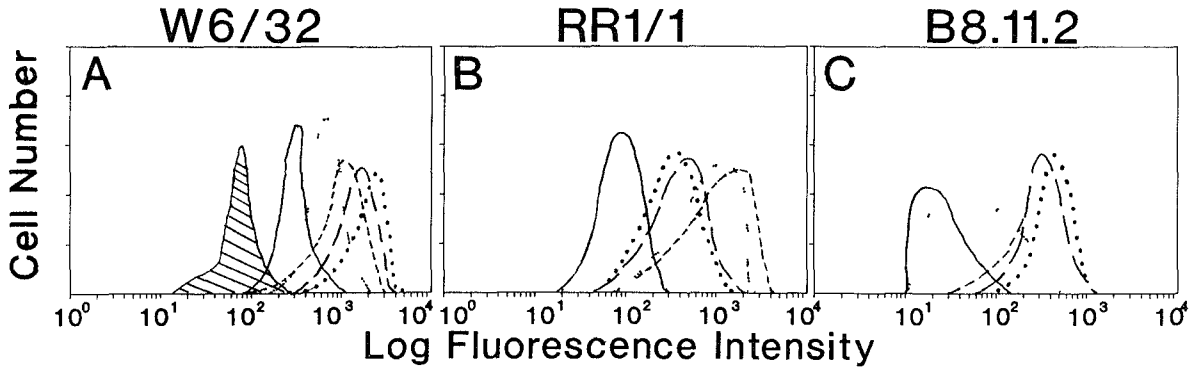

Figure 2 Cell surface expression of HLA class I (A), ICAM 1 (B) HLA DR (C) of cell suspensions of trypsinized keratinocyte cultures after incubation for zero (—), one (----), two (--), four (—) and seven ( ) days with 200 U/ml rIFNγ

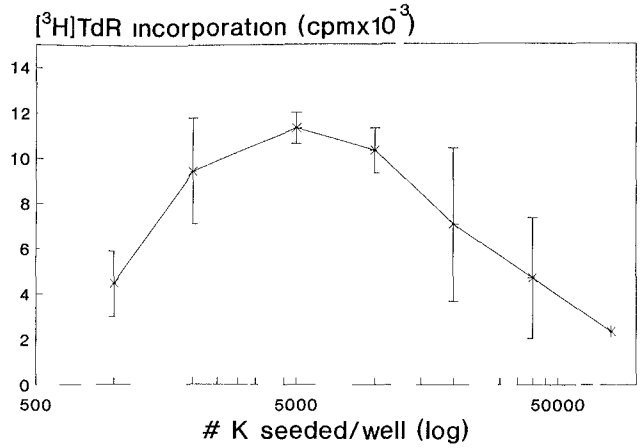

Figure 3 High as well as low densities of adherent KC/well are suboptimal for *in vitro* activation of allo reactive primed Th cells Mean values of [^{3}H]-thymidine incorporation by anti DR3 Th cells measured after coculture with four DR3+ KC cell lines in two experiments are given IFNγ pretreatment consisted of 6 d, 200 U/ml

Keratinocyte culture supernatants inhibit T cell proliferation in allo MLR

The reduced allo DR T cell proliferation at high KC cell densities could be caused by T cell inhibitory factors secreted by KC RPMI media conditioned for 24–72 hours on subconfluent layers of KC, were removed and compared with unconditioned medium for its ability to inhibit a primary allo MLR As shown in Table 3, the degree of inhibition of the MLR was dependent on the period the RPMI medium had been conditioned by KC Inhibition (up to 65% of control) was observed irrespective of whether KC had been IFNγ pretreated, irradiated or whether indomethacin had been added to the RPMI

The Th cell activating potential of HLA-DR+ ICAM-1+ keratinocyte layers is low compared to that of 'professional' APC

To address the relative antigen presenting potential of KC, values of T cell proliferation induced by intact layers of IFNγ-treated KC, suspensions of trypsinized IFNγ pretreated KC,

Table 3 KC culture supernatants inhibit T cell proliferation in allo MLR

KC pretreatment[a]			Conditioning[b] period (hours)	Proliferation[c] (cpm × 10^3)
IFNγ, 3 d 200 U/ml	3000 rad irradiation	Indomethacin 1 μg/ml		
–	–	–	—	25 3 ± 2 2
+	+	–	24	15 7 ± 1 2
			48	10 6 ± 0 2
			72	9 0 ± 1 3
–	+	–	24	13 6 ± 0 3
			48	11 2 ± 0 7
			72	12 7 ± 2 4
+	–	–	24	13 8 ± 1 8
			48	9 0 ± 1 1
			72	9 2 ± 1 2
+	+	+	24	19 1 ± 2 3
			48	12 2 ± 1 7
			72	10 9 ± 3 0

[a] Following IFNγ pretreatment and/or irradiation as indicated, subconfluent KC layers were incubated in RPMI + 15% HS in the presence of absence of indomethacin

[b] Samples of RPMI preconditioned by ±4 × 10^4 KC per 0 2 ml RPMI were drawn after the indicated period, filtered, adjusted to pH – 7 0–7 2 and added to MLR

[c] Mean [^{3}H]thymidine incorporation of triplicate wells of two experiments, in which 10^5 PMNC were cocultured with 10^5 2000 rad allogeneic PMNC for five days in a volume of 150 μl conditioned RPMI

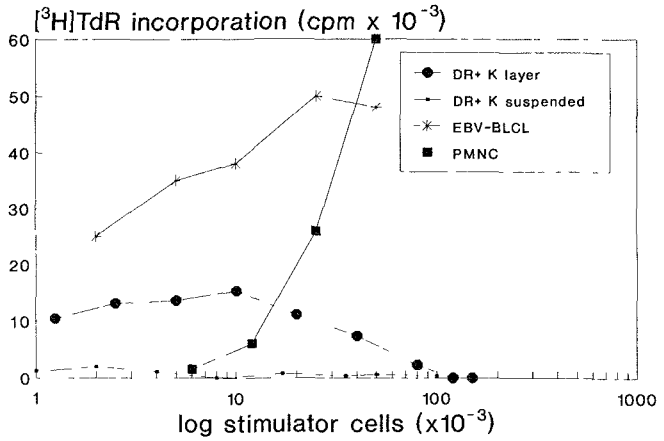

Figure 4 Comparison of the T cell activating potential of keratinocytes and specialized APC. αDR3 T cells were stimulated with varying numbers of PMNC (■,6–50 × 10^3, 2000 rad), EBV-BLCL (✳,2–50 × 10^3, 7500 rad), and trypsinized and suspended 6 d 200 U/ml IFNγ-treated KC (♦, 0.8–100 × 10^3, 3000 rad) in a conventional PLT. Also layers of KC generated by seeding 1,2–200 × 10^3 K/well, incubated with 200 U/ml rIFNγ for six dyas, were used as SC (●,3000 rad). All cell types were obtained from the same HLA-DR3+ donor.

EBV-BLCL and PMNC were compared as SC in PLT. Figure 4 represents one of two experiments where distinct cell types of the same DR3+ donor were used as SC for an αDR3 Th cell clone. T cell activation induced by DR+ KC layers was significant (≤13 × 10^3 cpm) but lower than T cell activation induced by PMNC (≤60 × 10^3 cpm), or EBV-BLCL (≤47 × 10^3 cpm), even at optimal KC densities. Surprising results were obtained using single cell suspensions obtained via trypsin detachment of IFNγ-treated KC cultures. Irradiated (Figure 4) as well as unirradiated (not shown) suspensions of the same DR3+ KC line did not result in significant [^{3}H]-thymidine incorporation (≥1 × 10^3 cpm) by αDR3 T cells in the concentration range tested (0.8–100 × 10^3 KC/w). Similarly, proliferation by αDR2 and αDR5 Th cell clones never exceeded 0.5 × 10^3 cpm when cocultured with antigen-positive suspensions of KC (data not shown).

IFNγ-stimulated keratinocyte layers activate MHC restricted Th cell clones specific for H-Y, but not Th cell clones specific for two other mH antigens

In a previous study we found that the presence of host mH antigen reactive proliferative rather than cytotoxic T cells in blood of patients after HLA-identical BMT was correlated to the development of graft versus host disease.[27] In GvHD, mH antigen reactive Th cells might be locally activated by antigen expressing keratinocytes in the GvHD affected skin.[32,33] As indicated in Figure 5, IFNγ pretreated keratinocytes of a male HLA-B60+ donor were able to induce proliferation of the H-Y/B60 CD8+ T cell clone R26, whereas IFNγ-treated female or B60-negative or IFNγ untreated cells could not (Figure 5A). Similarly, the H-Y/A2 specific CD4+ T cell clone R416 was only stimulated by IFNγ-treated male HLA-A2+ keratinocytes (Figure 5b). The failure of IFNγ untreated KC, which do express MHC class I (Figure 2), to activate R416 and R26 is compatible with our previous results.[10] CTLs specific for H-Y in the context of MHC class I only lysed KC after IFNγ treatment.[10] Activation presumably results only after an increase in the number of MHC class I/H-Y complexes and facilitated adhesion via ICAM-1 as a result of IFNγ treatment. Values of [^{3}H] thymidine incorporation by both male specific T cell clones were low (4.9 ± 0.5 and 5.8 ± 0.3 × 10^3 cpm respectively), but reproducable and represented 27% and 24% of the responses of these clones induced by PMNC. The MHC class I restriction of KC-induced proliferation of αH-Y/A2 Th cells was confirmed by antibody

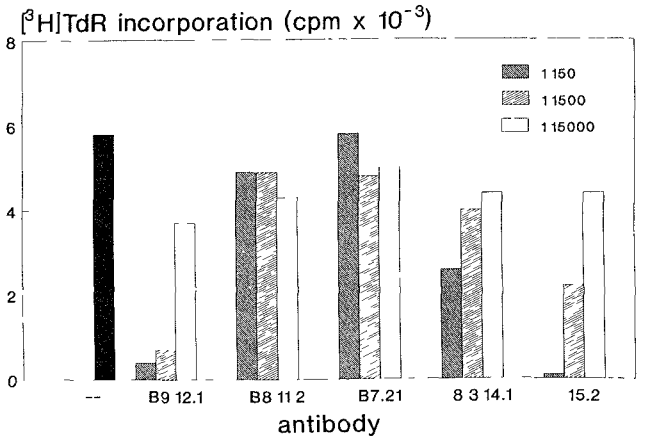

Figure 6 Effect of anti HLA class I (B9.12.1), DR (B8.11.2), DP (B7.21), and anti (LFA-1 (8.3.14.1) and ICAM-1 (15.2) mAbs on KC induced proliferation of H-Y/A2 specific Th clone R416. KC layers (10.000 kC/w; 6 d, 200 U/ml rIFNγ) of a HLA-A2+ male donor were used as SC in PLT.

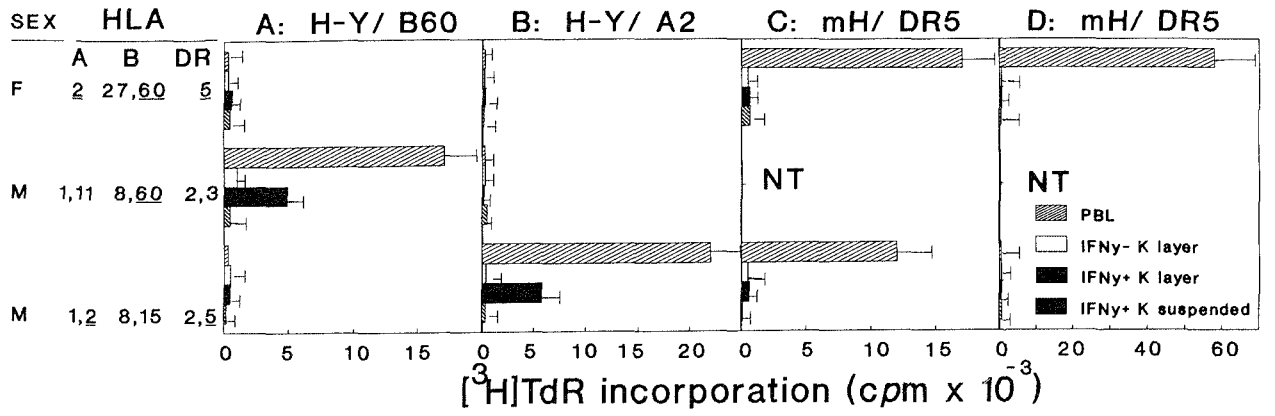

Figure 5 Keratinocytes induce activation of some mH antigen-specific, MHC-restricted T cell clones. Of three HLA typed individuals PMNC and IFNγ untreated and treated layers of KC, as well as suspensions of IFNγ treated KC were used to induce proliferation of an H-Y specific, HLA-B60 restricted Th cell clone R26 (A); an H-Y specific HLA-A2 restricted Th cell clone R416 (B); a DR3+ 5 restricted T cell line FAx (C); and a DR5 restricted Th cell line DAx (D) both reactive with nonsex linked unidentified mH antigens. Background values of RC and SC alone were always below 300 cpm.

inhibition experiments, which revealed a dose-dendent inhibition by αICAM-1, αLFA-1 (partial) and αMHC class I mAbs, but not by αMHC class II mAbs (Figure 6) In contrast, IFNγ treated keratinocytes of two HLA-DR5+ donors did not induce any measurable proliferation of the DR3/5 restricted mH antigen specific T cell line FAx ($\leq 0.3 \times 10^3$ cpm, Figure 5C), or similarly, of the mH epitope reactive, DR5 restricted line DAx (Figure 5D) As expected, suspensions of IFNγ treated KC (10 and 40×10^3 KC/w) did not stimulate any of the four Th cell clones at all

Absence of mH specific proliferation is not due to KC-induced T cell anergy

The failure of the two mH antigen specific Th cell lines FAx and DAx to be activated by KC can be explained in three ways First, the MHC class II restricted, mH antigen specific Th cells tested may have activation requirements different from the allo DR and H-Y specific Th cells tested Secondly, keratinocytes may not express the two DR restricted mH epitopes on their cell surface Thirdly, DAx and FAx Th cells may have been rendered anergic as a result of coculture with IFNγ KC The latter possibility was experimentally addressed in Figure 7 DAx (Figure 7) or FAx (not shown) T cells were preincubated for 24 hours or 48 hours in either medium alone, or on top of KC layers of a mH antigen positive donor (IFNγ treated or not), or of a mH antigen negative donor No difference in the proliferative responses was observed when these differently preincubated T cells (after extensive washing) were subsequently stimulated with adequate mH antigen positive APC in a conventional PLT assay (Figure 7) Thus, the absence of measurable proliferation of the mH specific T cell line DAx after coculture with IFNγ-treated KC layers potentially expressing the mH antigen, is not due to KC-induced unresponsiveness of these Th cells

Discussion

It is established that activated epidermal KC, even though secreting several lymphokines including IL-1 and expressing cell surface MHC class II and ICAM-1, are unable to induce primary T cell activation.[8 14 15 24 29] In the course of an ongoing inflammatory reaction, KC may encounter nonvirgin T cells which have previously been activated by bone marrow-derived 'professional' antigen presenting cells (APC) such as dendritic (Langerhans') cells Given that memory T cells are less stringent in the activation signals they require,[34 35] the question was asked by several investigators whether MHC class II+ KC, like several other MHC class II+ non bone marrow-derived cell types,[36 37] might be sufficient to stimulate established Th cell populations Thus far, controversial results have been reported on the capacity of *suspensions* of IFNγ-treated KC to induce proliferation of *allo* MHC class II reactive Th cell populations Gaspari *et al* reported on an αIA^k T cell line, which could be induced to proliferate in an MHC class II dependent fashion by coculture with MHC class II+ KC purified from skin of *in vivo* IFNγ-treated mice [8] Seemingly contrasting data are presented in this report (Figure 4) and in a previous report by Bal *et al* [25] showing that human MHC class II+ KC, obtained by trypsin-detachment of IFNγ pretreated cultures, were totally unable to stimulate cell division of allo HLA-DR Th cell clones Several factors may account for this difference First, the species difference, though this seems a trivial explanation Secondly, fresh

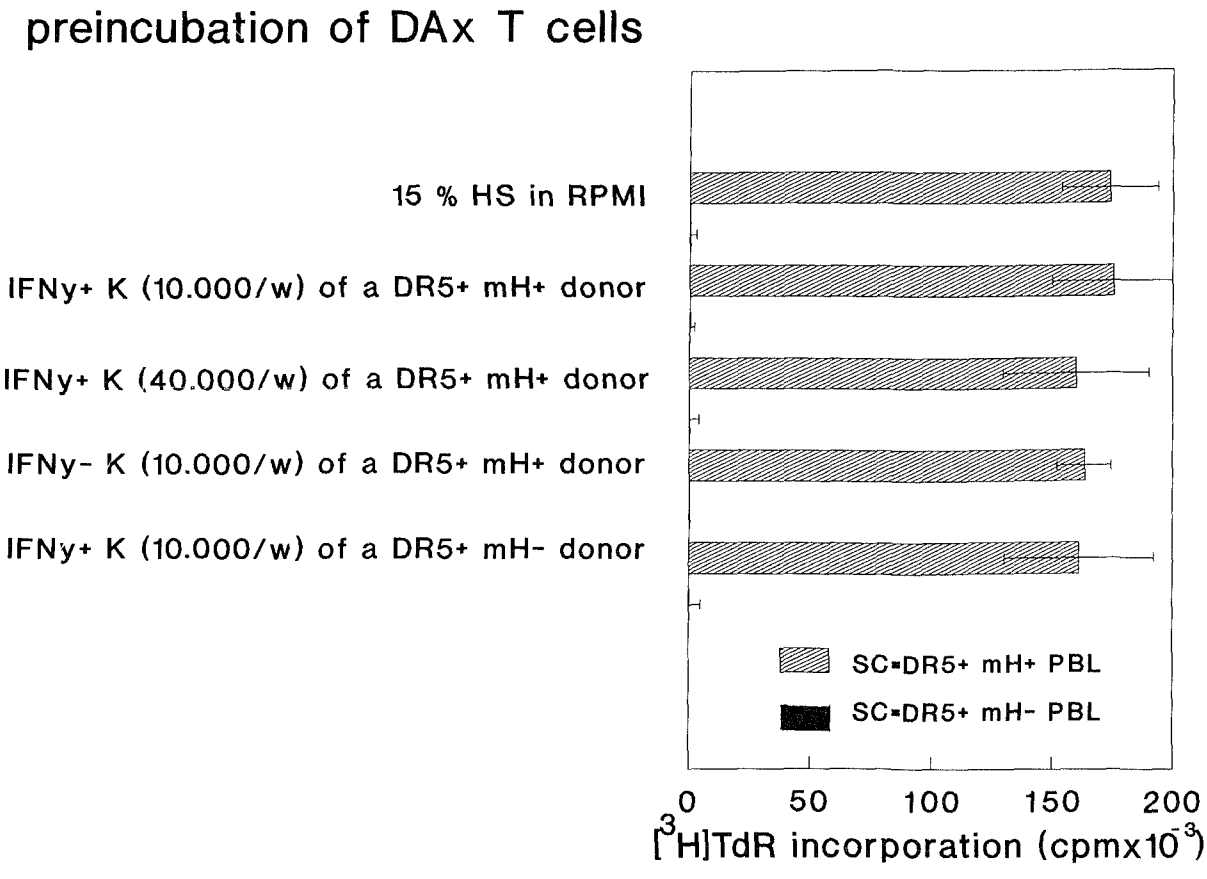

Figure 7 Absence of proliferation by the mH specific, DR5 restricted Th cell line DAx is not due to anergy 3×10^4 DAx T cells/w were preincubated for 24 hours at the indicated conditions Harvested T cells were tested for induced anergy in a conventional PLT using DR5+ mH+ and DR5+ mH− PMNC as SC

uncultured KC remain viable for a longer period when suspended in T cell culture medium than cultured KC [29] Likewise, terminal differentiation of detached KC (see below) may have distinct kinetics for purified versus cultured KC Thirdly, since KC were found to be inefficient APC compared to spleen cells,[8] they may only induce measurable activation of those Th cell clones with a very high proliferative potential

The potential role of *in vitro* KC treatment on allo MHC Th cell activation is most strikingly illustrated by the data shown here Whereas suspensions of class II+ KC, generated by trypsin-detachment of IFNγ treated cultures, were unable to induce allo DR specific T cell proliferation (Figure 4), intact layers of IFNγ treated KC at the right density activated these T cells in an ICAM 1 and HLA-DR dependent fashion (Table 1, Figure 1) Since most investigators thus far used suspensions of KC as stimulator cells, it is relevant to understand the apparent differential T cell activating capacity of suspended versus adherent KC First, the exposure of KC to trypsin is thought to induce rigorous membrane changes[38] and has been suggested by some authors to affect the ICAM-1 molecule [39 40] FACS data of trypsin treated KC cell suspen sions (Figure 2)[30] argue against trypsin sensitivity of the RR1/1 antibody binding site on ICAM 1 However, this does not exclude that the epitopes for LFA 1 binding to ICAM-1 might still be affected by trypsin treatment In contrast to when directly used as suspended SC, these potentially trypsin affected KC may, after the subsequent culture period (three to six days), express newly synthesized ICAM-1 molecules when used as intact layers Secondly, cultured KC, when detached from their substrate, rapidly undergo the destruc tive process of terminal differentiation [41 42] Within the first 12 hours in suspension RNA and protein synthesis have virtually stopped and keratin filaments of the extracellular matrix become cross linked These dying KC might fail to provide pivotal costimulatory signals which viable adherent KC do provide In addition, the changes in the extracellular matrix may negatively affect T cell binding to KC Whichever the exact mechanism causing the severely reduced T cell activat ing potential of suspended KC, intact layers of basal KC most probably represent a more adequate *in vitro* model to study KC-mediated T cell activation *in vivo*

We observed a striking variation of proliferation of anti DR Th clones with degree of confluence of the KC layers An increase in KC plated per well from the optimal number of 5×10^4 to 100×10^4 fully abrogated proliferation (Figure 4) Since KC-conditioned medium was found to inhibit T cell proliferation in an allo MLR (Table 3), the presence of T cell inhibitory factors in the assay medium is indicated to account for the observed dose-dependent reduction of T cell proliferation Additional experiments are required to identify these factors Previous reports have suggested that PGE_2 and TGFβ might be involved [29 43]

The data discussed thus far have indicated the relevance of the *in vitro* treatment of the KC cell population used as SC However, the controversial data on T cell activation by MHC class II+ KC cannot be solely attributed to experimental differences, since diversity in T cell activation has been observed within one single KC model Gaspari reported that KC activated an allo IA^k T cell ([8] see above), but not a TNP-specific IE^k restricted T cell clone [26] Instead, overnight co culture with antigen expressing KC rendered the latter T cell clone anergic, as marked by long-term unresponsiveness to subsequent triggering by adequate APC A similar observa tion of tolerance induction instead of activation was reported by Bal, involving a DR restricted influenza hemagglutinin peptide specific Th cell clone after coculture with peptide and IFNγ pretreated layers of human KC [25] Although the nature of the signals leading to T cell anergy is not yet fully clear, it is established that occupancy of the TCR by antigen in the absence of additional positive signals *in vitro* can induce this T cell state [34] Antigen positive MHC class II+ KC appear to anergize some and to activate other Th cell populations Differences between responder T cells in terms of (1) activation requirements and (2) antigenic specificity might account for this phenomenon It is well established that murine Th cell clones belonging to the Th1 subset have distinct requirements than Th2 cell clones[35] and that Th1 cells can be anergized under certain *in vitro* conditions [34 44] The allo MHC and mH antigen specific Th cell clones activated by KC (Table 1, Figure 5) have yet to be categorized as Th1 or Th2-like Both Th cell clones which were rendered anergic after coculture with antigen- and MHC class II-expressing KC were of the Th1 or (in human) of the Th1-like subset [25 26] In contrast, recent data by our group indicate that Th cells of the Th1-like subset can very well be activated by KC [12] The second aspect potentially influencing T cell activation versus inactivation is the antigen specificity of the TCR Evidently, cell surface expression of the antigenic epitope is a prerequisite to induce an antigen specific (either positive or negative) response in T cells Epitopes of soluble exogenous proteins which require processing,[8] but also mH epitopes, may not be presented by KC In previous reports we showed that the male specific mH antigen H-Y is expressed by activated KC,[10] in contrast to some autosomal mH peptides [11] The observation that KC of MHC-restriction and mH antigen positive donors failed to induce *either* proliferation *or* anergy in two mH antigen specific Th cells, is suggestive for the absence of these mH antigens on KC to provide a signal via the TCR However, the antigen specificity of the Th cell clones studied cannot easily explain the controversial results in T cell activation observed in the presence of antigen Whereas the Th cell clones rendered anergic were specific for TNP-modified IE^{k26} and an HLA-DR restricted viral peptide,[25] those activated recog nized allo DR[8] (Table 1) or a self class I restricted H-Y peptide (Figure 5), but also HLA-DR restricted HSP-65 peptides [12]

Evidently, a systematic analysis of a large panel of Th cell clones with well defined antigen specificities and known Th1/Th2 lymphokine production profiles is required within one *in vitro* KC model to settle the question of what determines if KC activates or inactivate Th cells Still, the data presented here serve to indicate that coculture of KC with allo MHC and mH antigen specific Th cell clones can result in antigen specific T cell stimulation instead of inactivation It is conceiv able that *in vivo* T cell-KC interactions can lead to either outcome, depending on multiple factors including antigen specificity, activation requirement of the T cells involved, but also local concentrations of antigen and lymphokines

Acknowledgements

We thank Dr A Termijtelen for providing the allo DR and DPw T cell clones and thank Drs F Koning and A Termijtelen for critical reading of the manuscript This work was sup ported by the Dutch 'Ziekenfondsraad'

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