Brief Definitive Report

SUSCEPTIBILITY OF HUMAN MALE KERATINOCYTES TO MHC-RESTRICTED H-Y-SPECIFIC LYSIS

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Cell-mediated immunity against polymorphic minor histocompatibility (mH) antigens is assumed to contribute to the development of graft-vs.-host disease (GVHD) and graft rejection in recipients of HLA-identical marrow grafts (1). Although the effector cell mechanisms underlying both events are not completely understood, it can be anticipated that the ultimate effect of T lymphocytes directed against mH antigens depends on the tissue distribution of these molecules.

One of the most extensively studied mH antigens is the male-specific antigen H-Y, which was first discovered as a transplantation barrier in a murine skin graft model by Eichwald and Slimser (2). In the mouse as well as in man the immune response to the H-Y antigen appears to be mainly, though not exclusively, mediated by MHC-restricted T cells (3–5). This poses major limits to the possibilities to perform tissue distribution studies for the human H-Y antigen, which thus depend on the usage of cellular techniques such as cell-mediated cytotoxicity. In 1977, Goulmy and co-workers isolated HLA class I-restricted CTLs specific for H-Y from a female patient after rejection of a marrow graft from her HLA-identical male sibling (5). This was actually the first report suggesting a role for the H-Y antigen in human bone marrow transplantation. In line with this notion, Storb et al. (6) and Kernan et al. (7) identified male donor sex as a risk factor for graft rejection and failure in transplantation for aplastic anemia and following T cell depletion. Recently, the role of H-Y in graft rejection was further clarified by Voogt et al. (8) who demonstrated that destruction of male hematopoietic progenitor cells can occur via H-Y-specific cytolysis.

Accordingly, cellular typing for the H-Y antigen on human skin cells may lead to a better understanding of the mechanism of GVHD. Human skin is extremely vulnerable to cell-mediated immunity during GVHD. In particular, young epidermal keratinocytes seem to be targeted in situ (9). In this article we have used different H-Y-specific CTL clones and cultured human keratinocytes as an in vitro model to investigate the susceptibility of male skin cells to H-Y-mediated cytolysis. In contrast to what has been found in the mouse (10), our results demonstrate that H-Y determinants in the human skin are functionally accessible to CTLs. Conceivably,

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the H-Y antigen can serve as a target structure in the local anti-host immune response during GVHD.

Materials and Methods

CTL Clones. The CTL clones, which were obtained by limiting dilution, were an allo-HLA-A2-specific clone designated IE2 derived from an in vitro MLR (11), an HLA-A2-restricted, H-Y-specific CTL clone "IR35," and an HLA-B7-restricted, H-Y-specific CTL clone ".5W4," both obtained from the peripheral blood of in vivo sensitized female patients (12).

Keratinocyte Cultures. Epidermal keratinocytes were obtained from three healthy volunteers (donor 1, male, HLA-A1,-A2,-B8,-B15; donor 2, male, HLA-A3,-A9,-B7,-B40; donor 3, female, HLA-A2,-A29,-B7,-B57) and were cultured using a Rheinwald-Green feeder layer technique (13) with small modifications (14).

Chromium-release Assay. Control cellular typing for HLA-A2 and H-Y of the skin donors was performed using their T lymphoblasts as target cells in a standard ^{51}Cr-release assay (15). Clone IE2 (i.e., HLA-A2 specific) significantly lysed lymphoblasts obtained from donors 1 and 3, clone IR35 (i.e., A2/H-Y specific) lysed lymphoblasts from donor 1, whereas clone .5W4 (i.e., B7/H-Y specific) lysed lymphoblasts from donor 2 (data shown in the legends to Figs. 1 and 2, and to Table I). Keratinocytes from these donors were used as target cells in a modified 4-h ^{51}Cr-release assay (de Bueger, M. M., C. A. C. M. van Els, J. Kempenaar, M. Ponec, and E. Goulmy, manuscript submitted for publication). Briefly, keratinocytes from subconfluent cultures were harvested and dispensed at 10,000 cells per well in 96-well flat-bottomed microtiter plates and allowed to attach for 48 h, either in the presence or absence of 250 U IFN-γ/ml for the last 18 h. Approximately 15% of the cells adhered. While adherent, keratinocytes were labeled and incubated in five replicate wells in the presence (*E*) or absence (*S*) of different numbers of effector cells, and in the presence of 1% Triton X-100 (*M*). Standard deviations of replicates were <15% and spontaneous release levels were between 10 and 20%. Specific cytolysis was calculated according to the amount of isotope released in *E*, *S*, and *M* in the following formula; $[(E-S)/(M-S)] \times 100\%$.

Results and Discussion

Detection of HLA-A2. Since the expression of MHC class I is a prerequisite for H-Y detection with the H-Y-specific CTLs, we first explored the cellular recognition of the HLA-A2 antigen on cultured keratinocytes using the HLA-A2-specific CTL clone IE2. As is illustrated in Fig. 1 *a*, keratinocytes of the HLA-A2+ve donors 1 and 3 were lysed in a dose-dependent manner, whereas keratinocytes of the HLA-A2−ve donor 2 were not. Herewith, the HLA allotyping on keratinocytes fully corresponded to typing on PHA blasts of the same individuals (see legend to Fig. 1). After treatment with IFN-γ (250 U/ml 18 h) the HLA-A2-specific lysis of keratinocytes from the HLA-A2+ve donors 1 and 3 was significantly enhanced (Fig. 1 *b*). Thus, in vitro cultured human keratinocytes are susceptible to cell-mediated lysis against major alloantigens.

Detection of A2/H-Y. We then established the cytotoxic activity of the HLA-A2-restricted, H-Y-specific CTL clone IR35 on keratinocytes of donors 1, 2, and 3 (Table I). In two of three experiments, 19–23% of H-Y killing was observed of untreated keratinocytes of the HLA-A2+ve male donor 1; in one of three experiments no lysis was obtained. Unstimulated keratinocytes from the HLA-A2−ve male donor 2 and the HLA-A2+ve female donor 3 were not lysed. After preincubation of the keratinocytes with IFN-γ (250 U/ml/18 h), H-Y-specific cytolysis of the HLA-A2+ve male keratinocytes of donor 1 was dramatically enhanced whereas no aspecific lysis of the other keratinocytes was induced.

Detection of B7/H-Y. To confirm the expression of the cellularly defined H-Y an-

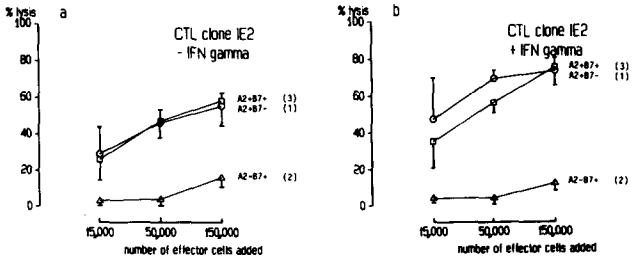

FIGURE 1. HLA-A2-specific cytolysis of cultured keratinocytes. Untreated keratinocytes from the HLA-A2+ve donors 1 (○) and 3 (□), and from the HLA-A2-ve donor 2 (△) were used as target cells for the anti-HLA-A2 CTL clone IE2. Only HLA-A2+ve keratinocytes were lysed in a dose-dependent manner (*a*). IFN-γ pretreatment (250 U/ml/18 h) of keratinocytes enhanced the susceptibility of the HLA-A2+ve keratinocytes to anti-A2 lysis (*b*). Mean percentages of specific kill ± SE of three experiments are shown. Cellular typing of T lymphoblasts from donors 1, 2, and 3 using CTL clone IE2 (E/T ratio, 30:1) gave the following results; 77 ± 2, 2 ± 2, and 82 ± 5% (mean of three experiments ± SE), respectively.

tigen on keratinocytes in conjunction with another restriction element, we performed a similar set of experiments using the H-Y-specific, HLA-B7-restricted CTL clone .5w4. In these experiments, untreated keratinocytes were not lysed (Fig. 2 *a*). However, after IFN-γ pretreatment, keratinocytes of the HLA-B7+ve male donor 2, but not of the HLA-B7−ve male donor 1 and the HLA-B7+ve female donor 3, were rendered susceptible to H-Y-mediated lysis (Fig. 2 *b*).

This study for the first time clearly demonstrates that the H-Y antigen can be cellularly detected on human keratinocytes by conventional H-Y-specific CTL clones. Furthermore, the recognition of H-Y was shown to take place in the context of two

TABLE I

Influence of IFN-γ Pretreatment on the Susceptibility of Keratinocytes to A2/H-Y-specific Lysis by CTL Clone IR35

	Targets			Number of effector cells added		
Exp.	Pretreatment with IFN-γ	Donor	Phenotype and sex	10,000	50,000	150,000
1	0	1	HLA-A2$^+$ ♂	0*	13	19
	250			11	46	70
	0	3	HLA-A2$^+$ ♀	0	−1	5
	250			−7	−6	NT
2	0	1	HLA-A2$^+$ ♂	2	23	NT
	250			32	68	NT
	0	2	HLA-A2$^-$ ♂	−2	3	NT
	250			−1	2	NT
	0	3	HLA-A2$^+$ ♀	0	1	NT
	250			1	17	NT
3	0	1	HLA-A2$^+$ ♂	−1	−1	−2
	250			22	26	42
	250	2	HLA-A2$^-$ ♂	−3	0	2

* Percentage of specific lysis of adherent keratinocytes.

‡ U/ml/18 hr; NT not tested.

Cellular typing of T lymphoblasts from donors 1, 2, and 3 using CTL clone IR35 (ET, 30:1) gave the following results respectively: 75 ± 8, 2 ± 3, and 0 ± 1% (mean of three experiments ± SE).

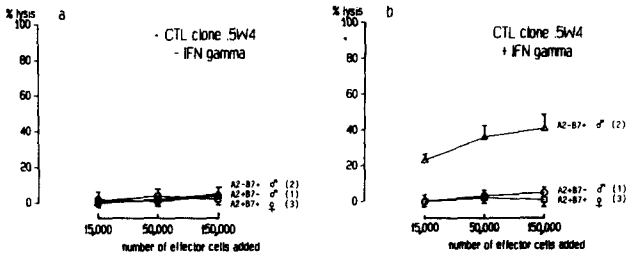

FIGURE 2. IFN-γ-mediated induction of B7/H-Y-specific cytolysis of cultured keratinocytes. Untreated keratinocytes of the three skin donors are not susceptible to HLA-B7-restricted, H-Y-specific lysis by CTL clone .5W4 (*a*). After pretreatment with IFN-γ (250 U/ml/18 h) keratinocytes from the HLA-B7 + ve male donor 2 (Δ), but not from the HLA-B7 − ve male donor 1 (O) and the HLA-B7 + ve female donor 3 (□) were lysed (*b*). Mean percentages of specific kill ± SE of three experiments are shown. Cellular typing of T lymphoblasts from donors 1, 2, and 3 using CTL clone .5w4 (E/T, 30:1) gave the following results, respectively: 9 ± 2, 87 ± 13, and 2 ± 2% (mean of three experiments ± SE).

different HLA class I antigens. The implications of these findings are twofold. First, the recognition of H-Y on keratinocytes through H-Y-specific CTL clones, which were induced and selected using APCs of lymphoid origin, implies that the cellularly defined human H-Y/class I structure apparently adopts a similar antigenic configuration on these different cell types. In mice, the same could be concluded from experiments carried out in the opposite direction, namely that by in vivo immunization for H-Y using male keratinocytes, CTLs were obtained that were capable of lysing male spleen cells (10). Second, the demonstration that destruction of keratinocytes can occur by H-Y-specific cytolysis clearly sustains the role of H-Y as a target cell structure in the epidermal effector phase of GVHD.

In the mouse, a role for mH antigen-specific CTL as proximal mediators in allograft immunity has strongly been promoted by the work of Steinmuller and colleagues on the skin-specific mH antigen Epa-1 (16, 17). These investigators reported the in vivo isolation of H-2-restricted, Epa-1-specific CTL lysing epidermal cells, fibroblasts, and activated macrophages while unaffecting lymphocyte targets. Epa-1-specific CTL could induce GVHD-like skin lesions when inoculated into the appropriate hosts. Unlike in the Epa-1 system, however, male murine skin cells were fully resistant to H-2-restricted, H-Y-specific lysis (10). This finding was even more puzzling because male epidermal cells were quite capable of priming syngeneic female lymphocytes in vivo for the subsequent generation of H-Y-specific CTLs. However, this apparent paradox stands not on itself since the failure to lyse murine epidermal cells with CTL was also found in other non H-2 antigenic systems (Steinmuller, D., personal communication). It remains unclear, however, why murine but not human keratinocytes would be refractory to H-Y-specific lysis. It might be possible that using keratinocytes in cell suspension induces resistance to cellular killing. In fact, refractoriness of nonadherent keratinocytes to lysis through conventional anti HLA CTL has been described in man also (18). Since our results to not agree with this latter finding our reasoning is that technical aspects such as clonal affinity and target cell circumstances are of major importance for the detection of HLA and or non-HLA antigens on nonconventional target cells (de Bueger, M. M., et al., submitted for publication).

Although we normally observed HLA-A2-directed cytolysis of non-IFN-γ-treated adherent human keratinocytes, in the majority of cases pretreatment with IFN-γ

was needed to upregulate H-Y-specific killing to detectable levels. It would be of interest to know whether the resistance to H-Y-specific CTLs of murine male keratinocytes (10) could be overcome by pretreatment with IFN-γ. If so, the accessibility of the H-Y antigen to CTLs in human and murine skin would not essentially differ. IFN-γ may enhance H-Y recognition by different mechanisms, such as upregulation of HLA restriction elements or of the H-Y antigen itself, but also other accessory structures such as ICAM-1 may play a role (19). Whatever mechanism(s) are involved, we propose that in situ release of IFN-γ, eventually produced by local immune T cells, may effectively enhance the CTL recognition of mH antigens in the human skin after allogeneic bone marrow grafting.

Summary

We studied the expression of the male-specific mH antigen H-Y on cultured human skin cells by investigating their susceptibility to H-Y-specific cytolysis using conventional class I-restricted CTL clones in a modified cell-mediated cytotoxicity assay. In contrast to what was found in the rodent system, we observed H-Y-specific lysis of human male keratinocytes. Susceptibility for H-Y-specific lysis was efficiently enhanced by exposure of the keratinocytes to IFN-γ. Our results demonstrate that human skin cells are equally sensitive for the activity of H-Y-specific CTLs as target cells of lymphoid origin. Finally, the cellular recognition of the H-Y mH antigen in the skin further supports its possible target function in the local graft versus host attack.

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