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TISSUE DISTRIBUTION OF HUMAN MINOR HISTOCOMPATIBILITY ANTIGENS

Ubiquitous versus Restricted Tissue Distribution Indicates Heterogeneity among Human Cytotoxic T Lymphocyte-Defined Non-MHC Antigens[1]

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We determined the tissue distribution of 7 human minor histocompatibility (H) Ag. Each of these Ag is defined by one or more MHC class I-restricted CTL clones, previously generated from PBL primed against minor H Ag by HLA-identical bone marrow transplantation (BMT). CTL-mediated lysis of tissue-derived cells and cultured cell lines was used as an in vitro assay for minor H Ag expression of several human tissues. The Ag HA-3 (HLA-A1-restricted), HA-4 (HLA-A2 restricted), HA-6 and HA-7 (HLA-B7 restricted), and the male-specific Ag H-Y (HLA-A2 and B7 restricted) were found to be expressed on cells of all tissues tested. In contrast, the HLA-A2-restricted Ag HA-1 and HA-2 were demonstrated on PHA-blasts, EBV-BLCL, purified T cells, B cells, monocytes, and immature thymocytes, but could not be demonstrated on skin-derived cultured fibroblasts, keratinocytes, melanocytes, cultured epithelial cells of kidney proximal tubili, and umbilical cord vein-derived endothelial cells. Incubation of the latter cell lines with rIFN-γ, rTNF-α, and/or rIL-1α, in concentrations shown to maximally increase their susceptibility to lysis by allo-MHC class I CTL, did not induce recognition by HA-1- and HA-2-specific CTL in vitro. These results indicate an ubiquitous tissue expression of the minor H Ag HA-3, -4, -6, -7 and H-Y in contrast to a to the hemopoietic cell lineage-restricted expression for HA-1 and HA-2. The heterogeneity in tissue expression of T cell-defined, class I-restricted non-MHC Ag implies that they might be derived from intracellular proteins with either an ubiquitous or a more specialized cell type-specific function.

Transplantation between individuals matched for the MHC genes can result in graft rejection and GvHD,[3] if donor and recipient differ at one or more minor H loci (1–4). Minor H genes have been initially identified by their ability to induce tumor (1) or skin (5) graft rejection between mice of congenic strains. Since that time over 40 genes have been localized on several chromosomes (6, 7). In humans, T cell responses in individuals grafted with HLA-identical bone marrow led to the description of a limited number of minor H Ag (4, 8–11), whose genes (with the exception of the male-specific H-Y Ag) (12), remain to be mapped.

Until recently, little was known about the molecular nature (6, 13) or the biologic properties of the minor H gene products. This mainly is due to the common feature of minor H Ag that they generally fail to be recognized by antibodies. To detect minor H gene products, in vivo T cell-mediated graft rejection and in vitro assays with MHC-restricted T cell clones can be used (8, 9, 14). With respect to the molecular nature of minor H Ag, recent evidence was obtained revealing that some murine minor H Ag represent short peptides, presumably derived from cellular proteins, which are presented to T cells by MHC class I molecules (15–17). With respect to their biologic properties, such as their tissue distribution, minor H Ag also remain ill characterized. To understand the impact of minor H Ag disparate organ or bone marrow grafting, it is essential to know the tissue distribution of these T cell epitopes. Some information on the tissue expression of minor H Ag has been obtained by studying in vivo rejection of minor H Ag disparate organs (18). This information is circumstantial because in vivo immunogenicity of an organ is not solely determined by minor H Ag expression (18). Recently Griem et al. (19), in an elaborate in vitro study, investigated the tissue distribution of three murine minor H Ag and found that they all had a MHC class I-like pattern of expression.

This study was performed to determine the expression of human minor H Ag on different tissues. Eleven cell types derived from several tissues were selected on the basis of their availability and suitability for use as targets in in vitro ^{51}Cr release assays. The human minor H Ag analyzed included the male-specific Ag H-Y and six minor H Ag HA-1, -2, -3, -4, -6, and -7, each defined by recognition by one or more MHC class I restricted CTL clones (4, 20). The minor H Ag HA-3, -4, -6, -7, and H-Y were detected on all tissues tested. In contrast, HA-1 and HA-2 could only be demonstrated on cell types of hemopoietic origin. The observed ubiquitous vs restricted tissue expression of these human minor H Ag not only gives useful information for transplantation, but also provides

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[3] Abbreviations used in this paper: GvHD, graft vs host disease; H, histocompatibility; BMT, bone marrow transplantation; PTEC, proximal tubular epithelial cells; HUVE, human umbilical venous endothelial cells; PE, phycoerythrin; PMNC, polymorphonuclear cell; HS, human serum.

a first indication for heterogeneity among CTL-defined human non-MHC Ag.

MATERIALS AND METHODS

CTL cultures. Minor H Ag-specific CTL lines were established as previously described (4). Briefly, PMNC of patients taken shortly after HLA genotypically identical BMT were stimulated with the patients' pre-BMT PMNC. Effector cells were further expanded by using pre-BMT feeder cells and rIL-2. CTL lines, obtained from six patients suffering from various grades of acute GvHD (4), were cloned by limiting dilution. All $CD8^+$ CTL clones selected for further analysis recognized non-MHC determinants in a MHC class I-restricted fashion. The eight clones used in this study display minor H Ag specificities designated HA-1 (clone 3HA15), HA-2 (5H17), HA-3 (5HO11), HA-4 (5G30), HA-6 (clone 21), HA-7 (clone 6), and H-Y (1R35 and 5W4), respectively, and have defined MHC class I restriction elements. HA-1, HA-2, and HA-4 use HLA-A2; HA-3 is HLA-A1-restricted (20); HA-6 and HA-7 are recognized in association with HLA-B7, and the male-specific Ag H-Y is recognized by CTL clones in the context of HLA-A2 (1R35) and B7 (5W4) (8). Phenotype frequencies of the minor H Ag in the healthy population are known (20), whereas their mode of inheritance and potential allelic relationships are still under study. Class I MHC-specific alloreactive CTL clones were used as control effector cells. The αHLA-A2 clone 3E7 and the αHLA-A1 clone 2 were generated in vitro by selective stimulation in MLR and subsequent limiting dilution. The HLA-B7-specific CTL clone KOR18 was kindly provided by Dr. B. Breur-Vriesendorp. Characteristics of the CTL clones used are summarized in Table I. The CTL clones were cultured in RPMI 1640 supplemented with antibiotics, 15% pooled HS, and 20 U/ml rIL-2, and they were expanded by weekly restimulation with irradiated EBV-BLCL plus freshly isolated PMNC of random donors. Cryopreserved CTL were thawed for immediate usage as effector cells in ^{51}Cr-release assays or kept for 2 to 4 days on 20 U/ml rIL-2 before use.

Isolation and culturing of distinct cell types. T cells, B cells, and monocytes were purified from Ficoll-isolated PMNC from healthy HLA and minor H Ag-typed donors ($n = 4$) by using a FACScan flow cytometer (Becton Dickinson, Palo Alto, CA). For T cell purification, PMNC were labeled by using an antibody mixture containing the FITC and PE-preconjugated mAb αCD20 and αCD15. The FITC and PE-negative cell fraction was sorted and collected and represented a >97% pure $CD3^+$ cell population. Similarly, highly pure B cell and monocyte suspensions were obtained after staining with αCD15-PE + αCD5-FITC or αCD16-PE + αCD5-FITC + αCD20-PE mAb, respectively (all mAb used were from Becton Dickinson). Monocyte and B and T cell suspensions were kept overnight in RPMI 1640 supplemented with FCS for immediate use as targets in ^{51}Cr-release assays. Thymocyte suspensions were generated by physical disruption of pieces of thymic material obtained by surgery from young children ($n = 8$). Thymic cell suspensions consisted of >75% double-positive immature thymocytes, expressing CD1 and low levels of MHC class I as determined by FACS analysis. Thymic cell suspensions were cryopreserved until use as targets in ^{51}Cr-release assays. Melanocytes were isolated from pieces of foreskin ($n = 10$) and cultured as adherent cell lines as previously described (22). Confluent cultures were trypsinized and frozen for later usage as target cell suspensions. Dermal fibroblast cultures ($n = 3$) were generated from shave skin biopsies of the upper arms of healthy donors as previously described (23). Briefly, fibroblasts were allowed to grow out of 1-mm explant sections of skin in petri dishes containing RPMI. Confluent fibroblast cultures were trypsinized and subcultured in DMEM (GIBCO) supplemented with glutamin, antibiotics, and 10% FCS. The 5th to 10th passage cultures consisted of 100% fibroblasts as indicated by cell morphology and 0% staining by anti-cytokeratin-reactive mAb. For use as adherent targets in ^{51}Cr-release assays, fibroblast suspensions were plated in flat bottom microtiter plates at 5000/well and grown to confluence. An aliquot of 200 U/ml rIFN-γ (Genetech, San Fransisco, CA) plus 5 ng/ml rTNF-α (Genzyme, Sanbio) was added during the last 48 h. Epidermal keratinocyte cultures ($n = 10$) were generated from shave skin biopsies by trypsinizing overnight (0.3% w/v trypsin, 0.1% glucose), and seeding the obtained cell suspension in culture flasks at $1.2 \times 10^3/cm^2$ on a feeder layer of irradiated mouse 3T3. Keratinocyte cell lines were maintained in a 3:1 mixture of DMEM and Ham's F12 (GIBCO), supplemented with 5% FCS, 10^{-6} M isoproterenol, 0.4 μg/ml hydrocortisone, and 10 ng/ml epithelial growth factor (all from Sigma, St. Louis, MO), and were subcultured weekly until the 4th to 6th passage as described elsewhere (24). For use as adherent targets in ^{51}Cr-release assays, keratinocytes were seeded in microtiter plates at 10^4 cells/well and allowed to adhere for 36 h. When the effect of cytokines was studied, rIFN-γ (Genentech), rTNF-α (Genzyme, Sanbio), and/or rIL-1α (Hoffmann-La Roche, Switzerland) were added to the wells during the last 24 h. PTEC ($n = 6$) were isolated from tubular epithelium of rejected kidneys and cultured as previously described (25, 26) in DMEM/Ham's F12 1:1, supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml), and epidermal growth factor (10 ng/ml) (all from Sigma) on a matrix of collagen type I (Vitrogen; Collagen Corp, Palo Alto, CA). PTEC monolayers, generated by seeding established PTEC lines in 48-well plates (the last 72 h in the presence of 200 U/ml rIFN-γ), were used after ^{51}Cr-labeling as targets in cell-mediated lysis. HUVE cells ($n = 8$) were isolated and cultured as described elsewhere (27). In short, cell suspensions obtained from cord veins by using collagenase (1 mg/ml, Sigma) were cultured on a matrix of 1% gelatin in M1999 with Earls salts (Seromed; Biochrom K.G., Berlin, FRG) supplemented with 20% HS and endothelial cell growth factor. Confluent endothelial cell cultures generated in 48-well plates (Costar 3548, Cambridge, MA) and incubated for the last 72 h with 200 U/ml rIFN-γ were used for ^{51}Cr-release assays. Established EBV-transformed B cell lines were used as control targets in cell-mediated lysis. To determine their minor H Ag phenotype, PHA-blasts were generated from all HLA-typed tissue donors (with the exception of the melanocyte donors) by culturing their PMNC for 7 days in RPMI 1640 plus 15% HS, 1% PHA and 20 U/ml rIL-2. A summary of the target cell types used is set out in Table II.

^{51}Cr-release assays. PHA-T cells, EBV-LCL, T cells, B cells, monocytes, thymocytes, and melanocytes were prepared and used as suspended targets at 5000/well in a standard 4 h ^{51}Cr-release assay. The numbers of ^{51}Cr target cells/well were adjusted for some cell types: 50,000/well for thymocytes and uncultured T cells and 20,000/well for monocytes. As previously described dermal fibroblasts (27), epidermal keratinocytes (28), PTEC (26), and HUVE (29) were tested as intact ^{51}Cr-labeled monolayers. Spontaneous ^{51}Cr release of all distinct cell types never exceeded 20% of the maximal release values measured in the presence of 1% Triton X100.

TABLE I

Characteristics of the minor H Ag-specific and allo-HLA class I-reactive CTL clones[a]

CTL Clone	HLA-Restriction/ Specificity	Minor H Ag	
		Code	Phenotype frequency[b]
3HA15	A2	HA-1	0.69
5H17	A2	HA-2	0.95
5HO11	A1	HA-3	0.88
5G30	A2	HA-4	0.16
Clone 21	B7	HA-6	>0.80
Clone 6	B7	HA-7	>0.80
1R35	A2	H-Y	±0.50
5W4	B7	H-Y	±0.50
3E7	Allo-A2		
Clone 2	Allo-A1		
Kor 18	Allo-B7		

[a] From Goulmy (4) and Van Els et al. (20).

[b] Phenotype frequencies of the minor H Ag in the HLA-A2.1 (HA-1, HA-2, HA-4)-, the HLA-A1 (HA-3)-, or the HLA-B7 (HA-6, HA-7)-positive population.

RESULTS

Susceptibility of distinct human cell types to MHC class I-specific lysis. We studied the tissue distribution of seven human minor H Ag by testing several available human tissues or tissue-derived cell lines for recognition by minor H Ag-specific CTL in ^{51}Cr-release assays. The eight MHC class I-restricted CTL clones defining these seven minor H epitopes have been previously described (4, 20) and are listed in Table I. To perform this in vitro CTL-mediated minor H Ag tissue typing, the various purified and/or cultured cell types (listed in Table II) should be suitable for use in ^{51}Cr-release assays, and in particular they should display sufficient susceptibility to MHC class I-restricted lysis. Therefore, conditions in ^{51}Cr-release assays were optimized for each cell type as described in *Materials and Methods.* For each tissue, cell lines from several donors ($n = 3$-10) were tested with alloreactive αHLA-A2, αHLA-A1, and αHLA-B7 CTL

TABLE II
Human target cell types tested

Cell Type (Code)	Tissue Source	In Vitro Isolation or Culturing (c)	In Vitro MHC Up-Regulation
T cells	PMNC	FACS purification	Na
PHA T blasts	PMNC	PHA stimulation (c)	
B cells	PMNC	FACS purification	Na
EBV-BLCL	PMNC	EBV transformation (c)	
Monocytes	PMNC	FACS purification	Na
Thymocytes	Thymus		Na
Melanocytes	Foreskin	Ref. 22 (c)	
Fibroblasts	Skin (dermis)	Refs. 23 and 28 (c)	IFN-γ + TNF-α
Keratinocytes	Skin (epidermis)	Refs. 24 and 29 (c)	IFN-γ, TNF-α,IL-1α
PTEC	Tubuli from rejected kidneys	Refs. 25 and 26 (c)	IFN-γ
HUVE	Umbilical cord veins	Ref. 27 (c)	IFN-γ

clones at several E:T ratios (i.e., 20, 2, 0.2:1). Nonspecific lysis by αMHC class I CTL was not observed with any of the cell types, as indicated by the absence of lysis (0–7%) of cell lines obtained from HLA-A2-, -A1-, or -B7-negative donors (data not shown). As Figure 1*A* indicates, the susceptibility of distinct cell types to lysis by αHLA-A2 CTL varied considerably. Whereas HLA-A2$^+$ EBV-BLCL, PHA-blasts, dermal fibroblasts, epidermal keratinocytes, PTEC, and HUVE were effectively lysed (51–98%), cultured melanocytes and purified T cell, B cell, thymocyte, and monocyte suspensions gave low values of HLA-A2-specific lysis (26–55%) at E:T ratio = 20. The observed

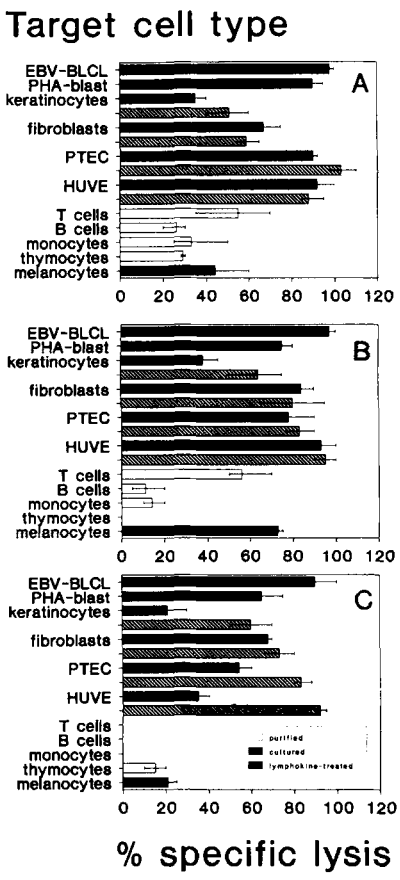

Figure 1. Susceptibility of distinct human cell types to lysis by α MHC class I CTL. Freshly isolated and purified cells (T, B, monocytes, and thymocytes) and cultured cell lines (melanocytes, fibroblasts, keratinocytes, PTEC, HUVE) from donors expressing the appropriate HLA Ag were used as suspended or adherent targets in optimized 4-h ^{51}Cr-release assays for αHLA-A2 (*A*), αHLA-A1 (*B*), and α HLA-B7 (*C*) CTL clones. Mean values + SD of Ag-specific lysis of cells of 2 to 20 HLA Ag$^+$ donors, each measured at E:T ratio = 20 in two experiments, are represented.

patterns of HLA-A1- and -B7-specific lysis of the distinct tissues revealed similar qualitative differences in susceptibility (Fig. 1, *B* and *C*). The effect of IFN-γ-induced up-regulation of MHC class I and ICAM-1 molecules was more pronounced on B7-specific lysis of keratinocytes, PTEC, and HUVE than on A2- or A1-specific lysis (Fig. 1, *C* vs *A* and *B*) also at lower E:T ratios (data not shown). Each of the cultured and uncultured cell types tested displayed a typical but in vitro detectable susceptibility to CTL-mediated class I lysis, and thus minor H Ag expression could be analyzed by using MHC class I-restricted CTL clones.

Minor H Ag-specific CTL clones differ in their capacity to lyse tissues of nonhemopoietic origin. To be able to determine the tissue expression of minor H Ag epitopes, the minor H phenotypes of the tissue donors had to be known. The absence of recognition of a certain tissue-derived cell line by minor H Ag-specific CTL may either result from 1) the absence of expression of the minor H Ag epitope on the tissue under study, or 2) absence of the minor H gene product in the tissue donor. All seven minor H epitopes studied here have previously been defined by CTL clones obtained by in vivo priming by HLA-identical BMT, followed by in vitro boosting by using PMNC and EBV-BLCL, and have been selected on the basis of reactivity toward PHA-T cells and EBV-BLCL targets (4, 20). Consequently, HA-1, -2, -3, -4, -6, -7, and H-Y are all expressed on PHA-T cells and EBV-BLCL. Thus, by testing their PHA-T cells for lysis by minor H Ag-specific CTL in ^{51}Cr-release assays, those tissue donors having the appropriate HLA could be assigned a minor H phenotype (+ or −) for each of the seven minor H Ag (data not shown).

The available cell lines (3-10/tissue) were then tested for their expression of the minor H epitopes by measuring their susceptibility to lysis by the minor H Ag-specific, MHC class I-restricted CTL clones as listed in Table I. Tissues from minor H Ag$^-$ donors (lacking either the MHC restriction molecule, the minor H Ag, or both) were never recognized (<5% lysis) by any of the minor H-specific CTL clones (data not shown). This excludes the possibility that any of these CTL clones might cross react with tissue specific epitopes. The results obtained with cell lines from all MHC restriction Ag$^+$, minor H Ag$^+$ donors are compiled in Table III. CTL clones specific for HA-3 (HLA-A1 restricted), HA-4 (HLA-A2 restricted), and H-Y (HLA-A2 and B7 restricted) induced significant lysis (>15%) of all cell types tested. Lysis values of uncultured cells and melanocytes were lower (15–58%) than lysis of the other cell types (34–95%), but these values are comparable to

TABLE III

Cell-mediated lysis of distinct human cell types by MHC class I-restricted, minor H Ag-specific CTL

Cell Type	rIFN-γ[a]	Percent Specific Lysis															
		HA-1		HA-2		HA-3		HA-4		HA-6		HA-7		H-Y[b]			
		Mean[c] (range)	n[d]	Mean (range)	n	Mean (range)	n	Mean (range)	n	Mean (range)	n	Mean (range)	n	Mean (range)	n	Mean (range)	n
T cells	NA[e]	41 (18–51)	4	28 (15–54)	4	24 (16–33)	4	58	1	ND[f]		ND		58 (48–69)	2	ND	
PHA-T cells	–	85 (64–100)	18	92 (83–100)	33	91 (70–100)	17	76 (70–93)	8	51 (35–57)	11	69 (59–85)	12	95 (87–100)	12	89 (75–93)	7
B cells	NA	23 (18–28)	2	ND		ND		50	1	ND		ND		35	1	ND	
EBV-BLCL	–	92 (87–100)	21	93 (91–100)	33	89 (85–100)	23	83 (75–100)	13	56 (39–73)	15	75 (50–90)	10	96 (85–100)	20	91 (73–100)	10
Monocytes	NA	36 (16–51)	3	40 (32–54)	3	26 (18–34)	2	51	1	ND		ND		49 (48–49)	2	ND	
Thymocytes	NA	34 (32–37)	3	25 (16–39)	6	ND		ND		ND		ND		38 (30–46)	5	15 (10–23)	3
Melanocytes[g]	–	0 (–3–7)	5	–1 (–1–1)	5	68 (43–82)	3	0[g] (–2–1)	4	18	1	42	1	57 (27–80)	7	28 (16–34)	3
Fibroblasts	–	1 (0–3)	2	1 (0–1)	2	84	1	ND		28 (24–31)	2	27 (24–28)	3	74 (56–91)	2	55 (45–79)	3
	+	–1 (–2–0)	2	–2 (–3–1)	2	76	1	ND		53 (48–57)	2	48 (41–58)	3	58 (51–64)	2	52 (45–58)	4
Keratinocytes	–	1 (–9–6)	4	–3 (–10–0)	5	59 (37–86)	5	19 (9–29)	2	14 (6–18)	3	14 (9–19)	3	21 (19–23)	2	–2 (–3–2)	2
	+	1 (–6–8)	4	–4 (–10–1)	5	82 (63–103)	5	51 (40–69)	3	60 (49–69)	3	73 (73–74)	2	69 (68–70)	2	34 (27–42)	2
PTEC	–	–3 (–6–0)	2	ND		73 (64–83)	2	69	1	2	1	17 (13–21)	2	70 (60–80)	2	ND	
	+	–2 (–5–1)	2	ND		66 (65–67)	2	95	1	15	1	34 (30–37)	2	72 (64–79)	2	ND	
HUVE	–	0 (–2–2)	3	2 (1–3)	3	92 (91–92)	2	ND		14	1	17	1	87	1	ND	
	+	–3 (–5–2)	3	0 (–4–3)	3	96 (92–97)	2	ND		38 (24–52)	2	75 (56–95)	2	89	1	71	1

[a] Preincubation of target cells with rIFN-γ at concentrations given in *Materials and Methods*.

[b] H-Y recognition was tested with an HLA-A2 restricted (left column) and a HLA-B7 restricted (right) anti-H-Y CTL clone.

[c] Percent specific lysis by the indicated α minor H Ag CTL clone at E:T ratio = 20.

[d] Number of cell lines tested of known minor H Ag + donors; each tested in at least two experiments.

[e] NA, not applicable.

[f] ND, not determined, no tissues of minor H Ag + donors available.

[g] Minor H Ag phenotype of melanocyte donors unknown.

the levels of lysis induced by α-allo-MHC CTL of these cell types (Fig. 1). The absence of measurable HA-4-specific lysis of HLA-A2$^+$ melanocytes (–2–1%, n = 5) does not necessarily rule out an ubiquitous expression of the HA-4 Ag on human tissues. Absence of expression could be due to lack of the HA-4 gene product in the melanocyte donors, whose minor H phenotype, in contrast to the donors of all other cell types tested, was unknown. Given the high probability of any HLA-A2$^+$ melanocyte donor of lacking the HA-4 phenotype (84%), melanocytes from more donors should be typed to enable one to make a significant statement on HA-4 expression on melanocytes. Both HLA-B7 restricted minor H epitopes HA-6 and HA-7 appeared to be recognized on all cell types, although on PTEC and HUVE preincubation with IFN-γ was required to induce significant values of HA-6- and HA-7-specific lysis. This effect may be explained by the slightly lower cytolytic potential of the HA-6- and HA-7-specific CTL clones used (see percent lysis of PHA-T cells and EBV-BLCL, Table III) or by a moderate recognition of HLA-B7 on IFN-γ-untreated PTEC and HUVE as was indicated by relatively low percentages of allo-B7-specific lysis (Fig. 1*C*). In contrast to the five minor H Ag (i.e., HA-3, -4, -6, -7, and H-Y) detectable on all tissues tested, the HLA-A2 restricted Ag HA-1 and HA-2 could be demonstrated only on T and B cells, monocytes, and thymocytes in addition to PHA-T cells and EBV-LCL. The levels of Ag-specific lysis induced by these CTL clones, which were strongly cytolytic for all other tissues, were uniformly below 5% (Table III). This discrepant capacity of minor H-specific CTL to lyse cells of nonhemopoietic origin is also illustrated by the data in Table IV. All distinct hemopoietic and nonhemopoietic cell types obtained from the same HLA-A1, -A2, -B7$^+$ male donor were lysed by the H-Y-, HA-3-, HA-6-, and HA-7-specific CTL. None of the cell types was recognized by the HA-4-specific CTL clone, indicating this individual failed to express the infrequent minor H Ag HA-4. However, only the PHA-T cells and EBV-B cells, but not keratinocytes or fibroblasts of the same donor were lysed by the HA-1- and HA-2-specific CTL (Table IV).

Absence of recognition of minor H Ag HA-1 and HA-2 on nonlymphoid tissues cannot be overcome by cytokines. The absence of HA-1- and HA-2-specific lysis of melanocytes but in particular of fibroblasts, keratinocytes, PTEC, and HUVE cannot be explained by an intrinsic resistance of the targets to CTL-mediated lysis, because CTL specific for allo-class I (Fig. 1) and other minor H Ag (Table III) effectively lysed these cell types. Lymphokine preincubation which maximally up-regulates surface expression of MHC class I and ICAM-1 molecules on fibroblasts (30, 31), keratinocytes (24, 32), PTEC (26), and HUVE (33) significantly enhanced recognition of minor H Ag HA-4, -6, and -7. In contrast, recognition by the strongly cytolytic αHA-1 and HA-2 CTL was not induced under these conditions (Table III). Because lymphokine-induced up-regulation of cell surface expression of HA-1 and HA-2 does not have to correlate with up-regulation of other minor H Ag, we further studied the effect of cytokine activation on HA-1- and HA-2-specific lysis of keratinocytes. rTNF-α (1-1000 ng/ml), rIL1-α (5-500 pg/ml) were tested singly and in combination with rIFN-γ (1-1000 U/ml) for their capacity to sensitize keratinocytes for lysis by αHA-1 and HA-2 CTL. As

TABLE IV

Distinct cell types of the same donor differ in expression of minor H Ag HA-1 and HA-2

Target Cell Type[a]	Percent Specific Lysis by CTL Clone										
	3E7 αHLA-A2	3HA15 αHA-1/A2	5H17 αHA-2/A2	5G30 αHA-4/A2	1R35 αH-Y/A2	Clone 2 αHLA-A1	5HO11 αHA-3/A1	Kor 18 αHLA-B7	Clone 21 αHA-6/B7	Clone 6 αHA-7/B7	5W4 αH-Y/B7
PHA-T cells	80[b]	75	87	−2	81	84	83	66	54	60	78
EBV-B cells	95	89	90	4	97	91	78	80	65	70	83
Keratinocytes	59	−6	−3	2	55	83	100	79	62	45	50
Fibroblasts	80	3	0	−2	75	73	76	92	27	58	70

[a] All target cell types were obtained from the same HLA-A1, -A2, -B7, -B52, -Cw7, -DR4, -DR5 male individual; keratinocytes and fibroblast layers were prepared as described in *Materials and Methods* and were pretreated with 100 U/ml rIFN-γ for 24 h and with 200 U/ml rIFN-γ + 5 ng/ml TNF-α for 48 h, respectively.

[b] Percent specific lysis by the indicated CTL clone at 20:1.

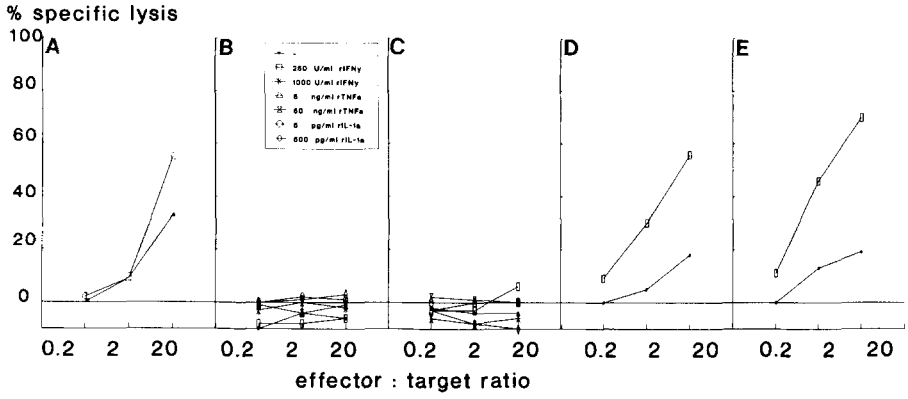

Figure 2. Absence of recognition of HA-1 and HA-2 on keratinocytes cannot be overcome by IFN-γ, TNF-α, or IL-1α. Keratinocytes of a HLA-A2, -B8, -B60, -DR5, and HA-1, -2, -4 expressing male individual were seeded in 96-well plates, allowed to adhere overnight, and preincubated with rIFN-γ, rTNF-α, or rIL-1α at the indicated concentrations for 24 h. ^{51}Cr-labeled layers were tested for lysis by a HLA-A2 (*A*), a HLA-A2/HA-1 (*B*), a HLA-A2/HA-2 (*C*), a HLA-A2/HA-4 (*D*), and a HLA-A2/H-Y (*E*) CTL clones in 4 h CML assay.

shown in Figure 2, *B* and *C*, HA-1- as well as HA-2-specific lysis of keratinocytes of an HA-1- and HA-2-positive donor could not be induced by any of the activation conditions tested.

DISCUSSION

CTL specific for the male-specific Ag H-Y and the minor H Ag HA-3, -4, -6, -7 lysed cultured and freshly isolated cell types of all tested human tissues in a MHC class I-restricted, Ag-specific fashion in vitro. By contrast, CTL clones defining two other minor H Ag HA-1 and HA-2 lysed cell types only of hemopoietic origin, i.e., purified T and B cells, monocytes, thymocytes, EBV B cell lines, PHA-T cells (reported here), and myeloid and lymphocytic leukemic cells (reported elsewhere) (34). The use of mostly cultured cell lines and CML assays as in vitro assay of expression has the disadvantage that no quantitative information is obtained on the levels of minor H epitope expression on the tissues in vivo. Griem et al. (19) in a recent study used an approach which did allow quantification of tissue expression of murine minor H Ag. These investigators observed that MHC class I expression of murine tissues determined the quantity of cell surface minor H epitopes that could be purified from each tissue. This is not surprising given the established view that minor H Ag T cell epitopes represent peptides brought to the cell surface by MHC class I molecules (15–17). To determine the potential of a cell type for presenting a minor H epitope to CTL in vivo, it might therefore be most informative to study tissue-derived cultured cell lines displaying maximal levels of surface MHC class I. These cultured cell lines could mimick cells in vivo during inflammatory reactions, such as activated keratinocytes in GvHD-affected skin (35). We therefore conclude that all human tissues tested may in vivo express the minor H Ag Ha-3, -4, -6, -7, and H-Y, whereas only the hemopoietic cell lineage can express HA-1 and HA-2.

Bone marrow grafting between HLA-identical individuals differing for multiple minor H Ag may induce CTL reactive to minor H Ag with an ubiquitous and to minor H Ag with a more limited tissue expression. CTL recognizing minor H Ag of the former category could in principle contribute both to the so-called "graft vs leukemia" effect (36) as well as to graft vs host pathogenesis (35). By contrast, the destructive effect in the GvHD target organs of CTL recognizing minor H Ag of the latter category, such as HA-1 and HA-2, is limited. Though these CTL could recognize eventual resident lymphoid cells of recipient origin within these target tissues, they could not mediate direct cell mediated destruction of the parenchymal target cells shown to lack these T cell epitopes. However, these CTL could fully contribute to the clearance of residual leukemic cells shown to express these epitopes (34). For clinical BMT the consequences of the existence of minor H Ag with distinct patterns of tissue expression will depend on the relative frequency of the CTL they induce and the existence and number of dominant minor H Ag within each category.

Our study of seven human class I-restricted minor H Ag reveals the existence of Ag with ubiquitous expression and Ag with expression only on hemopoietic cells. However, it is important to realize that the methods used to generate CTL codetermine the tissue distribution of the epitopes studied. The CTL clones used to define the minor H Ag described here were generated by in vitro boosting with PMNC and EBV-LCL of PMNC primed in vivo by HLA-identical BMT. In vitro effector cell selection for minor H specificity was done on the basis of cytotoxicity towards PHA-blasts (4, 20). Therefore, the tissue distribution of the Ag defined by these CTL clones would be expected to at least include (some) lymphoid cell types. This was indeed observed for all minor H antigens tested. The detection of some Ag on cell types not used for

immunization indicates that the generation of these epitopes in the context of class I does not involve a cell type specific process. Various immunization protocols have been applied in the mouse to generate minor H antigen specific CTL. Steinmuller et al. (37) identified a minor H Ag preferentially expressed on epidermal cells by utilizing epidermal cell suspensions for in vivo priming, in vitro boosting, and analysis of CTL function. The "classical" protocol used to identify the congenic strain defined BALB/c and C57BL/10 minor H Ag H-1 and H-3 to H-41 (1, 5) consisted of skin grafting, followed by in vitro boosting and CTL selection with splenic stimulator and target cells. The tissue distribution of most of these classical minor H Ag has not been addressed in direct assays, with the exception of the B cell lineage-specific H-40 (38) and H-4 and H-Y recently demonstrated to be expressed in all murine organs (19). The selective effect of the immunization protocol on the specificity of the detected CTL was most clearly illustrated by the work of Wettstein and Korngold (39, 40). CTL, generated by spleen cell injection of mice across a BALB/c minor barrier, expanded and selected by using spleen cells, did not recognize any of the known ubiquitously expressed BALB/c epitopes, but instead recognized "new" epitopes detected only on lymphoid cells (39, 40). Accordingly, it may be expected that human minor H Ag with other patterns of expression than presented here (e.g., leukemia-specific minor H Ag) will be detected when following distinct immunization and boosting protocols.

Recent studies have provided new insights into not only the molecular nature of the T cell epitopes seen by MHC class I-restricted, minor H Ag-specific CTL, but also into the proteins giving rise to these T cell epitopes. Recently it was shown that CTL defining the murine minor H Ag Mta (41), the socalled "Tum-" Ag (42) and the classical H-Y and H-4 Ag all recognized short peptides, typically 10 to 15 amino acids long, presented by the appropriate MHC class I molecules. The naturally occurring peptides were either purified from MHC molecules or from total extracts of minor H Ag^+ cells (15, 16), or mimicked by synthetic peptides based on the minor H Ag epitope encoding genes which had been obtained by sequencing (17, 43). The β2m membrane protein (13) and a mitochondrial protein MTF (17) were identified as proteins giving rise to classically defined minor H peptides. Recent attempts to create "new" minor H proteins (fulfilling the criteria of inducing MHC restricted CTL responses and skin graft rejection) revealed that introduction of genes encoding retroviral (44, 45), foreign (46) or polymorphic self proteins (47) could result in minor H epitopes.

These new insights may suggest that class I restricted CTL specific for murine minor H Ag recognize MHC class I bound short epitopes, which are uniform in length and hydrophobicity, but can be derived from a diversity of cellular proteins. The limited data available on the heterogeneity of human minor H proteins could very well be consistent with this concept. This model might raise the suggestion that the whole protein content of a cell would be presented to the immune system and therewith represent an enormous number of non-MHC transplantation Ag. Arguments against this suggestion are that quantitatively, only a fraction out of the total peptide pool in a given cell will have sufficient affinity for binding to MHC to result in the minimal number of MHC-peptide complexes at the cell surface required for T cell activation (48). T cell activation will then occur only if the peptide involved is derived from a polymorphic part of a cellular protein and if the MHC-peptide complex is immunogenic for T cells. The latter qualitative requirements will considerably reduce the number of cellular peptides which could qualify as minor H transplantation Ag. However, it seems important to realize that the repertoire of minor H peptides presented by a cells' MHC class I molecules is not static, but is continuously susceptible to environmental influences such as mutation (43), viral infection (44, 45), and lymphokine activation (47).

In conclusion, this report represents a first thorough analysis on the tissue distribution of human MHC class I restricted minor H Ag. Their observed heterogenic expression implies that minor H epitopes might be derived from intracellular proteins with either an ubiquitous or a more specialized cell type-specific function.

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