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Differential Recognition of the Serologically Defined HLA-A2 Antigen by Allogeneic Cytotoxic T Cells

II. Definition of Three HLA-A2 Subtypes by CTLs

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Abstract. A comprehensive analysis of human alloimmune cytotoxic T lymphocytes (CTLs) specific for the HLA-A2 antigen identified 11% of HLA-A2 positive cells as outliers. In total, 11 unrelated serologically indistinguishable, but distinguishable by cell-mediated lympholysis (CML) HLA-A2 positive outlier cells were identified. The outlier cells could be subdivided in two subgroups according to reactivity patterns obtained with CTLs directed against the HLA-A2 antigen of outlier cells and their inhibitory capacity in specific competitive inhibition experiments. Thus, the serologically defined HLA-A2 specificity can be divided into at least three subtypes using CTLs specific for the HLA-A2 antigen. Moreover, CTLs specific for an HLA-A2 subtype could be induced when responder cells expressed a different HLA-A2 subtype antigen. On the basis of several family studies, we conclude that the subtype HLA-A2 antigens are inherited in a codominant way.

Introduction

The major histocompatibility complex (MHC) codes for the highly polymorphic membrane antigens of the HLA series. The serologically defined HLA-A, -B, -C, and -D/DR antigens are thought also to be recognized by cytotoxic T lymphocytes (CTL) in cell-mediated lympholysis (CML). The question whether the HLA antigens themselves are the sole targets or whether other molecules controlled by closely linked loci are the real target antigens in CML remains (Kristensen et al. 1974, Mawas et al. 1975, Schendel et al. 1978, Christiansen et al. 1981). Discrepancies between the serologically defined HLA antigens and the HLA specificity recognized by CTLs have been documented (Goulmy et al. 1976, Bradley et al. 1978, Robinson

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et al 1978, Breuning et al 1982, Kato et al 1982) For the HLA-A2, specificity discrepancies were described in different test systems using virus-restricted CTLs, minor-restricted CTLs and alloimmune CTLs (Biddison et al 1980a, Goulmy et al 1982, Pfeffer and Thorsby 1982, Horai et al 1982) Biddison and co-workers (1980b) demonstrated that a structural difference in the HLA-A2 heavy polypeptide chain, as revealed by isoelectric focusing, might be responsible for the differences observed

In our previous paper (Horai et al 1982), evidence was presented that alloimmune CTLs specific for the HLA-A2 antigen identified five HLA-A2 outlier cells The outlier cells were defined on the basis of three criteria (1) they possessed the serologically defined HLA-A2 specificity based on serologic analyses (including absorption studies with HLA-A2 specific alloantisera), (2) they were lysed only weakly by HLA-A2 specific CTLs [i e, relative cytotoxic response (RCR) well below 60%], (3) they were unable to inhibit specific lysis as cold competitors of HLA-A2 specific CTLs

We reported previously that about 10% of the HLA-A2 seropositive individuals were identified as outlier cells The present paper confirms this finding in an extended population study In total, 11 unrelated HLA-A2 seropositive outlier cells were identified Evidence is presented that the serologically defined HLA-A2 specificity can be divided into at least three subtypes using CTLs raised against the HLA-A2 antigen of outlier cells Family studies show that the "subtype" HLA-A2 antigens segregate normally with the relevant *HLA* haplotypes

Furthermore, evidence is presented that CTLs specific for an HLA-A2 subtype could be induced when the responder cell expressed a different HLA-A2 subtype The implication of the differential recognition by CTLs of the HLA-A2 antigen will be discussed

Materials and Methods

Cell donors Cell donors were selected from our files of HLA-A -B -C and DR typed healthy blood donors Selection was either performed randomly or according to HLA phenotypes to obtain CTLs directed against the serologically defined HLA A2 antigen

CML technique CML was performed according to the European standard technique (Kristensen 1980) In brief, inducer cultures (i e standard mixed lymphocyte cultures) were established for 6 days followed by CML testing (4 h) in four different CTL dilutions against 10^4 ^{51}Cr labeled PHA stimulated (3 days) lymphoblasts

Cold target inhibition The CML inhibition capacity of selected cells was tested by addition of non ^{51}Cr labeled (cold) PHA stimulated cells to the specific combination (e g effector AB_X against ^{51}Cr-labeled target cells B) A fixed number of cold targets (10^5) was added to 10^4 hot targets at different CTLs/hot target cell ratios Control values were established by adding cold competitors autologous to either the responder or the stimulator cells

Calculation of results Cytotoxicity was calculated for each CTL target ratio according to the formula

$$\frac{\text{(Experimental spontaneous) cpm}}{\text{(Maximum spontaneous) cpm}} \times 100 = \text{Percent release}$$

The experimental results from different experiments were normalized to a percent RCR based on the specific response for a given CTL and calculated by the formula

$$\frac{\text{Percent release of experimental target}}{\text{Percent release of specific target}} \times 100 = \text{Percent RCR}$$

In all experiments described the percent RCR was calculated based on the percent release observed at a CTL/target cell ratio of 40 1

Results

Panel study with CTLs raised against the HLA-A2 antigen of "normal" and outlier cells

For an extended panel study, two HLA-A2-specific CTLs (control CTLs 1 and 2) were used as reference HLA-A2-specific CTLs (Table 1) Furthermore, CTLs 3 and 4 were generated against the HLA-A2 antigen of outlier cells to create reagents that recognized the HLA-A2 antigen of outlier cells specifically The HLA phenotypes of the responder and stimulator cells used in this study and the percent lysis of each CTL against autologous and specific targets are listed in Table 1 CTLs 3 and 4 were directed against lymphocytes carrying an outlier HLA-A2 antigen The results of the panel study in which CTLs 1 and 2 were tested on 97 HLA-A2-positive and 32 HLA-A2-negative target cells are presented in Figure 1 The anti-HLA-A2 outlier CTLs (i e, CTLs 3 and 4) were tested on 50 HLA-A2-positive and 13 HLA-A2-negative target cells

A clear-cut bimodal distribution of positive and negative values was observed for CTL 1 and CTL 2, which lysed, as expected, most of the HLA-A2-positive cells strongly (60% RCR or more) In total, 10 outlier cells that were lysed weakly (RCR below 40%) were identified, i e outlier cells designated LV 1, 2, 3, 4, 5, 6, 7, 8, 9, and 11

The anti-HLA-A2 outlier CTLs also showed a bimodal distribution outlier cells designated LV 2, 3, 5, 6, 10 and 11 were strongly lysed (65% RCR or more), while the majority of the "normal" HLA-A2-positive target cells were lysed much less efficiently (RCR below 55%)

Table 1 HLA phenotypes of responder stimulator combinations and percent CML against autologous and specific targets

Effector cells	Responder cells				Stimulator cells				Percent lysis[b]	
									Autologous	Specific[b]
CTL 1	A3	Bw35	Cw4	DR1	A2	B5	Cw2	DR4		
	A11	B5		DR4	A2	B5	Cw4	DR7	4	78
CTL 2	A1	B8		DR1	A1	B8		DR3		
	A1	Bw44		DR6	A2	Bw44	Cw5	DR3	0	67
CTL 3	A28	B27	Cw1	DR1	A2	B27	Cw2	DR6		
	Aw31	B37			A26	B37	Cw6	DR9	0	38
CTL 4	A28	B7	Cw6	DR2	A2	B7		DR6		
	Aw34	Bw58		DR8	A29	Bw58			5	44

[a] Percent lysis at effector to target ratio 40 1
Target cells from responder cell donor (i e autologous) and stimulator cell donor (i e specific)

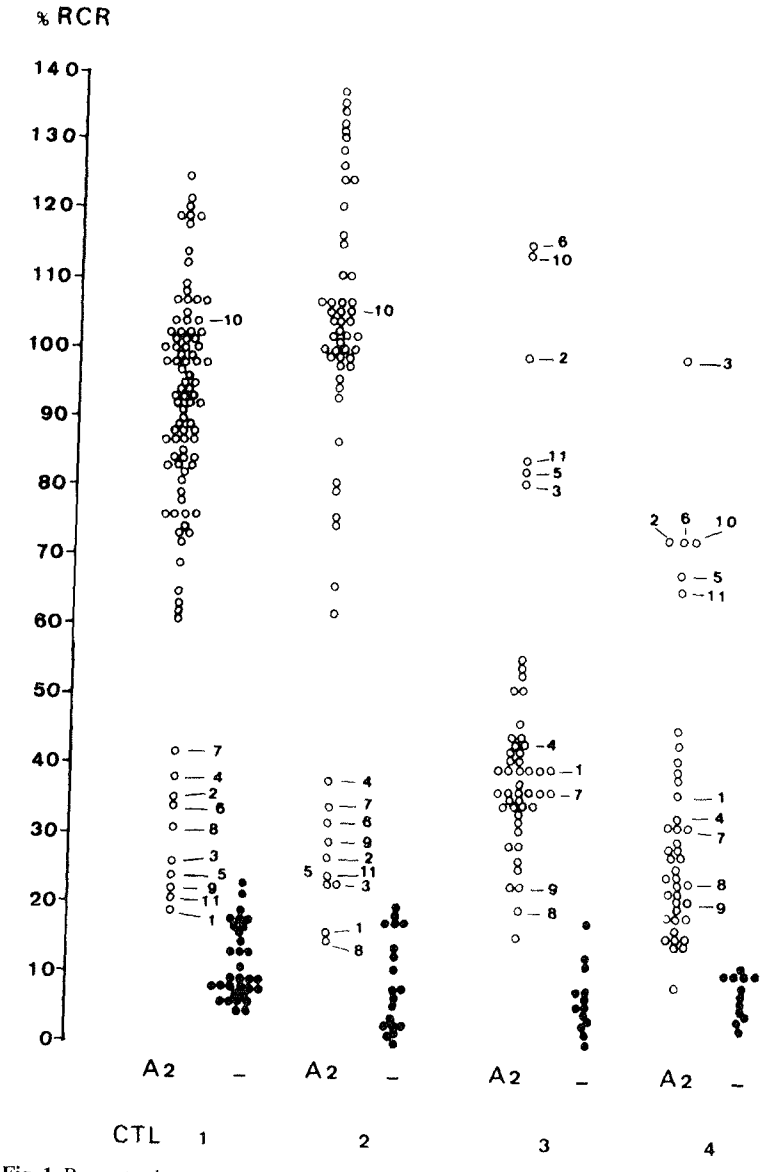

Fig. 1. Percent relative cytotoxic responses of HLA-A2 specific CTLs *Open circles* represent HLA-A2 seropositive target cells *Closed circles* represent HLA-A2 seronegative target cells
The outher HLA-A2 positive target LV1 to LV11 are numbered 1 to 11, respectively The HLA A, -B phenotypes are LV1=1, (−A1, −2, −B8, −w50), LV2=2, (−A2, −26, −B27, −37), LV3=3, (−A2, −29, −B7, −w58), LV4=4, (−A2, −3, −B8, −w35), LV5=5, (−A2, w31, −Bw44, −w50), LV6=6, (−A1, −2, −B8, −w50), LV7=7, (−A1, −2, −B8, −27), LV8=8, (−A2, −3, −Bw35, −w46), LV9=9, (−A2, −w24, −Bw38, −w60), LV10=10, (−A2, −2, −Bw39, −w58), LV11=11, (−A2, −3, −Bw35)

One additional outlier cell was identified (designated LV10) Interestingly, LV10 was HLA-A2 homozygous Since the latter target cell was lysed by all four CTLs, we assumed that LV10 possessed both a "normal" and an outlier HLA-A2 antigen Another important point is that the outlier cells LV1, LV4, LV7, LV8, and LV9 were only weakly lysed by CTLs 3 and 4 By definition these cells were only weakly lysed by the control CTLs 1 and 2 Consequently, the HLA-A2 antigens of these cells must therefore differ in some respect from those recognized by CTLs 1, 2, 3 and 4 (see also Table 2 and 3)

Table 2. Description of HLA-A2 subtypes

		HLA-A2 subtype		
Reagents	Target cells	A2 a "normal" LV10	A2 b LV 2, 3, 5, 6, 10*, 11	A2 c LV 1, 4, 7, 8, 9
CTL 1 and 2		+[†]	−[‡]	−
CTL 3 and 4		−	+	−
		major	minor	minor
	Frequency	89%	6%	5%

* Cell LV10 is serologically HLA-A2 homozygous, but possesses a subtype HLA-A2a and a subtype HLA-A2b antigen
[†] + RCR above 60%, inhibition of specific lysis
[‡] − RCR below 55%, no inhibition of specific lysis

Table 3. Family studies with HLA-A2 specific CTLs

Target cells	HLA-A2 subtype	HLA haplotypes	CTL1	CTL2	CTL3	CTL4
Family Nit						
Mother a/b	b	A2, Bw50, Cw6, DR7/A28, Bw62, Cw3, DR4	38*	19	113	84
Father c/d	a	A2, Bw39, DR5/Aw31, Bw44, DR1	90	93	26	28
Child 1 b/c	a		77	72	16	12
Child 2 a/d	b		23	15	90	70
Family Klo						
Mother a/b		A1, Bw8, DR3/Aw24, Bw35, Cw4, DR3	5	8	20	4
Father c/d	c	A2, Bw50, Cw6, DR7/Aw24, Bw55, Cw3, DR8	20	19	27	32
Child 1 a/c	c		19	12	35	35
Family Cla						
Mother a/b[†]		(A3, B7, DR2/Aw24, Bw35, DR2)	NT[‡]	NT	NT	NT
Father c/d	c	A2, B8, DR2/A3, Bw35, Cw4	33	27	35	30
Child 1 b/c	c		40	37	45	35
Child 2 b/c	c		43	46	48	42
Child 3 a/c	c		47	40	42	44
Child 4 a/d			3	1	10	7
Child 5 b/c	c		42	42	48	37

* Percent RCR at effector to target ratio 40 1
[†] The haplotypes of the mother were deducted from the HLA antigens present in the children
[‡] NT, not tested

Inhibition of specific lysis by "normal" and outlier cold competitor cells

The outlier cells designated LV1–10 and several randomly chosen "normal" HLA-A2 positive individuals were subsequently tested as cold competitors for their specific inhibitory capacity As shown in Figure 2A, five normal HLA-A2 positive target cells were able to inhibit specific lysis of the control CTL 2, including the outlier cell LV10 The other nine outlier cells tested did not block specific lysis, as HLA-A2 negative control target cells did not inhibit cytotoxicity

Inhibition of specific lysis of CTLs 3 and 4 was only seen with cells LV2, LV3, LV5, LV6, and LV10 (Figure 2B and 2C) Thus only target cells that were lysed strongly by CTLs 3 and 4 were capable of acting as cold competitors for anti-outlier CTLs 3 and 4 The outlier cells LV1, LV4, LV7, LV8, LV9 and "normal" HLA-A2 target cells showed no inhibition as did HLA-A2 negative control cells The presumption that LV10 carried both a "normal" and an outlier HLA-A2 antigen was confirmed by cold target inhibition experiments

Description of HLA-A2 subtypes

Based on the reactivity patterns in CML (see Fig 1) and the cold target competition experiments (see Fig 2), the HLA-A2 serologically defined specificity can thus be divided into three subtypes CTL 1 and CTL 2 defined the major HLA-A2 subtype, designated HLA-A2.a (Table 2). CTL 3 and CTL 4 defined a minor HLA-A2 subtype designated HLA-A2.b The third subtype designated HLA-A2.c is essentially defined as non-HLA-A2 a and non-HLA-A2.b The CTLs 1 and 2, on the one hand, and the CTLs 3 and 4, on the other, defined the mutually exclusive HLA-A2 subtypes a and b both in direct CML and cold target competition experiments. The target cell LV10 was a special case, which was shown by CTL analysis to possess both a subtype a and a subtype b HLA-A2 antigen.

Family studies

The families of three individuals that possessed an HLA-A2 subtype antigen were tested with HLA-A2-specific CTLs As shown in Table 3, the three HLA-A2 subtypes, present in the three families, segregated in a codominant way Family Nit demonstrated this well, since both the mother and the father carried an HLA-A2 antigen, one of subtype b and one of subtype a, respectively Child 1 inherited the subtype a antigen from the father, as demonstrated by the strong lysis by CTL 1 and CTL 2 On the other hand, child 2 inherited the subtype b antigen from the mother, as seen by the strong lysis by CTL 3 and 4 and the weak lysis by control CTLs 1 and 2 Although family Klo and Cla, who have the subtype c antigen, possessed only one HLA-A2 antigen, this antigen was inherited normally As shown in Table 3, none of the CTLs was able to lyse the HLA-A2 subtype c antigen strongly (i e , RCR above 60%).

Induction of HLA-A2 subtype specific CTLs

Three individuals representing the three different HLA-A2 subtypes were tested in a checkerboard fashion for induction of A2 subtype specific CTLs As shown in Table 4, HLA-A2 b and -A2 c positive responder cells could be triggered to a cytotoxic

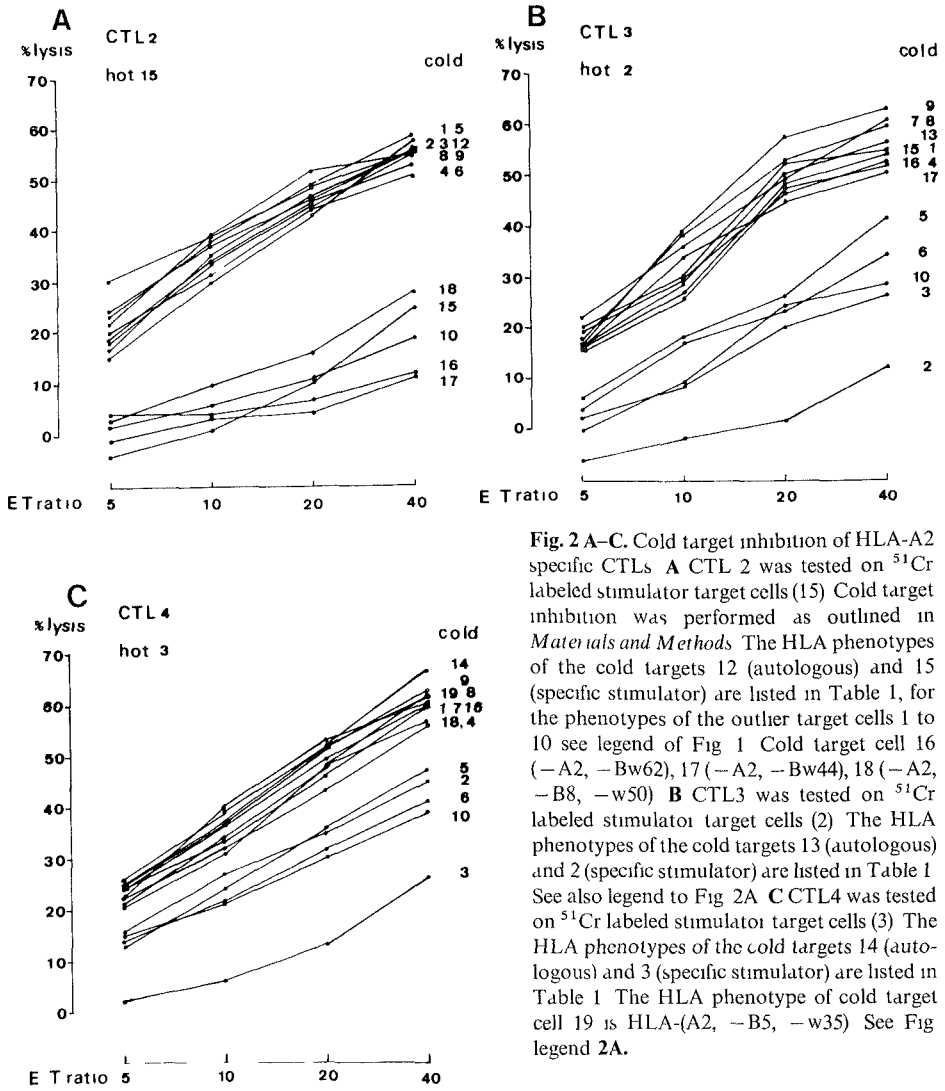

Fig. 2 A–C. Cold target inhibition of HLA-A2 specific CTLs **A** CTL 2 was tested on ^{51}Cr labeled stimulator target cells (15) Cold target inhibition was performed as outlined in *Materials and Methods* The HLA phenotypes of the cold targets 12 (autologous) and 15 (specific stimulator) are listed in Table 1, for the phenotypes of the outlier target cells 1 to 10 see legend of Fig 1 Cold target cell 16 (−A2, −Bw62), 17 (−A2, −Bw44), 18 (−A2, −B8, −w50) **B** CTL3 was tested on ^{51}Cr labeled stimulator target cells (2) The HLA phenotypes of the cold targets 13 (autologous) and 2 (specific stimulator) are listed in Table 1 See also legend to Fig 2A **C** CTL4 was tested on ^{51}Cr labeled stimulator target cells (3) The HLA phenotypes of the cold targets 14 (autologous) and 3 (specific stimulator) are listed in Table 1 The HLA phenotype of cold target cell 19 is HLA-(A2, −B5, −w35) See Fig legend **2A.**

response specific for the HLA-A2.a subtype Likewise, HLA-A2.a and A2.c positive responder cells developed a cytotoxic response specific for the HLA-A2.b subtype. However, no cytotoxicity specific for the HLA-A2 c antigen could be induced using HLA-A2 a- or-A2.b-positive responder cells. These data confirmed the mutually exclusive character of the HLA-A2 subtypes a and b as reflected by the cytotoxic patterns observed with CTLs 1 and 2 (defining subtype a) as opposed to CTLs 3 and 4 (defining subtype b)

Table 4. Induction of HLA-A2 subtype specific CTLs

Stimulator/Target/cells	HLA	A2, 2, B8, Bw50	A1, A2, B8, Bw50 (LV6)	A1, A2, B8, w50 (LV1)
Responder cells	subtype	A2.a	A2.b	A2.c
HLA-A2, 2, B8, w50	A2.a	–	+*	–
HLA-A1, A2, B8, w50 (LV6)	A2.b	+†	–	–
HLA-A1, A2, B8, w50 (LV1)	A2.c	+†	+*	–

+ Subtype specific cytotoxicity induced (between 40–60% CML on specific stimulator target cells).
– No cytotoxicity induced (below 10% CML on specific stimulator target cells).
* Strong lysis on HLA-A2.b positive target cells (LV2, 3, 5, 10 and 11).
† Strong lysis on HLA-A2.a positive target cells.

Discussion

In the present study 97 unrelated HLA-A2 seropositive individuals were tested with four CTLs specific for the HLA-A2 antigen. Eleven unrelated individuals possessing an HLA-A2 outlier antigen were identified. The frequency of outliers thus found was 11%. This confirms the estimated frequency in a smaller panel study published earlier by our group (Horai et al. 1982). The reported frequency is concordant with that recently published by Biddison and co-workers (1982) using virus immune CTLs.

Furthermore, evidence is presented that CTLs specific for the HLA-A2 antigen subdivided the serologically homogeneous HLA-A2 specificity into three subtypes (Table 2). The subdivision is based on the reactivity patterns observed with two pairs of CTLs and the ability of individual cells to inhibit specific lysis of these CTLs competitively. The subtype designated HLA-A2.a is by far the largest group, comprising 89% of the HLA-A2 seropositive individuals. The subtypes designated HLA-A2.b and -A2.c together formed the remaining 11% of the HLA-A2 serologically defined specificity.

Family studies demonstrated that the different HLA-A2 subtypes are inherited codominantly (Table 3). The third subtype (HLA-A2c) was essentially defined by absence of lysis by CTLs 1, 2, 3 and 4 as well as by absence of specific inhibitory capacity.

So far we have not been able to generate HLA-A2 subtype c specific CTLs. Two possible explanations can be formulated. First, A2 subtype c positive cells carry different amino acid substitutions (in comparison with HLA-A2 subtype a and b antigens) such that anti-A2 subtype a and anti-A2 subtype b CTLs are not able to recognize the changed HLA-A2 molecule. The latter modifications are evidently not interfering with the serologic recognition. Second, there is the possibility of a lysis incapability of the HLA-A2 subtype c target cells. This would imply that we are not dealing with another subtype of the HLA-A2 antigen but that we are confronted with a general defect of susceptibility to lysis. An approach to explain the first explanation will be given in an accompanying paper in which the combined biochemical and CTL analysis of all subtypes are described. The second explanation can be ruled out, since HLA-A2.c subtype positive target cells show a normal

susceptibility to lysis by CTLs recognizing other HLA antigens present on those target cells (data not shown)

The three HLA-A2 subtypes have apparently common determinant(s) recognized by HLA-A2-specific alloantisera. CTLs, however, recognized, at least in part, different determinant(s) or epitope(s) on the HLA-A2 molecule First, CTLs 1 and 2 (defining subtype a) and CTLs 3 and 4 (defining subtype b) seem to be mutually exclusive in their cytotoxic pattern. Second, HLA-A2 subtype specific CTLs could be induced when the responder cells expressed a different HLA-A2 subtype than the stimulator cells (Table 4). These data indicate that CTLs recognize different epitopes than antibodies on the HLA-A2 molecules (van Rood et al 1981) A further argument for this latter statement was reported previously Goulmy and co-workers (1982) suggest differential recognition of the HLA-A2 antigen by MHC-restricted H-Y specific antibodies as opposed to MHC-restricted H-Y-specific CTLs Consequently, it will be of interest to study whether alloimmune CTLs, virus-restricted CTLs and minor-restricted CTLs all respond in the same discriminating way towards the HLA-A2 subtypes (J J van der Poel, E Goulmy, J J van Rood, manuscript in preparation) The relevance of the additional polymorphism of the HLA-A2 antigen as recognized by CTLs will have to be investigated for its role in transplantation biology

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References

Biddison, W E , Ward, F E , Shearer, G M , and Shaw, S The self determinants recognized by human virus immune T cells can be distinguished from the serologically defined HLA antigens *J Immunol 124* 548–552, 1980a

Biddison, W E Krangel, M S Strominger, J L Ward, F E , Shearer, G M , and Shaw, S Virus immune cytotoxic T cells recognize structural differences between serologically indistinguishable HLA-A2 molecules *Hum Immunol 3* 225–232, 1980b

Biddison, W F , Kostyu, D D , Strominger, J L , and Krangel, M S Delineation of immunologically and biochemically distinct HLA-A2 antigens *J Immunol 129* 730–734, 1982

Bradley, B A , Goulmy, E , Schreuder, I , and Van Rood, J J Targets for killer T cells in human lymphocyte differentiation Its application to cancer *In* B Serrou and C Rosenfeld (eds) *INSERM SYMPOSIUM No 8*, pp 231–240 Elsevier/North Holland Biochemical Press, Amsterdam, 1978

Breuning, M H , Lucas, C J , Breur, B S , Engelsma, M Y , de Lange, G G , Dekker, A J , Biddison, W E , and Ivanyi, P Subtypes of HLA-B27 detected by cytotoxic T lymphocytes and their role in self recognition *Hum Immunol 5* 259–268, 1982

Christiansen, F T , Kim S J , Silver, D M , and Dupont, B Cytotoxic effector cells against HLA antigens in strong linkage disequilibrium identification of a strong, new CML determinant *Hum Immunol 2* 15–29, 1981

Goulmy, E , Termijtelen, A , Bradley, B A , and Van Rood, J J HLA restriction of non HLA-A, -B, C, and D cell mediated lympholysis (CML) *Tissue Antigens 8* 317–326 1976

Goulmy, E , van Leeuwen, A , Blokland, E , van Rood, J J , and Biddison, W E MHC restricted H-Y specific antibodies and cytotoxic T lymphocytes can recognize different self determinants *J Exp Med 155* 1567–1572, 1982

Horai, S , van der Poel, J J and Goulmy E Differential recognition of the serologically defined HLA-A2 antigen by allogeneic cytotoxic T cells I Population studies *Immunogenetics 16* 135–142, 1982

Kato, S , Ivanyi, P , Lacko, E , Breur, B du Bois, R , and Eijsvoogel, V P Identification of human CML targets HLA-B locus (B12) antigen variants defined by CTLs generated between B locus identical (B12) responder-stimulator pairs *J Immunol 128* 949–955, 1982

Kristensen, T , Grunnet, N , and Kissmeyer-Nielsen, F Cell mediated lympholysis in man Occurrence of unexpected HLA A (LA and Four) irrelevant lympholysis *Tissue Antigens 4* 378–382, 1974

Kristensen, T Human histocompatibility testing by T-cell mediated lympholysis A European standard CML technique Report from the European CML Study Group *Tissue Antigens 16* 335–367, 1980

Mawas, C , Charmot, D , and Sasportes, M Is the LD region of the human MHC a CML target ? *In* F Kissmeyer-Nielsen (ed) *Histocompatibility Testing 1975*, pp 855–857, Munksgaard, Copenhagen, 1975

Pfeffer, P F and Thorsby, E HLA-restricted cytotoxicity against male-specific (H-Y) antigen after acute rejection of an HLA-identical sibling kidney *Transplantation 33* 52–56, 1982

Robinson, M A , Noreen, H J , Amos, B A , and Yunis, E J Target antigens of cell mediated lympholysis discrimination of HLA subtypes by cytotoxic lymphocytes *J Immunol 121* 1486–1490, 1978

Schendel, D J , Wank, R , and Dupont, B Cell mediated lympholysis Examination of HLA genetic fine structure and complementation using cytotoxic lymphocytes *Eur J Immunol 8* 634–640, 1978

van Rood, J J , de Vries R R P , and Bradley, B A Genetics and biology of the HLA system *In* M Dorf (ed) The Role of the Major Histocompatibility Complex in Immunobiology pp 59–113, Garland Publishing Co , New York, 1981

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