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Recognition of Distinct Epitopes on the HLA-A2 Antigen by Cytotoxic T Lymphocytes

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ABSTRACT *Allosimmune CTLs specifically recognizing the HLA A2 3 subtype could be made besides the previously described HLA A2 1 and A2 2 subtype specific CTLs Examination of the fine specificity of 15 different CTLs directed against distinct HLA A2 subtypes demonstrated further complexity of antigenic epitopes present on the A2 molecule First epitopes could be defined which are unique for the HLA A2 1 A2 2 A2 3 and A2 4 subtypes Second epitopes could be defined which are shared between the HLA A2 1 A2 2 and A2 4 subtypes but which are not shared by the A2 3 subtype Analysis of the reactivity patterns of CTLs directed against the HLA A2 2 and A2 4 subtypes indicated that the observed cytotoxic response was dependent on the HLA type of the responder cell Biochemical analysis demonstrated the existence of isoelectric point variation in A2 heavy chains which deviated from the expected pIs for the A2 subtypes as described previously Individuals were identified who possessed A2 heavy chains typical for the A2 3 subtype antigen although the CTL analysis demonstrated the presence of an A2 1 subtype antigen*

ABBREVIATIONS

CML	cell mediated lympholysis	MoAb	monoclonal antibody
CTL	cytotoxic T lymphocyte	MHC	major histocompatibility complex
IEF	isoelectric focusing		
RCR	relative cytotoxic response	pI	isoelectric point

INTRODUCTION

The major histocompatibility complex (MHC) encoded class I antigens are homologous, polymorphic cell surface glycoproteins, which function as recognition structures (e g , in conjunction with foreign antigen) for cytotoxic T lymphocytes (CTL) The classical serology has documented the high polymorphism of the class I antigens, 23 alleles at the HLA A locus, 47 alleles at the HLA B locus, and eight alleles at the HLA C locus have been defined by alloantisera [1] The polymorphism of the class I antigens resides in the heavy polypeptide chain, which is associated with beta 2 microglobulin The recognition of the class I antigens by CTLs as opposed to (allo)antibody demonstrated that different functional epitopes are associated with the class I antigens (1) epitope(s) recognized by alloantibodies or monoclonal antibodies (MoAbs) [2] (2) epitope(s) recog

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nized by alloimmune CTLs [3], and (3) epitope(s) recognized as "self" by MHC restricted virus-specific or minor histocompatibility antigen-specific CTLs [4,5] Polymorphism of recognition sites on the HLA-A2 molecule has been demonstrated by HLA-A2 restricted influenza virus-specific CTLs [6,7], Epstein-Barr virus-specific CTLs [8], H-Y minor antigen specific CTLs [9,10], HLA-A2 specific alloimmune CTLs [11,12], and xenogeneic CTLs [13] In a combined CML and biochemical analysis, four distinct HLA-A2 subtypes could be distinguished [14] Analysis of the HLA-A2 heavy polypeptide chains on isoelectric focusing (IEF) gels, revealed variations in isoelectric point (pI) of the A2 heavy chains Three categories were distinguished as (1) a major HLA-A2 subtype, designated HLA-A2 1, found most frequently, (2) a minor HLA-A2 subtype, designated HLA-A2 2, possessing a more basic pI than the major HLA-A2 1 subtype, and (3) a minor HLA-A2 subtype, designated HLA-A2 3, having a more acidic pI than the major HLA-A2 1 subtype In addition, another minor subtype, designated HLA-A2 4, was found The latter subtype was detected by CML analysis, but was on IEF gels indistinguishable from the major HLA-A2 1 subtype [14]

Previously, only alloimmune CTLs specific for the HLA-A2 1 and -A2 2 subtypes could be made [12] Here we report on the generation of CTLs directed against the HLA-A2 3 and -A2 4 subtypes The reactivity pattern of CTLs directed against distinct HLA-A2 2 and -A2 4 positive stimulator cells were analyzed in detail and the influence of the type of responder cell on the reaction pattern of these CTLs was evaluated In total, 15 CTLs directed against the different HLA-A2 subtypes were tested on our panel of HLA-A2 subtype positive individuals The differences in recognition of epitopes on the A2 molecule by these alloimmune HLA-A2 subtype-specific CTLs will be discussed

Finally, the results of the analysis of A2 heavy chains on IEF gels are presented, demonstrating pI variation in A2 heavy chains which deviates from the expected pI for the distinct A2 subtypes as described previously [14]

MATERIALS AND METHODS

Alloimmune HLA-A2 subtype specific CTLs HLA-A2 subtype-specific CTLs were generated using the responder and stimulator cell combinations listed in Table 1 The standard CML assay has been described in detail previously [11,12] Cytotoxicity (i e , the amount of isotope released from ^{51}Cr-labeled target cells) was determined and calculated according to the method described [12] Normalization to a percent relative cytotoxic response (RCR) was performed as described [12] Experiments were repeated at least twice at five effector-to-target ratios

Competitive inhibition experiments In cold target inhibition experiments 10^4 ^{51}Cr-labeled target cells were mixed with 10^5 unlabeled target cells and cytotoxicity was measured after 4 hr of incubation at different effector-to-labeled target ratios

Biochemical analysis Procedures for cell culture, ^{35}S methionine-labeling, preparation of detergent extracts, immunoprecipitation, neuraminidase digestion, and isoelectric focusing (IEF) have been described in detail previously [14,15] The monomorphic anti-HLA backbone monoclonal, B9 12 1, was used for immunoprecipitation [16] Radioactive products were detected by fluorography on Kodak XAR film Identification of individual HLA-A,-B antigens was performed as described [14,15]

TABLE 1 HLA-A, B phenotypes of responder and stimulator cell combinations

CTLs	Responder cell	Stimulator cell	Specificity	Percent lysis[a]
CTL 1	A2 1 A24 B38 Bw60	A2 3 A24 B38 Bw60	A2 3	60
CTL 2	A28 A31 B27 B37	A2 2 A26 B27 B37	A2 2	38
CTL 3	A28 Aw34 B7 Bw58	A2 2 A29 B7 Bw58	A2 2	44
CTL 4	A1 A29 B7 Bw58	A2 2 A29 B7 Bw58	A2 2	53
CTL 5	A1 A3 B8 B35	A2 2 A3 B35	A2 2	70
CTL 6	A2 3 A3 B35 Bw46	A2 2 A3 B35	A2 2	64
CTL 7	A2 4 A3 B8 B35	A2 2 A3 B35	A2 2	72
CTL 8	A2 4 A1 B8 Bw50	A2 2 A1 B8 Bw50	A2 2	63
CTL 9	A2 1 A3 B7 Bw57	A2 2 A29 B7 Bw58	A2 2	40
CTL 10	A2 1 A29 B44 Bw50	A2 2 A31 B44 Bw50	A2 2	45
CTL 11	A2 1 A29 B44 Bw50	A2 2 A2 1 B44 Bw50	A2 2	60
CTL 12	A1 A24 B8 Bw50	A2 4 A1 B8 Bw50	A2 4	49
CTL 13	A1 B8 B35	A2 4 A24 B8 B35	A2 4	80
CTL 14	A1 A30 B8 B27	A2 4 A1 B8 B27	A2 4	65
CTL 15	A2 1 A24 B8 B35	A2 4 A24 B8 B35	A2 4	47

[a]Percent lysis at effector to target ratio of 40 1 on targets of the stimulator cell donor

RESULTS

Generation of HLA-A2.3 Subtype-Specific CTLs

Since the A2 3 subtype could only be identified by a combination of CML and biochemical analyses [14], we attempted to generate CTLs which specifically recognized the A2 3 subtype The HLA phenotypes of the responder and stimulator combinations used for the generation of HLA-A2 subtype specific CTLs are shown in Table 1 These CTLs were tested on our panel of A2 subtype positive individuals The HLA phenotypes and the HLA A2 subtypes of these donors are listed in Table 2 The reactivity pattern of CTL 1, directed against an

TABLE 2 HLA-A, B phenotypes of the A2 subtype target cell panel

Individual	HLA A	HLA B	Subtype CML	pI
1	A2 A26	B27 B37	A2 2	A2 2
2	A2 A29	B7 Bw58	A2 2	A2 2
3	A2 A31	B44 Bw50	A2 2	A2 2
4[a]	A2 A1	B8 Bw50	A2 2	A2 2
5[a]	A2 A2	B44 Bw50	A2 1 + A2 2	A2 1 + A2 2
6	A2 A2	B39 Bw58	A2 1 + A2 2	A2 1 + A2 2
7	A2 A3	B35	A2 2	A2 2
8	A2 A3	B35 Bw46	A2 3	A2 3
9	A2 A24	B38 Bw60	A2 3	A2 3
10	A2	Bw53 Bw60	A2 3	A2 3
11	A2 A1	B8 Bw50	A2 4	A2 1
12	A2 A24	B8 B35	A2 4	A2 1
13	A2 A1	B8 B27	A2 4	A2 1
14	A2 A31	B44 B51	A2 4	A2 1
15	A2 A3	B16 B36	A2 1	A2 3
16	A2 A3	B5 B7	A2 1	A2 3

[a]Individuals 4 and 5 are family members child and mother respectively

A2 3 subtype positive stimulator, is shown in Figure 1A The A2 3 subtype positive individuals are preferentially recognized by CTL 1, while low reactivity is observed on the A2 1, A2 2, and A2 4 subtype positive individuals

Previously, we have shown that individuals carrying the relevant A2 subtype were able to inhibit the CML activity of A2 1 and A2 2 subtype specific CTLs [12] Competitive inhibition experiments were performed to analyze the ability of the different A2 subtypes to inhibit the CML of the A2 3 subtype specific CTL 1 As shown in Figure 1B, lysis of CTL 1 could be inhibited efficiently by cold competitors of the specific stimulator (individual 9) and the A2 3 subtype positive individuals 8 and 10 No inhibition was observed when cold targets of A1 2, A2 2, or A2 4 subtype positive individuals were used

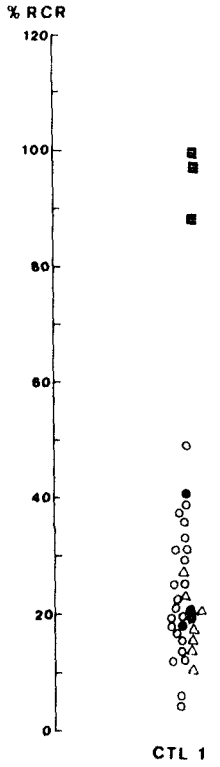
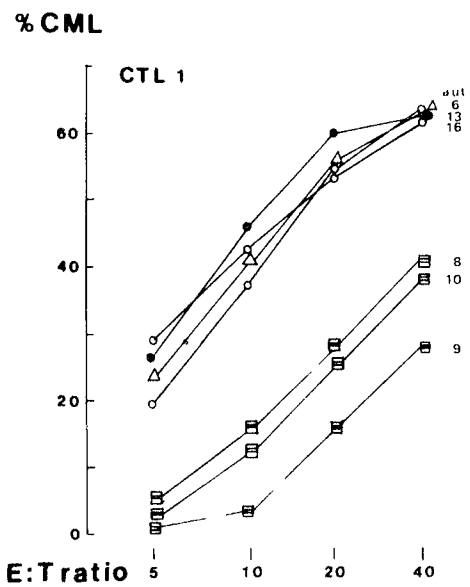


FIGURE 1(A) Reaction pattern of the HLA-A2 3 subtype specific CTL 1 The results are represented as %RCR (see Methods) CTL 1 is described in Table 1 Symbols ○ HLA A2 1 subtype positive individuals, △ HLA A2 2 subtype positive individuals, ⊟ HLA A2 3 subtype positive individuals, ● HLA A2 4 subtype positive individuals (B) Competitive inhibition of the A2 3 subtype-specific CTL 1 Cold target inhibition experiments were performed as outlined in Methods The results are presented as %CML The HLA phenotypes of cold autologous responder targets, indicated by aut , and the cold targets 6, 8–10, 13, and 16 are listed in Table 2 CTL 1 was tested on ^{51}Cr labeled stimulator cells (individual 9) Without addition of any cold targets, 66% CML was observed For explanation of the symbols, see legend of 1(A)



Analysis of A2 2 Subtype-Specific CTLs

Ten different responder–stimulator combinations were used to generate HLA A2 2 subtype specific CTLs Responder cells which were A2 negative as well as A2 1, A2 3, and A2 4 positive were used to test to what extent, if any the recognition of the A2 subtypes was influenced (see Table 1) Identity for the other HLA antigens, present on the responder and stimulator cells was considered to avoid additional CTL activity Although, perfect HLA identity was not always obtained, no interference was observed with respect to the recognition of the A2 2 subtype antigen

The reactivity patterns of the 10 CTLs are summarized in Table 3 All CTLs showed strong reactivity against the A2 2 subtype for which they were primed The overall concordance of the ten CTLs, as far as the recognition of the A2 2 subtype is concerned, is apparent from these data CTLs specifically recognizing the A2 2 subtype could be made as well with A2 seronegative responders (CTL 2–5) as with A2 seropositive responder cells (CTL 6–11), irrespective of their A2 subtype This is demonstrated by the reactivity patterns of CTL 9 10, 11 (A2 1 positive responders), CTL 7 and 8 (A2 4 positive responders), and CTL 6 (A2 3 positive responder) Apparently the A2 1, A2 3, and A2 4 subtype antigens are sufficiently different from the A2 2 subtype antigen to induce A2 2 subtype specific CTLs

The analysis of the A2 2 subtype positive individuals, using ten different CTLs, demonstrated no further heterogeneity within the A2 2 subtype However sev

TABLE 3 Reactivity patterns of HLA-A2 2 subtype-specific CTLs on the A2 subtypes

	A2 1	A2 2	A2 3	A2 4
CTL 2 (A28)[a]	36(10/10 55)[b]	96(11/83 100)	20(5/14 24)	34(10/25 45)
CTL 3 (A28)	27(11/7 54)	80(12/66 100)	16(4/13 19)	31(8/22 37)
CTL 4 (A2 ,A28)	40(20/5 70)	96(7/88 106)	30(9/20 37)	50(12/34 63)
CTL 5 (A2 A28)	44(12/24 57)	99(10/84 114)	32(11/23 44)	57(16/39 76)
CTL 6 (A2 3)	43(13/18 59)	94(8/83 106)	16(15/0 31)	56(14/37 69)
CTL 7 (A2 4)	20(14/5 54)	97(14/80 115)	24(11/12 34)	24(20/0 46)
CTL 8 (A2 4)	24(11/8 41)	91(10/79 100)	14(8/8 23)	15(15/0 30)
CTL 9 (A2 1)	16(12/0 38)	85(11/69 100)	13(5/8 18)	27(11/16 41)
CTL10 (A2 1)	24(13/0 35)	93(15/68 112)	15(4/11 19)	29(17/10 45)
CTL11 (A2 1)	12(7/0 28)	93(10/80 106)	17(12/6 30)	29(14/14 42)

[a]For description of CTLs see Table 1 Between brackets the presence or absence of the HLA A28 antigen or the distinct HLA A2 subtype antigens on the responder cell is indicated Reactivity on HLA A2 negative control cells ranged from 0 to 25% RCR

[b]Mean %RCR of the individuals tested with a given A2 subtype Between brackets the standard deviation and the range in %RCR is indicated

eral CTLs showed cross reactive lysis on the HLA-A2 1 and A2 4 subtypes the cross-reactivity appeared to be dependent on the type of responder cell used Namely, CTL 4, 5, and 6 showed cross-reactive lysis on the A2 1 subtype (>40% mean RCR) and the A2 4 subtype (>50% mean RCR) CTL 4 and 5 were generated using A2 seronegative responder cells and therefore one might expect some cross-reactivity CTL 2 and 3 showed less cross-reactivity on the A2 1 and A2 4 subtypes The responder cells of the latter CTLs were also A2 seronegative, but carried the HLA A28 antigen Thus, when using an A2 sero negative responder cell to generate an A2 2 subtype specific CTL, the presence or absence of the A28 antigen is presumably the difference which accounts for the cross-reactivity on other A2 subtypes Since the A28 antigen is highly homologous to the A2 antigen, this might be the reason why less cross-reactivity is observed

CTLs made with A2 subtype positive responder cells (CTL 6–11) showed, as expected, low cross-reactivity on A2 positive individuals except for CTL 6, for which an A2 3 subtype positive responder was used The latter CTL showed cross-reactivity on the A2 1 and the A2 4 subtype positive target cells In this respect, the A2 3 positive responder is comparable to an A2 and A28 negative responder (i e , CTL 4 and 5) Based on these observations, we conclude that the reaction pattern of A2 2 subtype specific CTLs is influenced by the type of responder cells used Furthermore, these data suggest that the epitopes on the A2 3 antigen, as seen by CTLs, are most distinct from the A2 2 antigen, whereas the epitopes on the A2 1, A2 2, and A2 4 subtype antigens seem to be related

Analysis of CTLs Directed Against A2 4 Subtype Positive Stimulators

The A2 4 subtype was essentially defined by a lack of recognition by A2 1, A2 2, and A2 3 subtype specific CTLs [12,14] Therefore, attempts were made to positively select for the A2 4 subtype CTLs were generated against A2 4 positive stimulator cells Four CTLs could be generated against three of the A2 4 subtype positive individuals (See Table 1) The reactivity patterns of these CTLs, as shown

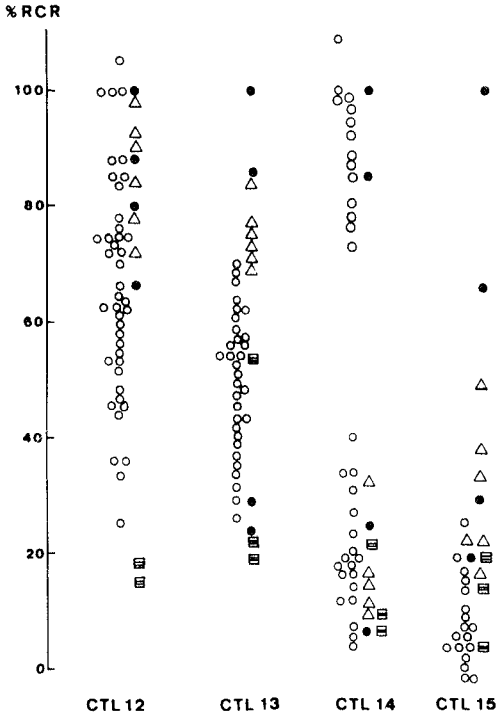


FIGURE 2 Reaction patterns of CTLs directed against the A2 4 subtype antigen The results are presented as %RCR (see Methods) CTL 12–15 are described in Table 1 For explanation of the symbols see Legend to Figure 1(A)

in Figure 2, were heterogeneous and complex When the reactivity within the A2 4 subgroup was analyzed, further heterogeneity was observed (Table 4) CTL 12 reacted with all four A2 4 subtype positive individuals CTL 13 reacted with the specific stimulator (individual 12) and individual 14, while the other A2 4 subtype positive individuals 11 and 13 were hardly recognized CTL 14 reacted with the specific stimulator (individual 13) and individual 12 Again, two of the A2 4 subtype positives (individuals 11 and 14) were hardly recognized CTL 15 which was also made against individual 12, showed the same reaction pattern as CTL 13 Thus, using these CTLs, the A2 4 subgroup could be subdivided according to the different types of reaction patterns observed Comparison of the reaction patterns of CTL 12 to 15 against the A2 4 subtype shows that the four A2 4 subtype positive individuals all differ from each other (Table 4)

CTL 12 and 13 showed considerable reactivity against most A2 1 and A2 2

TABLE 4 Reactivity patterns of CTLs directed against A2 4 subtype positive individuals within the A2 4 subgroup

Individual	CTL 12	CTL 13	CTL 14	CTL 15
11	+[a]	−[b]	−	−
12	+	+	+	+
13	+	−	+	−
14	+	+	−	+

[a] + denotes recognition by the indicated CTL

[b] − denotes absence of recognition by the indicated CTL

subtype positive individuals The reactivity pattern of CTL 14 differed from the former two CTLs, in that the reactivity on the A2 2 subtype was virtually absent The A2 1 subtype was only partially recognized by CTL 14, while CTL 12 and 13 showed variable reactivity on the A2 1 subtype All three CTLs had in common that the A2 3 subtype was hardly recognized

A responder cell effect is also observed in the generation of CTLs against the A2 4 subtype The same stimulator cells have been used for CTL 13 as well as CTL 15, i e , individual 12 (see Tables 1 and 2) The reactivity on the A2 1 subtype by CTL 15 is almost absent, as expected, and the reactivity on the A2 2 subtype is reduced The reactivity on one A2 3 subtype positive individual by CTL 13 might be explained by the fact that this CTL also recognized the HLA A24 antigen (see Table 1) The A2 3 positive individual 9 carried the A24 antigen and was therefore recognized by CTL 13 The A24 reactivity was absent in CTL 15, because the responder cell was itself A24 positive

The analysis of the reaction patterns of the CTLs directed against the A2 4 subtype suggests that the epitopes recognized by CTLs on the A2 1, A2 2, and A2 4 molecules are more related to each other than to the epitopes present on the A2 3 subtype molecule

Identification of Exceptional A2 Variants by Biochemical Analysis

Analysis of the A2 heavy polypeptide chains was performed on one dimensional IEF gels (see Materials and Methods) to directly compare the pIs of the heavy chains of the different A2 subtype positive individuals The A2 2, A2 3, and A2 4 subtype positive individuals listed in Table 2 possessed A2 heavy chains with the pIs expected for these A2 subtypes ([14], data not shown) However, individuals were identified where a variation of the pI of the A2 molecules was observed which deviated from the expected pI As shown in Figure 3, these individuals possessed A2 heavy chains with pIs identical to that of the A2 3 subtype, although these individuals were not recognized by the A2 3 subtype-specific CTL 1 Since the CML analysis indicated an A2 1 subtype for individuals 15 and 16, these individuals represent new types of A2 variants

DISCUSSION

The reactivity patterns of 15 CTLs generated against individuals carrying distinct A2 subtype antigens have been analyzed The results show that A2 3 subtype-specific CTLs could be made in addition to the previously described A2 1 and A2 2 subtype-specific CTLs These CTLs showed preferential recognition of the

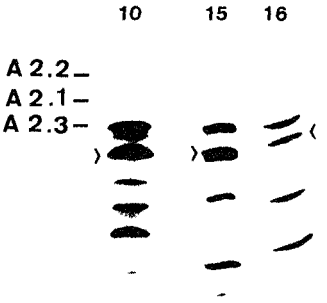


FIGURE 3 One dimensional IEF gel identifying two exceptional A2 variants HLA A B antigens were isolated as described in Methods The numbers indicated correspond to the A2 subtype panel listed in Table 2 The basic end of the gel is at the top The position of beta 2 microglobulin is indicated by an arrowhead The position of the A2 1 A2 2 and A2 3 heavy chains are indicated The A2 3 subtype positive individual 10 is shown as a control

relevant A2 subtype and the CML activity could be specifically inhibited by cold competitors carrying the relevant A2 subtype antigen ([12,14] and Figure 1)

Analysis of ten A2 2 subtype specific CTLs demonstrated that these CTLs clearly recognized the A2 2 subtype positive individuals (Table 3) The A2 2 subtype-specific CTLs could be generated using A2 seronegative as well as A2 seropositive responder cells It was found that the type of responder cell influenced the reactivity pattern on the A2 subtype panel Cross reactivity was observed on the A2 1 and A2 4 subtype when A2 and A28 seronegative responder cells were used This cross-reactivity was reduced when the responder cell carried an A28, A2 1, or A2 4 subtype antigen (Table 3)

The analysis of the reaction patterns of the A2 specific CTLs suggests that several distinct epitopes can be defined on the A2 molecule Some CTLs recognize epitopes which seem to be unique for a certain A2 subtype In this way the A2 1, A2 2, A2 3, and A2 4 subtypes could be defined ([12 14], Figure 1) The cross-reactivity exhibited by several CTLs (Table 3, Figure 2) suggests the existence of related or partially identical epitopes, which are present on the A2 1, A2 2, and A2 4 subtype molecules Since the A2 subtype-specific CTLs do not recognize the A2 3 subtype (with the exception of CTL 1), the latter subtype seems to be most distinct

The present study shows, that by using bulk cultures, complexity of epitopes on the A2 molecule can be demonstrated For precise definition of the different epitopes present on each A2 subtype antigen, the analysis has to be extended to the clonal level

As mentioned earlier the definition of the A2 4 subtype was based on lack of recognition by A2 1, A2 2 subtype specific CTLs [12,14] and A2 3 subtype-specific CTLs (Figure 1) Brenner et al independently confirmed that A2 4 subtype positive individuals were not lyzed by A2 1 specific CTLs, using an A2 1 subtype-specific CTL clone [17] Analysis of CTLs, which could be generated against the A2 4 subtype positive individuals, showed that the A2 4 subgroup was heterogeneous (Figure 2) Different types of reaction patterns were observed within the A2 4 subgroup (Table 5) In fact, all four A2 4 positive individuals differ from each other with respect to the epitopes recognized by the anti A2 4 CTLs So far, no CTLs could be made which selectively recognized all the A2 4 subtype positive individuals Moreover, extensive cross reactivity was found on A2 1 and A2 2 subtype positive individuals (Figure 2) Since the A2 4 subtype

turned out to be a heterogeneous collection of A2 variants rather than a homogeneous group as the other A2 subtypes, the designation A2 unclassified (A2u) is preferable to the designation A2 4

The results of the CML and biochemical analyses demonstrate that further heterogeneity may be defined, for instance within the A2 1 subtype First, CTL 14 discriminated between HLA-A2 1 subtype positive individuals (Figure 2) Second, biochemical analysis identified two individuals expressing A2 heavy chains identical in pI to the A2 3 subtype (Figure 3), although absence of recognition was observed with the A2 3 subtype specific CTL 1 It is reasonable to assume that in the case of individual 15 and 16 the amino acid substitution responsible for the pI shift is located at a position in the A2 molecule which leaves the A2 1 epitope(s) as defined by CTL recognition unchanged Individuals 15 and 16 are in fact comparable to one of the A2 mutants (8 21 1) obtained by immunoselection [18] The 8 21 1 A2 mutant also possessed a more acidic A2 heavy chain than the parental T5-1 B cell line used for immunoselection However, an A2 specific alloimmune CTL line still recognized the A2 mutant [19], as did Epstein-Barr virus specific, HLA-A2 restricted CTLs [20]

Primary amino acid sequence data of an A2 2 subtype antigen (M7) and an A2 3 subtype antigen (DK1) have localized the amino acid substitutions responsible for the shift in pIs of these subtype antigens [21,22] Based on these sequences it has been proposed that the region of the A2 molecule around amino acid 150 is important for CTL recognition [22] Information on the substitution(s) in the spontaneously occurring A2 variant molecule of individuals 15 and 16 may further strengthen this hypothesis The results of the analyses presented here predict that differences in amino acid sequence may be found within the A2u subgroup and between the A2u subgroup and the other A2 subtypes

Analysis of the A2 subtypes with HLA-A2 restricted minor histocompatibility antigen-specific CTLs and virus-specific CTLs demonstrated that these CTLs use, in general, the same epitope(s) for recognition as alloimmune CTLs [6–10] Interestingly, the minor Histocompatibility antigen-specific CTLs demonstrated that some individuals in the A2u subgroup had retained the epitope(s) necessary for associative recognition, while other individuals in the A2u subgroup, as expected, had lost such epitope(s) [10,23] Similarly, virus-specific, A2 2 restricted CTLs detect heterogeneity in the A2 2 subgroup [24], although alloimmune CTLs, as reported here, demonstrated no heterogeneity within the A2 2 subgroup These data illustrate that each specific way of analyzing the A2 subtype antigens presents a different image of variability Combination of analytical methods gives information concerning variability within the A2 antigens which would otherwise remain undetected

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