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Recognition by Class II Alloreactive T Cells of Processed Determinants from Human Serum Proteins

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Alloreactive T cells recognize a complex composed of an allogeneic major histocompatibility complex (MHC) molecule and a peptide derived from the processing of nonpolymorphic proteins. A sizable fraction of MHC class II alloreactive T cells is shown to recognize peptides derived from constitutive processing of human serum proteins. One such epitope is a fragment of human serum albumin. This epitope bound selectively to the human class II molecule DRw11 and was constitutively present on antigen-presenting cells in vivo. These data indicate that, in the case of MHC class II, peptides involved in allorecognition may originate from exogenous proteins.

IN ORDER TO TRIGGER HELPER T cells, exogenous protein antigens must be taken up by antigen-presenting cells (APC) and processed into peptides that bind to MHC class II molecules. Since APC cannot distinguish between self and foreign proteins, it is likely that self peptides are continuously generated and presented in association with class II molecules. Peptides have been found to constitutively occupy the antigen-binding groove of class II molecules (*1*), but the origin and nature of these peptides and their functions in alloreactivity (*2*) have not been established.

Human serum (HS) is the most abundant source of soluble proteins in vivo. If HS proteins are constitutively processed and presented by APC, a fraction of alloreactive T cells should be specific for peptides derived from HS presented by allogeneic class II molecules. These T cells should recognize allogeneic Epstein Barr virus–transformed B cells (EBV-B) grown in HS, but may not recognize the same cells grown in serum from a different source such as fetal calf serum (FCS), that provides a different set of peptides. Nineteen primary mixed lymphocyte reactions (MLRs) were set up with allogeneic peripheral blood mononuclear cells (PBMC) as stimulators, and 1489 $CD4^+$ alloreactive T cell clones were isolated. The clones were tested for their capacity to proliferate in response to allogeneic EBV-B cells that had been exposed to either HS or to FCS. Of these clones, 93.4% did not discriminate between HS-treated and FCS-treated B cells. However, 6.6% of the clones (from 2.2 to 10.1% in different MLRs) proliferated only in response to EBV-B cells that had been exposed to HS (Table 1). None of the alloreactive clones proliferated only in response to FCS-treated B cells.

The antigen-specificity and restriction of the HS-specific alloreactive clones were characterized. (i) All clones proliferated only in the presence of allogeneic EBV-B cells from the original allogeneic stimulator used in the primary MLR and 1 to 5% HS. (ii) The allodeterminants recognized were present on APC in vivo, since all T cell clones proliferated in response to allogeneic PBMC from the original stimulator. (iii) The stimulatory activity of HS was stable to heating for 30 min at 60°C and represented a protein because it was destroyed by treatment of HS with Pronase. (iv) The HS proteins recognized were not polymorphic, since serum autologous to the T cell clones was as effective as serum taken from other donors. (v) The clones displayed expected allospecificities toward MHC class II DR or DQ allelic products, as shown by inhibition of alloreactivity with monoclonal antibodies to DR or DQ molecules and by the use of homozygous EBV-B cells as APC. (vi) Different proteins were recognized by different alloreactive T cells. The proteins were separated by size and identified as distinct peaks by gel filtration chromatography of HS (*3*). These results indicate that approximately 7% of class II alloreactive T cells recognize determinants of proteins from HS presented by allogeneic class II molecules.

We tested the alloreactive clones for recognition of human serum albumin (HSA), a protein of known sequence that is available in sufficient amounts to allow epitope mapping. Two clones of the 98 tested recognized HSA (Table 2). These clones were isolated from two independent MLR's and were shown to be specific for HSA and restricted to DRw11. They proliferated in response to both purified and recombinant HSA (*4*), only in the presence of allogeneic DRw11 EBV-B cells or DRw11 L cell transfectants, but not in the presence of autologous EBV-B cells. In addition, these clones proliferated in response to DRw11 PBMC from the original stimulator that had been isolated and cultured in FCS, indicating that the HSA-derived allodeterminant was present on APC in vivo. HSA, like conventional antigens, had to be processed for at least 60 min at 37°C, and processing was inhibited by leupeptin or chloroquine (Table 2).

The HSA epitope recognized by clone AK42 was identified by proteolytic digestion of HSA that had been cleaved with cyanogen bromide (*5*). The epitope corre-

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sponded to residues 444 to 456 (Table 2). The peptide (TPTLVEVSRNLGK) (*6*) differed from the corresponding peptide of bovine serum albumin (which is not recognized by clone AK42) only by substitution of asparagine for serine at position 453. The other DRw11 alloreactive clone (NG74) did not recognize HSA (444–456), but it was stimulated by HSA from two different sources (purified from HS and recombinant HSA expressed in H yeast). This clone may recognize a different HSA epitope. These results indicated that constitutive processing of HSA in vivo generates at least one epitope that binds to DRw11 and can be recognized by alloreactive T cells.

HSA (444–456) is the first nonpolymorphic self-epitope to be mapped. We therefore compared the synthetic peptide with the naturally processed epitope. DRw11 EBV-B cells incubated with HSA or peptide had an identical stimulatory capacity that was comparable to that of freshly isolated PBMC (Fig. 1A). Although a precise comparison of the potency of the natural and synthetic peptide was difficult, because PBMC differ from EBV-B cells in the fraction and amount of DR expression and in their costimulatory capacity, these results suggest that the synthetic determinant has approximately the same activity as the naturally processed determinant. Furthermore, this allodeterminant appears to be stable because freshly isolated PBMC that had been cultured for 4 days in FCS were still fully stimulatory for clone AK42, an indication that this epitope remained associated with DRw11 for a long period of time (7).

In order to be triggered, clone AK42 required high concentrations of both HSA and peptide (Fig. 1B). These concentrations are 100 to 1000 times higher than those required for triggering tetanus toxin (tt)-specific T cells isolated from immunized individuals (*8*). To decide whether the requirement for high concentrations of HSA was due to low affinity of the T cell for the complex of DRw11 with the peptide or to

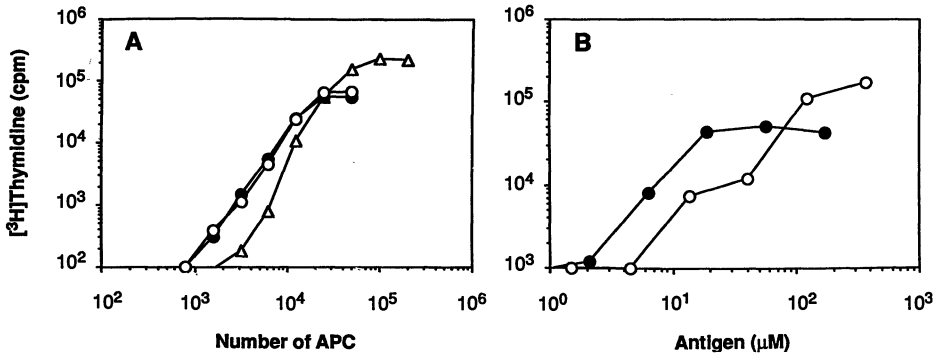

Fig. 1. Proliferation of clone AK42 in response to different APC's (**A**) or various concentrations of HSA or HSA 444 to 456 (**B**). AK42 was cultured in FCS medium with different numbers of irradiated allogeneic DRw11$^+$ cells: PBMC (△) or EBV-B cells treated either with HSA (50 μM) (○) or with HSA(444–456) (30 μM) (●) (A). Clone AK42 was cultured in RPMI-FCS with DRw11 EBV-B cells in the presence of various concentrations of purified HSA (○) or HSA(444–456) (●) (B). The data are representative of at least four different experiments.

Table 1. Recognition of HS determinants by alloreactive CD4$^+$ T cell clones. Alloreactive CD4$^+$ T cell clones were tested for their capacity to proliferate in response to EBV-B cells from the original allogeneic stimulator that were either treated with HS or cultured in FCS. The response of CD4$^+$ T cell clones to autologous EBV-B cells was monitored as a control. Clones were scored as positive when the stimulation index (ratio of the [^{3}H]thymidine incorporation in response to allogeneic EBV-B cells to the [^{3}H]thymidine incorporation in response to autologous EBV-B cells) was >5. The reactivity of the 98 HS-specific clones was confirmed in three separate experiments. To obtain alloreactive clones, 10^6 responder PBMC were cultured with 10^6 irradiated (3000 rad) allogeneic PBMC, in RPMI-1640 medium supplemented with L-glutamine (2 mM), nonessential amino acids (1%), sodium pyruvate (1%), and kanamycin (50 μg/ml) (complete RPMI) containing HS (5%) (Swiss Red Cross, Bern). Human recombinant IL-2 (50 U/ml) (Roche, Nutley, New Jersey) was added on day 6. After 3 to 5 days the activated cells were stained with fluorescein isothiocyanate-labeled monoclonal antibody to CD4 (Becton Dickinson, Mountain View, California), and the CD4$^+$ T cell blasts were sorted with a FACS 440 and cloned as described (*14*). To obtain allogeneic stimulators, EBV-B cells were produced and maintained in complete RPMI supplemented with FCS (10%). For T cell stimulation, the EBV-B cells were either left untreated in medium containing FCS or incubated overnight with HS (5%). In some experiments DRw11 L cell transfectants or fresh PBMC were used. To assay proliferation, cultures were prepared in RPMI-FCS (200 μl) in 96-well flat-bottom microplates containing 4×10^4 T cells and 2×10^4 irradiated (6000 rad) EBV-B cells or 10^5 irradiated (3000 rad) PBMC or 2×10^4 mitomycin C–treated L cells. After 48 hours, 1 μCi of [^{3}H]thymidine was added (Amersham, specific activity 5 Ci/mM) and the radioactivity incorporated was measured after 16 hours.

Stimulator (DR)	MLR (*N*)	Clones (*N*)	T cell clones (*N*) responding to		
			HS-treated and untreated B	HS-treated B only	Untreated B only
A (w13)	10	734	665 (90.6%)	69 (9.4%)	0
G (w11)	1	270	264 (97.8%)	6 (2.2%)	0
K (w11)	1	99	90 (89.9%)	9 (10.1%)	0
L (nd)	2	216	208 (96.3%)	8 (3.7)	0
GD (nd)	3	75	72 (92%)	3 (8%)	0
R (nd)	2	95	92 (96.8)	3 (3.2%)	0
Total	19	1489	1391 (93.4%)	98 (6.6%)	0

Table 2. Recognition by two alloreactive T cell clones of allodeterminants derived from constitutive processing and presentation of HSA. For experiment 1, T cell clones were cultured in RPMI-FCS with different APC and the following sources of antigen: autologous HS (5%) taken from the donors from which the clones were generated, HSA (5 mg/ml) (Sigma), recombinant HSA (5 mg/ml), synthetic peptides (30 μg/ml), or medium alone. For experiment 2, EBV-B cells were incubated with HSA or HSA 444 to 456 for the indicated times in the absence or presence of leupeptin (50 μg/ml) (Sigma) or chloroquine (10^{-4} M) at 37°C, washed, and fixed with glutaraldehyde (0.05%). Fixed APC (10^5) were cultured with the responding T cells. The results are representative of at least five different experiments.

Antigen	Proliferative response (cpm)	
	AK42	NG74
Experiment 1		
DRw11 EBV-B		
None	953	988
Autologous HS	44,212	104,820
Recombinant HSA	28,761	14,120
Autologous EBV-B		
Recombinant HSA	880	572
DRw11 PBMC		
None	31,742	57,618
DRw11 L cells		
None	22	24
Recombinant HSA	13,103	2,205
HSA (444–456)	34,876	20
tt 830–843	30	44
Experiment 2		
DRw11 EBV-B		
HSA (444–456) 10 min	6,402	101
HSA 20 min	669	532
HSA 60 min	4,586	5,121
HSA+chloroquine 60 min	154	121
HSA+leupeptin 60 min	100	142

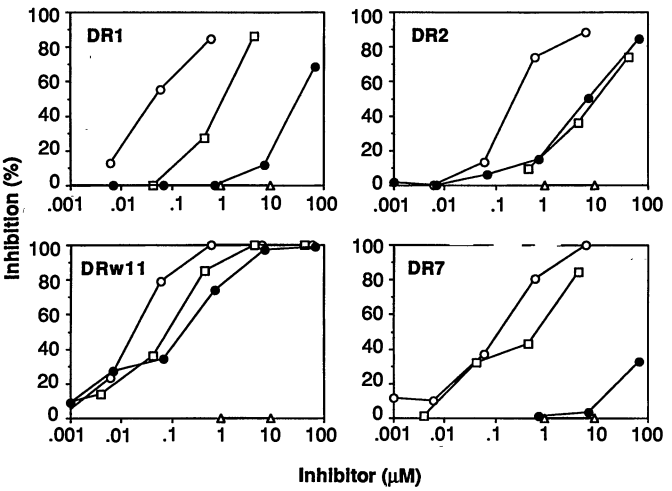

Fig. 2. Binding of HSA (444–456) to DRw11. The data represent the percent inhibition of binding of a ^{125}I-labeled peptide to four different purified DR molecules. The competing peptides were HSA 444 to 456 (●), and three tetanus toxin (tt) epitopes: tt 830 to 843 (○), tt 947 to 967 (□), tt 1274 to 1283 (△). The binding assay with purified and ^{125}I-labeled peptides has been described (*15*). DR molecules were purified from homozygous EBV-B cells (DR1, LG2; DR2, 3107; DRw11, SWEIG; DR7, DBB, or PITOUT) with the use of the monoclonal antibody LB3.1 covalently coupled to protein A–Sepharose CL-4B. Purified DR molecules (10 to 1000 nM) were incubated with 5 to 50 nM ^{125}I-labeled peptide (HA 307 to 319 for the DR1 assay; tt 830 to 843 for the DR2, DRw11, and DR7 assays) for 48 hours in the presence of different concentrations of the unlabeled inhibitor peptide in a mixture of protease inhibitors (1 mM PMSF, 1.3 mM 1,10-phenanthroline, 73 μM pepstatin A, 8 mM EDTA, 6 mM *N*-ethylmaleimide, and 200 μM *Nα-p*-tosyl-L-lysine chloromethyl ketone) containing NP-40 (0.05%) and DMSO (5%) in PBS. The DR-peptide complexes were separated from free peptide by gel filtration on Sephadex G-50 or TSK columns. Representative data from one of at least two experiments are shown. The variation of response to a half-maximal concentration of inhibitor was less than 50% of the mean.

poor binding of the peptide to DRw11, we measured peptide binding to purified DR molecules. HSA (444–456) bound to DRw11 with an affinity similar to that of other peptides that bind DRw11 efficiently (Fig. 2). Furthermore it bound specifically, since it bound 10- to 100-fold more avidly to DRw11 than to DR1 and DR2, while no binding was detected to DR7 (*9*). This pattern was different from that of two tetanus toxin epitopes that bound with high affinity to multiple DR molecules (Fig. 2). The results indicate that presentation of HSA (444–456) is mediated by DRw11 and that the high concentration required for T cell stimulation probably reflects the low affinity of the alloreactive T cell clone for the peptide-MHC complex.

The number (7%) of class II alloreactive T cells that recognized determinants generated by uptake and processing of monomorphic serum proteins in our studies may have been underestimated. Some low-affinity T cells might not have been detected because of the low concentration of human serum used for screening (5% versus 100% in vivo). Also, alloreactive T cells that recognize peptides that are shared or cross-reactive between human and bovine serum are undetectable. The results do not exclude the possibility that a proportion of class II alloreactivity may be directed against exogenously derived nonserum proteins such as cellular breakdown products, endogenously synthesized proteins, or class II molecules themselves.

These data show that allorecognition results, at least in part, from the presentation of a large number of different monomorphic self antigens, as originally suggested by Matzinger and Bevan (*10*). It is not surprising that for class II molecules the pathway of presentation of alloantigens follows the same rules as the presentation of foreign antigens (*11*). To date, peptides recognized by alloreactive T cells have been described only in very special cases in which the peptide was derived from the polymorphic part of one MHC molecule and presented by a different one (*12*). However, these alloreactive clones were derived by deliberate immunization, and it is therefore not clear whether these cases are relevant to alloreactivity, because alloreactivity usually maps to a single MHC allele. Heath *et al.* have described three class I alloreactive T cell clones from mice that recognize peptides derived from unidentified cellular proteins (*13*), but the epitopes recognized have not been characterized. The HSA (444 to 456) epitope is an example of a monomorphic self protein fragment that contributes to the formation of a specific allodeterminant. The identification of monomorphic self peptides that bind to self MHC should be useful in investigating the extent of self tolerance and may allow design of peptides that modulate the immune response in vivo.

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6. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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