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Immunology

HLA antigens expressed on murine cells are preferentially recognized by murine cytotoxic T cells in the context of the H-2 major histocompatibility complex

(histocompatibility antigens/major histocompatibility complex restriction/precursor frequency/T-cell repertoire)

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ABSTRACT **The frequency of murine cytotoxic T lymphocytes (CTL) capable of responding to HLA antigens expressed on human or murine cells was determined by limiting dilution analysis. HLA antigens expressed on human cells stimulated CTL with a precursor frequency of about 1 in 2 × 10^5 spleen cells in primed mice, over two orders of magnitude weaker than a primary allogeneic response. There was a 10-fold increase in the frequency of precursors responding to HLA antigens when they were expressed on murine cells. It was determined that the increased frequency of responders was due to CTL that could only recognize HLA antigens on the syngeneic murine line to which they had been stimulated and that these CTL could not lyse any other HLA expressing murine cells of different H-2 haplotypes. The lytic activity of these CTL was inhibited by H-2^b-specific antibodies. These results indicate that such CTL recognize HLA antigens in the context of the H-2 major histocompatibility complex. The magnitude and specificity of CTL responses to xenoantigens are discussed in the context of a model for T-cell interactions with major histocompatibility antigens.**

The class I transplantation antigens encoded in the major histocompatibility complex (HLA-A, -B, -C in the human and H-2K, -D, and -L in the mouse) are expressed on virtually all cells and exist as heterodimers consisting of a polymorphic 45-kDa integral membrane glycoprotein that is noncovalently associated with an invariant 12-kDa protein, β_2 microglobulin (1). These proteins are the principal targets for cell-mediated lysis by cytotoxic T lymphocytes (CTL) during the rejection of allogeneic and xenogeneic tissue transplants and function as restricting elements for CTL recognition and lysis of virally infected or tumor cells (reviewed in ref. 2).

This laboratory has studied the ability of murine CTL to recognize human histocompatibility antigens. Such xenogeneic murine CTL can distinguish between products of closely related *HLA* alleles and so seem to recognize determinants that are the same or are similar to those recognized by human CTL (3, 4, 14). It is also observed, however, that such a xenogeneic response is weak by comparison with the response to allogeneic H-2 antigens (5). In this paper, the response of mice to human histocompatibility antigens has been quantitated and compared to the allogeneic response by precursor frequency analysis. In addition, the effect on both the level and specificity of this response of presenting human histocompatibility antigens on the surface of transfected murine cells has been evaluated.

MATERIALS AND METHODS

Cell Lines. The human lymphoblastoid lines and their HLA types were as follows: JY (HLA-A2,2; -B7,7; -DR4,6), HSB (HLA-A1,2; -B12,17; class II negative), and JM (HLA-A3,25; B7,37; class II negative). The murine cell lines used were as follows: the T-cell lymphoma line, EL4 (H-2^b); the thymoma line R1.1 (H-2^k); a Rous sarcoma virus-transformed BALB/c line, BRS (H-2^d); a mastocytoma cell line, P815 (H-2^d); and a thymidine kinase negative L cell line, Ltk$^-$ (H-2^k). The HLA-expressing L cell transfectants 201 (HLA-A2) and 3.5 (HLA-B7) have been described (6).

DNA Transfections. Restriction fragments encoding the *HLA-A2* or *-B7* genes (6) were ligated into the *Eco*RI site of either pSV2gpt (7) or pSV2neo (8). The BRS cells were transfected as described (6), using the pSV2gpt plasmid carrying the *HLA-A2* or *-B7* genes and cloned in medium containing mycophenolic acid at 25 μg/ml (Eli Lilly), xanthine at 250 μg/ml, and HAT components (8). The murine lymphoid lines EL4 and R1.1 were transfected by a modified protoplast fusion method using pSV2neo derivatives (9, 10) and cloned by limiting dilution in medium containing Geneticin (400 μg/ml) (GIBCO). Expression of HLA-A2 or -B7 on transfected cells was evaluated by radioimmunoassay as described (6). The transfectants used in this study were as follows: EA6 (HLA-A2$^+$) and EB1 (HLA-B7$^+$), derived from EL4; RA6 (HLA-A2$^+$) and RB2 (HLA-B7$^+$), derived from R1.1; and BA3 (HLA-A2$^+$) and BB4 (HLA-B7$^+$), derived from BRS cells.

Limiting Dilution Analysis of Cytotoxic Activity. Mice were primed by intraperitoneal injection of 2 × 10^7 stimulator cells in some experiments. At least 1 month after immunization the mice were sacrificed, and various amounts of spleen cells were added to round bottom 96-well microtiter plates. Irradiated (2000 R; 1 R = 0.258 mC/kg), syngeneic spleen cells were added to give a total of 2.5 × 10^5 cells per well. Irradiated (5000 R) cells (1 × 10^4 cells) expressing the HLA antigen of interest and 25% (vol/vol) supernatant from concanavalin A-stimulated Lewis rat spleen cells (3) were added in a final volume of 200 μl. After 7 days, the same amounts of fresh irradiated spleen cells, irradiated stimulator cells, and concanavalin A supernatant were added to all wells. Three days later, aliquots of each well were assayed for cytotoxic activity in a ^{51}Cr-release assay (3). All plates were assayed on an HLA-expressing human cell line, an HLA-expressing EL4 transfectant, and untransfected EL4 cells. Two different methods were used to determine significant specific release values, and the larger value was used in all cases. In the first method, significant lysis was defined as being 3 SD above the mean spontaneous release value from four replicate wells containing only targets. In the second method, wells giving specific release values less than 0% were identified, and the most negative value was assigned as the parameter X. Only those specific release values that were outside the range −X to +X were considered significant. A

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Abbreviations: CTL, cytotoxic T lymphocyte(s); mAb, monoclonal antibody.

very small fraction of the responding wells showed significant lysis of the untransfected control line EL4. These wells were discounted when calculating precursor frequencies even if they showed significant reactivity on HLA-expressing targets. Precursor frequencies were determined as described (11).

RESULTS

Murine CTL Precursor Frequencies to HLA-A2 Expressed on Human or Murine Cells. Limiting dilution analyses were done to determine the frequency of cytotoxic precursors in H-2^b mice capable of responding to allogeneic (H-2^d) or xenogeneic (HLA-A2) histocompatibility antigens. In a typical experiment shown in Fig. 1, the frequency of cytotoxic precursors in naive C57BL/6 spleen cells capable of responding to the H-$2K^d$, -L^d, and -D^d antigens on P815 cells was 1 in 300. In different experiments, this value has ranged between 1 in 300 and 1 in 1000, in good agreement with results obtained by others (12, 13). In contrast, the frequency of cytotoxic precursors in naive animals capable of responding to HLA-A2 on a human lymphoblastoid cell line was not calculated because in several experiments the number of positive wells was too low, less than 1 in 10^6 spleen cells (data not shown). Therefore, spleen cells from an animal primed with the HLA-A2-bearing human line JY were tested for reactivity against HLA-A2 by restimulation *in vitro* with HSB, a human line that only shares HLA-A2 with JY. The frequency of HLA-A2 reactive precursors in this primed animal was 1 in 1.8×10^5 spleen cells (Fig. 1). In different experiments this value ranged between 1 in 10^5 and 1 in 5×10^5 spleen cells. These results indicate that the precursor frequency of HLA-A2-specific CTL in mice can be increased significantly by *in vivo* priming. However, the frequency is still between two and three orders of magnitude lower than that seen in naive animals for the allogeneic stimulator P815.

A number of factors could account for the quantitative differences between the responses to alloantigens and to xenoantigens. One possibility is that only a small fraction of murine T-cell antigen receptors are capable of recognizing determinants on HLA antigens. Another possibility is that stimulation requires a species-specific interaction between accessory molecules on the T cells and ligands on the stimulator cells. To investigate this latter possibility the cytotoxic response to an HLA-A2-expressing murine cell was tested. Spleen cells from a C57BL/6 mouse primed with the HLA-A2-expressing syngeneic transfectant EA6 were tested for their ability to lyse EA6 target cells after *in vitro* stimulation with EA6. Such CTL arose with a precursor frequency of 1 in 1.8×10^4 spleen cells (Fig. 1). In different experiments this value ranged between 1 in 1.8×10^4 and 3×10^4 spleen cells. Although this value is one order of magnitude higher than the frequency of responders to HLA-A2-expressing human cells in primed animals, it is still one to two orders of magnitude lower than the frequency of responders to the allogeneic target P815 in naive animals. This value is derived from wells that recognize EA6 but not the untransfected cell line EL4. Wells showing reactivity against EL4 represented only a small percentage of the total positive wells in all experiments. Thus, the HLA-A2-expressing murine transfectant EA6 can stimulate a better response than the HLA-A2-expressing human cell HSB.

The wells that had been stimulated with the HLA-A2-expressing murine transfectant EA6 were also assayed for their ability to lyse the HLA-A2-expressing human cell HSB. The frequency of CTL precursors that could lyse HSB in mice primed and restimulated with EA6 was one in 3.3×10^5 spleen cells, virtually the same value as that obtained in mice primed with HSB (Fig. 1). This result indicates that EA6 can prime cytotoxic precursors capable of recognizing HLA-A2-expressing human cells. However, the overwhelming number of T cells stimulated by EA6 recognize only the EA6 target and not HSB or EL4.

Specificity Analysis of Wells from Precursor Frequency Experiments. The ability of CTL in individual wells to lyse HSB and EA6 was compared in wells from dilutions that contained, on average, one or less CTL precursors per well. The results from an animal primed with JY and restimulated with HSB are plotted in Fig. 2. Most of the wells did not demonstrate significant cytotoxic activity against either target and formed a cluster of points around zero. Of the remaining 31 wells that showed significant lysis, 20 fell into a group that could lyse both HSB and EA6. Five wells showed significant lytic activity on HSB with no significant activity on EA6, and six wells showed lytic activity on EA6 alone. The majority of the CTL stimulated by an HLA-A2-

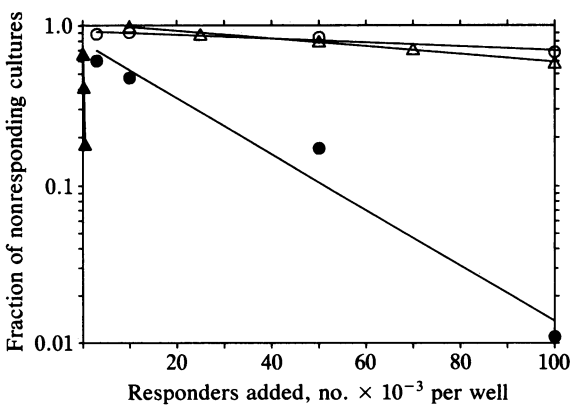

FIG. 1. Precursor frequency in C57BL/6 spleen cells was determined by limiting dilution analysis. Lines were defined by the method of least squares, and all had coefficients of determination greater than 0.91. ▲, Naive spleen cells stimulated with P815 and assayed on P815 targets; △, JY-primed spleen cells stimulated with HSB and assayed on HSB targets; ●, EA6-primed spleen cells stimulated with EA6 and assayed on EA6 targets; ○, EA6-primed spleen cells stimulated with EA6 and assayed on HSB targets.

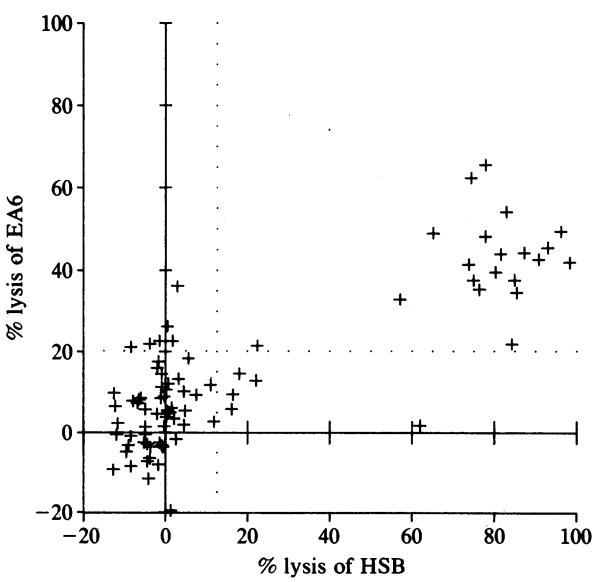

FIG. 2. Lytic activity against HSB and EA6 target cells in individual wells that initially contained 10^5 responder cells from a JY-primed animal and were stimulated with HSB. Dotted lines indicate significant lysis limits.

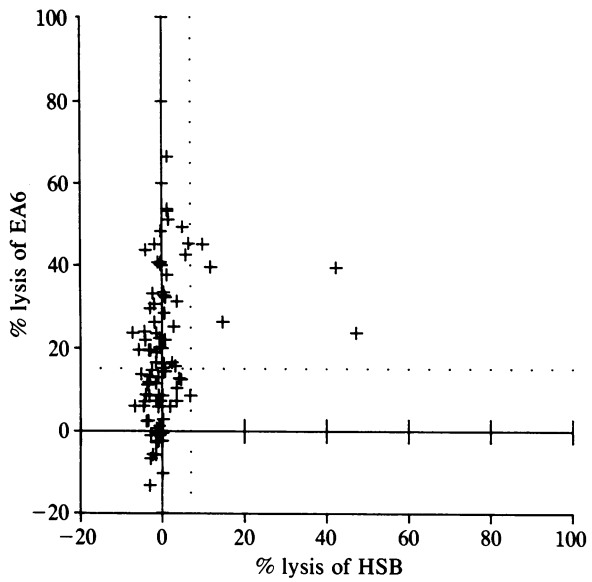

FIG. 3. Lytic activity against HSB and EA6 target cells in individual wells that initially contained 10^4 responder cells from a EA6-primed animal and were stimulated with EA6. Dotted lines indicate significant lysis limits.

expressing human cell were capable of lysing both murine and human cells expressing HLA-A2.

This pattern contrasted sharply with that observed using an animal that had been primed with EA6 and then restimulated with EA6 *in vitro* (Fig. 3). Out of 45 wells showing significant activity, cells from only 5 killed both HSB and EA6. The remaining 40 cultures exhibited cytotoxic activity against EA6 but were totally unreactive against HSB.

The majority of the CTL stimulated with an HLA-B7-expressing human cell could lyse the HLA-B7-bearing human target as well as the HLA-B7-expressing EL4 transfectant EB1 (data not shown). As with EA6, when EB1 was used as a stimulator, the majority of the wells exhibiting significant cytotoxic activity lysed EB1 and not a human cell line expressing HLA-B7, JM.

Reactivity of CTL on a Panel of HLA-Expressing Murine Cells. To further analyze the specificity of the CTL detected in limiting dilution experiments, cultures from representative wells exhibiting different reactivity patterns were expanded in culture and subcloned as described (3). The resulting subclones were then analyzed for their reactivity against human targets expressing HLA-A2 or -B7, as well as a series of murine transfectants representing cells of various H-2 haplotypes and tissue types. Several clones derived from mice that had been primed with HLA-A2-expressing human cells lysed both human and murine HLA-A2-expressing target cells (clones AX8.17 and AX9.6 in Table 1). Although the level of lysis of the murine transfectants was generally lower than that of human targets, this group of CTL gave comparable levels of lysis on all murine transfectants expressing the appropriate HLA antigen. Other clones derived from these animals lysed only human cells bearing HLA-A2 and did not lyse any of the murine transfectants (clones AH9.2 and AH10.4 in Table 1). The existence of murine CTL clones capable of lysing human but not murine target cells expressing the same HLA antigen has been reported (6, 14).

CTL clones derived from an animal primed with HLA-A2-expressing murine transfectants showed a different spectrum of reactivity patterns. A small number showed a reactivity pattern analogous to AX8.17 and AX9.6 above (data not shown). However, the majority were typified by the five clones in Table 1 (AM8.7, AM8.4, AM8.11, AM8.1, and AM17.3) that recognized only EA6. They did not recognize transfectants that expressed HLA-A2, but differed from EA6 in tissue and H-2 haplotype. A sixth clone, AM8.5, recognized an HLA-A2-expressing BRS (H-2^d) line, BA3, in addition to EA6. However, this clone did not recognize two other targets, one of lymphoid origin and one an L cell, that were of the H-2^k haplotype. None of the clones recognized any of the transfectants expressing HLA-B7, but all recognized other independently derived EL4 transfectants expressing HLA-A2 (data not shown).

Similar reactivity patterns were seen with CTL clones that had been derived from animals primed against HLA-B7 (Table 2). Clone BX9.88 was derived from an animal primed with human cells expressing HLA-B7. This clone recognized human cells expressing HLA-B7, as well as all murine cells that had been transfected with the HLA-B7 gene, but not those transfected with HLA-A2. In contrast, clones BM14.28 and BM15.84 recognized only the EB1 cell line against which they had been stimulated initially and did not recognize either HLA-B7-expressing human cells or HLA-B7-expressing murine transfectants other than EB1. None of these CTL recognized transfectants expressing the *HLA-A2* gene.

Monoclonal Antibody (mAb) Blocking of the Activity of CTL Clones. The existence of CTL that recognized only syngeneic target cells expressing an HLA antigen suggested that the structure being recognized required both the transfected gene product and either an additional cell-specific molecule or alteration of the transfected product to produce a new determinant. It had been reported (15) that HLA antigens introduced into EL4 via cell-liposome fusion were recognized by murine CTL in an apparently H-2-restricted fashion. To investigate this possibility, the ability of mAb specific for the H-2^b or the HLA antigens to block CTL lysis of EA6 or EB1 cells was evaluated.

Table 1. Specificity of CTL cloned from animals primed with human or murine cells expressing HLA-A2

	% specific ^{51}Cr release								
CTL	EA6 (b/+)	EL4 (b/−)	RA6 (k/+)	R11 (k/−)	201 (k/+)	LTK (k/−)	BA3 (d/+)	BRS (d/−)	HSB (NA/+)
AX8.17	51	−2	38	4	45	13	50	2	86
AX9.6	47	−8	ND	ND	31	3	21	7	68
AH9.2	3	0	ND	ND	0	1	−4	4	84
AH10.4	0	0	ND	ND	2	0	3	4	87
AM8.7	26	4	−1	5	4	6	4	4	4
AM8.4	19	3	−3	3	−3	−1	0	−2	3
AM8.11	28	8	−3	7	−2	3	2	−2	6
AM8.1	24	3	−5	2	0	−1	4	−6	7
AM17.3	43	−9	ND	ND	−1	−2	−1	−3	9
AM8.5	69	5	−3	0	−2	−1	35	−2	7

Following the name of the cell line in parentheses is the H-2 haplotype of the target cell and whether (+) or not (−) HLA-A2 is expressed. NA, Not applicable; ND, not determined.

Table 2. Specificity of CTL cloned from animals primed with human or murine cells expressing HLA-B7

CTL	% specific ^{51}Cr release								
	EA6 (b/A2)	EB1 (b/B7)	RA6 (k/A2)	RB2 (k/B7)	201 (k/A2)	3.5 (k/A2)	BA3 (d/A2)	BB4 (d/B7)	JM (NA/B7)
BX9.88	7	51	ND	ND	−2	36	28	54	96
BM14.28	0	45	2	1	−2	−1	11	18	0
BM15.84	4	41	1	8	4	8	2	4	1

Names of cell lines are followed in parentheses by the H-2 haplotype of the murine target cell and by the HLA antigen expressed by the target cells. NA, Not applicable; ND, not determined.

Clone AX9.6 recognized HLA-A2-bearing cells of both human and murine origin. Its lysis of EA6 was blocked by the HLA-A2-specific mAb A2,28.M1 but was not affected by the anti-HLA-B7 mAb ME.1 (Table 3). None of the anti-H-2^b mAb inhibited lysis by this clone. In contrast, the lytic activity of the clones AM8.1 and AM17.3, which recognized only HLA-A2-expressing EL4 cells, was inhibited by some of the anti-H-2^b antibodies (Table 3). The H-$2K^b$-specific mAbs 20.8.4 and 28.13.3 caused an 80%–90% inhibition of killing by the AM8.1 clone and a 50% inhibition of the lytic activity of AM17.3. The HLA-A2-specific mAb A2,28.M1 inhibited the lytic activity of AM8.1 and AM17.3 by about 60% and 40%, respectively. A different pattern of antibody blocking was seen with the CTL clone AM8.5, which recognized HLA-A2-expressing EL4 and BRS cells. Lytic activity was blocked by the H-$2K^b$-specific antibody 28.13.3, but 20.8.4 had no effect (Table 3). Lytic activity was also affected by the H-$2D^b$-specific mAb 28.11.5, which inhibited lysis by 40% at the higher concentration tested. The anti-HLA-A2 mAb A2,28.M1 slightly interfered with the lytic activity of AM8.5.

The same approach was used with several HLA-B7-specific clones. The clone BX9.88 recognizes both human and mouse cells expressing HLA-B7. The only antibody that blocked lysis by this clone was the HLA-B7-specific antibody ME.1 (Table 3). However, clones BM14.28 and BM15.84, which recognized only the HLA-B7-expressing murine target EB1, were blocked only by the H-$2K^b$-specific mAbs 20.8.4 and 28.13.3.

The antibody blocking results along with the specificity data suggest that the CTL clones that kill only the HLA-A2- or -B7-expressing H-2^b transfectants recognize the HLA antigens in the context of H-2^b restricting elements.

DISCUSSION

In this report, the influence of the cellular background on the stimulation of murine CTL by HLA antigens has been evaluated. The majority of the CTL stimulated by HLA-expressing human cells could lyse both murine and human targets bearing the appropriate HLA antigen. In contrast, the majority of the CTL stimulated by an HLA-expressing murine cell did not lyse human targets expressing the appropriate HLA molecule. The vast majority of such CTL lysed only the syngeneic transfectant to which they were stimulated and not allogeneic murine cell transfectants. This observation argues that the new determinant formed when HLA class I antigens are expressed on EL4 cells is not due to a murine-specific post-translational modification or to association with murine β_2 microglobulin. Furthermore, such reactivity cannot be explained by a species-specific interaction between murine accessory molecules, such as Lyt-2 or LFA-1, and their ligands. Instead, the antibody blocking data along with the target cell specificity results indicate that, for such CTL, H-2^b antigens are directly involved in the recognition of HLA class I antigens in a way that is completely consistent with H-2-restricted recognition of a nominal antigen. In the case of one clone, AM8.5, there appears to be some degeneracy in the use of restricting elements since the HLA-A2-expressing cell of the H-2^d haplotype (BA3) can be recognized and both H-$2D^b$- and K^b-specific mAb can interfere with its lytic activity. Importantly, this kind of degeneracy in restriction element specificity has been shown in other systems using different nominal antigens (18–20).

These results have confirmed and extended our earlier observations made with EL4 cells that had been fused with

Table 3. Inhibition of lysis by HLA-A2- and HLA-B7-specific CTL clones using mAb

CTL	Cells lysed	% inhibition of ^{51}Cr release					
		20.8.4 (D^bK^b)	28.13.3 (K^b)	27.11.3 (D^b)	28.11.5 (D^b)	A2.28.M1 (−A2)	ME.1 (−B7)
AX9.6 (50%)	All HLA-$A2^+$	−17	1	−5	3	58	−4
		−17	3	−3	4	58	−2
AM8.1 (30%)	EA6 only	93	80	25	25	61	9
		95	64	18	22	55	5
AM17.3 (52%)	EA6 only	47	48	−5	2	36	−1
		27	30	−10	3	33	−2
AM8.5 (30%)	EA6 and BA3	18	54	24	40	29	15
		8	53	30	12	35	5
BX9.88 (23%)	All HLA-$B7^+$	15	14	−2	−3	7	67
		−15	19	−2	7	−4	73
BM14.28 (32%)	EB1 only	89	74	16	25	25	24
		76	95	19	−3	24	10
BM15.84 (20%)	EB1 only	84	99	−2	3	−27	−9
		65	85	3	4	−24	−1

AX9.6, AM8.1, AM17.3, and AM8.5 were assayed on EA6 cells, while BX9.88, BM14.28, and BM15.84 were assayed on EB1 cells. Ascites fluids containing mAb specific for H-$2K^b$ and -D^b (25) were the gift of David Sachs (National Institutes of Health). A2,28.M1 (HLA-A2 specific), and ME.1 (HLA-B7 specific) were the gift of Peter Parham (Stanford University) (16, 17). They were affinity purified at an initial concentration of 1 mg/ml. The indicated mAbs were incubated with target cells on ice for 30 min and then at 37°C, in a humidified 4% CO_2/ 96% air atmosphere for 30 min. CTL clones were added, and the plates were incubated for 90 min at 37°C. EDTA (10 mM) was added, and incubation was continued for another 2 hr. ^{51}Cr release was measured in supernatants as described (3). For each clone, the upper row of values corresponds to 1:200 dilution of ascites and a 1:100 dilution of purified antibody. The lower values correspond to a 1:800 dilution of ascites and a 1:400 dilution of purified antibody. Specific ^{51}Cr release obtained in the absence of antibody is in parentheses under the CTL column.

HLA-expressing liposomes, which suggested that HLA antigens could be recognized in an H-2-restricted manner (15). Other workers have also observed that HLA antigens expressed on murine cells can be recognized as nominal antigens in the context of H-2-restriction elements (21, 22).

The weak T-cell response to xenogeneic antigens remains a paradox. Sequence homology between different H-2 alloantigens is in the range of 80–99%, while homology between H-2 and HLA antigens is usually 65–75%. Despite this, the murine T-cell response in naive animals to allogeneic H-2 molecules is at least three orders of magnitude greater than the response to xenogeneic HLA molecules. In primed animals, presentation of the HLA molecule on a murine cell surface does increase the response by an order of magnitude, but this appears to be due entirely to CTL that recognize the HLA as a nominal antigen in the context of syngeneic H-2 restriction elements. There is no change in the frequency of CTL that are capable of recognizing HLA antigens on human cells. This preferential recognition as a nominal antigen merely serves to emphasize how poorly HLA antigens function as histocompatibility antigens in stimulating murine T cells. Perhaps the weak response to xenogeneic antigens fails to develop during ontogeny when the T-cell repertoire is selected to recognize determinants that closely resemble syngeneic H-2 molecules. Therefore, CTL recognize allogeneic determinants more easily than more distantly related xenogeneic determinants. However, murine CTL capable of lysing HLA-expressing human and murine cells distinguish between different HLA alloantigens, indicating that allele-specific and not species-specific determinants are the principal polymorphic determinants recognized by such T cells (this paper and refs. 3 and 14). The observation that CTL recognize regions that are the most variable among both xeno- and alloantigens is difficult to reconcile with a model in which the repertoire develops to preferentially recognize structures that are the most closely related in evolutionary terms.

Another possible explanation is based on the observation that even across species barriers, T cells exclusively interact with histocompatibility antigens (5, 23). This suggests that histocompatibility antigens bear conserved determinant(s) that mark them as ligands for T cells. T cells would not interact as well with xenogeneic histocompatibility antigens if these molecules had undergone evolutionary modification of such determinants. The location of such determinants would presumably be in a portion of the histocompatibility antigen that is highly conserved within a species, but more divergent between species. Recognition of such a determinant could occur either through the T-cell receptor (4), or through accessory molecules such as Lyt2 or T8 (reviewed in ref. 24). A basic question that remains unanswered in cellular immunology is the mechanism by which T cells recognize and specifically interact with histocompatibility antigens. The xenogeneic system described here should be a good system to explore the role of species-specific determinants in T-cell interactions.

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