On the Nature of Peptides Involved in T Cell Alloreactivity

By Olaf Rötzschke, Kirsten Falk, Stefan Faath, and Hans-Georg Rammensee

From the Max-Planck-Institut für Biologie, Abteilung Immungenetik, D-7400 Tübingen, Germany

Summary

The strong reaction of T cells against foreign major histocompatibility complex (MHC) antigens, commonly termed "alloreactivity", is not only a nuisance for clinical organ transplantation; it also remains a puzzling question for immunologists. By making use of recent technical developments, alloreactive T cells nominally directed against a mutation in a single MHC class I molecule were found to fall into several major categories. One is recognizing peptides whose occurrence is dependent on one particular MHC allele, another is recognizing peptides supported by several MHC alleles, and a third is recognizing peptides occurring independently of MHC alleles. In a fourth category, the binding to MHC of any of a broad range of peptides appears sufficient. In addition, there are T cells for which no peptide involvement could be detected at all. Even within these categories, the heterogeneity of T cells is considerable: among 16 K^b-reactive T cells analyzed, 15 different modes of reactions were found.

The trait leading to the discovery of MHC genes 54 yr ago was the exquisite strength of graft rejection between members of a species expressing different MHC alleles (1, 2). This strong in vivo reaction, which contributed the affix "major" to MHC, found its in vitro correlate later in the strong activation of T cells confronted by foreign MHC antigen. The phenomenon, termed T cell alloreactivity, is manifested in high frequencies of precursors specific for a given foreign MHC, and in the property of unprimed T cells to become activated in vitro (2–4). Both characteristics contrast to "normal" T cell responses against foreign antigens, for example, of viral origin. In these instances, the frequency of antigen-specific precursors in unprimed T cells is very low or undetectable, and activation of unprimed T cells specific for physiologically presented foreign antigen is usually not observed. For physiological immune responses, it is now well established that T cells recognize foreign antigen as peptides processed by APC and presented by self MHC molecules (5–12). The peptide binding site of MHC class I molecules has been revealed by crystallography to be a cleft built up by heavy chain $\alpha 1$ and $\alpha 2$ domains (13, 14). In contrast to these relatively well-understood topics, the phenomenon of T cell alloreactivity is still a puzzling question for immunologists.

One paper with foresight (15) suggested that alloreactive T cells may recognize foreign MHC plus unknown cellular antigens, or peptides, as we would call it now. More recent reports indeed indicated, by indirect means, involvement of non-MHC antigens (16–18). Alloreactive T cells were shown to recognize synthetic peptides derived from MHC sequences (19–21). Another report indicated involvement of a non-MHC peptide in allorecognition in one case; the peptide in question, however, had also to be produced artificially (22). Alloreactivity has also been suggested to involve low affinity recognition of high-density alloantigens (23). It can be envisaged, for example, that T cells recognizing foreign MHC molecules independent of any peptide meet some 10^5 ligands per cell, whereas peptide-specific T cells meet a few only (24). Thus, for those not recognizing peptide, a much lower affinity will suffice for activation. Indeed, a recent report suggested that peptides need not be involved in alloreactivity (25).

We addressed the problem by taking advantage of two novel developments. One is the technique of extracting naturally processed peptides involved in the MHC class I–restricted antigen presentation pathway, as has been developed for minor histocompatibility and viral peptides (9–11). The other is the introduction of the mutant cell line, RMA-S, which appears to have a defect in peptide presentation; it can thus serve as a readout target for peptides that are otherwise intrinsically presented by all cells (26–30). Of the many possible MHC-incompatible mouse strain combinations that could be used to raise alloreactive CTL, we used B6.C-H-2^{bm1} anti-C57BL/6 (abbreviated, bm1 anti-B6) in most experiments. Both strains differ at a mutation in the H-2K gene; the K^b molecule expressed by C57BL/6 and the K^{bm1} molecule differ at three amino acid residues located in the peptide binding cleft (31).

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This strain combination has the advantage that it involves minimal differences in the MHC molecules of T cells and target cells but still features all classic aspects of alloreactivity (2–4, 31). One should keep in mind, however, that unrelated MHC molecules differ in many more amino acid residues, including those outside the peptide binding region. Thus, T cells raised against unrelated foreign MHC molecules might be more heterogeneous than those against an MHC mutation.

Our results indicate that even the antigens recognized by mutant-specific alloreactive T cells are of considerable heterogeneity. They can be classified into several distinct categories. To simplify discussions concerning this complex subject, we propose a systematic nomenclature for the categories of alloreactive T cells and the antigens they recognize.

Materials and Methods

Animals. C57BL/6 (abbreviated B6; H-2K^bD^b), B6.C-H-2^{bm1} (bm1; $K^{bm1}D^b$), B6.C-H-2^{bm3} (bm3; $K^{bm3}D^b$), BALB/c (K^dD^d), BALB.B (K^bD^b), BALB.5R (K^bD^d), BALB.HTG (K^dD^b), B10.BR (K^kD^k), B10.D2 (K^dD^d), and B10.S (K^sD^s) were bred and maintained at the animal facility of Max-Planck-Institut für Biologie. A 4.2-g specimen of *Lumbricus terrestris* was collected next to the parking lot of the institute.

Cell Lines. EL4, RMA, RMA-S (26) (all H-2^b), Jurkat, and Jurkat-K^b (22) cells were maintained in RPMI 1640 supplemented with 5% FCS at 37°C in a 5% CO_2 atmosphere. For mass cultures, cells were expanded in 1 liter of DMEM (Gibco Laboratories, Grand Island, NY) supplemented with 5% FCS in 2-liter roller bottles (Duran; Schott Mainz, FRG) at 37°C. "RMA-S(26°C)" or "cold RMA-S cells" indicates RMA-S cells precultured at 26°C for 24–48 h.

Acid Extraction of Peptides from Whole Cells. Two to four spleens of the mouse strains indicated, or 10^9 tumor cells suspended in 15 ml of 0.1% (vol/vol) TFA were homogenized by douncing (10 strokes) using a borosilicate glass dounce-homogenizer (15 ml; Braun, Melsungen, FRG). The suspension was further homogenized by ultrasonication (20 pulses of 1 s; sonifier model B15; Branson, Danbury, CT). The homogenate was stirred for 30 min at 4°C. pH was kept at 2.0 throughout this procedure by adding 1% TFA. Supernatant was collected after centrifugation (150,000 *g* for 30 min at 4°C). The remaining pellet was extracted again using 7 ml of 0.1% TFA. Combined supernatants of the first and second extraction were lyophilized overnight (model Gamma 1A; Christ, Osterode, FRG), resuspended in 2.5 ml of 0.1% TFA, and subjected to a Sephadex G25 coarse gel filtration column (Pharmacia Fine Chemicals, Piscataway, NJ) (bed volume, 75 ml). Material of M_r <5,000 was collected, lyophilized, and stored at −70°C, if not immediately used for HPLC separation.

Acid Extraction of Peptides from Purified MHC Class I Molecules. Two slightly different methods were used. For the first method, five BALB.B spleens were lysed in 20 ml of 1% NP-40 detergent in PBS containing 0.1 mM PMSF using the douncing procedure as described above. The resulting suspension was stirred for 30 min at 4°C and centrifuged (5 min, 250 *g*, 4°C). The resulting supernatant was centrifuged again (30 min, 150,000 *g*, 4°C). Supernatant from this step was incubated (under gentle agitation; 5–6 h at 4°C) with CNBr-activated Sepharose CL4B beads (Pharmacia Fine Chemicals), to which K^b-specific K9-178 (32) or D^b-specific B22-249 (33) antibodies had been covalently coupled according to manufacturer's protocol (0.5 ml of beads were coated using 0.5 to 1 mg/ml of antibody). Beads were washed twice in PBS/0.5% NP-40, once again in PBS, and were subjected to acid elution by vortexing in 3 ml of 0.1% TFA for 15 min at 4°C. For the second method, 6–8 × 10^9 RMA-S cells kept for 24 h at 26°C were detergent lysed (100 ml of PBS/1% NP-40) and treated as for the first method. The supernatant from the last ultracentrifugation step (100 ml) was first passed over a chromatography column filled with anti-K^b beads, then over a column with anti-D^b beads (bed volumes, 0.5 ml; flow rate, 0.25 ml/min; 4°C). Loaded beads were removed from the columns, washed as above, and subjected to acid extraction as above. Supernatant of both BALB.B- and RMA-S-derived material was lyophilized, resuspended in 1 ml of 0.1% TFA, and subjected to HPLC separation.

HPLC Separation of Acid Extracted Peptides. Extracts were solubilized in 1 ml of 0.1% TFA, subjected to a reverse-phase HPLC column (SuperPac PepS; Pharmacia LKB) (4.0 × 250 mm, 5-μm particles C2/C18), and were eluted using the following Pharmacia LKB equipment: HPLC-pump model 2248; low pressure mixer model 2248; variable wavelength monitor model 2141; fraction collector model Frac 100; HPLC Manager software for controlling elutions and for evaluating data. Elution gradient was as follows. Solution A, 0.1% TFA; solution B, acetonitrile containing 0.1% TFA. 0–5 min, 0% B; 5–40 min, linear increase to 60% B; 40–45 min, 60% B; 45–50 min, decrease to 0% B. Flow rate, 1 ml/min; fraction size, 1 ml, HPLC separations were done at room temperature. Individual fractions were collected into 1-ml Eppendorf tubes, dried by vacuum centrifugation (Speedvac; Savant, Farmingdale, NY), and stored at −70°C.

Cytotoxic T Lymphocytes. For generation of the CTL line 13V0-5, spleen cells from a bm1 mouse (preimmunized with 10^7 irradiated EL4 cells intravenously) were stimulated in vitro with irradiated (33 Gy from a ^{137}Cs source) B6 spleen cells in α-MEM medium supplemented with 10% FCS for 7 d. Thereafter, surviving cells were restimulated weekly using medium supplemented with Con A–induced rat spleen cell supernatant as a source of IL-2. Clones (designated 13V0-5-27.2 and so on) were derived from this line by limiting dilution at a seeding density of 27 or 9 cells per well, respectively, 10 d after the line's initiation. Growing cells were found in <37% of the cultures. Subclones derived from 27.B2 and 27.7 seeded at 0.5 or 1 cell per well showed the same reactivity pattern (as tested with extracted peptides, see Table 2) as their parental clones (not shown). The line 13V0-4 and its subline 81.13 was derived from a bm1 mouse preimmunized with 10^7 B6 cells. The 26T0 series are CTL lines derived from primary in vitro cultures. 26T0-1 is bm1 anti-B6, 26T0-3 is bm3 anti-B6, and 26T0-5 is B10.HTG anti-B6. For the minor H–specific 17S0 series, BALB.B mice were immunized intraperitoneally with 10^7 RMA cells (17S0-1) or RMA-S cells (17S0-3). Responder spleen cells were stimulated in vitro with RMA or RMA-S cells, respectively, in cultures supplemented with IL-2 after day 7. Both lines do not kill BALB.B targets, indicating that they are specific for B6 minor H antigens. Both lines do not lyse the natural killer cell targets K562. The H-4^b-specific line B21W9 and the H-Y-specific line 11P9 have been described (9, 34).

CTL Assays. Lysis of either tumor target cells or Con A–induced splenic blast cells in the absence of added peptide (applies only to Table 1) was tested in a standard ^{51}Cr release assay as described (35), using 4-h incubation of CTL and target cells. For the detection of CTL-recognized peptides, dried HPLC fractions were dissolved in 300–650 μl of PBS. 30–50 μl of this solution was used to incubate 10^4 ^{51}Cr-labeled RMA-S cells (grown at normal conditions; i.e., at 37°C), or EL4 cells (in Fig. 3 only) for 90 min in a total volume of 150 μl medium in round-bottomed wells of 96-well microtiter plates. CTL were added to give a total

volume of 200 μl. Plates were then incubated for 6 h at 37°C, followed by harvesting of supernatant and determination of released radioactivity. Percent specific release was determined according to standard methods (35).

A Consideration on the Yield of Extracted Peptides. Typically, ~10% of an HPLC fraction (e.g., a fraction was dissolved in 300 μl of PBS, and 30 μl of this was used) was used for CTL assays. As can be seen from Fig. 1, *b*, *d*, *f*, and *h*, or from Fig. 4, *a–e*, this material could be further diluted by a factor of 10 for many of the fractions. Thus, 1/100 or less of the material extracted from two to four spleens is still enough to sensitize 10,000 target cells, at least for some of the peptides, as seen by 9.6, 27.B2, 26T0-3, and 27.5 CTL. On the other hand, other peptides occur hardly over detection limit, e.g., fraction 24 of bm3 cells, as recognized by 26T0-3 CTL (Fig. 4 *b*). An absolute calculation on the yield of extracted peptides is not possible in the present cases, since their sequence is not known. Using the same extraction procedures, we have calculated the copy number of K^d- or D^b-restricted viral peptides occurring in influenza-infected cells to be between 200 and 500. Synthetic peptides admixed to noninfected cells followed by mock-extraction were recovered with a yield of 25–100% (36).

Results

CTL Lines. A set of 16 different mouse CTL lines and clones, all nominally directed against the MHC class I molecule K^b and all recognizing the K^b-expressing cell lines EL4 and RMA, were tested on mutant RMA-S targets. The latter cells have been reported to express only few functional class I molecules if cultured at 37°C. Upon culture at 26°C, the cells express what has been called "empty" MHC class I molecules (29). Some of the CTL lines recognized "warm" as well as "cold" RMA-S cells, confirming earlier data (30), whereas the remaining lines did not efficiently recognize warm RMA-S cells (Table 1). Since peptide-incubated warm RMA-S cells can be recognized by peptide-specific CTL (27, 29), most of the above CTL lines were suitable for analyzing peptide involvement in alloreactivity. The 13V0 series and 26T0-1 were produced in the mouse strain combination B6.C-H-2^{bm1} (bm1; H-2^{bm1}) anti-C57BL/6 (B6, H-2^b). 26T0-3 is bm3 anti-B6, and 26T0-5 is B10.HTG (K^dD^b) anti-B6. Table 1 shows the reactivity pattern of these CTL on several target cells.

Isolation of Naturally Processed "Allopeptides". We have recently described an acid extraction method allowing isolation and analysis of naturally processed minor histocompatibility and viral peptides, as recognized by MHC class I–restricted CTL (9, 11). This method was applied to extract naturally occurring peptides from K^b-expressing B6 and from K^b-negative BALB/c (H-2^d) spleen cells. The extracted

Table 1. *Recognition of Selected Target Cells by a Series of K^b-reactive and Minor H–specific CTL*

	Target cells*									
CTL	B6	bm1	bm3	B10.HTG	BALB/c	Jurkat	Jurkat-K^b	RMA	RMA-S	RMA-S (26°C)
Anti-K^b										
13V0-5	71/39/40	0/0/0	81/62/64	0/1/0	0/0/0	14/8/3	72/61/53	84/88/88	40/20/9	81/53/42
13V0-5-27.2	18/24/41	1/3/0	27/31/28	0/0/0	0/0/0	19/11/6	51/43/52	85/98/92	35/35/30	72/73/69
27.5‡	46/35/29	0/0/0	33/46/38	0/0/0	0/0/0	21/13/6	50/51/53	79/97/90	0/1/0	11/10/4
27.7	59/71/70	0/0/0	0/0/0	0/0/0	0/0/0	9/8/2	46/48/40	82/81/79	1/5/4	21/24/25
27.B1	65/59/43	0/0/2	0/0/0	0/0/0	0/0/0	24/8/3	92/83/66	86/75/64	1/1/0	11/9/3
27.B2‡	59/50/60	0/1/0	0/0/5	0/0/0	0/0/0	25/12/5	95/89/86	89/75/75	33/24/12	32/23/13
27.J	93/27/6	0/0/0	0/0/0	0/0/0	1/0/0	19/10/6	37/29/21	68/57/45	0/1/1	1/0/0
9.1	12/0/5	0/0/0	19/29/28	0/0/0	0/0/0	10/6/6	29/26/19	80/81/84	14/12/8	53/45/30
9.5	74/64/56	1/0/0	0/0/0	0/1/0	0/0/0	18/10/2	39/38/24	81/93/85	9/11/8	99/86/72
9.6	57/51/42	2/0/1	0/0/0	0/0/0	0/0/0	27/18/7	65/65/74	87/87/87	0/1/0	16/14/15
9.11	14/11/9	0/0/0	5/0/0	0/0/0	0/0/0	13/8/2	54/55/46	51/45/22	1/1/1	1/0/0
13V0-4	23/24/30	0/0/0	0/0/0	0/0/0	0/0/0	27/12/8	35/25/15	19/11/6	0/0/0	2/1/0
13V0-4-81.13	35/13/22	0/2/5	25/21/25	0/0/0	0/0/2	19/13/7	29/18/13	77/89/78	3/1/2	11/15/14
26T0-1	31/35/41	0/4/0	0/0/6	0/0/0	0/0/0	20/11/2	71/62/47	80/82/81	9/8/6	55/42/28
26T0-3	46/46/31	0/0/0	0/0/0	0/0/0	0/0/0	11/10/3	41/45/42	78/79/80	14/10/7	50/43/26
26T0-5	31/28/16	1/0/0	27/0/3	2/1/0	0/0/0	13/11/1	45/32/21	75/82/63	4/4/2	35/24/7
Minor H–specific CTL										
17S0-1	45/44/42	61/22/38	54/23/31	60/30/28	2/0/0	7/6/1	12/11/5	71/92/80	14/26/20	89/84/56
17S0-3	72/60/56	56/57/46	49/43/64	69/56/65	0/0/0	19/16/10	23/20/10	76/75/88	61/68/59	86/79/67

* Target cells were Con A–induced blasts from spleen cells of the strains indicated, or tumor cells. The numbers indicate specific lysis of target cells at relative E/T ratios of 1:1/1:3/1:9. Starting E/T ratios ranged between 3:1 and 12:1.

‡ The clones 27.5 and 27.B2 were also tested on B10.D2 and B10.S target cells, which were not killed.

peptides were separated by reverse-phase HPLC. Warm RMA-S cells were incubated with individual peptide fractions and tested for recognition by alloreactive CTL. The detailed recognition patterns of B6 and BALB/c extracts by some selected CTL lines are shown in Fig. 1; a summary of the behavior of all CTL lines tested appears in Tables 2 and 3. The data indicate that almost every one of the 16 CTL lines shows a unique antigen specificity, the majority with peptide involvement.

Peptides Dependent on Specific MHC Alleles. The clone 27.7 recognizes a peptide eluting at fraction 26 of B6, but not BALB/c extracts (Table 2, line 3). The occurrence of this peptide is MHC class I dependent (10), as indicated by its presence in BALB.B ($H\text{-}2^b$) and BALB.5R (K^bD^d), but not BALB.HTG (K^dD^b) extracts (Fig. 2, *a–c*). This is formally demonstrated by the presence of this peptide (fraction 26) in Jurkat-K^b, but not Jurkat cells (Fig. 2 *d*). The latter are human tumor cells with or without transfected K^b (22). The peptide is also detected in B10.129-H-4^b ($H\text{-}2^b$) and 129/Sv ($H\text{-}2^b$), but not in B10.BR ($H\text{-}2^k$), B10.D2 ($H\text{-}2^d$), or B10.S ($H\text{-}2^s$) extracts (Fig. 2 *g*; Table 2). Thus, the peptide is strictly dependent on expression of K^b molecules but appears independent of non-MHC background genes. The clone 9.6 is very similar in its reactivity pattern (Fig. 1 *a*; Table 2, line 9; Fig. 2, *e* and *h*); 9.6 and 27.7 actually represent the only recurrent specificities among the 16 CTL tested. The behavior of the two clones is reminiscent of that of minor H– or virus-specific CTL, which also recognize peptides absolutely dependent on specific MHC alleles (10, 11). Indeed, the peptide recognized by 9.6 as well as the K^b-restricted minor H peptide H-4^b could be eluted from purified K^b molecules (Fig. 3 *a*). By contrast, 9.6 did not recognize any peptide eluted from purified D^b molecules, although the D^b-restricted H-Y peptide could be detected (Fig. 3 *b*). The rela-

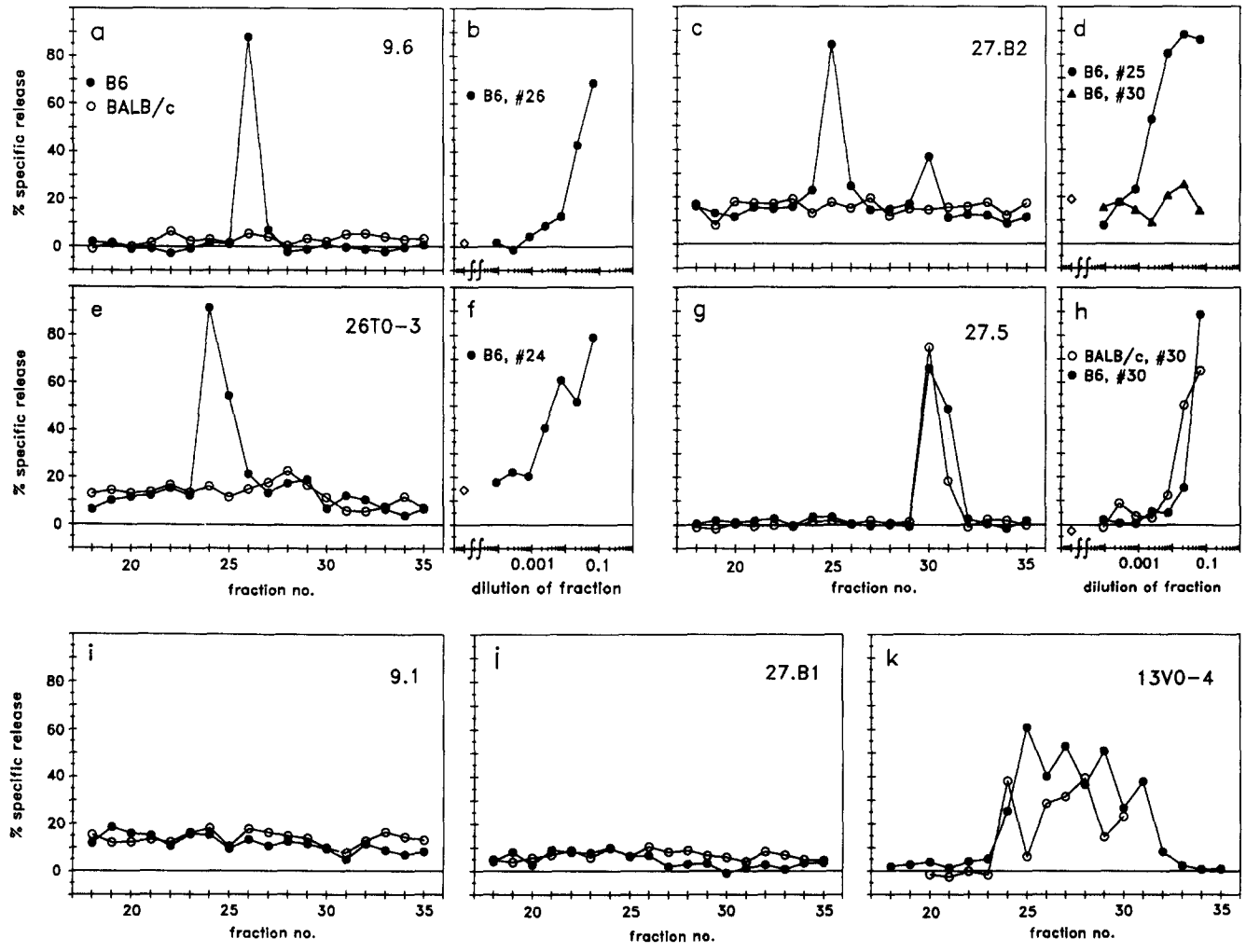

Figure 1. Screening of K^b-directed, alloreactive CTL on B6 and BALB/c peptide extracts. 9.6 (*a* and *b*), 27.B2 (*c* and *d*), 26T0-3 (*e* and *f*), 27.5 (*g* and *h*), 9.1 (*i*), 27.B1 (*j*), and 13V0-4 (*k*) CTL were tested for recognition of individual HPLC fractions of peptide extracts prepared from B6 (●) or BALB/c (○) spleen cells. *b*, *d*, *f*, and *h* show recognition of individual HPLC fractions in titrated concentrations. OD profiles of the separated material are not shown in this paper, since they look essentially identical to those published in references 9 and 10. We never found any correlation between any particular OD peak and an activity peak. E/T ratio was between 1:1 and 5:1, spontaneous release of target cells ranged between 17.9% and 30.7%.

Table 2. *Recognition of Peptide Extracts from Various Sources by K^b-reactive CTL*

CTL*	Extracts from: B6	bm1	bm3	BALB/c	B10.BR	B10.S	Jurkat	Jurkat-K^b	K^b	D^b	cat.
13V0-5	24(++),27(+)	26-30(+),34(+)	24-28(++),34(+)	(−)	ND	ND	ND	ND	ND	ND	pp
13V0-5-27.5	26(−/+),30/31(++)	30/31(++)	24/25(++),30/31(++)	30/31(++)	30/31(++)	30/31(++)	30/31(++)	30/31(++)	(−)¶	(−)	ip,pp
27.7‡	26(++)	(−)	(−)	(−)	(−)	(−)	29(+/−)	24(+),26(++),29(++)	ND	ND	fp
27.B1	(−)	ND	ND	(−)	ND	ND	(−)	(−)	ND	ND	?**
27.B2	25(++),30(+)	25(++)	25/26(++)	(−)	(−)	25(+)	(−)	24-26(++),29(+)	25/26(++)	(−)	pp
27.J	27(+/−)	ND	ND	(−)	ND	ND	ND	ND	ND	ND	pp
9.1	(−)	ND	ND	(−)	ND	ND	ND	ND	ND	ND	np
9.5	(−)	ND	ND	(−)	ND	ND	ND	ND	ND	ND	np
9.6	26(++)	(−)	(−)	(−)	(−)	(−)	(−)	26(++),29(+)	27(++)	(−)	fp
9.11	28(+)	ND	(−)	(−)	ND	ND	ND	ND	ND	ND	fp
13V0-4§	24-31(+)	ND	ND	24-31(+)	ND	ND	ND	ND	ND	ND	vp§
13V0-4-81.13	(−)	(−)	28/29(+)	(−)	ND	ND	(−)	(−)	ND	ND	pp
26T0-1	(−)	(−)	ND	(−)	ND	ND	ND	ND	ND	ND	np
26T0-3‖	24/25(++)	(−)	24(+)	(−)	(−)	(−)	(−)	24(+)	24–26,29/30(++)	30(+/−)	pp
26T0-5	(−)	ND	ND	(−)	ND	ND	ND	ND	ND	ND	np

Peptide extracts from the source indicated were tested for CTL recognition. Columns K^b and D^b indicate peptides eluted from purified MHC molecules of BALB.B spleen cells, as described in Fig. 3. The numbers indicate the fraction number(s) recognized by CTL; (++), (+), (+/−), or (−) indicate the degree of lysis. Compare, for example, the values for 27.B2 CTL with Fig. 1 *c*, Fig. 2 *a* and *k*, and Fig. 4 *a*. The column cat. indicates the classification of individual CTL into categories; see text for explanation.

* The K^b-reactive CTL are the same as in Table 1, except for 27.2, which is not included here due to its high lysis of RMA-S in the absence of added peptide.

‡ The clone 27.7 was tested for recognition of additional cell extracts. Fractions 26 each of both H-2^b strains B10.129-H-4^b and 129/Sv, but not B10.D2 (H-2^d), were recognized.

§ Shown is the behavior of this line between 7 and 10 wk after initial stimulation. In the meantime, the line matured into an fp or pp line, since it recognizes now only fraction 26 of B6 but not BALB/c extracts.

‖ The line 26T0-3 was also tested for recognition of B10.D2 extract, no fraction of which was recognized.

¶ The failure of detecting any peptide eluted from K^b molecules with this clone is probably due to the low amount of the pp peptide in K^b cells (fraction 24/25/26). If it is occasionally detected (see B6 column, and RMA-S (26°C) column in Table 3), it is barely over detection limit.

** 27.B1 CTL are peptide dependent, since they recognize fraction 32 of RMA extracts (see Table 3).

Table 3. *CTL Recognition of Peptide Extracts from RMA-S Cells*

CTL	Extracts from: RMA	RMA-S	RMA-S (26°C)
13V0-5-27.5	30/31(++)	30/31(++)	24(+/−),30/31(++)
27.7	26/27(++)	(−)	26(+),31(+)
27.B1	32(++)	ND	ND
27.B2	24-26(++),30(+)	(−)	(−)
13V0-4-81.13	29(+/−)	ND	ND
26T0-3	24-26(++)	(−)	(−)*

Peptides extracted from RMA or RMA-S cells cultured at 37°C, or from RMA-S cells cultured at 26°C, were tested for recognition by the CTL indicated. Presentation of the data is as in Table 2.
* See Fig. 3 *e*.

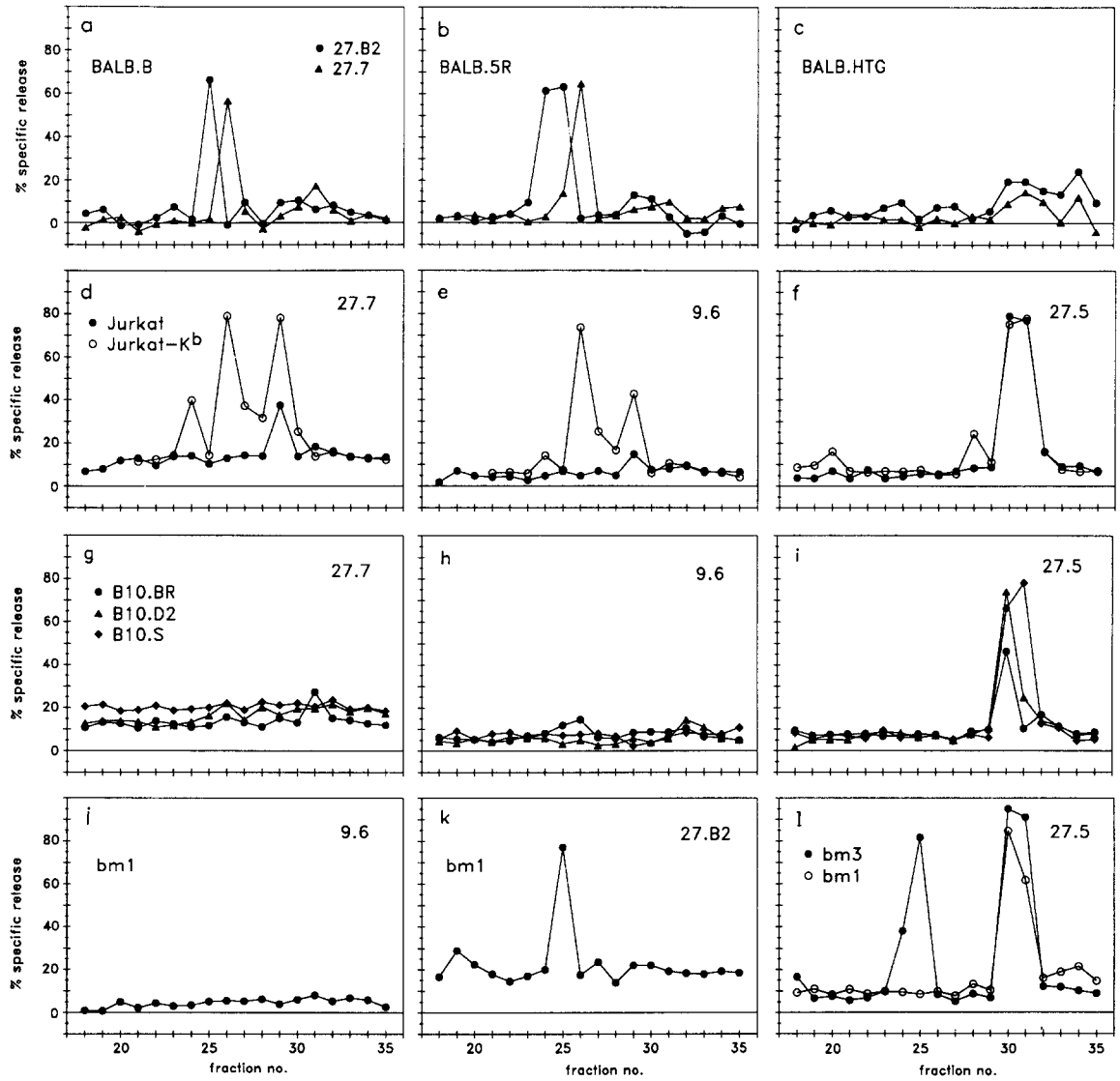

Figure 2. MHC dependency of allopeptides. Acid extracts from BALB.B (K^bD^b) (*a*), BALB.5R (K^bD^d) (*b*), BALB.HTG (K^dD^b) (*c*), B10.BR ($H\text{-}2^k$) (●), B10.D2 ($H\text{-}2^d$) (▲), or B10.S ($H\text{-}2^s$) (◆) (*g–i*), or bm1 (*j* and *k*), or bm3 (●) and bm1 (○) (*l*) male spleens, or Jurkat (●) and Jurkat-K^b (○) cells (*d–f*) were HPLC separated and tested for recognition by 27.B2 (●) and 27.7 (▲) CTL (*a–c*, *k*), or 27.7 (*d* and *g*), 9.6 (*e*, *h*, and *j*), or 27.5 (*f*, *i*, and *l*) CTL as in Fig. 1. E/T ratio was between 5:1 and 21:1; spontaneous release ranged from 15.9% to 28.7%.

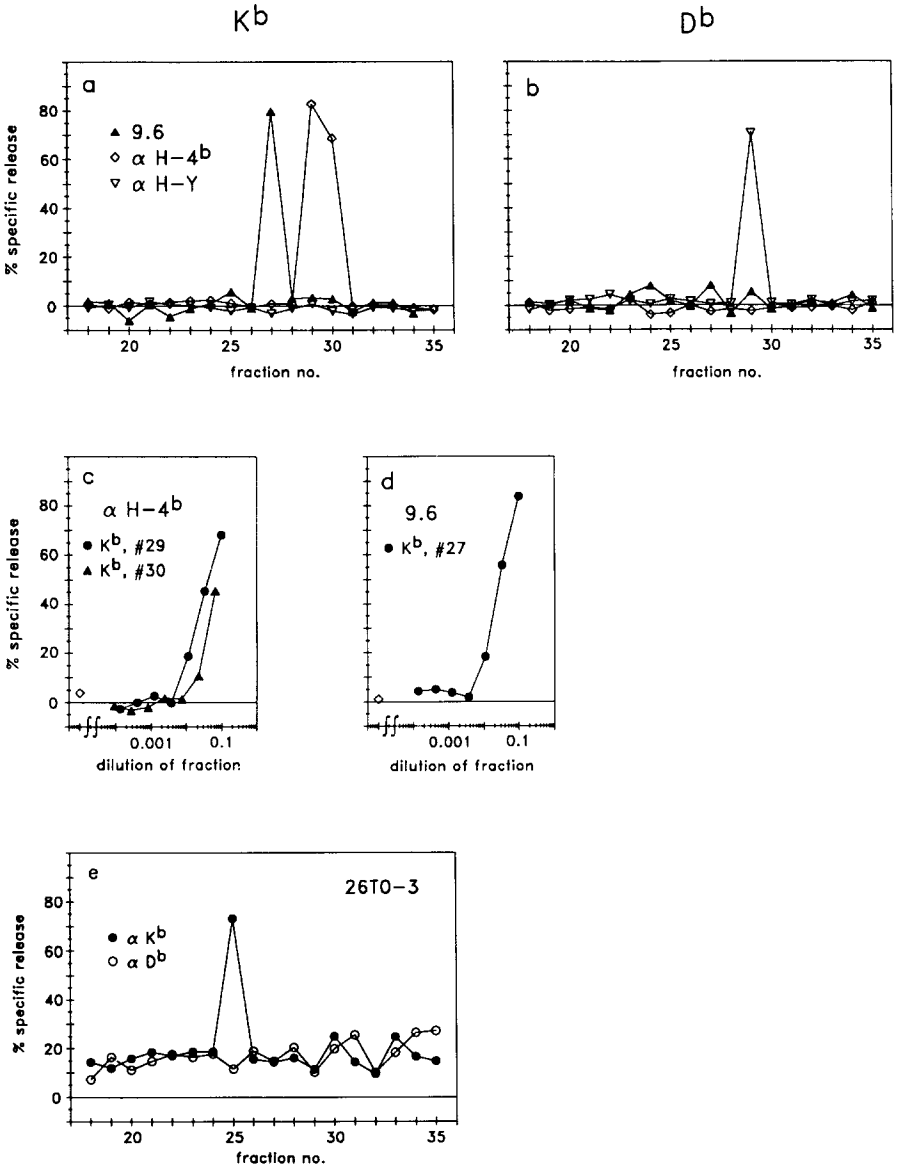

Figure 3. Extraction of peptides from purified MHC class I molecules. K^b (*a*) or D^b (*b*) molecules were immunoprecipitated from BALB.B male spleens. Peptides eluted from these preparations were HPLC separated and tested as in Fig. 1 for recognition by K^b-directed, alloreactive CTL 9.6 (▲) on RMA-S target cells, or by K^b-restricted, H-4^b-specific CTL B21W9 (◇), or by D^b-restricted, H-Y-specific CTL 11P9 (▽) on EL4 target cells (*a–d*). Fractions 29 (●) and 30 (▲) of *a* were assayed in titrated concentrations with B21W9 CTL (*c*), and fraction 27 of *a* was tested with 9.6 CTL (*d*). (*e*) K^b or D^b molecules immunoprecipitated from cold RMA-S cells were subjected to acid extraction. HPLC-separated fractions of K^b-eluted peptides (●) or D^b-eluted peptides (○) were incubated with ^{51}Cr-labeled RMA-S cells and assayed for recognition by 26T0-3 CTL. E/T between 2:1 and 12:1, spontaneous release between 17.3% and 30.7%.

tive quantities of the H-4^b minor H peptide and the allopeptide recognized by 9.6 do not differ, as indicated by the titration experiment in Fig. 3, *c* and *d*).

Peptides Supported by Several MHC Alleles. The clone 27.B2 finds its main peptide around fraction 25 of K^b-expressing B6 and RMA cells, as well as in bm1 and bm3 cells (Tables 2 and 3; Fig. 2 *k*). To a smaller extent, this applies also to B10.S (see the titration experiment in Fig. 4 *a*), but is not the case with B10.BR or BALB/c cells (Fig. 1 *c*; Table 2). The peptides eluting at fraction 25 of B6, bm1, or bm3 extracts, respectively, coelute if subjected to an HPLC gradient of high resolution power (not shown), suggesting that all three are identical. The peptide can also be eluted from purified K^b, but not from D^b molecules (Table 2). It is likely that this peptide is presented by K^b, K^{bm1}, K^{bm3}, and H-2^s molecules; the clone 27.B2, however, recognizes only B6 and not bm1, bm3, or B10.S target cells. 27.B2 recognizes, to a lesser extent, a peptide at fraction 29 or 30 in some cell extracts, for example in Fig. 1 *c*. This can, however, hardly be detected, as seen in the titration experiment in Fig. 1 *d*.

Line 26T0-3 recognizes a peptide around fraction 24 in B6, Jurkat K^b, and RMA cells, as well as in bm3 cells, but not in BALB/c, bm1, B10.BR, B10.D2, or B10.S cells (Tables 2 and 3). The peptide is elutable from purified K^b molecules (in addition to another peptide at 29/30, which is not detected in total cell extracts), but not from D^b molecules (Table 2). Thus, 26T0-3 recognizes a peptide supported by at least two MHC molecules, K^b and K^{bm3}.

Exchangeable Peptides? The line 13V0-4 recognizes peptides eluting from fractions 24 to 31 of both B6 and BALB/c extracts (Fig. 1 *k*). Since this line is uncloned, it is possible that this pattern reflects a multitude of peptide-specific clones

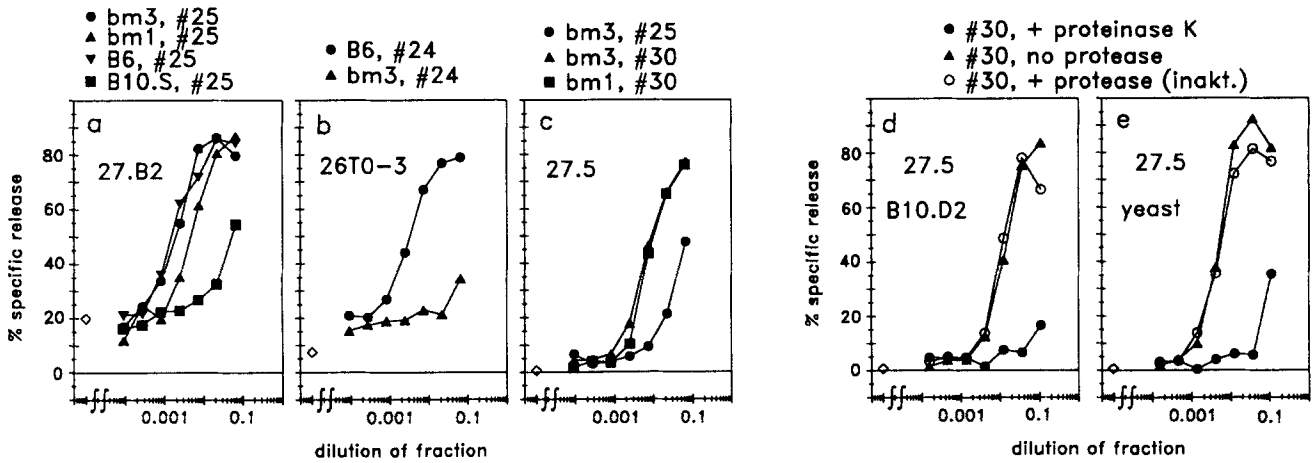

Figure 4. Relative quantities of peptides. RMA-S cells were incubated with serially diluted fractions and tested for recognition by the CTL indicated. (*a*) fraction 25 each of bm3 (●), bm1 (▲), B6 (▼), and B10.S (■) extracts were assayed with 27.B2 CTL. (*b*) fraction 24 each of B6 (●) or bm3 (▲) extracts assayed with 26T0-3 CTL. (*c*) fraction 25 of bm3 (●) and fraction 30 each of bm3 (▲) and bm1 (■) assayed with 27.5 CTL. (*d* and *e*) 27.5 CTL were tested for recognition of fraction 30 each of B10.D2 (*d*) or yeast (*e*) extracts. Before the assay, fractions were treated with Proteinase K followed by boiling (●), or by boiling alone (▲), or were mixed with self-digested Proteinase K inactivated by boiling to control for competing peptides stemming from Proteinase K (○). Proteinase K treatment was done at 0.5 mg/ml in PBS for 2 h at 37°C, followed by boiling for 3 min. E/T ratio, 3:1 to 6:1; spontaneous release, 15.9–28.0%.

present in this line. However, the MHC independency of the peptides involved may also suggest that this line recognizes K^b molecules occupied with any of a broad range of peptides.

Ubiquitous Peptide. The clone 27.5 shows an intriguing peptide specificity. It finds a peptide at fraction 30/31 in all cell extracts analyzed including RMA-S cells and nontransfected human Jurkat cells (Fig. 1, *g* and *h*; Fig. 2, *f*, *i*, and *l*; Tables 2 and 3). Thus, this rather ubiquitous peptide (which is a genuine peptide, as shown by 99% reduction of its activity after proteinase K treatment; Fig. 4 *d*) is completely MHC independent. Indeed, it cannot be eluted from purified K^b or D^b molecules (Table 2). In addition, however, the clone sometimes finds (to a marginal extent) a peptide at fraction 26 of B6 extracts, and 24 of cold RMA-S cells (Table 3). The clone efficiently detects a peptide at fraction 24/25

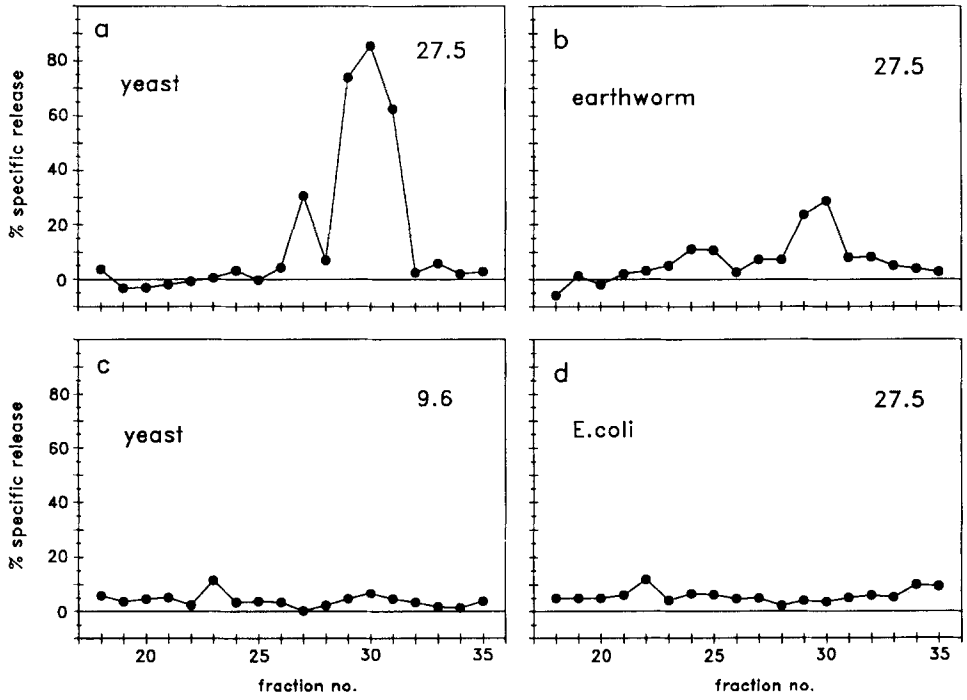

Figure 5. Peptides derived from other species. The ip clone 27.5 (*a*, *b*, and *d*) and the fp-clone 9.6 (*c*) were tested for recognition of HPLC-separated peptide extracts from yeast (*a* and *c*), an earthworm (*b*), or *E. coli* (*d*). For extraction, 2 g of *Saccharomyces cerevisiae* (baker's yeast) was frozen and thawed five times and sonicated in 0.1% TFA, and further processed as for spleen cells. A specimen of *Lumbricus terrestris* was chopped, mortared, and sonicated in 0.1% TFA. Half of this material was further processed as described for spleen cells. 1.5 g of *E. coli* strain C600 pellet was frozen and thawed five times, dounced and sonicated in 0.1% TFA, and further processed as for spleen cells. E/T ratio, 3:1 to 6:1; spontaneous release, 15.9–28.0%.

of bm3 but not bm1 extracts (Fig. 2 *l*). Thus, apart from the ubiquitous peptide, 27.5 recognizes an MHC class I–dependent peptide supported by bm3, and probably also by K^b. The two peptides might actually be related. The ubiquitous one, for example, could be a precursor of the MHC-dependent one. The MHC-dependent one (even if barely detectable, as in context of K^b) is probably the only one actually presented by cells, and thus the one inducing the respective alloreactive T cell.

Heteroclitic Peptides. The K^{bm3}-supported peptide recognized by 27.5 can be considered as heteroclitic because it is apparently more abundant in bm3 cells than in K^b-expressing cells, which were used as stimulators to produce this clone. Another heteroclitic peptide is recognized by the line 81.13 (also bm1 anti B6) at fraction 28/29 of bm3, but is not detectable in B6 extracts (Table 2). Both lines recognize B6 as well as bm3 target cells (Table 1).

Peptides: Self and Nonself. All peptides (unless eventually encoded by H-2K itself) recognized by the alloreactive CTL described in this study can be considered as self peptides from both the T cell's and the target cell's view, if one uses "self peptide" in the sense of "contained in a self protein sequence". The T cell compartment, however, accepts only those peptides as self that are actually processed and presented by self cells (37). For the sake of discussion, we shall use the term "self peptide" in the latter (i.e., in the T cell's) sense.

The clone 27.B2 (bm1 anti-B6) recognizes an MHC-dependent peptide eluting at fraction 25 from both B6 and bm1 extracts in similar relative quantities (Fig. 1, *c* and *d*; Fig. 2 *k*; Fig. 4 *a*). Thus, 27.B2 recognizes a self peptide. If the very same peptide can be eluted from both purified K^b and K^{bm1} molecules, MHC restriction of self tolerance (38–40) would finally have been proven on the molecular level. The line 26T0-3 (bm3 anti-B6) is similar to 27.B2 in this regard, since the MHC-dependent peptide recognized by the former line is produced by syngeneic bm3 cells as well. The relative quantity of this peptide (fr.24) is smaller in bm3 as compared to B6 cells (Fig. 4 *b*).

The ubiquitous peptide recognized by 27.5 (bm1 anti-B6) is also produced in bm1 cells syngeneic to the CTL. However, the MHC-dependent component, probably the one actually presented by intact B6 target cells, could not be detected in bm1 cells.

Peptides from Mice and Men. The clone 27.5 clearly recognizes a peptide (fr.30/31) present in the human cell line Jurkat (Fig. 2 *f*). Since this peptide shows the same elution behavior on the reverse-phase HPLC column as the one extracted from mouse cells (Table 2), and since it is recognized by the same clone, it is likely that the determinant recognized by 27.5 in fraction 30/31 is conserved between mouse and man.

The allopeptides extracted from Jurkat-K^b and B6 cells recognized by 26T0-3 CTL (Table 2) coelute on an HPLC gradient of high resolution (not shown). The same is found with Jurkat K^b and B6-derived peptides recognized by 9.6 CTL (not shown). Thus, both peptides are likely to be conserved between mouse and man. Alternatively, the MHC-dependent peptides detected by 9.6 and 26T0-3 (and also 27.7 and 27.B2; see Table 2) in Jurkat-K^b but not in Jurkat cells are derived from the transfected K^b itself.

Peptides from Baker's Yeast and Earthworms. Since 27.5 finds a peptide in both mouse and man, we were interested in the possible presence of this peptide in other taxa. The clone detects this peptide also in an annelide, *Lumbricus terrestris*, and very efficiently in *Saccharomyces cerevisiae*, but not in *Escherichia coli* (Fig. 5, *a*, *b*, and *d*). The material extracted from yeast, which is not recognized by the clone 9.6 (Fig. 5 *c*), is indeed of peptidic nature, since its antigenicity is destroyed by proteinase K (Fig. 4 *e*). Thus, the peptide recognized by clone 27.5 appears to be conserved throughout a wide range of eukaryotic taxa, but not to be present in prokaryotes.

Can T Cells Recognize Empty MHC Molecules? For the CTL clones 27.B1, 9.1, and 9.5, as well as for the lines 26T0-1 and 26T0-5, peptides were not detected in the initial screening, which used B6 and BALB/c extracts. Since all five CTL recognize, to a greater or lesser extent, cold RMA-S cells (Table 1), we considered them as candidates for CTL capable of recognizing empty MHC molecules. However, we found later that 27.B1 recognizes fraction 32 of RMA extracts (Table 3). Although this fraction yielded 90% lysis, it was only 10-fold over detection limit, as observed in a titration experiment (not shown). It is therefore possible that due to cell type–specific differences in quantities of peptide content, we could not detect this peptide in B6 spleen cells. Consequently, our inability to detect peptides recognized by the remaining four CTL does not establish that these recognize empty MHC molecules. The fact that the latter four CTL lines react against cold RMA-S cells is also inconclusive, since we have found that several of our peptide-dependent clones (27.5, 27.7, 27.B2, and 9.6) react with cold RMA-S cells (Table 1). The peptide-specific CTL line 26T0-3, which also recognizes cold RMA-S cells (Table 1), detects its peptide in material eluted from purified K^b molecules prepared from cold RMA-S cells (Fig. 3 *e*). The same is true for the clone 27.B2 (not shown). Furthermore, the peptide for 27.7 could be extracted from cold (but not warm) RMA-S cells, albeit hardly above detection limit (Table 3). In addition, the minor H–specific lines 17S0-1 (BALB.B anti RMA) and 17S0-3 (BALB.B anti RMA-S) also recognize cold as well as warm RMA-S cells (Table 1). Other minor H–specific CTL (A.BY anti RMA-S) recognize peptides eluted from K^b molecules prepared from cold RMA-S cells (not shown).

We conclude that not all of the MHC molecules of RMA-S coming out in the cold are empty (29), and that recognition of cold RMA-S cells by T cells does not indicate that these T cells recognize empty MHC molecules. In another recent report suggesting recognition of empty MHC molecules by T cells, the absence of peptides could not be formally excluded (25). Thus, the major question posed in this paragraph is still open.

Classification. The following attempt at classification of alloreactive T cells is partially hypothetical; we feel, however, that it will simplify future discussions.

The four lines without detectable peptide involvement are still candidates for the ability to recognize "empty" MHC

molecules. We propose to provisionally term this category of alloreactive T cells np (for no peptide detectably involved). Alloreactive T cells that are demonstrated to recognize empty MHC molecules may then be allocated to an as yet hypothetical mt category. The 11 peptide-dependent lines fall into at least four categories: those recognizing a peptide produced by the target cell in a strictly MHC allele–specific way (for example, 9.6, 27.7) are termed fp (for peptide faithfully MHC dependent); those recognizing peptides occurring in the context of more than one MHC molecule (for example, 27.B2) are termed pp (for dependent on promiscuous peptides); those for which various peptides suffice (for example, probably 13V0-4) are termed vp. Although the existence of this category is not proven by our present data, we include this category here for theoretical reasons. In addition, we now have preliminary data on a CTL clone that probably will prove the existence of this category (M. Opladen et al., unpublished data). Finally, those CTL that recognize a distinct, but MHC-independent peptide (for example, 27.5) are termed ip. The peptides recognized by alloreactive T cells should then be classified accordingly into fp, pp, vp, or ip peptides. The only T cell category whose members can belong to a second category as well is ip, as exemplified by 27.5, which recognizes a K^{bm3}-dependent peptide in addition to an ip peptide. More categories might be found when alloreactive T cells from other strain combinations are being tested.

Discussion

We have analyzed the relatively low number of 16 K^b-directed alloreactive CTL lines for their peptide involvement. Most of these CTL lines show peptide dependency; all CTL but one pair are different from each other. The number of different alloreactive CTL nominally reacting against a single MHC molecule, therefore, must be large, confirming the conclusion reached by analyzing alloreactive CTL by crossreactivity patterns (41, 42). It should be noted, however, that we have taken into consideration only CTL reactive against a single MHC molecule, and most of these CTL were derived in a single strain combination, bm1 anti-B6.

Taking into account the different peptides recognized by our alloreactive CTL, the enormous number of combinatorial possibilities for binding of cellular peptides to MHC molecules appears to be the reason for the complexity of alloreactivity (15). In addition, alloreactive T cells may recognize conformational differences between MHC molecules largely independent of the peptides bound (category vp), or even on empty MHC molecules, although no conclusive evidence has been found to formally demonstrate T cell recognition of empty MHC molecules. Thus, the density hypothesis (23, 24) and the peptide hypothesis (15) are not mutually exclusive; both phenomena might contribute to alloreactivity.

As in the case of minor H and viral peptides (10, 11), the MHC molecules themselves appear to be involved in the production, or at least in the maintenance, of a portion (the fp and pp categories) of the peptides involved in T cell allorecognition, and probably of all those peptides naturally presented by MHC class I molecules. Otherwise, it would be hard to understand why some peptides occur only in cells expressing particular MHC molecules. Note that most experiments in this paper involve peptide extracts from entire cells, and not from purified MHC molecules. Several models possibly explaining the MHC dependency of peptides occurring in cells (including determinant protection or the hypothesis of MHC being itself a protease) are put together in reference 10.

For the $H\text{-}4^b$ minor histocompatibility antigen, we detected two peptides. One is MHC dependent, the other is MHC independent (10). We speculated that the latter might be a larger precursor molecule from which the smaller, MHC-dependent, peptide is derived upon interaction with MHC. The same precursor-endproduct relation could apply for the MHC independent ip peptide at fraction 30/31 of all cell extracts, and the MHC-dependent pp peptide at fraction 25 of bm3 extracts, both recognized by 27.5 CTL.

It will be interesting to determine the relative frequencies of the categories of alloreactive CTL proposed above, and of other categories likely to be discovered. This should be carried out in several MHC-incompatible responder-stimulator combinations, the outcome of which might depend on the amount and quality of differences between MHC molecules of responder and stimulator cells. For example, the $H\text{-}2K^{bm1}$ molecule differs from K^b at three amino acid residues (31), all located at the supposed peptide binding sites (13, 14). Thus, it is possible that the combination bm1 against K^b or vice versa is prone to give rise to especially high frequencies of peptide-dependent alloreactive T cells. On the other hand, reactions across unrelated MHC alleles differing in as much as 40 amino acid residues (many of which are at sites not directly involved with peptide binding) might yield more T cells of the mt category (if it exists) or more of the vp type, or of as yet unknown categories. However, the strength of T cell reactions in vivo (graft rejection) and in vitro (precursor frequencies) across single–amino acid vs. 40–amino acid MHC differences are within the same range (2–4). This argues against a principal difference in alloreactivity across related vs. unrelated MHC incompatibilities and suggests that what we see in the combination bm1 anti-B6 is reflecting the general phenomenon.

For class I–restricted CTL, we have shown that the corresponding proteins giving rise to the peptides can be located in several cellular compartments (35). It is likely that the same applies to alloreactive CTL. Some T cells have been reported to recognize peptides derived from MHC molecules (19–21). The frequency of alloreactive CTL with specificity for MHC-derived peptides remains to be determined; in principle, there is no reason to assume that such T cells should occur with higher frequency than those specific for any other peptide.

One important problem pertinent to T cell alloreactivity, namely the reason for the strong primary in vitro response, is not solved by our results. One hypothesis invokes the high density of MHC determinants presented by a cell, as opposed to the relatively low density of determinants composed of MHC and any given particular peptide (23, 24). This explanation holds only for alloreactive mt or vp T cells. Since we find alloreactive T cells that recognize specific peptides no more abundant than minor histocompatibility peptides (Fig.

3, *c* and *d*), determinant density cannot explain alloreactivity entirely. Here it is important to note that while most of the CTL (the 13V0-5 series) tested here were derived from mice immunized once in vivo, the line 26T0-3, which is also peptide specific, was derived from a primary MLC. Thus, our data do not offer an explanation for the in vitro activation of unprimed alloreactive CTL, although the data explain the high frequency and complexity of alloreactive CTL by the large number of combinatorial self peptide/MHC possibilities.

Our data on RMA-S cells seem to contradict previously reported results. It has been reported that RMA-S cells are not recognized by bulk cultures of minor H–specific CTL, that they are not rejected by minor H–incompatible recipient mice, and that virus-infected RMA-S cells are not seen by virus-specific CTL (27–30). In addition, incubation of RMA-S cells with MHC-binding peptides increases the density of detectable cell surface class I molecules. If cultured at 26°C, RMA-S cells also increase expression of detectable MHC class I molecules, which then can be stabilized by adding MHC binding peptides (27, 29). It was concluded that RMA-S cells have a defect in peptide handling, and that they express empty MHC molecules, which are unstable at the cell surface at 37°C. The recognition of RMA-S cells by alloreactive CTL was taken as evidence for recognition of empty MHC molecules. Since we find recognition of RMA-S cells by peptide-specific alloreactive CTL and also by minor H–specific CTL, and since we can elute peptides from RMA-S-derived K^b molecules, we conclude that the previously observed failure of RMA-S cells to produce peptides in the MHC-restricted presentation pathway is not absolute. We should note here that RMA-S cells behave in our hands as described earlier, as far as immunofluorescence intensity tested with MHC-specific antibodies is concerned (not shown). In addition, minor H–specific CTL derived directly from bulk cultures (29) do not kill RMA-S cells also in our hands (not shown). One possibility for the apparent discrepancy is that RMA-S cells may have a defect limiting the amount of a given peptide to be presented, so that only the most abundant peptides are presented efficiently enough to be recognized by T cells. Another possibility is that RMA-S cells can present only a selected set of peptides, for example those derived from proteins present in the endoplasmic reticulum (ER).[1] It has been speculated that the defect in RMA-S cells lies in the failure of transporting peptides from the cytosol to the ER (27, 43). Regardless of the reason behind the defect of RMA-S cells, it is clear that these mutant cells are extremely useful. The presentation of a few peptides by these cells does not take away from their value for studying MHC biology.

One view of the interaction between TCR and MHC/peptide is that the TCR is in physical contact with both latter molecules (44). Another view is that the TCR touches only the peptide and that the apparent MHC specificity of T cells is imposed by the nature of the peptide selected by MHC molecules for binding (45). A third possibility is that the TCR touches only the MHC molecule, on which bound peptides can impose confirmational changes. Our data appear to argue against the second view, since we find that one peptide, which is presented by three different MHC molecules (K^{bm1}, K^{bm3}, and K^b), is recognized by the TCR of clone 27.B2 only in the context of one of these (K^b). One could still argue that MHC imposes a change in conformation upon bound peptides, which is then seen by the TCR. However, since MHC class I molecules generally present nonapeptides or octapeptides (this notion is based on the finding that most naturally processed viral and self peptides are nonapeptides or octapeptides; 11, 12, 46); the change in conformation would therefore have to be imposed on such small peptides.

A pertinent question is whether there is any physiological function for the obviously enormous number of different self peptides presented by normal cells. It is likely that that high number is a consequence of the cell's inability to discriminate between self and nonself: presentation of as many peptides as possible would represent selective advantage by increasing the chance of presenting peptides derived from pathogens. In addition, however, it is possible that the many different presented self peptides are the ones involved in positive selection of immature T cells in the thymus (47), since indirect evidence indicated involvement of peptides in positive selection (48, 49). If the set of self peptides presented by thymic epithelium, which induces positive selection (50), is roughly the same as found in spleen cells, for example, one would have to assume that immature T cells are selected to recognize this multitude of self peptides with low affinity, which is enough for inducing the differentiation signal required by the T cells at that stage, but not enough to trigger mature T cells. Thereby, T cells would be selected to preferentially recognize any peptide together with self MHC, under the assumption that those T cells with low affinity for self peptides presented on self MHC tend to crossreact with high affinity to other peptides presented by the same MHC. This model would imply that immature T cells are selected according to their ability to react with the allele-specific peptide motifs presented by self MHC class I molecules (46).

The conserved epitope recognized by the clone 27.5 appears to be a curiosity, since it is found in all the eukaryotes looked at (mouse, man, and annelide, and yeast) but not in a prokaryote. It will certainly be of academic interest to identify this peptide; candidates for it include peptides derived from conserved proteins such as histones or ubiquitin, which have then to be cut by MHC-independent proteases. Alternatively, the peptide might occur in cells as such. It is conceivable, for example, that a leader peptide, which is essentially a leftover product after a leader-containing protein has entered the ER and had its leader clipped off, binds to MHC molecules in the ER, and is then trimmed to the form finally presented. What the conserved peptide seen by 27.5 certainly illustrates, however, is the complexity of T cell alloreactivity. It appears that any self peptide, fulfilling the allele-specific requirements for MHC class I–restricted presentation (46), can be presented by MHC and serve as target for alloreactive T cells.

In conclusion, we have shown that alloreactive T cells nominally directed against a single MHC class I molecule show

[1] *Abbreviation used in this paper:* ER, endoplasmic reticulum.

a high degree of complexity in their peptide specificities. It will be of interest to identify the peptides involved using new technologies (11, 46). Molecular information on the peptides involved in allorecognition and the corresponding proteins should further our understanding of MHC-restricted antigen presentation, self tolerance, and, as mentioned before, of positive thymic selection.

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Address correspondence to Hans-Georg Rammensee, Max-Planck-Institut für Biologie, Abt. Immungenetik, Corrensstr. 42, D-7400 Tübingen, Germany.

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