On the Nature of Peptides Involved in T Cell
Alloreactivity

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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 Kb-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 an-
tigen. 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 α1 and α2 domains (13, 14). In contrast to
these relatively well-understood topics, the phenomenon of
T cell alloreactivity is still a puzzling question for immunol-
gists.

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 allorrecognition in one case; the peptide in ques-
tion, however, had also to be produced artificially (22). Al-
loreactivity has also been suggested to involve low affinity
recognition of high-density alloantigens (23). It can be en-
visaged, for example, that T cells recognizing foreign MHC
molecules independent of any peptide meet some 105 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-in-
compatible mouse strain combinations that could be used to
raise alloreactive CTL, we used B6.C-H-2<sup>bm1</sup> anti-C57BL/6
(abbreviated, bm1 anti-B6) in most experiments. Both strains
differ at a mutation in the H-2K gene; the K<sup>b</sup> molecule
expressed by C57BL/6 and the K<sup>bm1</sup> molecule differ at three
amino acid residues located in the peptide binding cleft (31).
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'Db), B6.C-H-2\textsuperscript{mix} (bm1; E\textsuperscript{bm}D\textsuperscript{b}), B6.C-H-2\textsuperscript{mix} (bm3; K\textsuperscript{bm}D\textsuperscript{b}), BALB/c (K\textsuperscript{d}D\textsuperscript{d}), BALB.B (K\textsuperscript{d}D\textsuperscript{d}), BALB.120R (K\textsuperscript{d}D\textsuperscript{d}), B10.D2 (K\textsuperscript{d}D\textsuperscript{d}), and B10.S (K\textsuperscript{d}D\textsuperscript{d}) 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\textsuperscript{a}), Jurkat, and Jurkat-K\textsuperscript{K} (22) cells were maintained in RPMI 1640 supplemented with 5% FCS at 37°C in a 5% CO\textsubscript{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), resuspended in 2.5 ml of 0.1% TFA, and subjected to acid extraction as above. A 4.2-g specimen of Lumbricus terrestris was collected next to the parking lot of the institute.

Acid Extraction of Peptides from Whole Cells. Two to four spleens of the mouse strains indicated, or 10\textsuperscript{6} 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), to which Kb-specific K9-178 (32) or D\textsuperscript{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\textsuperscript{6} RMA-S cells kept for 24 h at 37°C were centrifuged (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\textsuperscript{b} beads, then over a column with anti-D\textsuperscript{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\textsuperscript{7} irradiated EL4 cells intravenously) were stimulated in vitro with irradiated (33 Gy from a 137Cs source) B6 spleen cells in αMEM 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 B1.13 was derived from a bm1 mouse preimmunized with 10\textsuperscript{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\textsuperscript{7} RMA cells (1750-1) or RMA-S cells (1750-3). 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- 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 11Cr 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\textsuperscript{4} 11Cr-labeled RMA 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 PBS, and 30/µl of this was used) was used for CTL assays. Anti-Kb

| Table 1. | Recognition of Selected Target Cells by a Series of Kb-reactive and Minor H–specific CTL |
|----------|----------------------------------|
| CTL      | B6 | bm1 | bm3 | B10-HTG | BALB/c | Jurkat | Jurkat-Kb | RMA | RMA-S (26°C) |
| Anti-Kb  |     |     |     |         |        |        |           |     |               |
| 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 | 1/0/0 | 0/0/0 | 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 | 0/0/0 | 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 | 0/0/0 | 19/13/7 | 29/18/13 | 77/89/78 | 3/1/2  | 11/15/14 |
| 26T0-1  | 31/35/41 | 0/0/2 | 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 | 4/4/3  | 35/24/7 |

Minor H–specific CTL

| CTL     | B6 | bm1 | bm3 | B10-HTG | BALB/c | Jurkat | Jurkat-Kb | RMA | RMA-S (26°C) |
|---------|----|----|----|--------|--------|--------|-----------|     |               |
| 1750-1  | 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 |
| 1750-3  | 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.

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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-2b) and BALB.5R (Kvd6), but not BALB.HTG (KvdD6) extracts (Fig. 2, a–c). This is formally demonstrated by the presence of this peptide (fraction 26) in Jurkat-Kb, but not Jurkat cells (Fig. 2 d). The latter are human tumor cells with or without transfected Kb (22). The peptide is also detected in B10.129-H-4b (H-2b) and 129/Sv (H-2b), but not in B10.BR (H-2b), B10.D2 (H-2b), or B10.S (H-2b) extracts (Fig. 2 g; Table 2). Thus, the peptide is strictly dependent on expression of Kb 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 Kb-restricted minor H peptide H-4b could be eluted from purified Kb molecules (Fig. 3 a). By contrast, 9.6 did not recognize any peptide eluted from purified Db molecules, although the Db-restricted H-Y peptide could be detected (Fig. 3 b). The relat-

![Figure 1. Screening of Kb-directed, alloreactive CTL on B6 and BALB/c peptide extracts. 9.6 (a and b), 27.82 (c and d), 26T0-3 (e and f), 27.5 (g and h), 9.1 (i), 27.81 (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%.](image-url)
### Table 2. Recognition of Peptide Extracts from Various Sources by K<sup>b</sup>-reactive CTL

| CTL<sup>*</sup> | B6         | bm1      | bm3         | BALB/c     | B10.BR     | B10.S      | Jurkat     | Jurkat-K<sup>b</sup> | K<sup>b</sup> | D<sup>b</sup> | 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(+ +) | ND                   | ND             | ND          | fp   |
| 27.B1          | (−)        | ND       | ND          | (−)        | (−)        | 29(+/-)    | 24(+),26(+ +),29(+) | ND                   | ND           | ND          | pp   |
| 27.B2          | 25(+ +),30(+) | 25(+ +) | 25/26(+ +) | (−)        | (−)        | 25(+)     | 24-26( + +),29(+) | ND                   | ND           | ND          | ip, pp |
| 27.1           | 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(+)                  | (−)           | ND          | np   |
| 9.11           | 28(−)      | ND       | ND          | (−)        | ND         | ND         | ND         | ND                   | ND             | ND          | fp   |
| 13V0-45        | 24-31(+)   | ND       | ND          | 24-31(+)   | ND         | ND         | ND         | ND                   | ND             | ND          | vp<sup>3</sup> |
| 13V0-4-81.13   | (−)        | (−)      | 28/29(+)    | ND         | ND         | ND         | ND         | ND                   | ND             | ND          | vp<sup>3</sup> |
| 26T0-1         | (−)        | (−)      | ND          | (−)        | ND         | ND         | ND         | ND                   | ND             | ND          | np   |
| 26T0-3<sup>4</sup> | 24/25(+ +) | (−)  | 24(+)       | (−)        | (−)        | (−)       | 24(+)     | 24-26,29/30( + +) | 30(+/-)        | pp          |      |
| 26T0-5         | (−)        | ND       | ND          | ND         | ND         | ND         | ND         | ND                   | ND             | ND          | np   |

Peptide extracts from the source indicated were tested for CTL recognition. Columns K<sup>b</sup> and D<sup>b</sup> 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. 1c, Fig. 2a and k, and Fig. 4a. The column cat. indicates the classification of individual CTL into categories; see text for explanation.

<sup>*</sup> The K<sup>b</sup>-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.

<sup>1</sup> The clone 27.7 was tested for recognition of additional cell extracts. Fractions 26 each of both H-2<sup>b</sup> strains B10.129-H<sup>4</sup>b and 129/Sv, but not B10.D2 (H-2<sup>d</sup>), were recognized.

<sup>2</sup> 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.

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

<sup>4</sup> The failure of detecting any peptide eluted from K<sup>b</sup> molecules with this clone is probably due to the low amount of the pp peptide in K<sup>b</sup> 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               | 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(++)    | (-)          | (-)           |
| 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 (KbD b) (a), BALB.5R (KbD b) (b), BALB.HTG (KdD b) (c), B10.BR (H-2 k) (d), B10.D2 (H-2 k) (e), or B10.S (H-2 k) (f) male spleens, or Jurkat (g-i) cells (d-f) were HPLC separated and tested for recognition by 27.B2 (h) and 27.7 (g) or 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%.  

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Peptides Supported by Several MHC Alleles. The clone 27.B2 finds its main peptide around fraction 25 of Kb-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 Kb, but not from Db molecules (Table 2). It is likely that this peptide is presented by Kb, Km1, Km3, and H-2' 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 Kb, 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 Kb molecules (in addition to another peptide at 29/30, which is not detected in total cell extracts), but not from Db molecules (Table 2). Thus, 26T0-3 recognizes a peptide supported by at least two MHC molecules, Kb and Km3.

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 3. Extraction of peptides from purified MHC class I molecules. Kb (a) or Db (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 Kb-directed, alloreactive CTL 9.6 (△) on RMA-S target cells, or by Kb-restricted, H-4b-specific CTL B21W9 (●), or by Db-restricted, HY-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) Kb or Db molecules immunoprecipitated from cold RMA-S cells were subjected to acid extraction. HPLC-separated fractions of Kb-eluted peptides (△) or Db-eluted peptides (○) were incubated with 51Cr-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%.
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

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**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 (O). 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%.

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**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, doused 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 f). Thus, apart from the ubiquitous peptide, 27.5 recognizes an MHC class I-dependent peptide supported by bm3, and probably also by K\textsuperscript{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, in context of K\textsuperscript{b}) is probably the only one actually presented by cells, and thus the one inducing the respective alloreactive T cell.

**Heterologic Peptides.** The K\textsuperscript{bm3}-supported peptide recognized by 27.5 can be considered as heterologic because it is apparently more abundant in bm3 cells than in K\textsuperscript{b}-expressing cells, which were used as stimulators to produce this clone. Another heterologic 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\textsuperscript{b} and K\textsuperscript{bm3} 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\textsuperscript{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\textsuperscript{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\textsuperscript{b} but not in Jurkat cells are derived from the transfected K\textsuperscript{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\textsuperscript{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\textsuperscript{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 might be found when alloreactive T cells from other independent peptide (for example, 27.5) are termed ip. 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-2Kb bm1 molecule differs from Kb 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 Kb 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.
immunized once in vivo, the line 26T0-3, which is also pep-
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 pep-
tide 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 recip-
ient 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 den-
sity 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 sur-
face 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
Kb 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 addi-
tion, minor H-specific CTL derived directly from bulk cul-
tures (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 pep-
tides 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/pep-
tide 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 pep-
tides can impose conformational changes. Our data appear
to argue against the second view, since we find that one pep-
tide, which is presented by three different MHC molecules
(Kb1, Kb3, and Kd), is recognized by the TCR of clone
27.B2 only in the context of one of these (Kb). 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 selec-
tion of immature T cells in the thymus (47), since indirect
evidence indicated involvement of peptides in positive selec-
tion (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 recog-
nize 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 pep-
tides 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 ac-
tording to their ability to react with the allele-specific pep-
tide motifs presented by self MHC class I molecules (46).

The conserved epitope recognized by the clone 27.5 ap-
ppears 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 iden-
tify 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. Alter-
natively, the peptide might occur in cells as such. It is con-
ceivable, for example, that a leader peptide, which is essen-
tially 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 nomi-
nally 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.

We thank J. Klein for support, K. Kärre and G. J. Hämerling for RMA and RMA-S cells, L. A. Sherman for Jurkat and Jurkat-K° cells, C. Schönbach for E. coli cells, and H.-J. Wallny and L. Yakes for reading the manuscript.

This work was supported by Sonderforschungsbereich 120.

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Received for publication 28 May 1991 and in revised form 23 July 1991.

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