Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6949–6951, August 1993
Immunology

Alloantibodies can discriminate class I major histocompatibility complex molecules associated with various endogenous peptides

LINDA A. SHERMAN*†, SUSCHMITTA CHATTOPADHYAY*, JUDITH A. BIGGS*, ROBERT F. DICK, II‡, AND JEFFREY A. BLUESTONE‡

*Department of Immunology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037; and ‡University of Chicago, Ben May Institute, MC 1089, Department of Pathology, 5841 South Maryland Avenue, Chicago, IL 60637

Communicated by Herman N. Eisen, April 29, 1993

ABSTRACT **Molecules encoded by a single major histocompatibility complex class I gene can associate with any one of a large number of peptide ligands. T-cell receptors have the capacity to discriminate among these peptide–class I complexes and in many cases bind only a single peptide–class I complex with sufficient affinity to trigger effector function. In contrast, it is generally assumed that class I-specific alloantibodies are indifferent to peptide heterogeneity, being directed toward allele-specific determinants on the molecule. In this report, three monoclonal antibodies were used to precipitate K^b molecules from cell lysates. Surprisingly, in each case a different set of peptides was found to be associated with K^b as detected by peptide-dependent K^b-specific alloreactive cytolytic T lymphocytes or by biochemical resolution. These results demonstrate that the affinity of binding by alloantibodies can be affected by the endogenous peptide ligand.**

Stable expression of class I major histocompatibility complex (MHC) molecules is dependent on their association with short peptide ligands that are derived from proteolysis of endogenously synthesized proteins (1–6). As a result of sequence polymorphism within the peptide binding groove, each allelic form of class I binds a distinct set of peptides. The members of each set have a sequence motif in common that is sufficiently unrestrictive to be shared in theory by hundreds to thousands of peptides of similar length (3). This peptide diversity is the basis for enormous heterogeneity among cell surface class I molecules encoded by the same MHC gene.

Several cell lines, including the human cell line T2, have been described that carry mutations in genes that affect peptide loading of class I molecules and as a result express empty class I molecules (7–9). These molecules can be exogenously loaded with a single type of synthetic peptide thereby creating a situation in which all class I–peptide complexes on the cell surface are identical. In a set of recent reports, we (10) and others (11) observed that loading unoccupied K^b molecules with different synthetic peptide antigens altered their recognition by alloantibodies. For instance, the K^b-specific monoclonal antibody (mAb) 5F1.2 bound K^b-ovalbumin peptide complexes significantly better than empty K^b or K^b–Sendai (SV9) peptide complexes (10). In contrast, the K^b-specific mAb 100-30 reacted strongly to empty K^b or K^b–SV9 complexes but not to K^b–ovalbumin peptide complexes. These results suggested that the nature of the peptide bound to the class I molecule influenced alloantibody recognition. However, such results were obtained using synthetic antigens, some of which were much longer than endogenous peptides that are generally eight or nine residues long. Based on these results, as well as the observation by several laboratories that antibodies can be specific for certain MHC–antigen complexes (12–15), we investigated the possibility that naturally occurring endogenous class I peptides may also influence alloantibody recognition of MHC molecules expressed on cells.

MATERIALS AND METHODS

Mice. C57BL/6 mice (6–8 weeks of age) were purchased from the National Cancer Institute Animal Program at the Frederick Cancer Research and Development Center (Frederick, MD).

Cells. All K^b-specific alloreactive cytotoxic T-lymphocyte (CTL) clones used in this study and T2-K^b cells used as targets have been described (16). EL4 tumor cells were grown in C57BL/6 mice.

Preparation of Peptides. K^b molecules were purified from Nonidet P-40-solubilized cell lysates of 1.0×10^{10} EL4 tumor cells by the method of Van Bleek and Nathenson (17) using either 1 mg of antibody or 1 ml of ascites fluid coupled to protein A-Sepharose beads for immunoabsorption. The mAbs used in these studies included 5F1.2, Y3, and 100-30. All have been described (10). Cell surface binding of these IgG2 antibodies to EL4 cells indicated that 5F1.2 and 100-30 detect half the number of K^b molecules bound by Y3 as determined by indirect immunofluorescence using a fluorescent-labeled goat anti-mouse Fc-specific antibody. K^b–antibody complexes were dissociated in 0.2% trifluoroacetic acid. Low molecular weight material was separated by centrifugation through a Centricon 10 filter (Amicon), lyophilized, and resuspended in 1 ml of distilled water.

One-half of each peptide preparation was resolved by reverse-phase HPLC using a Supelco C_{18} column (220 mm × 2.1 mm). The column was equilibrated with 0.1% trifluoroacetic acid and developed with a continuous gradient of 0–60% (vol/vol) acetonitrile containing 0.08% trifluoroacetic acid over a period of 40 min. One-minute fractions were lyophilized and resuspended in 250 μl of water.

Cytotoxicity Assays. The indicated amount of peptide was incubated with 3×10^5 ^{51}Cr-labeled T2-K^b cells (16) for 1 hr in 100 μl of RPMI 1640 medium prior to dilution to a final volume of 3 ml in medium containing 5% (vol/vol) fetal bovine serum. Cytotoxicity assays were performed as described (16) using 10^4 target cells and an effector-to-target cell ratio of 10:1 except for bm8.10, which was used at a ratio of 1:1.

RESULTS AND DISCUSSION

As demonstrated (16, 18–21), the majority of K^b-specific alloreactive CTL clones are unable to recognize target cells that express "empty" K^b molecules. Such recognition can be

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: MHC, major histocompatibility complex; mAb, monoclonal antibody; CTL, cytotoxic T lymphocyte.

†To whom reprint requests should be addressed.

restored by incubating targets with peptides derived from purified K^b molecules (16, 18). Thus CTL clones can be an excellent tool to detect the presence or absence of a particular class I-associated peptide. As such, a panel of K^b-specific CTL clones was used to determine whether K^b-specific alloantibodies recognize all K^b molecules, regardless of the peptide ligand they contained, or whether each different K^b-specific mAb bound a different set of K^b molecules that was distinguishable on the basis of its peptide composition.

K^b molecules were purified from detergent lysates representing an equivalent number of EL4 tumor cells by using one of three K^b-specific mAbs, 5F1.2, Y3, and 100-30. These antibodies were selected based on the observation that Y3 appears to recognize all K^b–peptide complexes tested, whereas recognition by 5F1.2 and 100-30 varied depending on which peptide antigen was part of the complex (10). Peptides were acid-eluted from the class I molecules and used as a source of allopeptides to reconstitute CTL recognition of empty K^b molecules expressed on T2-K^b targets. Several K^b-specific clones were employed, each of which had been determined (16) to recognize a different endogenous peptide.

As represented in Fig. 1, mAb Y3 precipitated K^b molecules that contained peptides that could sensitize T2-K^b target cells for recognition by each of the CTL clones tested. In contrast, peptides extracted from K^b molecules bound by 5F1.2, while almost as efficient as Y3-extracted peptides at reconstituting recognition for some CTL clones (52 and 13), were far less efficient at reconstituting recognition by several other clones, including bm8.28, bm8.10, and M37. Likewise, the peptides extracted from K^b molecules purified using mAb 100-30 contained significantly less of the peptides required for recognition by CTL clones 52, 13, and bm8.28.

To facilitate quantitation and comparison of the amount of each peptide precipitated with each antibody, the amount of Y3 peptide required to achieve 50% lysis was assigned a value of 1, and the relative amount of peptide required to achieve a comparable level of lysis was calculated accordingly. These data are presented in Table 1. Based on these results, it can be concluded that for most K^b-associated peptides detected by our panel of CTL clones, Y3 is optimal in precipitating K^b molecules containing the relevant peptide. This suggests Y3 can recognize most, if not all, cell surface K^b molecules regardless of the associated peptide. In contrast, K^b molecules containing peptides recognized by clones 52 and 13 are more readily recognized by 5F1.2 than 100-30 whereas the reciprocal is true for clones bm8.10, 30, and M37. For clone bm8.28, there is no difference between the concentration of peptide precipitated by these two mAbs. However, both of these mAbs are >20-fold less efficient than Y3 at precipitating K^b molecules containing the peptide recognized by CTL clone bm8.28.

Table 1. Relative amount of peptide required for comparable levels of lysis

Clone	Relative amount		
	Y3	5F1.2	100-30
bm8.10	1	8.25	0.8
bm8.28	1	23	23
30	1	4	0.6
52	1	1.2	8.3
13	1	2.9	14.3
72	1	4	2.3
M37	1	5	1

mAbs Y3, 5F1.2, and 100-30 were used. Essentially identical results were obtained when this experiment was repeated using a second set of peptides prepared by the same procedures.

As significant differences were observed in the amount of certain peptide–MHC complexes recognized by the various mAbs, it was of interest to determine whether these differences could be observed by comparing the HPLC profiles of peptides obtained by immunoprecipitation of H2-K^b with each antibody. Fig. 2 *A–C* presents the elution profiles of peptides in the portion of the gradient known to contain allopeptides. Arrows denote peptide peaks that are present in Y3-extracted peptides yet are either greatly reduced or absent in one or both of the other peptide preparations. These results suggest that at least some K^b-associated peptides that are represented at particularly high concentrations are differentially precipitated by the various alloantibodies. It should be noted, however, that allopeptides recognized by the CTL clones used in these studies are probably not represented by these dominant peptide peaks. CTL clones were used to detect the presence of the specific allopeptides recognized by these clones. Individual fractions from the three HPLC gradients were used to load K^b molecules on the T2-K^b cells, and these cells were used as targets for CTL clones. As presented in Fig. 2 *D–F*, the elution position of the peptide seen by each CTL clone remained the same irrespective of the mAb used for peptide purification; however, as anticipated based on the results obtained with unfractionated material (Fig. 1), considerable variation was observed in the amount of peptide precipitated with the various alloantibodies. In addition, for several clones, a second minor peak of peptide activity was detected. These may represent alternatively processed forms of the peptides recognized by these clones.

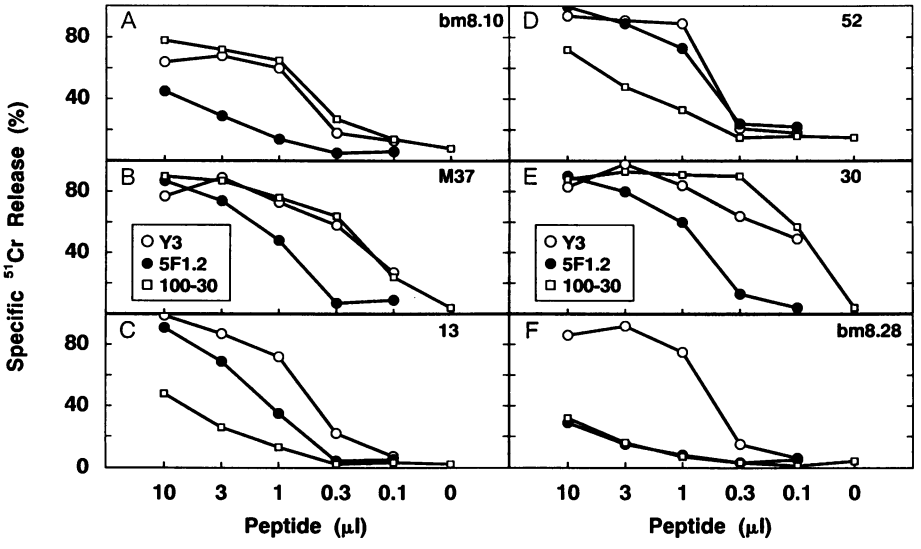

FIG. 1. Comparison of allopeptides associated with K^b molecules purified with mAbs Y3, 5F1.2, and 100-30. Various amounts of peptide extracted from K^b molecules purified with the indicated mAb were incubated with ^{51}Cr-labeled T2-K^b cells. These cells were used as targets for lysis by the following CTL clones. (*A*) bm8.10. (*B*) M37. (*C*) 13. (*D*) 52. (*E*) 30. (*F*) bm8.28.

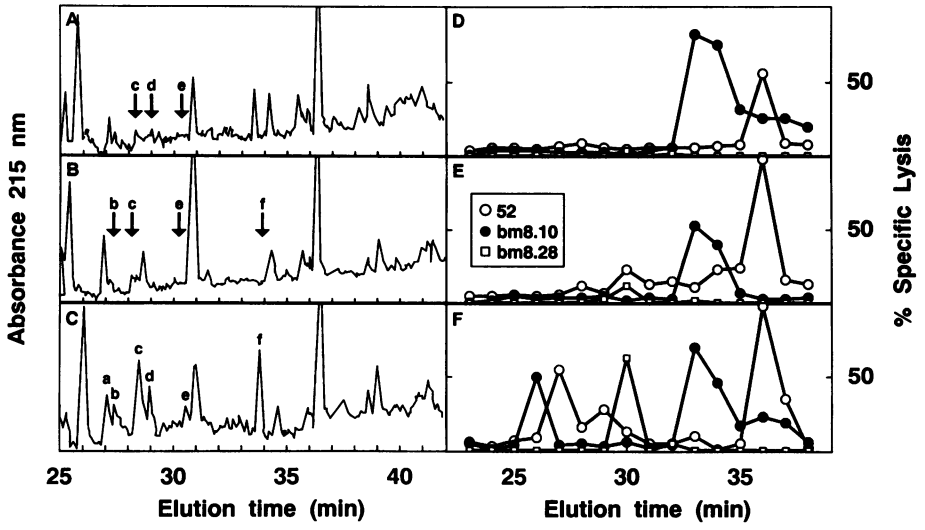

FIG. 2. Resolution by reverse-phase HPLC of K^b-associated peptides. (*A–C*) Peptides extracted from K^b molecules purified with mAbs 100-30, 5F1.2, and Y3, respectively, were resolved by reverse-phase HPLC. Materials that varied between the different preparations were designated peaks a–f for the purpose of comparison. (*D–F*) T2-K^b target cells were incubated with 10 μl of the indicated fraction as shown in *A–C*, respectively, and used as targets for the indicated CTL clones.

An important consideration in these studies is the fact that, in general, the variations observed using the different mAbs represent a preference of a particular antibody to bind particular K^b complexes, rather than an absolute inability to bind certain complexes. This preference is consistent with a difference in binding affinity for the class I molecule attributable to conformational changes induced by peptide rather than direct interaction of antibody with bound peptide (10). Recent x-ray crystallographic studies (22) of the K^b molecule demonstrate subtle changes in the conformation of the α-helices of K^b molecules bound by different peptides. It is possible that the affinity of antibody binding is too high to demonstrate the type of absolute, all or none, binding and peptide dependence that is characteristic of T-cell receptors. This may be a consequence of the higher binding affinity characteristic of antibodies compared to T-cell receptors (23, 24). The fact that the ability of alloantibodies to bind MHC can be affected by bound peptide suggests that it may be possible to derive antibodies with peptide specificity that is equivalent to the specificity of T cells by appropriate types of immunization and screening. In support of this possibility, it should be noted that a class II-specific mAb that specifically binds a particular MHC–peptide complex has been reported (25).

In view of the fact that many investigators utilize MHC-specific alloantibodies in their studies, it is important to consider that the type of preference for certain MHC–peptide complexes that has been observed for many K^b-specific mAbs may also apply to other class I- and class II-specific antibodies. Often, variation is observed in the level of cell surface binding by different mAbs specific for the same MHC molecule. Although in some cases this may genuinely reflect affinity differences by mAbs specific for the same epitope on the MHC, our results present the alternative possibility that in some cases such differences may be attributable to binding of select subsets of MHC–peptide complexes.

We thank C. Wood for excellent secretarial assistance. This work was supported by Grants CA 52856 and AI 29531 from the National Institutes of Health. J.A.B. is an American Cancer Society Faculty Scholar.

1. Townsend, A., Ohlen, C., Bastin, J., Ljunggren, H.-G., Foster, L. & Karre, K. (1989) *Nature (London)* **340**, 443–448.
2. Townsend, A. & Bodmer, H. (1989) *Annu. Rev. Immunol.* **7**, 601–624.
3. Falk, K., Rotzschke, D., Stevanovic, S., Jung, G. & Rammensee, H.-G. (1991) *Nature (London)* **351**, 290–296.
4. Van Bleek, G. M. & Nathenson, S. G. (1990) *Nature (London)* **348**, 213–216.
5. Schumacher, T. N. M., Heemels, M.-T., Neefjes, J. J., Kast, W. M., Melief, C. J. M. & Ploegh, H. L. (1990) *Cell* **62**, 563–567.
6. Elliott, T., Cerundolo, V., Elvin, J. & Townsend, A. (1991) *Nature (London)* **351**, 402–406.
7. Monaco, J. J. (1992) *Immunol. Today* **13**, 173–179.
8. Hosken, N. A. & Bevan, M. J. (1990) *Science* **248**, 367–370.
9. Cerundolo, V., Alexander, J., Anderson, K., Lamb, C., Cresswell, P., McMichael, A., Gotch, F. & Townsend, A. (1990) *Nature (London)* **345**, 449–452.
10. Bluestone, J. A., Jameson, S., Miller, S. & Dick, R. (1992) *J. Exp. Med.* **176**, 1757–1761.
11. Catipovic, B., Porto, J. D., Mage, M., Johansen, T. E. & Schneck, J. P. (1992) *J. Exp. Med.* **176**, 1611–1618.
12. Wylie, D. E., Sherman, L. A. & Klinman, N. R. (1982) *J. Exp. Med.* **155**, 403–414.
13. Froscher, B. G. & Klinman, N. R. (1986) *J. Exp. Med.* **164**, 196–210.
14. McLain, L. & Dimmock, N. J. (1993) *J. Immunol.* **150**, 3421–3426.
15. Murphy, D. B., Lo, D., Rath, S., Brinster, R. L., Flavell, R. A., Slanetz, A. & Janeway, C. A., Jr. (1989) *Nature (London)* **338**, 765–768.
16. Heath, W. R., Kane, K. P., Mescher, M. W. & Sherman, L. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5101–5105.
17. Van Bleek, G. M. & Nathenson, S. G. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 11032–11036.
18. Rotzschke, O., Falk, K., Faath, S. & Rammensee, H.-G. (1991) *J. Exp. Med.* **174**, 1059–1071.
19. Crumpacker, D. B., Alexander, J., Cresswell, P. & Engelhard, V. H. (1992) *J. Immunol.* **148**, 3004–3011.
20. Guimezanes, A., Schumacher, T. N. M., Ploegh, H. L. & Schmitt-Verhulst, A.-M. (1992) *Eur. J. Immunol.* **22**, 1651–1654.
21. Aosai, F., Ohlen, C., Ljunggren, H.-G., Hoglund, P., Franksson, L., Ploegh, H., Townsend, A., Karre, K. & Stauss, H. J. (1991) *Eur. J. Immunol.* **21**, 2767–2774.
22. Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A. & Wilson, I. A. (1992) *Science* **257**, 919–927.
23. Weber, S., Traunecker, A., Oliveri, F., Gerhard, W. & Karjalainen, K. (1992) *Nature (London)* **356**, 793–796.
24. Matsui, K., Boniface, J. J., Reay, P. A., Schild, H., Fazekas De St. Groth, B. & Davis, M. M. (1991) *Science* **254**, 1788–1791.
25. Rudensky, A. Y., Rath, S., Preston-Hulbart, P., Murphy, D. B. & Janeway, C. A., Jr. (1991) *Nature (London)* **353**, 660–662.