MURINE CYTOTOXIC T LYMPHOCYTE RECOGNITION OF INDIVIDUAL INFLUENZA VIRUS PROTEINS
High Frequency of Nonresponder MHC Class I Alleles

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A central aim of immunology is to understand how the polymorphism of class I genes relates to their sole known function as restriction elements for CTL. In the present study, we used CTL populations specific for five influenza virus proteins to determine the frequency of non-responder alleles to individual foreign proteins. Our findings indicate that, despite the large size of these proteins (ranging from 230 to 759 amino acids), the frequency of nonresponder alleles is very high. These results provide a basis for understanding the original detection of MHC-linked non-responsiveness to viruses using polyclonal antiviral CTL populations (1), and imply that responsiveness to foreign antigens could be a major selective force in maintaining MHC polymorphism in animal populations.

Materials and Methods

**Mice.** 8-12-wk-old BALB/c, CBA/J, A/J, C3H-H-2^d^/SJL/JSe^b^ (C3H.OH), C3H/HeJ x DBA/2J F1 (C3D2F1), BALB/c-DM2, and B10.D2-DM1 mice were purchased from The Jackson Laboratory, Bar Harbor, ME.

**Viruses.** Influenza viruses A/Puerto Rico/8/34 (H1N1) (PR8), A/Japan/305/57 (H2N2) (JAP), A/Northern Territories/60/68 (H3N2) (NT60), and A/Hong Kong/107/68 (H3N2) (HK) were used. The VAC recombinants used, shown in Table I, were kindly provided by Geoffrey Smith (Cambridge University, Cambridge, England) and Bernard Moss (NIAID, Bethesda, MD) (2).

**Cells.** The following established cell lines were used. P815 mastocytoma cells (derived from DBA/2 mice), L929 fibrosarcoma cells (derived from C3H mice), and NA neuroblastoma cells (derived from A/J mice). Additionally, cell lines were established from C3D2F1 and C3H.OH mice by transformation of primary lung cultures with SV40 as previously described (3).

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CTL Assays. CTL were derived from secondary in vitro cultures as described (4). CTL activity was determined by a 4-h 51Cr-release assays as described (3). Data are expressed as percent specific release defined as: [(experimental cpm) – (spontaneous [no splenocytes] cpm)]/[(total [detergent] cpm) – (spontaneous cpm)].

Results and Discussion

We previously showed that BALB/c (H-2^d) anti-influenza CTL recognize HA, NP, NSI, and PB2, but not PB1, while CBA anti-influenza (H-2^k) CTL recognize HA, NP, NSI, and PB1, but not PB2 (3). To determine the H-2K versus D-L end-restriction pattern for CTL specific for these individual proteins, secondary anti-influenza CTL populations from CBA or BALB/c mice were tested for lysis of target cells of recombinant haplotypes infected with PR8, VAC, or VAC recombinants expressing cloned influenza virus genes. BALB/c CTL lysed NA cells (H-2K^dD^dL^d) infected with PR8, NSI-VAC, or PB2-VAC, but not cells infected with NP-VAC, or PB1-VAC (Table II). The same target cells were lysed by CBA/J CTL when infected with PR8, NP-VAC, PB1-VAC, or NSI-VAC, but not PB2-VAC (Table II). C3H.OH target cells (H-2K^dD^k) were tested in an identical experiment. BALB/c CTL lysed C3H.OH cells infected with PR8 or NP-VAC, but not PB1-VAC, PB2-VAC, or NSI-VAC. CBA/J CTL lysed the same target cells infected with PR8 and PB1-VAC, but not NP-VAC, PB2-VAC, or NSI-VAC. None of the CTL populations tested specifically lysed allogeneic target cells infected with PR8 or any of the VAC recombinants (not shown), which further established the MHC-restricted nature of recognition.

The allele mapping of CTL recognition of individual proteins was confirmed at the level of CTL stimulation. Identical patterns of recognition of individual influenza virus proteins were obtained when anti-influenza CTL from A/J (H-2K^dD^dL^k) or C3H.OH (H-2K^dD^d) mice were tested on L929 (H-2^k) or P815 mastocytoma (H-2^d)-infected target cells (Table III).

### Table II

| Effectors | Percent specific 51Cr release with infected target cells |
|-----------|---------------------------------------------------------|
| Strain (KDL) | H-2 | Target cells | PR8 | NP-VAC | PB1-VAC | PB2-VAC | NSI-VAC |
| BALB/c^d | ddd | NA^t (H-2K^dD^dL^d) | 58 | 62 | 7 | 5 | 0 | 4 | 30 | 25 | 33 | 42 |
| CBA/J^f | k^k | | 53 | 56 | 57 | 55 | 31 | 27 | 0 | 3 | 37 | 32 |
| BALB/c^* | ddd | C3H.OH^f (H-2K^dD^d) | 41 | 57 | 42 | 38 | 0 | 2 | 0 | 1 | 4 | 0 |
| CBA/J^t | k^k | | 41 | 31 | 5 | 6 | 67 | 52 | 5 | 2 | 7 | 0 |

* Control lysis on VAC-infected targets has been subtracted from all percentages.

1 Target cells were tested using splenocytes from PR8 immunized mice restimulated in vitro with JAP at the E/T ratios indicated.

2 Control lysis on VAC-infected targets was 8% and 3% at 20:1 and 7:1 E/T ratios, respectively.

3 Control lysis on VAC-infected targets was 7% and 3% at 20:1 and 7:1 E/T ratios respectively.

4 Target cells were tested using splenocytes from PR8-immunized mice restimulated in vitro with NT60 at the E/T ratios indicated.

5 Control lysis on VAC-infected targets was 11% and 6% at 20:1 and 7:1 E/T ratios respectively.

6 Control lysis on VAC-infected targets was 11% and 0% at 20:1 and 7:1 E/T ratios respectively.

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**CTL Assays.** CTL were derived from secondary in vitro cultures as described (4). CTL activity was determined by a 4-h 51Cr-release assays as described (3). Data are expressed as percent specific release defined as: [(experimental cpm) – (spontaneous [no splenocytes] cpm)]/[(total [detergent] cpm) – (spontaneous cpm)].
In a similar set of experiments performed with CTL populations containing HA-specific CTL (derived by priming and stimulating CTL with PR8), we found that the PR8 HA is recognized solely in conjunction with K^d by H-2^d-restricted CTL and with K^k by H-2^k-restricted CTL (not shown).

To further dissect H-2^d-restricted recognition of PB2 and NS1, we used L929 cells transfected with genes encoding H-2^D^d (L929-DM1 cells) or H-2^L^d (L929-LM1 cells) (4) (Table IV). BALB/c antiinfluenza CTL recognized PB2-VAC and NS1-VAC solely with D^d and L^d, respectively. These patterns of restriction were confirmed by two additional experiments. First, PR8-infected L929-DM1 and L929-LM1 cells were used for in vitro stimulation of splenocytes from C3D2F1 (H-2^kxD^d) mice primed with PR8 (Table V). CTL restimulated with L929-DM1 lysed PB2-VAC but not the other VAC recombinants. L929-LM1-restimulated CTL lysed NS1-VAC but not the other VAC recombinants. As expected, CTL stimulated with either cell line lysed L929 cells infected with H1-VAC, NP-VAC, NS1-VAC, or PB1-VAC. In the second experiment, we determined the viral proteins recognized by CTL from BALB/c-DM2 and B10.D2-DM1 mutant mice (Table VI). These mice fail to express functional L^d, or both D^d and L^d molecules, respectively (5). Antinfluenza CTL from both strains recognized HA and NP. CTL from BALB/c-DM2 mice failed, however, to recognize NS1, while CTL from B10.D2-DM1 mice failed to recognize PB2 and NS1.

Our results indicate that, with a lone exception (H-2^k-restricted recognition of PB1), responses to individual influenza virus proteins in the H-2^d and H-2^k haplotypes are restricted at both stimulator and responder levels to a single class I locus (Table VII). This confirms and extends the findings of Pala and Askonas (6) regarding H-2^k-, H-2^d-, and H-2^k-restricted recognition of NP. We conclude that class I MHC alleles are frequently associated with low or absent responses in conjunction with individual viral gene products, despite the fact that the protein may consist of >750 amino acids. This conclusion is consistent with the idea that responsiveness to foreign antigens is a factor in maintaining the high degree of MHC class I polymorphism in outbred populations.

### Table III

| Strain (KDL) | Target cells | Percent specific 51Cr release* for infected target cells: |
|--------------|--------------|----------------------------------------------------------|
| A/J^kdd      | L929 (H-2^KkD^k) | PR8: 76 48 60 47 24 12 0 0 30 18 |
| C3H.OH^d     |              | PR8: 69 42 5 3 65 63 4 0 4 0 |
| A/J^kdd      | P815 (H-2^KdD^d) | PB1-VAC: 51 45 0 0 0 0 48 43 43 36 |
| C3H.OH^d     |              | PB2-VAC: 63 62 63 72 0 0 0 0 0 0 |
|              |              | NS1-VAC: 63 62 63 72 0 0 0 0 0 0 |

* Target cells were tested using splenocytes from PR8 immunized mice restimulated in vitro with HK at the E/T ratios indicated below. Control lysis on VAC-infected targets has been subtracted from all percentages.

1 Control lysis on VAC-infected targets was 20% and 6% at the 15:1 and 1:1 E/T ratios shown.
2 Control lysis on VAC-infected targets was 23% and 4% at the 20:1 and 1:1 E/T ratios shown.
3 Control lysis on VAC-infected targets was 26% and 3% at the 15:1 and 1:1 E/T ratios shown.
4 Control lysis on VAC-infected targets was 26% and 4% at the 20:1 and 1:1 E/T ratios shown.
Control lysis on VAC-infected targets has been subtracted from all percentages.

**Table IV**

BALB/c Anti-influenza CTL Lysis of Virus-infected L929 Cells Transfected with H-2D<sup>d</sup> (L929-DM1) or H-2L<sup>d</sup> (L929-LM1)

| Strain   | Target cells                  | Percent specific 51Cr release<sup>*</sup> for infected target cells |
|----------|-------------------------------|---------------------------------------------------------------------|
| BALB/c   | L929-DM1 (H-2K<sup>kd</sup>D<sup>d</sup>)<sup>1</sup> | PR8 34 17 33 16 0 0                               |
|          | L929-LM1 (H-2K<sup>kd</sup>L<sup>d</sup>)<sup>1</sup> | PR8 31 30 0 0 68 48               |

* Control lysis on VAC-infected targets has been subtracted from all percentages.

1 Splenocytes from BALB/c mice immunized with PR8 were restimulated in vitro with HK and tested at E/T ratios of 20:1 and 7:1.

**Table V**

In Vitro PR8 Stimulation of H-2D<sup>d</sup> and H-2L<sup>d</sup>-restricted C3D2F1 CTL

| PR8-infected stimulators<sup>2</sup> | Target cells                  | Percent specific 51Cr release<sup>*</sup> for infected target cells |
|-------------------------------------|-------------------------------|---------------------------------------------------------------------|
| C3D2F1<sup>5</sup>                 | P815 (H-2K<sup>kd</sup>D<sup>d</sup>L<sup>d</sup>) | PR8 41 6 11 4 27 9 0 0 8 2 28 3  |
| L929-DM1<sup>6</sup>                 | 29 3 0 1 0 4 0 0 24 10 3 0  |
| L929-LM1<sup>6</sup>                 | 48 22 0 0 4 0 0 0 0 0 33 13  |

* Control lysis on VAC-infected targets has been subtracted from all percentages.

1 Splenocytes from C3D2D1 mice immunized with PR8 were restimulated in vitro with autologous C3D2F1-PR8-infected spleen cells, L929-DM1-PR8-infected cells, or L929-LM1-PR8-infected cells. E/T ratios of 7:1 and 1:1.

5 Control lysis on VAC-infected targets was 7% and 3% at 20:1 and 7:1 E/T ratios, respectively.

6 Control lysis on VAC-infected targets was 12% and 4% at 20:1 and 7:1 E/T ratios, respectively.

**Table VI**

Influence Virus Protein Recognition by CTL from BALB/c-DM2 and B10.D2-DM1 Mutant Mice

| Effectors<sup>1</sup> | Target cells                  | Percent specific 51Cr release<sup>*</sup> for infected target cells |
|------------------------|-------------------------------|---------------------------------------------------------------------|
| BALB/c-PR8<sup>5</sup> | P815 (H-2K<sup>kd</sup>D<sup>d</sup>L<sup>d</sup>) | PR8 87 52 43 10 63 22 53 23 41 14  |
| BALB/c-DM2-PR8<sup>6</sup> | 75 25 28 5 45 11 37 9 2 1  |
| B10.D2-DM1-PR8<sup>6</sup> | 79 23 25 3 41 11 2 0 4 0  |

* Control lysis on VAC-infected targets has been subtracted from all percentages.

1 Splenocytes from mice immunized with PR8 were restimulated in vitro with autologous PR8- or VAC-infected spleen cells at E/T ratios of 20:1 and 2:1.

5 Control lysis on VAC-infected targets was 9% and 1% at 20:1 and 2:1 E/T ratios, respectively.

6 Control lysis on VAC-infected targets was 8% and 1% at 20:1 and 2:1 E/T ratios, respectively.

4 Control lysis on VAC-infected targets was 7% and 3% at 20:1 and 2:1 E/T ratios, respectively.
Summary

We determined the MHC restriction of CTL responses to five individual influenza virus proteins. Four viral proteins failed to be recognized in conjunction with three of the five class I alleles of the H-2^k and H-2^d haplotypes, while the fifth was recognized only in conjunction with a single allele. This indicates that there is a significant chance that a given class I allele will be associated with low responsiveness or non-responsiveness for a given foreign protein. This explains, at least in part, why MHC-linked nonresponsiveness is frequently detected in polyclonal antiviral CTL responses. Most importantly, these findings support the idea that responsiveness to foreign antigens is a critical factor in maintaining the high degree of MHC class I polymorphism in outbred populations.

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