DIFFERENT D END-DEPENDENT ANTIGENIC DETERMINANTS ARE RECOGNIZED BY H-2-RESTRICTED CYTOTOXIC T CELLS SPECIFIC FOR INFLUENZA AND BEBARU VIRUSES

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Murine cytotoxic thymus-derived (T) lymphocytes (Tc cells) that recognize modified self cells are specific for both the foreign antigen (X) and a self component dependent on gene(s) in either the K or the D region of the H-2 complex (1), i.e. they are “H-2 restricted.” Studies with several mutants of the H-2Kb region have shown that with a variety of viruses and minor histocompatibility (H) antigens (2-5), the genetic unit coding for the polypeptide chain of the classic, serologically defined H-2K glycoprotein is also responsible for a restriction antigen, though most of the mutants are largely, if not completely, undetectable by serological means (6, 7). The specificity of H-2-restricted Tc-cell discrimination between wild-type and mutant H-2K molecules is exquisite (2, 3, 6, 8-10), and seems similar, regardless of the X antigen involved, thus suggesting that the same antigenic determinant or area of the same H-2K molecule is recognized in each case. This idea is reinforced by the finding that of the first 10 mutants in the same genetic unit in the H-2Kb region, 3 are phenotypically identical (6, 11). Provided these three mutants arose independently, this implies that only small section(s) of the H-2K polypeptide sequence are relevant to Tc-cell recognition.

Thus far, there are insufficient mutants of other K or D regions to know how far the conclusions drawn from the H-2Kb mutants can be extrapolated. However, in contrast to H-2Kb mutants, the BALB/c-H-2dm2 mutation (formerly H-2dm) has generated data suggesting that the D end contains two genetic units concerned with H-2 restriction (12-14). This mutation has revealed a new locus (H-2L) that is apparently responsible for a molecule similar in many respects to the serologically defined H-2K and H-2D molecules (15, 16). All three are ≈45,000 mol wt, are associated in the cell membrane with β-2 microglobulin, are recognized by allogeneic Tc cells, and share certain serological public specificities, though major private specificities are carried by H-2K and H-2D, not H-2L. Evidence to date suggests that for ectromelia virus and some minor H antigens, H-2L does not act as a restriction antigen (13), i.e., it is not recognized by H-2-restricted self Tc cells, whereas it may be so recognized with influenza virus antigens (14).

An H-2L locus may be present in other H-2 haplotypes as illustrated by recent

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Abbreviations used in this paper.

BEB, Bebaru virus; JAP, influenza A JAP virus; MLR, mixed lymphocyte reaction; T cell, thymus-derived lymphocyte; Tc cell, cytotoxic T cell; WSN, influenza A WSN virus; X, foreign antigen.
information on the D end of H-2<sup>k</sup> (17). We report here that monoclonal antibody that binds to molecule(s) dependent on gene(s) in the D end of H-2<sup>k</sup> can block lysis by Tc cells specific for these H-2 antigen(s) and influenza virus, but not Bebaru virus. This indicates that two different D end-dependent antigenic determinants, possibly carried on two different H-2 molecules, are recognized by Tc cells specific for the two different viruses.

Materials and Methods

**Animals.** CBA/H, BALB/c, and C3H.OH and BIO.A mice were bred at the John Curtin School and were used in experiments at 8 wk of age.

**Viruses.** Ectromelia virus, Sendai virus, Bebaru virus (BEB), and influenza A strains JAP (JAP) and WSN (WSN) were grown, titrated, and used as described in detail previously (18).

**Generation of Tc Cells.** Alloreactive Tc cells were generated in primary mixed lymphocyte reactions (MLR) as described (18, 19). Secondary antiviral Tc cells were produced by culturing responder spleen cells from mice primed intraperitoneally with virus 3 wk previously with virus-infected syngeneic spleen cells; the complete method is given elsewhere (19).

**Target Cells.** L929 cells were grown overnight in spinner culture after routine harvest of monolayers (18). They were labeled with <sup>51</sup>Cr and used in cytotoxicity assays either uninjected or infected with viruses as described (18).

**Blocking with Monoclonal Anti-H-2 Antibodies.** The monoclonal antibodies 27R9 and 30R3 used were produced and described in detail by Lemke et al. (20). Briefly, these antibodies were stimulated by H-2<sup>k</sup> antigens (BALB/c anti-CBA), and they bind to molecules coded in both the K and D ends (24).

Triplicate samples of 50 μl containing 2 × 10<sup>4</sup> <sup>51</sup>Cr-labeled spinner L929 target cells of different categories (uninfected or 1.5 h after virus infection) were incubated (30 min at 37°C) with 50 μl of serial two-fold dilutions of the monoclonal antibodies (from 1:50 to 1:1,600) in flat-bottomed wells of 96-hole Linbro trays (Linbro Chemical Company, Hamden, Conn.). Tc cells of appropriate specificity were then added (10<sup>5</sup> cells in 100 μl/well), and the cytotoxicity assay was run for 6 h at 37°C.

Results

**Blocking with Monoclonal Anti-H-2 Antibodies of Target Cell Lysis Mediated by Various Tc Cells Specific for H-2K<sup>k</sup> or H-2D<sup>k</sup>.** Four different Tc-cell populations specific for K<sup>k</sup> antigens were generated in vitro and assayed on appropriate L929 (K<sup>d</sup>, D<sup>k</sup>) target cells. For the sake of brevity, we have used K<sup>k</sup> and D<sup>k</sup> to designate H-2 antigens recognized by Tc cells and dependent on genes in the K and D ends of the H-2 complex (including both H-2D and H-2L genes in the D end).
**H-2 RESTRICTION SPECIFICITIES DIFFER FOR TWO VIRAL GENERA**

Figure 1. Inhibition by monoclonal antibodies 27R9 (○) and 30R3 (■) of specific lysis of L929 target cells mediated by K^k-specific Tc cells. (a) Anti-K^k alloreactive Tc cells on uninfected targets; (b) K^k-ectromelia-immune Tc cells on ectromelia-infected targets; (c) K^k-Sendai-immune Tc cells on Sendai-infected targets; (d) K^k-WSN-immune Tc cells on WSN-infected targets or (e) on JAP-infected targets. Details of Tc-cell generation are given in the text and Materials and Methods. The horizontal lines show the level of specific lysis in the absence of antibodies. A killer:target ratio of 5:1 was used throughout.

1 a–e). 30R3 consistently blocked lysis by all of the K^k-specific Tc cells, though its activity was more pronounced with alloreactive (Fig. 1 a) than with virus-immune cells (Fig. 1 b–e). 27R9 blocked alloreactive Tc cells significantly, though less than 30R3 (Fig. 1 a), and showed little if any activity with virus-immune cells (Fig. 1 b–e).

The same monoclonal antibodies were tested for blocking of lysis mediated by various D^k-specific Tc cells. Anti-K^k alloreactive Tc cells were included for comparison with anti-D^k alloreactive cells, but responses to ectromelia and Sendai viruses in association with D^k are weak (21–23) and were omitted. Instead, Tc cells were generated against BEB, an α-virus with which D^k antigens give good responses (19).

Thus, the four different Tc-cell populations used in this second experiment were as follows:

(a) Anti-K^k; C3H.OH (K^k, D^k) anti-CBA/H (K^k, D^k) MLR.
(b) Anti-D^k; BALB/c (K^k, D^k) anti-C3H.OH (K^k, D^k) MLR.
(c) Anti-D^k-BEB; secondary C3H.OH (K^k, D^k) anti-BEB-infected C3H.OH.
(d) Anti-D^k-JAP; secondary C3H.OH (K^k, D^k) anti-JAP-infected C3H.OH.

As in the previous experiment, the Tc cells were assayed against L929 targets that were left uninfected for the alloreactive Tc cells (a and b), infected with BEB (c), or infected with JAP (d), and additional WSN-infected L929 targets were used with anti-JAP Tc cells to reveal the cross-reactive (Fig. 2 e) subset. Lysis of uninfected control targets by the antiviral Tc cells was again negligible at the killer:target ratio chosen, and the data given (Fig. 2) for these Tc cells concern infected targets only. 30R3 blocked anti-K^k Tc cell-mediated lysis very efficiently (Fig. 2 a) as expected, but showed little activity with anti-D^k alloreactive Tc cells (Fig. 2 b), and did not block any lysis by Tc cells recognizing D^k plus any of the viral antigens (Fig. 2 c–e). In
Fig. 2. Inhibition by monoclonal antibodies 27R9 (●) and 30R3 (■) of specific lysis of L929 target cells mediated by K\textsuperscript{k} and D\textsuperscript{k}-specific Tc cells. (a) Anti-K\textsuperscript{k} alloreactive Tc cells on uninfected targets; (b) anti-D\textsuperscript{k} alloreactive Tc cells on uninfected targets; (c) D\textsuperscript{k}-BEB-immune Tc cells on BEB-infected targets; (d) D\textsuperscript{k}-JAP-immune Tc cells on JAP-infected targets or (e) on WSN-infected targets. Details of Tc-cell generation are given in the text and Materials and Methods. The horizontal lines show the level of specific lysis in the absence of antibodies. A killer:target ratio of 5:1 was used throughout.

Table I

| Killer:target ratio | Male targets | | Female targets |
|---------------------|--------------|------------------|----------------|
|                     | Uninfected   | Infected         | Uninfected     | Infected       |
|---------------------|--------------|------------------|----------------|
| 4:1                 | 18.0         | 58.1             | 27.6           | 66.2           |
| 2:1                 | 19.8         | 37.1             | 27.4           | 31.1           |

* Secondary Tc cells were generated in vitro by using infected female stimulator cells as described in Materials and Methods. Data given are means of triplicates, and SEMs were never >4.8%.

Striking contrast, 27R9 blocked anti-K\textsuperscript{k} Tc-cell lysis less than 30R3 (Figs. 1 and 2 a), blocked anti-D\textsuperscript{k} lysis more than 30R3 (Fig. 2 b), blocked anti-D\textsuperscript{k}-JAP Tc-cell lysis assayed on both JAP- and WSN-infected targets (Fig. 2 d, e), and did not block anti-D\textsuperscript{k}-BEB lysis (Fig. 1 c).

No cross-reactivity between H-Y and influenza virus with female influenza (JAP)-immune Tc cells restricted to D\textsuperscript{k}. Inasmuch as Fischer Lindahl and Lemke (24) have shown that 27R9 blocks H-Y specific Tc cells restricted by D\textsuperscript{k}, we tested the possibility, perhaps remote that H-Y and influenza produce cross-reactive antigenic moieties in association with D\textsuperscript{k} antigens. Female CBA/H (K\textsuperscript{k}, D\textsuperscript{k}) JAP-immune secondary Tc cells were assayed on JAP-infected and uninfected female and male C3H.OH (K\textsuperscript{d}, D\textsuperscript{b}) macrophage targets (Table I). Infected targets were lysed significantly more than uninfected targets, regardless of whether they were from male or female mice.
Uninfected male targets were lysed less than uninfected female, clearly indicating that JAP-immune female Tc cells do not cross-react with H-Y antigen.

Discussion

In the case of interactions between Tc cells and a variety of target cells studied previously (12, 24-26), anti-H-2 antibodies could apparently block killer-target interaction by binding to the target not the killer cell. This seemed to be true in the present experiments, because lysis mediated by Tc cells from C3H.OH mice was blocked in some cases and not others, depending upon the nature of the target cell antigen being recognized (Fig. 2). The degree of blocking obtained with a particular anti-H-2 antibody in a killer-target system should therefore depend upon the concentration of antibody, its affinity for a certain H-2 determinant on the target cell, and the spatial relationship between that determinant and the determinant recognized by the Tc-cell receptor. Steric hindrance must be a possible cause of blocking, but the data in Fig. 2 (a, b) strongly suggest that for blocking to occur the determinant(s) recognized by antibody and Tc-cell receptor must be on the same H-2 molecule, because the same target cell-antibody combination showed different degrees of blocking when the Tc cells used were of different specificity. Thus, 30R3 antibody blocked anti-K$^k$ Tc cells strongly but showed little activity with anti-D$^k$ Tc cells. Conversely, 27R9 antibody blocked anti-D$^k$ Tc cells strongly and had weaker effects with anti-K$^k$ Tc cells.

All K end-specific virus-immune Tc cells were blocked in a similar manner by 30R3. Thus, there was no indication that antigenically different K end-dependent molecules were recognized by Tc cells in the different viral systems. However, there was a suggestion that blocking by 30R3 and 27R9 of alloreactive Tc cell-mediated lysis of uninfected targets was slightly more efficient than blocking of lysis of infected targets by the various virus-immune cells (Fig. 1). This may be a reflection either of hindrance of antibody access to H-2 determinants by adjacent viral antigens, or of allosteric change in H-2 determinants on molecules complexed with virus-specified molecules.

The findings with D end-specific virus-immune Tc cells contrasted strikingly with K end-specific cells. 27R9 antibody strongly blocked Tc cell-mediated lysis specific for antigens dependent on influenza plus the D end of H-2$^k$ thus indicating that 27R9 binds to an H-2 determinant that is identical to, or is located on the same molecule as, a determinant recognized by influenza-specific Tc cells. However, lysis mediated by Tc cells specific for antigens dependent on BEB plus the D end of H-2$^k$ was not blocked by 27R9.

Two quite separate explanations may be considered. First, it could be that different D end-dependent determinants are recognized by BEB-immune and influenza-immune Tc cells. The former would need to be sufficiently distant from the site of 27R9 binding so that the IgG2a molecule does not sterically inhibit access of the Tc-cell receptor. One form of this model would be that different D end-coded molecules serve as restriction antigens with influenza and BEB. The BALB/c-H-2$^{d^2}$ mutation has shown that H-2L as well as H-2D molecules are coded in the D end of H-2$^d$ (12-16), and evidence exists that H-2$^d$ may similarly code for two different D end molecules (17). Also, blocking studies with anti-H-2L$^d$ antibodies suggest that H-2L$^d$ may act as a restriction antigen with influenza virus (14). Antibodies specific for H-2D$^k$ and H-2L$^k$ should be able to test this idea.
A second explanation allows both BEB-immune and influenza-immune Tc cells to recognize the same D end-coded molecule, even the same area of that molecule, but postulates that when complexed with BEB antigen (not influenza antigen) there is sufficient allosteric change in the determinant bound by 27R9 to render it unrecognizable. This idea appears to be difficult to test experimentally and seems less likely than the first explanation.

Our results complement those of Fischer Lindahl and Lemke (24) who have shown that 27R9 blocks Tc cell-mediated lysis specific for H-Y plus Dk-dependent antigens, but not other minor H antigens. This raised the possibility that influenza antigens and H-Y form similar, cross-reactive complexes with a D-end antigen, both of which are reactive with 27R9 antibodies. However, no evidence was found for any cross-reactivity between influenza-infected female cells and uninfected male cells with respect to Dk-restricted Tc cells.

We showed that 27R9 antibodies strongly blocked JAP-immune Tc-cell lysis of both JAP-infected and WSN-infected target cells. Thus, a cross-reactive Tc-cell subset that recognizes an identical or related determinant common to JAP and WSN, possibly on the viral matrix protein (27, 28), was definitely blocked. From the data presented (Fig. 1 d and e), it is not possible to say whether a JAP-specific Tc-cell subset, probably recognizing a determinant on the hemagglutinin molecule, was also blocked. We are attempting to clarify this point by using specific anti-JAP or anti-WSN Tc cells and antibodies to various virus-specified antigens. Together with the answer to the question concerning which of the H-2 antigens (H-2Dk or H-2Lk) are recognized together with the various viral antigens, this information may allow better evaluation of current models of H-2-linked “Ir gene” control of H-2 restricted Tc-cell responses (29, 30).

Summary

Two different BALB/c anti-CBA(H-2k) monoclonal antibodies that bind to Kk and Dk antigens blocked Tc cell-mediated lysis of L929 (Kk, Dk) target cells, but with quite different specificity. One antibody (30R3) powerfully blocked Kk-specific lysis mediated by alloreactive or Kk-restricted Tc cells immune to ectromelia, Sendai, or influenza viruses. The other antibody (27R9) blocked these anti-Kk Tc cells much less than 30R3, but in contrast, 27R9 blocked anti-Dk lysis much more than 30R3. Most importantly, 27R9 strongly blocked Dk-restricted anti-influenza Tc cells, but did not significantly block Dk-restricted anti-Bebaru (BEB) lysis. This result indicated that different H-2 determinants coded in the D end of H-2k were recognized by influenza- and BEB-immune Tc cells. These determinants may be carried on two different molecules coded by the H-2D and H-2L loci, but other possibilities are not yet excluded.

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