CYTOTOXIC T-CELL RESPONSE TO ECTROMELIA VIRUS-INFECTED CELLS

Different H-2 Requirements for Triggering Precursor T-Cell Induction or Lysis by Effector T Cells Defined by the BALB/c-H-2<sup>db</sup> Mutation

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Significant portions of the amino acid sequences of H-2 and HLA antigens correspond, thus suggesting that the basic form of these antigens has been conserved through a considerable part of the evolution of vertebrates (1, 2). Despite this, some antigenic determinants borne on products of the major histocompatibility complex (MHC)<sup>1</sup> are highly polymorphic, and the genes coding for these antigens are apparently mutating at an extraordinary rate (3). Thus, there seems to be evolutionary pressure to change certain antigenic features of MHC products, while conserving their basic form and function. Perhaps the search for T-cell-mediated mechanisms allowing recovery from potentially lethal viral infections, such as ectromelia infection in mice (4) is a part of this evolutionary process. This idea is supported by findings that virus-immune cytotoxic T (T<sub>c</sub>) cells that specifically lyse virus-infected target cells in vitro (reviewed in reference 5), and promote recovery from primary viral infection in vivo (4) recognize H-2 antigens together with virus-specific antigens on infected cell surfaces (4-11). The relevant genes in the H-2 complex map in either the K or the D region, but not in the I region (4, 5, 10, 12, 13). Extensive experiments with five mutants of the H-2<sup>K<sub>b</sub></sup> region and ectromelia, vaccinia, and lymphocytic choriomeningitis viruses (4, 5, 10, 14-16) have shown that a single gene in H-2<sup>K<sub>b</sub></sup>, probably a cistron coding for an H-2 polypeptide antigen (17) makes an essential, direct contribution to the antigenic patterns recognized by T<sub>c</sub> cells specific for H-2<sup>K<sub>b</sub></sup> plus virus. Evidence from the H-2<sup>K<sub>b</sub></sup> mutants (16) and recent work with Sendai virus (18) suggests that H-2 antigens become physically associated with viral antigens in infected cell membranes, but the way this happens is unclear. Therefore, another important feature of H-2 antigens may be their capacity to form compound or interaction antigens by associating with viral gene products (5, 6, 16, 19, 20).

The present experiments examine further the role of H-2 molecules in ectromelia virus-induced antigenic changes. We have used a new H-2 mutant,

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<sup>1</sup>Abbreviations used in this paper: H, histocompatibility; i.v., intravenous; MHC, major histocompatibility complex; NIAID, National Institute of Allergy and Infectious Diseases; PFU, plaque-forming unit; T cell, thymus-derived lymphocyte; T<sub>c</sub> cell, cytotoxic T cell.
BALB/c-\(H-2^{ab}\), that is unique among \(H-2\) mutants characterized thus far (16, 21). It is carried in an inbred strain cosogenic with the parental strain (BALB/cKh). The mutation is of the loss type since BALB/c-\(H-2^{ab}\) reject BALB/cKh skin grafts, but not vice versa. Complementation studies have localized the mutation to the same locus in \(H-2D\) as B10.D2-\(H-2^{a}\) (M504, see references 16, 22), a different gain and loss mutation. Serological analysis indicates that structure(s) identified by antibodies against the \(H-2.28\) family of public specificities have been lost in BALB/c-\(H-2^{ab}\), all other \(H-2\) and Ia specificities being intact (21). Biochemical studies (23) show that the \(H-2D^d\) region apparently codes for two similar proteins, each of about 45,000 mol wt. One of these bears serologically-defined private \(H-2\) specificities; the other does not, but reacts with certain antibodies contained in anti-\(H-2.28\) sera. It is the latter molecule which is not expressed in BALB/c-\(H-2^{ab}\). This molecule has been designated \(D'\) by Hansen et al. (23). We report here evidence which suggests that \(D'\) may be involved in a physical association between \(H-2D^d\)-coded and viral antigens that is in turn required for efficient induction of precursors of \(T_c\) cells. In contrast, \(D'\) is apparently nonessential for target cell recognition and lysis by effector \(T_c\) cells.

**Materials and Methods**

*Mice.* A.TH, A.TL, A.TFR2, A.TFR5, B10.A(2R), DBA/1, DBA/2, BALB/c, SJL/J, D2.GD, CBA/H, C3H.OH, C3H.OL, C57BL/6, and various \(F_1\) hybrids were bred at The John Curtin School. BALB/c-\(H-2^{ab}\) (mutant) and the cosogenic BALB/cKh (wild type) mice were bred at the Austin Hospital and shipped to the John Curtin School. All mice were used at 6-12 wk of age.

*Virus.* Stocks of attenuated (Hampstead egg) and virulent (Moscow) strains of ectromelia virus were grown, titrated, and stored as described (24).

*Immunization.* Mice were injected intravenously (i.v.) with \(10^3-10^4\) plaque-forming units (PFU) of attenuated virus; the \(T_c\)-cell response was not affected by virus dose within this range (25). Spleens were taken for \(T_c\) cell assay on day 6, at the peak of the primary response (24, 25), or after at least 3 wk when they were used as a source of memory \(T_c\) cells for secondary responses in vitro (26, 27).

**Secondary \(T_c\)-Cell Response In Vitro to Virus-Infected Cells.** The basic methods used have been described in detail (26, 27). When the genotype of responder and stimulator cells was different (as in Table IV), infected macrophages were used as stimulators with procedures designed to minimize escape of virus particles, or virus-specified antigens from the nominated stimulators into the responder population, which could in turn act as stimulators. Therefore, macrophage stimulators were infected in suspension with \(\gamma\)-irradiated Moscow virus for 40 min at 37°C (\(10^6\) cells/ml; 1 PFU/cell). They were then washed three times in cold medium and dispensed into tissue culture flasks. The flasks were incubated at 37°C for 2-3 h followed by vigorous washing (with warm medium) to remove nonadherent cells and residual virus before culture with memory \(T_c\) cells (responders). In addition, carbonyl iron-adherent (phagocytic) cells were removed from the responder population before culture. Responder:stimulator ratio was 8:1 and the cultures were held at 39°C (nonpermissive for viral replication) for 5 days before harvesting and assaying for \(T_c\)-cell activity.

When spleen stimulator cells were used (as in Table VI), the cells were infected with \(\gamma\)-irradiated Moscow virus, washed three times with warm medium, left for 1.5-2 h at 37°C, and then washed three more times before culturing for 5 days at 39°C with memory \(T_c\) cells at a responder:stimulator ratio of 10:1.

**Response to Minor Histocompatibility (H) Antigens.** The method used was similar to that of Bevan (20). DBA/2 mice were primed by i.v. injection of \(10^7\) spleen cells from either BALB/c or BALB/c-\(H-2^{ab}\). After 14-16 days spleen cells from the primed mice were stimulated a second time in vitro by coculturing for 5 days with irradiated BALB/c or mutant stimulator cells, and \(T_c\)-cell activity against minor H antigens was then assayed against the appropriate macrophage target cells.

**Cytotoxicity Assay with Macrophage Target Cells.** The method has been described in detail elsewhere (15). Data given have had spontaneous release subtracted and are the means of
TABLE I

Effect of the BALB/c-H-2<sup>db</sup> Mutation on T-Cell-Mediated Lysis of Virus-Infected Macrophages

| Donors of virus-immune T cells* | Specific lysis of infected targets$ | Strain H-2 map | A.TH | A.TFR5 | BALB/c-H-2<sup>db</sup> | BALB/c-Kh |
|--------------------------------|----------------------------------|---------------|------|--------|----------------------|-----------|
|                                |                                  | K I-A I-B I-C | S   | D   |                     |           |
| 1 A.TH                         | s      a      a      a      d    | 24.0  | 12.5 | 32.7 | 20.4                 |           |
| A.TFR5                         | t      t      t      t      k    | 35.6  | 24.0 | 33.0 | 41.2                 |           |
| BALB/c-H-2<sup>db</sup>        | d      d      d      d      d    | 3.2§  | 0§   | 47.7 | 43.6                 |           |
| BALB/c-Kh                      | d      d      d      d      d    | 36.8  | 31.3 | 46.7 | 42.1                 |           |
| 2 A.TL                         | s      k      k      k      d    | 41.3  | 27.8 | 24.7 | 34.7                 |           |
| A.TFR2                         | f      f      f      f      a    | 36.0  | 33.9 | 42.9 | 35.2                 |           |

* T cells obtained from pools of two spleens at the peak of the primary response in vivo 6 days after i.v. immunization with attenuated virus.

† Percent 51Ch release from ectromelia-infected targets over a 16-h period with spontaneous release and release from uninfected controls subtracted. Killer:target ratio was 30:1. Means of triplicates are given. SE of means were never larger than 2.9 (exp. 1) and 2.5 (exp. 2).

§ Not significantly greater lysis than uninfected control targets. All other killer:infected target combinations gave significant lysis ($P < 0.01$).

Results

Effect of the BALB/c-H-2<sup>db</sup> Mutation on Virus-Specific T-Cell-Mediated Lysis. BALB/c-H-2<sup>db</sup>, BALB/cKh, and a number of other strains with recombinant H-2 haplotypes were immunized with ectromelia virus. Virus-immune T<sub>C</sub> cells from A.TH, A.TFR5, A.TL, and A.TFR2 mice, which share only the D region with BALB/cKh (wild type) or BALB/c-H-2<sup>db</sup> (mutant) lysed infected targets of either mutant or wild type with similar efficiency (Table I). In contrast, virus-immune T<sub>C</sub> cells from BALB/c-H-2<sup>db</sup> mice gave little or no significant lysis of infected A.TH or A.TFR5 targets (Tables I and II), but did kill infected self or infected BALB/cKh targets, presumably through H-2K<sup>d</sup> sharing (Table I). Thus the mutation did not seem to directly affect the virus-induced...
antigenic change(s), dependent upon the \( H-2^d \) region, which are required for recognition and lysis by virus-immune \( T_c \) cells. But these same antigenic change(s) apparently induced only a very weak \( T_c \)-cell response in BALB/c-\( H-2^{db} \) mice.

\( F_1 \) hybrids between BALB/c-\( H-2^{db} \) and B10.A(2R), DBA/1, or SJL were also tested; these last three strains all possess a gene for members of the H-2.28 family of specificities (21). After immunization with virus, the \( F_1 \) hybrids gave little or no significant \( T_c \)-cell-mediated lysis of infected A.TH or A.TFR5 target cells (Table II). Thus, introducing genes for other versions of H-2.28 was insufficient to overcome the effect of the BALB/c-\( H-2^{db} \) mutation.

**Capacity of BALB/c-\( H-2^{db} \) Mice to Respond to Virus-Induced Antigenic Changes Associated with Their Own H-2D Region.** Although BALB/c-\( H-2^{db} \) mice generated few virus-immune \( T_c \) cells that recognized wild-type \( H-2^d \) determinants plus virus-specific antigen, they may have responded to virus-induced changes specific for mutant \( H-2^d \) antigenic patterns. This was tested by taking pools of BALB/c-\( H-2^{db} \) and D2.GD virus-immune \( T_c \) cells and assaying them for a short time at various killer:target ratios against BALB/c-\( H-2^{db} \) and C3H.OH macrophage targets. Lysis of infected C3H.OH targets by these two \( T_c \) cell pools was similar at 10:1 (BALB/c-\( H-2^{db} \)) and 5:1 (D2.GD) (Table III). This lysis would be caused by \( T_c \)-cell clones recognizing virus-specific antigenic changes associated with \( H-2K^d \) (12, 13). If the BALB/c-\( H-2^{db} \) \( T_c \)-cell pool contained additional clones recognizing virus-induced changes specific for the mutant \( H-2^d \) region, then it should cause more lysis of infected BALB/c-\( H-2^{db} \) targets than the D2.GD \( T_c \)-cell population. This was not the case; killing was again similar (Table III), thus suggesting a poor response to mutant \( H-2^d \) region-associated, virus-induced antigenic patterns. This is not definitive, however, since in our hands, killer:target dose-response relationships are not reproducible with macrophage target cells (15). Attempts to resolve the issue with the cold target competition approach (14) were also unsuccessful.
### TABLE III
Relative Efficiency of Lysis of Infected Self or Infected H-2K-Compatible Targets by BALB/c-H-2<sup>db</sup> Virus-Immune T Cells

| Donors of virus-immune T cells | Killer target ratio | Specific lysis of macrophage targets |
|-------------------------------|----------------------|-----------------------------------|
|                              | Infected | Uninfected | Infected | Uninfected | Infected | Uninfected |
| BALB/c-H-2<sup>dd</sup>       | 10:1     | 26.0 ± 1.7 | 2.5 ± 0.1 | 21.6 ± 3.1 | 0        | 2.5 ± 1.1 | 1.2 ± 0.5 |
| (ddddd"db")                  |          |            |          |            |          |            |
| D2.GD                         | 5:1      | 20.6 ± 1.7 | 0        | 24.4 ± 2.6 | 0        | 0        |
| (ddbbb)                       |          |            |          |            |          |            |

* As for Table I.
† H-2 map regions as for Table I.
§ Assay run for 4 h. Data given are means of triplicates ± SE of the mean with spontaneous release subtracted.

### TABLE IV
Capacity of Infected BALB/c-H-2<sup>db</sup> Macrophages to Stimulate Cytotoxic T-Cell Clones that Recognize Virus-Induced Antigenic Patterns Associated with Either H-2K or H-2D

| Exp. | Responder T cells* | Infected macrophage stimulator cells | Yield | Killer target ratio | Specific lysis of infected target |
|------|--------------------|------------------------------------|-------|---------------------|----------------------------------|
| 1 (CBA/H × BALB/c:F<sup>1</sup>)<sup>‡</sup> | BALB/c (ddddd) | 32 | 5:1 | 31.6 ± 2.8 | 19.7 ± 1.3 | 4.7 ± 1.5 |
| (CBA/H × BALB/c:F<sup>1</sup>)<sup>‡</sup> | BALB/c-H-2<sup>dd</sup> (ddddd"db") | 32 | 2:1 | 26.2 ± 0.9 | 16.8 ± 1.9 | 2.1 ± 1.3 |
| 2 (C57BL/6 × BALB/c:F<sup>1</sup>)<sup>‡</sup> | BALB/c (ddddd) | 44 | 8:1 | 27.1 ± 1.1 | 23.5 ± 1.0 | 0 |
| (C57BL/6 × BALB/c:F<sup>1</sup>)<sup>‡</sup> | BALB/c-H-2<sup>dd</sup> (ddddd"db") | 38 | 2:1 | 27.8 ± 1.8 | 10.1 ± 0.9 | 0 |

* Cytotoxic T cells were generated in secondary responses in vitro (see Materials and Methods).
† Number of viable responder cells after 5 days in vitro expressed as a percentage of the original responder number set up.
‡ H-2 map regions as for Table I.
§ Assay run for 6 h at killer-target ratios indicated; data given are means of triplicates ± SE of the mean; otherwise as for Table I.
* Significantly less lysis than that caused by T cells stimulated by infected BALB/c macrophages and assayed on infected A.TH or A.TL targets at the same killer target ratio (P < 0.001).

Capacity of Infected BALB/c-H-2<sup>db</sup> Macrophages to Stimulate T<sub>c</sub>-Cell Responses. While infected BALB/c-H-2<sup>db</sup> macrophages display virus-induced antigens recognizable by virus-immune T<sub>c</sub> cells from wild-type H-2D<sup>d</sup> mice (Table I), the T<sub>c</sub>-cell response against these antigens in BALB/c-H-2<sup>db</sup> mice is weak or absent. The ability of infected BALB/c-H-2<sup>db</sup> macrophages to stimulate wild-type H-2D<sup>d</sup> T<sub>c</sub> cells was therefore tested. Memory T<sub>c</sub> cells from virus-primed (CBA/H × BALB/c:F<sup>1</sup>) mice were used as responders, to detect any escape of virus-specific antigen from the nominated stimulators into some responder cells.

BALB/c and BALB/c-H-2<sup>db</sup>-infected macrophages stimulated similar responses against infected C3H.OL targets, where lysis is caused by T<sub>c</sub> cells recognizing virus-specific plus H-2K<sup>d</sup> antigens (Table IV). But BALB/c-H-2<sup>db</sup> stimulators were significantly inferior to BALB/c in stimulating T<sub>c</sub> cells that
recognize virus-specific plus H-2D\textsuperscript{d} antigens on infected A.TH targets (Table IV, Exp. 1). Minor escape of virus-specific antigen into the (CBA/H × BALB/c)\textsubscript{F\textsubscript{1}} responder cells may have occurred, since slight lysis of infected CBA/H targets was seen (Table IV, exp. 1). A second, similar experiment with (C57BL/6 × BALB/c)\textsubscript{F\textsubscript{1}} responders gave similar results (Table IV, exp. 2) except that no escape of antigen into responder cells was detected. Thus, it seems that infected BALB/c-H-2\textsuperscript{ab} cells are significantly inferior to wild type in terms of stimulating a T\textsubscript{c}-cell response against virus-specific plus H-2D\textsuperscript{d}-coded determinants. These data suggest the possibility that the physical relationships between virus-specific and H-2D\textsuperscript{d} molecules that may be required for efficient induction of virus-immune T\textsubscript{c} cells could be impaired by the absence of D' (23) from the surfaces of BALB/c-H-2\textsuperscript{ab} cells.

Capacity of Uninfected BALB/c-H-2\textsuperscript{ab} Cells to Stimulate T\textsubscript{c}-Cell Responses to Minor H Antigens. The T\textsubscript{c}-cell response to minor H antigens exhibits H-2 restriction (20). Thus, it offers a means to test whether the stimulation defect of BALB/c-H-2\textsuperscript{ab} reflects an intrinsic failure of D region-coded molecule(s) to provide the antigenic configurations needed for T\textsubscript{c}-cell stimulation, or whether the defect lies in a failure of BALB/c-H-2\textsuperscript{ab} molecule(s) to associate adequately with virus-specific antigen.

Spleen cells from DBA/2 (H-2\textsuperscript{d}) mice primed with either BALB/c or BALB/c-H-2\textsuperscript{ab} cells, were stimulated a second time in vitro with irradiated BALB/c or BALB/c-H-2\textsuperscript{ab} cells, respectively. T\textsubscript{c}-cell activity was then assayed against C3H.OL (K\textsuperscript{d}, D\textsuperscript{b}) and A.TL (K\textsuperscript{a}, D\textsuperscript{a}) macrophage targets that would display minor H antigens shared with the BALB/c background in association with either H-2K\textsuperscript{d} (C3H.OL) or H-2D\textsuperscript{d} (A.TL) determinants. At a given killer:target ratio, C3H.OL and A.TL targets were lysed to a similar extent, regardless of whether stimulation was from BALB/c or BALB/c-H-2\textsuperscript{ab} cells (Table V). CBA/H (K\textsuperscript{k}, D\textsuperscript{b}) targets were not lysed, and SJL/J (K\textsuperscript{d}, D\textsuperscript{a}) targets were lysed slightly. These data confirm that the T\textsubscript{c}-cell response to minor H antigens is H-2 restricted to some extent (20) and also indicate that little, if any, of the lysis of C3H.OL and A.TL targets is due to nonspecifically activated T\textsubscript{c} cells that recognize H-2K or H-2D antigens alone. Apparently, therefore, minor H antigens are as immunogenic in association with H-2D-coded antigens of BALB/c-H-2\textsuperscript{ab} as they are with wild type. Similar results have been obtained by using trinitrophenyl-modified cells (data not shown). These results reinforce the possibility that the defect in the T\textsubscript{c}-cell response to ectromelia infection in BALB/c-H-2\textsuperscript{ab} mice may be caused by inefficient physical association between mutant H-2D-coded and virus-specific molecules.

Specific Blocking of T\textsubscript{c}-Cell-Mediated Lysis with Anti-H-2K and Anti-H-2D Sera. Specific anti-H-2 sera against the determinants H-2.4 (D\textsuperscript{a}, private) H-2.28 (D\textsuperscript{d}, public, borne on D') and H-2.31 (K\textsuperscript{d}, private) were used. Virus-infected BALB/c or BALB/c-H-2\textsuperscript{ab} macrophages were attacked by two separate subsets of virus-immune T\textsubscript{c} cells, one subset from D2.GD mice that recognized virus-induced plus H-2K\textsuperscript{d} antigens (12, 13) and the second subset from A.TH mice that recognized virus-induced plus H-2D\textsuperscript{d} antigens (12, 13). Lysis of both BALB/c and BALB/c-H-2\textsuperscript{ab} targets by D2.GD T\textsubscript{c} cells was blocked significantly more by anti-H-2.31 than by anti-H-2.4 and anti-H-2.28 (Table VI). Conversely, lysis of BALB/c targets by A.TH T\textsubscript{c} cells was blocked more by anti-H-2.4 and anti-H-
TABLE V

Cytotoxic T-Cell Response to Minor H Antigens Stimulated by BALB/c and BALB/c-H-2<sup>db</sup> Cells

| Responder<sup>*</sup> T cells | Irradiated stimulator cells Yield | Killer: target ratio | Specific lysis of macrophage target<sup>§</sup> |
|-------------------------------|---------------------------------|---------------------|-------------------------------|
| DBA/2 BALB/c                 | 51 (dddddd)                     | 7.5:1               | 30.9 ± 2.6 (ddddkkk)         |
| DBA/2 BALB/c-H-2<sup>db</sup> | 90 (dddddd)                     | 2.5:1               | 10.5 ± 2.3 (ddddkkk)         |
| DBA/2 BALB/c-H-2<sup>db</sup>| 6.8:1                           | 3.5 ± 1.9           | 19.5 ± 1.9 (ddddkkk)         |

* Cytotoxic T cells were generated in secondary responses to minor H antigens in vitro (see Materials and Methods).

** H-2 regions as for Table I.

§ Assay run for 16 h; data given are means of triplicates ± SE of mean. Since the culture with BALB/c-H-2<sup>db</sup> stimulators gave a higher yield and more powerful cytotoxicity, data presented are from killer:target ratios where lysis caused by both T-cell populations is similar.

TABLE VI

Blocking of T-Cell-Mediated Lysis of Virus-Infected Macrophages by "Monospecific" Anti-H-2 Sera

| Donors of virus-specific T cells<sup>*</sup> | H-2 region shared with target | Specificity of blocking antiserum<sup>‡</sup> | Specific lysis of infected targets<sup>§</sup> |
|--------------------------------------------|-------------------------------|---------------------------------------------|---------------------------------------------|
| D2.GD (ddbbbb)                             | K<sup>‡</sup>                 | H-2.4 (D<sup>+</sup>)                        | 25.9 ± 2.0 (dddbbb)                         |
| A.TH (assssss)                             | D<sup>‡</sup>                 | H-2.4 (D<sup>+</sup>)                        | 25.1 ± 2.4 (ddbbkkk)                        |
|                                            |                               | H-2.31 (K<sup>−</sup>)                      | 19.4 ± 1.7 (ddbbkkk)                        |
|                                            |                               | Nil                                          | 35.2 ± 0.6 (ddbbkkk)                        |
|                                            |                               | H-2.28 (D<sup>−</sup>)                      | 7.3 ± 2.4** (ddbbkkk)                       |
|                                            |                               | H-2.31 (K<sup>−</sup>)                      | 9.6 ± 1.5** (ddbbkkk)                       |
|                                            |                               | Nil                                          | 17.4 ± 0.9 (ddbbkkk)                        |

* Cytotoxic T cells were generated in secondary responses in vitro to syngeneic ectromelia-infected cells (see Materials and Methods).

| Blocking antiserum was added to targets before addition of T cells. Full procedure given in Materials and Methods. Nil, no serum.

§ Assay run for 4 h at a killer target ratio of 2:1. Data given are means of triplicates ± SE of mean with spontaneous release subtracted. Lysis of uninfected controls was negligible.

** Significantly less lysis than with anti-H-2.4 and anti-H-2.28 sera (P < 0.05).

+++ Significantly less lysis than with anti-H-2.31 serum (P < 0.05).

2.28 than by anti-H-2-2.31 (Table VI). Most importantly, anti-H-2.28 failed to block lysis of BALB/c-H-2<sup>db</sup> targets by A.TH T<sub>c</sub> cells while anti-H-2.4 was active (Table VI). This is consistent with the loss of D<sup>+</sup> from the surfaces of mutant BALB/c-H-2<sup>db</sup> cells (21, 23), and also suggests that the blocking activity of the sera was directed at target cells, not T<sub>c</sub> cells, since A.TH T<sub>c</sub> cells possess D<sup>+</sup>.

The specificity of blocking (Table VI) indicated that T<sub>c</sub> cells recognized virus-induced antigenic changes which incorporated, or were physically close to,
either H-2K or H-2D molecules bearing serologically defined determinants. In the case of H-2D\(^d\) antigens on BALB/c cells, a molecule with private determinants (H-2.4) and the D' molecule were apparently both involved.

**Discussion**

The present experiments showed that ectromelia-infected cells of the BALB/c-H-2\(^{db}\) line display virus-induced antigenic patterns associated with the H-2D region, but BALB/c-H-2\(^{db}\) T\(_c\) cell precursors seem to respond weakly, if at all, to these antigenic patterns. BALB/c-H-2\(^{db}\) is a loss mutation apparently in a gene required for production or expression on cell membranes of a 45,000 mol wt molecule, designated D' by Hansen et al. (23), that bears certain H-2 public specificities (21). No other H-2 or Ia antigens seem to be affected (21). Hypotheses to account for the defective response to ectromelia virus infection were considered in this context. First, has there been deletion or suppression of a gene for a T\(_c\)-cell receptor needed for recognition of H-2D\(^d\) antigenic determinants on virus-infected cells? This seems unlikely, since variable regions of T\(_c\)-cell receptors defined thus far are not coded by MHC-linked genes (8, 30). T\(_c\)-cell recognition structures physically associated with D' also seem to be disqualified, since anti-H-2 antibody blocked T\(_c\)-cell-mediated lysis by binding to target cells, not to T\(_c\) cells (Table VI).

Second, does the absence of the D' molecule in BALB/c-H-2\(^{db}\) expose on infected cells other H-2D antigenic determinants, recognizable by T\(_c\) cells, that are not normally exposed on infected wild-type cells? These exposed determinants, plus virus-specific determinants, could then be the predominant inducers of virus-immune T\(_c\) cells in BALB/c-H-2\(^{db}\) mice. There are several points of evidence against this hypothesis, though none of them are definitive. It seems unlikely that new H-2D determinants unique to BALB/c-H-2\(^{db}\) should be present on infected cells, in view of the findings that uninfected BALB/c-H-2\(^{db}\) cells show no such determinants on the basis of serological or skin graft criteria (21). Also, this hypothesis predicts that BALB/c-H-2\(^{db}\) mice should generate a normal level of virus-immune T\(_c\)-cell response against their own H-2D determinants plus viral antigen; such a response is not evident (Table III). Finally, and most importantly, it does not account for the demonstrated defect in the capacity of infected BALB/c-H-2\(^{db}\) macrophages to stimulate virus-immune memory T\(_c\) cells that recognize H-2D\(^d\) determinants (Table IV), despite observations that such infected macrophages are lysed to the same extent as wild-type targets by T\(_c\) cells recognizing wild-type H-2D antigens plus virus-specific determinants, and that BALB/c-H-2\(^{db}\)-infected macrophages are not defective in stimulating virus-immune T\(_c\) cells that recognize H-2K\(^d\) determinants.

The possibility exists, therefore, that the minimum requirements for precursor T\(_c\)-cell induction (including memory T\(_c\) cells) are more rigorous than those for the process of antigen recognition by effector T\(_c\) cells that leads to lysis of infected targets. It seems that the presence of the D' molecule, plus the molecule-bearing H-2D\(^d\)-coded private determinant(s), plus virus-specific antigen on the infected cell surface are all required for precursor T\(_c\)-cell induction, but D' is nonessential for effector T\(_c\)-cell recognition and lysis. When tested for capacity to stimulate T\(_c\)-cell responses against minor H antigens or trinitrophenyl in association with H-2D\(^d\)-coded determinants, BALB/c-H-2\(^{db}\) cells were as effi-
cient as BALB/c cells. Thus, there seems to be no intrinsic defect in the molecule-bearing H-2Dd-coded private determinants in terms of Tc-cell induction. This suggests that the poor induction capability of infected BALB/c-H-2dh cells with respect to virus-specific Tc cells may reside in the manner in which the viral and the two H-2D-coded molecules physically associate in infected cell membranes. The D' molecule may play a crucial role in this association. That a close physical relationship does exist between both of the H-2Dd-coded molecules and virus-specific determinants on the surface of infected cells was shown by the specificity of blocking of virus-immune Tc-cell-mediated lysis by monospecific anti-H-2 sera directed against both private and public determinants (Table VI).

The foregoing evidence, taken together with findings from the H-2Kb mutations (16) suggests that there are two features of H-2 molecules that are pertinent to the H-2 restriction phenomenon. The first is a structure bearing antigenic determinant(s) that are recognized by receptors on Tc cells; such determinant(s) are affected by the H-2Kb mutants (16), but do not seem to be affected in BALB/c-H-2dh. The second may be a structure required for physical association between the H-2 determinant(s) and a virus-specified antigen molecule; such association seems necessary for efficient induction of virus-specific Tc cells. This class of structure is unaffected by the H-2Kb mutations (16) but appears to be affected in BALB/c-H-2dh. In the case of products of the H-2Dd region, this structure is apparently embodied in a second molecule (D') that does not bear private H-2 specificities, but can be identified by antibodies in anti-H-2.28 sera (21, 23, 31, 32). The unusual distribution of the genes for the H-2.28 and H-2.1 families of public specificities among murine H-2K and H-2D regions discussed by Snell et al. (33, 34) and Démant et al. (31) also deserves comment in this connection. It seems that the H-2.28 and H-2.1 specificities are never both coded in the same H-2K or H-2D region, but either one or the other is invariably present (31, 33, 34). This raises the possibility that an H-2.1-bearing molecule serves a similar function in other mouse strains to that ascribed to D' in the present experiments with BALB/c. Testing this possibility may depend upon the isolation of appropriate loss mutations.

Summary

The Tc-cell response to ectromelia virus infection was studied in BALB/c-H-2dh mice which carry a loss mutation in the H-2D region that results in the absence from cell surfaces of a molecule (D') bearing certain public H-2 specificities. When infected, these mice showed a poor response of Tc cells that recognize H-2D plus virus-specific determinants on infected macrophage targets, but gave a normal response to H-2K plus virus-specific antigens. However, their own infected macrophages do display wild-type antigenic patterns involving virus and H-2D since they were killed as efficiently as wild-type (BALB/c,H-2dh)-infected cells by Tc cells specific only for H-2D plus viral antigens. When tested in vitro, infected BALB/c-H-2dh cells stimulated a poor Tc-cell response to H-2D plus virus-specific antigens, but stimulated a normal response (in comparison with infected BALB/c macrophages) to H-2K plus viral antigens. Uninfected BALB/c-H-2dh cells stimulated a normal Tc-cell response to minor H antigens or trinitrophenyl in association with H-2D, thus suggesting that the defective response to infection may reside in a failure of the relevant H-2D
antigens of mutant cells to physically associate with viral antigens. Close
association of viral and H-2D-coded molecules was also suggested by ability of
specific anti-H-2K or -H-2D to partially block Tc-cell-mediated lysis of infected
targets.

These results were interpreted to mean that H-2Dd-dependent, virus-immune
Tc cells recognized an antigenic pattern consisting of virus-specific and H-2Dd
determinants with the latter borne on an H-2D molecule carrying serologically-
defined H-2Dd private specificities. A second H-2Dd-coded molecule (D') was not
required for recognition and lysis by activated Tc cells, but was apparently
necessary for efficient stimulation of precursor Tc cells, perhaps by promoting
appropriate physical association of viral and H-2Dd molecules.

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References
1. Silver, J., and L. Hood. 1976. Structure and evolution of transplantation antigens:
partial amino acid sequences of H-2K and H-2D alloantigens. Proc. Natl. Acad. Sci.
U. S. A. 73:599.
2. Terhorst, C., P. Parham, D. L. Mann, and J. L. Strominger. 1976. Structure of the
HLA antigens: amino-acid and carbohydrate compositions and NH2-terminal se-
quences of four antigen preparations. Proc. Natl. Acad. Sci. U. S. A. 73:910.
3. Melvold, R. W., and H. I. Kohn. 1975. Histocompatibility gene mutation rates: H-2
and non-H-2. Mutat. Res. 27:415.
4. Kees, U., and R. V. Blanden. 1976. A single genetic element in H-2K affects mouse T-
cell antiviral function in poxvirus infection. J. Exp. Med. 143:450.
5. Doherty, P. C., R. V. Blanden, and R. M. Zinkernagel. 1976. Specificity of virus-
immune effector T cells for H-2K or H-2D compatible interactions: implications for
H-antigen diversity. Transplant. Rev. 26:89.
6. Zinkernagel, R. M., and P. C. Doherty. 1974. Immunological surveillance against
altered self components by sensitized T lymphocytes in lymphocytic choriomeningi-
as. Nature (Lond.). 251:547.
7. Doherty, P. C., D. Gotze, G. Trinchieri, and R. M. Zinkernagel. 1976. Models for
recognition of virally-modified cells by immune thymus-derived lymphocytes. Immu-
nogenetics. 3:517.
8. Raff, M. 1976. T cell recognition at Cold Spring Harbor. Nature (Lond.). 263:10.
9. Janeway, C. A., H. Wigzell, and H. Binz. 1976. Two different Vhi gene products make
up the T-cell receptors. Scand. J. Immunol. 5:993.
10. Blanden, R. V., T. E. Pang, and M. B. C. Dunlop. 1977. T cell recognition of virus-
infected cells. In Cell Surface Reviews. G. Poste and G. L. Nicolson, editors. North-
Holland, Amsterdam. 2:249.
11. Langman, R. E. 1977. The role of the major histocompatibility complex in immunity:
a new concept in the functioning of a cell-mediated immune system. Rev. Physiol.
Biochem. and Pharmacol. In press.
12. Blanden, R. V., P. C. Doherty, M. B. C. Dunlop, I. D. Gardner, R. M. Zinkernagel,
and C. S. David. 1975. Genes required for cytotoxicity against virus infected target
cells in K and D regions of H-2 complex. Nature (Lond.). 254:269.
13. Zinkernagel, R. M., M. B. C. Dunlop, R. V. Blanden, P. C. Doherty, and D. C.
Shreffler. 1976. H-2 compatibility requirement for virus-specific T-cell-mediated cytolysis. Evaluation of the role of H-2I region and non-H-2 genes in regulating immune responses. J. Exp. Med. 144:519.

14. Zinkernagel, R. M. 1976. H-2 compatibility requirement for virus-specific T-cell-mediated cytolysis. The H-2K structure involved is coded for by a single cistron defined by B6 (Hzl) and B6 (Hzl70) H-2K* mutant mice. J. Exp. Med. 143:437.

15. Blanden, R. V., M. B. C. Dunlop, P. C. Doherty, H. I. Kohn, and I. F. C. McKenzie. 1976. Effects of four H-2K mutations on virus-induced antigens recognized by cytotoxic T cells. Immunogenetics. 3:541.

16. McKenzie, I. F. C., T. Pang, and R. V. Blanden. 1977. The use of H-2 mutants as models for the study of T cell activation. Immunological Reviews. In press.

17. Brown, J. L., and S. G. Nathenson. 1977. Structural differences between parent and mutant H-2K glycoproteins from two H-2K gene mutants: B6.C-H-2m (Hzl) and B6-H-2m (M505). J. Immunol. 118:98.

18. Schrader, J. W., and G. M. Edelman. 1977. Joint recognition by cytotoxic T cells of inactivated Sendai virus and products of the major histocompatibility complex. J. Exp. Med. 145:523.

19. Schrader, J. W., B. A. Cunningham, and G. M. Edelman. 1975. Functional interactions of viral and histocompatibility antigens at tumor cell surfaces. Proc. Natl. Acad. Sci. U. S. A. 72:5068.

20. Bevan, M. J. 1975. Interaction antigens detected by cytotoxic T cells with the major histocompatibility complex as modifier. Nature (Lond.). 256:419.

21. Schrader, J. W., G. M. Morgan, R. W. Melvold, and H. K. Kohn. 1977. BALB/c-H-2D*: a new H-2 mutant in BALB/cKh that identifies a locus associated with the D region. Immunogenetics. 4:333.

22. Egorov, I. K. 1967. A mutation of the histocompatibility-2 locus in the mouse. Genetika. 3:136.

23. Hansen, T. H., S. E. Cullen, R. Melvold, H. Kohn, L. Flaherty, and D. H. Sachs. 1977. Mutation in a new H-2-associated histocompatibility gene closely-linked to H-2D. J. Exp. Med. 145:1550.

24. Gardner, I. A., N. A. Bowern, and R. V. Blanden. 1974. Cell-mediated cytotoxicity against ectromelia virus-infected target cells. I. Specificity and kinetics. Eur. J. Immunol. 4:63.

25. Blanden, R. V., and I. D. Gardner. 1976. The cell-mediated immune response to ectromelia virus infection. I. Kinetics and characteristics of the primary effector T cell response in vivo. Cell. Immunol. 22:271.

26. Gardner, I. D., and R. V. Blanden. 1976. The cell-mediated immune response to ectromelia virus infection. II. Secondary response in vitro and kinetics of memory T cell production in vivo. Cell. Immunol. 22:283.

27. Pang, T., and R. V. Blanden. 1976. The cell-mediated immune response to ectromelia virus infection. Secondary response in vitro: specificity, nature of effector and responder cells and the requirements for induction of antigenic changes in stimulator cells. Aust. J. Exp. Biol. Med. Sci. 54:255.

28. McKenzie, I. F. C., and G. D. Snell. 1973. Comparative immunogenicity and enhanceability of individual H-2K and H-2D specificities of murine histocompatibility. J. Exp. Med. 138:250.

29. Hansen, T. H., S. E. Cullen, and D. H. Sachs. 1977. Immunochemical evidence for an additional H-2 region closely-linked to H-2D. J. Exp. Med. 145:438.

30. Hammerling, G. J., S. J. Black, C. B. K. Eichmann, and K. Rajewsky. 1976. Idiotype analysis of lymphocytes in vitro. II. Genetic control of T-helper cell responsiveness to antiidiotype antibody. J. Exp. Med. 143:861.
31. Démant, P., G. D. Snell, M. Hess, F. Lemonnier, C. Neauporte-Sautes, and F. Kourilsky. 1975. Separate and polymorphic genes controlling two types of polypeptide chains bearing the H-2 private and public specificities. *J. Immunogenetics*. 2:263.

32. Lemonnier, F., C. Neauporte-Sautes, F. M. Kourilsky, and P. Démant. 1975. Relationships between private and public H-2 specificities on the cell surface. *Immunogenetics*. 2:517.

33. Snell, G. D., M. Cherry, and P. Démant. 1973. H-2: Its structure and similarity to HL-A. *Transplant. Rev.* 15:3.

34. Snell, G. D., P. Démant, and M. Cherry. 1974. Hemagglutination and cytotoxic studies of H-2. V. The anti-27,28,29 family of antibodies. *Folia Biol. (Prague)*. 20:145.