NONRANDOM INCLUSION OF $H-2K$ AND $H-2D$ ANTIGENS
IN FRIEND VIRUS
PARTICLES FROM MICE OF VARIOUS STRAINS*

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Murine cytotoxic T lymphocytes whose activity is subject to H-2 restriction appear to recognize two different specificities on target cell surfaces: a nonself antigen and a self H-2 antigen (1-5). Understanding this phenomenon will depend upon elucidation of both the nature of T-cell receptor(s) and the nature of target cell antigen(s). The two antigens may be either presented separately on target cell surfaces and recognized separately by T cells or combined in some way which permits the recognition of a new interaction antigen. In this latter case, the properties of the molecules involved may allow them to come into contact on the membrane surface creating the target antigen either by means of conformational changes or by formation of a complex.

In instances where the nonself antigen in question is virus-induced, this hypothesis has been tested in the past by the use of anti-virus and anti-H-2 antibodies in studies employing both immunofluorescence and lysostrip techniques to establish whether the two sets of antigens interact physically on cell surfaces. Studies with EL4 cells have shown that $H-2K^b$ and $H-2-D^b$ antigens comigrate in the plane of plasma membrane with an endogenous murine leukemia virus (MuLV)$^\dagger$-specified gp70 glycoprotein under the influence of either anti-virus or anti-H-2 antibodies (6, 7). Studies in our laboratory have demonstrated a similar relationship between antigens induced by Friend MuLV (FV) and H-2 antigens on the surfaces of infected spleen cells, since capping of FV antigens caused partial capping of H-2 antigens. However, in the case of FV, the association between viral and H-2 antigens appeared to be selective: not all allelic products of the $H-2K$ and $H-2D$ loci were observed to cocap with virus antigens (8, 9).

Type C MuLV such as FV mature by budding at the host cell surface, acquiring in this process an envelope consisting of cell membrane (10) bearing virus-specific glycoprotein. If viral glycoprotein molecules are associated with H-2 molecules on the host cell surface at the site of virus budding, then these H-2 molecules might also be detectable in completed virions. To investigate this possibility, we have isolated FV from the serum of infected mice of various H-2 types and examined it for the presence of H-2 antigens. As previously reported (11), disrupted virus from BALB.B ($H-2^b$) mice demonstrated $H-2D^b$

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$^\dagger$ Abbreviations used in this paper: EDTA, ethylenediamine tetra-acetic acid; 199-FCS, medium 199 plus 20% heat-inactivated fetal calf serum; FV, Friend murine erythroleukemia virus; MuLV, murine leukemia virus; NP-40, Nonidet P-40.
but not H-2K\(^k\) antigenic activity, and virus from BALB/c (H-2\(^c\)) animals contained no detectable H-2. In this report we extend these findings to include virus from mice of several other H-2 haplotypes, including H-2\(^k\) and the recombinant haplotypes H-2\(^\alpha\) and H-2\(^\omega\). Of the six H-2K or H-2D private specificities examined, only two, H-2D\(^b\) and H-2K\(^k\), were found in Friend virus particles which must be disrupted to express this activity.

**Materials and Methods**

**Mice.** All animals used in these experiments were from our own colony of highly inbred animals. The congenic BALB.B (H-2\(^\alpha\)) strain was established from the (BALB/c × C57BL/10) F\(_1\) cross as previously described (12). Similarly the congenic BALB.G (H-2\(^b\)) and BALB.K (H-2\(^*\)) strains were established from the (BALB/c × HTG) F\(_1\) cross and (BALB/c × C3H/An) F\(_1\) cross, respectively. C3H.OL mice were originally obtained from Dr. Donald C. Shreffler.

**Viruses.** The variant N → NB-tropic strain of FV was used throughout these studies. This virus strain was derived from the original N-tropic FV by forced passage in BALB/c mice (13). Before each experiment, virus normally passaged in BALB/c mice was passed once through mice of the same strain as the ultimate recipient. For each experiment, virus was obtained from groups of 12-18 mice infected i.p. with ~10\(^5\) spleen focus-forming units (SFFU) of FV. At the peak of viremia 8-10 days later the animals were bled on 3 consecutive days and the serum pooled. Serum prepared in this way characteristically contained 5 × 10\(^5\) SFFU/ml.

**Spleen Membrane Preparation.** A membrane fragment preparation from spleens of normal BALB.B mice was made by homogenization in saline (9 ml/g of spleen); after centrifugation at 6,800 g for 15 min, the supernate was stored at -70°C.

**Virus Purification.** 15 ml of serum clarified by centrifugation at 6,800 g for 15 min was layered over a 30-40% discontinuous sucrose (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. ultrapure) gradient. The virus was banded at the interface by ultracentrifugation in a Beckman SW27.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 16,000 rpm (40,000 g) for 180 min. 1 ml of the interface was collected, diluted to 5 ml with phosphate-buffered saline, transferred to a Beckman SW50.1 rotor, and the virus pelleted by ultracentrifugation at 30,000 rpm (100,000 g) for 90 min. The virus pellet was then resuspended in 0.05 ml PBS with 1 mM ethylenediamine tetra-acetic acid (EDTA) to which was added 0.01 ml of a 1.5% Nonidet-P-40 detergent (NP-40) solution. The virus was allowed to sit for 1 h on ice after which time 0.06 ml of a 25% solution (wt/vol) of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) was added to diminish the effect of the NP-40. After adjusting to the desired volume with medium 199 (Microbiological Associates, Bethesda, Md.) containing 20% heat inactivated fetal calf serum (FCS) (199-FCS), the virus preparation was ready for use. Sham preparations were made similarly but starting with serum from uninfected mice.

**Antisera.** Anti-H-2 antisera specific for a single H-2K or H-2D gene product were produced by hyperimmunization of recipient mice with allogeneic spleen cells as indicated in Table I. The antisera were routinely assayed by cytotoxicity to verify their specificity and to establish the absence of cytotoxic anti-FV antibodies.

**Direct Antibody-Dependent Cytotoxicity.** H-2 antigen activity was detected by its capacity to inhibit lysis of normal mesenteric lymph node cells in the direct antibody-dependent cytotoxicity assay (14). To tubes containing 0.1 ml of serially diluted virus or control preparation was added 0.1 ml of anti-H-2 antiserum diluted in 199-FCS to a concentration producing about 80% of maximum lysis in the absence of competing antigen. This mixture was allowed to stand for 10 min at room temperature, and then 0.05 ml of \(^{51}\)Cr-labeled mesenteric lymph node cells (10\(^6\)/ml) and 0.05 ml of diluted guinea pig serum (Grand Island Biological Co., Grand Island, N. Y.) as a complement source were added. After 1 h incubation at 37°C in an atmosphere containing 5% CO\(_2\), the reaction was terminated by the addition of 0.3 ml of a 10 mM EDTA solution in saline. The tubes were centrifuged for 5 min at 300 g and 0.3 ml of supernate collected for counting \(^{51}\)Cr radiation. 100% killing was defined as counts per minute (minus the spontaneous release) contained in 0.3 ml of uncentrifuged \(^{51}\)Cr-labeled target cell suspension.

**Virus Neutralization.** Attempts to detect neutralization by anti-H-2 antisera (Table I) of the spleen focus-forming virus component of FV were performed with the spleen focus assay (15) as
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Results

Virus collected and partially purified from the infectious sera of several H-2-congenic strains of mice was tested for content of H-2 antigenic activity by determining its capacity to inhibit the lytic activity of various anti-H-2K and anti-H-2D antisera. Virus prepared from the serum of BALB.B mice (H-2^b) inhibited the cytotoxicity of anti-H-2D^b antibodies but did not inhibit anti-H-2K^b antibodies (Fig. 1). Virus from BALB/c mice (H-2^d) did not inhibit anti-H-2D^d or anti-H-2K^d antibodies, nor did it inhibit antisera specific for H-2^b antigens (Fig. 2). As previously reported (11), virus preparations from heterozygous H-2^d/H-2^b mice, either (C57BL/6 × DBA/2)F_1 or (BALB/c × BALB.B)F_1, demonstrated H-2D^b antigenic activity to the exclusion of the three other H-2K and H-2D specificities of the host. The H-2^o haplotype of BALB.G mice represents a recombinant haplotype produced originally in an H-2^d/H-2^b heterozygote and which comprises the K, I, and S regions of the H-2^d haplotype and the D region of the H-2^b haplotype. Virus collected from these mice showed inhibition of anti-H-2D^b but not anti-H-2K^o antibodies (Fig. 3).

Virus prepared from the serum of infected BALB.K mice (H-2^k) was found to inhibit anti-H-2K^k antiserum but showed little or no inhibition of anti-H-2D^k
antiserum (Fig. 4). This pattern of inhibitory activity was the inverse of that seen with virus from BALB.B mice.

Three controls important for validating these results were performed. To insure that the selective inhibition of anti-\(\mathrm{H}-2D^b\) antibodies was not a unique function of a particular anti-\(\mathrm{H}-2D^b\) antiserum, three alloantisera specific for \(\mathrm{H}-2D^b\) produced by three different alloimmunizations (Table I) were used, and the results were similar in all cases. The possibility that the selective inhibition observed might be due to unequal sensitivity of the various antisera to competitive inhibition was minimized by the finding that membrane preparations, presumed to include equal amounts of \(\mathrm{H}-2K\) and \(\mathrm{H}-2D\) antigen activities, from spleens of normal BALB.B mice showed identical patterns of inhibition of
both anti-\(H-2K^a\) and anti-\(H-2D^b\) antisera (data not shown). To determine if the antigen activity detected was present in some other sedimentable component of mouse serum, normal sera from uninfected BALB.B and BALB.K mice were used to produce sham virus preparations, and no H-2 activity was detected (data not shown).

Since not more than a single \(H-2\) antigenic specificity had been detected in virus particles in the preceding experiments, two further experiments were carried out to rule out the possibility that the incorporation of one \(H-2\) antigen in virus precludes the incorporation of a second \(H-2\) antigen. First, we examined FV from infected C3H.OL mice bearing the \(H-2^{a1}\) haplotype, which is a recombinant comprising the \(K\) and \(I\) regions of \(H-2^d\) and the \(S\) and \(D\) regions of
H-2^k, and Fig. 5 indicates that no H-2K^d activity and at most a trace of H-2D^k activity were detected. This finding indicates that the lack of H-2D^k activity in virus from BALB.K mice was not due to its exclusion by the presence of H-2K^k activity. Second, FV prepared from (BALB.G x BALB.K)F_1 mice showed both H-2D^b and H-2K^k antigenic activity (Fig. 6), thus demonstrating that it is possible to detect more than one specificity in a single FV preparation.

As cellular constituents, H-2 molecules are detected most readily on plasma membranes. If the inclusion of these molecules into virus particles is the result of H-2/virus molecular interaction on the cell surface at the site of virus budding, then the probable location of H-2 molecules in the virion is on the virus envelope. To test this hypothesis, experiments were performed with gradient purified virus taken directly from the 30-40% sucrose interface and not subjected to disruption with NP-40 detergent. Virus prepared in this way from either BALB.B or BALB.K mice showed little or no H-2 antigenic activity (Figs. 7 and 8). A second approach was to determine whether H-2 molecules in intact virions were accessible to anti-H-2 antibodies as measured by the capacity of antisera to neutralize the virus. Infectious serum from (BALB.B x BALB.K)F_1 mice was incubated for one-half hour with either antiserum to the K or D specificities of H-2^b or H-2^k antigens or no serum as a control. Despite previous experiments demonstrating that disrupted virus isolated from this
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Fig. 6. Lysis of BALB.G (left) or BALB.K (right) lymph node cells by either anti-H-2\(^b\) or anti-H-2\(^k\) antisera, respectively, in the presence of either disrupted FV from (BALB.G x BALB.K) F\(_1\) mice (---) or control preparations (-----).

Fig. 7. Lysis of BALB.B lymph node cells by anti-H-2\(\text{D}^b\) (top) or anti-H-2\(\text{K}^b\) (bottom) antisera in the presence of either gradient-purified, undisrupted FV from BALB.B mice (---) or control preparations (-----).

serum contained H-2\(D^b\) and H-2\(K^b\) antigens (data not shown), antiserum to these H-2 antigens had no effect on the spleen focusing-forming activity (Table II). In experiments not shown, further efforts were made to detect neutralization of virus activity with anti-H-2 antisera by the addition of either goat antismouse gammaglobulin serum or rabbit or guinea pig serum as complement sources. No neutralization was detected.

Discussion

Disrupted FV particles isolated from the serum of viremic mice displayed H-2\(D^b\) or H-2\(K^b\) antigen activity when the H-2 haplotype of the host animal included the alleles governing either or both of those antigens. H-2\(K^b\), H-2\(D^b\),
Fig. 8. Lysis of BALB.K lymph node cells by anti-H-2K\(^k\) (top) or anti-H-2D\(^k\) (bottom) antisera in the presence of either gradient-purified, undisrupted FV from BALB.K mice (---□--) or control preparations (--O--).

### Table II

| Antiserum | Mean no. of foci ± SD  |
|-----------|-----------------------|
| Exp. 1    |                       |
| None      | 40.0 ± 3.5            |
| * Anti-H-2D\(^b\) | 37.4 ± 5.9        |
| † Anti-H-2K\(^k\) | 37.8 ± 2.9        |
| Exp. 2    |                       |
| None      | 24.0 ± 3.7            |
| § Anti-H-2D\(^k\) | 25.3 ± 4.7         |
| || Anti-H-2K\(^k\) | 21.8 ± 1.3         |

* BALB/c anti-BALB.G.
† (A x BALB.G)F, anti-BALB.B.
§ (C3H/SW x A)F, anti-BESS (C3H/An).
|| C3H.OL anti-BESS (C3H/An).

H-2K\(^d\), or H-2D\(^k\) antigens were not detected in virus from mice carrying those alleles. The selective inclusion into FV of only two of six H-2 antigens examined implies a nonrandom association of FV and H-2 molecules on the host cell surface, presumably at the site of virus budding. The antigens were detected by the capacity of virus preparations to inhibit cytolysis mediated by anti-H-2 sera. The observed antigenic activity was not the result of a cross-reaction with virus antigens because virus preparations from H-2\(^d\) mice did not demonstrate H-2D\(^k\) antigen activity (Fig. 2), and none of the H-2 sera contained FV-specific cytotoxic activity (14). The selectivity of detection was not due to peculiarities of the antisera used as three separate anti-H-2D\(^b\) antisera raised in three different alloimmunizations (Table I) all gave similar results during
the course of this study. Also, in control experiments, the anti-H-2 sera were found to be inhibited equally when normal spleen cell membrane preparations were used as the source of H-2. Occasional augmentation of lysis over controls was observed in both virus and sham normal mouse serum preparations, suggesting that there was a normal mouse serum component present in addition to the virus. The effect of the contaminant was not immunologically specific, however, as preparations from H-2d mice augmented lysis by anti-H-2b antisera in the same manner (Fig. 2).

For a given haplotype, the appearance of one H-2 antigen to the exclusion of the other did not appear to be the result of competition between products of the K and D loci for virus molecules. H-2Dk was not detected in virus from H-2k mice in which H-2Kk was detected, but it was also not detected in virus from mice with the recombinant H-2d1 haplotype governing the nonincluded H-2Kd molecule (Fig. 5). In a second experiment, both H-2Db and H-2Kk antigens were found in virus from (H-2d/H-2k)F1 mice (Fig. 6), suggesting that the association of virus molecules on the cell surface with one H-2 antigen does not preclude the association with a second H-2 antigen. Although it is not possible to say whether both antigens can be found on a single virion, it is reasonable to suspect that associations are formed solely on the basis of the capacity of H-2 molecules to bind with virus antigens. Hecht and Summers (16) have reported similar experiments in which purified vesicular stomatitis virus grown in L cells (H-2k) showed incorporation of both H-2Kk and H-2Dk antigens. Because of the detection of both antigens, they were unable to determine whether the inclusion of H-2 antigens was due to random distribution of H-2 molecules on cell membrane destined to become virus envelope or, as reported here, a selective process.

To detect H-2 activity, it was necessary to disrupt the virions with NP-40 detergent; intact virions displayed little or no H-2 antigenicity (Figs. 7 and 8). Since H-2 molecules are membrane proteins, it is reasonable to anticipate that H-2 found in virus would be associated with the virus envelope. The inability to detect H-2 antigens on the surface of intact virus particles may be due to steric inhibition of this 45,000 dalton glycoprotein caused by the close packing of the larger gp69 virus envelope molecules. The viral membrane protein p15 may be inaccessible on intact virions to antibodies for similar reasons. Schwarz et al. (17) have proposed that the failure of anti-p15 antiserum to neutralize intact FV is due to the p15 molecule being covered by glycoprotein knobs of gp69. Even if the steric inhibition of virus-associated H-2 antigens is incomplete, it may be of sufficient magnitude to decrease the amount of H-2 antigen exposed on intact virus below the level of detection of the cytotoxicity inhibition assay. Indeed, the insensitivity of the assay leaves open the question of whether the undetected H-2 antigens may, in fact, be present in small amounts in virions. In other enveloped virus, normal cell membrane components have been demonstrated to be present in progeny virus envelopes (18). Unless those H-2 molecules which fail to complex with virus molecules are actively excluded from the assembling virion it may be presumed that allelic differences in H-2 antigenicity are quantitative rather than all-or-none.

Our studies concerning the presence of H-2 in virus do not indicate at which
point the presumed molecular interaction occurs, and, in the absence of biochemical studies, we cannot exclude the possibility that interaction occurs at the level of transcription with the production of one gene product expressing both virus and H-2 antigens. This possibility seems unlikely, however, in light of recent findings by two groups of investigators (19, 20). They found that T cells from mice immunized with Sendai virus lysed uninfected syngeneic target cells onto which had been adsorbed inactivated Sendai virus. Because neither viral replication nor the synthesis of new proteins was necessary for target cells to become susceptible to lysis, they concluded that the target antigen was not formed by alteration of H-2 or virus antigens during biosynthesis.

We cannot conclude from this study alone that the cell surface H-2/virus complex which leads to inclusion of H-2 in the virion is the same structure which serves as the target antigen for killer T cells. However, there are several lines of evidence which suggest that this is the case. Those strains of mice whose progeny virions incorporate H-2, BALB.B, BALB.G, and BALB.K, are the same strains which are able to reject inocula of syngeneic FV-induced tumor cells of the HFL series and generate tumor-specific cytotoxic T cells (21, 22). BALB/c mice, on the other hand, produce virions with no detectable H-2, are unable to reject syngeneic HFL cells, and fail to generate anti-FV T cells. In studies on the effect of treating infected spleen cells with anti-FV antibodies, capping of FV antigens was found to induce co-capping of both H-2K and H-2D antigens (8, and unpublished observations). Finally, H-2D but not H-2Kb or H-2Kb antigens are recognized on target cells by T cells from BALB.B and BALB.G mice immunized with syngeneic HFL cells (21). (Analogous studies in BALB.K mice have not been done). These findings all suggest the existence of a cell surface H-2/virus molecular complex which acts as the target antigen for H-2-restricted T lymphocytes.

Such an H-2/virus molecular complex may serve not only as a target antigen for H-2-restricted T lymphocytes but also as a stimulating antigen in the generation of these T cells. This may be the mechanism by which the Rfv-1 gene (23) confers relative resistance to the FV disease in mice carrying the H-2Db haplotype. Presentation of H-2D and virus antigens in a complex may be the signal which elicits cytotoxic T-lymphocyte formation and thus protects the host from the lethal effects of the disease syndrome.

Summary

Friend murine leukemia virus (FV), isolated from infectious serum of several mouse strains, has been examined for the presence of H-2 antigens. Following banding of the virus on a discontinuous sucrose gradient, pelleting, and disruption with Nonidet P-40 detergent, virus preparations were tested for their capacity to inhibit the lysis of target cells mediated by various anti-H-2K or anti-H-2D antisera. Virus from mice homozygous for the H-2b, H-2d, H-2g, H-2k, and H-2t haplotypes or heterozygous for the H-2b/H-2k, H-2d/H-2k, and H-2g/H-2k haplotypes was used. Of the six H-2D or H-2K alleles examined, the products of only two, H-2Db and H-2Kk were detected. Virus preparations contained no, one, or both antigens, depending on the genotype of the host animal. Control preparations from normal mouse serum and preparations in
which the virus had not been disrupted demonstrated no H-2 activity. Furthermore, attempts to neutralize FV spleen focus forming activity with anti-H-2D<sup>b</sup> or anti-H-2K<sup>k</sup> antisera yielded negative results.

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