

MOUSE H-2^k-RESTRICTED CYTOTOXIC T CELLS
RECOGNIZE ANTIGENIC DETERMINANTS IN BOTH THE
HA1 AND HA2 SUBUNITS OF THE INFLUENZA A/PR/8/34
HEMAGGLUTININ

BY KEITH G. GOULD, HILARY SCOTNEY, ALAIN R. M. TOWNSEND,*
JUDY BASTIN,* AND GEORGE G. BROWNLEE

*From the Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE; and
the *Nuffield Department of Medicine, John Radcliffe Hospital,
Oxford OX3 9DU, United Kingdom*

CTL specifically recognize and lyse virus-infected target cells generally in conjunction with self class I MHC molecules (1). Infection by influenza A viruses produces a vigorous CTL response in both mice and humans (2), and this may be important in limiting the spread of viral infection and in viral clearance (3, 4). Influenza A virus hemagglutinin (HA)¹ is known to serve as a target antigen for influenza-specific murine CTL (5–7), although the nucleoprotein, rather than the HA, appears to be the major influenza protein recognized by CTL (7, 8).

The HA molecule consists of a heavy chain subunit (HA1) of ~330 amino acid residues and a light chain subunit (HA2) of ~220 amino acid residues held together by a single disulfide bond (9). The antigenicity of the HA for B lymphocytes has been well studied, and a number of dominant antigenic regions have been localized within the HA1 subunit (10, 11). In contrast, much less is known about the sites within the HA molecule recognized by CTL.

Recently, recombinant vaccinia viruses expressing individual influenza genes have been shown to prime and stimulate specific CTL responses in mice (8, 12, 13). Cells infected with recombinant vaccinia viruses can also serve as targets for MHC-restricted CTL specific for the product of the inserted gene (8, 12, 13). We and others, using vaccinia viruses expressing influenza A/PR/8/34 or A/JAP/305/57 HA, have confirmed that HA is a target antigen for polyclonal CTL (5, 12, 14), and have also shown that there is very little crossreaction of CTL between different HA subtypes (5, 14). This was confirmed in an analysis of the specificity of 10 HA-specific CTL clones (15). Minimal crossreaction of CTL occurs between HAs of H1 and H3 subtypes, as expected from the relative amino acid homologies between different HA subtypes (16, 17). We have taken advantage of this specificity to test whether determinants in the A/PR/8/34 HA for H-2^k-restricted polyclonal CTL occur in the HA1, HA2, or in both subunits. We chose to construct genes coding for intact HA molecules in which the HA1 subunits of A/PR/8/34 (H1 subtype) and A/NT/60/68 (H3 subtype) were

¹ *Abbreviation used in this paper:* HA, hemagglutinin.

exchanged to give two chimeric HA molecules. These chimeric genes were used to construct recombinant vaccinia viruses expressing HA polypeptides. In one chimera the HA1 subunit is of H1 subtype and the HA2 subunit is of H3 subtype, and in the second chimera the HA1 subunit is of H3 subtype and the HA2 subunit is of H1 subtype. Although these molecules do not exist naturally, they might be expected to form structures similar to naturally occurring HA molecules, and to behave in a similar manner in the infected cell. We have used the recombinant vaccinia viruses expressing chimeric HAs to prime mice and to test the specificity of any CTL response. In addition, cells infected with the recombinant vaccinia viruses have been tested as targets for HA-specific CTL.

Materials and Methods

Recombinant Vaccinia Viruses Containing HA Gene Chimeras. A full-length cDNA clone of the HA of human influenza A/NT/60/68 (H3 subtype) was constructed using the same method (7) as previously used for the HA of influenza A/PR/8/34 (H1 subtype, Mount Sinai strain). A *Stu* I restriction enzyme site was introduced into the cDNA clone of the A/PR/8/34 HA at the junction between the HA1 and HA2 subunits (17) using a 22-nucleotide-long mismatch oligonucleotide (18) to direct (19) the synthesis of the required mutation in the HA clone, inserted in M13mp8. A *Stu* I site exists naturally in the equivalent position in the A/NT/60/68 HA gene (20). The chimeric genes KG11 and KG12 were constructed by ligating the required restriction fragments, isolated from agarose gels, with *Hind* III–*Bam* HI cut pKG5 plasmid (pKG5 [Gould, K., unpublished data] is a derivative of pSVtkneo (7), containing extra cloning sites). In the case of the cDNA clone derived from A/NT/60/68, a partial *Hind* III digest was required to isolate the region encoding the entire HA1 subunit because of the presence of an internal *Hind* III site. Similarly, a partial *Bam* HI digest was required for the isolation of the region encoding the HA2 subunit. Recombinant vaccinia viruses expressing the two chimeric HAs, the H1 subtype HA, and the H3 subtype HA were made by standard methods (21, 22). The viruses were designated KG11-VAC, KG12-VAC, H1-VAC, and H3-VAC, respectively (Fig. 2). Plasmid pGS62 (23) was used as the insertion vector, except for the construction of H1-VAC. In that case a new vector, pKG18 (Gould, K., unpublished data), a derivative of pGS62 using the same vaccinia promoter for expression but with extra cloning sites, was used.

Immunoprecipitation. Confluent 25-cm² flasks of CV-1 cells were infected with vaccinia viruses at 30 PFU/cell. After 1 h at 37°C the inoculum was removed and replaced by methionine-free DME medium with 5% FCS. After a further 2 h at 37°C, 100 μCi of [³⁵S]methionine was added to each flask. 1.5 h later cells were harvested, washed once with PBS, and treated as described previously (7, 22).

Cytotoxic T Cell Cultures In Vitro and CTL Assay. 3–6-mo-old female CBA mice (H-2^k) were primed intravenously by injecting 10⁷ PFU of recombinant vaccinia virus in 0.1 ml of PBS into the tail vein (12). 2 wk later their spleens were removed and the spleen cells were stimulated in vitro, as described previously (7). Briefly, 1.2 × 10⁷ spleen cells were mixed with 2 × 10⁶ syngeneic, 2,000 rad irradiated, A/PR/8/34 virus-infected feeder spleen cells in 15 ml of complete medium (RPMI 1640, 10% FCS, 5 × 10⁻⁵ M 2-ME). Cultures were harvested after 5 d at 37°C for testing in a standard 6-h ⁵¹Cr-release assay, with modifications for the use of adherent L929 target cells (H-2^k), as described (7). 2 × 10⁶ target cells were infected with 0.2 ml of infectious allantoic fluid or 2 × 10⁷ PFU vaccinia virus and labeled with 125 μCi of ⁵¹Cr for 90 min at 37°C. Percentage of specific chromium release was calculated as follows: Percent release = 100 × [(release by CTL) – (medium release)]/[(2.5% Triton release) – (medium release)].

Each point was measured in duplicate against quadruplicate controls.

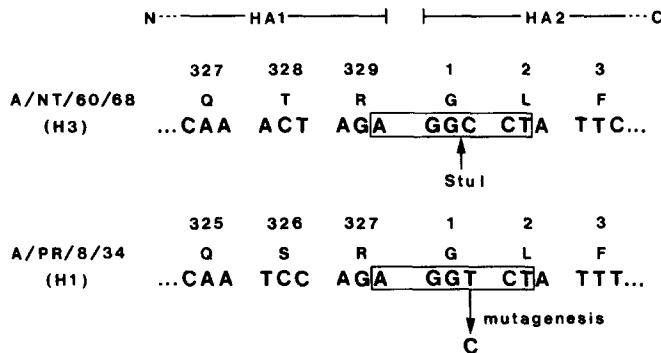


FIGURE 1. Strategy for the construction of a *Stu* I restriction enzyme site at the junction between the HA1 and HA2 subunits of the A/PR/8/34 HA gene.

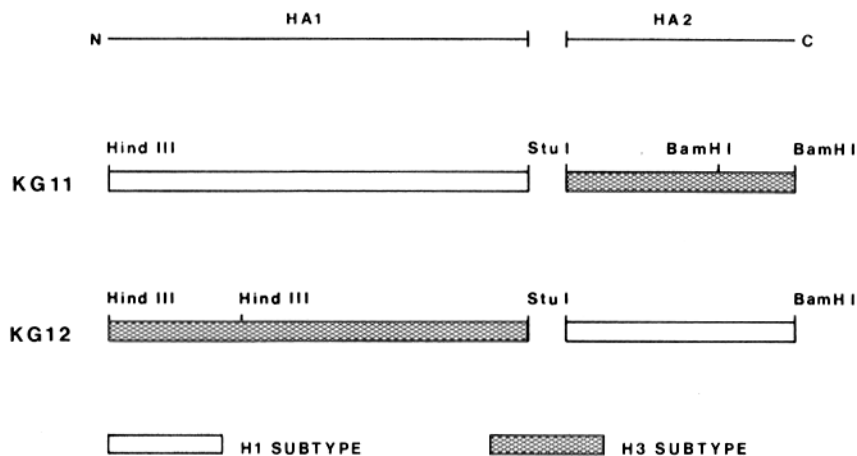


FIGURE 2. The design and construction of chimeric HA genes. The appropriate gel-purified restriction fragments were ligated together as shown, together with Bam HI + Hind III double-cut pKG5 plasmid vector, as described in Materials and Methods. The chimeras were designated KG11 and KG12, as indicated.

Results

Design and Construction of Chimeric HA Genes. A naturally occurring and unique *Stu* I restriction enzyme recognition site (Fig. 1) is present at the precise junction of the HA1 and HA2 subunits in the cDNA clone derived from the HA of influenza A/NT/60/68 (20), and allowed us to design and construct artificial HA1-HA2 chimeras between influenza strains of H1 and H3 subtype. For the design (see below) to work, we first had to mutate a T → C in the HA clone derived from influenza A/PR/8/34 to generate a *Stu* I site, using site-specific mutagenesis (see Materials and Methods).

The design of the chimeras KG11 and KG12 is shown in Fig. 2 and involves ligation of the relevant restriction fragments, isolated from the wild-type cDNA clone of the A/NT/60/68 HA and from the mutant HA clone of A/PR/8/34, with the plasmid pKG5 (see Materials and Methods). Recombinant vaccinia viruses containing these chimeric HAs as well as the wild-type HAs were con-

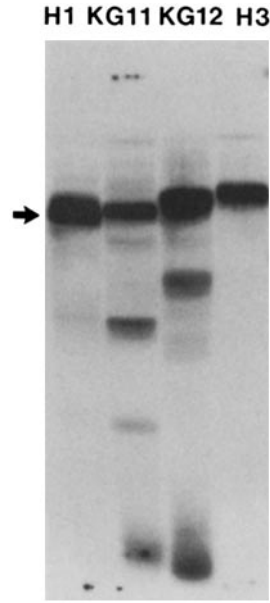


FIGURE 3. Immunoprecipitation of HA polypeptides from cells infected with vaccinia viruses. Cells were infected with vaccinia viruses and labeled with [³⁵S]methionine as described in Materials and Methods. Polyclonal antiserum R401 (H1 subtype) was used for immunoprecipitation from H1-VAC- and KG11-VAC-infected cells, and antiserum X31 (H3 subtype) for immunoprecipitation from KG12-VAC- and H3-VAC-infected cells. Proteins were resolved on a 10% SDS polyacrylamide gel, the gel was treated with sodium salicylate, dried down, and autoradiographed for 49 h. The arrow indicates the position of H1 HA immunoprecipitated from influenza A/PR/8/34-infected cells. Immunoprecipitation of cells infected with nonrecombinant vaccinia virus strain WR gave no visible bands (not shown).

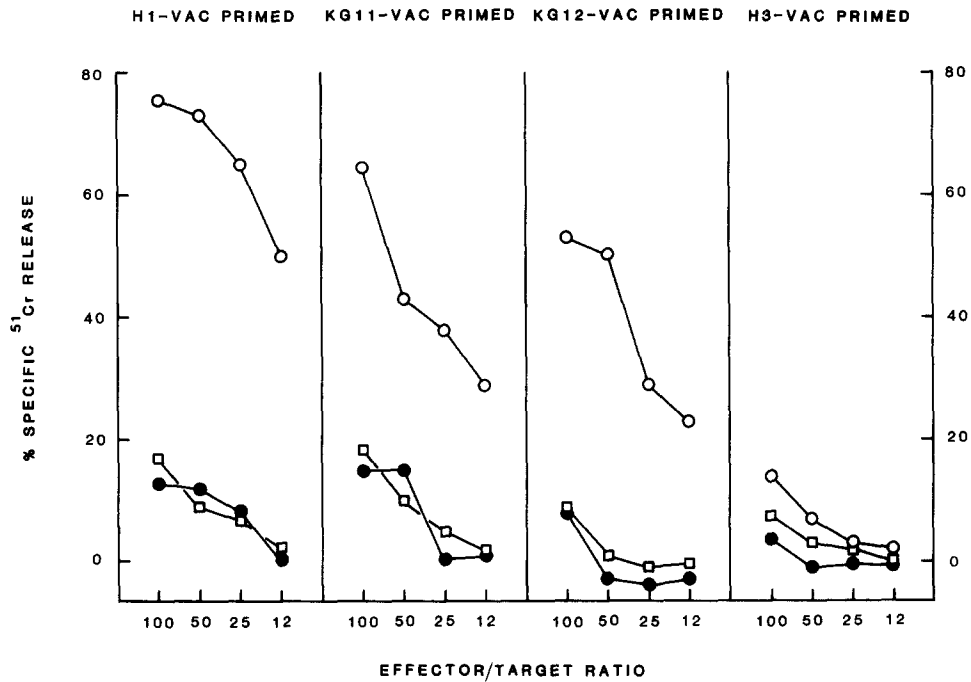


FIGURE 4. Specificity of CTL generated by priming CBA mice with recombinant vaccinia viruses. Mice were primed as described in Materials and Methods, the resulting splenocytes were stimulated in vitro with influenza A/PR/8/34 (H1 subtype), and were tested in a chromium-release assay using L929 target cells infected with (○) influenza A/PR/8/34 (H1 subtype); (●) influenza A/NT/60/68 (H3 subtype); (□) uninfected. Spontaneous release from labeled target cells in medium alone was 24–28% that released by Triton X-100.

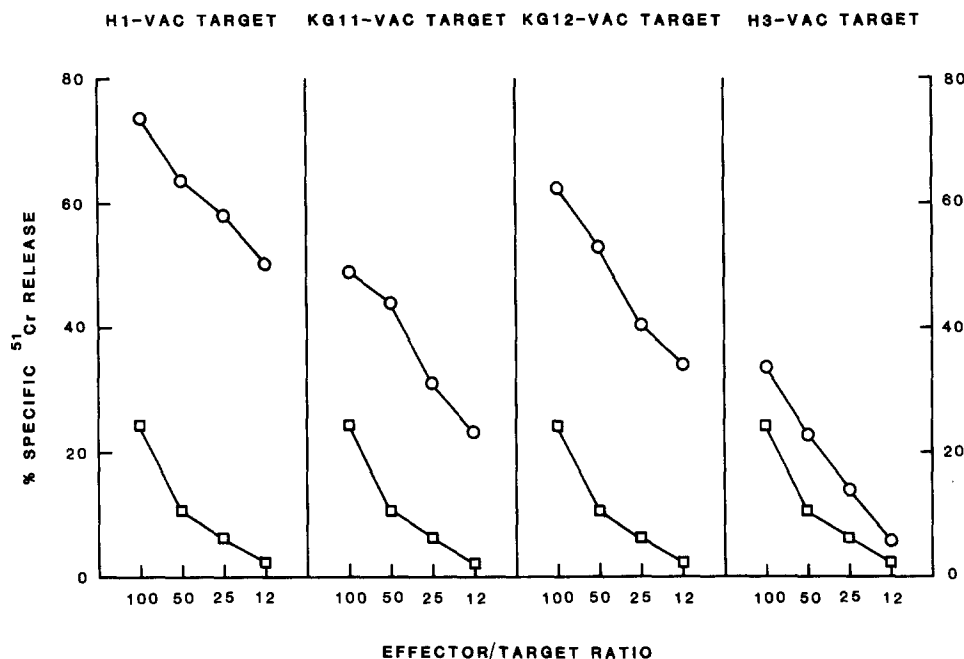


FIGURE 5. Specificity of CTL generated by priming CBA mice with H1-VAC. Mice were primed as described in Materials and Methods, the resulting splenocytes were stimulated in vitro with influenza A/PR/8/34 (H1 subtype), and were tested in a chromium-release assay. L929 target cells were (○) infected with the indicated recombinant vaccinia virus at 10 PFU/cell; (□) uninfected. Spontaneous release from labeled target cells in medium alone was 16–24% that released by Triton X-100.

structed (see Materials and Methods) and are referred to as KG11-VAC, KG12-VAC, H1-VAC, and H3-VAC.

Expression of HA Polypeptides by Recombinant Vaccinia Viruses. Immunoprecipitation of polypeptides from cells infected with the four recombinant vaccinia viruses showed that HA molecules of the expected molecular weights are synthesized in good yield (Fig. 3). The two chimeric HAs are recognized by antisera specific for the subtype of the HA1 subunit. The slight difference in mobility of the intact HAs in the four tracks is probably related to differing extents of glycosylation, although all the HAs have the same number of potential glycosylation sites. The minor low molecular weight bands in the KG11 and KG12 tracks presumably reflect HA breakdown products, and their absence in the H1 and H3 tracks indicates decreased stability of the chimeric HAs. Expression of HA polypeptides on the cell surface was confirmed by FACS profiles of recombinant virus-infected cells stained with HA-specific mAbs (data not shown).

Both Subunits of the HA Molecule Prime Mice for an HA Subtype-specific CTL Response. As expected, priming mice (see Materials and Methods) with control H1-VAC generates H1 subtype-specific polyclonal CTL that do not lyse H3 influenza virus-infected target cells (Fig. 4). Priming with control H3-VAC and stimulating with H1 influenza virus generates barely detectable influenza-specific CTL. In contrast, priming with vaccinia viruses expressing the two chimeric HAs

(KG11-VAC and KG12-VAC) gives a strong H1-specific CTL response in both cases (Fig. 4). These results clearly show that both the HA1 and HA2 subunits of the A/PR/8/34 HA molecule contain determinants for generating CTL.

Both Subunits of the HA Molecule Are Targets for HA-specific Polyclonal CTL. Cells infected with each of the four recombinant vaccinia viruses were tested as targets for CTL generated by priming mice with H1-VAC (Fig. 5). As expected from previous results (7, 14), control H1-VAC CTL recognize H1-VAC-infected cells but only show a small amount of crossreaction with control H3-VAC-infected cells. Here we observe that both chimeric HA molecules (KG11 and KG12 targets) are recognized efficiently (Fig. 5). In three different experiments, including that shown in Fig. 5, lysis of targets expressing the HA2 subunit of H1 subtype (i.e., KG12-VAC) was higher than lysis of targets expressing the HA2 subunit of H3 subtype (i.e., KG11-VAC).

Discussion

In this report we show that both subunits of the HA of influenza A/PR/8/34 (H1 subtype) contain an antigenic determinant or determinants for CTL in CBA (H-2^k) mice. This is in agreement with other work (15) that suggested that CTL derived from H-2^d mice recognize up to four distinct antigenic sites within the HA molecule of A/JAP/305/57 (H2 subtype). Recently, it has been shown (24) that the epitopes of influenza nucleoprotein recognized by class I MHC-restricted CTL can be defined with short synthetic peptides. These and other experiments (25, 26) suggest that class I-restricted recognition by CTL might involve processed antigenic fragments, as is the case for class II-restricted recognition of antigen by helper T cells (27).

We have shown previously (14) that HA lacking an NH₂-terminal signal peptide is efficiently recognized by CTL. This HA molecule is not transported to the cell surface but is rapidly degraded within the cytosol (14), and thus it is unlikely that the sites recognized by CTL within the HA are dependent on the correct conformation of the molecule. Studies with cyanogen bromide fragments derived from the HA of influenza virus A/JAP/305/57 suggested that recognition by CTL could be localized to subpolypeptide regions (28). In an extension of these experiments, two peptides, HA1 (amino acids 181–204) and HA2 (amino acids 103–123), were found to generate murine secondary antiinfluenza CTL in vitro (29, 30). Different work using engineered proteins produced in *Escherichia coli* suggested that the HA2 subunit of influenza virus A/PR/8/34 could stimulate the production of secondary CTL in vitro (31, 32). However, this was only true if the HA2 polypeptide chain was fused to 81 amino acids of another influenza protein, the nonstructural protein NS1. Other workers failed to show the induction of a secondary CTL response using purified HA2 subunit derived from influenza virus A/FPV/Rostock/34 (H7 subtype) (33). Our results clearly show that for CBA mice there is an immunogenic site or sites for CTL within each subunit of the H1 subtype HA molecule.

As it now seems that recognition by both CTL and helper T cells involves antigen processing (24, 26), it is interesting to consider whether both types of T cell recognize the same sites within the HA molecule. A recent study (34) showed that there are determinants for proliferative T cells on both the HA1 and HA2

subunits of H2 and H3 subtype HAs (34). However, the three recognition sites for helper T cells within the A/PR/8/34 HA (H1 subtype) that have been defined in detail so far are all in the HA1 subunit (35). Clearly, further work using CTL clones is necessary to define precisely the sites within the A/PR/8/34 HA molecule that are recognized. Recently, it has been shown (36) that T cell antigenic sites tend to be amphipathic structures. This allows predictions to be made about the location of T cell recognition sites within proteins. The chemical synthesis of peptides corresponding to these regions and their use in chromium-release assays should allow precise identification of CTL recognition sites within the HA molecule.

Summary

We have constructed two chimeric influenza hemagglutinin (HA) genes in which the HA1 and HA2 subunits of the HA molecule have been interchanged between influenza A/PR/8/34 (H1 subtype) and A/NT/60/68 (H3 subtype). These genes were used to construct recombinant vaccinia viruses that expressed intact chimeric HA. These recombinant viruses were used to test whether murine CTL recognize antigenic determinants in either the HA1, HA2, or both subunits. We found that both subunits of the HA molecule contain determinants for CTL. This implies that CTL have, at least in part, separate antigenic determinants from B lymphocytes, which recognize mainly epitopes within the HA1 subunit.

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