Influenza virus infection elicits class II major histocompatibility complex-restricted T cells specific for an epitope identified in the NS1 non-structural protein

Charles J. Hackett,† Daniel Horowitz, Maria Wysocka and Susan B. Dillon

Introduction

Antiviral immune responses are directed not only to components of virus particles, but also to virus-encoded non-structural proteins that appear only in virus-infected cells. For influenza A virus, the non-structural protein NS1 is produced in abundance within infected cells, but is not incorporated into virions (Lamb, 1989; Richardson & Akkina, 1991). Immune responses to NS1 are of interest, since they are elicited exclusively by active virus infection, and their importance in protective immunity to influenza virus is not yet well understood. Class I major histocompatibility complex (MHC)-restricted cytotoxic T lymphocytes (CTLs) to NS1 have been characterized (Bennink et al., 1987; Yewdell et al., 1988; Yewdell & Hackett, 1989) and shown to reduce lung virus titres in experimental infections (Kuwano et al., 1990). Additionally, an L3T4+ T cell clone responsive to influenza virus NS1 has been described, providing evidence that helper T cells may also be directed to NS1 (Kuwano et al., 1990). To evaluate the potential role of NS1-specific T helper cells in immune responses to influenza virus, we undertook the identification of an NS1 protein sequence presented by class II MHC and studied recognition of this antigenic region by T cells of virus-infected mice.

Methods

Viruses and viral antigens. Influenza viruses A/Puerto Rico/8/34 (PR8; H1N1), A/USSR/90/77 (H1N1), A/Udorn/72 (H3N2), J1 (H3N1) (Bennink et al., 1982) and B/Lee/40 were grown in embryonated hen's eggs and purified where required by sedimentation and banding on sucrose density gradients. Virus concentration in haemagglutinating units (HAU) was determined by chicken erythrocyte agglutination (Fazekas de St Groth & Webster, 1966). Virus was inactivated by short-wave u.v. light using a UVGLD58 Mineralight lamp (Ultraviolet Products) at 240 μW/cm² for 15 min. Inactivation was verified by infecting L929 cell monolayers and staining with a monoclonal antibody (MAb) to the NS1 protein (provided by Dr J. W. Yewdell, NIH, Rockville, Md., U.S.A.). Recombinant vaccinia virus expressing the NS1 gene of PR8 influenza virus (NS1-vac) has been described (Smith et al., 1987). Vaccinia virus recombinants were grown in BSC cells, harvested and stored frozen at -70 °C as crude virus stocks in Hanks' balanced salt solution containing 0.1% bovine serum albumin (BSA). Isolated influenza PR8 virus NS1 protein produced in Escherichia coli (Young et al., 1983) was provided by Dr James F. Young. Recombinant E. coli proteins NS1181-HA21222, NS1147-HA21222 (having serine rather than the native cysteine at position 13), and also a control E. coli protein extract were supplied by Dr Alan Shatzman, SmithKline Beecham Pharmaceuticals. Synthetic peptides were produced using tert-butoxycarbonyl-protected amino acid deriva-
tives by solid-phase methods and purified by reversed-phase liquid chromatography. Peptide composition was confirmed by amino acid analysis.

Medium. Iscove's modified Dulbecco's medium (IMDM) (Gibco) supplemented with 5% foetal bovine serum was used for in vitro cultures.

Immunizations and T cell hybridomas. Viruses and recombinant products used in the following immunization scheme each derive their NS1 gene from PR8 virus. For intranasal immunization, adult female BALB/c mice were lightly anaesthetized and administered 1 HAU/mouse infectious allantoic fluid of PR8 virus diluted in 20 μl of PBS. To produce hybridomas, adult female BALB/c mice were immunized intraperitoneally, initially with 500 HAU of infectious PR8 virus, boosted 5 weeks later with 500 HAU of infectious influenza reassortant J1 virus (Bennink et al., 1982), and then 16 weeks later with 1 × 10^7 p.f.u. of NS1-vac. Splenocytes were taken 12 days after the last inoculation and cultured 3 days in vitro in the presence of recombinant E. coli-derived NS1. These cultures were fused as described (Hurwitz et al., 1984), using a T cell receptor α-β-BW5147 parent (White et al., 1989). Hybridomas secreting lymphokines in response to recombinant E. coli-derived NS1 were cloned by limiting dilution. Class II-MHC-restricted BALB/c T hybridomas specific for influenza virus nucleoprotein (NP) residues 50 to 65 (NP 10-3.1; I-Ad-restricted) and neuraminidase (NA) amino acids 79 to 93 (Hackett et al., 1991d) (NA 7/11 6.1; I-E-restricted) were used as controls.

Tcell stimulation assays. Hybridomas were tested in cultures containing 2 × 10^4 to 1 × 10^5 cells/well, plus antigen dilutions, and T hybridoma cells (2 × 10^4 to 1 × 10^5 cells/well). These were cultured in serum-free IMDM containing 0.05% BSA at 37°C/6% CO₂ for 48 to 72 h. To identify the class II MHC restriction of the T cells, antibodies 14-4-4, and MKD6 (Kappel et al., 1981) were included in some experiments at 10 μg/ml. Supernatants of T cell cultures were assayed for lymphokine release using the indicator cell lines CTLL-2, HT-2 or DA-1 (Gillis et al., 1978; Palaszynski & Ihle, 1984). Viability of indicator cells in culture supernatants was quantified by a modification of the original method of Mosmann (1983), Tada et al., 1986, Heeg et al., 1985) using cleavage of the dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Colour development (absorbance, A) was measured using a Biotek EL309 plate reader at 570 nm.

In vitro T cell responses of intranasally immunized mice to peptides were measured using proliferation assays. Briefly, splenocyte suspensions were cultured (4 × 10^6/ml) in serum-free IMDM containing 0.5% (v/v) Nutridoma SP (Boehringer Mannheim) for 3 days with dilutions of various antigens. Cultures were then tested for proliferation by uptake of [3H]thymidine.

Results and Discussion

For clonal analysis of class II MHC-restricted T cell responses to NS1, T hybridomas were produced from BALB/c mice that had been immunized with infectious PR8 and J1 influenza virus, boosted in vivo with NS1-vac, and restimulated in vitro with recombinant E. coli NS1 protein. T hybridoma clone 27-2, derived from such fusions, responded to influenza virus-infected APC as shown in Table 1. Purified infectious PR8 virus was recognized, whereas u.v.-inactivated virus was non-stimulatory. In contrast, a class II MHC-restricted T hybridoma specific for influenza virus NP recognized both infectious and u.v.-treated preparations equally well. Non u.v.-treated purified B/Lee/40 virus was not recognized by hybridoma 27-2. Infectious PR8 virus in the form of unpurified allantoic fluid was also recognized by clone 27-2 (see Table 3). The hybridoma was found to be restricted to the class II I-E<sup>d</sup> molecule; the I-E<sup>d</sup>-specific MAb 14-4-4, and not the I-A<sup>d</sup>-binding MKD6, inhibited antigen recognition (data not shown).

Definitive data showing that viral NS1 was recognized relied upon recombinant E. coli proteins. Hybrid 27-2, and not a T hybridoma specific for NA, responded to full-length PR8 virus NS1 in the absence of other influenza proteins (Table 2). No responses were seen

![Table 1. Infectious, but not u.v.-inactivated, PR8 virus is specifically recognized by T hybridoma 27-2](image1)

![Table 2. T hybridoma 27-2 recognizes recombinant NS1 protein in the 1 to 42 amino acid region](image2)
Table 3. Localization of the T cell epitope to residues 13 to 32 of NS1 by differential recognition of H1 and H3 viruses and synthetic peptides

Amino acid sequence of residues 1 to 42 of influenza virus NS1 proteins (data from Krystal et al., 1983) (differences from PR8 virus are shown, in single-letter code):

<table>
<thead>
<tr>
<th>Virus</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Udorn/72 (H3N2)</td>
<td>S Q V</td>
</tr>
<tr>
<td>A/USSR/90/77 (H1N1)</td>
<td>Q</td>
</tr>
<tr>
<td>A/PR8 (H1N1)</td>
<td>MDPNTSSFQDVDCFLWHVRKRQADQELGDAPFLDLRRDQKS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Virus†</th>
<th>Stimulation (net A570)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/Udorn/72† (H3N2)</td>
<td>-0.014 0.148</td>
</tr>
<tr>
<td>A/USSR/90/77 (H1N1)</td>
<td>0.245 1.518</td>
</tr>
<tr>
<td>A/PR8 (H1N1)</td>
<td>0.595 1.303</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antigen§</th>
<th>Stimulation (net A570)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NS1 1–15 peptide</td>
<td>0.070 0.041</td>
</tr>
<tr>
<td>NS1 13–32 peptide</td>
<td>0.870 1.041</td>
</tr>
<tr>
<td>NS1 1–15 + NS1 13–32 peptide</td>
<td>0.983 -0.054</td>
</tr>
<tr>
<td>A/PR8 virus</td>
<td>0.315 1.003</td>
</tr>
</tbody>
</table>

* Cleavage of MTT by viable indicator cells, detected at 570 nm, indicates T cell lymphokine release in response to antigen and APC. Data are means of triplicate wells. Values reported are the maximum obtained in a dilution series. T cell NA 7/11 6.1 is specific for influenza virus NA.
† Infectious allantoic fluid was used at 240 HAU/ml dilution to pulse APC, which were washed and serially diluted for T cell stimulation cultures.
‡ A/Udorn/72 (H3N2) virus was not recognized by the N1-specific T hybridoma NA 7/11 (above) but was recognized by the subtype cross-reactive T hybridoma NP 10-3.1 (specific for residues 50 to 69 of NP), giving a net A570 of 1.053 in the presence of 0.5 HAU/ml of PR8 virus allantoic fluid.
§ Peptides were used at concentrations of 4 μg/ml or, for NS1 13–32, at 8 μg/ml when cocultured with peptide NS1 1–15. PR8 virus allantoic fluid was used at 3 HAU/ml.

with partially purified E. coli protein controls. However, hybrid E. coli proteins NS11–81–HA21–222 (data not shown) and NS11–42–HA21–222 (Table 2), known to elicit or restimulate class I MHC-restricted CTLs in BALB/c mice (Yamada et al., 1985; S. B. Dillon et al., unpublished observations), were recognized by clone 27-2. The relevant epitope therefore resides within the first 42 amino acids of NS1.

Studies with heterosubtypic virus and synthetic peptides localized the T cell epitope to a short segment of NS1 differing in sequence between H1 and H3 influenza viruses. Table 3 shows that hybridoma 27-2 T cells did not recognize subtype H3 A/Udorn virus, but responded to the related H1 strain A/USSR/90/77. Amino acid changes between H1 and H3 NS1 molecules in the first 42 amino acids are known at positions 3 and 23 (Buonagurio et al., 1986). Accordingly, peptides encompassing residues 1 to 15 and 13 to 32 were synthesized. Table 3 shows that only the 13 to 32 amino acid segment was recognized by 27-2, and did not stimulate a NA-specific T hybridoma. Truncated peptides are needed to delineate further the minimally sized determinant within this region, and peptide analogues bearing the A/Udorn/72 substitution of valine for alanine at position 23 are required to verify the effect of that alteration on T cell recognition. It should be noted that the T cell epitope defined here appears to be different from that discussed by Kuwano et al. (1990), which cross-reacted with H3 strains, and whose location was known only to be within the first 81 amino acids of NS1. This region is also distinct from that recognized by a class I MHC-restricted NS1-specific CTL clone (residues 50 to 68) (Kuwano et al., 1991).

We could then ask whether the NS1 13 to 32 region is a significant target in mice infected with influenza virus. BALB/c mice were primed intranasally with PR8 virus, and 9 to 15 days later draining lymph node and spleen cells were tested in vitro for recognition of peptide in proliferation assays. Table 4 shows that a single immunization with live virus induced proliferating cells responding to peptide NS1 13–32. These proliferating cells released interleukin 3 (data not shown), confirming that T cells were being stimulated. Non-immunized mice did not respond to NS1 13–32 nor to other known antigenic influenza virus peptides. The T cell response to NS1 13–32 compared favourably in magnitude with that.
analysing various antigen-processing routes that the recombinant required in endosomes or biosynthesized in the cytosol of APCs (Germain, 1986; Long, 1989; Jaraquemada virus-infected cells, as well as from exogenously added Hackett presentation depending upon whether antigen is ac-

1990; Nuchtern protein. Current information suggests that different endogenously synthesized antigen or as an exogenous MHC class II molecules when provided either as an

was found in responses to such diverse viruses as dengue (Kurane, 1991) and human immunodeficiency virus type I (Bahraoui et al., 1990), as well as to influenza virus. Therefore, helper T cells to non-structural proteins may be a common feature of immune responses to many viruses.

seen to NA 79–93 and HA 111–129 (Table 4), which are known to be major I-Eα-restricted T cell epitopes of PR8 virusNAH and HA3 (Hackett et al., 1991a; Gerhard et al., 1991). Although quantitative data would be required to establish rigorously the frequency of NS1 13–32-specific T cells in virus-immunized mice, these data suggest that we have identified an epitope of NS1 which serves as a significant target antigen for class II MHC-restricted T cells responding to virus infection. T cell responses to non-structural proteins of several other viruses have frequently been found to be class I MHC-restricted (Bukowski et al., 1989; Del Val et al., 1988; Borysiewicz et al., 1988; Martin et al., 1988; Koenig et al., 1990); however, class II MHC-restricted T cells to non-

structural proteins have been found in responses to such diverse viruses as dengue (Kurane et al., 1991) and human immunodeficiency virus type I (Bahraoui et al., 1990), as well as to influenza virus. Therefore, helper T cells to non-structural proteins may be a common feature of immune responses to many viruses.

Table 4. Mice immunized with a single dose of infectious A/PR8 virus recognize peptide 13–32 of NS1

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>PR8 virus-immunized</th>
<th>Unimmunized</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4090</td>
<td>3614</td>
</tr>
<tr>
<td>PR8 virus</td>
<td>44230</td>
<td>4484</td>
</tr>
<tr>
<td>NSI 13–32</td>
<td>14815</td>
<td>3016</td>
</tr>
<tr>
<td>NA 79–93</td>
<td>19429</td>
<td>3470</td>
</tr>
<tr>
<td>HA 111–120</td>
<td>11863</td>
<td>3616</td>
</tr>
<tr>
<td>HA 79–91</td>
<td>4266</td>
<td>3016</td>
</tr>
</tbody>
</table>

* All peptides were present at 2.5 μg/ml, and infectious PR8 virus at 0.6 HAU/ml. Control peptides NA 79–93 and HA 111–120 represent known I-Eα-restricted T cell epitopes of influenza virus glycoproteins, whereas HA 79–93 is an I-Aβ-restricted T cell determinant of HA which does not elicit a T cell response in H-2b mice. Cultures of concanavalin A-stimulated unprimed mice incorporated 17470 c.p.m. (non-optimized), demonstrating the potential of T cells in those cultures to be activated.

molecule may encounter. Additionally, these observations suggest that cells should also be able to present foreign genes packaged and expressed under the control of NS1 genetic elements in recombinant influenza virus (Luytjes et al., 1989), which could be employed to study antigen presentation of various viral and non-viral proteins biosynthesized, as NS1, within virus-infected APCs.

Availability of an NS1 helper T cell epitope peptide should enable further studies of the role of NS1 in immunity to influenza. These include quantitative estimates of the precursor frequency and genetic diversity of NS1-specific T cells, and the effect on protective immunity of intentionally priming NS1-specific helper T cells. Significant for vaccine design, this study identifies a class II MHC-restricted T cell determinant which could not be elicited by immunization with inactivated virus or by virion subunit vaccines, indicating that live virus or constructs including at least portions of NS1 may be needed to elicit the full repertoire of T helper cells that function in an influenza virus challenge.

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References


MHC-restricted recognition of influenza virus


