Preliminary analysis of murine cytotoxic T cell responses to the proteins of the flavivirus Kunjin using vaccinia virus expression

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A series of recombinant vaccinia viruses expressing various parts of the entire Kunjin virus (KUN) coding region was used to analyse the cytotoxic T (Tc) cell responses to KUN. CBA/H mice inoculated with KUN or West Nile virus were shown to develop responses to KUN or various vaccinia virus expression constructs in either primary cytotoxic assays, or after secondary stimulation of the Tc cells in vitro with KUN antigens. Tc cells from CBA mice showed the strongest response to target cells infected with recombinant vaccinia viruses expressing parts of the KUN NS3 and NS4A proteins, and only a weak response to the other structural or non-structural proteins. Further analysis of deleted versions of the NS3-NS4A region showed that the main epitope recognized was derived from a sequence of 99 amino acids spanning parts of NS3 and NS4A. No other major epitopes were detected by Tc cells from CBA mice in the remaining 3333 amino acids of the KUN polypeptide.

Introduction

Kunjin virus (KUN) is a member of the family Flaviviridae, which includes many different human and animal pathogens, including yellow fever (YF) virus, West Nile (WN) virus and dengue (DEN) viruses (Westaway et al., 1985). KUN is widely distributed in Australia and New Guinea, and infection normally results in a mild or subclinical syndrome, although clinical disease is occasionally observed in humans and other animals (Monath, 1986). KUN is closely related to WN virus antigenically (De Madrid & Porterfield, 1974) and the viruses show 93% amino acid sequence similarity (Speight et al., 1988). Almost the entire genome of KUN has been cloned and sequenced, including the entire coding region of 10302 nucleotides (Coia et al., 1988; Speight et al., 1988), and the N-terminal amino acids of all the viral proteins, with the exception of NS1, have been confirmed by protein sequencing (Speight et al., 1988; Speight & Westaway, 1989). The order of the genes on the genome is 5' C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5 3'.

The various immune responses induced by a flavivirus infection include antibody responses to various structural and non-structural proteins, natural killer (NK) cell responses and a variety of T cell responses. Antibodies against either of the structural proteins prM or E have been shown to neutralize flaviviruses (Bray et al., 1989; Gould et al., 1986) and these antibodies, as well as antibodies against the non-structural protein NS1, have been shown to protect mice against challenge with DEN or YF viruses in the homologous reaction (Falgout et al., 1990; Gould et al., 1986; Kaufman et al., 1989; Schlesinger et al., 1985, 1987; Zhang et al., 1988). An immune response is clearly required for recovery from flavivirus infection; mice treated with cyclophosphamide (which primarily affects B cells), sub-lethal X-irradiation, or T cell-depleting antiserum all have altered susceptibility to flavivirus infection (Bhatt & Jacoby, 1976; Cole, 1968; Hirsch & Murphy, 1967; Webb et al., 1968).

T cell responses to flaviviruses demonstrated in in vitro assays include major histocompatibility complex (MHC) class I-restricted cytotoxic T (Tc) cell responses (Bukowski et al., 1989; Gadjosova et al., 1981; Kesson et al., 1987, 1988; Kurane et al., 1984; Liu et al., 1989; Sheets et al., 1979) and MHC class II helper/delayed-type hypersensitivity (Kurane et al., 1984; Rothman et al., 1989; Uren et al., 1987) responses. Antibody responses to the E protein have been suggested to enhance the pathogenesis of DEN and YF viruses (Gould et al., 1987; Halstead, 1991 SGM
1988), and T cell responses may play a role in the pathogenesis of WN virus encephalitis in mice (Liu et al., 1989).

Mice infected with WN virus develop a severe encephalitis characterized by a mononuclear cell infiltrate in the meninges, Virchow-Robin space and brain parenchyma (Liu et al., 1989; Monath, 1986). Lymphocytes recovered from the brains of affected mice include both NK cells and Tc cells. Of the inflammatory cells, 14 to 40% are Thy1+, of which 3% are Lyt-2+, which would recognize antigens in association with MHC class I antigens; no L3T4+ cells were detected (Liu et al., 1989).

The roles of cell-mediated responses and the proteins which give rise to epitopes recognized by T cells in protective or immunopathological responses to flavivirus infection have not yet been fully defined. In the only previous report of Tc cell responses to flavivirus gene products (Bukowski et al., 1989), human lymphocytes recognized targets composed of the DEN-4 virus E protein and at least one of proteins NS2B, NS3, NS4A and NS4B expressed from recombinant vaccinia viruses.

We prepared a series of vaccinia viruses containing and expressing cDNAs of the entire KUN coding region, and initially characterized the sources of the KUN epitopes recognized by the T lymphocytes of CBA/H mice. We used these vaccinia viruses to define some of the epitopes which are the targets of murine MHC class I-restricted specific Tc cell responses to KUN.

**Methods**

**Viruses and cells.** KUN MRM 61C (Westaway, 1973) and WN virus (strain Sarafend) were prepared as 10% suspensions of infected suckling mouse brains. Vero cells were grown in roller bottles in medium 199 with 10% foetal bovine serum (FBS) and after infection were maintained in Eagle's MEM containing 0.1% bovine serum albumin.

143B cells were maintained in MEM containing 5% FBS, with 25 μM-5-bromo-2′-deoxyuridine (BUdR); prior to use they were grown in medium without BUdR for two or three passages. Vaccinia viruses (strain WR and recombinants) were grown in 143B or CVL cells in culture using standard procedures (Boyle et al., 1985; Mackett et al., 1985) and stocks were stored frozen as suspensions of infected cells.

**Plasmids, cloning and sequences.** The preparation, cloning and sequencing of plasmids containing cDNA from KUN have been described (Coia et al., 1988). A variety of subclones were prepared from these plasmids in vectors pUC18 and pUC19 (Yanisch-Perron et al., 1985) to assemble larger portions of the KUN sequence (see Fig. 1).

A plasmid containing a multiple cloning site within the thymidine kinase (TK) gene (HindIII I fragment) of vaccinia virus (pBCB06*) was obtained from Dr D. Boyle (Boyle et al., 1985). All plasmids were propagated in Escherichia coli strain JM101 and purified by banding to equilibrium on CsCl gradients using standard procedures (Maniatis et al., 1982).

**Vaccinia virus cloning and expression.** A series of vaccinia virus plasmid clones was prepared in the multiple cloning site of vector pBCB06*, downstream of the vaccinia virus 7.5K early-late promoter (Boyle et al., 1985). Clones were prepared by cleavage and ligation of cDNA fragments from a series of 10 cDNA clones spanning the entire coding region of the KUN genome (Coia et al., 1988) (Fig. 1).

The 5′ end of the genome, encoding primarily the structural proteins, was assembled from a number of cDNA clones spanning nucleotides (nt) 1 to 4790 of the published KUN sequence, and therefore included the 5′ leader sequence of 75 nt and the coding sequences of the C, prM, E, NS1, NS2A and NS2B proteins, and that of 67 amino acids from the amino-terminal region of NS3. This clone was prepared by isolation and ligation of four fragments into one plasmid (pD20), and of three fragments into a second clone (pC6), from which the two large fragments were re-isolated and ligated into a further plasmid (pF6) (Fig. 1a). The entire sequence was cloned in vector pBCB06* to give pKV1031 (Fig. 1a).

The remainder of the genome was reconstructed from several fragments recovered from plasmids pKV61, pKV61 and pKV62, as shown in Fig. 1(b). An initial clone (pKV1022), expressing primarily the NS5 gene, was constructed from the PvuII-EcoRV fragment of pKV61 ligated to the EcoRV–PstI fragment from pKV62 and was dependent on a pre-existing translation initiation site at nt 7582, prior to the start of NS5. A second clone (pKV1023) encoded the carboxy-terminal region of NS2B, as well as the entire NS3 gene and the amino-terminal 65 amino acids of NS4A; this clone was prepared using firstly a synthetic adaptor, which included the consensus sequence for translation initiation (Kozak, 1984), ligated to the Styl site at nt 4531, and a second adaptor, which encoded a translation termination codon, ligated to the NcoI site at nt 6638. A clone spanning the entire NS3–NS5 region was prepared from pKV1023 and pKV1022 by the addition of the intervening sequences (from pKV61) between the HindIII and EcoRV sites in the two clones, including the adaptor from the beginning of pKV1023; this clone (vKV1024) contained an open reading frame terminating at the KUN termination codon at nt 10375.

Plasmids prepared were transfected into cultures of 143B cells and resulting TK− vaccinia viruses were selected by incubation with 25 μg/ml BUdR, using standard procedures (Boyle et al., 1985; Mackett et al., 1985). TK− plaques were picked and screened for recombinants by hybridization, and the positive plaques were cloned three times. DNA was prepared from virus recovered from 64 cm² dishes of 143B cells (Rice et al., 1985), digested with restriction enzymes and electrophoresed on 0.7% agarose gels. The DNA was transferred to nitrocellulose membranes by Southern blotting and probed with labelled DNA from plasmids containing the entire vaccinia virus TK gene, as well as the appropriate DNA insert.

**Protein labelling.** Dishes (9 cm²) of 143B cells were inoculated with either 5 or 20 p.f.u./cell of either vaccinia virus strain WR or one of the recombinant viruses. Either 6 h (20 p.f.u./cell) or 24 h (5 p.f.u./cell) after inoculation, the medium was removed from the cells and the cultures were incubated at 37 °C with 1 ml MEM per dish with one-hundredth of the normal concentration of methionine. After 30 min the medium was changed for the same medium containing 25 μCi/ml [35S]methionine and the cultures were incubated for a further 45 min at 37 °C. The cells were washed with phosphate-buffered saline (PBS), scraped into 1·5 ml PBS and pelleted in a microcentrifuge for 5 min at 15000 g. The pellet was lysed into 0·4 ml SDS sample buffer (50 mm-Tris–HCl pH 6·8, 1% v/v SDS, 1% 2-mercaptoethanol) and 1 mm-PMSF, passed through a 26-gauge needle five times to shear the cellular DNA and stored at −70 °C. Samples were electrophoresed in 7·5 to 15% gradient polyacrylamide gels, fixed, fluorographed and exposed to pre-photographed film at −70 °C.

**Western blotting.** Cells were infected at a multiplicity of about 5 p.f.u./cell, incubated for 24 h at 37 °C and then recovered into PBS, washed and suspended in 10 mm-NaCl, 10 mm-Tris–HCl pH 7·5, 1·5 mm-MgCl₂ and 1 mm-PMSF. After lysing the cells by passing them...
**Kunjin virus T cell epitopes**

Fig. 1. Construction of clones expressing the entire KUN polyprotein in a series of recombinant vaccinia viruses. The genetic map of the KUN genome from Coia et al. (1988) and Speight et al. (1988) is shown above the diagram. Nucleotide positions are numbered in kb from the first nucleotide in the published sequence. The locations of restriction enzyme sites used in the preparation of the various expression clones from the original cDNA clones are shown. (a) Preparation of vKV1031 from seven different cDNA subclones. Initially four fragments were ligated to form clone pD20 and three fragments were ligated to form pC6. These fragments were then ligated into pF6, using a KpnI site in the multiple cloning site of plasmid vector pUC19, then into the vaccinia virus expression vector pBCB06* and recombinated into vaccinia virus to form vKV1031. The translation initiation codon was the authentic KUN initiating codon, whereas the termination codon was derived from the polylinker of plasmid pUC19 during subcloning. (b) Preparation of clones expressing the right-hand (3') portion of the KUN genome. A series of clones were prepared by reconstructing the original cDNA clones using the restriction sites shown. A synthetic adaptor added to a SphI site at nt 4531 within the NS2B coding region encoded the translation start. A termination codon was added in an adaptor ligated to the NcoI site at nt 6638. Clones were inserted into vector pBCB06* and recombinated into vaccinia virus to form vKV1021. The translation initiation codon was the authentic KUN initiating codon, whereas the termination codon was derived from the pBCB06* polylinker.
through a 26-gauge needle 10 times, the nuclei were pelleted, and supernatant proteins were prepared in SDS sample buffer, electrophoresed on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The transferred proteins were detected with a rabbit antiserum against KUN-infected suckling mouse brain.

Indirect immunofluorescence assay (IFA). Vero cells were infected with KUN as described previously (Westaway & Goodman, 1987) and fixed for staining 24 h after infection. Cells were infected with vaccinia virus and fixed with acetone at 4 °C for 1 min after 20 to 24 h incubation. Antisera used have been described (Smith & Wright, 1985; Westaway & Goodman, 1987), and included a rabbit antiserum against purified KUN E protein and another against DEN-2 NS1 protein purified by PAGE. Antibodies were detected with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antiserum.

Cellular cytotoxicity assays. Specific pathogen-free CBA/H (H-2^k) mice were supplied by the Animal Breeding Establishment at the John Curtin School of Medical Research. The mice were immunized with virus at the age of 6 weeks or older. The TK^- derivative of vaccinia virus strain WR (vv-WR-TK^-) was used as a control and was a gift from Dr D. Boyle. Vaccinia virus stocks (TK^- and KUN recombinants) were prepared as crude lysates of CV1 cells, titrated on I43B cells, stored at -70 °C in aliquots and sonicated prior to use. Secondary vaccinia virus-, KUN- and WN virus-immune Tc cells were generated basically as described by Mullbacher et al. (1983) and Kesson et al. (1988). Briefly, CBA/H mice were injected intravenously with 10^7 p.f.u. of vaccinia virus strain WR or 10^6 p.f.u. of KUN or WN virus. The animals were killed 6 to 10 days later and single cell suspensions of splenocytes were prepared. One-tenth of the spleen cells was incubated with 5 × 10^5 p.f.u. vaccinia virus strain WR per spleen, 2 × 10^5 p.f.u. KUN per spleen, or 5 × 10^5 p.f.u. WN virus per spleen, for 1 h and then added to the remainder of the splenocytes. The dose of virus used was determined to adequately boost homologously primed Tc cell populations. After 5 days in culture at 37 °C, viable spleen cells were used as secondary immune effectors in cytotoxicity assays.

Primary KUN-immune Tc cells were generated by the method described for WN virus by Kesson et al. (1987). Briefly, adult mice were injected intravenously with 10^7 p.f.u. of KUN and 5 days later the animals were killed and single cell suspensions of splenocytes were prepared; these were centrifuged over Ficoll-Hypaque. The cells recovered from the interface were then used as primary KUN-immune effector cells in cytotoxicity assays.

Target cells for cytotoxicity assays were either L929 fibroblasts (H-2^k) or CBA/H peritoneal macrophages (Kesson et al., 1988). For the latter, peritoneal exudate cells were obtained 5 days after intraperitoneal injection of mice with 1 ml thioglycollate fluid broth (Difco) prepared at 59.6 g/l; these cells were more than 95% macrophages as determined by morphology. Macrophage targets were incubated with 20 to 50 p.f.u./cell vv-WR-TK^- and 51Cr for 1 h, and washed twice prior to use in the assay. Macrophage targets were also incubated with 100 to 300 p.f.u./cell WN virus, or 25 to 100 p.f.u./cell of KUN, in 1 ml of medium for 1 h, and then incubated in 50 ml of medium in spinner culture overnight, before labelling with 51Cr as above.

For cytotoxicity assays a standard 51Cr release assay was used. Effector cells generated as described above were diluted to the required concentration, usually 10^7 cells/ml, or cells derived from a culture of one spleen were resuspended in 3 ml of medium, and titrated in round-bottomed 96-well tissue culture plates to give triplicate cultures of four threefold dilutions; 2 × 10^4 target cells were added per well. Medium without effector cells was added to target cells to give spontaneous 51Cr release values and 1% Triton X-100 was added to other cultures to determine the maximum releasable 51Cr. The assay was incubated at 37 °C in 5% CO_2 for 4 to 6 h, the plates were spun and 0.1 ml of supernatant was harvested from each well; the c.p.m. of each sample was determined. The percentage specific lysis (%SL) was calculated as [(c.p.m. experimental – spontaneous 51Cr release)/(c.p.m. maximum – c.p.m. spontaneous 51Cr release)].

Results

Plasmid clones and vaccinia virus expression

From cDNA clones spanning the entire KUN coding sequence we prepared in vaccinia virus a series of plasmids expressing the entire sequence (Fig. 1). The structural protein genes and genes encoding NS1, NS2A and NS2B were all expressed from a single plasmid clone (pKV1031) which included nt 1 to 4790 of the reported KUN sequence and 75 nt of the 5' non-translated region, to allow initiation of translation.

The sequences between nt 4532 and 10664 encoded the region from the carboxyl terminus of NS2B to the end of NS5 and were expressed in their entirety from vKV1024; a synthetic linker was added to provide sequences required for translation initiation and the KUN termination codon was added to terminate translation (Fig. 1). Subclone vKV1023 encoded the carboxyl terminus of NS2B, all of NS3 and the amino terminus of NS4A, and initiated at the same site in the linker as pKV1024; a stop codon was added to this sequence after an NcoI site, as shown in Fig. 1(b). vKV1022 encoded the carboxyl-terminal 50 amino acids of NS4B, as well as the NS5; two in-frame AUG sequences occur 8 and 28 codons upstream of the defined cleavage site of the N terminus of the NS5 gene (Speight et al., 1988). The translation termination codon used was the KUN termination codon.

After initial testing of the expressed products for reactivity with Tc cells from CBA/H mice we revealed strong responses to sequences expressed by vKV1023, three subclones containing deletions of this sequence (pKV1039, pKV1040 and pKV1041) were prepared to allow more detailed mapping of the epitope(s).

Vaccinia virus cloning and protein analysis

Expression from vKV1031, which contains the region encoding the structural proteins and NS1, NS2A and NS2B, was confirmed by Western blot analysis which revealed the presence of a protein the size of the KUN E protein, although no other proteins were observed (Fig. 2). The E and NS1 proteins were observed by IFA using polyclonal rabbit antibodies prepared against the KUN E protein and the DEN-2 virus NS1 protein (Smith & Wright, 1985; Westaway & Goodman, 1987) (Fig. 3). Expression of prM was not detected by the Western blot and could not be confirmed by IFA because the polyclonal anti-prM serum used for staining the vaccinia
vKV1023 sequence (vKV1039, vKV1040 and vKV1041) (results not shown).

**Lysis of vaccinia virus–KUN recombinant-infected cells by KUN-immune Tc cells**

Secondary *in vitro* KUN-immune Tc cells, generated from spleen cells of CBA/H mice, lysed KUN- and WN virus-infected L929 fibroblasts (Table 1). The closely related flavivirus WN virus was used in this initial experiment since it infected L929 cells more efficiently. The same effector population showed strong specific lysis of CBA/H mouse macrophages infected with vKV1023 and vKV1024, but did not kill target cells infected with vv-WR-TK−, vKV1031 or vKV1022. Adequate target cell infection by the vaccinia viruses was shown by efficient lysis by CBA/H mouse secondary vaccinia virus-immune Tc cells. These results therefore mapped the immunogenic determinant(s) for CBA/H mouse Tc cells to NS3 or the small flanking regions from NS2B or NS4A. In other experiments the specificity of H-2 restriction was demonstrated by the absence of specific lysis of recombinant vaccinia virus-infected H-2 mismatched target cells (data not shown). The effector population was shown to be Thy-1+, Lyt-2+ and L3T4− by inhibition of lysis by treatment with monoclonal antibodies 53.6.7 (anti-Lyt-2), Thyl.1 (anti-Thy-1.1), or GK1.5 (anti-L3T4), and complement (data not shown).

To exclude a bias in the determinants recognized due to *in vitro* expansion of the secondary effector Tc cell population, we tested the ability of splenocytes taken from an animal immunized 5 days previously with KUN to lyse recombinant vaccinia virus-infected target cells. Table 2 shows that these primary Tc cells also killed target cells infected with vKV1023 and vKV1024, but not vv-WR-TK−, vKV1031 or vKV1022, indicating that the pattern of determinants for the primary *in vivo* response was the same as the secondary *in vitro* response.

**Further mapping of the epitopes**

To define further the epitope recognized in vKV1023, we tested the ability of secondary *in vitro* KUN-immune Tc cells to lyse cells infected with deleted versions of vKV1023, i.e. vKV1039, vKV1040 and vKV1041. Table 3 shows that targets infected with vKV1039 or vKV1040 were efficiently lysed, whereas those cells infected with vKV1041 were not. All targets were adequately infected, as shown by lysis with vaccinia virus-immune Tc cells.

Although the demonstration of KUN protein products from these vaccinia viruses has been difficult (see above), we believe that the deletion constructs derived

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**Fig. 2. Western blot analysis of proteins expressed from vKV1031 which encodes C, prM, E, NS1, NS2A, NS2B and part of NS3.** Proteins from cells 24 h after infection with vKV1031 (lane 1), vv-WR (lane 2), KUN (lane 3) or mock infection (lane 4) were electrophoresed on a 10% acrylamide gel, transferred to nitrocellulose and reacted with a rabbit antiserum prepared against KUN-infected suckling mouse brain. The bound antibody was stained with a horseradish peroxidase-conjugated goat anti-rabbit antiserum and visualized with 4-chloro-1-naphthol. Arrowhead indicates the E protein band detected.

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Expression of proteins from vKV1022 or vKV1023, which contain sequences from between the NS2B and NS5 coding regions, was shown by direct analysis of labelled vaccinia virus-infected cell lysates (Fig. 4); specific label was also immunoprecipitated with polyclonal antisera prepared against KUN-infected suckling mouse brain (not shown). No specific products could be detected by radioimmunoprecipitation or by direct analysis of labelled proteins from vKV1024-infected cells (Fig. 4), or in cells containing the deleted versions of the virus-infected cells reacted strongly with non-recombinant vaccinia virus strain WR.
Fig. 3. Indirect immunofluorescent antibody staining of I43B cells 24 h after mock infection (a and e), infection with non-recombinant vaccinia virus WR (b and f), infection with recombinant vaccinia virus vKV1031 (c and g) or infection with KUN (d). (a to d) Incubated with a rabbit antiserum against purified KUN E protein and counterstained with an FITC-conjugated antiserum against rabbit IgG; (e to g) incubated with a rabbit antiserum against polyacrylamide gel-purified DEN-2 virus NS1 protein and then with an FITC-conjugated antiserum against rabbit IgG. Staining and photographic exposures of all infections within each set (a to d, or e to g) were performed under equivalent conditions.

Table 1. Lysis of flavivirus-infected and vKV-infected targets by CBA/H mouse secondary anti-KUN Tc cells and anti-vaccinia virus Tc cells

<table>
<thead>
<tr>
<th>Target cells/ Infecting virus</th>
<th>Medium release (%)</th>
<th>CBA/H secondary in vitro anti-KUN Tc cells</th>
<th>CBA/H secondary in vitro anti-vaccinia virus Tc cells</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>E:T ratio 50:1 16:1 6:1</td>
<td>E:T ratio 30:1 10:1 3:1</td>
</tr>
<tr>
<td>L929/uninfected</td>
<td>11</td>
<td>14 6 2</td>
<td>9 4 3</td>
</tr>
<tr>
<td>L929/KUN</td>
<td>23</td>
<td>23 10 3</td>
<td>34 25 15</td>
</tr>
<tr>
<td>L929/wn virus</td>
<td>13</td>
<td>34 10 3</td>
<td>41 34 23</td>
</tr>
<tr>
<td>CBA/H mφ/wn/uninfected</td>
<td>19</td>
<td>2 -2 0</td>
<td>9 4 3</td>
</tr>
<tr>
<td>CBA/H mφ/vv-WR-TK</td>
<td>24</td>
<td>7 4 1</td>
<td>34 25 15</td>
</tr>
<tr>
<td>CBA/H mφ/vKV1031</td>
<td>30</td>
<td>6 4 1</td>
<td>41 34 23</td>
</tr>
<tr>
<td>CBA/H mφ/vKV1024</td>
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<td>50 42 29</td>
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<td>29</td>
<td>8 4 2</td>
<td>43 40 28</td>
</tr>
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</table>

* Data are the mean of three experiments. The s.e.m. was less than 4% in all cases.
† Effector cell: target cell.
‡ Mφ, Macrophage.
Kunjin virus T cell epitopes

Fig. 4. Expression of KUN cDNA of genes encoding NS3 to NS5. Proteins in Vero cells infected with vKV1022 (lane 2), vKV1023 (lane 3) or vKV1024 (lane 4) were labelled with [35S]methionine 24 h post-infection, cell lysates were electrophoresed in a 7.5 to 15% gradient acrylamide gel and labelled proteins were detected by fluorography. The theoretical sizes of the expected KUN-encoded products are: vKV1022, 105K or 112K (depending on the initiation codon used), vKV1023, 76K, and vKV1024, 210K. Controls include cells infected with vaccinia virus strain WR (lane 1), mock-infected Vero cells (lane 6) and [35S]methionine-labelled lysate of KUN-infected Vero cells (lane 5).

Table 3. Mapping of epitopes within the sequence of vKV1023 (NS2B–NS4A region) of KUN virus by analysis of deletion constructs vKV1039 to 1041

<table>
<thead>
<tr>
<th>Infecting clone</th>
<th>Spontaneous release (%)</th>
<th>CBA/H primary anti-Kunjin Tc cells E:T ratio†</th>
<th>CBA/H secondary anti-vv Tc cells E:T ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>16</td>
<td>5:6</td>
<td>6:3</td>
</tr>
<tr>
<td>vv-WR-TK−</td>
<td>19</td>
<td>7:5</td>
<td>43:35</td>
</tr>
<tr>
<td>vKV1039</td>
<td>20</td>
<td>31:19</td>
<td>46:32</td>
</tr>
<tr>
<td>vKV1040</td>
<td>20</td>
<td>32:19</td>
<td>43:30</td>
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<tr>
<td>vKV1041</td>
<td>20</td>
<td>7:3</td>
<td>45:32</td>
</tr>
</tbody>
</table>

* Results are the means of triplicate experiments. The S.E.M. was less than 4% in all cases.
† Effector cell:target cell.

Table 2. Lysis of vKV-infected CBA/H mouse macrophages by CBA/H mouse primary in vivo derived anti-KUN Tc cells

<table>
<thead>
<tr>
<th>Infecting clone</th>
<th>Spontaneous release (%)</th>
<th>CBA/H primary in vivo anti-KUN Tc cells E:T ratio†</th>
<th>CBA/H secondary anti-vaccinia virus Tc cells E:T ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>16</td>
<td>−1:0</td>
<td>6:3</td>
</tr>
<tr>
<td>vv-WR-TK−</td>
<td>19</td>
<td>2:1</td>
<td>43:35</td>
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<td>vKV1031</td>
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<td>47:35</td>
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<tr>
<td>vKV1022</td>
<td>18</td>
<td>2:3</td>
<td>46:34</td>
</tr>
</tbody>
</table>

* Results are the means of triplicate experiments. The S.E.M. was less than 2% in all cases.
† Effector cell:target cell.

Discussion

The series of recombinant viruses expressing the region encoding all 3433 amino acids of KUN proved to be invaluable for the identification of a major epitope recognized by CBA/H mouse immune Tc cells, and also because SJL/J mouse KUN-immune Tc cells efficiently recognized vKV1041 (our unpublished results). vKV1041 contains cDNA for all but 99 amino acids of vKV1023, suggesting that the epitope recognized by CBA/H mouse KUN-immune Tc cells is contained within the last 34 amino acids of NS3 or the first 65 amino acids of NS4A.
determined to be within a 99 amino acid sequence near the carboxyl terminus of NS3. Expression of the KUN proteins by the recombinant vaccinia viruses was confirmed by Western blotting and IFA, or by direct analysis of [35S]methionine-labelled proteins.

Clones vKV1031 and vKV1024 contained sequences for the entire KUN open reading frame between nt 1 and 10664 of the published sequence, including the stop codon at 10375 (Coia et al., 1988; Speight et al., 1988). The E protein was detected by Western blotting, and the E and NS1 proteins by IFA of products from vKV1031, but no proteins could be readily identified in cells infected with vKV1024. As the initiation codon of vKV1024 was derived from vKV1023, which expressed a protein of the predicted size, it is likely that the additional sequences in vKV1024 resulted in the proteins being unstable, or expressed in very small amounts.

In vKV1022- and vKV1023-infected cells, proteins of sizes predicted from the nucleotide sequences cloned could be detected readily by direct labelling of the infected cells during the late phase of infection (24 h after inoculation). The proteins could be immunoprecipitated, albeit inefficiently, although that is likely to be due to the poor immunological reagents against KUN NS proteins. No KUN proteins could be detected in cells infected with the deleted versions of vKV1023, again suggesting that the proteins expressed were unstable. These results show that the sequences of vKV1031, vKV1022 and vKV1023 all allow expression of the cloned proteins.

Since Tc cells are known to recognize peptides from degraded proteins in complexes with MHC class I molecules, we examined the reactivity of Tc cells from KUN-infected CBA/H mice with MHC-compatible target cells infected with each of the various recombinant vaccinia viruses (Tables 1 to 3). The strongest reactivity was found with vKV1023, which encoded the carboxy-terminal 20 amino acids (15%) of NS2B, the entire NS3 gene (619 amino acids) and the amino-terminal 65 amino acids (44%) of NS4A (Chambers et al., 1989; Speight et al., 1988; Speight & Westaway, 1989). Further analysis of subclones of the vKV1023 sequences indicated that the Tc cells from the CBA/H mice react with sequences expressed from the carboxy-terminal 34 amino acids (5%) of the NS3 protein, or the amino-terminal 65 amino acids (44%) of the NS4A protein. Homologies between KUN sequences and those of other flaviviruses are given in Coia et al. (1988). The relationships between the C-terminal regions of NS3 and N-terminal regions of NS4A are given in Table 4. The reactivity of the pooled splenic Tc cells from inbred CBA/H mice with only a small region of 99 amino acids within the KUN polyprotein of 3433 amino acids is similar to that observed in Tc cell responses to other viruses (Rouse et al., 1988) and emphasizes the relative rarity of the recognized epitopes. Identifying these epitopes will allow experimental analysis of their roles in the protective or immunopathological responses to flavivirus infections.

The only comparison with other flaviviruses currently available was reported by Bukowski et al. (1989). Human skin fibroblast cultures were infected with recombinant vaccinia viruses containing gene sequences between C and NS4B of DEN-4 virus, and used as targets for autologous Tc cells. These effector cells were from peripheral blood mononuclear cells obtained from a donor immunized with an experimental live DEN-4 virus vaccine. Their results show that the E protein is a target protein and imply that at least one of the NS2B, NS3, NS4A and NS4B proteins is also a target, but exclude NS1 and NS2A as targets.

Our results using CBA/H mice do not exclude a role for the KUN E protein as a target protein in other strains or species, but focus more precisely on the non-structural protein region via the deletions. Localization of Tc cell responses in CBA/H mice to only one domain of less than 100 amino acids within the non-structural region of the very large KUN polyprotein facilitates precise identification of the relevant peptide and its possible role in immunity or immunopathogenesis. We are further investigating T cell responses in various inbred mouse strains to differentiate MHC and non-MHC genetic control of reactivity, and to examine the control of Tc cell responses to KUN proteins by MHC class I immune response genes.

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**References**


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**Table 4. Amino acid sequence similarities included in the 99 amino acid region mapped between KUN and other flavivirus NS3 and NS4A proteins**

<table>
<thead>
<tr>
<th></th>
<th>C-terminal NS3</th>
<th>N-terminal NS4A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(34 amino acids)</td>
<td>(65 amino acids)</td>
</tr>
<tr>
<td>KUN/WN virus</td>
<td>33/34* (97%)</td>
<td>57/65 (88%)</td>
</tr>
<tr>
<td>KUN/DEN 2</td>
<td>20/34 (59%)</td>
<td>26/65 (40%)</td>
</tr>
<tr>
<td>KUN/YF virus</td>
<td>18/34 (53%)</td>
<td>21/65 (32%)</td>
</tr>
</tbody>
</table>

* Number of identical amino acids/total number.
Kunjin virus T cell epitopes


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