Flavivirus-specific Murine L3T4+ T Cell Clones: Induction, Characterization and Cross-reactivity

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SUMMARY

Murine T cell clones specific for the Kunjin (KUN), West Nile (WN) and Murray Valley encephalitis (MVE) flaviviruses were generated in vitro following priming in vivo. Clones were isolated by limiting dilution and maintained in culture with antigen stimulation and interleukin-2 (IL-2). The cells were characterized as having the Thy 1+, L3T4+ and Lyt 2− phenotype by immunofluorescence. All clones proliferated strongly and secreted high levels of IL-2 and IL-3 in response to homologous antigen. Both KUN- and WN-specific clones showed extensive cross-reactivity to KUN and WN antigen, but recognized MVE to a lesser extent. In contrast, MVE-specific clones cross-reacted strongly with both KUN and WN. These data show that antigen-specific, major histocompatibility complex-restricted L3T4+ T cells are generated during flavivirus infection and are cross-reactive for viruses of the same subgroup.

INTRODUCTION

The specificity of the helper (h)/delayed-type hypersensitivity T lymphocyte response to flaviviruses has been investigated in our laboratory using polyclonal cell populations restimulated with virus in vitro (J. E. Allan & P. C. Doherty, unpublished observations). These studies suggest that there is some specificity in recognition by T cells of the closely related Kunjin (KUN), Murray Valley encephalitis (MVE) and West Nile (WN) viruses (Westaway et al., 1985). It has been reported for other flaviviruses, the dengue viruses, that there is also a dengue virus serotype-specific response (Pang et al., 1982) as shown by measurement of delayed-type hypersensitivity in vivo.

Cross-reactivity to flaviviruses is of particular interest because of the occurrence of dengue haemorrhagic shock syndrome (DHS), which appears to be antibody-mediated and to occur following infection with a dengue virus of a different serotype to that causing the initial infection (Halstead, 1982). Since antibody production is dependent on T cell activity we have developed flavivirus-specific T cell clones and have further addressed the question of cross-reactivity to flaviviruses by examining the response of cloned flavivirus-specific T cell lines. These clones will also be used to analyse the influence of T cell cross-reactivity on the specificity of antibody produced.

This paper describes the isolation and characterization of cloned, virus-specific L3T4+ T cell lines following priming of mice with KUN, WN or MVE virus. The reciprocal specificity profiles of these L3T4+ T cells have been analysed, and show patterns of cross-reactivity that have some similarities to those found by antibody neutralization tests (Madrid & Porterfield, 1974; Westaway, 1965). This is the first time that the specificity of flavivirus-immune L3T4+ T cells has been analysed.
METHODS

Animals. C57BL/6J, B10.D2, B10.BR, B10.A(2R), B10.A(SR), CBA/H, BALB/c, DBA/1 and SJL female mice, 6 to 8 weeks of age, were bred at The John Curtin School of Medical Research, Canberra, Australia.

Antigens. WN virus, Sarafend strain, KUN virus, MRM16 Prototype strain, MVE virus, 96961/53 Prototype strain, and Semliki Forest virus (SF virus), strain 25639, was a gift from Dr I. Marshall, Department of Microbiology, John Curtin School of Medical Research. These were provided as sucking mouse brain homogenates and were passaged a further three to four times by intracerebral inoculation into newborn CBA/H mice as described previously (Allan & Doherty, 1986). Virus used as stimulator antigen in the proliferation assays were diluted fivefold and 5 ml was irradiated with 6 x 10^{-3} J/s/cm^2 u.v. light in a 19.6 cm^2 glass Petri dish. Representative samples were inoculated intracerebrally into newborn mice to test the efficacy of the u.v. treatment. All mice survived without any sign of infection.

Assays for p.f.u. were carried out on Vero cells as described previously (Allan & Doherty, 1986). Cultures were incubated at 37 °C in 5% CO_2 for 3 days for SFV (10^{11.0} p.f.u./ml) and 5 days for WN (10^{9.0} p.f.u./ml), KUN (10^{9.5} p.f.u./ml) and MVE (10^{9.5} p.f.u./ml).

Immunization. Mice (C57BL/6J) were inoculated by the intraperitoneal (i.p.) route with 10^7 to 10^9 p.f.u./mouse in 0.2 ml.

Culture of spleen cells in vitro. Seven to 10 days after infection cell suspensions were prepared from spleens. Cells were resuspended to 10^7/ml in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal calf serum (FCS), 25 mM-HEPES buffer, 1 mM-sodium pyruvate, 5 x 10^{-5} M-2-mercaptoethanol, penicillin (100 units/ml), streptomycin (100 μg/ml) and 2 mM-L-glutamine and dispensed into 24-well Linbro plates (Flow Laboratories) at 1.5 ml/well. Antigen was added at 150 μl/well and the plates were incubated at 37 °C in 5% CO_2 for 10 days. After 7 days incubation 1 ml of medium was carefully removed from each well and replaced with fresh medium.

T cell growth factor (TCGF). Interleukin-2 (IL-2)-containing medium was obtained by stimulation of murine EL-4 tumour cells with phorbol myristate acetate (PMA) according to the method of Farrant et al. (1980). The supernatant was collected by centrifugation at 1200 g for 10 min and the PMA removed by mixing with dextran-coated activated charcoal (West Chem, Ca., U.S.A.) (Chen & Schwartz, 1982). The EL-4 supernatant was sterilized by passage through a 0.2 μm filter and stored at -20 °C.

Cloning of antigen-specific lymphocytes. Viable spleen cells were collected 10 days after stimulation in vitro by centrifugation through Ficol-Hypaque as described by Davidson & Parish (1975). After washing, 1 x 10^5 cells were incubated with 10^6 gamma-irradiated (2000 rad) syngeneic spleen (stimulator) cells and 150 μl of u.v.-irradiated antigen in 24-well trays (Linbro). After 10 days, live cells were harvested as before and cloned under limiting dilution conditions (33-3 cells/well) in 96-well flat-bottom trays (Nunc) as described elsewhere (Schreier & Tees, 1981). Rapidly growing colonies were transferred to 24-well trays and stimulated every 8 to 10 days with specific antigen and irradiated syngeneic spleen cells. TCGF was added every 2 to 3 days. Clones were grown for 6 to 8 days after addition of antigen before testing.

T cell depletion. T cells were depleted from stimulating cell populations by treatment with monoclonal anti-Thy 1.2 antibody (AT83A) and rabbit complement. Spleen cells (10^7 cells/ml) were incubated with antibody for 30 min at room temperature, washed once and resuspended in 3% rabbit serum for 45 min at 37 °C. Cells were washed twice before addition to culture.

Phenotypic analysis of T lymphocyte clones. Cells were incubated in 100 μl of monoclonal antibody at 4°C for 30 min, washed through FCS and incubated as before in 100 μl of fluorescein isothiocyanate (FITC) F(ab')_2-sheep anti-mouse IgG (1/10, Silenus Laboratories, Victoria, Australia). They were then centrifuged through FCS and resuspended in 0.5 ml medium containing 1% FCS. The surface phenotype was analysed using a fluorescence-activated cell sorter (FACS IV; B-D FACS Systems, Becton-Dickinson). The murine monoclonal antibodies were culture supernatants from cell lines: GK 1.5 (IgG2b anti-L3T4) (Dialynas et al., 1983), 53.6.7 (IgG2a anti-Lyt 2) (Sarmiento et al., 1980) and AT83A (IgM anti-Thy 1.2) (Sarmiento et al., 1980).

Proliferation assays. Four thousand cloned T cells in DMEM containing 10% FCS were added to 96-well, flat-bottom trays (Nunc) at 150 μl/well together with 5 x 10^5 stimulator cells (syngeneic spleen cells gamma-irradiated with 2000 rad) and 25 μl antigen. After 7 days incubation at 37 °C, 5 μg/ml MTT solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma cat no. M 2128)] was added at 10 μl/well and plates incubated for a further 4 h at 37 °C as described by Mosmann (1983). Acid-isopropanol (100 μl of 0.04 M-HCl in isopropanol) was added to all wells and mixed thoroughly to dissolve crystals. After a few minutes at room temperature the plates were read on a Dynatech MR600 Microelisa Reader using a test wavelength of 570 nm, a reference wavelength of 630 nm and a calibration setting of 1.99.

IL-2 and -3 assay. The ability of samples to support proliferation of the IL-2-dependent T cell line CTLL-2 (Gillis et al., 1978) and the IL-3-dependent bone marrow-derived cell line 32D (Greenberger et al., 1983) was measured by the MTT colorimetric assay. Microassays were performed by incubating 50 μl samples with 50 μl of media containing either 5 x 10^5 CTLL-2 or 2 x 10^4 32D cells overnight at 37 °C. Cultures were incubated with 10 μl/well MTT for 4 h before reading on a Dynatech Reader.
Flavivirus-specific T cell clones

RESULTS

Isolation of flavivirus-specific T cell clones

Following stimulation of primed spleen cells with virus, flavivirus-specific lymphoblasts were plated into 96-well plates together with T cell-depleted syngeneic spleen stimulator cells and virus as described in Methods. Approximately 25% of wells contained proliferating cells 10 days later. Although many T cell clones were isolated, only six were selected for each virus and further expanded using TCGF in the absence of virus. Clones were then maintained in culture by antigenic restimulation every 8 to 10 days in the presence of 1% TCGF.

Analysis of T cell phenotype

The phenotypes of all T cell lines maintained in continuous culture were determined by immunofluorescence using a panel of monoclonal antibodies. All clones were Thy 1+, L3T4+ and Lyt 2- as illustrated for one clone in Fig. 1, since all profiles were essentially identical.

Virus specificity

The specificity of individual T cell clones was confirmed by culturing cells with various antigens either with or without irradiated syngeneic stimulator cells (Table 1). All T cell clones showed maximal proliferation only when cultured in the presence of both virus and stimulator cells and there was little variation (<15% s.d.) in the response between clones. All clones were unreactive to the serologically unrelated SF virus and the control normal suckling mouse brain homogenate, and all increased activity could be attributed to stimulator cells alone.

Fig. 1. FACS analysis of KUN-specific clone F10.1. T cells (10^6) were treated with anti-Thy 1, anti-Lyt 2 or anti-L3T4 monoclonal antibodies followed by FITC-conjugated sheep anti-mouse immunoglobulin.
Fig. 2. MTT uptake by the IL-2-dependent cell line CTLL-2. Cells (5 \times 10^3/well) were cultured in microtitre wells containing 50 \mu l of supernatant taken from cultures of KUN-specific clones (\(\Delta\), F10.1; \(\triangle\), C27.2; \(\nabla\), E6.1). Clones were stimulated with (a) homologous virus and (b) heterologous WN virus, in the presence of irradiated T cell-depleted B6 spleen cells and the supernatants were harvested daily. Control cells were stimulated with normal mouse brain homogenate (\(\bullet\)) and stimulator cells and the results are plotted as the mean response.

Fig. 3. MTT uptake by the IL-3-dependent cell line 32D. Cells (10^4/well) were cultured in microtitre wells containing 50 \mu l of supernatant taken from cultures of WN-specific clones (\(\blacksquare\), D4.1; \(\square\), F16.1; \(\bigcirc\), F4.1; \(\bullet\), control). Clones were stimulated with (a) homologous WN and (b) heterologous KUN virus.

Table 1. Virus specificity of T cell clones*

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Stimulator cells</th>
<th>MTT cleavage (A_{570-630} \times 10^3)†</th>
<th>KUN</th>
<th>WN</th>
<th>MVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>−</td>
<td>20.0 ± 2.0</td>
<td>24.5 ± 4.9</td>
<td>22.1 ± 4.4</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>56.6 ± 7.7</td>
<td>52.6 ± 9.8</td>
<td>52.3 ± 7.6</td>
<td></td>
</tr>
<tr>
<td>Normal brain</td>
<td>+</td>
<td>49.6 ± 7.6</td>
<td>47.6 ± 7.8</td>
<td>55.3 ± 7.9</td>
<td></td>
</tr>
<tr>
<td>Homologous virus</td>
<td>−</td>
<td>20.6 ± 5.0</td>
<td>21.5 ± 5.8</td>
<td>22.5 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Homologous virus</td>
<td>+</td>
<td>195.0 ± 28.7</td>
<td>229.5 ± 35.1</td>
<td>207.6 ± 29.1</td>
<td></td>
</tr>
<tr>
<td>SFV</td>
<td>+</td>
<td>42.5 ± 8.0</td>
<td>54.0 ± 8.9</td>
<td>54.3 ± 7.6</td>
<td></td>
</tr>
<tr>
<td>TCGF</td>
<td>+</td>
<td>101.5 ± 8.5</td>
<td>89.5 ± 13.3</td>
<td>88.5 ± 8.5</td>
<td></td>
</tr>
</tbody>
</table>

* T cell clones (10^4/well) were cultured in the presence of stimulator cells (5 \times 10^5/well) and the extent of proliferation was assayed (see Methods).
† Values represent the mean (±S.D.) of the summed data from six clones in each group.

Production of lymphokines

All T cell clones were capable of IL-2 and IL-3 production (Fig. 2,3). Fig. 2 demonstrates the response of three KUN-specific T cell lines to stimulation with homologous and heterologous virus. The peak production of IL-2 occurred 24 to 48 h following restimulation with either KUN
Flavivirus-specific T cell clones

Table 2. Cross-reactivity patterns of T cell clones following stimulation with a standard virus dose*

<table>
<thead>
<tr>
<th>Clone no. † (specificity)</th>
<th>MTT cleavage ((A_{570-630} \times 10^3))†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KUN</td>
</tr>
<tr>
<td>C27.2 (KUN)</td>
<td>196</td>
</tr>
<tr>
<td>D10.2 (KUN)</td>
<td>185</td>
</tr>
<tr>
<td>E 6.1 (KUN)</td>
<td>150</td>
</tr>
<tr>
<td>E18.3 (KUN)</td>
<td>159</td>
</tr>
<tr>
<td>E10.1 (KUN)</td>
<td>223</td>
</tr>
<tr>
<td>F10.1 (KUN)</td>
<td>245</td>
</tr>
<tr>
<td>D 4.1 (WN)</td>
<td>230</td>
</tr>
<tr>
<td>E 7.1 (WN)</td>
<td>208</td>
</tr>
<tr>
<td>E36.2 (WN)</td>
<td>196</td>
</tr>
<tr>
<td>F 4.1 (WN)</td>
<td>152</td>
</tr>
<tr>
<td>F16.1 (WN)</td>
<td>213</td>
</tr>
<tr>
<td>G 1.2 (WN)</td>
<td>181</td>
</tr>
<tr>
<td>A16.1 (MVE)</td>
<td>115</td>
</tr>
<tr>
<td>B 2.3 (MVE)</td>
<td>95</td>
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<tr>
<td>B10.1 (MVE)</td>
<td>165</td>
</tr>
<tr>
<td>D 3.2 (MVE)</td>
<td>119</td>
</tr>
<tr>
<td>F11.1 (MVE)</td>
<td>162</td>
</tr>
<tr>
<td>F20.2 (MVE)</td>
<td>186</td>
</tr>
</tbody>
</table>

* Results expressed as the response of T cells to a titre of \(10^5\) p.f.u./ml of homologous and heterologous virus prior to u.v. inactivation. The results are derived from virus dose–response curves for each virus–T cell clone combination. Examples from this experiment are shown in Fig. 4.

† T cell clones \((10^4/well)\) were cultured in the presence of stimulator cells \((5 \times 10^5/well)\) and measured by cell yield.

‡ Values represent the mean absorbance of triplicate wells (<10% S.D.).

or WN virus. Similarly, three WN-specific clones stimulated with either homologous or heterologous virus showed peak levels of IL-3 production at 24 h (Fig. 3).

Virus cross-reactivity

All T cell clones demonstrated cross-reactivity to other flaviviruses (Table 2, Fig. 4). Maximum proliferation of the clones did not necessarily occur only in the presence of homologous virus. For example, the MVE-specific clone A16.1 responded more strongly \((P < 0.05)\) to WN virus than to the homologous MVE virus (Table 2). KUN-specific T cells demonstrated marked cross-reactivity to WN virus with a significantly lower \((P < 0.01)\) response to MVE. Similarly WN-specific clones proliferated strongly in response to both WN and KUN viruses but to a lesser extent \((P < 0.01)\) to MVE virus. In contrast, the MVE-specific clones proliferated as strongly to WN as to MVE. With the exception of clones B2.3 and D3.2, there was also marked stimulation by KUN virus. The response of MVE-specific clones to KUN and WN viruses was in marked contrast to the response of KUN and WN-specific clones to MVE virus.

The effect of virus dose on T cell specificity was investigated using 10-fold dilutions of virus. The dose–response curves of all three groups of clones followed a predictable pattern. The virus dose–response curves for the WN-specific clone E36.2, the MVE-specific clone A16.1 and the KUN-specific clone F10.1 are shown in Fig. 4 (a, b, c). At dilutions of virus as low as \(10^{-4}\) (equivalent to \(10^5\) to \(10^4\) p.f.u./ml prior to inactivation) there was still evidence of proliferation in response to homologous, and in some cases heterologous, virus (Fig. 4). However, it was evident that very high levels of virus \((10^8\) to \(10^9\) p.f.u./ml) are required to stimulate a strong response.

Stimulation by alloantigens

Spleen cells from four allogeneic strains of mice were used to determine whether the T cell clones would respond to a panel of allogeneic cells. T cell clones \((10^4/well)\) were incubated with
irradiated spleen cells (5 × 10⁵) from BALB/c, B10. BR, DBA/1 and SJL mice, representing the d,k,q and s H-2 haplotypes respectively, and assayed for cell yield on day 6. Pooled data are shown in Table 3 for brevity. There was no evidence of even weak allogeneic stimulation by any clone.

Genetic restriction of T cell clones

Following the loss of the first group of clones, a second group was used to analyse restriction by the major histocompatibility complex (MHC). These clones were also Lyt 2-, L3T4+ and flavivirus-specific, with cross-reactive specificities essentially the same as the first group (data not shown). Spleen cells from the five inbred mouse strains C57BL/6, B10.D2, B10.BR, B10.A(5R) and B10.A(2R) were used as a source of irradiated stimulator cells. Spleen cells from both the syngeneic C57BL/6J and also B10.A(5R) mice were able to stimulate the proliferation of T cells, indicating that they were restricted by the MHC (Table 4). Compatibility at H-2D [B10.A(2R)] alone did not cause stimulation. In contrast, matching at H-2K/IA was effective.

DISCUSSION

Virus-specific L3T4+ T cells were induced in vivo by i.p. inoculation with high titres of infectious flaviviruses, a technique used previously to stimulate polyclonal flavivirus-immune T cells (Allan & Doherty, 1986). Six cloned T cell lines for each virus were maintained in culture and further characterized although many more virus-specific T cell lines were isolated and cloned. All 18 clones were demonstrated to be L3T4+ and Lyt 2− by immunofluorescence, and
showed an absolute requirement for the inducing virus to be presented in association with accessory cells. The analysis of T cell cross-reactivity to dengue virus is still incomplete due to difficulties in stimulating murine cells with dengue virus and is therefore not reported here.

These studies used the MTT dye uptake assay, the sensitivity of which has recently been reported to be comparable with the measurement of [3H]thymidine uptake (Heeg et al., 1985; Mosmann, 1983). A number of preliminary experiments were undertaken to establish the optimal incubation period required to minimize background uptake of MTT by irradiated cells. This was established as day 7, and the assay was found to be an accurate alternative to the measurement of [3H]thymidine uptake (data not shown).

The T cell clones responded to antigen by proliferation and lymphokine production. Since IL-2 and IL-3 regulate the proliferation of T and B cells (Melchers & Andersson, 1986; Smith, 1984) and myeloid cells (Schrader, 1986), respectively, these data indicate that T cells could play an important role in the response to flavivirus infection. Investigations into the kinetics of IL-2 and IL-3 production showed a pattern which was in marked contrast to findings from restimulation of polyclonal T cells, when peak responses were observed on day 4. Maximal levels of both IL-2 and IL-3 were found within 24 to 48 h of restimulation, and had dropped to almost background levels by day 4. The presence of both types of lymphokine in the supernatants of T cell clones has been observed for alloantigen-responsive Th cells in other systems (Miller & Stutman, 1982; Mosmann et al., 1986). Additionally, the amount of IL-2 produced by T cell lines has been shown previously to correlate with IL-3 production by the same line (Mosmann et al., 1986).

Recently Mosmann et al. (1986) have suggested that there may be a preference for the type of T<sub>H</sub> cell obtained by different experimental protocols. It is possible that our protocol of priming with infectious virus in vivo and restimulation with inactivated virus may have preferentially selected L3T4<sup>+</sup> cells of a particular functional type.

The L3T4<sup>+</sup> cell specificity profiles described here indicate the existence of common or cross-reactive epitopes on KUN, WN and MVE. Recent work suggests that, serologically, antigenic epitopes of flaviviruses which map to the E glycoprotein are arranged as a continuum of overlapping domains (Henchal et al., 1985; Roehrig et al., 1983; Schlesinger et al., 1984), but there is no information on which antigens are recognized by T cells.

Serological cross-reactions among flaviviruses observed both in vitro (Madrid & Porterfield, 1974; Westaway, 1965) and in vivo (Henderson et al., 1970; Kayser et al., 1985; Price et al., 1963) have been proposed as the basis for cross-protection against flavivirus infection. The data presented here indicate that virus cross-reactivity is also found at the L3T4<sup>+</sup> T cell level but we have not demonstrated anything analogous to the completely cross-reactive helper T cell clones directed at shared, internal viral antigens that are recognized in the influenza system (Lamb et al., 1982).
Both WN and KUN viruses demonstrated one-way cross-reactivity with MVE virus as observed for the polyclonal T cell responses (data not shown). It is noteworthy that a one-way cross-reactivity has been described also for the neutralizing antibody response. Westaway (1965) found that rabbit antisera to MVE cross-reacted with WN and KUN to a greater extent than antibody to WN or KUN reacted with MVE, though another serological survey does not entirely agree (Madrid & Porterfield, 1974). Thus a direct comparison of T and B cell responses in mice with our stocks of virus would be necessary to make any definitive conclusions. In addition the use of purified antigen to study cross-reactivity patterns will be necessary to determine whether differences in stimulatory capacity reflect molar ratio differences in the amounts of epitopes expressed. They are also required to rule out alternative explanations, such as discrepancies between antigen concentrations and infectious virus titre. However, it is interesting to note that the cross-reactivities of the T cell clones suggest a closer relationship between KUN and WN than to MVE, a relationship previously suggested by peptide mapping of viral proteins (Wright et al., 1983).

Current experiments are concerned with determining the capacity of the cross-reactive cloned L3T4+ T cells to provide help to flavivirus-primed B cells. These data will be of particular interest considering the importance of cross-reactive antibody in the development of the dengue haemorrhagic shock syndrome.

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