Classification of Barmah Forest Virus as an Alphavirus Using Cytotoxic T Cell Assays

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SUMMARY

Barmah Forest virus, an arbovirus, does not cross-react convincingly with alpha-, flavi- or bunyavirus immune sera. Secondary cytotoxic T cells generated in vitro immune to a number of alphaviruses cross-lyse Barmah Forest virus-infected target cells. Flavivirus (West Nile and Kunjin)- and Bunyamwera virus-immune Tc cells lyse homologous virus-infected target cells, but not alphavirus-infected targets. Using cytotoxic T cell assays Barmah Forest virus can be classified as an alphavirus.

INTRODUCTION

The classification and identification of arboviruses still relies heavily on serological techniques, such as virus neutralization, haemagglutination inhibition and complement fixation tests (Casals, 1967).

Barmah Forest (BF) virus, a mosquito isolate (Marshall et al., 1982), was originally placed in the family Bunyaviridae because of a weak complement-fixing cross-reactivity with Umbre virus, a member of the Turlock group of viruses. Subsequently, haemagglutinin was prepared and, although this too cross-reacted weakly with Umbre virus antiserum, it also reacted to low titre with some alphaviruses, particularly Sindbis virus (Dalgarno et al., 1984). Antiserum to BF virus again failed to react with heterologous alphavirus haemagglutinins.

However, biological, molecular and biochemical investigations of BF revealed similarities with alphaviruses (Dalgarno et al., 1984; Bell et al., 1984). These results were based on morphological and structural analysis, the existence of a linear rather than segmented genome structure, protein structure and partial protein sequence analysis. Thus, BF virus has been reclassified as an alphavirus.

One means of classification of viruses which has so far been neglected is the use of a cell-mediated immune response as a tool in taxonomy, especially cytotoxic T cell (Tc) responses on virus-infected target cells. Unlike tests relying on serology which may give a wide range of specific and cross-reactive reactions to a virus within a group, Tc cell responses, whilst specific for a group of viruses, do not generally show specificity within groups, e.g. influenza A virus (Braciale, 1977; Zweerink et al., 1977), alphaviruses (Müllbacher et al., 1979; Wolcott et al., 1982), vesicular stomatitis viruses (Rosenthal & Zinkernagel, 1980), strains of lymphocytic choriomeningitis virus (Byrne et al., 1984) and poxviruses (Gardner et al., 1974). In the case of alphaviruses, cross-reactivity is absolute. Tc cells generated against any one member of the group will cross-react with all other members, even between serologically distant viruses such as Semliki Forest virus and Sindbis virus (Müllbacher et al., 1979; Wolcott et al., 1982).

We present here evidence that BF virus-infected targets are lysed by heterologous alphavirus immune Tc cells but not by Tc cells immune to other groups of arboviruses, thus placing BF virus firmly into the alphavirus group of arboviruses.
METHODS

Animals. CBA/H and outbred mice were obtained from the breeding unit of the John Curtin School. CBA/H mice were used from 6 to 20 weeks of age.

Viruses. The alphaviruses Bebaru (BEB), Getah (GET), Ross River (RR), Semliki Forest (SF) and Sindbis (SIN), the flaviviruses Kunjin (KUN) and West Nile (WN) and the bunyaviruses, Bunyamwera (BUN) and Umbre (UMB) were grown in outbred infant mouse brains 1 to 3 days of age, from mouse brain stock. BF virus was grown from a plaque-purified stock in either infant mouse brain [BF(m)] or in tissue culture on Vero cell monolayers [BF(v)]. Viruses were prepared by freeze–thawing of brain or cell cultures, sonication and differential centrifugation, and were titrated on Vero cell monolayers, as described previously (Taylor & Marshall, 1975).

Immunization. Eight- to 10-week-old CBA/H female mice were inoculated with $5 \times 10^6$ p.f.u. of BEB, GET, RR, SF, SIN, BF(m), BF(v), KUN, WN and UMB intraperitoneally (i.p.). In the case of mouse virulent BUN, animals were given initially $2 \times 10^7$ p.f.u. equivalent of inactivated virus i.p. ($1 \times 10^6$ rad from a $^{60}$Co source) and boosted 1 week later with $5 \times 10^7$ p.f.u. live virus.

Generation of secondary effector cells. The method has been described in detail elsewhere (Müllbacher & Blanden, 1978). In brief, $8 \times 10^7$ spleen cells from CBA/H mice immunized 2 to 4 weeks previously were co-cultured with $1 \times 10^7$ syngeneic virus-infected stimulator spleen cells for 5 days in 40 ml Eagle’s MEM (Gibco, cat. no. 15), supplemented with 5~ fetal calf serum (FCS), antibiotics plus $10^{-4}$ 2-mercaptoethanol, at 37°C in a humidified 5% CO₂ atmosphere.

Stimulator cells were infected with 2 p.f.u./cell of homologous virus for 1 h at $2 \times 10^7$ cells/ml.

Target cells. L929 tumour cells grown in Dulbecco’s modified Eagle’s medium H16 (Gibco) or thioglycollate-induced peritoneal macrophages, produced by the i.p. injection of 2.5 ml of 3% (w/v) thioglycollate (Difco) solution 4 to 10 days previously were infected in suspension with 10 p.f.u. of the relevant virus and labelled with $^{51}$Cr (Amersham) for 1 h, thoroughly washed twice, and resuspended at $2 \times 10^5$ cells/ml.

Cytotoxic assay. This has been described in detail elsewhere (Müllbacher & Blanden, 1978). In brief, effector cells were harvested and resuspended at $6 \times 10^6$ cells/ml in F15 medium containing 5% FCS. Aliquots (0.1 ml) of effector cells were titrated in threefold dilution steps in round bottom 96-well microtitre plates (Linbro) prior to the addition of 0.1 ml aliquots of target cells ($2 \times 10^4$ per well) to give effective killer to target (K : T) ratios of 30:1, 10:1, 3:3:1 and 1:1:1. After an incubation time of 6 h for macrophage targets or 14 h for L929 targets, 0.1 ml of culture supernatant was removed from each well and radioactivity measured.

Percent specific lysis was calculated as $[(\text{experimental release} - \text{spontaneous release})/(\text{maximum release} - \text{spontaneous release})] \times 100$.

Serum titration. Serum was obtained from the same animals used for memory spleen cells 2 weeks after priming. The serum was heat-inactivated at 56°C for 30 min. Plaque reduction neutralization (NT) tests were carried out on Vero cells as described in full elsewhere (Marshall et al., 1982).

RESULTS

Neutralization tests of Toga- and Bunyaviridae convalescent sera

Sera taken from animals primed for Tc cell responses were NT-tested against homologous and heterologous viruses. The results in Table 1 clearly show that BF antisera did not give cross-neutralization with any of the alpha-, flavi- or bunyaviruses used here. Neither did any convalescent sera of heterologous virus cause neutralization of BF virus. Some cross-neutralization was observed among the alpha- and flaviviruses tested here.

Cross-reactivity and specificity of Toga- and Bunyaviridae immune Tc cells

Secondary effector cells generated in vitro immune to alpha- (SF), flavi- (KUN, WN), bunyaviruses (UMB, BUN) and BF were tested against a panel of macrophage targets infected with homologous and heterologous viruses (Table 2, expt. 1). SF-immune T cells lysed SF- and BF(v)-infected targets efficiently at K : T ratios of 20:1 and 6:1. Flavivirus- and bunyavirus-infected targets were not lysed. BF(m)-immune T cells did not lyse any of the infected targets tested, even at the highest K : T ratio (20:1). The two effector populations of flavivirus (KUN and WN)-immune Tc cells gave low but significant lysis on both flavivirus-infected targets at high (20 : 1, K : T) ratios. Some slight cross-reactivity on BF(v)- and UMB-infected targets was observed in experiment 2 with WN-immune Tc cells. Such lysis above that of uninfected control targets was not observed in experiment 1 or other repeat experiments (data not shown).

Of the bunyavirus-immune Tc cells only BUN-immune effectors lysed homologous virus-infected targets. In six separate experiments using individual UMB-primed animals as the
source of spleen responder populations, we have failed to obtain a single active effector population of UMB-immune Tc cells.

To test whether alphavirus-immune TC effectors in general cross-lyse BF(v)-infected targets, BEB-, GET-, RR-, SF-, SIN- and BF-immune Tc cells were tested against a panel of infected macrophage target cells (Table 3). Strong reactivity against BF(v)-infected macrophage targets was observed with all except BF(m)-immune Tc cells (expt. 1). However, in a subsequent
Table 3. Cross-reactivity of alphavirus- and BF-immune Tc cells on heterologous and homologous virus-infected target cells

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>CBA secondary virus-immune effector</th>
<th>K : T ratio</th>
<th>% Specific lysis of macrophage targets*</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>U† SF BF(v) BEB GET RR SIN</td>
</tr>
<tr>
<td>1</td>
<td>SF</td>
<td>10 : 1</td>
<td>9 52 53 48 65 16 53</td>
</tr>
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<td></td>
<td></td>
<td>3 : 1</td>
<td>2 42 37 30 38 13 33</td>
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<td></td>
<td></td>
<td>1 : 1</td>
<td>0 15 12 13 16 5 8</td>
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<tr>
<td></td>
<td>BF(m)</td>
<td>10 : 1</td>
<td>19 10 19 12 12 8 8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 : 1</td>
<td>14 1 7 5 4 2 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 : 1</td>
<td>4 0 6 0 1 0</td>
</tr>
<tr>
<td>2</td>
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<td>10 : 1</td>
<td>12 53 65 62 82 30 53</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 : 1</td>
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<td>7 8 12 8 9 4 0</td>
</tr>
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<td></td>
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<td>37 56 57 52 72 33 60</td>
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<td>20 51 45 66 61 16 65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 : 1</td>
<td>7 26 24 23 33 8 24</td>
</tr>
</tbody>
</table>

*†† As for Table 2.

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experiment, BF(v)-immune Tc cells generated by priming and boosting splenic T cells with Vero-grown virus lysed both homologous BF(v)- and heterologous virus-infected targets (Table 3, expt. 2). RR-immune Tc cells did not lyse virus-infected targets as efficiently as other alphavirus-immune Tc cells. RR-infected targets were not susceptible to lysis by SF- or BF(m)-immune Tc cells.

DISCUSSION

The results described in this paper are an attempt to utilize cell-mediated immune mechanisms, e.g. cytotoxic T cells, as a means of classifying new isolates of arboviruses.

BF, as found by us and others (Dalgarno et al., 1984; Marshall et al., 1982) did not show any appreciable cross-reactivity in the virus haemagglutination inhibition, complement fixation or NT tests using hyperimmune and convalescent sera of alphavirus-, flavivirus- or bunyavirus-immunized mice. Thus, using conventional tests BF cannot convincingly be classified as belonging to any of the three major arbovirus groups.

However, cross-lysis of target cells was observed using secondary cytotoxic T cells generated in vitro from spleens of animals whose sera showed no cross-neutralizing activity. This cross-reactivity was observed exclusively between groups of viruses; alphaviruses including BF virus immune Tc cells lysed alphavirus- but not flavivirus- or bunyavirus-infected targets. Flavivirus-immune effectors lysed flavivirus-infected targets but did not distinguish between serologically distinct viruses such as KUN and WN. Thus, it appears that flavivirus-immune Tc cells show the same or a similar lack of specificity within this group as previously observed for the alphaviruses (Müllbacher et al., 1979; Wolcott et al., 1982). BUN virus-immune Tc cells lysed
only targets infected with the homologous virus. We were unable to generate UMB-immune Tc cells under the conditions used here and are therefore unable to make a statement regarding UMB-infected targets. One can however suggest from the high antibody titre obtained in the NT test, that UMB-primed animals were subject to a viraemia.

One noteworthy observation was the inability to sensitize macrophage targets with mouse-grown BF as compared to Vero cell-grown virus (Table 3). This difference was not observed on L929 tumour cell targets (data not shown). Furthermore, BF(m) failed to sensitize mice or re-stimulate responder memory T cell populations to give an effective Tc cell response. Vero cell-grown virus was, however, efficient in priming, re-stimulating and sensitizing target cells. One possible explanation for this finding is the presence of interferon(s) in high titre in BF virus-infected mouse brain homogenates, but not present in Vero cell culture virus stocks.

In conclusion, the data presented here show that BF virus, based on cellular immunological criteria, should be classified as an alphavirus and we would like to propose that cytotoxic T cell assays may be used as an additional convenient tool for the classification of togaviruses, especially in cases where serological means are inconclusive.

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REFERENCES


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