Cross-reactive Immunity among Different Serotypes of Virus Causing Haemorrhagic Fever with Renal Syndrome

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SUMMARY

Spleen cells primed by Prospect Hill (PH) or Puumala (Pu) virus could cross-react with Hantaan virus (HV) 76-118 strain-infected target cells after in vitro stimulation with HV-infected cells, although anti-PH or anti-Pu immune serum showed no cross-reactive neutralizing (NT) activity to HV without complement. These results and our previous findings with cross-reactive cytotoxic T lymphocytes (CTLs) suggest that some epitopes recognized by CTLs might be common among the hantavirus genus, while the epitopes related to NT activity were mainly specific to each virus of this genus. Next, to evaluate the cross-reactive immunities demonstrated by in vitro study, we investigated the effect of transferring T lymphocytes and sera from BALB/c mice immunized with PH or Pu virus into nude mice before HV inoculation. Transferring T lymphocytes primed by PH or Pu virus reduced HV titres in lungs and spleens of nude mice, corresponding with the results of the in vitro CTL assays. Transferring anti-Pu immune serum also decreased HV titres in nude mice, which seemed to reflect complement-dependent NT activity. Moreover ICR mice previously immunized with PH or Pu virus showed resistance to challenge with a lethal dose of the HV KHF strain, indicating that cross-reactive immunity induced by PH or Pu virus could protect ICR mice against pathogenic HV infection.

INTRODUCTION

Hantaan virus (HV) injection induces a severe systemic disease in humans known as Korean haemorrhagic fever (Lee et al., 1978). Many viruses that are antigenically related to this virus are widely distributed throughout the world and infect a variety of rodents as well as humans (Lee et al., 1981; Lee et al., 1982; Kitamura et al., 1983; Song et al., 1983; Yamanishi et al., 1983; Yanagihara et al., 1984). These viruses have been shown to be members of the family Bunyaviridae (McCormick et al., 1982; White et al., 1982; Hung et al., 1983, 1985; Schmaljohn & Dalrymple, 1983; Schmaljohn et al., 1983, 1985) and proposed to belong to a new genus, hantavirus. There are reports that these viruses can be classified into four groups by assays of neutralizing (NT) antibodies, immunoadherent haemagglutination, haemagglutination inhibition and immunoprecipitation (Lee et al., 1985b; Schmaljohn et al., 1985; Dantas et al., 1987; Sugiyama et al., 1987), i.e. HV (Apodemus type), Seoul virus (Rattus type), Puumala (Pu) virus (Clethrionomys type) and Prospect Hill (PH) virus (Microtus type). It is reported that the clinical manifestations of HV, Seoul virus and Pu virus are severe, intermediate and quite mild, respectively (Lähdevirta, 1982; Song et al., 1984). There is no evidence that PH virus shows any pathogenic effect in human infection.

Recently, T cell-mediated immunity was suggested to play a crucial role in the resistance of mice to HV infection (Nakamura et al., 1985a, b). Furthermore, we demonstrated that cytotoxic 
T lymphocytes (CTLs) were particularly important for the elimination of this virus and, additionally, indicated cross-reactivity of CTLs between HV and Seoul virus (Asada et al., 1987, 1988).

In this work, other serotypes of hantavirus, PH virus and Pu virus, were examined for their immunological cross-reactivity with HV by means of a CTL assay system and neutralization (NT) test. Moreover we investigated the effect of transferring T cells and sera from BALB/c mice immunized with PH or Pu into nude mice before HV infection to evaluate the results of the in vitro study. We also performed cross-protection experiments with PH or Pu against lethal infection with the HV KHF strain in ICR mice to determine whether the cross-reactive immunities induced by these mild or non-pathogenic viruses can protect against the clinical symptoms induced by pathogenic HV infection.

METHODS

Mice. BALB/c mice and athymic nude mice (BALB/c background) 6 to 8 weeks old, and ICR mice 4 weeks old, were obtained from CLEA Japan Incorporated.

Virus and cells. The HV 76-118 strain, KHF 83-61 BL (KHF) strain, Pu virus Hällnäs strain and PH virus were used. The 76-118 strain was originally isolated from lung tissue of Apodemus agrarius (Lee et al., 1978). The KHF strain was isolated from a blood sample of a patient by Dr H. W. Lee and passaged 10 times in the brains of newborn ICR mice in our laboratory and plaque-purified twice on Vero E6 cells. The Hällnäs strain was isolated from lung tissue of Clethrionomys glareorus in Sweden (Yanagihara et al., 1984). PH virus was isolated from lung tissue of Microtus pennsylvaniaicus (Lee et al., 1982, 1985a). All viruses were passaged in Vero E6 cells which were obtained from the American Type Culture Collection and virus titres were measured by the indirect immunofluorescence antibody (IFA) test (Yamanishi et al., 1983).

Preparation of macrophages as target cells. Target cells were prepared as described previously (Asada et al., 1988). Briefly, peritoneal exudate cells were obtained from BALB/c mice and non-adherent cells were removed. Adherent cells (referred to as macrophages) were then inoculated with the 76-118 strain at an m.o.i. of 0.5 f.f.u./cell and cultured for 5 days. The virus-infected macrophages were labelled with 100 μCi per 10^6 cells of sodium [51Cr]chromate (in saline) for 1 h. They were then washed three times and resuspended at a concentration of 10^5 cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS), l-glutamine, penicillin and 2-mercaptoethanol (complete RPMI medium).

Immunochemistry and preparation of spleen cells as effector cells. BALB/c mice were infected intra-peritoneally (i.p.) with 1 × 10^3 f.f.u. of PH virus or Pu virus. Two weeks after infection, their spleens were removed aseptically. Spleen cells were treated with 0.83% NH4Cl solution to remove erythrocytes, washed and resuspended in complete RPMI medium. For in vitro stimulation, 1 × 10^7 spleen cells were cultured with 5 × 10^3 HV-infected syngeneic macrophages at 37 °C for 5 days under 5% CO₂ in air.

Cytotoxicity assay. [51Cr]-labelled target cells were added to the wells (U-shaped) of microtitre plates (10^5 cells per well in 100 μl of complete RPMI medium). The effector cells in 100 μl of complete RPMI medium were added to the plates, which were then centrifuged at 1000 r.p.m. for 30 s before incubation. The microtitre plates were incubated under 5% CO₂ in air at 37 °C for 4 h, and then centrifuged at 1500 r.p.m. for 5 min; samples (50 μl) of the supernatants were counted in a gamma counter. The percentage specific [51Cr] release was calculated from specific release = experimental release - spontaneous release/total release - spontaneous release. The total release was defined as that obtained by exposing the target cells to 3% Triton X-100. CTL assays were carried out at different effector:target cell ratios and all assays were performed in triplicate.

Detection of serum antibodies. The sera from BALB/c mice, immunized i.p. with 1 × 10^4 f.f.u. of PH, Pu or HV 76-118 three times at 14 day intervals, were examined by IFA and neutralization assays against homologous and heterologous viruses. IgG antibodies against these viruses were tested by IFA as described previously (Asada et al., 1987). Complement-independent neutralizing (NT) antibody titres of heat-inactivated sera were measured by the peroxidase-anti-peroxidase technique as described previously (Tanishita et al., 1984) and expressed as the reciprocal of the highest serum dilution resulting in more than 80% reduction in the number of foci. Complement-dependent NT tests were also performed with 3% rabbit serum as a source of complement.

Passive transfer of immune sera and immune T lymphocytes. Immune sera and spleen cells were obtained from BALB/c mice immunized i.p. with 1 × 10^4 f.f.u. of PH or Pu three times at 14 day intervals. The anti-PH serum antibody titre was 1:5120 by IFA tests and 1:160 by NT tests against homologous virus. The anti-Pu serum antibody titre was 1:10240 by IFA tests and 1:160 by NT tests against homologous virus. These immune sera (300 μl/mouse) were injected intravenously (i.v.) into nude mice 1 day before i.p. inoculation with 1 × 10^3 f.f.u. of the HV 76-118 strain.

The PH or Pu virus-immune spleen cells described above were treated with 0.83% NH4Cl, washed and passed through a nylon wool column to obtain the immune T cell fractions. Contamination of these T cell fractions with B
Hantavirus cross-reactive immunity

Table 1. Cross-reactivity of antibodies

<table>
<thead>
<tr>
<th>Status of BALB/c mice†</th>
<th>NT antibody titre* against</th>
<th>IFA titre against</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HV</td>
<td>PH</td>
</tr>
<tr>
<td>HV-immune</td>
<td>160 (1280)†</td>
<td>&lt;10</td>
</tr>
<tr>
<td></td>
<td>320 (2560)</td>
<td>&lt;10</td>
</tr>
<tr>
<td>PH-immune</td>
<td>&lt;10 (&lt;10)</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>&lt;10 (&lt;10)</td>
<td>160</td>
</tr>
<tr>
<td>Pu-immune</td>
<td>&lt;10 (40)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>&lt;10 (80)</td>
<td>ND</td>
</tr>
<tr>
<td>Non-immune</td>
<td>&lt;10 (&lt;10)</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Titres are expressed as the reciprocal of the highest serum dilution giving definite fluorescence or resulting in more than 80% reduction in the number of foci.
† The sera from BALB/c mice that had been immunized i.p. with $1 \times 10^5$ f.f.u. of PH virus, Pu virus or HV 76-118 strain three times at 14 day intervals were examined by IFA and NT tests against homologous and heterologous viruses.
‡ Parentheses denote the antibody titre against HV in the presence of rabbit complement.
§ ND, Not determined.

cells was 3% as determined by the IFA technique. These immune T cells (4 x $10^7$ cells in 300 μl complete RPMI medium/mouse) were injected i.v. into nude mice 1 day before i.p. inoculation with $1 \times 10^5$ f.f.u. of HV 76-118. Non-immune serum and non-immune T lymphocytes were also prepared from non-immune BALB/c mice and injected i.v. into nude mice.

Virus titration in organs of nude mice. Lungs and spleens were removed from nude mice 6 days after HV inoculation. The organs were homogenized (10% homogenates) and centrifuged. The HV titres of the supernatants were determined as previously described (Asada et al., 1987).

Cross-protection study against lethal infection with the KHF strain. Four-week-old ICR mice were divided into four groups of seven animals. One group was immunized with a single i.p. inoculation of $1 \times 10^5$ f.f.u. of the PH virus. Another group was immunized i.p. with $1 \times 10^5$ f.f.u. of the Pu virus. Non-inoculated mice served as control groups. After 1 month mice were challenged by i.v. inoculation with $1 \times 10^5$ or $1 \times 10^6$ f.f.u. of the HV KHF strain. The ICR mice were observed daily for clinical symptoms.

RESULTS

Cross-reactivity of serum antibody for different serotypes of hantavirus

The sera from BALB/c mice immunized with PH, Pu or HV were examined by IFA and NT assays against homologous and heterologous viruses (Table 1). In the case of anti-PH immune serum, the titres of cross-reactive antibody against HV were low or undetectable, such as 1:80 to 1:160 by IFA tests and <1:10 by NT tests without complement, although antibody titres against homologous virus were significantly higher, such as 1:5120 by IFA tests and 1:160 by NT tests without complement. Even in the presence of complement, no cross-neutralizing activity could be observed. In the case of anti-Pu immune serum, the titres of cross-reactive antibody against HV were also low or undetectable, such as 1:80 to 1:160 by IFA tests and <1:10 by NT tests without complement, but antibody titres against homologous virus were high such as 1:5120 to 1:10240 by IFA tests and 1:160 by NT tests without complement. In contrast to anti-PH immune serum, however, anti-Pu immune serum showed some cross-neutralizing activity (1:40 to 1:80) in the presence of complement.

Cross-reactivity of CTLs for different serotypes of hantavirus

We examined the cross-reactivity of CTLs for different serotypes of hantavirus. Mice were infected with PH or Pu and 2 weeks later the spleen cells from the mice were cultured with HV-infected macrophages for 5 days. These spleen cells were tested for CTL activity against the target cells infected with HV. As shown in Table 2, some cross-reactive lysis was observed with
Fig. 1. Sera or T lymphocytes, obtained from BALB/c mice immunized i.p. with $1 \times 10^5$ f.f.u. of PH or Pu virus three times, were transferred i.v. into nude mice. The mice received an i.p. inoculation of HV ($1 \times 10^5$ f.f.u./mouse) the following day. Six days after inoculation, virus titres in lungs (a), and spleens (b) were measured. The dashed line represents the detection limit.

Table 2. Cross-reactivity of CTLs

<table>
<thead>
<tr>
<th>Status of BALB/c mice</th>
<th>In vitro stimulation</th>
<th>Specific $^{51}$Cr release from target cell (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E/T†</td>
<td>Uninfected</td>
</tr>
<tr>
<td>PH-immune</td>
<td>HV</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td>Pu-immune</td>
<td>HV</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.5</td>
</tr>
<tr>
<td>Non-immune</td>
<td>HV</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>12.5</td>
</tr>
</tbody>
</table>

* BALB/c mice were inoculated i.p. with $1 \times 10^5$ f.f.u. of PH or Pu virus. Two weeks later, the spleen cells from the mice were co-cultured with HV-infected macrophages for 5 days. After in vitro stimulation, spleen cells were assayed for cytotoxic activity. Spontaneous release was less than 20%.
† E/T, Effector:target cell ratio.

HV-infected targets and spleen cells primed by PH or Pu virus. In contrast, the cytotoxic activity was undetectable when spleen cells from uninfected mice were cultured with HV-infected macrophages. When CTL activity was assayed without in vitro restimulation, the spleen cells did not lyse HV-infected target cells (data not shown).
Table 3. Effects of immunization with PH or Pu virus against challenge with the HV KHF strain

<table>
<thead>
<tr>
<th>Status of ICR mice</th>
<th>Challenge* (f.f.u./mouse)</th>
<th>No. of mice (survivors/challenged)</th>
<th>Survival (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PH-immune</td>
<td>$1 \times 10^6$</td>
<td>6/7</td>
<td>86</td>
</tr>
<tr>
<td>Pu-immune</td>
<td>$1 \times 10^6$</td>
<td>7/7</td>
<td>100</td>
</tr>
<tr>
<td>Non-immune</td>
<td>$1 \times 10^6$</td>
<td>0/7</td>
<td>0</td>
</tr>
<tr>
<td>Non-immune</td>
<td>$1 \times 10^5$</td>
<td>3/7</td>
<td>43</td>
</tr>
</tbody>
</table>

* ICR mice were immunized i.p. with $1 \times 10^5$ f.f.u. of PH or Pu virus. After 1 month the mice, including non-immune controls, were challenged i.v. with $1 \times 10^6$ or $1 \times 10^5$ f.f.u. of the HV KHF strain. The survival rate was observed.

We also investigated the typing of cross-reactive cytotoxic effector cells. Treatment of effector cells with anti-Thy1.2 plus complement or anti-Lyt2.2 plus complement abolished cytolysis, but treatment with complement alone or anti-L3T4 plus complement did not reduce their cytotoxic activity (data not shown). Thus T cells expressing Lyt2+ on their surface have cross-reactive cytolytic activity.

Effect of transferring immune sera or immune T lymphocytes into nude mice

Sera or T lymphocytes, obtained from BALB/c mice immunized with PH or Pu virus, were transferred i.v. into nude mice 1 day before HV inoculation. Six days after inoculation, virus titres in the lung and spleen were measured (Fig. 1).

Although the transfer of T lymphocytes from the mice immunized with PH virus decreased HV titres in the lung and spleen of nude mice, sera from these mice did not protect against HV infection.

In contrast to the case of PH virus, both T lymphocytes and sera from the mice immunized with Pu virus significantly decreased HV titres in the lung and spleen of nude mice.

Effect of immunization with PH or Pu virus on lethal infection with the KHF strain

ICR mice that had been immunized with PH or Pu virus were challenged by i.v. inoculation of the HV KHF strain 1 month after immunization. As shown in Table 3, all of the mice immunized with Pu virus and six of the seven mice immunized with PH virus survived and showed no clinical symptoms when challenged with $1 \times 10^6$ f.f.u. of KHF. In contrast all non-immune mice injected with $1 \times 10^6$ f.f.u. of KHF showed ruffled fur and hypoactivity on day 6 or 7, and died on day 7. Even at the lower dose of $1 \times 10^5$ f.f.u. four of the seven non-immune mice died on day 7 or 8.

DISCUSSION

In this work, cross-reactive immunity to HV induced by PH or Pu virus was investigated by using both in vitro and in vivo assay systems. First, the cross-reactive NT activity to HV induced by PH or Pu virus was examined (Table 1). Neither anti-PH nor anti-Pu immune sera had NT activity to HV in the absence of complement. However, in the presence of complement anti-Pu immune serum had some NT activity to HV. Previously, some cross-reactive NT activity was observed between Seoul virus and HV even without complement (Yamanishi et al., 1984). It has been reported that antibodies concerned with NT activity (without complement) showed virus specificity (Lee et al., 1985b; Schmaljohn et al., 1985). Our present results were consistent with these observations.

Cross-reactivity of CTLs among different serotypes of hantavirus was then examined. The spleen cells primed by PH or Pu virus cross-reacted with HV-infected target cells after in vitro stimulation with HV-infected cells (Table 2). These results suggest that spleen cells primed by PH or Pu virus contain CTL precursors which recognize HV-infected cells. We recently observed that the CTLs induced by infection with HV and Seoul virus both cross-react with target cells infected with the heterologous viruses (Asada et al., 1988). CTLs showed more extensive cross-reactivity than NT antibody within the hantavirus genus. From these in vitro
studies, it seems that although the epitopes related to NT activity are not shared by all four of these hantaviruses and show virus specificity, some epitopes recognized by CTLs are separate from the critical NT sites and might be common to all viruses of this genus. In this system, macrophages infected with PH or Pu virus could not be used at target cells or stimulators because these two viruses replicate very slowly in macrophages and the proportion of infected cells was restricted to less than 1% even 2 weeks after inoculation. Similarly, in the case of influenza virus and rotavirus, it was demonstrated that CTLs could cross-react with serologically distinct viruses and that cross-reactive anti-influenza virus CTLs mainly recognized viral nucleoprotein (Zweerink et al., 1977; Effros et al., 1977; Yewdell et al., 1985; Offit & Dudzik, 1988). For hantaviruses, which determinant is recognized by cross-reactive mechanisms

In order to examine whether the cross-reactive immunities demonstrated by the in vitro studies are consistent with the in vivo phenomena, a cross-protection study was performed in vivo. We investigated the effect of transferring T lymphocytes or sera from BALB/c mice immunized with PH or Pu virus into nude mice before HV infection. Although T lymphocytes primed by PH virus showed a cross-protective effect against HV infection, anti-PH immune serum had no effect. On the other hand, not only T lymphocytes primed by Pu virus, but also anti-Pu immune serum showed a cross-protective effect against HV infection (Fig. 1). These in vivo findings correspond to the results of the in vitro studies, i.e. part of the cross-protection mediated by immune T lymphocytes may be due to cross-reactive CTLs and some of the cross-protective effect of anti-Pu serum seems to reflect complement-dependent NT activity. However, it is possible that immune T lymphocytes and immune serum may protect via other immunological mechanisms in vivo; for example, T lymphocytes could mediate help in antibody production, or mediate a delayed type hypersensitivity reaction, involving the production of lymphokines including interferon. Antibodies could protect via the enhancement of phagocytosis and/or antibody-dependent cellular cytotoxicity. These mechanisms are under investigation.

Finally it was observed that ICR mice previously immunized with PH or Pu virus showed no clinical signs on challenge with a lethal dose of the HV KHF strain (Table 3). These findings indicate that cross-reactive immunity induced by PH or Pu virus could protect ICR mice from lethal infection with HV. Additionally it might be considered that Pu or PH viruses, which are believed to be mildly or non-pathogenic in human infections, could be used as models for live vaccines against pathogenic HV infection. In order to confirm this possibility, we have to establish animal models that manifest clinical symptoms similar to human infection such as haematological disorders and renal failure.

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