Cell-mediated Immunity to Virus Causing Haemorrhagic Fever with Renal Syndrome: Generation of Cytotoxic T Lymphocytes

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SUMMARY

Cytotoxic T lymphocytes (CTLs) were generated when spleen cells from mice infected with viruses causing haemorrhagic fever with renal syndrome were stimulated in vitro with syngeneic cells infected with viruses. These cytotoxic effector cells, with Lyt2+ L3T4− markers on their surface, demonstrated H-2 restriction. CTLs induced by Hantaan virus (76-118 strain) or Seoul virus (B-1 strain) showed cross-reactivity with infected target cells. Hantaan virus infection induced a higher CTLs response than Seoul virus infection, although the antibody responses to these two viruses and the replication of the two virus strains in athymic nude mice were not significantly different. Viral antigen detected with a monoclonal antibody reacting with nucleocapsid antigen was observed mainly in the cytoplasm of macrophages infected with Hantaan virus, but in the nucleus of cells infected with Seoul virus. The major viral antigens recognized by CTLs are discussed on the basis of these findings.

INTRODUCTION

Viruses causing haemorrhagic fever with renal syndrome are widely distributed throughout the world and infect various rodents as well as humans (Gajdusek, 1962; Lee, 1978). Hantaan virus (HV), the aetiologic agent of Korean haemorrhagic fever, was isolated in 1976 from Apodemus agrarius (Lee et al., 1978), and subsequently viruses that are antigenically related to HV were isolated from rodents and patients (Lee et al., 1981, 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 antibodies, immunoadherent haemagglutination, haemagglutination inhibition and immunoprecipitation (Lee et al., 1985; Schmaljohn et al., 1985; Dantas et al., 1987; Sugiyama et al., 1987), i.e. Apodemus type, Rattus type, Clethrionomys type and Microtus type.

Some groups have attempted to infect various rodents with these viruses (Lee et al., 1981a, b; Lee et al., 1981; Tsai et al., 1982; Kurata et al., 1983; Yamanouchi et al., 1984; Kim & McKee, 1985; Yanagihara et al., 1985a, b; Tanishita et al., 1986). It has recently been suggested that T cell-mediated immunity plays a crucial role in resistance of mice to HV infection (Nakamura et al., 1985a, b). Furthermore, we demonstrated persistent infection with HV in congenitally athymic nude mice, but only transient infection in immunocompetent BALB/c mice. We also found by transferring T cell subsets into HV-infected nude mice that T cells possessing Lyt2+ L3T4− markers on their surface, which are considered to constitute the majority of cytotoxic/suppressor T cells, were especially important for elimination of infectious virus in vivo (Asada et al., 1987).
Here we report the generation of cytotoxic T lymphocytes (CTLs) by in vitro restimulation of spleen cells from hantavirus-infected mice. In this work we also investigated the cross-reactivity of CTLs between HV strain 76-118 (Apodemus type) and Seoul virus B-1 strain (Rattus type) infections and compared the intensity of CTL induction by these two viruses. We believe this is the first report of CTL activity in rodents following hantavirus infection.

**METHODS**

**Mice.** BALB/c mice (H-2k) and athymic nude mice (BALB/c background) 6 to 8 weeks old were obtained from CLEA Japan Inc. BALB.K (H-2k) and BALB.B (H-2k) mice 6 to 8 weeks old were obtained from Dr H. Yamamoto (Kochi Medical School, Kochi, Japan).

**Viruses and cells.** The HV 76-118 and Seoul virus B-1 strains of hantavirus were used. The 76-118 strain, originally isolated from *A. agrarius*, was obtained from the American Type Culture Collection. The B-1 strain was isolated from a laboratory rat in Vero E6 cells as reported (Yamanishi *et al.*, 1983). Both 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.** Peritoneal exudate cells were obtained by washing the peritoneal cavity with cold Eagle's MEM supplemented with 10% foetal calf serum (FCS), penicillin, streptomycin and L-glutamine. After incubation for 3 h at 37 °C in 6 cm plastic dishes, non-adherent cells were removed. Adherent cells (referred to as macrophages) were then infected with either the 76-118 strain or the B-1 strain at an m.o.i. of 0.5 f.f.u./cell. The cells were incubated at 37 °C for 2 h, washed and cultured in fresh medium for 5 days. At this time, 100% of the cells contained viral antigen detected by the IFA test. The virus-infected macrophages were removed from the dishes and labelled with 100 μCi per 10⁶ cells of sodium [51Cr]chromate (in saline) for 1 h. They were then washed three times and resuspended at a concentration of 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, L-glutamine, penicillin and 2-mercaptoethanol (complete RPMI medium). Uninfected macrophages were labelled in the same way.

**Immunization of mice and preparation of spleen cells.** Mice were infected intraperitoneally (i.p.) with 1 x 10⁴ f.f.u. of the 76-118 strain or B-1 strain. At various times 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 x 10⁷ spleen cells were cultured with 5 x 10⁷ virus-infected syngeneic macrophages at 37 °C for 5 days under 5% CO₂ in air.

**Treatment of effector cells with various monoclonal antibodies (MAbs) against T cell subsets.** After in vitro secondary stimulation, the effector cells were treated with rabbit complement (C), monoclonal anti-Thy1.2 + C', anti-L3T4 + C' or anti-Lyt2.2 + C' as described previously (Asada *et al.*, 1987). The viabilities of the effector cells after treatments with C', anti-Thy1.2 + C', anti-L3T4 + C' and anti-Lyt2.2 + C' were 83%, 10%, 33% and 43%, respectively, as judged by the trypan blue dye exclusion test.

**Cytotoxicity assay.** [51Cr]-labelled target cells were added to the wells (U-shaped) of microtitre plates (10⁴ cells per well in 100 μl of complete RPMI medium). The effector cells in 100 μl of complete RPMI medium were introduced into microtitre plates, which were then centrifuged at 1000 r.p.m. for 30 s before incubation. CTL assays were carried out at different effector:target cell ratios, and all assays were performed in triplicate. 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 as follows: percentage 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.

**Inhibition of cytotoxicity by unlabelled target cells.** Cytotoxicity assays were carried out in the presence of various numbers of unlabelled competitor cells, which were 76-118-infected, B-1-infected or uninfected macrophages. Unlabelled competitor cells were mixed with the effector cells and 100 μl samples of the mixtures in medium were promptly added to wells containing 1 x 10⁴ [51Cr]-labelled target cells in 100 μl of medium.

**Virus replication in macrophages.** Macrophages grown in plastic plates were infected with the 76-118 strain or B-1 strain at an m.o.i. of 0.5 f.f.u./cell, and after adsorption for 1 h at 37 °C the cells were kept in a CO₂ incubator. Culture fluid was harvested every 2 days and stored at -70 °C until titration on Vero E6 cells.

**Detection of serum antibodies.** The sera from infected BALB/c mice were examined by IFA and neutralization assays. IgG antibodies against the 76-118 strain and B-1 strain were tested by IFA as described previously (Asada *et al.*, 1987). Neutralizing (NT) antibody titres 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 90% reduction in the number of foci.
RESULTS

Generation of cytotoxic T cells in response to HV 76-118 strain infection

Spleen cells from mice infected with the 76-118 strain or from uninfected mice were cultured with infected syngeneic macrophages. After in vitro stimulation for 5 days, spleen cells were harvested and used for the cytotoxicity assay. As shown in Table 1, potent cytotoxic activity was observed with effector cells from infected mice and infected syngeneic target cells. The degree of cytotoxicity was related with the effector : target cell ratio. When effector cells from uninfected mice or uninfected target cells were used, the cytotoxic activity was low or undetectable. Moreover, when CTL activity was assayed without in vitro restimulation, spleen cells from infected mice also did not lyse infected target cells (data not shown).

The H-2 restriction of cytotoxicity was then evaluated. Table 2 shows that effector cells from BALB/c mice (H-2^d) lysed target cells from the same strain but not those from BALB.K (H-2^k) or BALB.B (H-2^b) mice, which are identical to the BALB/c strain except that they have different H-2 gene products. Similarly, effector cells from BALB.K mice lysed target cells from the same substrain but not those from BALB/c or BALB.B mice. Thus, only target cells with the same H-2 antigens as the effector donors were lysed specifically.

We also investigated the antigen type of cytotoxic effector cells. Table 3 shows that treatment of effector cells with anti-Thy1.2 + C' or anti-Lyt2.2 + C' abolished cytolysis, but that treatment with C' alone or anti-L3T4 + C' did not reduce their cytotoxic activity. Thus T cells expressing Lyt2^+ on their surface have cytotoxic activity against HV-infected cells.

Table 1. Lysis of HV-infected and uninfected syngeneic macrophages by cytotoxic effector cells

<table>
<thead>
<tr>
<th>Effector cells*</th>
<th>E/T †</th>
<th>Uninfected</th>
<th>HV-infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-immune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>1</td>
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</tr>
<tr>
<td>25</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Immune</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>1</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>0</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

* BALB/c mice were injected i.p. with 1 x 10^4 f.f.u. of HV. After 2 weeks, their spleen cells were cultured with HV-infected syngeneic macrophages. After in vitro stimulation for 5 days, spleen cells were used for the cytotoxicity assay.
† E/T, Effector : target cell ratio.
‡ Spontaneous release was less than 25%.

Table 2. H-2 specificity of cytotoxic effector cells from HV-infected mice*

<table>
<thead>
<tr>
<th>Donor of effectors</th>
<th>Specific ^51^Cr release from target cells (%) ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E/T †</td>
</tr>
<tr>
<td>BALB/c (H-2^d)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>25</td>
</tr>
<tr>
<td>BALB.K (H-2^k)</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>25</td>
</tr>
</tbody>
</table>

* BALB/c or BALB.K mice were treated i.p. with 1 x 10^4 f.f.u. of HV; spleen cells were restimulated in vitro with HV-infected syngeneic macrophages and assayed on syngeneic or allogeneic infected target cells.
† E/T, Effector : target cell ratio.
‡ Lysis of uninfected targets was less than 5%. Spontaneous release was less than 29%. 

CTLs in hantavirus infection
Table 3. Effects of monoclonal antibodies to T cell surface antigens on cytotoxic effector cells

<table>
<thead>
<tr>
<th>Treatment of effector cells*</th>
<th>E/T†</th>
<th>Specific 51Cr release from HV-infected target cells (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>70</td>
</tr>
<tr>
<td>C'</td>
<td>25</td>
<td>57</td>
</tr>
<tr>
<td>Anti-Thy1.2 + C'</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Anti-L3T4 + C'</td>
<td>25</td>
<td>0</td>
</tr>
<tr>
<td>Anti-Lyt2.2 + C'</td>
<td>100</td>
<td>1</td>
</tr>
</tbody>
</table>

* BALB/c mice were treated i.p. with 1 × 10⁴ HV, and spleen cells were restimulated in vitro with HV-infected syngeneic macrophages. After secondary in vitro stimulation, the spleen cells were treated with C', anti-Thyl.2 + C', anti-L3T4 + C' or anti-Lyt2.2 + C' and assayed for cytotoxic activity.
† E/T, Effector : target cell ratio.
‡ Spontaneous release was less than 32%.

Table 4. Cytotoxic T lymphocytes and antibody responses induced by hantavirus strains 76-118 and B-1

<table>
<thead>
<tr>
<th>Status of mice</th>
<th>Mouse no.</th>
<th>E/T‡</th>
<th>Uninfected</th>
<th>76-118-infected</th>
<th>B-1-infected</th>
<th>Antibody response†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IF titre against 76-118 B-1</td>
</tr>
<tr>
<td>Uninfected</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>&lt;10 &lt;10 &lt;5 &lt;5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>&lt;10 &lt;10 &lt;5 &lt;5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>&lt;10 &lt;10 &lt;5 &lt;5</td>
</tr>
<tr>
<td>76-118-infected</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>76</td>
<td>58</td>
<td>1280 1280 40 20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>83</td>
<td>67</td>
<td>2560 2560 40 20</td>
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<tr>
<td></td>
<td>3</td>
<td>ND‡</td>
<td>ND</td>
<td>73</td>
<td>57</td>
<td>2560 2560 40 20</td>
</tr>
<tr>
<td>B-1-infected</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>2560 5120 10 80</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>14</td>
<td>7</td>
<td>5120 10240 20 80</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>5</td>
<td>5120 10240 20 80</td>
</tr>
</tbody>
</table>

* BALB/c mice were treated i.p. with 1 × 10⁴ f.f.u. of 76-118 or B-1, and spleen cells were restimulated in vitro with homologous virus-infected macrophages. After secondary stimulation, spleen cells were assayed for CTL activity against target cells infected with homologous or heterologous hantavirus.
† The antibody titres in the sera of BALB/c mice used as CTL donors were determined by IFA and NT test. Titres are expressed as the reciprocal of the highest serum dilution giving definite fluorescence or resulting in more than 90% reduction in the number of foci.
‡ E/T, Effector : target cell ratio.
§ ND, Not determined.

Cross-reactivity of CTLs for different serotypes of hantavirus

We evaluated the cross-reactivity of CTLs with different serotypes of hantavirus (strains 76-118 and B-1). BALB/c mice were infected with 1 × 10⁴ f.f.u. of 76-118 or B-1 and 14 days later their spleen cells were cultured with homologous virus-infected macrophages for 5 days. The spleen cells were then tested for CTL activity against target cells infected with the 76-118 or B-1 strain. As shown in Table 4, some cross-reactive lysis was observed with cells infected with the 76-118 or B-1 strain. Unlabelled target cell competition experiments were performed to determine whether the killing of target cells infected with homologous virus or with heterologous virus was mediated by the same cytotoxic T cell population (Fig. 1). Effector cells induced by the
CTLs in hantavirus infection

Fig. 1. Inhibition of cytotoxicity by unlabelled competitors. Spleen cells from BALB/c mice were obtained 2 weeks after injection i.p. of $1 \times 10^4$ f.f.u. of the 76-118 strain and restimulated in vitro with homologous virus-infected macrophages. After in vitro stimulation, the spleen cells were assayed for cytotoxic activity on $^{51}$Cr-labelled infected target cells (a, 76-118; b, B-1) in the presence of increasing numbers of unlabelled competitors. The ratio of effectors to targets was 30:1. The ratio of unlabelled competitors to labelled targets was as indicated. Unlabelled competitors were macrophages infected with strain 76-118 (O) or B-1 (△), or uninfected cells (■). Asterisks indicate the percentage specific $^{51}$Cr release in the absence of competitor cells.

Fig. 2. Time course of generation of CTL precursors in BALB/c mice infected i.p. with $1 \times 10^4$ f.f.u. of the 76-118 strain (O) or B-1 strain (△). Spleen cells obtained on the indicated day were stimulated in vitro with macrophages infected with homologous virus and cytotoxic activity against macrophages infected with homologous virus was measured. The effector:target cell ratio was 100:1.

76-118 strain were tested for cytotoxic activity on $^{51}$Cr-labelled homologous or heterologous targets in the presence of increasing numbers of unlabelled target cells either uninfected or infected with strain 76-118 or B-1. With target cells infected with the 76-118 strain, inhibition was much greater with competitors infected with this same strain than with those infected with the B-1 strain. However, with target cells infected with the B-1 strain, competitors infected with either the 76-118 strain or the B-1 strain inhibited lysis to the same extent. Uninfected competitors caused no significant inhibition. These results suggest that two distinct subpopulations of cytotoxic T cells were generated in response to immunization with the 76-118 strain of hantavirus: one population was specific for the 76-118 strain, while the other cross-reacted with target cells infected with the B-1 strain. CTLs induced by the B-1 strain could not be examined by competition experiments because the level of cytotoxicity was low even in the absence of competitors.

Comparison of the CTL responses induced by different serotypes of hantavirus

The 76-118 strain induced higher CTL activity than the B-1 strain even when target cells infected with the latter were used (Table 4). We next measured CTL activity at various times after infection by these two viruses (Fig. 2). In both cases, cytotoxic activity reached a maximum 15 days after virus inoculation and the level of cytotoxicity persisted for a further 15 days at least. Throughout the observation period the CTL level induced by the 76-118 strain was significantly higher than that induced by the B-1 strain. On the other hand, the antibody titres in the sera of BALB/c mice infected with the B-1 strain were the same or slightly higher than those in the sera of such mice infected with the 76-118 strain as determined by IFA and NT tests (Table 4).
Fig. 3. Virus titres in spleens of nude mice. Three nude mice were treated i.p. with $1 \times 10^4$ f.f.u. of the 76-118 strain (○) or B-1 strain (△) and were sacrificed on the indicated day. Spleens were homogenized in Eagle's MEM and centrifuged at 2000 g for 10 min. Virus titres in the supernatants were measured.

Fig. 4. Replication of the 76-118 strain (○) and B-1 strain (△) in macrophages. Peritoneal macrophages were infected with the 76-118 strain or B-1 strain at an m.o.i. of 0.5 f.f.u./cell. Fluids from infected cultures were harvested on the indicated day and virus titres were determined by focus formation on Vero E6 cells.

Table 5. Cytotoxic activities of immune spleen cells after secondary in vitro stimulation with homologous and heterologous virus

<table>
<thead>
<tr>
<th>Source of spleen cells</th>
<th>In vitro stimulation*</th>
<th>E/T †</th>
<th>Specific $^{51}$Cr release from BALB/c target cells (%)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Uninfected 76-118-infected B-1-infected</td>
</tr>
<tr>
<td>76-118-immune</td>
<td>76-118</td>
<td>30</td>
<td>0 36 19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0 15 8</td>
</tr>
<tr>
<td>76-118-immune</td>
<td>B-1</td>
<td>30</td>
<td>1 29 18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0 14 10</td>
</tr>
<tr>
<td>B-1-immune</td>
<td>B-1</td>
<td>30</td>
<td>0 7 10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0 6 6</td>
</tr>
<tr>
<td>B-1-immune</td>
<td>76-118</td>
<td>30</td>
<td>1 22 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>0 12 5</td>
</tr>
</tbody>
</table>

* BALB/c mice were treated i.p. with $1 \times 10^4$ f.f.u. of the 76-118 strain or B-1 strain. Two weeks later, spleen cells were restimulated in vitro with homologous or heterologous virus-infected macrophages. After secondary stimulation, the spleen cells were assayed for CTL activity.
† E/T, Effector:target cell ratio.
‡ Spontaneous release was less than 21%.

In addition, to determine whether the difference in the CTL response depended on virus replication in vivo, we measured the virus titres in spleens of nude mice (BALB/c background) at various times after infection. No significant difference in virus titres was observed in the spleens of mice infected with either the 76-118 strain or the B-1 strain (Fig. 3). Next, to determine whether the conditions of priming and/or secondary in vitro stimulation were related to the difference in the CTL response, we investigated the cytotoxic activity of immune spleen cells.
after in vitro stimulation with the homologous and heterologous strain. Cytotoxic activities in spleen cells either primed by the 76-118 strain after in vitro stimulation with the B-1 strain, or primed by the B-1 strain after in vitro stimulation with the 76-118 strain were higher than that of spleen cells primed by the B-1 strain after in vitro stimulation with the homologous strain and lower than that of spleen cells primed by the 76-118 strain after in vivo stimulation with the homologous strain (Table 5). Therefore, the difference in cytotoxic activities induced by the 76-118 and B-1 strains depended on both the conditions of priming in vivo and stimulation in vitro.

**Expressions of viral antigens in macrophages infected with the two strains of hantavirus**

Mouse macrophage cells were infected with the 76-118 strain or B-1 strain at an m.o.i. of 0.5 f.f.u./cell, and fluid from infected cultures was harvested for virus titration. Both viruses began to replicate within 1 day and the virus titres reached a maximum 4 days after infection (Fig. 4). Next, viral antigens were stained in an immunofluorescence test with MAb 80A, which reacts with the nucleocapsid polypeptide, or MAb B-102, which reacts with a glycoprotein (G2) (Fig. 5) (Yamanishi et al., 1984; Dantas et al., 1986). In studies carried out 2 to 7 days after
infection, in cells infected with the B-1 strain MAb 80A reacted with an antigen that was mainly located in the nucleus and appeared to have a fibrous form. On the other hand, in cells infected with the 76-118 strain, fluorescence was seen throughout the cytoplasm. When MAb B-102 was used to stain viral antigens, no difference in the amount or location of antigens was observed in cultures infected with either of the two strains. These data showed that in infected macrophages the location of viral nucleocapsid protein of each of the two strains was different.

**DISCUSSION**

In this work we first demonstrated that cytotoxic cells against the 76-118 strain of hantavirus were generated when spleen cells from mice infected with the 76-118 strain were stimulated in vitro with syngeneic cells infected with virus. The cytotoxic effectors lysed target macrophages infected with the 76-118 strain (Table 1), but only those with the same H-2 antigens as the effector donors (Table 2); they did not lyse uninfected macrophages (Table 1). These results confirmed the requirements for both virus antigen and expression of the same H-2 antigen for effector–target cell interactions. Furthermore, treatment of effector cells with C' alone, anti-Thyl.2 + C', anti-L3T4 + C' or anti-Lyt2.2 + C' showed that the cytotoxic effector cells were T lymphocytes with Lyt2+ L3T4− markers on their surface (Table 3). Since Lyt2+ L3T4− T cells are reported to recognize major histocompatibility complex (MHC) class I antigen (Swain, 1981, 1983), these cytotoxic effectors were considered to be T lymphocytes restricted with MHC class I antigen.

We recently demonstrated, by experiments on immune T cell subset transfer into nude mice infected with the 76-118 strain (Asada et al., 1987), that Lyt2+ L3T4− T lymphocytes were important for clearance of HV. We also reported that hantavirus mainly infects macrophages, and that the macrophages transport the virus systemically to target organs (Nagai et al., 1985). Macrophages were therefore used as target cells in this study. As shown in this work, lysis of virus-infected target cells by CTLs can be regarded as an important mechanism for clearance of virus in vivo.

We observed that the CTLs induced by infection with the 76-118 strain and the B-1 strain both cross-react with target cells infected with the heterologous virus strains (Table 4). Furthermore, unlabelled target cell competition experiments indicated that two distinct populations may be induced in CTLs of mice infected with the 76-118 strain: one is strain-specific and the other cross-reacts with target cells infected with the B-1 strain (Fig. 1). In the case of CTLs induced by the B-1 strain, because CTL activity was low it was difficult to determine whether there were two populations of CTLs or only the cross-reactive one. We have previously observed cross-reactivity between antibodies to the 76-118 strain and B-1 strains of hantavirus (Yamanishi et al., 1984; Dantas et al., 1987). Our results suggested cross-reactivity not only of humoral immunity but also of cellular immunity directed against these two types of hantavirus.

The 76-118 strain induced a higher CTL response than the B-1 strain (Table 4, Fig. 2). Stimulation in vitro and priming in vivo both appeared to be involved in this difference in CTL activity judging from the results of experiments on secondary stimulation with the heterologous strain (Table 5). However, replication of the 76-118 strain in nude mice was similar to that of the B-1 strain (Fig. 3) and the antibody responses against these two strains were not significantly different (Table 4). Moreover, for stimulation in vitro, in all experiments almost 100% of the macrophages were infected with virus. When macrophages were infected with these two strains and viral antigens were stained in the immunofluorescence test using MAbs, nucleocapsid protein was observed throughout the cytoplasm of cells infected with the 76-118 strain, and antigen was seen in the nuclei of cells infected with B-1 strain (Fig. 5). Viral antigens such as nucleocapsid or matrix proteins have been reported to be recognized by cytotoxic T cells (Townsend & Skehel, 1984; Townsend et al., 1984, 1985; Yewdell et al., 1985, 1986). Although it is not clear from our study, nucleocapsid protein might be expressed on the cell surface and thus be recognized by CTLs. This would explain why CTL activity was higher in 76-118-infected than in B-1-infected rodents. In general, the symptoms of patients with virus transmitted from
rats are mild, whereas those of patients with virus transmitted from A. agrarius are severe (Lähdevirta, 1982). At present it is not known whether this difference in cellular immunity on infection with the two strains is related to the difference in severity of hantavirus infection. This problem must be examined using other hantaviruses.

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