Role of T Lymphocyte Subsets in Protection and Recovery from Hantaan Virus Infection in Mice

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SUMMARY

Adult athymic nude (BALB/c background) mice or inbred BALB/c mice were inoculated intraperitoneally with Hantaan virus (HV), and attempts were made to isolate the virus from brain, lung and spleen. Virus was isolated from the organs of BALB/c mice for only a short time after infection but was isolated from various organs of nude mice consistently for at least 84 days after infection. Viral antigen was also detected in various organs of nude mice for a long time after infection. The effects of adoptive transfer of immune serum or immune T cells from BALB/c to nude mice before or after virus inoculation were examined. Before transfer, the T cell fraction was treated with complement (C') (group 1), anti-L3T4 + C' (group 2), anti-Lyt1.2 + C' (group 3) or anti-Lyt2.2 + C' (group 4). When transferred before virus inoculation to test the effects on protection against infection, immune serum and T cells of groups 1, 2 and 4 were effective. When transferred after HV inoculation to test the effects on clearance of virus, group 1 was the most effective followed by group 2. These results suggest that humoral and cellular immunity both have roles in protection against HV infection, and that T cells possessing L3T4−Lyt2+ markers on the cell-surface are especially important for elimination of infectious virus in vivo.

INTRODUCTION

Hantaan virus (HV) is the aetiological agent of Korean haemorrhagic fever (Lee et al., 1978), and is designated as the type member of the Hantavirus genus of the family Bunyaviridae from electron microscopical observations (White et al., 1982; McCormick et al., 1982; Hung et al., 1983, 1985) and RNA analysis (Schmaljohn et al., 1983a, b, 1985).

Several groups have attempted to infect various rodents with the virus (Lee, H. W. et al., 1981a, b; Lee, P. W. et al., 1981). Infection of newborn rodents with hantaviruses was fatal (Tsai et al., 1982; Kurata et al., 1983; Yamanouchi et al., 1984; Kim et al., 1985; Tanishita et al., 1986), but infection of adults with these viruses resulted in prolonged infection without any clinical symptoms (Lee, P. W. et al., 1981; Yanagihara et al., 1985a, b). Recently, T cell-mediated immunity was suggested to play a crucial role in resistance of mice to HV infection (Nakamura et al., 1985a, b). Immune T cells mediate many kinds of immunological responses, such as a cytotoxic T cell response, help in antibody production, induction of delayed type hypersensitivity reactions, and production of lymphokines including interferon. However, the mechanism of T cell-mediated immunity in HV infection is poorly understood.

In this paper, we describe persistent infection of congenitally athymic nude mice, but only
transient infection of immunocompetent BALB/c mice after HV inoculation. We found also that the persistent infection in nude mice could be prevented by transfer of immune serum or immune T cells from BALB/c mice. These results show that T cell-mediated immunity was essential for recovery from HV infection. To determine which subset of T lymphocytes was important for protection against HV and recovery from infection, we transferred immune T lymphocytes treated with complement (C') only, anti-L3T4 + C', anti-Lyt1 + C' or anti-Lyt2 + C' to nude mice before or after HV inoculation. The immune mechanisms of resistance against HV infection are discussed on the basis of these results.

METHODS

Mice. BALB/c mice and congenitally athymic nude (nu/nu) mice with a BALB/c background (all 6 weeks old) were obtained from CLEA Japan Inc.

Cells and viruses. HV (strain 76-118) was obtained from the American Type Culture Collection and passaged in Vero E6 cells. The virus titre was measured by the indirect immunofluorescent antibody (IFA) technique using monoclonal antibody clone 80A, which reacts with HV nucleocapsid protein (Yamanishi et al., 1984), and fluorescein isothiocyanate (FITC)-labelled goat antibodies to mouse IgG (Cappel Laboratories) as described previously (Yamanishi et al., 1983).

Virus isolation and detection of viral antigen in infected BALB/c mice and nude mice. HV (1 × 10^4 f.f.u./mouse) was inoculated intraperitoneally (i.p.) into BALB/c mice and nude mice. At various times from 1 to 84 days after infection, three BALB/c and three nude mice were sacrificed. The organs removed from the mice were homogenized in Eagle's MEM (2 ml/0.2 g of tissue) and centrifuged at 2000 g for 10 min to obtain 10% suspensions. Volumes of 100 μl of serial tenfold dilution of the suspension were inoculated onto monolayers of Vero E6 cells in eight-chamber plates (Miles Laboratories). After an absorption period of 2 h at 37°C in a CO₂ incubator, the monolayers were rinsed with MEM and then overlaid with MEM supplemented with 10% foetal calf serum (FCS) and 1.3% methyl cellulose, and incubated at 37°C in a CO₂ incubator for 3 days. The cells were then fixed and stained by the IFA technique with monoclonal antibody clone 80A, which reacts with HV nucleocapsid protein (Yamanishi et al., 1984), and anti-Thy-1.2 monoclonal antibody (MIH 010-220, Meiji Nyugyo Inc. Japan) + C', anti-L3T4 (YTS 191.1, Sera-Lab, Crawley Down, U.K.) + C', anti-Lyt1.2 (MIH 020-220, Meiji Nyugyo) + C' and anti-Lyt2.2 (MIH 040-440, Meiji Nyugyo) + C', respectively. For these treatments, 1.0 × 10^6 spleen cells were incubated with 1 ml of monoclonal anti-Thy-1.2 (diluted 1:1000), anti-mouse L3T4 (diluted 1:200), Lyt1.2 (diluted 1:500) or Lyt2.2 (diluted 1:2000) for 1 h at 4°C with occasional mixing. The cells were then washed and incubated in 10 ml of 10% dilution of C' for 1 h at 37°C with occasional mixing. After C' treatment the cells were washed and adjusted to numbers appropriate for adoptive transfer.

Preparation of splenocytes and immune serum, and adoptive transfer. Spleen cells, obtained from BALB/c mice 14 days after i.p. injection of 2 × 10^6 f.f.u. of HV, were treated with 0.83% NH₄Cl to remove erythrocytes, washed and resuspended in RPMI 1640 containing 10% heat-inactivated FCS. A non-immune spleen cell fraction was also obtained from non-immune BALB/c mice and treated in the same way as immune cells. The immune spleen cells were passed through a nylon wool column to obtain the immune T cell fraction. Contamination of this T cell fraction with B cells was 2 to 3% as determined by the IFA technique with FITC-labelled goat antibodies to mouse IgG. This T cell fraction was treated with rabbit C' (Low-Tox-M rabbit C' from Cedarlane Laboratories, Hornby, Ontario, Canada), anti-Thy-1.2 monoclonal antibody (MIH 010-220, Meiji Nyugyo Inc. Japan) + C', anti-L3T4 (YTS 191.1, Sera-Lab, Crawley Down, U.K.) + C', anti-Lyt1.2 (MIH 020-220, Meiji Nyugyo) + C' and anti-Lyt2.2 (MIH 040-440, Meiji Nyugyo) + C', respectively. For these treatments, 1.0 × 10^6 spleen cells were incubated with 1 ml of monoclonal anti-Thy-1.2 (diluted 1:1000), anti-mouse L3T4 (diluted 1:200), Lyt1.2 (diluted 1:500) or Lyt2.2 (diluted 1:2000) for 1 h at 4°C with occasional mixing. The cells were then washed and incubated in 10 ml of 10% dilution of C' for 1 h at 37°C with occasional mixing. After C' treatment the cells were washed and adjusted to numbers appropriate for adoptive transfer. The viability of the spleen cells after treatment with C', anti-Thy-1.2 + C', anti-L3T4 + C', anti-Lyt1.2 + C' and anti-Lyt2.2 + C' were 80%, 2%, 49%, 24% and 61% respectively, as judged by the trypan blue dye exclusion test. The purity of T cells after treatment in each group was determined by staining with homologous antibody by the IF technique and was more than 95%.

Anti-HV immune mouse serum was obtained from BALB/c mice that had been immunized with 2 × 10^6 f.f.u. of HV three times at 10 day intervals. The antibody titre against HV was 1:20480 by IFA and 1:160 by neutralization assay. Immune serum (300 μl/mouse) was injected intravenously (i.v.) into nude mice 1 day before or 10 days after virus inoculation.

Immune T cells treated with C', anti-L3T4 + C', anti-Lyt1.2 + C' or anti-Lyt2.2 + C' were injected into nude mice 1 day before or 10 days after i.p. inoculation of 1 × 10^6 f.f.u. HV; 1 × 10^7 viable T cells in 300 μl of RPMI 1640 supplemented with 10% FCS were injected i.v. Tissues from these nude mice were tested for virus infectivity and their sera were examined by IFA and neutralization assays.

Detection of serum antibodies. Serial twofold dilutions of sera were tested for class-specific antibodies to HV by the IFA technique. HV-infected A549 cells on spot slides served as antigen. FITC-labelled goat antibodies to mouse IgG or IgM (Cappel Laboratories) were used as secondary antibody. IFA titres were expressed as the reciprocal of the highest serum dilution giving specific fluorescence.

Neutralizing (NT) antibody titres were measured by the peroxidase-anti-peroxidase technique as described previously (Tanishita et al., 1984).
RESULTS

Virus isolation from various organs and distribution of viral antigen in HV-infected BALB/c and nude mice

BALB/c and nude mice were sacrificed on different days after virus inoculation for studies on isolation of virus from brain, lung and spleen. Virus was isolated only from lung and spleen of BALB/c mice and only on day 5. In contrast, it was consistently isolated from all these organs of nude mice for more than 84 days after its inoculation (Fig. 1).

In pathological studies, viral antigen was not detected in any organ of BALB/c mice, at any time after virus inoculation, but was consistently detected in the lung, spleen and brain of nude mice from 7 days after HV inoculation. None of the mice showed clinical signs and all remained healthy for the entire 84 day observation period. These data showed that nude mice were persistently infected with HV.

Serum antibody responses in BALB/c and nude mice

In BALB/c mice, IgM antibody was first detected by the IFA test as early as day 5 after infection, but in nude mice, it was first detected 10 days after infection. In both groups, IgM antibody became undetectable by day 28. IgG antibody appeared on day 7 in BALB/c mice and on day 10 in nude mice, and its titre increased to a maximum on day 14 in BALB/c mice and on day 56 in nude mice. In BALB/c mice, neutralizing antibody became detectable on day 5 reaching a maximum level on day 28. On the other hand, in nude mice, it became detectable at only low titre by day 14 and soon disappeared (Fig. 2).

Characterization of effective factors for protection and clearance

As described above, BALB/c mice recovered from HV infection, but nude mice became persistently infected. The persistent infection in nude mice was prevented by transfer of immune spleen cells from BALB/c mice (data not shown). Therefore, it was thought that immune T lymphocytes might be important for resistance against HV infection. To confirm this possibility, the T cell fraction was treated with C' or anti-Thy-1 + C', and adoptively transferred into nude mice. One day later, mice were inoculated with virus and virus isolation from lung, spleen and brain was attempted. As shown in Fig. 3, no virus was isolated from brain, lung and spleen of mice receiving the T cell fraction treated with C' only. On the other hand, there was no substantial difference in virus titres between the organs of mice treated with anti-Thy-1 and the organs of untreated mice. To determine which T cell subset was important for viral clearance and protection against HV infection, we tested the effects of transfer of immune T cells treated with C' only, anti-L3T4 + C', anti-Lyt1 + C' or anti-Lyt2 + C'. In addition, we transferred immune serum to examine whether anti-HV antibodies are also involved in viral clearance and protection.

Serum or spleen cells were transferred adoptively on nude mice 1 day before HV inoculation and the virus titres in the lung and spleen were measured. As shown in Fig. 4, the virus titres in the lung and spleen were greatly reduced by transfer of immune serum. In particular, no virus was detected in the lung after transfer of immune serum. The transfer of immune T cells treated with C', anti-L3T4 or anti-Lyt2 reduced the virus titres in the lung and spleen significantly; the C' treatment was the most effective. In contrast, the virus titres in the lung and spleen of nude mice that had received immune T cells treated with anti-Lyt1 were as high as those of mice that had not received a transfer.

The serum antibody titres of nude mice were measured 11 days after adoptive transfer (10 days after virus infection). As shown in Fig. 5, the serum NT and IgG antibody titres were higher after immune serum transfer than after transfer of T cells. In the groups that received T cells treated with C' or anti-Lyt2, the NT antibody and IgG antibody titres increased slightly, but in the group that received no transfer and in the groups that received T cells treated with anti-L3T4 or anti-Lyt1, NT antibody titres were undetectable (less than 1:5), and the titres of IgG antibody were low (1:20). IgM antibody was detectable in all mice.

When adoptive transfer was carried out 10 days after virus inoculation, immune serum
Fig. 1. Virus titres in (a) brain, (b) lung and (c) spleen of BALB/c (○) and nude (▲) mice. Mice received HV i.p. (1 × 10⁶ f.f.u./mouse) and were sacrificed on the indicated day. Each point represents the geometric mean titre of samples from three mice.

Fig. 2. (a) The NT antibody response; (b) IgM and (c) IgG antibodies detected by the IFA test in BALB/c (○) and nude (▲) mice. HV (1 × 10⁴ f.f.u.) was inoculated i.p. 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.

reduced the virus titres in brain, lung and spleen significantly, but had less effect than when transferred before virus inoculation. The transfer of immune T cells treated with C' reduced the virus titres in brain, lung, and spleen to almost zero. The virus titres in these organs were also decreased by transfer of immune T cells treated with anti-L3T4 or anti-Lyt2, but neither T cell fraction was sufficient to induce viral clearance, at least within 26 days after virus inoculation. Immune T cells treated with anti-L3T4 seemed more effective than those treated with anti-Lyt2. On the other hand, immune T cells treated with anti-Lyt1 did not reduce the virus titre in any organ (Fig. 6).

The serum antibody titres were measured 7 days and 16 days after adoptive transfer (17 days and 26 days respectively after virus infection). As shown in Fig. 7, in the groups that received immune T cells treated with C' or anti-Lyt2, the NT and IgG antibody titres increased significantly in 7 days and 16 days to titres of 1:5 and 1:1280, respectively. On the other hand, in the group without transfer and the groups that received immune T cells treated with anti-L3T4 or anti-Lyt1, no NT antibody was detectable and the IgG antibody titres were lower than in the groups that received T cells treated with C' or anti-Lyt2. After transfer of immune serum, the NT and IgG antibody titres decreased gradually during the 16 day observation period.

DISCUSSION

In this work, we demonstrated a difference in viral infections of immunocompetent BALB/c mice and athymic nude mice. HV became undetectable in all organs of BALB/c mice by 10 days after inoculation, but was consistently isolated from all organs of nude mice throughout the observation period (Fig. 1). However, the nude mice showed no clinical symptoms during the 84 day observation period. It has been reported that nude mice (14, 21, 28 and 42 days old)
Fig. 3. Effect of adoptive transfer of T cells. T cells were treated with C' only or anti-Thy-1 + C', and treated cells were transferred into nude mice. The mice received an i.p. inoculation of HV (1 × 10^4 f.f.u./mouse) the following day. Organs were removed 20 days after virus inoculation and virus titres were determined in (a) brain, (b) lung and (c) spleen. Columns represent mean titres of two mice.

Fig. 4. Effects of adoptive transfer of serum and T cells. Immune serum or 1 × 10^7 immune T lymphocytes treated with C', anti-L3T4 + C', anti-Lyt1 + C' or anti-Lyt2 + C' were transferred into nude mice 6 weeks old. The mice received an i.p. inoculation of HV (1 × 10^4 f.f.u./mouse) the following day. Organs were removed 10 days after virus inoculation and virus titres were determined in (a) lung and (b) spleen. Columns represent mean titres of two mice. The experiments were attempted twice and data are representative of two experiments.

Fig. 5. (a) Neutralizing antibody tests and (b) IgM and (c) IgG titres determined by IFA in nude mice 10 days after i.p. inoculation of 1 × 10^4 f.f.u. HV. The day before HV inoculation, the mice received an i.v. transfer of immune T lymphocytes or immune serum. Columns represent mean antibody titres of two mice.
Fig. 6. The effects of adoptive transfer of serum and T cells. Nude mice (6 weeks old) were treated i.p. with HV (1 × 10⁴ f.f.u./mouse), and 10 days later received an i.v. transfer of immune serum (●) or 1 × 10⁷ immune T lymphocytes treated with C' (○), anti-L3T4 + C' (△), anti-Lyt1 + C' (□) or anti-Lyt2 + C' (▲). Control values of untreated mice are also included (■). Points represent mean virus titres of two mice in (a) brain, (b) lung and (c) spleen. The experiments were done twice and data are a representative of these.

Fig. 7. (a) Neutralizing antibody tests and (b) IgM and (c) IgG titres determined by IFA in nude mice after i.p. inoculation of 1 × 10⁴ f.f.u. of HV. Immune serum (●), or immune T lymphocytes treated with C' (○), anti-L3T4 + C' (△), anti-Lyt1 + C' (□) or anti-Lyt2 + C' (▲) were transferred i.v. 10 days after HV inoculation. Control values of untreated mice are also included (■). Points represent mean antibody titres of two mice.
inoculated intracerebrally with HV showed clinical symptoms 40 days later and subsequently died (Nakamura et al., 1985a). This discrepancy might be explained by the different route of virus inoculation or the different viral titre inoculated.

As shown in Fig. 2, antibodies could be detected by IF tests in both nude mice and BALB/c mice. However, NT antibody was induced only transiently and at low titre in the nude mice, and so the virus could not be eliminated from these mice. This finding suggested that since antibodies against glycoproteins, such as neutralizing or haemaglutination inhibiting antibodies, are not induced in nude mice, T cells are necessary to induce these antibodies which are important for viral clearance.

To determine which subset of T lymphocytes was important for protection of mice against HV infection and recovery from infection, we transferred immune T lymphocytes treated with C' only, anti-L3T4 + C', anti-Lyt1 + C' or anti-Lyt2 + C' from BALB/c mice immunized with HV to nude mice before or after HV inoculation. We also transferred immune serum from BALB/c mice to nude mice. There are reports that resistance to various viral infections can be induced by transfer of antibodies (Rager-Zisman & Allison, 1973; Kohl et al., 1984), macrophages (Hirsch et al., 1970; Stohlman et al., 1980; Kohl et al., 1986) and T cells (Zinkernagel & Welsh, 1976; Nagafuchi et al., 1979; Kai et al., 1981; Jacoby et al., 1980; Nakamura et al., 1985b). There are also reports of separation of immune T cells with monoclonal antibodies of T cell surface markers and their transfer to infected animals. Some workers found that T cells possessing the Lyt1 marker were effective in inducing resistance to infectious viruses (Erle, 1981; Nagafuchi et al., 1982; Kyuwa & Fujiwara, 1984; Larsen et al., 1984; Kohl et al., 1986; Stohlman et al., 1986), whereas others reported the effectiveness against some viruses of T cells with the Lyt2 marker on their surface (Larsen et al., 1983; Byrne & Oldstone, 1984; Allan & Doherty, 1985). From these results, it seems that the effective immune mechanism is different depending on the virus. In our study, in the case of adoptive transfer before HV inoculation, T cells expressing either the L3T4 or Lyt2 marker were almost equally effective in protecting nude mice against HV infection. On the other hand, in the case of transfer after HV inoculation, L3T4 - T cells were more effective than Lyt2 - T cells for viral clearance. Mature T cells have been found to express either L3T4 antigen or Lyt2 antigen, but not both (Dialynas et al., 1983a, b). Moreover L3T4 + Lyt2 - T cells are restricted by major histocompatibility complex (MHC) class II antigens and seem to constitute the majority of helper/inducer cells, whereas L3T4 - Lyt2 + T cells recognize MHC class I antigens and apparently constitute the majority of cytotoxic/suppressor cells (Swain, 1981, 1983; Dialynas et al., 1983a; Wilde et al., 1983). Therefore, in our experiments, both helper/inducer T cells and cytotoxic/suppressor T cells were important for protection against HV infection. On the other hand, cytotoxic T cells are more important than helper/inducer T cells for clearance of virus. Although NT antibodies were induced in nude mice, after transfer of T cells treated with anti-Lyt2 antibody, no NT activity was found in mice after transfer of T cells treated with anti-L3T4. This finding supports the idea that antibody has some role in eliminating virus in the transfer of Lyt-2 T cells. In both protection and clearance experiments, T cells treated with C' were more effective than an equal number (1 x 10^7 cells/mouse) of L3T4 - T cells alone or Lyt2- T cells alone. Thus L3T4 + Lyt2 + T cells and L3T4 + Lyt2 - T cells may have cooperated in protecting the mice against virus infection and in clearance of HV. However, Lyt1 - T cells had no role in resistance against HV infection, as shown in Fig. 4 and 6. Previously, helper/inducer T cells were thought to express Lyt1 but not Lyt2, whereas cytotoxic/suppressor T cells express Lyt2 but not Lyt1 (Cantor & Boyse, 1976). However, more recent results showed that cytotoxic/suppressor T cells also have a little Lyt1 antigen, and all T cells express some Lyt1 antigen (Mathieson et al., 1979; Ledbetter et al., 1980). Therefore, in our study, treatment with anti-Lyt1 + C' probably killed not only helper/inducer T cells but also cytotoxic/suppressor T cells.

Immune serum from BALB/c mice also induced both protection and clearance, although it was more effective in protection. Since we used hyperimmune serum, it is uncertain whether immune serum acts naturally in vivo, as expected from our experiment. If so, vaccination against HV should be useful in protection against HV infection, provided the vaccine can induce a sufficiently high level of NT antibody.
In addition to the in vivo studies described in this paper, in vitro studies are needed to determine the exact mechanisms of resistance against HV infection.

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T cell subsets in Hantaan virus infection

1969


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