Interactions between equine herpesvirus type 1 and equine herpesvirus type 4: T cell responses in a murine infection model

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Interactions involving the immune responses to equine herpesvirus types 1 and 4 (EHV-1 and EHV-4) were studied in a murine infection model. When mice were inoculated intranasally with EHV-1, virus replication occurred in the respiratory tract and clinical signs were produced. In contrast, mice that were similarly inoculated with EHV-4 produced no evidence of virus replication and showed no clinical signs. When mice that had been inoculated with live EHV-4 were challenged 1 month later with EHV-1 they were partially protected. Although clinical signs were apparent on re-infection, virus replication in the respiratory tract was reduced in these mice compared with control mice that had not been previously immunized. Mice primed with heat-inactivated EHV-4, however, were not so protected. Live EHV-4-primed mice developed very low levels of antibody to EHV-1 and the humoral response could not account for this protection. However, the infected mice did give a strong delayed-type hypersensitivity reaction in a skin test using either EHV-1 or EHV-4 antigen. Spleen cells from EHV-4-primed donors provided a source of immune cells, including T cells which were used for transfer to recipient mice which were then challenged with EHV-1. The cells were protective; there was a reduction of virus replication on challenge with EHV-1 which correlated with the number of cells transferred. Modulation of the protective effect of primed cell populations was tested after depletion in vivo by means of complement-mediated lysis. The depletion of CD4-bearing cells produced the least effect on the protection afforded by cell transfer. In contrast, depletion of CD8-bearing cells markedly reduced the protection in recipients. EHV-1 and EHV-4 are widespread in horses and cross-infections are common. These results gained from a murine model indicate that important interactions occur at the level of T cell immunity between the two virus types which warrant further investigation in the natural host.

Introduction

Equine herpesvirus type 1 (EHV-1) and equine herpesvirus type 4 (EHV-4) are important causes of respiratory disease in horses world-wide (Allen & Bryans, 1986). The natural route of infection by these viruses is via the respiratory tract and EHV-4 causes disease which is normally restricted to this area. However, EHV-1 is also associated with neurological disease and abortion in mares. Serological studies have shown extensive cross-reaction exists between the two virus types (Fitzpatrick & Studdert, 1984; Crabb & Studdert, 1990). EHV-1 and EHV-4 glycoproteins share common antigenic sites; however, the serological cross-reactive response measured by neutralizing antibody or immunoprecipitation was shown recently to be relatively low in uncomplicated primary single infections in specific pathogen-free foals (Gibson et al., 1992; Tewari et al., 1993). Using the same experimental system, prior exposure to EHV-4 was found to confer partial protection against subsequent experimental challenge with EHV-1 (Tewari et al., 1993). Thus, the interactions between the two viruses during infection and the development of the immune responses are poorly understood. Commercial vaccines for EHV-1 derived from the homologous virus are available but have not been fully protective (Burrows et al., 1984; Allen & Bryans, 1986; Burki et al., 1990).

Intranasal (i.n.) inoculation of mice has been described as a model for EHV-1 infection (Awan et al., 1990; Field et al., 1991). EHV-1 replicates in, and causes damage to, the epithelial lining of the respiratory tract, producing clinical signs of respiratory disease. The possibility of using this model in the study of protective immunization against EHV-1 has been reported recently (Azmi & Field, 1993).

Mice that recovered from primary infection with EHV-1 were protected from subsequent challenge with a moderate dose of the same virus for up to 6 months (Awan et al., 1990). The antibody response to EHV-1 in this model following i.n. inoculation was relatively poor and neutralizing antibody was not detected in serum, implying that local responses or cellular immunity are likely to be more important factors in protection.

We have extended the study of protection to EHV-1
infection by testing the effects of a priming inoculation with the heterologous virus, EHV-4, either live or heat-inactivated. Although mice were found to be apparently non-permissive for EHV-4 replication, the results obtained in the present study provide strong evidence for the important role of cell-mediated responses in the induction of protective immunity to EHV-1 and also suggest a role for T cells in the induction of immune pathology.

Methods

Cells and viruses. EHV-1 strain AB4 (Awan et al., 1990) was grown in rabbit kidney (RK-13) cells, whereas EHV-4 strain 1778 (obtained from Dr J. Mumford, Animal Health Trust, Newmarket, U.K.) was grown in equine embryo lung (EEL) cells; in both cases the growth medium was Eagle’s MEM (EMEM) supplemented with 8% fetal calf serum. Cell cultures were maintained at 37 °C in a humidified incubator in an atmosphere of 5% CO₂.

Virus assay. EHV-1 and EHV-4 were titrated by plaque assay using 24-well plates of RK-13 and EEL cell monolayers respectively. To determine the growth of virus in mouse organs, mice were killed and tissue samples were collected in 1 ml EMEM. The samples were homogenized and clarified, and the suspension was 10-fold serially diluted. Virus titres were determined by plaque assay in RK-13 or EEL cells for EHV-1- and EHV-4-infected tissues respectively as described previously (Awan & Field, 1993); the results were expressed as the geometric mean (log₁₀ p.f.u./organ) obtained from four mice tested independently. The lower limit of detection was 10 p.f.u./organ.

Viraemia was detected by infectious centre assay using previously described methods (Slater et al., 1993).

Inoculation of mice with EHV-4 and -1. Four-week-old female BALB/c mice (Banti & Kingman) were i.n. inoculated with 5 × 10⁶ to 1 × 10⁷ p.f.u. live EHV-1, EHV-4, or a similar amount of heat-inactivated virus in 40 µl EMEM. Virus was inactivated by heating at 56 °C for 30 min and then the suspension was tested for the absence of surviving virus by plaque titration. Control mice were mock-infected with uninfected EEL cell lysate. Groups of four mice were killed on days 3 and 5 post-inoculation (p.i.) and tissues including lungs, turbinates, liver, spleen and brain were tested for the presence of virus by plaque titration as described above.

Four weeks after the primary inoculation, all mice were challenged by i.n. inoculation with 5 × 10⁸ p.f.u. EHV-1. The mice were killed on days 3 and 5 p.i. and nasal turbinate and lungs were tested for the presence of virus.

Delayed-type hypersensitivity (DTH) test. The antigen used in the DTH test was heat-inactivated virus (EHV-1 or -4). Virus was purified by means of 10 to 50% potassium tartrate density gradient centrifugation, then heated at 56 °C for 30 min. A skin test was carried out in mouse ear pinnae as follows. Firstly 20 µl of the inoculum, containing 10⁸ virus titres p.f.u. was inoculated intradermally into the left ear pinna of anaesthetized mice and then a similar quantity of uninfected EEL cell lysate suspension was inoculated at the same time into the right ear pinna as a control. Mice that had been primed with uninfected EEL cell lysate were inoculated with virus antigen as a further control. All DTH tests were carried out in quadruplicate. The ear skin thickness was measured at daily intervals with an engineer's micrometer screw gauge: the details of this technique have been described previously (Nash et al., 1980).

Adoptive transfer of spleen cells. Mice that had been i.n. inoculated 3 weeks previously with 5 × 10⁸ p.f.u. live EHV-4 (or a similar amount of heat-inactivated EHV-4 or with uninfected EEL cell lysate) were used to provide the donor cells. Spleens from groups of 15 mice were pooled and gently homogenized. The cell suspensions were filtered through fine sterile muslin gauze and centrifuged at 1500 r.p.m. for 10 min. The cells were treated with distilled water for 15 s to lyse erythrocytes. The osmotic balance was then restored in RPMI 1640 medium and the cells were washed twice. Approx. 5 ml samples of the cell suspensions (containing 10⁷ cells) were added and allowed to adhere to Petri dishes by incubation at 37 °C for 1 h. Non-adherent cells (mostly lymphocytes) were harvested and pooled. The cell suspensions were centrifuged at 1500 r.p.m. for 10 min and resuspended in a small volume of RPMI 1640 medium. The number of cells and their viability (normally > 95%) were determined by counting in the haemocytometer using the trypan blue exclusion method. The concentration of the cells was adjusted in RPMI 1640 as required for inoculation. Then 200 µl of the cell suspension was injected into the tail vein of recipient mice; later on the same day, within 4 h of cell transfer, all recipients were challenged by i.n. inoculation with EHV-1 at a dose of 5 × 10⁸ p.f.u./mouse.

Depletion of CD4 and CD8 T cells. Depletion of T cell populations was carried out in vivo by inoculation with monoclonal antibodies (MAbs) YTS 191 (anti-CD4) and YTS 169 (anti-CD8) according to the technique previously described (Cobbold et al., 1984). To study the effects of cell depletion on protective immunity, mice (4 weeks after immunization with EHV-4) were given 100 µg of the appropriate MAb intravenously (i.v.) 2 days before and 2 days after EHV-1 challenge. Samples of spleen cells from four mice given each single MAb or MAb combination were analysed by fluorescence-activated cell sorter (FACS) scanning. In non-depleted mice, the proportions of CD4⁺ and CD8⁺ were approx. 25% and 13% respectively. After administration of the appropriate antibody, satisfactory depletion of the relevant T cell populations was confirmed to the level of sensitivity of the assay (≤ 1/100 000 cells).

For adoptive transfer of spleen cells, the donor mice, immunized 3 weeks previously with live EHV-4, were given the same amount of each MAb and MAb combination were analysed by fluorescence-activated cell sorter (FACS) scanning. In non-depleted mice, the proportions of CD4⁺ and CD8⁺ were approx. 25% and 13% respectively. After administration of the appropriate antibody, satisfactory depletion of the relevant T cell populations was confirmed to the level of sensitivity of the assay (≤ 1/100 000 cells).

Results

Viral infectivity in lungs following i.n. inoculation with EHV-1 and -4

The pathogenesis of EHV-1 in mice following i.n. inoculation with doses of 1 × 10⁶ to 1 × 10⁷ p.f.u. EHV-1 (AB4) has been described (Awan et al., 1990; Field et al., 1991, 1993). Briefly, infected mice developed clinical signs, including ruffled hair, respiratory distress (dyspnoea and tachypnoea), weight loss and, in some cases, death. Virus was recovered from nasal turbinates and lungs from the time of infection for up to 8 days p.i. with maximum virus titres of approx. 1 × 10⁸ to 1 × 10⁹ p.f.u./organ and viraemia was detected during the acute disease. In contrast, i.n. inoculation with 1 × 10⁶ to 1 × 10⁷ p.f.u. EHV-4 produced no clinical signs and no virus was isolated from any of the organs normally infected following EHV-1 inoculation nor was viraemia detected.
EHV-1 and EHV-4 interactions

Fig. 1. Skin test for DTH response in EHV-4 primed mice. Mice were inoculated 3 weeks previously with $5 \times 10^6$ p.f.u. live EHV-4; control mice were similarly inoculated with uninfected cell lysate. The increase in ear thickness was measured on days 1 to 3 following inoculation with EHV-4 (■) or EHV-1 (△) antigens. Increase in ear thickness was also measured in control mice that were inoculated with EHV-4 (○) or EHV-1 (□) antigens. Bars represent s.d. of the mean from groups of four mice.

DTH response

Mice were immunized i.n. with live EHV-4. Four weeks later a skin test was carried out in the ear pinnae using EHV-1 or EHV-4 antigen to measure the DTH response. A swelling response of up to 0·37 mm was detected from 24 h.p.i. (Fig. 1). The response to the homologous antigen (EHV-4) was significantly higher than that to the heterologous (EHV-1) antigen, with a difference between the swelling responses to the antigens of 0·14 mm on day 2. Mice primed with heat-inactivated EHV-4 showed a quantitative lower swelling response to both EHV-4 and EHV-1 antigens with a maximum swelling of 0·27 mm and 0·20 mm respectively on day 1 (data not shown). A very low (< 0·08 mm) response to viral antigens was observed in control mice that had been primed with mock-infected cells. Swelling responses to EEL and RK-13 cell antigens in EHV-4-primed mice were also very low, giving an increase in ear thickness of < 0·05 mm.

Protection against EHV-1 challenge following i.n. immunization with EHV-4

Protection against EHV-1 infection was studied in mice that had been (i) immunized with live EHV-4, (ii) immunized with heat-inactivated EHV-4 or (iii) unimmunized by being given uninfected EEL cell lysate, the latter serving as controls. Following EHV-1 challenge, mice from all groups became ill as early as 1 day p.i. Typical clinical signs (described above) were observed in all cases. Separate cages of 10 mice were used to determine mortality which occurred in all experimental groups and 25 to 50% mice died within 8 days. Turbinates and lungs from groups of four mice were tested on days 3 and 5 p.i. for virus replication (Fig. 2).

Adoptive transfer of spleen cells

In order to investigate the ability of EHV-4-primed immune cells to clear EHV-1 from the site of infection, the mice primed with EHV-4 or uninfected EEL cell lysate were used as donors for the adoptive transfer of spleen cells. The number of spleen cells transferred was varied from $1 \times 10^7$ to $4 \times 10^7$ per recipient. Recipient mice gave a positive response in the DTH test. For $4 \times 10^7$ cells transferred, the increase in skin thickness was 0·38 ± 0·06 mm 48 h after EHV-1 antigen inoculation. This was similar to the maximum value observed in the standard test (Fig. 1). Following EHV-1 i.n. challenge inoculation (carried out on the day of cell transfer) all mice developed clinical signs and the mortality rate was approx. 30% in all groups irrespective of the priming inoculation. Turbinates and lungs from groups of four mice were tested for the presence of infectious virus on days 3 and 5 p.i. Virus was recovered from turbinates and lungs of all mice and the corresponding virus titres are shown (Fig. 3).
Fig. 3. Antiviral effects of spleen cell transfer from EHV-4-primed donors to recipient mice challenged with EHV-1. Donor mice were immunized 3 weeks previously with live EHV-4 (group A) or uninfected EEL cell lysate (group B) and then 1 x 10^7 (■) or 4 x 10^7 (□) spleen cells from group A or 4 x 10^7 (■) spleen cells from group B were transferred i.v. to recipient mice. All recipient mice were then challenged i.n. with 5 x 10^6 p.f.u. EHV-1. Data points represent the geometric mean virus titres/organ with s.D. in turbinates (left panel) and lungs (right panel) from groups of four mice and an asterisk denotes significantly different (P < 0.05) values.

In all cases the transfer of EHV-4-primed spleen cells resulted in reduced virus titres in both lungs and turbinates. When 1 x 10^7 cells were transferred, the observed reductions in virus titre were not significant in all cases at day 3 (Fig. 3); however, significant reductions (10^5- and 10^5-fold respectively) were observed in lungs and turbinates on day 5 p.i. in mice that received 4 x 10^7 spleen cells from live EHV-4-primed mice. No reductions in virus titre were observed, however, in mice receiving donor cells from heat-inactivated EHV-4-primed mice (data not shown).

Effect of in vivo depletion of CD4+ and/or CD8+ T cells

Mice were immunized by i.n. inoculation with live 1 x 10^7 p.f.u. EHV-4. Four weeks later they were challenged with 5 x 10^6 p.f.u. EHV-1. Two days before and 2 days after challenge they were inoculated with MAb to deplete CD4+ and/or CD8+ T cells.

The lungs and turbinates were tested, as above, on days 3 and 5 p.o.i. to determine the virus titres. The controls for this experiment were mice inoculated with uninfected cell lysate and mice immunized with EHV-4 but given no MAb. As shown previously (Fig. 2) the immunized mice given no depleting antibody showed similarly reduced virus titres which were approx. 10^5-fold lower than the titres observed in respiratory tissues of unimmunized mice. When MAb with the ability to deplete CD4+ cells were administered, this had a minimal effect on the reduction in virus titre observed in either tissue. However, when antibodies that deplete CD8+ or both CD8+ and CD4+ cells were employed, this markedly reduced the protective effect of immunization; virus titres were increased by approx. 10-fold toward the control values (Fig. 4). The depletion of T cells was also reflected in a reduction in the DTH response assessed by means of the skin test. The skin thickness increases observed 48 h after EHV-1 or -4 antigen inoculation are shown (Table 1).

Adoptive transfer of spleen cells with depletion of CD4+ and/or CD8+ T cells

In a final experiment, immunized MAb-treated mice were used as sources of donor spleen cells. These were given by adoptive transfer to test the ability of the depleted cell populations to protect recipient mice from EHV-1 challenge. In this case the recipient mice were given X-irradiation to avoid complicating immune
Table 1. The effect of T cell depletion on the DTH response to EHV-1 or -4 antigen measured by means of a skin test

<table>
<thead>
<tr>
<th>Status of mice</th>
<th>Depletion*</th>
<th>Skin thickness</th>
<th>Increase†</th>
</tr>
</thead>
<tbody>
<tr>
<td>EHV-4-primed</td>
<td>None</td>
<td>27 (3-6)</td>
<td>40 (3-7)</td>
</tr>
<tr>
<td>CD4</td>
<td>18 (4-3)</td>
<td>21 (2-3)</td>
<td></td>
</tr>
<tr>
<td>CD8</td>
<td>22 (4-3)</td>
<td>27 (4-3)</td>
<td></td>
</tr>
<tr>
<td>CD4 and CD8</td>
<td>8 (2-9)</td>
<td>9 (2-5)</td>
<td></td>
</tr>
<tr>
<td>Unprimed or cell lysate</td>
<td>None</td>
<td>≤ 8.0</td>
<td>≤ 8.0</td>
</tr>
</tbody>
</table>

* MAb regimen designed to deplete the cell types indicated.  † Mean skin thickness (S.E.M.) x 10^-3 mm, measured 48 h after EHV-1 or -4 antigen inoculation.

Discussion

The most important findings in this paper are as follows. (i) Exposure of mice to EHV-4 did not produce detectable virus replication in respiratory or any other tissue. Such exposure, however, conferred protective immunity to challenge with the heterologous virus, EHV-1. (ii) The observed protection appeared to be dependent on cell-mediated responses rather than on antibody. (iii) Evidence was obtained which suggested that CD8+ T cells have an important role in exerting the protective effect. (iv) The protective effects measured by reductions in virus titre were not necessarily reflected in reduction in clinical signs and mortality. Although histological findings are not described here, our preliminary observations suggest that the immunizing infection with EHV-4 also resulted in sensitizing mice such that damaging responses to challenge infection contributed to lung pathology and resulted in clinical signs.

We have shown previously that a sublethal i.n. dose of EHV-1 induces a state of protective immunity to re-infection lasting several months (Azmi & Field, 1993). When the priming virus was given by the i.v. route the resultant protection was much less despite higher serum antibody levels (Field et al., 1993). Similar findings were reported for influenza A virus in mice where i.n. inoculation with vaccinia recombinant influenza virus induced protection to challenge with influenza A virus infection (Small et al., 1985; Taylor et al., 1991). Transferred immune cells were found to clear the virus responses, hence the virus replication data are not directly comparable with those of the previous experiments.

When cells were transferred from EHV-4-immunized mice that had not been treated with MAb, clear reductions in virus titre were observed as shown previously. The reductions were 10^8- and 10^6-fold on days 3 and 5 p.i. respectively in turbinates (Fig. 5a) and 10^7- and 10^5-fold in lungs (Fig. 5b) on the respective days.

Following transfer of spleen cells from antibody-treated immunized mice, little change was observed in the virus titres in the turbinate samples although the turbinates from mice that received cells from the depleted donors showed a trend to more virus irrespective of the MAb employed (i.e. the titres were less reduced compared with mice that received undepleted immunized donor cells). In the lungs (Fig. 5b), a significant loss of protection was observed in mice that received donor cells from which CD8+ cells or both CD4+ and CD8+ cells had been removed. Depletion of CD4+ cells alone, however, appeared to have much less effect on virus reduction produced by the transfer of immune cells.
from the lung more effectively than from the nasal turbinate. This leaves open the possibility that local immunity in the upper respiratory tract may have an important role in protection in this site. The potential role of IgA in exerting antiviral effects at the upper respiratory sites as shown for influenza virus (Liew et al., 1984) will be the subject of future investigation in the murine EHV-1 model.

In contrast to EHV-1, EHV-4 was found to be apathogenic in BALB/c mice. This is consistent with many previous reports that EHV-4 has a much narrower host range than EHV-1 both in tissue culture and experimental animals (Yeaggar et al., 1985). Following i.n. inoculation of mice there was no evidence of virus replication and we were not able to determine the precise fate of EHV-4 in relation to stimulation of immune responses. It was of interest that heat-inactivated EHV-1 given by the same route also failed to induce protective immunity (Field et al., 1993), and u.v.-inactivated EHV-1 given by i.n. inoculation was also not protective (M. Azmi & H. J. Field, unpublished). The mechanisms by which the antigens of the live virus are processed and presented to the immune system are unknown. Following hyperimmunization of mice (three subcutaneous injections of purified virus with complete and incomplete Freund’s adjuvant) we have detected antibody to EHV-4 which cross-reacts with EHV-1. However, our previous results suggest that the serum antibody response is a less important mechanism of protection in this model (Field et al., 1993). Moreover, cell-mediated immunity has already been shown to be the dominant mechanism of protection following EHV-1 priming (Azmi & Field, 1993). Elimination of virus-infected cells mediated effectively by class I major histocompatibility complex (MHC)-restricted CD8+ effector T cells (cytotoxic lymphocytes) has been reported for other diseases (Koszinowski et al., 1991). Class II MHC-restricted CD4+ helper cells modulate T cell responses through their lymphokines, such as interleukin 2 and γ-interferon (Doherty, 1985; reviewed by Nasir & Cambouropoulos, 1993). In the present study, the prominent role of T cells is shown by means of a DTH test, adoptive transfer of spleen cells and depletion of CD4+ or CD8+ T cells.

Those mice which had been immunized 4 weeks previously with EHV-4 responded strongly to both EHV-1 and -4 antigens in the skin test for DTH although the response to the heterologous antigen was approx. 50% less. A potential problem with all the experimental work was the possibility that responses to cellular antigens contained in the virus inoculum may interfere with the specificity of the results. In practice, no evidence for this was observed. Indeed the DTH response to cellular antigens was found to be very low, confirming the specificity of the test for virus. The DTH response observed in mice immunized with heat-inactivated EHV-4 was significantly lower and these mice were not protected from challenge with EHV-1.

Notwithstanding the fact that mice immunized with live EHV-4 showed protection against EHV-1 challenge in terms of virus replication in the respiratory tract, this did not prevent the development of clinical signs of disease. Histological findings in the lung are not the subject of this manuscript, but the lungs of the immunized mice were found to be markedly consolidated following challenge. This suggests that the protective responses were also associated with immunopathology which may differ from the direct pathology that results from virus replication in the respiratory tract during the primary virus inoculation. Immunopathology in the lung could occur by two mechanisms, primary infiltration of cytotoxic lymphocytes into the lung in response to infected cells bearing EHV-1 antigens or secondary infiltration of leukocytes attracted by cytokines produced by CD4+ or CD8+ cells that have been focused to the antigen-containing tissue. Both mechanisms may occur simultaneously. CD4+ cells may have a more important role in mediating these effects. The distinct patterns of protective and damaging responses in mice immunized against respiratory syncytial virus have been studied in some detail by Openshaw and colleagues (Alwan & Openshaw, 1993) and these hypotheses are currently the subject of further investigation.

Experiments in which lymphocyte subpopulations were depleted provided direct evidence that T cells account for the protective effects and that CD8+ cells are most important in mounting the protective effects. In turn, this suggests that cytotoxic lymphocytes are most likely to account for protection. Experiments are in hand to confirm the activity of cytotoxic lymphocyte in a functional assay. Nevertheless, our results suggest that complete protection requires both CD4+ and CD8+ T cells, and this has been reported for other viral diseases (Leung & Ada, 1982; Hom et al., 1991; Jennings et al., 1991). Further circumstantial evidence for the essential role of T cells comes from the observation that suppression of T cells with cyclosporin A treatment results in a heightened and much protracted phase of acute EHV-1 replication in the mouse (M. Azmi & H. J. Field, unpublished).

The sera of specific pathogen-free foals that had been previously exposed to single experimental infection with EHV-1 or -4 demonstrated only weak cross-reaction in tests for neutralizing antibody (Gibson et al., 1992). However, little data have yet been published on T cell cross-reactive responses to EHV-1 in the natural host. Nevertheless, we have demonstrated in the murine model that EHV-1 and -4 are cross-reactive at the level of T cell immunity, by means of a DTH test and adoptive cell
transfer. As shown previously for influenza virus (Townsend et al., 1985, 1986), EHV-1 and -4 may share internal proteins that elicit the T cell responses which induce protection. Stimulation of T$_{H1}$ cells by viral internal proteins probably account for the DTH response as shown for other related viruses (Leung & Ada, 1980; Nash et al., 1980).

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References


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