Enhancement of Antibody Formation against Herpes Simplex Virus in Mice by the T-Cell Mitogen Bestatin

By ANDREAS KNOBLICH, WERNER E. G. MÜLLER, VERONIKA HARLE-GRUPP AND DIETRICH FALKE*

Institut für Medizinische Mikrobiologie, Abteilung für Experimentelle Virologie and 1 Institut für Physiologische Chemie, Abteilung 'Angewandte Molekularbiologie', Johannes-Gutenberg-Universität, 6500 Mainz, Federal Republic of Germany

(Accepted 27 June 1984)

SUMMARY

The influence of the T-lymphocyte-stimulating dipeptide bestatin on the induction of neutralizing antibodies against herpes simplex virus type 1 in the mouse was investigated. Dose-response experiments revealed two active ranges from 1 ng/kg to 100 ng/kg and from 10 μg/kg to 10 mg/kg or more. Bestatin (10 mg/kg) enhanced antibody levels after primary infection, if injected between day 5 and day 8 after infection with a maximum effect at day 5. Following secondary infections, bestatin was most effective at day 1 after secondary infection. Moreover, the antibody-generating potency of a formalinized herpes simplex virus type 1 vaccine was elevated considerably. Bestatin and silica seemed to be effective systemically. Treatment of mice with silica before virus infection and additionally with bestatin at day 1 after infection resulted in an additive effect on antibody production. Comparable effects could be obtained when polynosinic acid·polycytidylic acid or indomethacin was combined with bestatin at day 1. It was assumed that certain factors released by macrophages 'sensitize' the antibody-producing system for the enhancing activity of bestatin at day 1. Indeed, culture fluids of macrophages obtained from mice either pretreated with silica or infected by herpes simplex virus were active in enhancing antibody formation upon injection into mice at day 1 in combination with bestatin. Bestatin did not induce interferon activity. No influence of bestatin on the virus content of organs or on mortality was observed.

INTRODUCTION

The development of safe and effective vaccines against certain virus diseases is one of the main tasks of virology. To achieve better protection it is possible either to use higher doses of antigen or to potentiate the efficacy of immunization by the use of non-specific adjuvants or by drugs known specifically to stimulate certain compartments of the immune system.

Antibody production against herpes simplex virus (HSV) by B-lymphocytes is governed by T-helper lymphocytes (Burns et al., 1975). The interaction of T-cells with Ia⁺ antigen-presenting cells results in secretion of II-1 and subsequently in clonal proliferation of T-cells. These cells in turn are able to activate resting B-cells to proliferate and to produce antibodies.

Bestatin is a chemically well-characterized microbial product, [(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine; mol. wt. 309 (Nishizawa et al., 1977), which has been shown to enhance immune responses both in vitro and in vivo (Müller, 1981; Nishizawa et al., 1977; Saito et al., 1977; Umezawa et al., 1976a). In aged mice, immune responses are restored by bestatin (Bruley-Rosset et al., 1979). Moreover, bestatin appears to exhibit antitumour effects both in animals and in humans (Ishikawa et al., 1978; Ishizuka et al., 1980a; Umezawa, 1981).

Recently, the mode of action of bestatin was investigated more thoroughly. It is a strong inhibitor of the aminopeptidase B and leucine aminopeptidase present on the surface of mammalian cells (Suda et al., 1976; Umezawa et al., 1976b). Ishizuka et al. (1980b) have
demonstrated that bestatin stimulates T-cells through the activation of macrophages. Bestatin enhances the activity of DNA polymerase \( \alpha \) and of the terminal deoxynucleotidyl transferase in murine T-cells (Müller et al., 1979). Bestatin binds to macrophages five times more strongly than to T-cells, whereas B-cells bind only tiny amounts (Müller et al., 1982).

Our present work refers to the question of whether bestatin influences antibody formation following primary and secondary HSV infections of mice. Further experiments were carried out to elucidate the most effective regimen of bestatin administration and to see whether there is an effect on antibody formation after immunization with a formol-inactivated HSV-1 vaccine.

**METHODS**

**Cells and viruses.** Titrations and neutralization tests were done on confluent monolayers of either secondary rabbit kidney cells or 2-day-old Vero cells essentially as described earlier. For most experiments, the Lennette strain (Len) of HSV-1 was used (Knoblich et al., 1983). Some experiments required the use of a formol-inactivated HSV-1 vaccine prepared from primary rabbit kidney cells infected with three different strains of HSV-1 (Andonov et al., 1978); its protein content was 300 \( \mu \)g in 0.2 ml.

**Mice.** NMRI outbred mice were purchased from Ivanovas (Kislegg/Allgäu, F.R.G.). When used for experiments, mice were 5 to 7 weeks of age. For most experiments, female mice were used because of their stronger antibody response (Knoblich et al., 1983).

**General experimental design.** Female or male mice (five animals per group) were infected with \( 3 \times 10^3 \) p.f.u. HSV-1 (Len) or immunized with the vaccine. Infection was done intraperitoneally (i.p.) or into the hind footpad. Twenty-one days after infection, animals were bled retroorbitally under ether anaesthesia. Blood was allowed to clot overnight at 4 °C and centrifuged afterwards. Sera were stored at -20 °C separately for each mouse until use.

**Neutralization test.** Titres of neutralizing antibodies were determined by a 50% endpoint method as described earlier (Knoblich et al., 1983). Briefly, diluted sera were mixed with a virus suspension (30 to 50 p.f.u. per dilution). After incubation for 1 h at 37 °C, samples of this mixture were pipetted onto cell monolayers in roller tubes. Virus that had not been neutralized by antibodies was allowed to adsorb. Two days later, plaques were counted and the titres were calculated in relation to the virus control. The results are expressed as average values ± standard deviation. Statistical evaluation was done by Student's t-test (\( P < 0.05 \)). Results from representative experiments are presented.

**Drugs.** Bestatin was kindly provided by H. Umezawa, Institute of Microbial Chemistry, Research Foundation, Tokyo, Japan. The compound was dissolved in basal minimum essential medium (BME). Bestatin was used, if not otherwise stated, at a concentration of 10 mg/kg i.p. Amorphous silica was purchased from Sigma. Silica was suspended in Hanks' solution at 60 mg/ml. The particle size was 0.5 to 10 \( \mu \)m. The interferon inducer polyinosinic acid-polycytidylic acid [poly-I:C(LC)] was kindly provided by Dr H.B. Levy (NIH, Bethesda, Md., U.S.A.). The compound was diluted in Hanks' solution. Indomethacin obtained in saline at alkaline pH.

**Interferon assay.** Interferon activity in serum specimens was tested on secondary mouse embryo fibroblasts (MEF) of BALB/c mice. 1.5 × 10^5 MEF were seeded into 24-well plates (Nunc). One day after seeding, 0.5 ml of dilutions of active mouse serum were added to each well. After incubation overnight at 37 °C in a CO, incubator, the fluid was washed off and the wells were infected with vesicular stomatitis virus (Indiana serotype) at 20 to 50 p.f.u. per well. After 2 h incubation at 37 °C, the non-adsorbed virus was washed off and the cells were overlayed with agar medium. The results were read 48 h after infection. As a positive interferon control we used serum from mice injected with 100 \( \mu \)g poly-I:C(LC)/mouse 1 day before bleeding.

**Anti-sheep red blood cell (SRBC) haemolysin assay.** SRBC in Alsever's solution obtained from Biologische Arbeitsgemeinschaft (Lich, F.R.G.) were washed three times in veronal-buffered saline (VBS) [5 mm-barbitone sodium, 142 mm-NaCl, 3.55 mm-CaCl\(_2\), 50 mm-MgCl\(_2\), 6 H\(_2\)O in distilled water made to pH 7.4 with HCl and containing 1 mg gelatin per l (Serva, Heidelberg, F.R.G.)]. A 5% solution of SRBC in VBS was prepared and filtered through a cotton-wool layer to remove aggregates. Guinea-pig serum absorbed three times with SRBC served as complement source and was diluted 1:15.

Serial twofold dilutions of sera to be tested (250 \( \mu \)l) were mixed with the same volume of 5% SRBC solution and incubated at 37 °C for 15 min. After addition of 125 \( \mu \)l guinea-pig complement, incubation was continued for a further 15 min exactly. Then the lysis reaction was stopped by addition of 125 \( \mu \)l ice-cold citrate saline (15 mm-sodium citrate, 120 mm-NaCl). SRBC that had not been lysed were pelleted and the haemoglobin concentration in the supernatant was determined photometrically at 540 nm. Zero % lysis controls (250 \( \mu \)l 5% SRBC, 125 \( \mu \)l complement, 1500 \( \mu \)l citrate saline) and 100% lysis controls (250 \( \mu \)l 5% SRBC, 1625 \( \mu \)l 0.1% Na\(_2\)CO\(_3\)) were included for each test. Titres were calculated as for neutralizing antibody as the dilution of serum lysing 50% of the SRBC. Statistical evaluation was done by Student's t-test (\( P < 0.05 \)).
Enhancement of antibody formation by bestatin

RESULTS

Enhancement of antibody formation by bestatin after primary infection with HSV-1

In order to elucidate whether bestatin is able to enhance antibody formation following primary HSV-1 infection, 10 mg/kg of this drug were injected i.p. into groups of female and male mice at various times before and after infection with HSV-1.

From Fig. 1 it can be seen that bestatin significantly enhanced antibody levels determined at day 21 p.i., if injected once between days 5 and 8 after infection. The greatest increase was achieved by injection 5 days after infection. Bestatin was not effective when administered earlier than day 5 or later than day 8 after infection. In our system antibody production can be detected from day 5 onwards (Knoblich et al., 1983).

To find the time at which the amplification of antibody synthesis by bestatin becomes detectable, female NMRI mice were infected i.p. with HSV-1. Five days after infection, half of the animals received bestatin (10 mg/kg) i.p.; the others served as controls. Groups of control and bestatin-treated mice were bled daily from day 5 onwards.

Fig. 2 shows antibody levels in bestatin-treated mice to be always somewhat higher than in control animals. Later than day 12 after infection there was a strong and significant increase in antibody titres in the bestatin-treated mice compared to the non-treated mice.

The following set of experiments was done to analyse whether the bestatin-induced increase of antibody formation is dose-dependent. As can be seen from Fig. 3, a moderate increase of antibody levels was achieved by administration of as little as 1 ng/kg bestatin. Consistently, significantly higher levels of neutralizing antibodies could be produced using doses above 10 μg/kg. Thus, there seem to exist two effective dose ranges of bestatin, yielding two ranges of increased antibody levels. No toxic effects have been reported after injection of up to 1·2 mg/mouse (Ishizuka et al., 1981).
Fig. 2. Influence of bestatin on the development of neutralizing antibody levels after infection. Two groups of female NMRI mice were infected i.p. with HSV-1 (3 × 10³ p.f.u.). Five days later, the animals of one group received a single i.p. dose of bestatin (10 mg/kg) (▲), the other group served as controls (●). Sera were taken at the days indicated on the abscissa.

Fig. 3. Dose-dependence of the bestatin-induced increase of neutralizing antibody formation. Female NMRI mice were infected i.p. with HSV-1 (3 × 10³ p.f.u.). Five days after infection, mice of the respective groups received a single dose of bestatin (100 fg to 10 mg/kg) i.p. Sera were taken 21 days after infection.
Enhancement of antibody formation by bestatin

Fig. 4. Influence of bestatin on neutralizing antibody formation following a second i.p. infection (†, 3 × 10^3 p.f.u.) with HSV-1. Three weeks after primary i.p. infection with HSV-1, female (a) and male (b) NMRI mice were re-inoculated i.p. with 3 × 10^3 p.f.u. of HSV. At the days indicated on the abscissa, mice of one group received a single dose of bestatin (10 mg/kg) i.p. Sera were taken 21 days after secondary infection. Mice infected with HSV-1 twice but which did not receive bestatin at any time served as controls (□, ■).

Influence of bestatin on the secondary antibody response to HSV-1

To elucidate whether bestatin is also able to influence antibody levels in secondary infections, female and male NMRI mice were re-infected i.p. 3 weeks after primary infection with the same dose of HSV-1. As shown in Fig. 4, bestatin was also effective in secondary infections. In contrast to the effect on the primary response, however, the enhancement of a secondary anti-HSV antibody response was most efficient if bestatin was given 1 day after secondary infection and not at day 5 as found for the primary response.

Influence of silica on the bestatin-induced enhancement of antibody formation

Macrophages are involved in initiation and regulation of immune responses. They are thought to mediate the bestatin-induced stimulation of T-lymphocytes (Ishizuaka et al., 1980b). Treatment of mice with silica results in destruction of macrophages thereby reducing the resistance of mice to infection with HSV-1 (Zisman et al., 1970) and enhancing the antibody response (Knoblich et al., 1983). We therefore tested the influence of silica on the effects achieved by treatment with bestatin.

From Fig. 5 it can be seen that after i.p. pretreatment with silica, antibody levels were increased both in bestatin-treated and in control mice. Compared to mice that did not receive silica, bestatin given i.p. was very effective at only 1 day after infection in the silica-treated group.

Table 1 shows the influence of bestatin on the antibody response generated by injection of a formalin-inactivated HSV-1 vaccine (Andonov et al., 1978). Clearly, bestatin enhanced antibody titres after immunization with an inactivated HSV-1 vaccine. This effect was achieved after administration of bestatin at day 5 but not at day 1 after infection. Similarly, after pretreatment with silica (4 h before infection) a significant increase of antibody levels could be seen (see also Fig. 5). A combination of silica (−4 h) and of bestatin given at day 5 increased antibody production. As would be expected from Fig. 5, the combination of silica (−4 h) and administration of bestatin at day 1 post-infection resulted in antibody levels higher than that effected by either substance alone.
Female NMRI mice were infected i.p. with HSV-1 (3 × 10^3 p.f.u.; †, day of infection). Four hours before infection, some groups received silica (1 g/kg mouse i.p.) (II), and on the day indicated on the abscissa silica-treated (II) and non-treated (A) mice were given a single dose i.p. of bestatin (10 mg/kg). Sera were taken 21 days after infection and assayed for neutralizing antibody.

Table 1  
Influence of silica and bestatin on antibody formation after injection of an HSV vaccine

<table>
<thead>
<tr>
<th>Treatment and time</th>
<th>Neutralizing antibody titre†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine control</td>
<td>48 ± 17</td>
</tr>
<tr>
<td>+ silica −4 hours</td>
<td>178 ± 71‡</td>
</tr>
<tr>
<td>+ bestatin + day 1</td>
<td>70 ± 31§</td>
</tr>
<tr>
<td>+ bestatin + day 5</td>
<td>230 ± 57‡</td>
</tr>
<tr>
<td>+ bestatin + day 1</td>
<td>232 ± 56‡§</td>
</tr>
<tr>
<td>+ silica −4 hours</td>
<td>294 ± 111‡</td>
</tr>
</tbody>
</table>

* Animals received silica (1 g/kg mouse) i.p. and/or bestatin (10 mg/kg) i.p. at the hours or day before (−) or after (+) injection of a formalin-inactivated HSV-1 vaccine. Female NMRI mice were used at 4 to 6 weeks old; five mice per group.
† 21 days after infection.
‡ Significant difference compared to control.
§ Significant difference between the pair labelled thus (Student’s t-test, P < 0.05).

Enhancing activity of macrophage culture fluids on antibody formation

The rationale of our protocol was that pretreatment of macrophages in vivo by silica and the consequences of i.p. HSV infection might stimulate macrophages to produce substances in vitro which in turn could be able to sensitize the antibody-producing system to the amplifying potency of bestatin upon re-injection into mice. Indeed, this seemed to be the case.

It was seen (Table 2) that the supernatants of macrophages pretreated in vivo with silica or with HSV exerted a slight antibody-enhancing activity. Culture fluids from macrophages, the
Enhancement of antibody formation by bestatin

Table 2. Changes in time dependence of the bestatin-mediated increase of anti-HSV antibody levels after application of mouse peritoneal macrophage culture fluids

<table>
<thead>
<tr>
<th>Treatment of HSV-1-infected mice*</th>
<th>Antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no additional treatment)</td>
<td>205 ± 45</td>
</tr>
<tr>
<td>Bestatin day 1 p.i.</td>
<td>198 ± 53</td>
</tr>
<tr>
<td>Bestatin day 5 p.i.</td>
<td>826 ± 138†</td>
</tr>
<tr>
<td>CF resident</td>
<td>228 ± 53</td>
</tr>
<tr>
<td>CF silica</td>
<td>462 ± 194†</td>
</tr>
<tr>
<td>CF HSV-1 (day 1)</td>
<td>303 ± 121†‡</td>
</tr>
<tr>
<td>CF HSV-1 (day 3)</td>
<td>428 ± 377†§</td>
</tr>
<tr>
<td>CF HSV-1 (day 5)</td>
<td>313 ± 69†</td>
</tr>
<tr>
<td>Bestatin day 1 p.i. + CF resident</td>
<td>226 ± 85</td>
</tr>
<tr>
<td>Bestatin day 1 p.i. + CF silica</td>
<td>333 ± 377†</td>
</tr>
<tr>
<td>Bestatin day 1 p.i. + CF HSV-1 (day 1)</td>
<td>562 ± 79†‡</td>
</tr>
<tr>
<td>Bestatin day 1 p.i. + CF HSV-1 (day 3)</td>
<td>234 ± 46§</td>
</tr>
<tr>
<td>Bestatin day 1 p.i. + CF HSV-1 (day 5)</td>
<td>332 ± 193</td>
</tr>
</tbody>
</table>

* Female NMRI mice were infected i.p. with 3 × 10^3 p.f.u. HSV-1. Some experimental groups received 10 mg/kg bestatin i.p. as indicated. Two h post-infection (p.i.) several groups were injected with cell-free 24 h culture fluids (CF) of mouse peritoneal macrophages (1·5 × 10^7 adherent peritoneal macrophages in 10 ml basal Eagle’s medium; 2 ml per mouse). These culture supernatants were taken either from resident macrophages (CF resident), from macrophages the donors of which had been treated with silica 1 g/kg i.p. on the day before rinsing the peritoneal cavity (CF silica) or from mice where the macrophages were harvested 1, 3 or 5 days after i.p. infection with 3 × 10^3 p.f.u. HSV-1 (Len) (CF HSV-1 day 1/day 3/day 5). Three weeks after infection, animals were bled.
† Significant difference compared to control.
‡, § Significant difference between the pairs labelled thus (Student’s t-test, P < 0.05).
I Significant difference compared to bestatin day 1 p.i.

Table 3. Influence of poly-I:C(LC) or of indomethacin on the bestatin-mediated increase of HSV-induced antibody levels*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Len i.p.</td>
<td>271 ± 46</td>
</tr>
<tr>
<td>Len i.p. + bestatin, day 1 p.i.</td>
<td>221 ± 48</td>
</tr>
<tr>
<td>+ bestatin, day 5 p.i.</td>
<td>908 ± 173†‡</td>
</tr>
<tr>
<td>+ poly-I:C(LC), day 1 p.i.</td>
<td>345 ± 93†‡§</td>
</tr>
<tr>
<td>+ poly-I:C(LC), day 5 p.i.</td>
<td>562 ± 162†‡‖</td>
</tr>
<tr>
<td>+ poly-I:C(LC), day 1 p.i. + bestatin, day 1 p.i.</td>
<td>787 ± 197†‡§</td>
</tr>
<tr>
<td>+ poly-I:C(LC), day 5 p.i. + bestatin, day 5 p.i.</td>
<td>827 ± 92‖</td>
</tr>
<tr>
<td>+ indomethacin, day 1 p.i.</td>
<td>345 ± 93‡§</td>
</tr>
<tr>
<td>+ indomethacin, day 1 p.i. + bestatin, day 1 p.i.</td>
<td>621 ± 134‡§</td>
</tr>
</tbody>
</table>

* Female NMRI mice were challenged i.p. with 3 × 10^3 p.f.u. HSV-1 (Len). At the time post-infection (p.i.) indicated, they were injected with bestatin (10 mg/kg, intramuscularly to avoid local interactions with the second compound), indomethacin (10 mg/kg i.p.) or poly-I:C(LC) (8 mg/kg i.p.). Mice were bled 21 days after infection.
† Significant difference compared to control.
‡, § Significant difference between the pairs labelled thus.
I, ‖ Significant difference compared to bestatin day 1 p.i.

Donors of which had been pretreated with silica or had been infected with HSV-1 on the day before peritoneal cell harvest rendered the antibody-producing system susceptible to the action of bestatin at day 1 post-infection. In contrast, supernatants from macrophages obtained 3 or 5 days after infection of mice with HSV failed to do so. However, the active supernatants did not seem to contain all the factors for optimal sensitization of the antibody-producing system, because the titres they generated in combination with bestatin at day 1 were still lower than those induced by bestatin at day 5. The culture supernatants of resident macrophages in these experiments did not depress antibody formation, as shown in earlier studies (Knoblich et al., 1983).
Table 4. Influence of application of bestatin by different routes on antibody formation after infection into footpad*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Bestatin†</th>
<th>Antibody titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plantar</td>
<td>–</td>
<td>113 ± 16</td>
</tr>
<tr>
<td>Plantar</td>
<td>Day 1 i.p.</td>
<td>111 ± 46</td>
</tr>
<tr>
<td>Plantar</td>
<td>Day 5 i.p.</td>
<td>419 ± 71§</td>
</tr>
<tr>
<td>Plantar</td>
<td>Day 5 plantar</td>
<td>246 ± 68§</td>
</tr>
</tbody>
</table>

* Female NMRI mice (4 to 6 weeks old), five mice per group.
† Bestatin (10 mg/kg) was injected 1 or 5 days after infection. Inoculum of HSV: 3 × 10^3 p.f.u. into the footpad.
§ Significant difference compared to control.
§§ Significant difference between the pairs labelled thus.

Table 5. Influence of silica and bestatin administration on anti-SRBC haemolysin titres*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Haemolysin titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12 ± 8</td>
</tr>
<tr>
<td>Bestatin day 1 p.i.</td>
<td>26 ± 14†</td>
</tr>
<tr>
<td>Bestatin day 5 p.i.</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>Silica (4 h)</td>
<td>32 ± 4†</td>
</tr>
<tr>
<td>Silica (4 h) + bestatin day 1 p.i.</td>
<td>35 ± 8†</td>
</tr>
</tbody>
</table>

* Female NMRI mice immunized i.p. with 0-4 ml 10% SRBC in Hanks’ solution. On the days indicated, animals received 1 g/kg silica and/or 10 mg/kg bestatin i.p. Three weeks after infection, animals were bled.
† Significant difference compared to control.

Influence of poly-I:C(LC) and of indomethacin on antibody levels when combined with bestatin

Interferons and prostaglandins (PG) are known to be secreted by macrophages during the course of an immune response. Molecules belonging to each group have been found to exert various effects on immunocompetent cells. Therefore, it was of interest to see whether either reduction of PG synthesis by injection of indomethacin or enhancement of interferon levels by application of poly-I:C(LC) would be able to influence the effect of bestatin on antibody formation.

In Table 3, it can clearly be seen that both poly-I:C(LC) and indomethacin acted comparably to the effect of silica on antibody formation; this was especially true for their capability to render the antibody-producing system susceptible to bestatin at day 1. Thus, both combinations induced much higher antibody levels when injected at day 1 compared to the effect of a single compound given at day 1.

Influence of the route of infection and the route of bestatin administration on the antibody response to HSV-1

In our previous studies (Knoblich et al., 1983) we established that identical doses of virus when injected i.p. resulted in higher antibody levels than injection into the footpads. We therefore examined whether the route of administration of bestatin also influences the antibody titres. It was shown (Table 4) that bestatin given at day 5 post-infection into the footpads or i.p. significantly elevated the levels of neutralizing antibody after plantar infection. Intraperitoneal injection of bestatin was more effective than application into the footpad. No increase was seen if bestatin was given at day 1.

The influence of administration of bestatin and silica on haemolysin titres against SRBC

Now it was important to see whether our observations for HSV-1 infection also applied to immune reactions induced by non-viral antigens. For that reason, mice were immunized with SRBC. Bestatin and silica were then injected according to the schedules used in the experiments above with HSV-1.

As can be seen from Table 5, bestatin also increased haemolyzing antibodies against SRBC. However, in contrast to the situation in HSV-1 infections, the compound acted best when given...
on day 1 after immunization. There was a greater increase in antibody levels after silica treatment compared to bestatin, but the combination of silica and bestatin yielded no further enhancement of antibody titres.

The effect of bestatin on virus content of visceral organs

To exclude the possibility that bestatin has some unknown effect on the virus content of visceral organs which could influence our results, we tested the virus content of liver, spleen, thymus and spinal cord. It was found that no influence of bestatin on the virus content of these organs could be detected, when compared to the non-treated controls (data not presented).

Interferon levels in the sera of bestatin-treated mice

An increase in HSV-1 neutralizing antibody levels was observed when the interferon inducer poly-I:C(LC) was administered into HSV-infected mice. We could not exclude the possibility that bestatin might similarly enhance antibody formation, i.e. by induction of interferon. We therefore collected sera from mice pre-treated with bestatin at various intervals. Assays for interferon activity included positive control sera obtained from C57BL/6 mice after pretreatment with 100 μg poly-I:C(LC). No biological activity of interferon was present in any of the sera from mice pretreated with bestatin, whereas high interferon levels (up to 1:3200) could be detected in the poly-I:C(LC) control sera.

DISCUSSION

In this study the effects of bestatin on the formation of neutralizing antibodies against HSV-1 are described. Our experiments have shown that this dipeptide considerably enhances the formation of neutralizing antibodies against HSV-1 in mice. Its effect is most prominent at the 5th day after primary infection; in secondary infections, bestatin acts most efficiently if given on the day following re-infection. These results strongly suggest a selective mechanism of the action of bestatin. The drug seems to stimulate (a) certain component(s) of the network involved in induction of antibody formation, being effective at different points of time in the course of primary and secondary HSV infections. In earlier experiments (Knoblich et al., 1983), we demonstrated marked differences between the antibody responses generated by female and those by male mice following primary infection with HSV-1. Application of silica resulted in an amplification of antibody titres which was much stronger in males than in females. This difference in antibody titres between females and males was abolished by silica treatment. In contrast to the effect of silica, bestatin augmented antibody levels in female and in male mice to about the same degree.

The extent of the increase in antibody levels by bestatin is dose-dependent, showing two dose ranges. The shape of the curve indicates an all-or-none response in both dose ranges. Very high titres of antibodies are produced at doses of 10 μg/kg or more, but a significant increase is already achieved at 1 ng/kg. Ishizuka et al. (1980a) found that more than 1 μg bestatin/mouse i.p. was necessary to increase thymidine incorporation into lymphocytes in the absence of lectins. More than 100 μg/mouse increased the number of IgM plaque-forming cells to heterologous red blood cells and 0.1 to 100 μg/mouse increased the delayed-type hypersensitivity response to SRBC. According to Müller et al. (1979), more than 0.5 mg/kg was necessary to induce significantly higher activities of DNA polymerase α in T-lymphocytes.

Comparison of antibody levels during 4 weeks after infection with HSV-1 revealed that the bestatin-treated (day 5 post-infection) animals always had higher antibody titres than the non-treated controls. From day 12 after infection, the difference became more and more pronounced.

The effects of bestatin and of silica seem to be systemic, because they enhance antibody formation irrespective of the site of their application. Injection of bestatin does not only enhance antibody levels after infection with live virus but it is also effective after immunization with a formol-inactivated HSV-1 vaccine.

The results concerning the combined effects of bestatin and silica require special mention. In contrast to the observation that bestatin applied alone acts best when given at day 5, an additive effect on the increase of antibody levels is achieved by bestatin application even at day 1 when
mice were pretreated with silica. Comparable results were also obtained by the combination of poly-I:C(LC) or of indomethacin with bestatin at the same time. These results can be interpreted to mean that silica, poly-I:C(LC) and indomethacin when given at day 1 sensitize the antibody-producing system for the enhancing activity of bestatin. Silica is known to destroy macrophages (Allison et al., 1966). Early after infection (day 1) silica, by eliminating the barrier of resident macrophages, allows HSV to replicate to higher titres, to increase mortality and to provide a stronger antigenic stimulus to the immune system. Previously, we have shown (Knoblich et al., 1983) that silica also increases antibody levels if given late (up to day 12) after infection when neither infectious virus nor HSV-specific antigens were detectable. It can be concluded that this effect is due to release of certain soluble factors (e.g. II-1) from macrophages which in turn amplify antibody production. These factors are supposedly also synthesized early after infection (day 1) and may then be released through silica in this way sensitizing the antibody-producing system for the effect of bestatin. This assumption is supported by the observation that culture fluids of peritoneal macrophages from mice pretreated in vivo with silica or infected with HSV contain factors able to enhance antibody formation if re-injected into mice. Another mechanism could be the elimination by silica of certain macrophage-derived suppressor activities (Kirchner, 1978) which may account for the delay in sensitivity to bestatin up to day 5.

Suppression may at least in part be due to secretion of prostaglandins (Humes et al., 1977). Prostaglandins of the E type are known to inhibit antigen- or mitogen-stimulated lymphocyte transformation, to suppress antibody production or antibody release by plasma cells (Trang, 1980) and to reduce the expression of Ia antigens on macrophages (Snyder et al., 1982). In our experiments bestatin was found to be effective on day 1 post-infection after inhibition of prostaglandin synthesis by indomethacin. Thus, prostaglandins appear to be involved in those mechanisms causing the physiological delay of the time at which bestatin may act (days 4 to 8).

Poly-I:C(LC) is a potent interferon inducer (Levy, 1977). Twenty-four h after i.p. infection, high levels of interferon were detected in the sera of mice. As poly-I:C(LC) exerted an effect comparable to that of silica and indomethacin with respect to the action of bestatin it may be assumed that 'interferon' accelerates the process that renders the immune system susceptible to the effect of bestatin. It is likely that comparable mediators are present in the macrophage culture supernatants, which were shown to sensitize the antibody-producing system for bestatin at day 1.

Interferon increases immune responses when given at the same time as, or after, immunization or infection (Friedman & Vogel, 1983). This suggests that bestatin itself might act by induction of interferon. After all, the stimulation of T-lymphocytes by bestatin requires the presence of macrophages. However, we were unable to detect interferon in sera of mice treated with bestatin.

To see whether the time dependence of the bestatin-mediated amplification of the antibody response was a feature specific for HSV, we examined the effect of silica and of bestatin on haemolytic activity to SRBC. Compared to the situation in HSV-1 infections, the effect of bestatin was less marked in the haemolytic antibody system and was detectable at day 1 rather than at day 5 post-infection. Silica increased haemolysin titres more than bestatin did. We conclude that our observations concerning the effect of bestatin on antibody formation in primary HSV-1 infections were specific for this virus system, whereas in other situations, such as the antibody response to SRBC, differences in extent and in time dependence of bestatin action were noted.

Investigations by Müller et al. (1979) have shown that maximal stimulation of deoxynucleoside incorporation into T-lymphocytes from thymus and spleen as well as maximal induction of DNA polymerase α occurs 5 to 15 h after i.p. injection of bestatin into mice. The limited span of time when bestatin is effective in our system may be the phase in which T-lymphocytes are able to exert an amplifying influence on the HSV-induced antibody response. This helper effect of bestatin seems to be effective comparatively late (day 5) after primary infection when a multitude of events is needed for the induction of an antibody response. In secondary infections,
the induction of an immune response is achieved by different mechanisms (including memory cells) allowing bestatin to be effective at day 1 after re-infection.

Bestatin may serve as a good tool to evaluate T-lymphocyte involvement in B-cell responses, particularly to compare the timing of antibody production against replicating HSV and non-replicating antigens (SRBC). In our HSV-1 mouse system, it has proven to be a good immunoenhancing drug which enables us to obtain high-titrated antisera.

We are grateful to B. Schambach and M. Engel for technical assistance during the experiments and to I. Albrecht for preparation of the manuscript.

REFERENCES


*(Received 25 April 1984)*