Immune Control of Herpes Simplex Virus Infections

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Summary

The role of cell-mediated protection to herpes simplex infection was studied in vivo and in vitro. Sensitized spleen cells, obtained from syngeneic mice immunized with herpes simplex virus, protected recipient mice against lethal challenge with herpes simplex virus. Survival was significantly greater in challenged mice that received washed spleen cells from donor animals immunized with herpes simplex than from control donor animals. This increase in survival after challenge was associated with a significant decrease in virus titers in the brains of mice that had received specifically sensitized cells. There was no obvious association between increased survival and the levels of serum antibody and interferon in the challenged animals. In vitro studies demonstrated control of herpes simplex infection in human and murine cell cultures by sensitized spleen cells from specifically immunized mice. Control of infection required intact, immune, non-glass-adhering lymphoid cells.

Introduction

The importance of host factors in control and recovery from herpes simplex virus infections has not been completely delineated. In general, homotypic antibody can protect against subsequent viral challenge (1), but it does not prevent recurrent infections (15) or aid in recovery if passively administered after infection has commenced (5). Interferon treatment also is generally unsuccessful if initiated following viral challenge (2). Hirsch et al. (9) have reported an adverse effect of immunosuppression, by treatment with antilymphocyte and antimacrophage sera, in mice challenged with herpes simplex. This paper summarizes the results of experiments on the prevention of spread of herpes simplex virus in tissue culture by immune spleen cells and serum after initiation of infection, and the protection conferred by immune spleen cells in vivo against lethal viral challenge, in an attempt to delineate the role of host factors in recovery from herpes simplex infection.

Materials and Methods

Virus. Herpesvirus hominis (herpes simplex), strain Rhodanus, was obtained from Dr. S. Kibrick. The virus was isolated from the lung of an infant with eczema herpeticum and had been passed 18 times in human amnion tissue culture. Supernatant fluid from the 18th passage was removed and, after centrifugation to remove debris, was stored at −70°C until used.

Mice. Six-week-old white male inbred CFW mice were purchased from Carworth Farms, New City, N. Y. They possessed no plaque-neutralizing antibodies against herpes simplex after shipment. Animals were kept in a room separate from the infected-animal room until after virus administration. Challenge with 5 LD₅₀ of virus was given in 0.1 ml i.v. in the tail vein. Donor mice were immunized with 0.1 LD₅₀ in 0.1 ml of virus, diluted in HBSS and 1% FCS, s.c. in the hind leg, followed by 0.1 LD₅₀ in 0.1 ml i.v., 4 and 5 weeks after primary immunization. Spleens were removed approximately 2 weeks after the 2nd booster. Donor spleen cells did not contain herpes simplex virus, as determined by isolation attempts in clone 1 5-C 4 cells. Spleens were removed from mice following cervical fracture, and the spleen was extruded from its capsule. After being minced with scissors, it was filtered through cotton gauze to remove large particulate material. Cells were washed 3 times in HBSS and 1% FCS and were then counted in a hemocytometer. Eosin-excluding spleen cells (3 × 10⁶) were then used in plaque studies or were given to recipient mice by tail vein injection. At similar intervals, control mice of the same strain, age, and sex were given injections of the same diluent not containing herpes virus, or of 1000 PFU of the AoNWS strain of influenza virus (obtained from Dr. E. D. Kilbourne, Mt. Sinai School of Medicine, New York, N. Y.) grown in the allantoic fluid of hens' eggs and diluted in HBSS and 1% FCS, and their spleen cells were prepared in an identical fashion. Recipient animals were sacrificed at intervals after viral challenge by cervical fracture. Brains were removed and were homogenized in 1.8 ml cold HBSS and 1% FCS. They were frozen and thawed 3 times before viral assay (this resulted in higher virus titers than with homogenization alone or with only 1 freeze-thawing). Blood was removed from the heart with Pasteur pipets, and red blood cells were separated. Sera were stored at −20°C before antibody or interferon assay.

Plaque Assays. Clone 1 5-C 4 cells, a derivative of Chang's human conjunctival cell line, were obtained from Dr. E. D. Kilbourne, and were grown in Medium 199 with 10% FCS. Mouse L 929 cells were obtained from Dr. S. Cooperband, Boston Univ. School of Medicine, Boston, Mass., and were grown in Eagle's MEM and 10% FCS. Plaque studies were performed on monolayers of these
cells in 60-mm plastic dishes. Each dish was infected with 50 PFU of virus in 0.2 ml of HBSS with 1% FCS, and was incubated at 36° for 45 min. After this incubation period, monolayers were washed twice with 5 ml of PBS, pH 7.2. Then 0.1 ml of immune or nonimmune serum diluted 1:10 in PBS, 0.1 ml of spleen cells containing 3 × 10^7 eosin-excluding cells, or 0.1 ml of PBS was added to monolayers. The dishes were reincubated at 36° for 45 min, followed by addition of 10 ml of an agar overlay, without removing serum or cells. The agar overlay that was added to the clone 1 5-C 4 cells contained Medium 199 and has been described (16). A similar agar overlay, substituting Eagle’s MEM for Medium 199, was added to the dishes containing L 929 cells. After the agar solidified at room temperature for 15 min, dishes were incubated at 36° in a humidified atmosphere containing 5% carbon dioxide. Three to 4 days later, agar was removed from the dishes, and monolayers were stained with 0.1% crystal violet in 95% ethanol. Plaques were measured on an inverted tissue culture microscope, with a linear grid in the eye piece. The number of cells in uninfected monolayers was determined after trypsinization by counting the number of eosin-excluding cells. The average plaque area was calculated after measuring 50 plaque diameters to determine the average plaque diameter. The number of cells in an average plaque was determined by the formula:

\[
\frac{\text{av. plaque area}}{\text{total monolayer area}} = \frac{\text{no. of infected cells in average plaque}}{\text{total no. of cells in monolayer}}
\]

Statistical analysis of plaque diameters was performed by means of the Mann-Whitney U test.

**Antiserum Preparation.** Herpes simplex antiserum was prepared in rabbits by administering, into the footpad, 0.4 ml of virus in HBSS and 1% FCS containing approximately 1 × 10^8 PFU of virus, without adjuvants, at 6 and 2 weeks before bleeding. This serum titrated 1:128 in a standard plaque neutralization assay, and was used at a dilution of 1:10 in the plaque experiments described below. Normal rabbit serum with a herpes simplex plaque neutralization titer of < 1:8 was used as the nonimmune serum also at a dilution of 1:10 in PBS. All sera were inactivated by heating at 56° for 30 min. Preliminary experiments revealed that addition of complement had no effect on plaque development; therefore, none was added to the plaque system. Antisera to the AoNWS influenza virus were obtained from mice following immunization, as described above, when they were sacrificed for spleen cell samples.

**Antibody Assays.** Sera from challenged mice were diluted in sterile PBS (pH 7.2), and an equal volume of virus, diluted in HBSS and 1% FCS to yield 100 PFU of virus, was added to each serum dilution. The mixture of serum dilution and virus was incubated at 36° in a water bath for 30 min before 0.2 ml from each mixture was added to 60-mm plastic dishes of clone 1 5-C 4 cells for virus titrations, as described above. Sera were not heat inactivated because early herpes simplex antibodies are complement dependent (13), and heating lowered antibody titers.

**Lymphotoxin Assay.** Virus was diluted in HBSS and 1% FCS, and 0.2 ml containing 500 PFU of virus was added to monolayers of clone 1 5-C 4 cells in 35-mm plastic dishes. After 45 min of incubation at 37°, 1.8 ml of Medium 199 with 2% FCS were added to the dishes, which were reincubated at 37° in a humidified atmosphere containing 5% CO₂. Twenty-four hr after virus inoculation, the medium was removed and monolayers were washed twice with PBS before receiving either 5 × 10^7 viable spleen cells in 0.5 ml HBSS and 1% FCS (obtained from control mice, or immune mice that had been immunized as described above) or diluent. After 30 min of incubation at 37°, 3.5 ml of Medium 199 and 2% FCS were added to the dishes in each of the 4 experimental groups, i.e., (a) virus, no spleen cells; (b) virus, immune spleen cells; (c) virus, control spleen cells; and (d) no virus, immune spleen cells. Five-tenths ml of supernatant fluid was removed from each dish at 12, 36, and 60 hr after the addition of cells to the infected monolayers. Samples from each group at each interval were pooled, centrifuged to remove cell debris, and stored at 4° for 48 hr. Live virus was inactivated in these samples by heating at 56° for 120 min, which treatment does not destroy lymphotoxin. The samples were then diluted 1:4 in Medium 199 and 2% FCS, and this mixture was added in 2.0-ml volumes to fully grown monolayers of primary rhesus monkey kidney, WI-38, and primary rat embryo cells, purchased from Microbiological Associates, Inc., Bethesda, Md., and to mouse L 929 cells. These cell cultures were examined daily for 10 days for cytotoxicity and cytopathic effect, as were cell cultures that contained media alone, without added samples from the above experiment.

**Interferon Assays.** L 929 cells in 60- x 15-mm plastic petri dishes were treated for 24 hr with a serial 2-fold dilution of samples in MEM and 10% FCS. Supernatant fluid was then removed and 50 PFU of vesicular stomatitis virus were added to each dish; after 1 hr of adsorption at 36°, an agar overlay with MEM and 5% FCS was added. Forty-eight hr later, agar was removed and monolayers were stained with 0.1% crystal violet in 95% ethanol. A 50% decrease from the average number of plaques counted in 3 virus control dishes was interpreted as being due to interferon at that dilution. Samples that contained this activity were pooled, and activity persisted after dialysis at pH 2 and pH 10 for 24 hr and was decreased by heating at 56° for 30 min.

**RESULTS**

**In Vivo Studies**

Initial studies were directed at delineation of the development of encephalitis in adult mice after challenge with herpes simplex virus. These investigations demonstrated that intracerebral challenge with 10 to 100 PFU of virus reproducibly produced encephalitis and death in 50% or more of challenged animals. The LD₅₀ increased to 1000 PFU of virus when i.v. challenge was utilized. The results of experiments with i.v. challenge will be summarized here.

**Challenge i.v.** In the 1st experiment, the i.v. viral challenge was given 3 days after cell transfer, when adoptive immunity against intracerebral challenge had been demon-
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This experiment revealed significant protection, so in the next experiment, viral challenge was given on the same day (12 hr later) as cell transfer. Table 1 depicts the percentage survival in this experiment. The group of animals that received immune cells had significantly increased survival throughout the experiment (Day 5, $\chi^2 = 14.3; p < 0.001$; Day 10, $\chi^2 = 8.2, p < 0.01$; Day 21, $\chi^2 = 8.0, p < 0.01$) over the group that received control cells.

**Antibody, Interferon, and Brain Virus Titers in Recipients of Immune and Control Spleen Cells.** Animals were sacrificed randomly during the experiment (Table 1) on Days 3, 7, and 10 after viral challenge and cell transfer for determination of neutralizing antibody, serum interferon, and brain virus titers; these results are shown in Chart 1. Brain virus titrations were performed because preliminary experiments had revealed that virus could be detected in the brains only at that interval after challenge, when no virus could be detected in the blood, spleen, heart, or liver. At each interval, 12 different mice from the group that received cells from herpes virus-immunized donor animals were studied, as were 8 mice from the group of animals that received cells from influenza-injected donors. An additional control group received spleen cells and was observed for possible graft-versus-host reactions, which did not develop. Since this group did not receive challenge with virus, they were not sacrificed for assay of virus, antibody, or interferon.

There is no detectable antibody present in any of the animals 3 days after viral challenge. Antibody is present in most of the animals in both groups by Day 7, and in all animals by Day 10. There is some difference in the geometric mean antibody titers between the groups on Day 7 but not on Day 10. Serum interferon levels are not shown in Chart 1. No interferon was detectable in any of the animals 3 and 10 days after challenge; however, 4 of 12 animals that had received sensitized spleen cells from virus-immunized donors did have detectable interferon on Day 7, although not to a high titer.

There is a significant decrease in the amount of virus in the brains of mice that had received immune spleen cells, at both 3 and 7 days after challenge. This is the interval when many deaths occur. All animals have herpes virus in their brains on Day 7, at a time when none is present in blood or other organs; however, several recipients of nonsensitized cells have 10- to 100-fold more PFU of virus than do the recipients of sensitized spleen cells. The difference in virus titers on Day 3 ($U = 20.5; p < 0.05$) and on Day 7 ($U = 13.5; p < 0.02$) between recipients of sensitized and control spleen cells is significant. There is no significant difference in virus titers in the brains of animals sacrificed on Day 10 ($U = 32.0; p < 0.05$).

**In Vitro Studies**

**Effect of Antibody.** Specific antibody to herpes simplex, when added to monolayers of the 2 types of cell lines tested, decreased the subsequent plaque size. Infection does spread from the initially infected cell, but there is herpes virus PSR
in the presence of immune rabbit serum. Table 2 demonstrates the significant PSR and the number of cells subsequently infected by the initial virus-infected cell. The number of cells infected by the originally infected cell was significantly less in cell monolayers treated with immune serum, compared with those receiving nonimmune rabbit serum. Table 2 illustrates the decrease in number of virus-lyzed cells between monolayers treated with immune serum and those receiving nonimmune serum.

Antibody also caused an increase in the size of multinucleated giant cells in clone 1 5-C 4 cells, although total plaque size (lytic area and giant cell combined) was diminished (Table 2). Thus, it appears that antibody enhances the fusion of clone 1 5-C 4 cells infected with herpes simplex, which form smaller giant cells when antibody is not present. Antibody did not induce giant cell formation in herpes-infected L 929 cells, although it did cause PSR in these cells, also. This may be associated with the complete absence of giant cell formation by herpesvirus in L 929 cells when antibody is not present.

Effect of Immune Spleen Cells. The contribution of immune lymphoid cells to the control of herpes infection in cell culture was then evaluated. Sensitized lymphoid cells did not neutralize herpes simplex in a cell-free suspension as determined by preadsorption incubation of virus and sensitized cells before plaque virus titration (unpublished observation). However, sensitized cells do cause a reduction in spread of infection after herpesvirus infects cells. There was a significant reduction in the number of cells infected by 1 virus particle in L 929 cells by sensitized cells alone, and this was increased to a 96% reduction when both sensitized cells and immune sera were present. The immune cells alone significantly decreased the number of cells infected by the initially infected cells (Z = 4.7; p < 0.001); moreover, the combination of immune cells and sera was additive in limiting the number of infected cells than was either alone (Z = 6.0; p < 0.001). Most plaques developing under agar-containing immune sera and sensitized cells in L 929 cells are too small to be seen without a microscope (average, 0.08 mm, with a range between 0.04 and 0.14 mm in diameter). Antibody or sensitized cells alone resulted in somewhat larger plaques (average, 0.16 mm), and control cells and sera allowed plaque development similar to that of untreated virus controls (average, 0.40 mm). The control of infection in 1 5-C 4 cells was similarly decreased (p < 0.01) by immune cells and serum (average, 0.20 mm) from the nonimmune cells and serum (average, 0.60 mm).

This control of herpes simplex infection by sensitized spleen cells and serum was specific. Table 3 demonstrates that spleen cells obtained from mice immunized in a similar fashion with the AoNWS strain of influenza did not cause herpes simplex PSR, unlike the action of specifically sensitized spleen cells or serum.

The role of antibody in the immune lymphoid cell-mediated plaque size reduction was analyzed. There are 2 markers for antibody activity on herpes simplex plaques in clone 1 5-C 4 cells, namely, PSR and enhancement of giant cell development in the plaque. If immune cells cause PSR by release of antibody, similar plaque changes might occur in immune cell-treated plaques; however, there was no increase in the plaque giant cell size associated with immune cell treatment, as opposed to treatment with antibody alone. The difference in the giant cell diameters between antibody- and immune cell-treated dishes is significant (Z = 5.56; p < 0.001), although both reduce total plaque diameter over control dishes treated with nonimmune serum or cells.

Elaboration of lymphotoxin, a factor released from sensitized lymphocytes which can destroy nonspecific target cells, was sought in the supernatant fluids of herpes simplex-infected clone 1 5-C 4 cells, after addition of spleen cells from immune and control animals. This factor is reportedly released by the interaction of sensitized lymphocytes with the specific antigen to which they have been sensitized, viral (10) or nonviral (14). Supernatant fluids from herpes-infected tissue culture cells, exposed to sensitized and nonsensitized mouse spleen cells, were all negative for lymphotoxin when assayed on a variety of target cells, as described in "Materials and Methods."

The PSR caused by immune spleen cells was studied in an experiment designed to decrease the number of macrophages present in the spleen cell population. One group of infected monolayers received 3 \times 10^7 spleen cells prepared in the usual method; another group of dishes received 3 \times 10^7 cells from supernatant that had been passed 3 times, at 15-min intervals, on glass Petri dishes. This treatment removed over 90% of macrophages from this sample; however, this macrophage depleted population decreased the mean plaque size from 0.44 to 0.15 mm (Z = 5.39; p < 0.001), which is similar to that accomplished by the original macrophage-rich population of immune spleen cells.

Table 2

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Total plaque diameter (mm)</th>
<th>No. of lysed cells/plaque</th>
<th>Giant cell size/plaque (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-herpes simplex</td>
<td>0.53</td>
<td>482</td>
<td>0.21</td>
</tr>
<tr>
<td>Anti-influenza (AoNWS)</td>
<td>0.65</td>
<td>1009</td>
<td>0.05</td>
</tr>
<tr>
<td><em>p &lt; 0.05</em></td>
<td><em>p &lt; 0.01</em></td>
<td><em>p &lt; 0.01</em></td>
<td></td>
</tr>
</tbody>
</table>

* Antibody was added after virus adsorption and was not removed, as described in "Materials and Methods."
* Average of 50 plaques/group, measured on an inverted microscope 3 days after infection.
* Statistical analysis of 50 plaques/group by Mann-Whitney U test.

Table 3

<table>
<thead>
<tr>
<th>Spleen cells</th>
<th>Av. plaque diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herpes simplex</td>
<td>0.35</td>
</tr>
<tr>
<td>Influenza (AoNWS)</td>
<td>0.55</td>
</tr>
<tr>
<td>None, virus control</td>
<td>0.60</td>
</tr>
</tbody>
</table>

* Monolayers of clone 1 5-C 4 cells, after adsorption of herpes simplex virus, received 3 \times 10^7 spleen cells from mice immunized with herpes simplex or influenza (AoNWS) viruses as described in "Materials and Methods."
mm). The need for intact sensitized cells in this reduction of plaque size was then demonstrated. Aliquots of immune cells were disrupted by freeze-thawing rapidly 3 times in a Dry Ice bath, and these fragmented cells did not cause any reduction in plaque size. The mean plaque size was 0.38 mm in dishes receiving lyzed immune cells, 0.42 mm for dishes receiving intact control lymphocytes, and 0.13 mm ($p < 0.01$) for dishes treated with intact sensitized spleen cells.

The number of sensitized cells needed to limit the spread of herpes simplex infection in clone 1 5-C 4 cells was studied. Preliminary experiments revealed that >60 x 10^6 mouse spleen cells, obtained from other control or herpes-immunized mice, would be toxic to the monolayer cells if left under the agar for 4 days. Therefore, serial dilution of immune spleen cells was performed in HBSS and 1% FCS. It required 15 x 10^6 spleen cells, obtained from specifically sensitized mice, to significantly reduce herpes simplex plaque size when spleen cells were added to a 6.0-mm Petri dish containing 5 x 10^4 clone 1 5-C 4 cells.

DISCUSSION

These experiments indicate that immune spleen cells aid in the recovery of mice from primary systemic challenge with herpes simplex. Animals that received immune spleen cells have less virus in their brains than mice that received nonimmune spleen cells. There is no difference in the development of serum-neutralizing antibody levels on Day 3, when brain virus titers are already different, between animals that received sensitized or nonsensitized spleen cells at the time of viral challenge. The lack of difference in Day 10 virus titers may be secondary to the fact that animals sacrificed on Day 10 are more likely to survive than animals sacrificed on Days 3 and 7. There is a difference in antibody titers on Day 7 between the groups, but specimens from individual mice do not indicate that serum antibody titers are related to the brain virus titers.

Some interferon was detected in the serum of several animals that had received sensitized spleen cells; none was detected in control animals. Interferon levels of less than 80 units could not be detected because of the decision to assay individual, unpooled sera. The animals with detectable interferon did not have any lower brain virus titer or antibody titer than most of the animals without detectable interferon, so any importance of serum interferon in the recovery of mice from primary systemic challenge with herpes simplex is not obvious (4). The increase in production of serum interferon after challenge with homotypic virus in recipients of immune spleen cells was not unexpected. Glasgow (6) demonstrated an increased production of interferon by immune peritoneal leukocytes challenged with Chikungunya virus. A similar increased production of interferon by immune lymphocytes following nonviral secondary antigenic challenge was reported by Green et al. (7). The increased production of interferon by immune lymphoid cells might explain the presence of more serum interferon in the recipients of immune spleen cells. The data do not support a determining role for serum interferon in lowering brain virus titers, and the problem of local production of interferon by lymphocytes was not evaluated in this in vivo model. Catalano and Baron (2) demonstrated protection against 1 LD_{50} of herpes simplex virus challenge in mice given 200 μg of double-stranded polynucleoside polycytidylic RNA i.p. 18 hr before viral challenge, and 100 μg 3 times/week for 5 doses after challenge. This regimen did not protect mice given challenge with more virus, despite the ability of a single 200-μg dose to yield 1000 units of interferon in the sera and brains of mice. Challenged mice were not examined for antibody, interferon, or virus titrations. Although protection was demonstrated against 1 LD_{50} of herpes simplex by treatment with the interferon inducer, the experiments reported here demonstrate protection with higher doses of challenge virus, without high levels of serum interferon. It appears that both interferon induction and sensitized spleen cells can protect mice challenged with herpes simplex, but the mechanisms of protection seem dissimilar. The in vitro studies also suggest the control of herpes virus infection by washed sensitized spleen cells (12). This control of spread of herpesvirus infection appeared to be independent of interferon activity because immune spleen cells inhibited the spread of herpesvirus infection in cell lines derived from both human and murine tissues. Moreover, this inhibition appeared to be distinct from antibody control because it caused plaque size reduction or control of herpes infection without causing an increase in the plaque multinucleated giant cells, unlike the increased polykaryon size caused by antibody in the cell line (clone 1 5-C 4) used. In addition, washed immune spleen cells do not neutralize herpesvirus. These in vitro studies do not exclude the possibility that local antibody production by the immune cells is the mechanism of survival in the mouse experimental model. They do, however, support the in vivo data which show no obvious role for serum antibody in the observed cell-mediated protection.

In addition to the production of interferon and antibody, sensitized lymphocytes release a factor, lymphotoxin, which can destroy target cells. This is not a species-specific effect. Lysis of target cells in the area of viral-infected cells that are exposed to sensitized lymphoid cells could then result in a decrease in viral spread. Supernatant fluids were obtained from herpes simplex-infected tissue cultures after the addition of sensitized lymphoid cells; however, these fluids did not contain lymphotoxin when assayed on cell cultures of human, monkey, rat, and mouse cells (3). An alternative explanation for the mechanism of protection conferred by sensitized spleen cells could be target cell lysis of host cells with viral antigen on their surface. Structural herpes viral antigens develop as early as 2 hr after infection, and viral antigens appear on the surface of infected cells shortly thereafter (11). The number of cells with herpes simplex antigens rises steeply and peaks before 8 hr, when new herpes infectious virus is first detected. Cells with herpes surface antigens could possibly then be attacked by sensitized lymphoid cells and destroyed before producing infectious virus. Immune lymphocyte-mediated cell lysis of virus-infected cells has been reported with lymphocytic choriomeningitis virus (12). It is also conceivable that the sensitized lymphocytes release a factor that depresses synthesis of viral DNA, analogous to proliferation inhibition factor, which is released from sensitized human lym-
phocytes and suppressed cellular DNA synthesis (8). The precise mechanism of the observed protection against herpesvirus conferred by the passive transfer of immune lymphoid cells awaits further delineation.

These studies on the host defense mechanisms of protection and recovery from lytic herpetic infections should be extended to the role of host factors in tumor systems associated with infections with herpes simplex virus. Herpes simplex type 2 infection occurs significantly more frequently in individuals with cervical carcinoma. Delineation of the host factors that protect against infection with this agent, and the role of these factors, humoral and cellular, in the resistance to or enhancement of tumor spread, should be attempted in patients with this common malignant tumor.

References

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