Factors Enhancing the Host-Cell Penetration of *Toxoplasma gondii*

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**ABSTRACT**

The penetration into HeLa cells of *Toxoplasma gondii* was studied with a cell culture technique. The influence on the rate of penetration and the number of penetrating *Toxoplasma* parasites was tested by use of preparations of disintegrated parasites mixed with test parasites. These preparations were found to contain factors enhancing the penetrating rate of the parasites. This effect was demonstrable by use of untreated parasites as well as parasites lacking active motility owing to a previous exposure to Formalin. The preparations of disintegrated parasites contained, in addition, components inhibitory to the penetration-enhancing factors. These inhibitory components were able to reduce the penetrating capacity of normal *Toxoplasma* parasites, suggesting that the studied enhancing factors may play a role in the natural process of penetration. The efficacy of various techniques for disintegration of *Toxoplasma* parasites was investigated for release of penetration-enhancing factors from *Toxoplasma* parasites. The methods used resemble those used for liberation of lysosomal enzymes. Reduced osmotic pressure was obviously not adequate for release of enhancing factors, whereas the freezing and thawing procedure, sonic treatment, and irradiation produced high yields. It was difficult to evaluate the effect of incubation at acid pH on release of enhancing activity, because the penetration-promoting factors seemed unstable on both the acid and the alkaline sides of pH 7.6.

Observations on cultured cells incubated with *Toxoplasma* parasites indicated that even parasites which lacked any demonstrable active motility were able to infect the cells. This suggested that reasons other than strictly mechanical ones could determine whether or not the parasites passed intracellularly. Some mucopolysaccharidases were found capable of promoting the penetration of *Toxoplasma* (8; R. Norrby, E. Lund, and E. Lycke, unpublished data). Moreover, preparations of lyed *Toxoplasma* parasites proved to contain not only enzymelike factors which enhanced the penetration, but also components which inhibited the enhancement of penetration (9). The promoting factors and the inhibitory ones could be separated, and the activities were individually tested.

The present study describes the effect of the promoting factors on the number of penetrating parasites and on the rate of parasite penetration. This information was considered important also for the study of the yield of penetration-enhancing activity released from parasites by various techniques for disintegration. The methods used for disintegration were similar to those used for the extraction of lysosomal enzymes from mammalian cells (2).

**MATERIALS AND METHODS**

*Parasite suspension.* The RH strain of *Toxoplasma gondii* was used. Parasite suspensions were prepared from mouse peritoneal exudate according to a technique described previously (9). The parasite suspensions were used for obtaining preparations of disintegrated parasites, and as the source for test parasites in assays of penetration-enhancing activity.

*Preparation of lyed parasites (PLP).* Parasite suspensions containing about 20 million parasites per milliliter were frozen and thawed three times, sonicated in a Raytheon 200-w, 10-kc oscillator for 15 min, and centrifuged at 20,000 × g for 45 min. The supernatant fluid obtained is referred to as PLP. In some cases, PLP was centrifuged at 80,000 × g for 45 min to give PLPsup and PLPsub.

The sonic treatment was performed in an ice bath, and the centrifugations, at 2 C. The first centrifugation providing PLP removed most of the cell debris and the undisrupted parasites. The centrifugation of PLP...
separated the penetration-enhancing factors and the inhibitory components. The supernatant fluid containing the enhancing activity is called PLP_{sup} and the resuspended sediment with the inhibiting activity is referred to as PLP_{sed}. Concentration of PLP_{sup} was achieved by lyophilization. All of the preparations were adjusted to pH 7.6 before use in assays of penetration-enhancing or -inhibiting activity.

Assay of penetration-enhancing or -inhibiting activity. The methods for culturing Hela cells in Gey chambers, determination of the number of penetrating parasites by use of the cell cultures, and the accuracy of these determinations have been described elsewhere (6, 9). Samples of standardized suspensions of test parasites were mixed with the preparation of disintegrated parasites to be tested by use of 0.2 ml of both the suspension of test parasites and the preparation. Each of three cultures was inoculated with 0.4 ml of the mixture. The cultures were incubated at 37°C for 19 hr or the times stated. One of the controls, included in all experiments, contained Hanks basal salt solution (BSS), pH 7.6. Instead of a preparation of disintegrated parasites, Hanks BSS was mixed with test parasites, and was added to the cultures. The results of the reading of the cultures were expressed in terms of relative number of infective units (RNIU), i.e., 100 times the number of parasites, found to have penetrated, divided by the number of exposed cells. The enhancing or inhibiting activity of a preparation was demonstrable as the differences in RNIU values between the tests and the controls.

RESULTS

Enhancement of penetration and the penetration rate of Toxoplasma. The first series of experiments demonstrated the influence of preparations of disintegrated parasites on the penetration of Toxoplasma. In Fig. 1, the number of penetrating parasites is plotted against the concentration of the penetration-enhancing preparation (PLP_{sup}). If the PLP_{sup} is concentrated, the effect on the enhancement is directly proportional to the degree of concentration. This is in accord with the finding that dilution of the enhancing preparation results in a proportionally large reduction of the number of penetrating parasites (9).

When PLP_{sup} concentrated six times or more, was added to the cell cultures, lysis of the cells occurred. No corresponding destruction of parasites was observed. Enhancement of the penetration in the presence of PLP_{sup} was evident, not only from the total number of penetrating parasites, but also from an increased rate of penetration (Fig. 2). At 1 hr after the inoculation, about 50% more parasites penetrated the host cells if the cultures had received PLP_{sup} instead of Hanks BSS as supplement. At 19 hr after the inoculation, this difference amounted to 24%. Thus, it seemed possible that, by the addition of PLP_{sup}, Toxoplasma parasites with reduced capacity of penetration became able to invade the host cells.

Formalin treatment of Toxoplasma parasites effectively impairs the motility and, thus, also affects the penetration of the parasites. In the following experiment, Formalin-treated parasites were used to study the relation between the
viability of the test parasites and the effect of the penetration-enhancing preparation. Parasites were treated with 10% Formalin in Hanks BSS for 20 min at room temperature, and were subsequently washed three times by low-speed centrifugation and resuspension in BSS. After this treatment, only 0.5% of the parasites were capable of reproduction, whereas 43.4% of control parasites were found to have divided after 19 hr of incubation.

In the experiment illustrated in Fig. 3, the same number of Formalin-treated and control parasites were used. Figure 3 shows the effect on the rate of penetration of PLP$_{sup}$ by use of Formalin-treated or untreated parasites. Addition of PLP$_{sup}$ to the control parasites resulted in a 45% increase in penetration at 1 hr after the parasite inoculation, whereas the corresponding increase at 9 hr was 35%. With Formalin-treated Toxoplasma parasites, the figures were 10 and 45%, respectively. Thus, there was an obvious promoting effect of PLP$_{sup}$ also on the penetration of parasites lacking the capacity of active motility, although a prolonged time was required before the enhancement was clearly demonstrable.

Inhibition of penetration of normal Toxoplasma parasites. As mentioned above, preparations of lysed Toxoplasma parasites contain, in addition to factors enhancing the penetration, components able to inhibit that enhancement. The effect of these components on the penetration of normal parasites, i.e., without addition of any preparation with enhancing activity, was studied.

PLP$_{sed}$ was washed twice by centrifugation at 80,000 × g and resuspension in Hanks BSS. After each centrifugation, the resuspended sediment was sonic-treated, frozen, and thawed. In this way, remaining traces of enhancing activity were removed. Samples of PLP$_{sed}$ were then mixed with test parasites, incubated at 37 C for various periods of time, and inoculated into cell cultures. As controls, mixtures of Hanks BSS and test parasites were treated in the same way. The influence on the penetration of Toxoplasma is shown in Fig. 4.

At the addition of PLP$_{sed}$ to the test parasites, reduction of the number of penetrating Toxoplasma parasites was observed. Preincubation of parasites with PLP$_{sed}$ caused a further pronounced inhibition of penetration.

Effect of freezing and thawing, and sonic treatment on yield of penetration-promoting activity. Samples of a suspension of Toxoplasma parasites were submitted to the treatments listed in Table 1. After the treatment, the samples were centrifuged at 2 C for 45 min at 80,000 × g to remove cell debris, not disintegrated parasites and the inhibitory components.

Table 1 demonstrates that more enhancing activity was released when the parasite suspension was frozen and thawed three times instead of once. Additional freezing and thawing resulted only in a slightly increased yield. Sonic treatment alone caused some release of activity, but, when performed after freezing and thawing, it contributed effectively to release of penetration-promoting activity.

Effect of reduced osmotic pressure. Four preparations were each dialyzed against distilled water for 2 hr. The preparations were: intact parasites; parasites disintegrated by freezing and thawing and sonic treatment; PLP, i.e., the supernatant fluid obtained after centrifugation of disintegrated parasites at 20,000 × g for 45 min; and PLP$_{sup}$. The dialysis was performed at 2 C by use of 3-ml volumes of the material to be dialyzed and repeated replacements of large volumes of the distilled water. After the dialysis, the materials were centrifuged at 80,000 × g for 45 min, and NaCl was added to the supernatant fluids to compensate.
The penetration-enhancing factors did not pass the dialyzing membrane.

Lyophilization of dialyzed material could be performed without noticeable loss of activity.

**Effect of irradiation.** Samples of a parasite suspension and of various preparations of disintegrated parasites were submitted to irradiation from a 20-ma, 200-kv X-ray source. Before and after the irradiation, the samples were kept in an ice bath. The longest period of irradiation was 17 min (20,000 rad). A rise in temperature during the irradiation to 20 C was then observed.

Before and after the irradiation, samples of the parasites were stained with methylene blue and were counted in a hemocytometer. About one-third of the parasites receiving 20,000 rad became lysed, and one-third of the remaining parasites could not be vitally stained. Thus, at least about 50% of the parasites seemed to be affected by the irradiation.

After irradiation, the samples of parasites and PLP were centrifuged at 80,000 × g for 45 min. The supernatant fluids obtained, as well as the irradiated PLPsup and PLPsed, were each mixed with test parasites, and, after incubation, the number of penetrating parasites was determined. The results were compared with those obtained with identically treated, but not irradiated, control preparations. Table 2 shows the release of enhancing activity from irradiated parasites and the changes induced by irradiation in the enhancing or inhibiting activities.

In an additional experiment, the irradiation dose was further increased to 30,000 rad; however, this did not significantly enlarge the release of activity.

For the release of enhancing activity from parasites in suspension, an irradiation dose of at least 20,000 rad was most effective. The release seemed to occur concomitantly with the disruption of the parasites. A dose of 20,000 rad to PLP caused a marked lowering of the penetration-enhancing factors.

<table>
<thead>
<tr>
<th>Freezing and thawing (no. of times)</th>
<th>Sonic treatment</th>
<th>RNIU</th>
<th>Increased penetration</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>54.4</td>
<td>6.7</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>63.0</td>
<td>23.5</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>57.3</td>
<td>12.4</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>62.7</td>
<td>22.9</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>71.6</td>
<td>40.4</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>70.0</td>
<td>37.3</td>
</tr>
<tr>
<td>Control*</td>
<td></td>
<td>51.0</td>
<td>-</td>
</tr>
</tbody>
</table>

*Hanks BSS.

for the reduced salinity. The supernatant fluids were then tested for penetration-enhancing activity.

Reduction of the osmotic pressure by dialysis insignificantly influenced the release of penetration-enhancing activity. This was the case also when intact parasites were used, although most of the parasites were lysed by the dialysis. The maximal increase in activity noted after dialysis was 5.6% obtained with the crude preparation of disintegrated parasites.

TABLE 1. Relative number of infective units (RNIU) and calculated increased penetration of Toxoplasma parasites owing to activity released from parasites by freezing and thawing with and without sonic treatment

| Table 2. Changes in enhancement and inhibition activity caused by irradiation* |
|-----------------------------|---------|------------------|
| Prepn | Rad | Change in effect (per cent) |
|       |       | Enhancement | Inhibition |
| Parasites | 200 | 4.9 | |
|          | 2,000 | 8.7 | |
|          | 20,000 | 31.5 | |
| PLP | 20,000 | -21.6 | |
| PLPsup | 20,000 | 2.3 | |
| PLPsed | 20,000 | 7.2 | |

* Supernatant fluids were tested after centrifugation of irradiated preparations.
enhancing activity. This did not seem to be a result of a destruction of the enhancing factors, because the irradiation of PLP_{sup} had no deteriorating effect. The increase in the inhibitory capacity of PLP_{sed} after irradiation suggested that the changes in the activity of PLP probably were secondary to an increased inhibitory activity.

Penetration-enhancing activity and pH. Autolysis at acid pH is a useful method for releasing lysosomal enzymes from rat liver lysosomes (2). In the following experiment, the influence of pH on release of penetration-enhancing activity in preparations of lysed Toxoplasma cells was studied. By means of adding crystalline citric acid or sodium bicarbonate, samples of a preparation of disintegrated parasites with a range of pH from 4.8 to 9.1 were obtained. The samples were incubated at 37 C for 1 hr, and the pH of all the samples was adjusted to 7.6. They were subsequently mixed with test parasites, and the penetration-enhancing activity was assayed.

Incubation at low pH reduced the penetration-enhancing activity (Table 3). A reason for this reduction is probably the instability of the factors responsible for the promoting activity. Maximal activity of PLP_{sup} was obtained only within a narrow range of pH on the alkaline side.

**DISCUSSION**

Toxoplasma gondii is known to multiply only inside a living host cell. Thus, it must be able to penetrate the host cell wall or stimulate the cell to incorporate the parasite. Two phases of the cell-penetrating process may be discerned. In the infected cell culture, the parasites are actively motile shortly after the liberation from a rupturing infected cell. Time-lapse studies have shown that they move at a speed of 3 mm, corresponding to 400 times the parasite length, per sec (6). The force then developed seems to enable the parasites to break through the host cell wall. Parasites which have not succeeded in reaching an intracellular position a few minutes after the liberation seem to lose their active motility. These parasites can be seen in the microscope attached with their anterior tip to the cell surface. In this end, inside the conoid of the parasite, the anterior parts of the paired organelle are seen. Electron microscopy "clearly indicates the secretory nature of this structure" (3). After a certain period of time, the attached parasites have penetrated and appear in the cytoplasm of the host cells.

A faster, as well as a slower, evolving phase is also distinguishable in the diagrammatic representation of the kinetics of parasite penetration. With the cell culture technique employed, about 25% of the inoculated parasites are found intracellularly at 1 hr after the inoculation. At 3 to 4 hr, 30 to 40% of the parasites are found inside the cells, and from then on there seems to be no further penetration. Thus, about 60% of the parasites appear to be noninfective, as they are not recovered as intracellularly located parasites even after 19 hr of incubation. This is found to be the result in spite of the fact that there is almost the same number of cells per culture as the number of inoculated parasites, that each cell can be infected by a great number of parasites, and that all the cells of a studied culture seem to be equally sensitive to the parasite infection.

The presence of an enzymatic activity of *Toxoplasma* affording a means of cell penetration also for parasites with reduced motility is suggestive. In fact, it was demonstrated that preparations of disintegrated *Toxoplasma* parasites contain factors which, if mixed with parasites, enhanced the penetration (9). As shown in the present report, the enhancing effect was reflected in an increased rate of penetration as well as in the number of penetration parasites. In the presence of enhancing factors, parasites lacking demonstrable active motility were also able to pass intracellularly at an increased rate. The promoting effect of the preparations used seemed to be proportional to the concentration of the factors responsible for the enhancement of penetration.

When preparations concentrated six times or more were used, lysis of the host cells but not of the parasites was observed. This suggests that the action of the promoting factors was selectively directed against the host cell walls.

Although the penetration-enhancing activity in the preparations studied is unequivocal, it may be argued that it is artificially obtained, and that the released factors have no biological function in the natural process of parasite penetration. Also, at the present stage, such a function cannot be proved. However, as already mentioned, preparations of disintegrated parasites contain, in addition to penetration-enhancing factors, com-

**Table 3. Change in penetration-promoting activity caused by incubation of PLP at 37 C for 1 hr at different pH levels**

<table>
<thead>
<tr>
<th>pH</th>
<th>Change in enhancement</th>
</tr>
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<tbody>
<tr>
<td>4.8</td>
<td>-0.4</td>
</tr>
<tr>
<td>5.7</td>
<td>-1.0</td>
</tr>
<tr>
<td>7.0</td>
<td>7.1</td>
</tr>
<tr>
<td>7.6</td>
<td>18.8</td>
</tr>
<tr>
<td>8.1</td>
<td>16.0</td>
</tr>
<tr>
<td>9.1</td>
<td>-0.8</td>
</tr>
</tbody>
</table>

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Although the penetration-enhancing activity in the preparations studied is unequivocal, it may be argued that it is artificially obtained, and that the released factors have no biological function in the natural process of parasite penetration. Also, at the present stage, such a function cannot be proved. However, as already mentioned, preparations of disintegrated parasites contain, in addition to penetration-enhancing factors, com-
ponents which can inhibit the promoting activity. When the inhibitory components were separated and freed from enhancing factors, they were capable of reducing the capacity of penetration of normal *Toxoplasma* parasites. This finding may suggest that factors occur normally which react in a manner similar to that of the penetration-enhancing factors studied, and that these factors therefore have a biological function in the natural process of penetration.

Enzymes, such as lysozyme, hyaluronidase, \(\beta\)-glucuronidase, and \(\beta\)-galactosidase, acting on mucopolysaccharides are all able to increase the number of penetrating parasites, if added to infected cultures (8; Norrby, Lund, and Lycke, unpublished data). These enzymes occur as lysosomal enzymes (1, 10). Acid phosphatases, also occurring in lysosomes, have been demonstrated histochemically in *Toxoplasma* parasites (5). These observations focused the interest on the relation of the penetration-enhancing factors to the lysosomal enzymes. The yield of activity by use of various methods of releasing lysosomal enzymes from mammalian cells was therefore investigated.

High yields of penetration-enhancing activity were achieved when the parasites were irradiated with 20,000 rad or exposed to freezing-thawing treatments. Especially effective were the combined treatments of freezing-thawing and sonic treatment. From our previous work, it is known, however, that the yields can be further improved if the preparation is subsequently incubated at 37°C for 1 hr. These results resemble those obtained on release of lysosomal enzymes (4, 10).

Insignificant release of activity was encountered after exposure of the parasites to reduced osmotic pressure, and no penetration-enhancing activity was demonstrable after treatment at acid pH. Triton X-100, a detergent reported effective for releasing lysosomal enzymes, was also used. However, because the detergent could not be effectively removed before determining the penetration-enhancing activity, the cell cultures were damaged and assays could not be made. The finding that reduced osmotic pressure was less effective is somewhat surprising, because the *Toxoplasma* parasites are unstable in a hypotonic milieu and become disrupted (7). Probably a more complete disintegration of the parasites is required for release of the penetration-enhancing activity. The reason for the negative result with acid pH treatment may be explained by the sensitivity of the enhancing factors to changes in pH outside a narrow range around 7.6.

If the penetration-enhancing activity does depend on the release of lysosomal enzyme(s), the responsible enzyme(s) does not seem to be \(\beta\)-galactosidase, \(\beta\)-glucuronidase, or acid phosphatases, because the penetration-enhancing factors were unstable at an acid pH. The enhancing effect is probably due to lysozyme or hyaluronidase activities or both. At the present stage, however, information is insufficient to permit more than vague speculations.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**