Effect of Mycoplasmas on Natural Cytotoxic Activity and Release of Tumor Necrosis Factor Alpha by Spleen Cells

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It has been reported that mycoplasma-infected cells are more sensitive to lysis by natural cytotoxic (NC) effector cells and that splenic NC cells release tumor necrosis factor (TNF-α) when they lyse sensitive target cells. Here we showed that spleen cells released TNF-α when they were incubated with NC-sensitive cells that were infected with mycoplasmas or when they were incubated with mycoplasmas alone, but did not release TNF-α when incubated with NC-sensitive cells that were not infected with mycoplasmas. Thus, in the presence of mycoplasmas, spleen cell cultures contain both NC effector cells and free TNF-α. Because NC-sensitive cells are also sensitive to free TNF-α, when mycoplasma-infected cells were incubated with spleen cells, they were lysed by the combination of NC cells and free TNF-α. When NC-sensitive cells that were not infected with mycoplasmas were incubated with spleen cells, they were lysed only by NC effector cells and thus appeared to be less sensitive than mycoplasma-infected cells. These results also suggested that the release of TNF-α may be part of a host protective response to mycoplasmas.

MATERIALS AND METHODS

Cell lines. The fibroblast cell line L929 was derived from the L-M strain of NCTC clone 929. The cell line referred to as L929(A) was obtained from the American Type Culture Collection (Rockville, Md.) (ATCC CCL12); it is free mycoplasmas. The cell line referred to as L929(S) was obtained from The Sloan-Kettering Cancer Center (Rye, N.Y.); it is mycoplasma infected. The cell line L929(A)-I was derived from L929(A) by exposing L929(A) to mycoplasma-containing medium from L929(S); it is mycoplasma infected. The cell lines L929(A)-I-C and L929(S)-C were derived from L929(A)-I and L929(S), respectively, following antibiotic treatment (B-M Cycline: Boehringer GmbH, Mannheim, Federal Republic of Germany). L929(A)-I-C and L929(S)-C are free of mycoplasma infection. The fibroblast cell line 10ME was derived by us from a BALB/c fetus. It is NC sensitive (3, 11) and is not infected with mycoplasmas.

All cell lines were maintained at 37°C in humidified incubators containing 10% CO₂ and 90% air. The growth medium was Dulbecco modified Eagle medium supplemented with fetal calf serum (10%), penicillin (450 units/ml), streptomycin (40 μg/ml), and l-glutamine (5 mg/ml). The presence of mycoplasma infection was determined by a fluorescent Hoechst 33258 staining procedure (2).

In vitro assay of NC/TNF-α-mediated lysis. For each experiment, various numbers of spleen cells from groups of two 6- to 12-week-old immunologically naive BALB/c mice which were not infected with mycoplasmas, titrated concentrations of spleen cell culture supernatant fluids, or recombinant human TNF-α (rTNF-α) were mixed with 10⁶ ⁵¹Cr-labeled targets in 96-well microtiter plates containing a final volume of 0.1 ml per well. ⁵¹Cr release was determined after 18 h. The percent specific lysis was calculated by the following formula: percent specific lysis = 100 × ([sample cpm - spontaneous cpm]/[total cpm - spontaneous cpm]), where cpm is counts per minute. Each datum point represents the average of triplicate determinations, with the cpm of the triplicates within 10% of each other; this includes sample cpm and spontaneous cpm. In all experiments, the

Mycoplasmas are the smallest and simplest procaryotes capable of self-replication. Certain species of mycoplasmas are normal inhabitants of the oropharynx and genital tract; the major human pathogen in this group, Mycoplasma pneumoniae, is the causative agent of atypical pneumonia (10). In addition to their role as pathogens, mycoplasmas are a common contaminant of cell cultures.

Natural cytotoxic (NC) activity is one of several naturally occurring cytotoxic activities found in the spleen, lymph nodes, and peripheral blood of normal individuals. There is now considerable evidence that both human and murine NC effectors use tumor necrosis factor alpha (TNF-α) to mediate target lysis (8, 11). Recently, it has been shown that spleen cells incubated with some NC-sensitive cells release TNF-α into the medium (8, 9). This has led to the hypothesis that NC effector cells mediate lysis by secreting free TNF-α (8, 9).

In this report, we show that spleen cells release free TNF-α into the medium when incubated with mycoplasmas or with mycoplasma-infected L929 cells. In contrast, uninfected L929 cells do not cause spleen cells to release TNF-α despite the fact that they are sensitive to spleen cell-mediated NC lytic activity. It has been reported that mycoplasma-infected cells express an increased sensitivity to lysis in the presence of spleen cells and that this lysis is mediated by NC activity (6). The results of our analysis indicated that the increased lysis of mycoplasma-infected cells by spleen cells is mediated by the mycoplasma-induced release of TNF-α from spleen cells and probably does not require the recognition of targets by NC effectors. Since our experiments indicate that the release of TNF-α from spleen cells is not required for the NC lysis of some cells, we suggest that NC cells use a cell-associated TNF-α to mediate lysis. Additionally, these results suggest that the release of TNF-α is involved in host protection against infection with mycoplasmas.

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spontaneous release of $^{51}$Cr from all targets ranged between 26 and 34% of the total.

**Culture supernatant fluid and reagents.** Supernatant fluids from L929(A) and L929(S) were obtained from 20-h-old cultures established by plating cells in 60-mm dishes containing 5 ml of RPMI 1640 medium supplemented as previously described (5). Supernatant fluids from pooled spleen cells from two mice that were not infected with mycoplasmas were obtained from cultures of 2 x $10^7$ BALB/c spleen cells plated in 60-mm dishes containing 2 ml of supplemented RPMI 1640 medium. Supernatant fluids from spleen cells cultured with medium in which L929(S) had been grown were obtained by incubating 2 x $10^7$ BALB/c spleen cells in 60-mm dishes containing 1 ml of fresh medium and 1 ml of medium from 3-day-old cultures of L929(S). Supernatant fluids from cocultures of spleen cells and L929(S) or L929(A) were obtained from cultures initially plated with 1 x $10^5$ L929 and 2 x $10^7$ BALB/c spleen cells in 60-mm dishes containing 2 ml of supplemented RPMI 1640 medium. Unless otherwise indicated, all supernatant fluids were obtained after 20 h of incubation at 37°C in humidified air containing 5% CO$_2$. Cultures containing spleen cells were incubated on a rocking platform. Supernatant fluids were used after they were centrifuged two times at 200 x g to remove cells.

Pure cultures of mycoplasmas were obtained by inoculating Mycotrim plates (Hanna Biological, Alameda, Calif.) as described by the manufacturer for the detection of mycoplasmas in mammalian cell cultures. Five days after the Mycotrim plates were inoculated, mycoplasma colonies were scraped from the agar plates with a 1-mm loop and incubated with or without spleen cells as described above. Supernatant fluids were collected from these cultures as described above.

rTNF-α was obtained from Cetus Corp. (Emeryville, Calif.). We refer to units of TNF-α as originally determined by Cetus Corp. Rabbit anti-murine TNF-α sera and normal rabbit serum were provided by Genentech, Inc. (South San Francisco, Calif.). The neutralizing activity of the anti-TNF-α antiserum was determined with recombinant murine TNF-α.

**Statistics.** The percent specific lysis of targets by rTNF-α or supernatant fluids was compared by Student $t$ tests, and the percent specific lysis of targets incubated with spleen cells was compared by analysis of covariance.

## RESULTS

**L929 cells infected with mycoplasmas are lysed to a greater extent in the presence of spleen cells than are uninfected cells.** We have previously shown that the cell line L929 is sensitive to NC- and TNF-α-mediated lysis (11). To determine whether the infection of these cells with mycoplasmas affects the extent to which they are lysed in the presence of spleen cells, infected and uninfected L929 cell lines were incubated with spleen cells and the level of lysis was determined after 14 h of incubation. The cell line L929(A) is not infected with mycoplasmas; the cell line L929(S) was discovered to be infected with mycoplasmas after being maintained in our laboratory for several months. The medium in which L929(S) cells have been grown contains mycoplasmas and was used to infect L929(A) cells. The mycoplasma-infected L929(A) cells are referred to as L929(A)-I. Subpopulations of the L929(A)-I cell line and the L929(S) cell line were cured of the mycoplasma infection (see Materials and Methods); these cured cell lines are referred to as L929(A)-I-C and L929(S)-C. The two mycoplasma-infected cell lines, L929(A)-I and L929(S), were lysed to a significantly greater extent ($P < 0.001$) in the presence of spleen cells than were the uninfected cell lines, L929(A), L929(A)-I-C, and L929(S)-C (Fig. 1). By comparing the number of spleen cells required to give an equivalent level of lysis, we found that there was between 10 and 30 times more lytic activity against infected cells than against uninfected cells. These results suggest that mycoplasma infection has a marked effect on either the sensitivity of L929 cells to NC-mediated lysis or the level of lytic activity in these cultures.

**Mycoplasmas cause spleen cells to release TNF-α.** We next did experiments to ascertain whether the increased spleen cell activity against mycoplasma-infected L929 cells was cell mediated or mediated by the release of a diffusible factor. Also, because mycoplasma-infected cells release free mycoplasmas into the culture medium, experiments were performed to determine whether the increased lytic activity requires, in addition to the presence of mycoplasmas, the recognition of L929 target cells. Supernatant fluids from cultures containing only L929(A), L929(S), or spleen cells did not lyse L929(A) cells (Table 1). Moreover, the supernatant fluid from spleen cells incubated with L929(A) did not contain detectable lytic activity. This latter finding is in contrast to similar experiments by others using WEHI-164 cells incubated with spleen cells, in which high levels of TNF-α were detected in the medium (8, 9). However, supernatant fluid from the incubation of spleen cells with the L929(S) cells (mycoplasma infected) did contain a diffusible factor that lysed L929(A) cells. It was not necessary that mycoplasma-infected cells be incubated with spleen cells for the supernatant to contain cytotolytic activity; the supernatant fluid from spleen cells incubated with medium containing mycoplasmas [i.e., medium in which L929(S) cells had been cultured] or pure cultures of mycoplasmas incubated with spleen cells was also able to affect the lysis of L929(A) cells (Table 1). These results show that the lytic activity of these supernatant fluids is derived from spleen cells and is only
produced when mycoplasmas (or mycoplasma-infected cells) are present.

Rabbit anti-TNF-α antiserum containing enough antibody to neutralize 10 units of recombinant murine TNF-α was able to block the lytic activity of NC effectors (3) and the activity of the supernatant fluids (Table 2). Normal rabbit serum did not block the activity of the lytic supernatant fluids (data not shown). These results indicate that TNF-α is responsible for the lytic activity of supernatant fluids and that in the presence of mycoplasmas, spleen cells release TNF-α. By comparing the lytic activity of rTNF-α with that of the supernatant fluid prepared by incubation of L929(S) cells (or mycoplasmas grown in pure culture) and spleen cells, we determined that supernatant fluid contains 1 to 2 units of TNF-α per ml (Table 2).

**Infection with mycoplasmas does not increase the sensitivity of cells to lysis by TNF-α.** By assaying the lytic activity of culture supernatant fluids, we showed that mycoplasmas cause the release of TNF-α from spleen cells. It is likely that the release of TNF-α accounts for the increased lysis of mycoplasma-infected cells as compared with uninfected cells. However, it is also conceivable that the infection of cells with mycoplasmas makes them more sensitive to NC-mediated lysis, as has been suggested by others (6); such an increase in sensitivity could be caused by an increase in TNF-α receptors or by affecting some other step in the TNF-α-mediated lytic process. Because NC activity is mediated by TNF-α (3, 8, 9, 11), if infection with mycoplasmas causes cells to be more sensitive to NC-mediated lysis, then infection with mycoplasmas would also cause them to be

![Graph](A)![Graph](B)

FIG. 2. Mycoplasma-infected cells are lysed to a greater extent than uninfected cells when incubated with spleen cells (A), but not when incubated with rTNF-α (B). Symbols: ○, L929(A) cells; ▲, L929(S) cells; Δ, L929(S)-C cells. The data represent the mean of triplicate samples, and the standard deviation of the mean for all data is less than 10%.

come more sensitive to TNF-α. Although the mycoplasma-infected cell line L929(S) was lysed to a greater extent by spleen cells than either L929(S)-C or L929(A) cells, it was not more sensitive to lysis by rTNF-α (Fig. 2). This indicates that infection with mycoplasmas does not result in increased sensitivity to TNF-α. Further, because NC activity is medi-

### TABLE 1. Mycoplasmas and mycoplasma-infected L929 cells induce the release of a cytotoxic factor from spleen cells

<table>
<thead>
<tr>
<th>Source of supernatant fluid</th>
<th>% Specific lysis with the following percentage of supernatant fluid in the assay:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25%</td>
</tr>
<tr>
<td>L929(A) cells</td>
<td>NDa</td>
</tr>
<tr>
<td>L929(S) cells</td>
<td>0</td>
</tr>
<tr>
<td>Spleen cells</td>
<td>0</td>
</tr>
<tr>
<td>L929(A) + spleen cells</td>
<td>4</td>
</tr>
<tr>
<td>L929(S) + spleen cells</td>
<td>24b</td>
</tr>
<tr>
<td>L929(S) supernatant + spleen cells</td>
<td>26b</td>
</tr>
<tr>
<td>Mycoplasmas</td>
<td>ND</td>
</tr>
<tr>
<td>Mycoplasmas + spleen cells</td>
<td>ND</td>
</tr>
</tbody>
</table>

a ND, Not determined. 
b The probability that the percent specific lysis of control [L929(A)] equals the percent specific lysis of experimental; P < 0.001.

### TABLE 2. Anti-TNF-α serum blocks the lytic activity of supernatant fluids prepared by cocultivation of mycoplasma-infected cells and spleen cells

<table>
<thead>
<tr>
<th>Source of lytic activity</th>
<th>Anti-TNF-α antibody</th>
<th>% Specific lysis with the following percentage of supernatant fluid in the assay:</th>
<th>With the following no. of units of TNF-α per well:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5%</td>
<td>50%</td>
</tr>
<tr>
<td>Supernatant from L929(A) + spleen cells</td>
<td>–</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Supernatant from L929(S) + spleen cells</td>
<td>–</td>
<td>7</td>
<td>61a</td>
</tr>
<tr>
<td>Supernatant from L929(S) + spleen cells</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>rTNF-α</td>
<td>–</td>
<td>5</td>
<td>11</td>
</tr>
</tbody>
</table>

a The probability that the percent specific lysis of control [L929(A) + spleen cells] equals the percent specific lysis of experimental; P < 0.001.
ated by TNF-α, this result suggests that infection with mycoplasmas does not increase sensitivity of infected cells to lysis by NC activity.

**DISCUSSION**

It is now well established that NC effector cells utilize TNF-α to lyse sensitive cells (3, 8, 9, 11). Because TNF-α has been identified in the supernatant fluids from spleen cells incubated with some NC-sensitive cells, it has been suggested that NC cells mediate cytotoxicity by releasing TNF-α, which in turn lyases sensitive cells (8, 9). The data presented here show that NC-sensitive L929 cells that are not infected with mycoplasmas do not induce the release of TNF-α from spleen cells (Table 1). However, mycoplasma-infected L929 cells or mycoplasmas alone caused a significant amount TNF-α to be released from spleen cells (Table 1). The amount of TNF-α released in a 20-h period (1 to 2 units/ml) is enough to cause a high level of lysis of NC-sensitive cells (Table 2). Our inability to detect the release of free TNF-α from spleen cells incubated with L929(A) cells indicates that NC activity is not necessarily mediated by freely diffusible TNF-α and that a cell-associated TNF-α similar to that described for activated macrophages (1, 4, 7) might mediate NC activity.

It has been reported that infection of cells with mycoplasmas increases their sensitivity to NC-mediated lysis (6). Because lysis by NC effector cells is mediated by TNF-α (3, 8, 9, 11), if infection with mycoplasmas increases the sensitivity of infected cells to NC-mediated lysis, it should also increase their sensitivity to lysis mediated by TNF-α. However, we observed no difference between the sensitivity of mycoplasma-infected and uninfected cells when lysis was mediated only by free TNF-α (Fig. 2B). Thus, the higher level of lysis of mycoplasma-infected versus uninfected L929 cells (Fig. 1) is probably not due to an increased sensitivity of the infected cells to NC- or TNF-α-mediated lysis; more likely, the lysis of mycoplasma-infected cells in the presence of spleen cells is due to the combination of NC activity and the mycoplasma-induced release of TNF-α. Because uninfected L929 cells do not cause the release of TNF-α, their lysis by spleen cells is mediated only by NC effectors and is, therefore, less than that of the mycoplasma-infected cells. Because macrophages have been shown to secrete TNF-α in response to a variety of stimuli (4, 12), it is possible that the TNF-α released in response to mycoplasmas is derived from a monocytic subpopulation of the spleen cells. It is, however, also possible that while NC cells do not release TNF-α in response to NC-sensitive cells, they do release TNF-α in response to mycoplasmas.

Similar experiments with another NC-sensitive cell line, 10ME (3, 11), which is not infected with mycoplasmas, also showed that TNF-α is not released by spleen cells despite the fact that 10ME is lysed by spleen (NC) cells (data not shown). Because we tested only a limited number of NC-sensitive cell lines, we cannot determine whether these are general properties of all NC-sensitive cell lines. Nevertheless, the fact that L929(A), L929(S)-C, and 10ME are NC sensitive yet do not cause the release of TNF-α from spleen cells indicates that some cells can be NC sensitive without being infected with mycoplasmas and that for these NC-sensitive cells to be lysed by NC effector cells, it is not necessary that TNF-α be released from NC effector cells.

The data presented here do not directly address the role of TNF-α as an in vivo protection mechanism against mycoplasma infection. Indeed, we do not know whether the in vitro release of TNF-α by spleen cells is induced by all species of mycoplasmas or whether the mycoplasma species involved in this study is pathogenic. Nonetheless, our findings are consistent with the hypothesis that in vivo, cells of the immune system respond to mycoplasmas by releasing TNF-α and that TNF-α may be either directly or indirectly involved in the elimination of the mycoplasmas.

**ACKNOWLEDGMENT**

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**LITERATURE CITED**