Reduction in Cellular Tumorogenicity after Mycoplasma Infection and Elimination of Mycoplasma from Infected Cultures by Passage in Nude Mice

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SUMMARY

The mouse L-cell derivative A9, deficient in hypoxanthine phosphoribosyltransferase and previously shown to be highly tumorigenic in nude mice, was infected deliberately with known mycoplasma species and transplanted in nude mice in order to determine the effect of the adventitious organisms on tumor initiation and growth rate. Two of the most common mycoplasma strains found in contaminated cell cultures, Mycoplasma hyorhinis and Acholeplasma laidlawii, have been used, since both of these possess high levels of endogenous hypoxanthine phosphoribosyltransferase activity that can be utilized as a quantitative measure of cell contamination. The presence of mycoplasma particles in the infected cultures was verified independently by electron microscopy and a fluorescent staining technique. The results indicate that contamination of cell lines by mycoplasma can significantly alter the tumor initiation and growth rate of normally tumorigenic cells injected in nude mice. In addition, at least for the 2 mycoplasma strains described here, the passage of contaminated cells in nude mice invariably resulted in their complete elimination from the cultures. Since many animal cell lines are tumorigenic in nude mice, this observation suggests that passaging cells in vivo in nude mice may be a simple and effective means of obtaining mycoplasma-free cells from contaminated cell cultures.

INTRODUCTION

Inadvertent contamination of animal cell cultures by mycoplasmas is a widespread problem that often complicates genetic and biochemical studies on cellular metabolism and function (21). Especially troublesome are those species of mycoplasma that cause no overt cytopathic effects on the parasitized cells, are difficult to detect by the agar plate test (8), and yet can significantly influence many aspects of cellular physiology (26). For instance, a few select strains of Mycoplasma hyorhinis cannot be cultured in the absence of animal cells (9), and such mycoplasmas undoubtedly remain unrecognized in many laboratories. In 1 recent survey involving more than 3000 cultures submitted from various laboratories, it has been reported that at least 8% of all cultures were contaminated with mycoplasma (11).

Elimination of mycoplasmas from contaminated cultures has been achieved by treatment with antibiotics (13) or various chemical agents (18, 23), but mycoplasmas can develop resistance to each of these agents (21). Specific antisera can also be used for successful elimination of mycoplasma from cell cultures (12). This last method, however, requires the prior identification of the contaminating mycoplasma species and the availability of a specific antiserum. In any case, at least with some strains of M. hyorhinis, a specific antiserum to the mycoplasma can prevent the initiation of a new round of infection but it cannot eliminate one that is already established (Ref. 26; G. J. McGarrity, personal communication).

Here we report a potentially useful technique of eliminating mycoplasmas from contaminated animal cell cultures by transplantation in the immunodeficient nude mouse. Nude mice are genetically devoid of cell-mediated immune functions but still are capable of recognizing foreign antigens and mounting a humoral immune response. Although highly susceptible to pathogenic microorganisms, these mice are by no means incapable of a minimum level of defense against at least some of them (14). In fact, a relatively large colony of nude mice has been maintained in our laboratory under isolated but nonsterile conditions in which the presence of a spectrum of microbial organisms was demonstrated.

In previous studies, it has been shown that there exists a general correlation between the ability of cells in vitro to form growing colonies in a semisolid medium and their ability to produce tumors in vivo when injected into nude mice (6, 19). Heteroploid animal cell lines, the majority of which are able to grow in semisolid medium containing soft agar or methyl cellulose and are tumorogenic in nude mice, can be transplanted in these mice and grown to large tumors in a relatively short period of time without causing alterations in the genetic identity of the transplanted cells (5).

Fogh (4) reported earlier that malignant human cells infected with certain strains of mycoplasma are less tumorogenic than the uncontaminated cells when injected in the...
Syrian hamster cheek pouch. However, these tumors always regressed completely, and thus it was not possible to recover the original human cells for further analysis after they had proliferated in the heterologous host. In contrast, tumors produced by injected cells in nude mice do not as a rule regress, and pure cell cultures free of the host cells can easily be reestablished from the tumors for genetic and biochemical characterization (5, 6).

It seemed probable that the transplantation in nude mice of tumorigenic cell lines contaminated with mycoplasma might result in the growth of the animal cells as tumors, while at the same time the host defense mechanisms selectively prevented the proliferation of the adventitious microorganism or even eliminated it entirely. To test this possibility, we deliberately infected the mouse A9 cells (10) with 2 common species of mycoplasma that are found in contaminated cell cultures and we transplanted the cells in nude mice.

We have taken advantage of the fact that the mouse A9 cells contain barely detectable levels of HPRT* (EC 2.4.2.8.) activity, whereas both M. hyorhinis and Acholeplasma laidlawii have high levels of endogenous HPRT activity that can be utilized as a quantitative indicator of their presence in cell cultures (26). In addition, scanning electron microscopy (26) and the fluorescence staining technique described by Chen (2) were used to monitor the presence of mycoplasma particles in the cultures. We have shown previously that these 3 techniques together provide an extremely sensitive and accurate measure of mycoplasma contamination in A9 cells that can be used to detect a low level of infection which otherwise escaped conventional diagnostic tests for mycoplasma (26).

Our results show that A9 cells deliberately infected with mycoplasmas have significantly reduced tumor-forming capacity. Passage of these cells in nude mice invariably results in the complete elimination of the mycoplasmas from the infected cells.

MATERIALS AND METHODS

Cell Culture Conditions. All cultures were maintained in McCoy’s Medium 5a (Grand Island Biological Co., Grand Island, N. Y.) plus 10% fetal bovine serum prescreened for the absence of mycoplasma. No antibiotics were used.

Standard Strains of Mycoplasma. M. hyorhinis and A. laidlawii strains used in this study were kindly provided by Dr. Gerard J. McGarrity, Institute for Medical Research, Camden, N. J.

The Nude Mouse Colony. The nude mouse colony was established from a stock backcrossed in BALB/c and maintained in sterile-air laminar-flow cage racks (Lab Products, Inc., Garfield, N. J.), as described previously (6).

Transplantation and Reinitiation of Cell Lines after Passage in Nude Mice. These procedures have been described previously (5, 6). Briefly, cells were trypsinized, counted, and resuspended in phosphate-buffered saline; and 0.2-ml aliquots were injected s.c. at a single site. Trypsinization reduced the cell-associated mycoplasma titers considerably but did not eliminate them completely from the cells. The original maximal titers were regained within 4 days after trypsinization if the cells were kept in culture (26). When progressively growing tumors developed in injected mice, tumors were removed aseptically from the animals, washed in phosphate-buffered saline, minced, and sieved through a stainless steel mesh to produce single-cell preparations. The cells were then plated in the culture medium. Unattached cells were washed away after overnight incubation. Vigorous monolayer cultures were obtained in all cases.

Assay of HPRT. Confluent cell cultures grown in 100-mm Petri dishes (Falcon Plastics, Oxnard, Calif.) were trypsinized and divided 1/20. After 4 days, when the cultures reached confluency again, culture medium was removed by suction, and the cells were lysed in situ with the addition of 0.2 ml of 20 mM potassium phosphate buffer, pH 7.6, containing 5 mM MgCl₂, 5 mM β-mercaptoethanol, and 1% Triton X-100 (17). HPRT activity in the lysate was then measured with [¹⁴C]hypoxanthine according to the DEAE-cellulose paper chromatography method previously described (20).

Electron Microscopy. For scanning electron microscopy, cells were grown on glass coverslips, fixed in 2.5% collidine-buffered glutaraldehyde, dehydrated in ethanol series, and transferred to acetone. Coverslips were critical-point dried with liquid CO₂ in a Sorvall Critical Point Drying System, coated with gold with an Edwards 306 coater, and viewed in an ETEC Autoscan. For transmission electron microscopy, cells were grown on Falcon plastic Petri dishes. Cells were fixed in 2.5% collidine-buffered glutaraldehyde, postfixed in 1% OsO₄, dehydrated through alcohol, and embedded in Epon.

Staining of Cells with Bisbenzimid (Hoechst 33258) for Mycoplasma. Cells were seeded on glass coverslips and allowed to incubate for at least 4 days before examination to maximize the mycoplasma titer. The procedures for fixing and staining with the bisbenzimid dye (Hoechst 33258) described by Chen (2) were followed. The stained cells were screened under a Zeiss fluorescence microscope.

RESULTS

Tumorigenicity of A9 Cells Deliberately Infected with Mycoplasma. Uninfected control A9 cells are highly tumorigenic in nude mice and produce tumors in 100% of animals given injections of at least 10⁶ cells/mouse. At lower cell doses, the frequency of tumor formation decreases, but as few as 100 cells/mouse can still produce tumors in about one-half of the injection-treated mice (5). As Table 1 demonstrates, the lag time between the injection of cells and the first appearance of a tumor nodule is also a function of the cell dose. When 10⁶ A9 cells were injected, for instance, a detectable nodule appeared at the site of injection within 7 to 13 days; but when 10⁸ cells were injected, this lag time increased to 21 to 28 days. Since the cell-doubling time for
A9, at least in vitro, is about 16 hr, this increase in lag time is unlikely to be due solely to the time it takes for the $10^6$ injected cells to expand to $10^9$ cells.

More significantly, however, data given in Table 1 show that A9 cells deliberately infected with M. hyorhinis (A9Mh) and A. laidlawii (A9AI) have a slightly longer lag time for tumor initiation and significantly slower tumor growth compared to the uninfected controls. In fact, tumors developed from A9Mh cells all became growth arrested and never reached the usual large sizes regularly observed with the uninfected A9 cells. Many of the mice given injections of mycoplasma-infected A9 cells developed cachexia and a severe hepatitis-like syndrome. A similar wasting syndrome has been described previously in nude mice infected with M. hyorhinis on A. laidlawii. The increase in this HPRT activity is correlated with the increase in the number of mycoplasma particles in the culture as determined by scanning electron microscopy on the DNA-specific binding of the fluorescent dye Hoechst 33258 (see Fig. 4).

Changes in A9 Cells following Infection with M. hyorhinis and A. laidlawii. Both M. hyorhinis and A. laidlawii can establish a persistent covert infection in A9 cells without causing detectable alterations in cell-doubling time, plating efficiency, or gross morphology when examined under the low-magnification phase optics (O. P. van Diggelen and S. Shim, unpublished observations). However, when glutaraldehyde-fixed cells are examined by electron microscopy, striking changes in the cell surface morphology are evident (Figs. 1 and 2).

In Fig. 1 are the scanning and thin-section electron micrographs of A9Mh cells, which were infected with M. hyorhinis 4 days previously and reached the maximal titer of the mycoplasma particles as determined by mycoplasma-specific HPRT activity (see below). The most striking feature of A9Mh cells is that the exposed surfaces of all interphase cells are literally saturated with the pleomorphic, roughly spherical particles (Fig. 1, A to D), which are present only on the external side of the cell membrane and have diffuse non-electron-dense cores characteristic of mycoplasmas (Fig. 1, E and F) (1). These particles can be released from the cells by a gentle trypsinization, and the culture medium can contain as many as $10^9$ infectious particles per ml 4 days after the cultures are freshly infected with mycoplasma.

A9AI cells, A9 cells infected with A. laidlawii, are shown (Fig. 2, A and B). There are numerous particles on the cell-free areas of the glass coverslips on which the cells were grown, but compared to A9Mh cells, there are fewer particles in actual contact with the cell membrane. This difference between A9Mh and A9AI cells probably reflects the fact that, whereas A. laidlawii can be isolated easily as pure cultures in the liquid broth medium for mycoplasma (11), M. hyorhinis often has an absolute requirement for animal cells for growth (9).

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Both of these mycoplasma strains possess high levels of endogenous HPRT activity (22), which can be distinguished from the mouse HPRT by several criteria. We have therefore used this enzyme activity as a quantitative measure of the infection of A9 cells by the 2 strains of mycoplasma. As shown in Table 2, uninfected control A9 cells have negligible HPRT activity but acquire the mycoplasma-specific HPRT activity following a deliberate infection with M. hyorhinis or A. laidlawii. The increase in this HPRT activity is correlated with the increase in the number of mycoplasma particles in the culture as determined by scanning electron microscopy or the DNA-specific binding of the fluorescent dye Hoechst 33258 (see Fig. 4).

Elimination of Mycoplasmas from Infected Cells by Passage in Nude Mice. A9Mh and A9AI cells were infected into nude mice, and tumors that developed were removed from the mice and processed to reinitiate cultures. A total of 9 tumor-derived cultures, 4 from the A9Mh series and 5 from the A9AI series injected with $10^6$ cells/mouse (see Table 1), were examined for evidence of mycoplasma by HPRT activity, DNA staining, and electron microscopy. Results are presented in the lower one-half of Table 2, which demonstrates that each of the 9 cultures reestablished from tumors is completely devoid of mycoplasma particles. These cultures remained free of mycoplasma until the experiment was terminated 4 to 8 weeks later, providing a definite

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin of cell line</th>
<th>No. of cells injected/mouse</th>
<th>Days before tumor first detected</th>
<th>Final wt of tumor (g)*</th>
<th>Duration of experiment (days)*</th>
<th>No. of mice that developed tumor/no. injected</th>
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<tr>
<td>A9</td>
<td>Uninfected control</td>
<td>$10^3$</td>
<td>21-28</td>
<td>3</td>
<td>50</td>
<td>3/5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^4$</td>
<td>15-19</td>
<td>3</td>
<td>45</td>
<td>10/10</td>
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<tr>
<td></td>
<td></td>
<td>$10^5$</td>
<td>10-20</td>
<td>5</td>
<td>40</td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$10^6$</td>
<td>7-13</td>
<td>11-11</td>
<td>35</td>
<td>15/15</td>
</tr>
<tr>
<td>A9Mh</td>
<td>A9, infected with M. hyorhinis</td>
<td>$2 \times 10^6$</td>
<td>14-22</td>
<td>0.04-0.2</td>
<td>38-52</td>
<td>4/6</td>
</tr>
<tr>
<td>A9AI</td>
<td>A9, infected with A. laidlawii</td>
<td>$10^6$</td>
<td>10-16</td>
<td>0.2-1.5</td>
<td>33</td>
<td>5/5</td>
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<td>10, 16</td>
<td>1.6, 2.0</td>
<td>35</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4 \times 10^6$</td>
<td>12, 14</td>
<td>0.3, 2.5</td>
<td>36</td>
<td>2/2</td>
</tr>
</tbody>
</table>

* Final weight of tumors when each series was terminated. Single values represent the average of the group; otherwise, the range of weights is given.

* Days postinjection, when the tumors were recovered or the experiment was terminated.
Elimination of mycoplasmas from infected A9 cells by passage in nude mice

The absence of mycoplasma particles from the cells recovered after the passage in nude mice is particularly evident when the "cured" cells are compared to the original infected cells by scanning electron microscopy. Fig. 3 shows the scanning electron micrographs of A9Mh-nu1. A comparison with A9Mh cells shown in Fig. 1 demonstrates that a single passage of A9Mh cells in the nude mouse has resulted in the complete elimination of the M. hyorhinis particles from the culture. Essentially identical results were obtained also with the A9Al cells; Fig. 2, C and D, shows the mycoplasma-free A9Al-nu1 cells derived from a tumor of A9Al cells, shown in Fig. 2, A and B. The scanning electron microscopy data have been confirmed independently by the fluorescent staining of mycoplasma, demonstrated in Fig. 4 for A9Mh-nu1 and A9Mh.

When the "cured" cells recovered from the tumors, A9Mh-nu1 and A9Al-nu1, were once again injected in nude mice, the lag time for tumor initiation and subsequent tumor growth rate were now comparable to those of the uninfected control A9 cells. Therefore the failure or delay in tumor growth following the 1st injection of A9Mh and A9Al cells is due to the presence of mycoplasmas in the cultures and is not caused by an irreversible change in cellular tumorigenicity.

DISCUSSION

Our observation that mycoplasma-infected A9 cells have a markedly reduced tumorigenic potential compared to the uninfected controls confirms and extends the earlier report by Fogh (4), who studied the tumor formation by a group of human cell lines in the cheek pouch of cortisone-treated young Syrian hamsters. In Fogh's study, however, even the uninfected cells produced only small tumors that eventually regressed completely. Progressively growing lethal tumors were apparently never produced. It is thus unclear whether the growth of mycoplasma-infected xenogeneic cells in immunologically protected sites such as hamster cheek pouch or in artificially immune-suppressed hosts will also result in the elimination of mycoplasmas.

Nude mice have often been used for analysis of cellular tumorigenicity and malignant transformation (6, 19, 24, 25) and as carriers of heterologous tumor transplants in a number of laboratories (7, 16). Inadvertent contamination of the transplanted cells by mycoplasmas may significantly lower the incidence of tumor formation even when the cells are otherwise highly tumorigenic.

Our data presented above show that a single passage of A9 cells infected with 2 common species of mycoplasmas may result in the complete elimination of the microorganism from the animal cells (9 of 9 trials). Whether this observation is applicable to other cell lines as well as to other species of mycoplasma remains to be established. The nude mouse, once restricted to a few breeding laboratories with a pathogen-free environment, is becoming more widely available. The ability to rescue a critical cell line from a contaminated culture by using the relatively simple procedure reported here may prove to be of general usefulness.

ACKNOWLEDGMENTS

We wish to thank Dr. Gerard J. McGarrity for many helpful discussions and for the standard strains of mycoplasma used in this study, and André Brown and Susan Mahler for expert technical assistance.
REFERENCES


Fig. 1. Electron micrographs of A9Mh. Mouse A9 cells were deliberately infected with M. *hyorhinis*, allowed to reach the saturating level of mycoplasma titer, and fixed and stained as described in "Materials and Methods." A to D, scanning electron micrographs of cells grown on glass coverslips. Two pairs of recently divided cells can be seen in C; they appear to have fewer mycoplasma particles attached to the cell surface. E and F, same cells in thin sections, fixed *in situ* on plastic Petri dish.
Fig. 2. Scanning electron micrographs of A9AI (A9 cells deliberately infected with A. laidlawii) (A and B), and of A9AI-nu1 cells derived from a tumor produced by injection of A9AI cells in a nude mouse (C and D). External blebs sometimes appear on uninfected cells, as in D, but these structures can be distinguished from the spherical mycoplasma particles, which are smaller and more uniform in size and shape.
Fig. 3. Scanning electron micrographs of A9Mh-nu1 cells, derived from A9Mh cells after a single passage in nude mice. Note the striking difference in surface morphology compared with the original infected cells that are shown in Fig. 1.

Fig. 4. Fluorescence micrographs of the "cured" A9Mh-nu1 cells derived from a tumor (A) and the original mycoplasma-infected A9Mh cells before injection into nude mice (B). Cells were grown for 4 days on glass coverslips, fixed, and stained with the DNA-specific dye Hoechst 33258. Mycoplasmas appear as brightly fluorescent particles localized exclusively in extracellular space, while mycoplasma-free cells show only nuclear fluorescence.
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