Selective association of murine T lymphoblastoid cell surface alloantigens with *Mycoplasma hyorhinis*

(Thy-1.1 alloantigen/H-2 histocompatibility antigen/murine leukemia virus/cell surface receptors)

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**ABSTRACT** *Mycoplasma hyorhinis*, isolated by isopycnic centrifugation from supernatants of a persistently infected murine T lymphoblastoid cell line, demonstrated the presence of the Thy-1.1 differentiation alloantigen and H-2K*^b^* histo compatibility antigens. The murine leukemia virus-related gp70 antigen also present on the surface of these lymphoblastoid cells was absent from mycoplasma preparations. Quantitative assessment of Thy-1.1 present in preparations of *M. hyorhinis* revealed a specific activity greater or equal to that of membrane preparations from lymphoblastoid cells, suggesting a marked accumulation of this T lymphocyte antigen by membrane-associated mycoplasmas. The accumulation of the Thy-1,1 antigen in association with purified mycoplasmas was also demonstrated in lymphoblastoid cells experimentally infected with a defined culture of *M. hyorhinis*.

Mycoplasmas produce a wide variety of diseases in animals and man, including pneumonia, arthritis, and disorders of the urogenital tract (1). Many of these diseases share the common characteristics of chronicity and autoimmune reactions (2). Two hallmarks of these agents are avoidance of the immune response and close association with host cell surfaces (2). *Mycoplasma hyorhinis*, which causes chronic arthritis of swine, exhibits both of these features (3, 4). This organism is also one of the major contaminants of many established cell lines (5).

A murine lymphoblastoid cell line chronically infected with *M. hyorhinis* has provided an ideal in vitro model to explore two aspects of mycoplasmal infections which may be relevant to the immunological features of chronic disease in vivo. First, quantitation of a number of antigens associated with mycoplasmas isolated from these cultures suggests that these organisms are capable of acquiring certain host cell surface antigens. Second, evidence is presented suggesting that the Thy-1.1 T cell differentiation alloantigen may be highly concentrated in association with these organisms. Mycoplasmal acquisition of host antigens, particularly those of the T cell surface, may represent important mechanisms by which these agents avoid or alter the host immunological response. This process most likely depends on the intimate interaction between these organisms and the host cell membrane.

**MATERIALS AND METHODS**

Mycoplasma-Infected Lymphoid Cells. The acquisition and maintenance of the BW5147 murine T lymphoblastoid cell line has been described (6-9), as well as the expression on these cells of the cell surface antigens Thy-1.1 (6, 10), H-2K*^b^* (10, 11), gp70 (12), and Gross cell surface antigen, GCSA (12, 13).

After several passages in our laboratory, BW5147 cells were screened for mycoplasmas by (i) standard culture procedures (14), (ii) inoculation of a 3T6 indicator cell line for isolation and detection of fastidious and cell-adapted mycoplasma strains (5, 14), (iii) fluorescent bisbenzimide staining (15), (iv) electron and immunofluorescent microscopy (14), and (v) the uridine-uracil assay (16) modified for lymphoblastoid cell cultures. The cells were shown to be mycoplasma positive by all of the procedures except cultivation on artificial media. Inoculation of BW5147 cells onto an indicator cell line and use of an unnocculated indicator culture as a negative control enabled detection of mycoplasmas by fluorescent DNA staining (15) and provided confirmation for identification of mycoplasmas using standard typing sera (National Institutes of Health Research Resource Branch) in an indirect immunofluorescent assay (14). The contaminating strain was identified by the latter technique as *M. hyorhinis* strain GDL both in our laboratory and that of R. A. Del Giudice (Frederick Cancer Center, Frederick, MD). Unsuccessful attempts in both laboratories to cultivate the organism on artificial media indicated that this particular strain of *M. hyorhinis* was "non-cultivable." The "cell-dependence" of this strain was not surprising because *M. hyorhinis* detected in many chronically infected cell lines commonly demonstrate this property (5, 14). This mycoplasma-infected BW5147 line is referred to in this report as naturally infected. Separate cultures of BW5147 cells, consistently shown to be free of mycoplasmas, were used as uninfected controls.

Control *M. hyorhinis* Culture. *M. hyorhinis* strain GDL was obtained from R. F. Ross, Iowa State University, Ames, IA, as a lymphoplated culture; it was reconstituted and passaged three times in Hayflick's medium containing 20% horse serum (17). Aliquots of this stock culture containing 10^4.5^ mean colorchanging units (CCU) or ml (18) were stored at -70°. These organisms served as control mycoplasmas, antigenically similar to the mycoplasma detected in BW5147 cultures, but grown in the absence of lymphoid cells. These will be referred to as broth-grown *M. hyorhinis*.

Experimentally Infected BW5147 Cultures. Approximately 1.5 X 10^5 mycoplasma-free BW5147 cells were experimentally infected with 10^4.8^ CCU of broth-grown *M. hyorhinis*. After five serial passages, cells were pelleted at 400 X g for 10 min and resuspended in fresh medium for further passage. Mycoplasmas from culture supernatants were readily cultivated in Hayflick's medium (typically 10^5.8^ CCU/ml) but not in cell-free tissue culture medium.

Antiserum. Mule typing antiserum to *M. hyorhinis* (GDL strain, catalog number M781-511-569) and preimmune mule serum (M-713-501-569) were obtained from the Research Resources Branch, National Institute of Health, Bethesda, MD. Goat antiserum to purified gp70 of Bauscher murine leukemia virus (MuLV) was obtained from the Office of Program Resources and Logistics of the National Cancer Institute through

Abbreviations: MuLV, murine leukemia virus; ADs, absorption dose resulting in 50% reduction in assay reaction; CCU, mean color changing unit.

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R. Wilsnack, Huntingdon Research Center, Brooklandville, MD. Antiserum to Thy-1.1, Thy-1.2, and H-2K<sup>k</sup> antigens have been previously described (6, 10, 11).

Mice and Tumors. AKR/J and C57BL/6J (B6) mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and AKR/Cum mice from Cumberland Farms, Clinton, TN. The E<sup>2</sup> C2 Gross MuLV-induced leukemia of B6 mice was kindly provided by F. A. Boyse (Sloan-Kettering Cancer Center, New York, NY) and maintained in B6 mice.

Cytotoxicity and Inhibition Assays. Cytotoxicity and quantitative absorption assays were performed with slight modifications of procedures previously described for assessment of Thy-1.1 (10), Thy-1.2 (6, 10), H-2K<sup>k</sup> (10, 11), and gp70 (12) antigens. The concentration of absorbing material added to a reaction resulting in a 50% reduction in specific 51Cr release was designated the absorption dose<sub>50</sub> (AD<sub>50</sub>).

Isolation of <i>M. hyorhinis</i> from BW5147 Cultures. BW5147 cells growing in exponential phase (7) and demonstrating greater than 97% viability (by trypsin blue exclusion) were centrifuged at 400 x g for 10 min at 4° to pellet cells. The supernatant was centrifuged at 1800 x g for 60 min at 4°, and the resultant supernatant was further centrifuged at 100,000 x g for 45 min at 4° to pellet mycoplasmas and MuLV virions. This pellet was suspended and applied to a linear potassium tartrate (K tartrate) gradient as previously described (19). After isopycnic centrifugation at 100,000 x g for 90 min at 4°, clearly visible bands were recovered and rinsed by centrifugation at 100,000 x g for 60 min. Pellets were then fixed for electron or fluorescent microscopic studies, or suspended in phosphate-buffered saline for determination of protein (20), or for absorption of antisera used in cytotoxic reactions.

Uridine Labeling of Mycoplasma in BW5147 Cultures. Mycoplasma-infected or uninfected BW5147 cells were grown as described above for 20 hr in exponential phase at a density of 1-2 x 10<sup>6</sup> cells per ml in the presence of [5-<sup>3</sup>H]uridine (New England Nuclear, Boston, MA; specific activity 25 Ci/mmol) at 20 μCi/ml. Mycoplasmas were isolated by isopycnic centrifugation, and fractions were collected and their densities were calculated from refractive indices. A portion (0.2 ml) of each fraction was precipitated with cold 5% trichloroacetic acid onto glass fiber filter discs which were dried, placed in glass scintillation vials, and incubated at 55° for 45 min with 0.2 ml of Protosol (New England Nuclear). After subsequent addition of 5 ml of ScintiPrep scintillation cocktail (Fisher Scientific, Fairlawn, NJ), the radioactivities in the vials were measured in a Delta 300 liquid scintillation counter at approximately 40% efficiency.

RESULTS

Isolation of <i>M. hyorhinis</i> from BW5147 Cultures. The presence of <i>M. hyorhinis</i> in naturally infected BW5147 cells was demonstrated by a number of criteria. Immunofluorescent and electron microscopic analysis showed mycoplasmas intimately associated with the lymphoid cell periphery. In addition, biosynthetic labeling of naturally infected BW5147 cell cultures resulted in the incorporation of [5-<sup>3</sup>H]uridine into supernatant material having a buoyant density on K tartrate gradients of approximately 1.22 g/cm<sup>3</sup>, characteristic of mycoplasmas (21, 22). No labeled material was obtained from identically treated uninfected BW5147 cultures (Fig. 1A). Application of this technique to BW5147 cultures experimentally infected with <i>M. hyorhinis</i> yielded similar results (Fig. 1B). Under conditions identical to those used for naturally infected cultures, mycoplasmas from experimentally infected BW5147 cells showed uridine incorporation into material of the same density. Compared to naturally infected cultures, approximately 1000-fold more label was obtained from the same volume of experimentally labeled cultures, in accord with the much greater quantity of material recovered from these supernatants.

It proved possible to isolate a highly enriched preparation of mycoplasmas from the supernatant of infected BW5147 cell cultures by differential centrifugation followed by isopycnic sedimentation on K tartrate gradients. A pronounced, flocculent band at p = 1.20-1.22 g/cm<sup>3</sup> was clearly separated from a lighter MuLV-containing band at p = 1.15-1.17 g/cm<sup>3</sup>. Transmission electron micrographs prepared from the heavier material revealed a marked predominance of typical mycoplasma structures (Fig. 2A). As a comparison, micrographs of broth-grown <i>M. hyorhinis</i> prepared analogously revealed a very similar morphology (Fig. 2B). The K tartrate gradient-purified mycoplasma from BW5147 culture supernatants demonstrated intense fluorescence when stained with anti-<i>M. hyorhinis</i> antiserum followed by fluorescein-conjugated rabbit anti-equine IgG, whereas treatment with control sera gave negative results. The specific reaction of mycoplasmas isolated from BW5147 cultures with the standard typing serum for <i>M.
hyorhinis further confirmed the strain identification of these organisms.

Lymphoid Cell Surface Antigens Associated with M. hyorhinis Grown in BW5147 Cells. To evaluate the association of lymphoblastoid cell surface antigens with M. hyorhinis recovered from BW5147 cultures, the mycoplasma fraction isolated by K tartrate gradient centrifugation was analyzed by quantitative absorption of cytotoxic reactions for membrane antigens.

Fig. 3 depicts the absorption analysis for the Thy-1.1 antigen. Mycoplasmas isolated from naturally infected cell cultures demonstrated the presence of Thy-1.1 alloantigen, with an AD50 of approximately 15 μg of protein per ml. Broth-grown M. hyorhinis failed to inhibit this reaction significantly at concentrations over 1000 μg/ml. This indicated (i) that the inhibition of cytotoxicity in the Thy-1.1 assay is not due to a component of M. hyorhinis antigenically similar to this T-cell alloantigen and (ii) that M. hyorhinis is not anti-complementary in this assay, because this would appear as an inhibition of complement-dependent cytotoxicity. Furthermore, BW5147 cell-grown M. hyorhinis isolated from K tartrate gradients did not inhibit the standard, analogous cytotoxic reaction for the alternate Thy-1.2 allelic specificity, which is not expressed on BW5147 cells (Fig. 3). This ensured that mycoplasmas isolated from culture supernatants were not themselves anti-complementary, and that the inhibition observed in the Thy-1.1 assay was in fact due to the presence of the alloantigen in the purified mycoplasma material.

The specific activity of Thy-1.1 associated with purified mycoplasmas from naturally infected cultures was strikingly high. As a comparison, multiple determinations utilizing purified membranes prepared from BW5147 cells resulted in an AD50 from 15 μg/ml (shown in Fig. 3) to over 100 μg/ml (10), a specific activity equal to or lower than that of purified mycoplasmas. This suggested that Thy-1.1 may be "concentrated" in these mycoplasma preparations. Interestingly, M. hyorhinis isolated from experimentally infected cultures demonstrated a specific activity nearly identical to that of mycoplasmas from naturally infected cells (Fig. 3). This suggested that acquisition of host cell antigens occurred both with "cell adapted" and with "cultivable" strains of this organism.

The presence of H-2K1 alloantigens in the mycoplasma fraction prepared from BW5147 cultures was similarly demonstrated by quantitative absorption (Fig. 4). Broth-grown M. hyorhinis failed to inhibit this reaction, again arguing against an antigenic relationship between M. hyorhinis and H-2K1 alloantigens, and also against an anti-complementary explanation for this inhibition.

In contrast to H-2K1 and Thy-1.1, the MuLV-related gp70 molecule also residing on the surface of BW5147 cells was not detected in the same mycoplasma preparations even at high protein concentrations (4000 μg/ml) of absorbing material (Fig. 5). The inability to detect this surface marker in mycoplasma preparations from BW5147 cultures initially suggested a selective association of Thy-1.1 and H-2K1 surface antigens with these organisms. The lack of gp70 antigen was not due to its inactivation or removal during the K tartrate gradient purification.
cation procedures used to prepare mycoplasmas, because it was present (i) on purified MuLV virions isolated from the same K tartrate gradient (ρ = 1.15–1.17 g/cm³) used for mycoplasma purification (Fig. 5), and (ii) on membranes prepared from BW5147 cells and purified on gradients (ρ = 1.12–1.13 g/cm³) (Fig. 5). Thus, gp70 was stable to these procedures, and its absence in the mycoplasma fraction could not be attributed to its lability under conditions of purification.

Selective Association of Lymphoid Surface Antigens with M. hyorhinis. The selectivity of antigen association with M. hyorhinis from these cultures could be further established by considering quantitative features of antigen distribution in mycoplasma fractions.

One source of lymphoid cell surface antigens in mycoplasma preparations could have been the presence of membrane fragments in culture supernatants co-isolating with these organisms. Two observations argued against this possibility: (i) The buoyant density of membranes prepared from disrupted BW5147 cells was between 1.12 and 1.135 g/cm³, well separated from the heavier mycoplasma material found at 1.20–1.22 g/cm³ on K tartrate gradients. (ii) When supernatants from uninfected BW5147 cells were processed for mycoplasma purification, only a single band of 1.15 g/cm³ was observed on K tartrate gradients, which contained MuLV particles. No membrane material at lower density or mycoplasma material at higher density was observed.

While co-isolation of membrane fragments was probably not a source of lymphoid cell surface antigens in purified mycoplasmas, the binding of such fragments to mycoplasmas could still have provided a mechanism by which these antigens were acquired. However, the high specific activity of Thy-1.1 in mycoplasma isolated from BW5147 cultures (Fig. 3), equal to that obtained with purified BW5147 membranes, argues against simple binding of membranes to mycoplasmas as a source of this antigen. Furthermore, the lack of cell membrane-associated gp70 in mycoplasma preparations, and the observation that membranes from BW5147 cells completely absorb anti-gp70 sera (Fig. 5) imply that the degree of contaminating membranes (or MuLV particles bearing gp70) would be negligible.

M. hyorhinis as a Surface Constituent of BW5147 Cells. While immunofluorescent and electron microscopy indicated a close association between mycoplasmas and cell periphery, further evidence supporting their interaction was found by the lymphoid cell membrane was provided by studying the complement-dependent cytotoxic properties of anti-mycoplasma antibodies on infected BW5147 cells labeled with 51Cr. Antiserum against M. hyorhinis was cytotoxic for BW5147 cells infected with this organism, whereas no cytotoxicity was observed with normal serum (Fig. 6). When assayed on uninfected BW5147 cells, antiserum to M. hyorhinis showed no cytotoxic activity (Fig. 6). That 51Cr release was due to cell lysis and not to lysis of surface-associated mycoplasmas (which may have accumulated 51Cr) was confirmed by the demonstration that infected cells treated with anti-M. hyorhinis were over 95% dead as determined by trypan blue exclusion (not shown). This demonstrated that a specific cytolytic reaction could be mediated by recognition of M. hyorhinis antigens on the surface of infected BW5147 cells and suggested a close relationship between the host cell membrane and mycoplasma antigens.

DISCUSSION

While the precise events resulting in selective association of host cell surface antigens with M. hyorhinis were not fully revealed by these experiments, the following possible mechanisms can be considered in light of the present findings: (i) Membrane fragments in the supernatant of cell cultures could bind to mycoplasmas after the organisms detach from cells. This seemed unlikely because (a) membrane fragments were not detectable (by K tartrate gradient isopycnic centrifugation) in BW5147 cell culture supernatants from either uninfected or mycoplasma-infected cultures and (b) attachment of membranes should result in the acquisition of all membrane antigens assayed and should not result in the asymmetric distribution of host antigens that was observed in mycoplasma preparations. (ii) Soluble host cell antigens present in the supernatant could selectively bind to mycoplasmas. We have examined the possibility that Thy-1.1 antigen exists in a soluble form in infected BW5147 cell cultures by analysis of material obtained from cell supernatants that were passed through a hollow fiber filter system retaining 100,000-dalton macromolecules and concentrated 4000-fold by further filtration with a 10,000-dalton retention filter and acetone precipitation (procedures known to select for the 25,000-dalton Thy-1.1 molecule, ref. 10). No Thy-1.1 activity was demonstrable in these concentrates. While we have been unable to demonstrate "soluble" Thy-1.1 activity in BW5147 cultures, high molecular weight material (>2 × 10⁶) precipitable by anti-Thy-1 alloantisera from low-speed supernatants of lymphoblastoid cell lines has recently been reported as "shed" Thy-1 (23). In light of our present findings, a cautionary note may be appropriate in interpreting the phenomenon of shedding of macromolecules in any cell culture not rigorously shown to be free of mycoplasmas, even when grown in the presence of putative mycoplasma inhibitors. (iii) The interaction of mycoplasmas with host cell surface components could result in the selective acquisition of host cell membrane antigens. Mycoplasmas are known to attach readily to a variety of cell types, including lymphoid cells, suggesting a strong interaction between cell surface components and constituents on the mycoplasma surface (34). If these lymphoid surface components were laterally mobile in the membrane, multiple molecules could interact with an organism on the cell surface and a "capping" or "patching" phenomenon might.
result (25, 26). This hypothesis provides an attractive mechanism for selective acquisition of host cell surface components because the area adjacent to surface-associated mycoplasmas would necessarily be enriched in mobile receptor molecules interacting with multivalent organisms. This explanation would also provide a means of concentrating antigens that are associated with mycoplasmas on the cell surface. It has been reported that the predominant Thy-1 molecule of T lymphoid cells can be "capped" and is thus laterally mobile (26). Within this context, the large quantities of Thy-i. cells can be "capped" and is thus laterally mobile (26). This explanation would account for the antigenic diversity associated with multivalent organisms. This explanation would also explain the association of these organisms with the cell surface where virus particles are present. Mycoplasma antigens may reflect an interaction between the cell surface and mycoplasmas. Antiserum to M. hyorhinis has shown to inhibit the interaction of these antigens with Thy-i. cells, an observation suggesting that these antigens are coordinate. In MuLV-producing cells, the viral envelope antigen has been shown to reside both on budding virions (28) and in areas of the cell surface where virus particles are absent (29). Lack of this antigen in mycoplasma preparations may reflect an interaction of the organisms with areas of the cell surface devoid of this viral component. It has been postulated, however, that antigens of the H-2 complex may be associated with gp70 on the cell surface and may even serve as a receptor for this envelope glycoprotein (30, 31). If the H-2Kk antigens detected in the present study are associated with gp70, this interaction is apparently not preserved in the course of H-2Kk acquisition by mycoplasmas.

Antiserum to M. hyorhinis was shown to mediate a specific complement-dependent cytolysis of infected lymphoblastoid cells, an observation suggesting that association of the organisms with the host cell membrane may have important immunological consequences. These experiments did not reveal whether lysis resulted from recognition of M. hyorhinis antigens integrally inserted into membranes of these cells, or from "innocent bystander" damage arising from complement activation by antigen-antibody complexes formed on organisms residing on the host cell membrane. The phenomenon did, however, suggest a mechanism by which tissue damage may result from an immunological reaction to surface-associated mycoplasmas in vivo.

Anti-mycoplasma antibody-mediated cytolysis of host cells, acquisition of host cell antigens by these organisms, and selective association of T lymphoid cell surface antigens represent important functions that may be biologically significant in chronic mycoplasmal disease. It will be important to determine the general nature of this phenomenon, and how the antigens of the major histocompatibility complex may interact with mycoplasmas in chronically infected individuals. Further investigation and assessment of these processes will be facilitated by use of the model system described herein.

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