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Immunology

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^k histocompatibility 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^k (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 bisbenzimidazole 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 uninoculated indicator culture as a negative control enabled detection of mycoplasmas by fluorescent DNA staining (15) and provided reinforcement 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 lyophilized culture; it was reconstituted and passaged three times in Hayflick's medium containing 20% horse serum (17). Aliquots of this stock culture containing $10^{4.8}$ mean color-changing units (CCU_{50}) per 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 1.5×10^8 mycoplasma-free BW5147 cells were experimentally infected with $10^{4.8}$ CCU_{50} of broth-grown *M. hyorhinis*. After five serial passages, cells were pelleted at $400 \times g$ for 10 min and resuspended in fresh medium for further passage. Mycoplasmas from culture supernatants were readily cultivated in Hayflick's medium (typically $10^{5.9}$ CCU_{50}/ml) but not in cell-free tissue culture medium.

Antisera. Mule typing antiserum to *M. hyorhinis* (GDL strain, catalog number M781-511-569) and preimmune mule serum (M-713-801-569) were obtained from the Research Resources Branch, National Institutes of Health, Bethesda, MD. Goat antiserum to purified gp70 of Rauscher murine leukemia virus (MuLV) was obtained from the Office of Program Resources and Logistics of the National Cancer Institute through

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Abbreviations: MuLV, murine leukemia virus; AD_{50}, absorption dose resulting in 50% reduction in assay reaction; CCU_{50}, mean color changing unit.

R. Wilsnack, Huntingdon Research Center, Brooklandville, MD. Antisera to Thy-1.1, Thy-1.2, and H-2K^k 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 Eδ G2 Gross MuLV-induced leukemia of B6 mice was kindly provided by E. 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^k (10, 11), and gp70 (12) antigens. The concentration of absorbing material added to a reaction resulting in a 50% reduction in specific ^{51}Cr release was designated the absorption dose$_{50}$ (AD$_{50}$).

Isolation of *M. hyorhinis* from BW5147 Cultures. BW5147 cells growing in exponential phase (7) and demonstrating greater than 97% viability (by trypan blue exclusion) were centrifuged at 400 × *g* for 10 min at 4° to pellet cells. The supernatant was centrifuged at 1800 × *g* for 60 min at 4°, and the resultant supernatant was further centrifuged at 100,000 × *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 × *g* for 90 min at 4°, clearly visible bands were recovered and rinsed by centrifugation at 100,000 × *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 × 10^6 cells per ml in the presence of [5-^{3}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 4.5 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 *M. hyorhinis* from BW5147 Cultures. The presence of *M. hyorhinis* 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 [^{3}H]uridine into supernatant material having a buoyant density on K tartrate gradients of approximately 1.22 g/cm^3, characteristic of mycoplasmas (21, 22). No labeled material was obtained from identically treated uninfected BW5147 cultures (Fig. 1*A*). Application of this technique to BW5147 cultures experimentally infected with *M. hyorhinis* yielded similar results (Fig. 1*B*). 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.

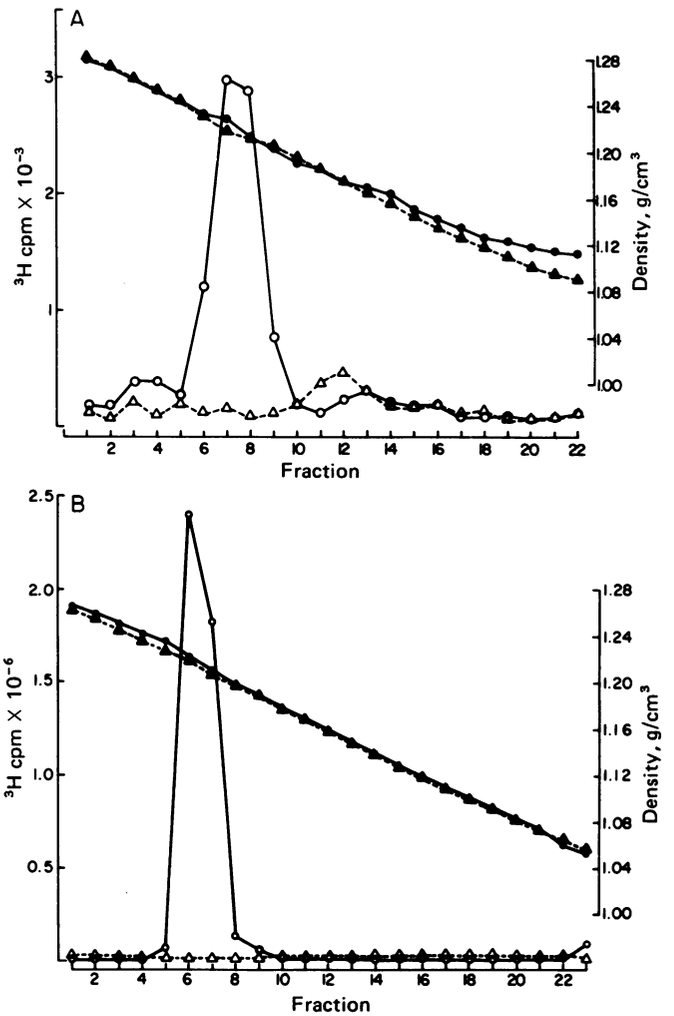

FIG. 1. Isopycnic sedimentation of [5-^{3}H]uridine-containing material from BW5147 cell culture supernatants. Fractions collected after isopycnic sedimentation of supernatant material from [5-^{3}H]-uridine-labeled BW5147 cultures were analyzed for the amount of trichloroacetic acid-precipitable ^{3}H. Uridine incorporation into supernatant material from uninfected (▲- - -▲) control BW5147 cultures is compared to that from naturally (*A*) or experimentally (*B*) infected BW5147 cultures (○—○). Densities are indicated for fractions obtained from infected (●—●) and uninfected (▲- - -▲) cultures.

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 ρ = 1.20–1.22 g/cm^3 was clearly separated from a lighter MuLV-containing band at ρ = 1.15–1.17 g/cm^3. Transmission electron micrographs prepared from the heavier material revealed a marked predominance of typical mycoplasma structures (Fig. 2*A*). As a comparison, micrographs of broth-grown *M. hyorhinis* prepared analogously revealed a very similar morphology (Fig. 2*B*). The K tartrate gradient-purified mycoplasma from BW5147 culture supernatants demonstrated intense fluorescence when stained with anti-*M. hyorhinis* 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 *M.*

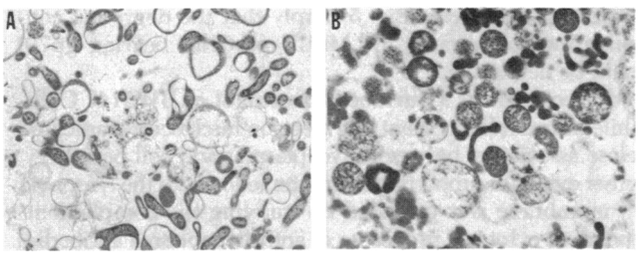

FIG. 2. Electron micrographs of mycoplasmas obtained from naturally infected BW5147 supernatants and purified by isopycnic sedimentation on K tartrate gradients (*A*); and *M. hyorhinis* analogously prepared from serially passaged, broth-grown stock cultures (*B*). Preparations were fixed with glutaraldehyde and osmium tetroxide in phosphate buffer. (×7000.)

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 AD_{50} 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.

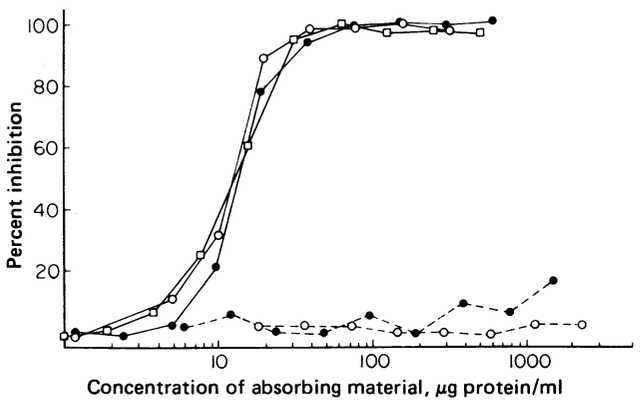

FIG. 3. Quantitative absorption of cytotoxic antiserum against Thy-1.1 antigen. Absorption of the Thy-1.1 cytotoxic reaction was carried out with increasing amounts of various absorbing materials: mycoplasma isolated from naturally infected BW5147 cells (●—●); broth-grown *M. hyrohinis* (○- - -○); *M. hyorhinis* from experimentally infected BW5147 cells (□—□); and BW5147 membranes isolated by isopycnic centrifugation (19) (○—○). Absorption of the cytotoxic reaction for Thy-1.2 antigen by mycoplasma isolated from naturally infected BW5147 cells is also shown (●- - -●).

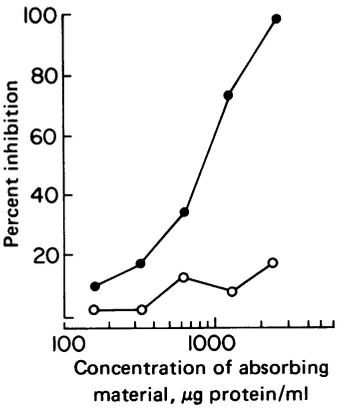

FIG. 4. Quantitative absorption of cytotoxic antiserum against H-2K^k alloantigens. Absorptions were performed as in Fig. 3 to quantitate H-2K^k antigens associated with mycoplasma isolated from naturally infected BW5147 cells (●) and broth-grown *M. hyorhinis* (○).

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 AD_{50} 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-2K^k 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-2K^k alloantigens, and also against an anti-complementary explanation for this inhibition.

In contrast to H-2K^k 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-2K^k surface antigens with these organisms. The lack of gp70 antigen was not due to its inactivation or removal during the K tartrate gradient purifi-

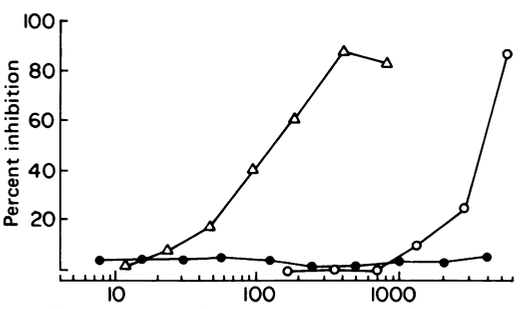

FIG. 5. Quantitative absorption of cytotoxic antiserum against gp70 antigen. Absorptions were performed as in Fig. 3 to quantitate gp70 associated with mycoplasmas isolated from naturally infected BW5147 cells (●), BW5147 membranes (○), and MuLV from BW5147 cultures isolated by isopycnic sedimentation on K tartrate gradients (△).

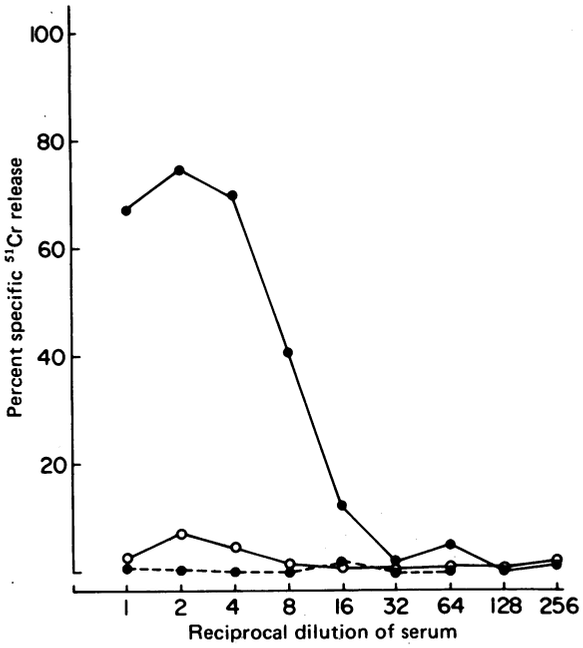

FIG. 6. Cytotoxic effect of antiserum to *M. hyorhinis* on mycoplasma-infected BW5147 cells. Various dilutions of mule antiserum to *M. hyorhinis* (—) or preimmune mule serum (- - -) were assessed for complement-dependent cytotoxic activity, using ^{51}Cr-labeled BW5147 cells, either uninfected (○) or naturally infected (●) with *M. hyorhinis*.

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/cm^3) used for mycoplasma purification (Fig. 5), and (*ii*) on membranes prepared from BW5147 cells and purified on gradients (ρ = 1.12–1.13 g/cm^3) (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 1.13 g/cm^3, well separated from the heavier mycoplasmal material found at 1.20–1.22 g/cm^3 on K tartrate gradients. (*ii*) When supernatants from uninfected BW5147 cells were processed for mycoplasma purification, only a single band of 1.16 g/cm^3 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 with the lymphoid cell membrane was provided by studying the complement-dependent cytotoxic properties of anti-mycoplasma antibodies on infected BW5147 cells labeled with ^{51}Cr. 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 ^{51}Cr release was due to cell lysis and not to lysis of surface-associated mycoplasmas (which may have accumulated ^{51}Cr) 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 × 10^6) 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 (24). 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-1.1 antigen associated with mycoplasmas derived from BW5147 cells may reflect the interaction of this T cell alloantigen with *M. hyorhinis* at the cell surface. The use of immunoferritin electron microscopic techniques to establish the precise location of mycoplasma-associated Thy-1.1 could help in providing a mechanistic explanation of this phenomenon. Elucidation of these points is important because the ability of any infectious agent to alter the distribution of T cell surface components may have a profound effect on the nature of the host response to this agent.

The absence of MuLV gp70 in purified mycoplasmas prepared from infected cell cultures, but its presence in membrane and virus preparations derived from these cells, may also be viewed in terms of specific interactions between mycoplasmas and the lymphoblastoid cell surface. Blocking studies suggest that Thy-1 and H-2 antigens may be spatially associated on the lymphoid cell surface (27). It might be expected then that mycoplasmal acquisition of these antigens is 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-2K^k antigens detected in the present study are associated with gp70, this interaction is apparently not preserved in the course of H-2K^k 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 organism 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 generality 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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