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Solid-Phase Micro-Radioimmunoassay to Measure Immunoglobulin Class-Specific Antibody to *Mycoplasma pulmonis*

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A solid-phase radioimmunoassay (RIA) was used for the detection and quantitation of immunoglobulin class-specific antibodies to *Mycoplasma pulmonis* in mouse sera. The RIA was found to be more sensitive than the single radial haemolysis (SRH) test for detecting antibodies in sera from mice at intervals after inoculation with *M. pulmonis,* since antibody was detected in 26 out of 29 serum samples by RIA but in only 8 out of 29 samples by SRH. A method is described for quantitating immunoglobulin class-specific antibody in test sera by reference to a standard serum. The RIA should be useful for the study of the relative importance of various immunoglobulin classes in resistance to mycoplasma infections.

A comparison of the ability of serum and cells to passively immunize against mycoplasma-induced respiratory infections has led to the suggestion that antibody is an important factor in mediating resistance (17). However, there is often a poor correlation between the levels of serum antibodies as measured by a variety of techniques and resistance (14, 19). In addition, attempts to demonstrate specific antibodies to mycoplasmas in respiratory tract secretions by routine serological tests have not been very successful (8, 9). It seems, therefore, that the tests commonly used to measure antibodies to mycoplasmas are not very sensitive or do not detect those antibodies that mediate resistance, or both. For these reasons more sensitive techniques such as indirect immunofluorescence (1, 5), radioimmunoprecipitation (3), indirect staphylococcal radioimmunoassay (4), and enzyme-linked immunosorbent assay (11) have been used to measure antibody to mycoplasmas. These sensitive tests also detect the immunoglobulin class of the antibody. However, the indirect immunofluorescence test is time consuming, and estimations of antibody titers are subjective. Although the radioimmunoprecipitation and indirect staphylococcal radioimmunoassay tests are quantitative, they are also time consuming and the antigen needs to be radiolabeled to a high specific activity. Therefore the solid-phase micro-radioimmunoassay (RIA) described by Rosenthal, Hayashi, and Notkins (16) was adapted for measuring immunoglobulin class-specific antibodies to mycoplasmas. The advantages of this assay over radioimmunoprecipitation assays are the greater speed in processing samples and, like the enzyme-linked immunosorbent assay, the ease of labeling antibody compared with labeling mycoplasmas. The triple incubation technique described by Charlton and Blandford (6), using a single ^{125}I-labeled reagent, was used so that more accurate comparisons of immunoglobulin class-specific antibody levels could be made than if each anti-immunoglobulin class serum were labeled with ^{125}I. The assay described below was set up to measure levels of immunoglobulin class-specific antibody to *Mycoplasma pulmonis* in mouse sera.

MATERIALS AND METHODS

Preparation of antigens. The JB strain of *Mycoplasma pulmonis* and the Ab/1 strain of *Mycoplasma bovis,* which have been described previously (13, 18), were used as antigens. Both mycoplasma species were grown in liquid medium (10) containing 10% heat-inactivated horse serum. Mycoplasma antigens were prepared from an overnight culture of organisms in 5 liters of medium. The mycoplasmas were sedimented at 15,000 × *g* for 30 min, washed three times with phosphate-buffered saline (PBS) at pH 7.2, resuspended in 5 ml of PBS (total yield was 30 to 50 mg of protein), and stored at −70°C.

Antisera. Pooled, convalescent mouse serum was obtained from 60 specific-pathogen-free CBA mice (Institute for Research on Animal Diseases, Compton) which had been inoculated intranasally (i.n.) 3 weeks previously with 2 × 10^6 CFU of *M. pulmonis* strain JB. Pooled, control serum was prepared from similar but noninfected mice.

Goat antisera specific for mouse immunoglobulin G1 (IgG1), IgG2, IgM, and IgA (GAM IgG1, GAM IgG2, GAM IgM, GAM IgA) were obtained from Meloy (Springfield, Va.). The antisera appeared to be specific when examined by immunoprecipitation techniques.

Donkey antiserum specific for goat IgG (DAG) was obtained from Wellcome Research Laboratories (Beckenham, England) and labeled with ^{125}I. Purified donkey IgG was prepared from DAG serum by chromatography on a DE52 (Whatman) ion-exchange column using 0.01 M phosphate buffer at pH 8.0. Fractions which contained IgG were concentrated to the original volume by ultrafiltration (Amicon B15) and stored at −20°C. The procedure used for iodination of the donkey IgG was similar to that described by Purcell et al. (15). Antibody preparations had a specific activity of approximately 0.5 μCi/μg of protein.

Assay procedure. The RIA procedure was similar to that reported elsewhere (6, 16). Polyvinyl U-bottom microtiter plates (Cooke Engineering Co., Alexandria, Va.) served as the solid phase for the assay. The wells of the microtiter plate were coated with 100 μl of an appropriate dilution (usually approximately 100 μg of protein/ml) of mycoplasmas from a constantly stirred suspension. Immediately before use, the concentrated mycoplasma suspension was diluted in PBS + 0.1% azide and sonicated for 3 s to disperse clumps. The plates were sealed, incubated overnight at 37°C, shaken to remove remaining fluid, and washed 6 times with PBS containing 0.05% Tween 20 (Sigma). Serial twofold dilutions of 100 μl each of mouse sera were added to each well, the plates were sealed and incubated at 37°C for 1 h, and the washing procedure was repeated. A 100-μl portion of a 1:1,000 dilution of either GAM IgA, IgG1, IgG2, or IgM serum was added to each well, and the plates were resealed. After 1 h of incubation at 37°C, the plates were washed again and ^{125}IDAG was added in a volume of 100 μl. The dilution of ^{125}IDAG used was calculated to give 40,000 to 70,000 cpm per well. After an additional 1 h of incubation at 37°C, the plates were washed and the wells were cut out and counted in an NE 8311 automatic gamma counter (Nuclear Enterprises Ltd.).

Calculation of results. Binding ratios (B/B_o) were calculated for each dilution of mouse sera, by dividing the counts per minute, minus electrical background, obtained with the test mouse serum (B) by the counts per minute, minus electrical background, obtained with wells not exposed to the test serum (B_o). Binding ratios of 2.1 or greater were considered indicative of the presence of specific mycoplasmal antibody. In experiments designed to set up the assay, the endpoint titer of a test serum was the reciprocal of the dilution of the serum which gave a binding ratio of 2.1.

SRH. The single radial hemolysis (SRH) test was based on that described by Howard, Collins, and Gourlay (12). *M. pulmonis* was coupled to sheep erythrocytes as follows: 0.6 ml of a 1/8 dilution of *M. pulmonis* antigen in PBS, 0.6 ml of 5% (vol/vol) sheep erythrocytes in PBS, and 0.3 ml of carbodiimide (Sigma) in PBS (400 mg/ml) were mixed together and incubated at 20°C for 1 h. The erythrocytes were washed three times with PBS and resuspended in 1.5 ml of barbitone buffer. After overnight incubation at 4°C, the suspension was warmed to 44°C and suspended in agarose, and the test was performed as described previously (12). Areas of hemolysis produced by mouse sera were measured after 1 h of incubation at 37°C. Gels containing erythrocytes that had not been sensitized with mycoplasmas were used as controls.

RESULTS

Preparation of solid-phase mycoplasma antigen. Various conditions that may affect the attachment of mycoplasma antigen to polyvinyl wells were examined. Since mycoplasmas aggregate, the effect of a 3-s sonication was examined. With a sonicated suspension of organisms the titer of a hyperimmune mouse serum was 4 times greater than with nonsonicated organisms; therefore a sonicated suspension was used. Wells were coated "wet," as described in Materials and Methods, fixed with ethanol, and then dried onto the plate by incubating unsealed plates at 37°C overnight or dried and then fixed with ethanol. There was no difference in titer of the hyperimmune serum when the wells were coated with wet antigen or with wet and fixed antigen. In contrast, there was a marked reduction in titer of the serum when dried antigen or dried and fixed antigen was used. Therefore wells were coated with wet antigen. There was little difference in the titer of the hyperimmune mouse serum with antigen suspended in buffers with pH values ranging from 7.0 to 8.7; therefore pH 7.2 buffer was chosen for convenience.

To determine the optimum amount of antigen to use, a checkerboard titration was performed with various antigen dilutions against dilutions of convalescent mouse serum. The dilution of antigen chosen was approximately 100 μg of protein/ml.

Titration of immunoglobulin class-specific antibody. To determine the optimum dilutions of GAM immunoglobulin class-specific sera to use, a checkerboard titration was performed of various dilutions of convalescent and normal mouse sera against dilutions of each GAM immunoglobulin class-specific serum. The dilution of the anti-immunoglobulin class-specific serum chosen was that which gave the highest titer of antibody in the convalescent mouse serum. In contrast to convalescent mouse serum, specific antibody to *M. pulmonis* was not detected in normal mouse serum at any dilutions of GAM immunoglobulin class-specific sera with the exception of GAM IgM. The dilution (10^{-3}) of GAM IgM serum that gave the highest titer of antibody to *M. pulmonis* in the convalescent mouse serum also gave a titer of $\log_{10}$ 1.0 for normal mouse serum. However, this dilution of GAM IgM serum was used and titers $\leq \log_{10}$ 1.0 were considered not significant.

Specificity of the assay. As shown in Table 1, IgG1 antibodies in hyperimmune mouse se-

rum against mycoplasma medium and *M. bovis* were detected at a titer of $\log_{10}$ 1.0. Similarly, IgG2 antibodies were detected at a titer of $\log_{10}$ 1.0, whereas IgM and IgA antibodies were $<\log_{10}$ 1.0. In contrast, antibodies were not detected when medium containing serum fraction instead of horse serum was used as antigen.

The assays shown in Table 2 were performed to check the specificity of each stage of the procedure. It can be seen that the binding of GAM IgG1 serum to *M. pulmonis* was not significant. Similar results were obtained with the other anti-immunoglobulin class-specific sera. The ^{125}IDAG did not bind directly to the antigen or to the mouse serum.

Standardization of results. To overcome day-to-day variation of the assay resulting from decay of the ^{125}I-labeled serum and new batches of antigen, the titers of antibody in test sera were determined by reference to a standard convalescent mouse anti-*M. pulmonis* serum. The standard hyperimmune serum was titrated on eight occasions, using two different batches of antigen and several preparations of ^{125}IDAG sera, and the endpoint titers for each immunoglobulin class were determined as described in the text. The results from each assay were used to calculate the geometric mean immunoglobulin class-specific antibody titer of the serum. Thus, the units (U) of antibody activity of the reference serum for IgG1, IgG2, IgM, and IgA were $\log_{10}$ 3.93, $\log_{10}$ 4.02, $\log_{10}$ 3.41, and $\log_{10}$ 2.90, respectively. Each time an assay was performed, a standard curve was obtained by plotting B/B_o against the units of antibody at each dilution of the reference serum (Fig. 1a). A regression analysis was performed on the linear part of the curve and the B/B_o value corresponding to $\log_{10}$ 1.0 U of antibody was calculated; e.g., this value was 7.5 in Fig. 1a. Seven twofold dilutions of the test sera were made, and the B/B_o value for each dilution was determined by RIA. The dilution of each test serum giving a B/B_o value equivalent to $\log_{10}$ 1.0 U of the reference serum was calculated from a regression analysis performed on the linear part of the test serum curve of B/B_o against the reciprocal of dilution. From this value the units of antibody in the test serum were determined; e.g., from Fig. 1b the dilution of a test serum which gives a B/B_o value of 7.5 is $\log_{10}$ 2.15; thus, the $\log_{10}$ 2.15 dilution contains 10 U of antibody. This procedure was performed for each immunoglobulin class. One serum was tested seven times over a period of 6 weeks to evaluate the reproducibility of the assay. The geometric mean titer of antibody and its standard deviation for seven determinations was 3.10 ± 0.29 for IgG1, 3.59 ± 0.21 for IgG2, 2.59 ± 0.27 for IgM, and 1.12 ± 0.35 for IgA.

TABLE 1. *Specificity of IgG1 antibody[a] in mouse sera*

Antigen	Mouse serum	$\log_{10}$ titer
M. pulmonis	Convalescent	2.85
	Normal	<1.0
Medium + horse serum	Convalescent	1.0
	Normal	<1.0
M. bovis	Convalescent	1.0
	Normal	<1.0
Serum-fraction broth	Convalescent	<1.0
	Normal	<1.0

[a] GAM IgG1 used at 1:200 dilution.

Sensitivity of RIA to detect antibody. Of 29 sera obtained from mice at various intervals after inoculation with *M. pulmonis,* antibody was detected in 26 using RIA and in only 8 by SRH. In no instance was antibody detected by SRH and not by RIA. In general, antibody was not detected by the SRH test in sera which contained less than $\log_{10}$ 2.4 U of IgG2 or less than $\log_{10}$ 2.7 U of IgM antibody as measured by RIA.

Detection of immunoglobulin class-specific antibody to *M. pulmonis*. The ability of the assay to measure the development of serum antibody in mice after infection with *M. pulmonis* was examined. Pooled sera were obtained from groups of 10 mice at intervals following intravenous (i.v.) inoculation with 2×10^6 colony-forming units (CFU) of *M. pulmonis*. As shown in Fig. 2, at week 1 after infection, antibody to *M. pulmonis* was predominantly IgM. The level of specific IgM remained constant over

TABLE 2. *Determination of nonspecific binding of immunoglobulin class-specific and radiolabeled sera*

Reagents				cpm × 10^3	B/B_o[a]
Solid-phase antigen	Mouse serum	GAM/IgG1	^{125}IDAG		
M. pulmonis	1:20 Convalescent	+	+	12.13 (*B*)	8.94
M. pulmonis	1:20 Normal	+	+	1.75 (*B*)	1.12
M. pulmonis	−	+	+	1.59 (B_o)	−
M. pulmonis	−	−	+	0.58	−
M. pulmonis	1:20 Convalescent	−	+	0.91	−

[a] B/B_o = (counts per minute bound by test serum − electrical background)/(counts per minute bound at zero dose of test serum − electrical background).

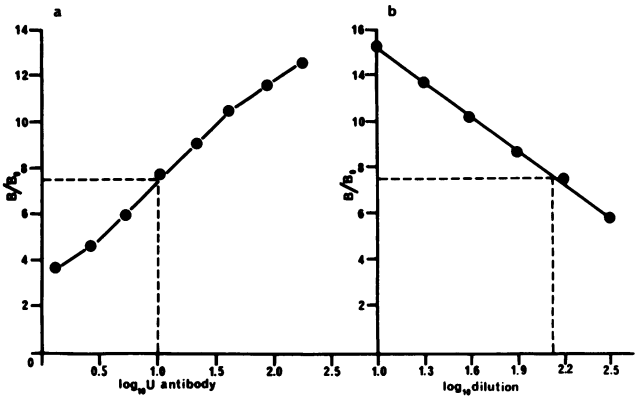

FIG. 1. *Standardization of results by reference to a standard serum. (a) Standard curve for IgG1 obtained by plotting binding ratio (B/B_o) against units of antibody of the standard serum, $\log_{10}$ 1.0 U of antibody = B/B_o of 7.5. (b) Titration of a test serum. The dilution of serum giving a B/B_o value of 7.5 is $\log_{10}$ 2.15.*

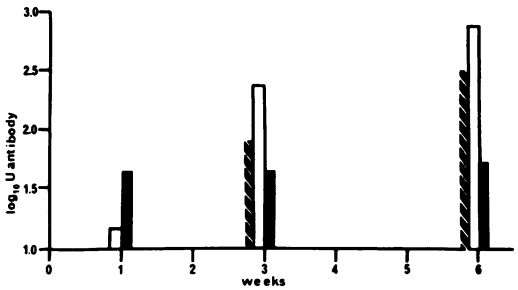

FIG. 2. *Immunoglobulin class-specific antibody to M. pulmonis in sera obtained from mice inoculated i.v. with 2 × 10⁶ CFU of M. pulmonis. IgG2 (▨); IgG1 (□); IgM (■).*

TABLE 3. *Immunoglobulin class-specific antibody levels in pooled sera obtained from groups of 20 mice*

Route	Time after inoculation (weeks)	IgG1	IgG2	IgM	IgA
i.v.[a]	6	2.50[b]	2.87	1.73	<1.0
i.v.	6	2.77	3.27	2.15	<1.0
i.v.	3	1.92	2.38	1.65	<1.0
i.n.[c]	3	3.40	3.45	2.25	2.09
i.n.	3	2.90	2.99	2.33	1.73

[a] Intravenous inoculation with 2 × 10⁶ CFU of *M. pulmonis*.
[b] $\log_{10}$ units of antibody.
[c] Intranasal inoculation with 5 × 10⁵ CFU of *M. pulmonis*.

the 6-week period of observation. At week three after inoculation, specific antibody was predominantly of the IgG2 class. Specific IgA antibody was not detected in sera from these mice at any time after i.v. inoculation with *M. pulmonis*. In contrast, specific IgA was detected in pooled sera from groups of 20 mice inoculated i.n. 3 weeks previously with 5 × 10⁵ CFU of *M. pulmonis* (Table 3). As shown in Table 3, there appear to be higher levels of IgG2 than IgG1 specific antibody in sera from mice inoculated i.v. with *M. pulmonis*, whereas the levels of these antibodies were equivalent in sera from mice that had been inoculated i.n.

DISCUSSION

As with other solid-phase micro-RIA and like the enzyme-linked immunosorbent assay, the assay described above is economical of reagents, rapid, specific, and very sensitive. The main disadvantage is that new batches of ^{125}I-labeled reagent have to be prepared every 3 to 4 weeks and titrated to determine the optimum dilution to use in the test.

Because the antigen coating the wells is predominantly whole mycoplasmas, the assay is probably measuring those antibodies directed to surface antigens. However, mycoplasma antigen preparations frequently contain medium components (2); therefore, mice inoculated with a suspension of *M. pulmonis* will probably make antibodies to medium components. In addition, the antigen used in the assay will also contain some medium components. In an attempt to determine the contribution made by antibodies to medium components in the assay, mycoplasma broth and *M. bovis* grown in the same medium as *M. pulmonis* were used as antigens. However, the amount of medium components that absorbed to the well when broth was used as antigen and the amount present in suspensions of *M. bovis* and *M. pulmonis* antigens are not known and may not be the same. Despite this, antibody was detected at a titer of only $\log_{10}$ 1.0 when either mycoplasma medium or *M. bovis* was used as antigen, suggesting that the contribution by antibody to medium compo-

nents is not very significant in this assay. The antibody detected was probably directed against the horse serum component of the medium since there was no detectable antibody activity when broth containing serum fraction instead of horse serum was used as antigen.

It was found that for each immunoglobulin class assay, the linear part of the curves B/B_o against reciprocal of dilution, for sera obtained at various times after infection, were not always parallel. Because of these differences it was not possible to calculate the titers of test sera by applying the B/B_o value obtained at each dilution of a test serum directly to the standard curve as described by Charlton and Blandford (7). To overcome problems associated with the slope of the curves, the method described in Results was therefore used to calculate units of antibody in test sera.

The SRH test was chosen for comparison with RIA because it is simple to perform and is more sensitive than either indirect hemagglutination or complement fixation for the detection of antibody to mycoplasmas (12). However, the SRH test failed to detect antibody to *M. pulmonis* in 18 serum samples that were shown to contain antibody by the RIA test. Although the SRH test detects only those antibodies that activate complement, it was not sensitive enough to detect antibody in sera that contained moderate levels of IgG2 and IgM antibody, as detected by RIA, which are the immunoglobulin classes in the mouse which fix complement.

The RIA was used in preliminary experiments with sera obtained from mice at various intervals after i.v. inoculation with *M. pulmonis*. The titers of antibody in the various immunoglobulin classes fluctuated independently of each other, indicating that the GAM immunoglobulin class sera were specific. It was interesting to note that only mice that had been inoculated i.n. with *M. pulmonis* developed IgA antibodies to the mycoplasma. By RIA, IgA antibodies to *M. pulmonis* were detected in sera at fairly high levels (i.e., about 1:100) 3 weeks after i.n. inoculation of CBA mice. This is in contrast with the findings of Cassell et al. (5) who demonstrated IgA antibodies at very low levels (i.e., 1:4) in sera of CD-1 mice 3 weeks after i.n. inoculation with *M. pulmonis,* using the immunofluorescence test. These differences may be due to the greater sensitivity of RIA and/or to a different type of antibody response in CBA, compared with CD-1 mice.

Further work is in progress to determine the sequence and relative titers of immunoglobulin class-specific antibodies to *M. pulmonis* in sera and lung washings of experimentally infected and vaccinated mice in an attempt to elucidate the relative importance of the various immunoglobulin classes in the development of resistance to mycoplasma-induced respiratory infections.

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