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Specific Secondary Biological Properties of Purified Rabbit Immunoglobulin M and Immunoglobulin G Antibodies to *Brucella abortus* and *Bordetella pertussis*

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Rabbit immunoglobulin (Ig)M and IgG antibodies to *Brucella abortus* and *Bordetella pertussis* were isolated as purified products and their specific secondary biological activities were compared. IgM antibodies were found to be more active than IgG proteins in inducing agglutination and sensitization of *B. abortus* for the complement-dependent bactericidal effect and in inhibiting *B. pertussis*-induced lymphocytosis in the mouse. IgM and IgG antibodies were found to be equally effective in inducing agglutination of *B. pertussis* suspended in a colloidal solution. These data parallel previous work to indicate that IgM antibodies to bacterial surface antigens are more efficient than IgG molecules in initiating biological processes concerned with the inactivation of these pathogens.

Despite their identical immune specificity, the secondary biological activities of antibodies have been shown to differ (4, 7, 11-14, 17, 23, 29, 35, 36). Antibodies have been isolated to the somatic antigen of *Salmonella typhimurium* and to the capsular polysaccharide of *Diplococcus pneumoniae* type 1 (7, 11, 30). In these reports, immunoglobulin (Ig) M antibodies isolated from hyperimmune serum were shown to have 250- to 1,000-fold greater activity, in such secondary biological activities as complement-dependent bacterial reaction, opsonization, and agglutination reactions, than IgG immunoglobulins of identical immune specificity.

To test whether these early observations permit a general principle to describe the secondary biological activities of antibacterial immunoglobulins, the IgM and IgG antibodies specific to the surface antigen of two other microorganisms, *Brucella abortus* and *Bordetella pertussis*, were isolated. Analyses of the secondary biological activities of these purified proteins showed that IgM antibodies to the somatic antigen of *B. abortus* have a higher specific agglutination and bactericidal activity than IgG immunoglobulins. In another biological assay, the inhibition of lymphocytosis, IgM anti-*B. pertussis* antibodies had a higher specific activity than IgG antibodies.

MATERIALS AND METHODS

Antigens and antisera. *B. abortus* strain 19 kindly supplied by Dale E. Bordt, Pittman-Moore Division of Dow Chemical Company, was used as the immunogen. A sample of the bacterial suspension was heated at 60 C for 2 hr, centrifuged at 6,000 × *g* at 4 C, and resuspended in 0.15 M NaCl to a final concentration of 10^{10} organisms/ml. Organisms (5×10^9) were injected intradermally into multiple sites in the toepads of randomly bred adult rabbits. Three weeks later, 5×10^9 organisms were injected intravenously, and these injections were repeated every third day for three doses. At 7 days after the last injection, the animals were bled from the marginal ear vein twice weekly for 2 consecutive weeks. Immunization was then repeated by using 10^{10} live organisms injected intraperitonealy every third day for three doses. A week after the last injection, the animals were exsanguinated by cardiac puncture. *B. abortus* strain 19 of low virulence for humans (kindly donated by Norman McCullough of the National Institute of Allergy and Infectious Diseases, Bethesda, Md.) was prepared to be used as an immunoadsorbent (10).

B. pertussis. Pertussis Vaccine Fluid (kindly supplied by F. B. Peck, Jr., Eli Lilly & Co.) was used as antigen and immunoadsorbent. The preparation was diluted to contain 10^{10} organisms/ml and then twice centri-

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fuged at 7,000 × *g* at 4 C for 20 min and resuspended in 0.15 M NaCl before use. Immunization of six randomly bred, adult rabbits was performed according to the schedule outlined for animals immunized with *B. abortus*.

After incubation of the collected blood at 37 C for 1 hr and at 4 C for 18 hr, the sera was separated by centrifugation at 1,000 × *g* for 30 min and stored at −20 C without preservative.

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Purification of antibodies. The "hyperimmune" sera were separated by anion-exchange chromatography [diethylaminoethyl (DEAE) cellulose, type 40, 0.87 mEq/g, Schleicher & Schuell Co., Keene, N.H.] into IgG- and IgM-rich fractions (30). Adsorption of the antibodies from these fractions to the bacterial antigen and subsequent elution were performed according to published methods (7, 30). The antibodies eluted from each fraction were further purified by using anion-exchange chromatography (DEAE cellulose; Whatman column chromedia; DEAE 32; 1.0 mEq/g; H. Reeve Angel & Co., Inc., Clifton, N.J.) for the IgG antibodies and Sephadex G-200 (Pharmacia, Piscataway, N.J.) for IgM antibodies (30).

Characterization of antibodies. Physiochemical and immunochemical techniques designed to check the purity and antigenic characteristics of the purified antibodies were performed by the published methods summarized in a preceding publication (30).

Specificity of antibodies. Double diffusion in agar gel was performed by using standard methods. *Brucella melitensis* endotoxin, prepared according to the Boivin procedure and kindly supplied by Wesley W. Spink of the University of Minnesota School of Medicine, was solubilized by phenol extraction (37), and a phenol-extracted endotoxin of *B. pertussis* (kindly supplied by John Munoz of the U.S. Public Health Service Rocky Mountain Laboratory, Hamilton, Mont.) was used as antigen.

Bacterial adsorption studies were performed by using *B. abortus* and *B. pertussis* as well as *Escherichia coli* and *S. typhimurium* cells (30).

Bacterial agglutination. The suspending medium for the agglutination assays was 0.145 M NaCl-0.005 M potassium phosphate, *p*H 7.0 (PBS). A modification of the standard tube agglutination test for anti-*B. abortus* antibody was performed (37). Briefly, twofold serial dilutions of 0.25 ml of the preparation (or a commercially available standard serum) to be tested were mixed with equal volumes of the *B. abortus* antigen containing 10^9 organisms/ml. The reaction mixtures were incubated at 37 C for 1 hr and at 4 C for 18 hr, during which time the tubes were occasionally shaken to resuspend the bacterial antigen. The specific agglutination activity was recorded as the highest dilution of the antibody-containing solution that induced macroscopic agglutination. Augmentation of antibody-induced agglutination was performed by using the monospecific goat and anti-rabbit immunoglobulin sera described previously (2).

A modification of the reported tube agglutination test for *B. pertussis* was done (18, 19). Briefly, twofold serial dilutions of 0.25 ml of the preparation to be tested were mixed with equal volumes of *B. pertussis* suspensions containing 2 × 10^9 organisms/ml. The suspending medium was 0.05% gelatin (Difco) in PBS. The tubes were agitated and the titer was recorded as the highest dilution of the antibody-containing solution yielding macroscopic agglutination after incubation of the reaction mixtures for 4 hr at 25 C. Augmentation of agglutination by using antiglobulin reagents was performed as described for *B. abortus* with the suspending medium of 0.05% gelatin in PBS.

Each serum specimen was assayed at least three times in duplicate to minimize the effects of errors in the tube dilution procedures. The variation in titers for a single sample was within one-tube dilution. A standard reference serum [*B. abortus* titered control serum (human)-Hypertussis, Cutter Laboratories, Berkeley, Calif.] and a normal rabbit serum were used throughout to verify the stability of the antigen.

Complement-dependent bactericidal assay. The complement-dependent bactericidal activity of the antibody solution for *B. abortus* was assayed by the method described for *S. typhimurium* (15) except that the media used were Tryptose broth and Tryptose agar (Difco). The incubation time for the reaction mixture containing antibody, complement, and bacteria was extended to 2 hr because of the growth characteristics of *B. abortus*. Appropriate controls in which either antibody or complement were lacking were used. The control containing complement but no antibody was assumed to represent 100% survival. The control containing antibody but no complement also gave 100% survival, indicating the response assayed was a bactericidal one and not an effect of clumping.

Passive protection against Bordetella pertussis-induced lymphocytosis in mice. The inhibition of *B. pertussis*-induced lymphocytosis in mice by passively administered antibody was assayed by a modification of the procedure described by Morse and Riester (19). Peripheral blood for leukocyte counts was obtained from 4- to 6-week-old, pathogen-free mice (CD strain, Charles River Mouse Farms, Charles River, Mass.) by severing the tail a few millimeters from the distal end, and differential counts were performed on smears stained with Wright's stain (8). Groups of six mice were injected in the lateral tail vein with 0.2 ml of mixtures containing anti-*B. pertussis* antibodies and bacteria and appropriate controls. Leukocyte and differential counts were performed the day of injection and at intervals of 2, 5, and 10 days. In this assay the specific activity of the anti-*B. pertussis* antibodies was reported as the minimal amount of antibody necessary to induce a lymphocyte response which was 50% less than the response in animals injected with organisms alone (28).

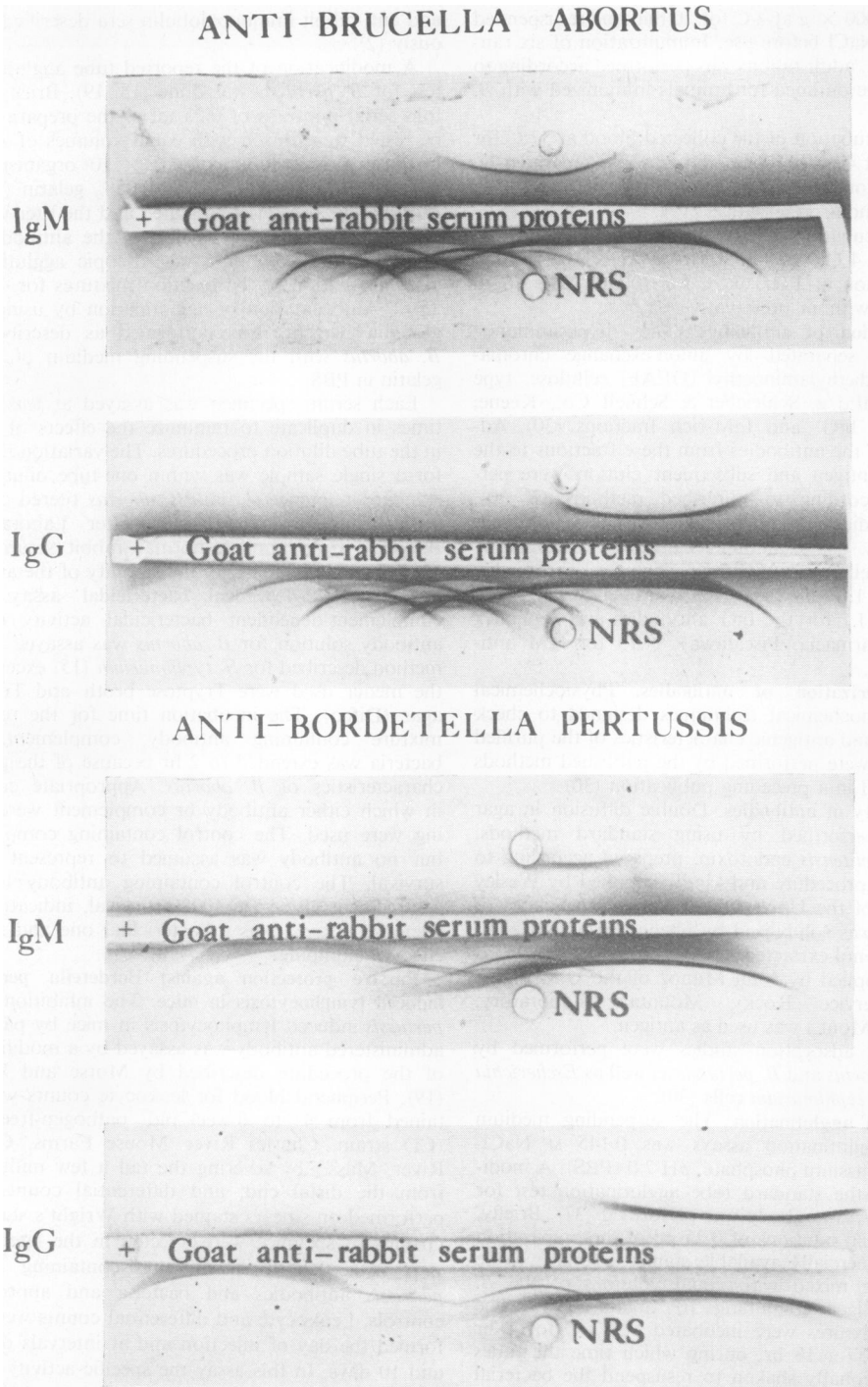

FIG. 1. *Immunoelectrophoresis of the purified rabbit antibodies and normal rabbit serum (NRS). The precipitin arcs were developed with a goat polyvalent anti-rabbit serum protein reagent.*

RESULTS

Analysis of antibody-containing solutions. The purity of the antibodies prepared in two separate experiments for each bacterial antigen was determined by four methods. A solution containing 5.0 mg of each antibody preparation per ml showed a single homogeneous component during analytical ultracentrifugation for 200 min at 56,100 rev/min at 21 C. The calculated $S_{20,w}$ values for the antibodies were 6.7*S* for the IgG preparations and 17.8*S* for the IgM preparations and were consistent with reported values for these rabbit immunoglobulins (16, 33).

Immunoelectrophoretic analyses of solutions containing from 0.5 to 4.0 mg of protein/ml by using anti-rabbit serum protein sera from two goats revealed a single precipitin band for each antibody preparation (Fig. 1). All of the purified antibodies showed marked electrophoretic homogeneity.

The immunological specificity of the antibodies was studied by two methods. Gel diffusion analyses, shown in Fig. 2, revealed two common lines of precipitation formed by the anti-*B. abortus* IgM and IgG antibodies and the *B. melitensis* endotoxin, reflecting at least two antigenic components present in the endotoxin reactive with both classes of antibodies. The faint precipitin band closest to the antigen well was only demonstrable when using a 1% concentration of the endotoxin. The major precipitin component showed a single line at concentrations of the endotoxin from 0.1 to 1.0%. A single common line of precipitation was formed by the IgM and IgG antibodies with the endotoxin extracted from *B. pertussis*.

Adsorption studies (Table 1) showed that the bacteria adsorbed approximately 80 to 85% of the specific antibody protein. In contrast, *E. coli* and *S. typhimurium* cells, having somatic antigens unrelated to the immunogens, adsorbed 4 to 7% of the protein from the antibody preparations. Anti-*B. abortus* and anti-*B. pertussis* antibodies revealed a similar degree of nonspecific adsorption when incubated with unrelated bacteria.

Specific activity of purified rabbit anti-B. abortus immunoglobulins. (i) *Bacterial agglutination.* IgM and IgG anti-*B. abortus* antibodies, prepared in two separate experiments, were assayed for agglutination activity and for antiglobulin-enhanced agglutination activity (Table 2). During the antibody purification procedure in the first experiment, it was found that a significant amount of agglutinating activity remained in the serum fractions separated by chromatography (DEAE cellulose). Accordingly, a second ad-

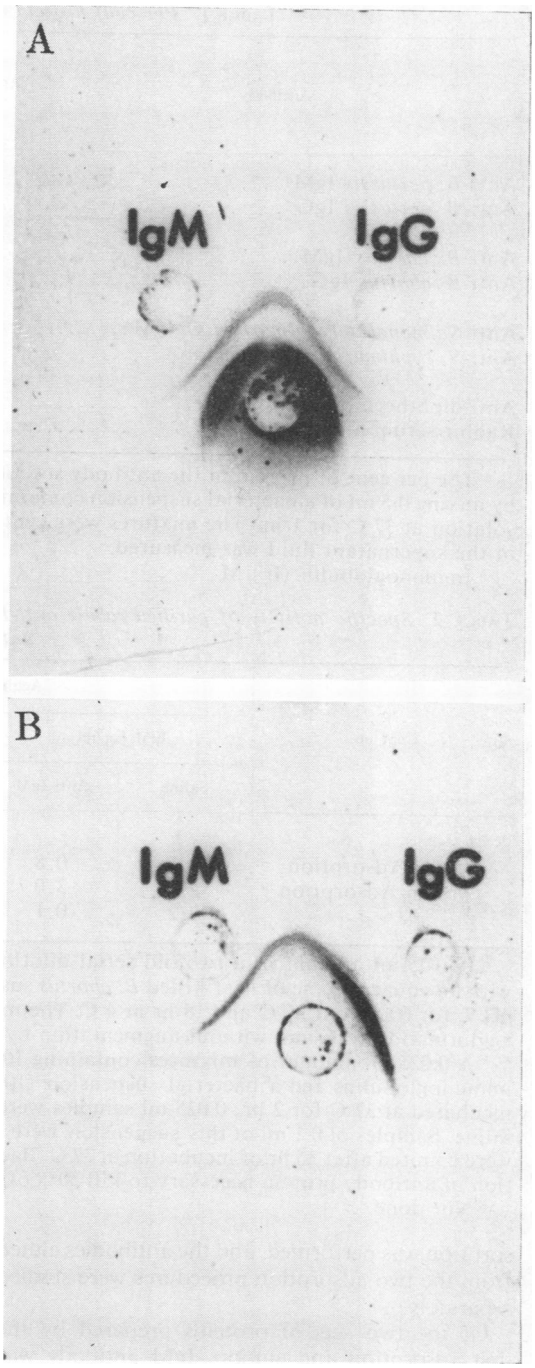

FIG. 2. *Immunodiffusion studies of purified antibacterial antibodies and soluble bacterial endotoxins. The upper wells contain the antibody preparations (5.0 mg/ml) and the bottom wells contain 1% solutions of (A) Brucella melitensis endotoxin and (B) Bordetella pertussis endotoxin. For clarity in reproduction, the immunodiffusion lines have been accentuated.*

TABLE 1. *Per cent protein adsorbed by bacterial antigens*[a]

Antibody	Antibody concn (mg/ml)	Bacterial antigens		
		Bordetella pertussis	*Brucella abortus*	*Escherichia coli*
Anti-*B. pertussis* IgM[b]	0.08	84.0	4.6	5.1
Anti-*B. pertussis* IgG	0.12	85.3	5.2	3.8
Anti-*B. abortus* IgM	0.16	2.9	93.0	1.3
Anti-*B. abortus* IgG	0.21	3.0	95.8	4.6
Anti-*Salmonella typhimurium* O IgM	0.13	3.8	4.7	3.6
Anti-*S. typhimurium* O IgG	0.20	1.9	5.8	6.0
Anti-diphtheria toxoid IgG	0.13	3.2	4.0	5.6
Rabbit serum albumin	0.13	0.5	6.9	3.2

[a] The per cent of protein in the antibody solutions reacting with the specific antigen was determined by mixing 0.5 ml of a bacterial suspension containing 5×10^9 organisms/ml with 0.5 ml of each antibody solution at 37 C for 1 hr. The mixtures were centrifuged at 12,000 × *g* for 1 hr at 0 C and the protein in the supernatant fluid was measured.

[b] Immunoglobulin (Ig) M

TABLE 2. *Specific activity of purified rabbit anti-Brucella abortus (19) immunoglobulin (Ig) M and IgG antibodies*

Expt no.	Agglutination[a]				Complement-dependent bactericidal activity[b]	
	IgM (μg/ml)		IgG (μg/ml)		IgM (μg/ml)	IgG (μg/ml)
	Saline	Anti-IgM	Saline	Anti-IgG		
1						
1st Adsorption	1.5	0.8	11.8	5.9	0.0072	0.44
2nd Adsorption	5.9	3.0	35.0	17.5	0.0040	7.6
2	0.2	0.1	8.0	2.2	ND[c]	ND

[a] A 0.25-ml amount of a twofold serial dilution of anti-*B. abortus* immunoglobulins was incubated with an equal volume of heat-killed *B. abortus* suspended in 0.145 M NaCl–0.005 M potassium phosphate, *p*H 7, for 60 min at 37 C and 18 hr at 4 C. The minimal concentration necessary to cause macroscopic agglutination with and without augmentation by antiglobulin reagents was determined.

[b] A 0.025-ml amount of mixtures containing 10^9 heat-killed *Bordetella pertussis* and anti-*B. pertussis* immunoglobulins and a bacterial suspension (10^5 cells/ml) were mixed in Perspex plastic plates and incubated at 37 C for 2 hr; 0.025-ml samples were removed by calibrated loop and diluted in 1.0 ml of saline. Samples of 0.1 ml of this suspension were plated directly on Tryptose agar plates and colonies were counted after 36 hr of incubation at 37 C. Bactericidal activity was expressed as minimal concentration of antibody protein necessary to kill 50% of the original inoculum.

[c] Not done.

sorption was performed, and the antibodies eluted from the two adsorption procedures were studied separately.

Of the two sets of proteins prepared by the first adsorption and elution, IgM antibody was more efficient (0.2 mg/ml) in inducing agglutination of *B. abortus*. Approximately a 290-fold difference was observed when the activities of the two pairs of IgM and IgG antibodies were compared on a molar basis. A twofold degree of enhancement of agglutination activity was observed for both classes of immunoglobulins after the addition of antiglobulin reagents. Comparable results were obtained with the anti-*B. abortus* antibodies isolated in the second experiment.

Antibodies of both immunoglobulin classes isolated from the second adsorption procedure in the first experiment were less active than were the comparable immunoglobulins isolated from the first adsorption, i.e., three- to fourfold decrease in activity.

(ii) *Complement-dependent bactericidal activity.* Table 3 shows that as little as 0.0072 μg of IgM anti-*Brucella* antibody per ml induced a 50% re-

TABLE 3. *Specific activity of purified hyperimmune rabbit anti-Bordetella pertussis antibodies*

Expt no.	Agglutination reaction[a]				Passive protection against pertussis induced lymphocytosis in mice[b]	
	IgM[c] (μg/ml)		IgG (μg/ml)		IgM (μg/ml)	IgG (μg/ml)
	Saline	Anti-IgM	Saline	Anti-IgG		
1	25	25	3.8	0.9	13	77
2	18	18	3.3	0.7	ND[d]	ND[d]

[a] A 0.25-ml amount of twofold dilutions of anti-*Bordetella pertussis* immunoglobulins was incubated with an equal volume of heat-killed *B. pertussis* suspended in 0.05% gelatin in 0.14 M NaCl–0.05 M potassium phosphate, *p*H 7, for 240 min at 25 C. The minimal concentration necessary to cause macroscopic agglutination with and without augmentation by antiglobulin reagents was determined. A standard reference serum was used throughout the assay to verify the stability of the antigen.

[b] A 0.20-ml amount of mixtures containing 10^9 heat-killed *B. pertussis* and anti-*B. pertussis* immunoglobulins was injected into the lateral tail vein of pathogen-free mice. Leukocyte counts and differential counts were performed on peripheral blood on the day of injection and at intervals of 2, 5, and 10 days. The amount of anti-*B. pertussis* immunoglobulins necessary to induce a 50% reduction in the concentration of peripheral lymphocytes is shown.

[c] Immunoglobulin (Ig) M.

[d] Not done.

duction in the number of viable organisms in the presence of complement in contrast to 0.44 μg of IgG antibody protein per ml, i.e., 300-fold difference in activity on molar basis. The specific activities of the first and second adsorption products isolated in the first experiment differed. IgG isolated during the first adsorption was 17 times more active than the second adsorption product. In contrast, the IgM antibodies isolated from both the two adsorption procedures in experiment 1 had similar specific bactericidal activities.

Specific activity of purified rabbit anti-B. pertussis immunoglobulins. (i) *Bactericidal agglutination.* The sensitivity of agglutination assay for anti-*B. pertussis* antibodies was less than the agglutination assay for anti-*B. abortus* or *S. typhimurium* immunoglobulins. As much as 18 to 25 μg of IgM antibody per ml and 3.3 to 3.8 μg of IgG antibody per ml were necessary to induce macroscopic agglutination in two separate experiments. The specific activities of both classes of antibodies were approximately equal when compared on the basis of molar concentration. IgG antibodies to *B. pertussis* responded to antiglobulin enhancement with a fourfold increase in agglutination activity. In contrast, IgM anti-*B. pertussis* antibodies showed no enhancement after the addition of the antiglobulin reagent.

(ii) *Inhibition of B. pertussis-induced lymphocytosis.* A lymphocytosis of approximately 25,000 mm^3 was induced by the intravenous injection of 10^9 organisms into pathogen-free mice. The peripheral lymphocyte count rose after 24 hr, reached peak concentrations at 48 to 72 hr, and then returned to initial levels 5 to 7 days later. To identify the inhibitory activity of the purified anti-*B. pertussis* antibodies, mixtures of organisms and antibodies were made, incubated at 37 C for 30 min, and injected into the lateral tail vein of the mice.

The bleeding procedure induced a small but statistically significant rise in the peripheral leukocyte count. To compensate for this nonspecific effect, the mean normal leukocyte level at each interval was determined by performing counts on 10 mice that had received an injection of sterile saline. This figure was subtracted from the leukocyte count for each experimental group to determine the corrected leukocyte count.

Results summarized in Table 3 show that 13 μg of IgM anti-*B.pertussis* antibody and 77 μg of IgG antibody caused a 50% inhibition of pertussis-induced lymphocytosis, i.e., approximately a 30-fold difference in specific activity. The specificity of this protective effect was demonstrated by the uninhibited lymphocytosis which occurred when anti-*S. typhimurium* "O" antibodies were substituted for the anti-*B. pertussis* antibodies in the assay.

DISCUSSION

The antibody purification technique resulted in the recovery of 15 to 20% of the total IgM and IgG antibodies in the serum as determined by the final yield of agglutination activity (30). In all experiments there were approximately 10 to 15 times more IgG antibody recovered than IgM. Therefore, the proportion of IgM to IgG antibodies induced by these two bacteria are comparable to values reported for immunoglobulins for another microorganism, anti-*S. typhimurium* "O," as well as for antibodies to other antigens (7, 12, 14, 30).

The two classes of rabbit immunoglobulins produced in response to the same bacterial antigen were identical in their immune specificity but revealed marked differences in their secondary

activities. A 40- to 200-fold higher agglutination activity was detected for IgM anti-*B. abortus* antibodies as compared to IgG proteins which is similar to the values reported for anti-*S. typhimurium* "O" immunoglobulins (7, 30). The agglutination activity of both classes of anti-*B. abortus* antibodies was increased by the addition of the antiglobulin reagents.

In the complement-dependent bactericidal reaction IgM anti-*B. abortus* antibody was approximately 300 times more active than IgG antibody. The difference in the specific activity of the two classes of immunoglobulins parallels the values reported for complement-dependent reactions mediated by antibodies to *Salmonella* erythrocytes and haptens (12, 14, 29, 30, 31). Studies of the hemolytic complement systems have shown that a single molecule of IgM antibody bound to a cell surface antigen was capable of activating two molecules at C1, whereas at least two molecules of IgG antibody in close proximity to each other at the cell surface were necessary to activate two molecules of C1 sufficient to induce cell lysis (5). A morphological lesion induced by immune hemolysis has been postulated to be due to the binding of three IgM molecules to the cell surface (12), whereas approximately 3,000 IgG molecules have been deduced to cause the same effect. The difference observed for the specific complement-dependent activities of rabbit IgM and IgG antibodies indicate a similar relationship between the efficiency of the two anti-*B. abortus* immunoglobulins.

Due to the incomplete extraction of all the antibodies during the initial purification of the anti-*B. abortus* immunoglobulins, a further adsorption and elution procedure was done which yielded more antibody. IgG anti-*B. abortus* antibodies isolated during the first adsorption procedure had an approximately 20-fold higher specific complement-dependent bactericidal activity than the IgG molecules prepared in the second adsorption. Subfractionation procedures, similar to those described in experiments utilizing antihapten, antidextran, and anti-*S. typhimurium* "O" antibodies, have permitted the isolation of molecules of one immunoglobulin class showing heterogeneity of biological function (9, 26, 32). However, IgM anti-*B. abortus* antibodies isolated by successive adsorption and elution procedures had almost identical specific complement-dependent bactericidal activity. We have as yet no explanation for the similarity in specific activities of the two IgM elution products in the bactericidal reaction.

When the specific agglutination activities of the anti-*B. pertussis* antibodies were compared, the IgG anti-*B. pertussis* antibodies were found to have a slightly higher activity than IgM antibodies. The agglutinating activity of the IgG antibodies was enhanced by the antiglobulin reagent, whereas the activity of the IgM anti-*B. pertussis* molecules remained unchanged. These observations differ from those reported in most bacterial agglutination assays where IgM antibodies are more active than IgG antibodies (7, 30).

One explanation for the observed similarity in specific agglutinating activities of the classes of anti-*B. pertussis* antibodies is the use in this assay of a suspending medium containing 0.05% gelatin, necessary to prevent nonspecific agglutination of *B. pertussis* cells. Although the mechanism for antibody-induced agglutination is unknown, recent studies have proposed that agglutination is the result of at least two forces (27). The first, a force exerted by the electrostatic charge on the cell surface (zeta potential), tends to prevent agglutination. This repelling force is reduced when colloid is added to the reaction mixture or when the surface of the cell is altered. The second force, the intercellular bridge formed by antibodies, is the "attractive" force inducing agglutination. Thus, the similar agglutination activities of IgM and IgG anti-*B. pertussis* antibodies may be due to the altered surface of the bacterial cell suspended in a colloid medium. The importance of the surface antigen in determining the agglutination activity of immunoglobulins may be illustrated by the comparable activities of IgM and IgG anti-A blood group substance antibodies (14).

The results of these experiments again reveal that the two major classes of serum antibodies specific for surface antigens of bacteria differ in their secondary biological activities. The significance of these differences in biological activities may be related to distribution of these two classes of immunoglobulins within the body fluids. IgM and at least one complement component, B_1C, are predominantly intravascular molecules (1, 3, 6). Similarly, complement-mediated systems related to the inactivation and elimination of particulate antigens are largely intravascular phenomena, whereas biological activities such as neutralization of toxins, liberation of vasoactive substances, and tissue fixation are characteristic of IgG antibodies and closely parallel the predominantly extravascular distribution of this immunoglobulin (25, 26).

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