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Shared Antigens Between Heterologous Bacterial Species

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Sera from normal rabbits were shown to have antibodies that bound radiolabeled test antigens derived from many taxonomically unrelated bacteria. Sera from rabbits that had been immunized with sonically treated material of 12 different bacteria, including *M. bovis* strain BCG, had antibodies that bound not only radiolabeled homologous test antigens but also radiolabeled antigens from many unrelated bacteria. Binding by normal and immunized sera to radiolabeled test antigens was inhibited by homologous unlabeled test antigens but not by substances such as bovine serum albumin, polyvinylpyrrolidone, sheep erythrocytes, and endotoxin. The broad range of shared or cross-reactivity among antigens in bacteria may explain the presence of antibodies to many bacteria in sera from normal humans and previously unimmunized experimental animals. The presence of these antibodies raises the question whether resistance to many bacterial infections may be partly due to immune mechanisms, whether cellular or humoral, that have been stimulated by unrelated bacteria.

Previous studies from this and other laboratories have shown that sera from normal persons have antibodies that bind to components derived from a variety of bacteria and fungi (2, 3, 5, 6, 11, 12, 17, 22, 23, 25). Similar observations have been made using sera from unimmunized laboratory animals. Antigens in these studies included components from *Mycobacterium tuberculosis*, *Aspergillus fumigatus*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Listeria monocytogenes*, and *Brucella abortus*. The immunogens responsible for the antigenic stimulation of these antibodies in normal sera have not been determined.

Mycobacteria and *L. monocytogenes* have in the past been found to have antigenic components in common with each other and with a number of other taxonomically unrelated bacterial species (22). The experiments described in this paper were undertaken to investigate further the extent to which antibodies raised after stimulation by one organism will react with antigens derived from unrelated organisms. It might then be possible to explain the presence of antibodies to pathogenic bacteria in normal sera as due to antigenic stimulation by organisms found in normal human microbial flora and in the external environment.

MATERIALS AND METHODS

Bacteria and growth conditions. *M. bovis* strain BCG was obtained from the U.S.-Japan Medical Science Program. *M. tuberculosis* strain H37Rv was obtained from the Trudeau Institute. Cultures of *B. abortus* S.19 and of *L. monocytogenes* were kindly provided by B. A. Hatten, Dallas, and S. Raffel, Stanford, respectively. *Butyrivibrio* sp. Nor37, *Butyrivibrio* sp. 1L6-31, and *Bacteroides* sp. R2 were kindly provided by B. Reiter and M. E. Sharpe of the National Institute for Research in Dairying, Reading, England. These are anaerobic bacteria isolated from the rumens of cattle. *Corynebacterium diphtheriae* ATCC 11913, and *Bordetella pertussis* ATCC 9797 were obtained from the American Type Culture Collection. The following bacteria were obtained from stock cultures maintained at this institution: *Salmonella typhimurium*, *Pseudomonas* sp., *Corynebacterium* sp., *Escherichia coli*, *H. influenzae*, *Staphylococcus epidermidis*, and *Streptococcus viridans*. *B. pertussis* was grown on Bordet-Gengou medium for 72 hr in a 5% CO_2 atmosphere at 37 C. *H. influenzae* was grown in Eugon broth supplemented with 2.5% Fildes extract for 48 hr with gently shaking at 37 C. *M. tuberculosis* H37Rv was grown on a modified Wong medium as previously described (21), and the other bacteria were grown in Tryptose phosphate broth (Difco) at 37 C overnight with vigorous shaking for aeration. All cultures were heat killed by inspissation at 80 C and

washed with three volumes of pyrogen-free water (21, 22).

Preparations of antigens. Suspensions of killed, washed strains BCG and H37Rv were sonically treated for 60 min, and portions of sonically treated material were used to immunize rabbits (21). Non-acid-fast bacteria were similarly prepared, sonically treated for 5 min, and injected into groups of rabbits. The suspensions of rumen bacteria were sonically treated for 5 min. Test antigens were prepared from the sonically treated bacteria, their nitrogen (N) content was determined, and some were labeled with ^{125}I or ^{131}I as previously described (22). They are referred to as ^{125}I-*M. bovis* strain BCG, ^{125}I-*S. typhimurium*, etc. The labeled mycobacterial antigen has previously been shown to be a protein component closely associated with an anionic mucopolysaccharide (20, 21). Similar information regarding the labeled antigens derived from non-acid-fast organisms is not presently available. The labeled test antigens were diluted in 1:100 normal rabbit serum (NRS) to concentrations that ranged from 0.05 to 0.5 μg of N/ml. The counts per minute of the test antigens employed ranged from 5,000 to 15,000. These were tested for their solubility in 50% saturated ammonium sulfate (SAS/2). ^{125}I-*B. abortus* and ^{125}I-*Pseudomonas* sp. were prepared after SAS/2-precipitable components of test antigens were removed as follows. Equal volumes of labeled antigen and NRS diluted 1:10 were incubated for 30 min at 4 C with SAS so that the final ammonium sulfate concentration was 50%. After centrifugation, the supernatant fluid was dialyzed repeatedly in borate buffer, *p*H 8.3, ionic strength 1 (9). If the supernatant solution was soluble in SAS/2, it was employed as the radiolabeled antigen for the ammonium sulfate test (9, 19). ^{125}I-*B. abortus* and ^{125}I-*Pseudomonas* sp. antigen-antibody complexes were precipitated with SAS/2. For all other test antigens, anti-rabbit gamma globulin (anti-RGG) was employed. Using a labeled bovine serum albumin (BSA) antigen, previous studies have shown that SAS/2 and anti-immunoglobulin G (IgG) are essentially identical in their capacity to precipitate radiolabeled antigen-antibody complexes (18).

Preparation of antisera. Each of the organisms mentioned above, except for the rumen bacteria, was suspended in incomplete Freund's adjuvant (IFA) and injected intramuscularly into groups of four to seven New Zealand White rabbits. Injections consisted of approximately 1 mg of nitrogenous material and were given three times at 3-week intervals. The antisera are referred to as anti-*M. bovis* BCG, anti-*S. typhimurium*, etc. Control groups of rabbits were injected with heterologous serum albumins (bovine, sheep, and human) in IFA, with 2×10^7 sheep red blood cells (SRBC) in IFA, or with IFA alone. Sera were collected from all animals prior to immunization and 1 week after the final injection. Three rabbits received no injections, and NRS were obtained from these animals at similar intervals. Two animals were similarly immunized with BSA suspended in complete Freund's adjuvant (CFA), and two animals received injections of CFA alone.

Measurement of antibodies. The primary interactions between labeled test antigens and antibodies in antisera were studied by precipitating ^{125}I-labeled antigen-antibody complexes with either goat anti-RGG (21) or SAS/2. For labeled antigens insoluble in SAS/2, sera were diluted 1:5 in borate buffer, and 0.1-ml fractions were reacted with 0.1 ml of the ^{125}I-labeled test antigens. After overnight incubation at 4 C, anti-RGG was added. The tubes were incubated at 37 C for 1 hr, then at 4 C overnight. After centrifugation, the tubes were allowed to drain overnight, and the radioactivity in the unwashed precipitates was determined. Anti-RGG was prepared by repeated injections of goats with RGG mixed with IFA. The precipitating capacity of anti-RGG was determined by incubating varying amounts of this antiserum with 0.1 ml of a 1:5 dilution of rabbit serum trace-labeled with ^{125}I-rabbit IgG. To insure an excess of precipitating reagent, 50% more anti-RGG than the amount needed to precipitate all the gamma globulin in this dilution was used in each experiment, usually 0.2 to 0.3 ml. The immunoglobulin levels in 30 sets of paired sera from normal and immunized rabbits were studied by cellulose acetate electrophoresis to confirm that immunoglobulin levels were at suitable concentrations for the amount of anti-RGG employed. In some experiments, samples of antisera were incubated separately with trace-labeled ^{125}I-RGG, as well as with the radiolabeled test antigens, after which anti-RGG was added to all the preparations. Radioactivity in the precipitates of tubes containing ^{125}I-RGG verified the effectiveness of anti-RGG in precipitating all the gamma globulin in the sera studied.

When labeled antigens were soluble in SAS/2 (e.g., ^{125}I-*B. abortus* and ^{125}I-*Pseudomonas* sp.) the antigen-antibody complexes were precipitated with SAS/2; samples of 0.5 ml of 1:5 dilutions of antisera in borate buffer and labeled antigens were employed.

Inhibition studies. Antisera were preincubated with unlabeled homologous test antigens and with a variety of potentially inhibitory substances. Twenty-four hours later, labeled test antigens were added to all such preparations, and their binding capacities determined. The concentrations of unlabeled test antigens were far in excess of that of the labeled test antigens. Binding by antisera preincubated with unlabeled test substances was always compared to binding by antisera which were simultaneously preincubated with buffer only. In addition to the test antigens, unlabeled substances used to control these experiments were endotoxin derived from *S. typhimurium* (100 μg; item no. 3125-25, Difco Laboratories; 5% solution of polyvinylpyrrolidone [PVP; molecular weight of 700,000; PDH Chemicals]), BSA (1 mg, Armour Pharmaceutical), 2×10^7 SRBC, and samples of culture media.

In some experiments, sera were preincubated with dilutions of unlabeled homologous and heterologous test antigens. The degree of inhibition by heterol-

ogous antigens was compared with the inhibition obtained by adding known amounts of unlabeled homologous test antigen, and with control preparations where equivalent volumes of borate buffer were added (20, 21).

RESULTS

Binding of labeled antigens by sera from unimmunized animals. Sera were obtained from each of the rabbits before they were immunized and were tested for binding by the labeled test antigens. The greatest amount of binding was observed when these "normal" sera were tested with ^{125}I-*Corynebacterium* sp. antigens (Table 1). Lesser, but significant, radioactivity was also observed with the other test antigens. A wide range in the amount of radioactivity in precipitates using individual normal sera with each labeled test antigen was seen, suggesting that there were individual differences among these animals in their past history of exposure to environmental immunogens or in their genetic backgrounds.

Inhibition of binding by sera from unimmunized animals. Inhibition studies were carried out to confirm the specificity of the binding seen by sera from the unimmunized animals. Sera from 16 rabbits were preincubated with excessive quantities of unlabeled homologous test antigens as well as with a variety of potentially inhibitory substances, and the labeled antigens were added 24 hr later. The radioactive counts in the precipitates of sera containing unlabeled homologous antigens were compared with those of sera that were not inhibited. Determinations were carried out in duplicate or triplicate. Averages of the data obtained using two rabbit sera for each labeled test antigen are shown in Table 2. Results are also expressed as inhibition ratios: counts per minute of inhibited preparation per counts per minute of noninhibited preparation.

TABLE 1. *Binding of labeled test antigens by sera from rabbits prior to immunization*

^{125}I-Test antigen	No. of sera	Avg percent radioactivity in precipitates
M. bovis BCG	55	12.4 (8.5–17.1)
L. monocytogenes	54	13.1 (7.4–23.1)
B. abortus	67	16.5 (7.3–26.3)
E. coli	30	11.5 (8.3–17.3)
Salmonella typhimurium	14	22.1 (16.2–36.1)
Staphylococcus epidermidis	30	12.0 (9.2–16.8)
Pseudomonas sp.	75	11.5 (6.7–19.7)
H. influenzae	30	15.8 (19.0–25.1)
Corynebacterium sp.	56	25.4 (14.9–43.9)

A ratio of less than 1.0 indicates that unlabeled substances were occupying sites which would otherwise be binding labeled antigen. A ratio less than 0.9 indicated 10% inhibition and was considered significant. Binding by normal sera was partially inhibited by excessive amounts of all the homologous unlabeled antigens. Endotoxin, suspensions of SRBC, PVP, and BSA, and culture media did not appreciably alter this binding, and in no case was the ratio less than 0.9. Endotoxin was used as a control to test the possibility that it was a component of the antigens shared between the various bacterial species employed. Relatively large amounts of endotoxin failed to inhibit the reactions observed, and it is, therefore, not likely that endotoxin is involved in the reactions between the labeled bacterial antigens and the antibodies to which they were bound. However, the direct binding between endotoxin and antibodies could not be demonstrated because of the difficulty of labeling endotoxin with radioactive iodine.

One of the normal sera was selected for more detailed inhibition studies using a number of unlabeled test antigens at various concentrations. Increasing amounts of unlabeled *E. coli*, *B. abortus* S.19, *M. bovis* BCG, *Corynebacterium* sp., and *L. monocytogenes* test antigens in 0.1- to 0.2-ml volumes were added to 0.1 ml of a 1:5 dilution of the normal serum. Equivalent volumes of borate buffer were added to control tubes. After 24 hr, 0.1 ml of ^{125}I-*L. monocytogenes* containing 0.09 μg of N/ml was added to all tubes. Binding by all the preparations was then determined, and the results are illustrated in Fig. 1. Small amounts of homologous unlabeled *L. monocytogenes* readily inhibited the binding. All of the heterologous test antigens partially reduced the binding by the normal rabbit serum to ^{125}I-*L. monocytogenes* when added in significantly greater concentrations.

Binding of heterologous labeled test antigens by sera from immunized animals. Nine of the ^{125}I-labeled test antigens were reacted with antisera from groups of rabbits that were immunized with the 12 different sonically treated bacteria, as well as with antisera from the control groups of rabbits. Binding by serum from an immunized animal was always compared with its matching initial serum obtained before immunization. The binding capacity of a given immune antiserum to a radioactive antigen is expressed as the ratio of the counts per minute in the precipitate to that from the corresponding initial bleeding. A ratio of 1.0, for example, represents no increase, and a ratio of 1.4 is a 40% increase.

Results of experiments using all the labeled test antigens are summarized in Table 3. Aver-

TABLE 2. *Inhibition of binding of labeled test antigens to normal sera by unlabeled homologous antigens*

^{125}I-Test antigen concn[a]	Counts per min precipitated by normal sera		
	No inhibitor added	After incubation with homologous unlabeled antigen	Inhibition ratio[b]
M. bovis BCG (0.009)	898	532 (5.0)[a]	0.59
	671	490	0.73
L. monocytogenes (0.009)	2,722	1,930 (5.0)	0.71
	1,920	1,086	0.57
B. abortus (0.018)	2,913	2,565 (11.4)	0.88
	3,906	2,049	0.52
E. coli (0.051)	1,601	1,318 (16.3)	0.86
	1,521	1,170	0.77
Salmonella typhimurium (0.030)	1,325	718 (12.6)	0.54
	1,285	692	0.54
Staphylococcus epidermidis (0.016)	2,381	1,645 (10.0)	0.69
	2,326	1,833	0.79
Pseudomonas sp. (0.016)	2,362	1,890 (16.1)	0.80
	1,516	1,356	0.89
Corynebacterium sp. (0.019)	2,703	1,846 (21.0)	0.68
	2,572	1,750	0.68

[a] Concentration (in parentheses) at micrograms of N per test.
[b] Inhibition ratio = counts per minute of inhibited preparation/counts per minute of noninhibited preparation.

ages of the ratios and the standard deviation (SD) values obtained when a labeled test antigen was reacted with all the sera from a group of animals immunized with the same sonically treated bacteria are shown. Also shown are averages of the ratios and the SD values when each labeled test antigen was reacted against the control groups of sera. Differences between the ratios of experimental and control groups were statistically analyzed by the Student *t* test. Significant values at $P = <0.002$ are indicated. Some of the data in this table using ^{125}I-*M. bovis* strain BCG and ^{125}I-*L. monocytogenes* were previously reported (22). Sera from animals immunized with sonically treated *M. bovis* BCG bound labeled antigens derived from strain BCG, *L. monocytogenes*, *B. abortus*, *Pseudomonas* sp., and *Corynebacterium* sp. The labeled strain BCG test antigen in turn bound to antibodies in sera from rabbits immunized with sonically treated material derived from strain BCG, *L. monocytogenes*, *S. typhimurium*, *B. abortus*, *Pseudomonas* sp., *S. viridans*, *Corynebacterium diphtheriae*, and *B. pertussis*. Sera from unimmunized animals and sera from rabbits immunized with SRBC, various albumins, and IFA only, showed only minimal changes in their binding ratios. Similar relationships between other test antigens and antisera that were raised after stimulation by heterologous organisms were also seen. Although not shown in Table 3, normal and immune sera from each

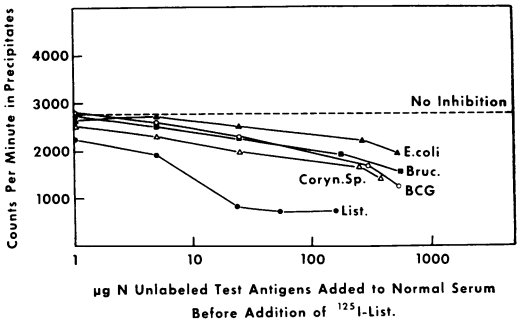

FIG. 1. *Inhibition of reaction between ^{125}I-List (^{125}I-L. monocytogenes) test antigen and antibodies in serum from a normal rabbit by unlabeled test antigens derived from Escherichia coli (E. coli), B. abortus (Bruc), M. bovis strain BCG, Corynebacterium sp. (Coryn sp.) and List. There was no inhibition by unlabeled BSA, PVP, SRBC, or endotoxin.*

group of animals that received sonically treated bacteria were also tested for their capacity to bind ^{125}I-BSA. No significant differences between the pre- and postimmunization sera were observed.

Data from some individual sera are shown in Fig. 2A and B. The test antigens were ^{125}I-*B. abortus* and ^{125}I-*Pseudomonas*. There were only trace differences in binding to these test antigens between the initial and final bleedings of all the control sera (Table 3). All the sera from rabbits immunized with sonically treated *B. abortus* and

TABLE 3. *Average of binding ratio when nine labeled test antigens were reacted with all the sera from animals immunized with 12 sonically treated bacteria*

Determinations	Avg of binding ratios[a]																	
	M. bovis BCG[b]		*L. monocytogenes*		*S. typhimurium*		*B. abortus*		*Pseudomonas*		*Corynebacterium*		*E. coli*		*S. epidermidis*		*H. influenzae*	
	Ratio	SD[c]	Ratio	SD	Ratio	SD	Ratio	SD	Ratio	SD	Ratio	SD	Ratio	SD	Ratio	SD	Ratio	SD
Antisera to sonically treated:																		
M. bovis BCG	*4.39*[d]	± 1.11	1.50[d]	± 0.05	1.35	± 0.24	1.31[d]	± 0.154	1.48[d]	± 0.21	1.45[d]	± 0.22	1.38	± 0.11	1.43	± 0.27	1.26	± 0.14
L. monocytogenes	1.91[d]	± 0.50	*3.89*[d]	± 1.54	1.80[d]	± 0.26	1.49[d]	± 0.20	2.23[d]	± 0.68	1.57[d]	± 0.27	1.67[d]	± 0.20	2.81[d]	± 0.44	1.28	± 0.22
Salmonella typhimurium	1.74[d]	± 0.46	1.36[d]	± 0.13	*2.01*[d]	± 0.35	1.29	± 0.15	2.01[d]	± 0.35	1.50[d]	± 0.20	1.70[d]	± 0.30	1.40	± 0.24	1.25	± 0.08
B. abortus	1.50[d]	± 0.23	1.20[d]	± 0.19	1.54[d]	± 0.37	*2.88*[d]	± 0.44	1.31	± 0.19	1.35	± 0.24	1.26	± 0.25	1.21	± 0.14	1.07	± 0.13
Pseudomonas sp.	1.63[d]	± 0.18	1.50[d]	± 0.10	1.69[d]	± 0.25	1.55[d]	± 0.11	*3.69*[d]	± 0.54	1.41	± 0.11	1.81[d]	± 0.23	1.61[d]	± 0.18	1.49[d]	± 0.11
Corynebacterium sp.	1.22	± 0.18	1.47	± 0.34	ND[e]		1.24	± 0.11	1.51	± 0.37	*2.01*[d]	± 0.42	1.47[d]	± 0.22	1.49	± 0.44	1.23	± 0.15
E. coli	1.44	± 0.48	1.33[d]	± 0.22	2.35[d]	± 0.24	1.22	± 0.12	1.98[d]	± 0.46	1.29	± 0.11	*2.76*[d]	± 0.70	1.61[d]	± 0.18	1.34	± 0.15
Staphylococcus epidermidis	1.27	± 0.14	2.39[d]	± 0.51	ND		1.27	± 0.12	1.28	± 0.14	1.37	± 0.19	1.23	± 0.38	*2.85*[d]	± 0.21	1.07	± 0.20
H. influenzae	1.31	± 0.29	1.39[d]	± 0.16	1.77[d]	± 0.20	1.31	± 0.18	1.39[d]	± 0.18	1.32	± 0.29	1.27	± 0.11	1.33	± 0.31	*1.78*[d]	± 0.12
Streptococcus viridans	1.32[d]	± 0.14	3.15[d]	± 0.85	1.46[d]	± 0.12	1.22	± 0.07	1.36	± 0.19	1.75[d]	± 0.30	1.31	± 0.13	2.65[d]	± 0.57	ND	
C. diphtheriae	1.40[d]	± 0.23	1.74[d]	± 0.24	1.29	± 0.15	1.13	± 0.05	1.08	± 0.23	1.61[d]	± 0.10	1.12	± 0.16	ND		ND	
B. pertussis	1.67[d]	± 0.39	1.25	± 0.29	1.73[d]	± 0.43	1.09	± 0.14	1.24	± 0.23	1.40	± 0.28	1.13	± 0.26	1.24	± 0.29	1.06	± 0.17
Control antisera to:																		
SRBC	1.02		0.95		1.12		0.99		1.10		1.01		1.12		0.9		1.0	
Albumin	1.07		1.05		1.02		1.04		1.08		1.14		1.08		1.22		1.07	
NRS	0.93		1.0		0.97		1.04		1.11		1.21		1.01		1.02		1.0	
ICF	1.05		1.04		1.10		1.07		1.08		1.19		1.11		1.08		1.14	
SD of control sera	0.15		0.14		0.14		0.12		0.12		0.13		0.20		0.16		0.15	

[a] Binding ratio = counts per minute final bleeding/counts per minute initial bleeding.
[b] ^{125}I-labeled antigens.
[c] SD, standard deviation.
[d] $P = <0.002$.
[e] ND, Not done.

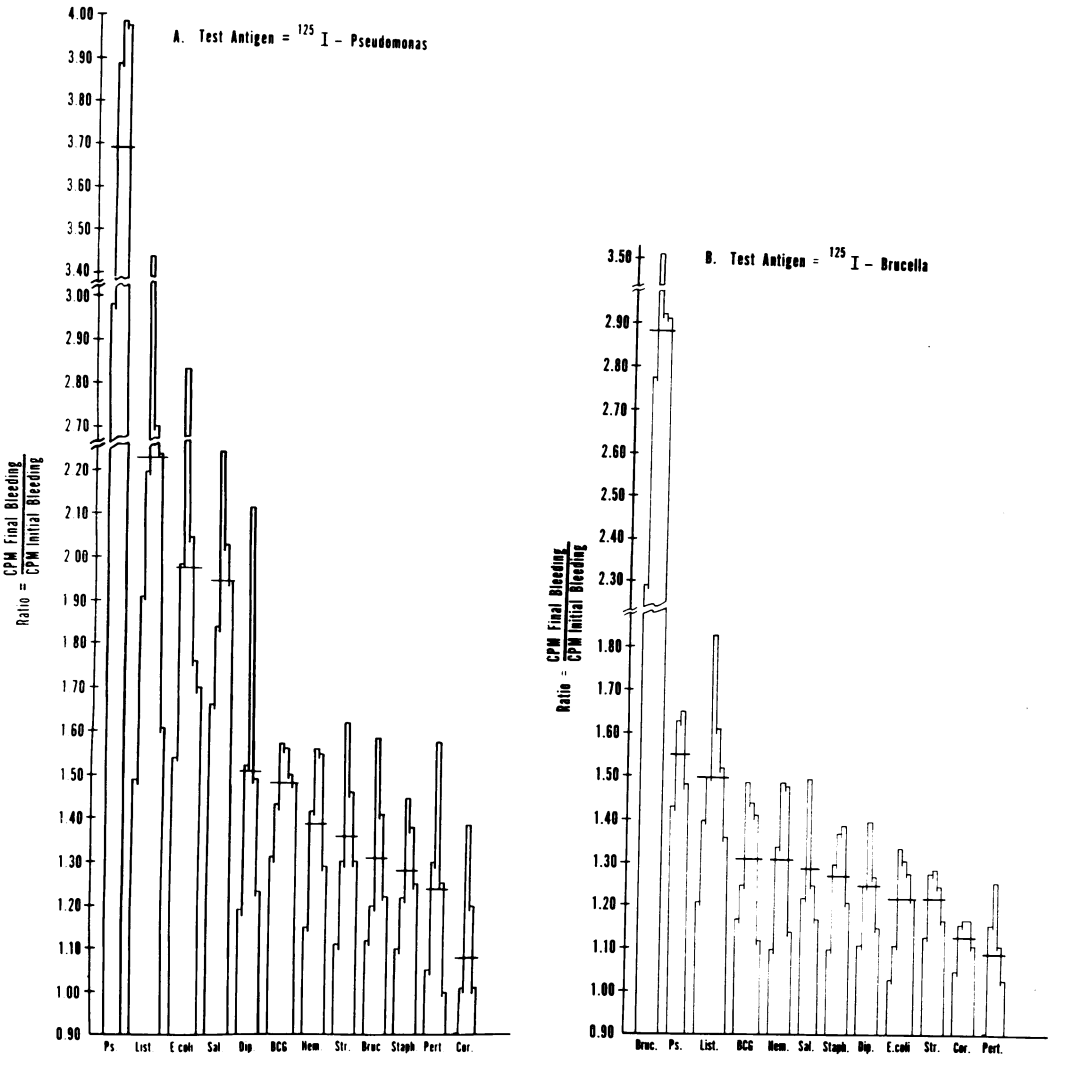

FIG. 2. *A*, ^{125}*I-labeled Pseudomonas test antigen was used. The ordinates show the ratio of the radioactivity (counts per minute) in precipitates derived from sera of final bleedings to that in serum obtained before the animals were immunized. Sera were obtained from rabbits immunized with sonically treated Pseudomonas sp. (Ps), L. monocytogenes (List), Escherichia coli (E. coli), S. typhimurium (Sal), Corynebacterium sp. (Dip), M. bovis strain BCG (BCG), Haemophilus influenzae (Hem), S. viridans (Str), B. abortus (Bruc), S. epidermidis (Staph), B. pertussis (Pert), and Corynebacterium diphtheriae (Cor). B,* ^{125}*I-labeled B. abortus test antigen was used.*

Pseudomonas sp. bound increased amounts of the homologous labeled test antigens. Animals immunized with sonically treated *L. monocytogenes, E. coli, S. typhimurium, Corynebacterium* sp., *M. bovis* BCG, and *H. influenzae* also bound considerable amounts of ^{125}I-*Pseudomonas* sp., and animals immunized with sonically treated *Pseudomonas* sp., *L. monocytogenes, M. bovis* BCG, and *H. influenzae* bound significantly increased amounts of ^{125}I-*B. abortus*.

When sera from the rabbits that were injected with CFA alone, or BSA suspended in CFA, were tested, all the sera bound significant amounts of the ^{125}I-*M. bovis* BCG test antigen. The animals that received BSA also bound increased amounts of ^{125}I-BSA.

Inhibition of binding by sera from immunized animals. Similar to the inhibition experiments with normal sera, studies were carried out to confirm the specificity of the binding shown by sera from the immunized animals. Data were obtained from tests using sera from 16 rabbits,

two in each group, tested with the homologous labeled test antigen and are shown in Table 4. Binding was partially inhibited by all the homologous unlabeled test antigens but not by unrelated substances such as endotoxin, SRBC, PVP, or BSA.

One serum was studied in greater detail. Dilutions of five unlabeled antigens were incubated with 0.1 ml of a 1:5 dilution of an anti-*M. bovis* BCG serum. After 24 hr, 0.1 ml of ^{125}I-*M. bovis* BCG containing 0.09 μg of N/ml was added to all the tubes. Results of this experiment are shown in Fig. 3. As little as 1 μg of N of the unlabeled homologous strain BCG test antigen partially inhibited the reaction. Heterologous antigens partially reduced this binding but only when excess concentrations were employed.

Inhibitory effects of rumen bacteria. Antiserum from a rabbit that had been immunized with sonically treated *M. tuberculosis* H37Rv organisms was diluted so that it bound 50% of 0.02 μg of N/ml of an ^{131}I-labeled strain H37Rv test antigen. The inhibitory effect of progressive increments of unlabeled strain H37Rv test antigen, and test antigens derived from the three rumen bacteria, were then compared (Fig. 4).

TABLE 4. *Inhibition of binding of labeled test antigens to immune sera by unlabeled homologous antigens*

^{125}I-Test antigen[a]	Counts per min precipitated by immune sera		
	No inhibitor added	After incubation with homologous unlabeled antigen[a]	Inhibition ratio[b]
M. bovis BCG	4,906	1,017	0.21
	7,685	1,953	0.25
L. monocytogenes	7,028	1,553	0.22
	4,051	1,725	0.43
B. abortus	7,529	2,822	0.38
	7,757	3,396	0.44
E. coli	3,981	2,431	0.61
	4,658	2,163	0.67
Salmonella typhimurium	9,955	2,867	0.29
	9,697	1,810	0.19
Staphylococcus epidermidis	4,882	3,015	0.62
	4,974	2,888	0.58
Pseudomonas sp.	7,651	4,245	0.55
	8,136	4,796	0.59
Corynebacterium sp.	5,412	3,628	0.67

[a] Antigen concentrations were the same as those shown in Table 2.

[b] Inhibition ratio = counts per minute of inhibited preparation/counts per minute of noninhibited preparation.

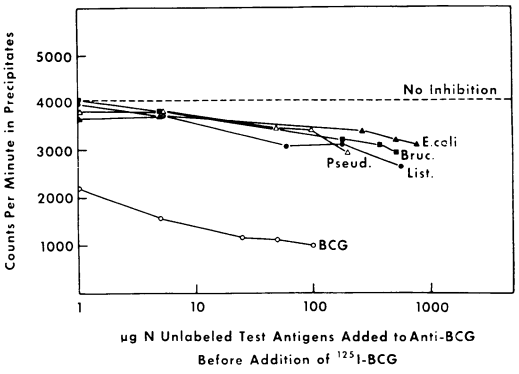

FIG. 3. *Inhibition of reaction between ^{125}I-M. bovis BCG test antigen and antibodies in serum from a rabbit that had been immunized with sonically treated M. bovis BCG by unlabeled test antigens derived from Escherichia coli (E. coli), B. abortus (Bruc), L. monocytogenes (List), Pseudomonas sp. (Pseud), and M. bovis strain BCG. There was no inhibition by unlabeled BSA, PVP, SRBC, or endotoxin.*

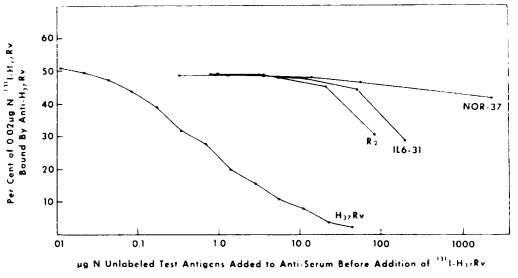

FIG. 4. *Inhibition of reaction between ^{131}I-M. tuberculosis H37Rv test antigen and anti-strain H37Rv. A 0.45-μg amount of N from unlabeled strain H37Rv reduced the binding from 50 to 30%. A 2,000-μg amount of N from Butyrivibrio sp. 1L6-31 and 84 μg of N from Bacteroides sp. strain R2 produced the same effect. 2400 μg of N from Butyrivibrio sp. strain Nor 37 reduced binding only minimally.*

When unlabeled strain H37Rv was used, 0.45 μg of N reduced the binding from 50 to 30%. An equal amount of inhibition was obtained when 2,000 μg of N from *Butyrivibrio* sp. 1L6-31 and 84 μg of N from *Bacteroides* sp. R2 were used. A 2,400-μg amount of N from *Butyrivibrio* Nor37 only reduced the binding to 41.5%.

DISCUSSION

Antibodies in sera from normal rabbits were heterogeneous with respect to their capacity to bind with nine radiolabeled test antigens derived from taxonomically unrelated organisms (Table 1). Sera from normal humans have previously been shown to have antibodies that

bind to antigens derived from tubercle bacilli (3, 23), aspergilli (2), *Neisseria meningitidis* (12), *N. gonorrheae* (11), *Haemophilus influenzae* (6), and enteric gram-negative bacteria (25). In frequent observations (5, 17), antibodies in sera from normal humans and animals have often been referred to as "nonspecific" or natural antibodies. In the present study, binding of normal sera was inhibited by unlabeled homologous antigens and was partially inhibited by heterologous unrelated organisms (Fig. 1), but not by nonmicrobial substances such as BSA, PVP, or SRBC. In previous studies, binding by antigens from fungi and bacteria to normal human and rabbit sera was demonstrated to the Fab or antigen-binding portion of IgG, indicating that the binding by normal sera was a true immunological reaction. It was also shown that IgG, as well as immunoglobulin M, from normal sera bound these microbial antigens (2, 3, 20, 22, 23). In every way, except in degree, antibodies in normal sera appeared to have the same immunological characteristics as did antibodies in sera from immunized animals.

Sera from animals that had been immunized with different sonically treated organisms readily bound homologous labeled test antigens as expected. However, sera from rabbits that had been immunized with unrelated bacteria also bound less, but significant, amounts of the heterologous antigens. It is noteworthy that the labeled test antigen prepared from *L. monocytogenes* bound antisera from almost all the groups tested. This finding is in accord with previous observations that *L. monocytogenes* has antigenic components in common with many other gram-positive (26, 32) and gram-negative organisms (13) and protozoa (28). Many of the data in Table 3 were highly significant statistically ($P = <0.002$).

Some individual animals bound significantly greater amounts of antigen than did others in the same group (Fig. 2A and B). This variation, similar to that noted by normal sera, is again probably a reflection of the genetic background and past history of the rabbits, with respect to previous natural exposure. The binding was readily inhibited by homologous unlabeled antigens (Table 4) and in one case, which was studied in greater detail using ^{125}I-strain BCG, was partially inhibited by unrelated test antigens as well (Fig. 3).

Antigens shared among gram-positive organisms (27) and among gram-negative enterobacteria (15, 33) have previously been described, and the present study presents evidence of antigenic determinants shared between gram-positive and gram-negative organisms as well as mycobacteria. This broad range of shared or cross-reactivity may explain the presence of antibodies to many bacteria in sera from normal humans and experimental animals. The possibility that resistance to many bacterial infections may be partly due to immune mechanisms, whether cellular or humoral, that have been stimulated by unrelated bacteria would appear to merit consideration. In addition, the concept that resistance to many infections is sometimes due entirely to nonspecific, nonimmunological mechanisms (7, 16, 30) should be reevaluated.

Enhanced resistance to tuberculous infections has frequently been observed following immunization with unrelated bacteria, and in this and a previous study strain BCG was shown to share antigenic components with some of these unrelated organisms (22). There are also examples of enhanced resistance to infections after immunization by some of the non-acid-fast bacteria used in the present study (1, 4, 8, 14, 31). Whether shared antigenic components in these bacteria are responsible for any of the protective effects observed is not known. More intriguing, however, is the possibility that immune responses evoked by antigens associated with nonpathogenic bacteria such as *E. coli* and *S. epidermidis* may be protective against infections with virulent bacteria.

Of special interest in this regard are the studies involving the anaerobic rumen bacteria. It has been frequently observed that human strains of tubercle bacilli rarely cause disease in cattle (10). Studies by Sharpe et al. have established that rumen bacteria engender antibodies that are readily detected in bovine sera (29). When extracts of rumen bacteria were tested for their capacity to inhibit the reaction between a rabbit antiserum to a virulent strain of *M. tuberculosis* H37Rv and a radiolabeled component of this organism, two of the rumen bacteria partially inhibited the reaction. The amounts of *Butyrivibrio* 1L6-31 (2,000 μg of N), *Bacteroides* R2 (84 μg of N), and unlabeled H37Rv (0.45 μg of N) needed to reduce the binding of the ^{131}I-labeled strain H37Rv-anti-H37Rv system from 50 to 30% suggest that antigenic material shared with strain H37Rv antigen was 0.02% for *Butyrivibrio* and 0.5% for *Bacteroides*. This indicates the extremely small amount of shared antigens needed to be manifest serologically and possibly biologically.

It is not surprising that sera from animals that were immunized with CFA alone produced antibodies that bound the labeled *M. bovis* BCG antigen. This is also true of sera from animals that were immunized with

BSA which had been suspended in CFA to enhance immunological reactivity. Increased responsiveness after CFA has usually been attributed soley to nonspecific mechanisms (24). This is probably true for nonbacterial antigens such as BSA, but may not be entirely true for all bacterial antigens in view of the cross-reactivity between BCG and other bacteria. It would appear that the use of CFA to enhance the immunological response against bacterial antigens could lead to confusing results.

The widespread cross-reactivity between microorganisms suggests that immunological responses to bacteria may sometimes represent an elevation of an isoimmune state rather than a new actively acquired immune response. Resistance to infectious diseases may in part depend upon a preexisting state of sensitization to bacteria that are ubiquitous in the environment.

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