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NATURAL RESISTANCE OF GERM-FREE MICE AND COLOSTRUM-DEPRIVED PIGLETS TO GROUP A STREPTOCOCCI[1]

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Human immunity to infection with Group A streptococci is generally considered to be type specific, that is, dependent upon the action of antibodies directed against the type of M protein produced by the invading organism (2). It is not clear, however, to what extent antibodies to other antigenic determinants of the Group A streptococcus' cell wall may be part of the host's defense against this agent. Most experimental studies which have demonstrated the type-specific nature of immunity to Group A streptococci have used mice as the host species and have employed highly virulent strains. These strains usually were rich in M protein and contained large hyaluronic acid capsules. Studies of human antibodies to M protein have frequently been made by some variation of Todd's original bactericidal test (3), a procedure which also requires the use of strains maintained in a highly virulent state by frequent mouse passage (4).

When such strains dissociate, losing their capsules and diminishing their M protein content, resistance to phagocytosis decreases markedly. It has been shown that in the presumed absence of serum opsonins, polymorphonuclear leucocytes easily recognize and ingest unencapsulated Group A streptococci on filter paper (5). There is, however, an opsonic effect of human and animal serum against these less virulent strains which suggests the presence of non-type-specific antibodies. Thermolabile opsonin-enchancing factors in human serum directed against encapsulated strains have been demonstrated recently (4, 6). These appear to be "natural" factors, however, rather than conventional acquired antibodies, and the role of the latter in immunity to M-negative, capsule-negative streptococci remains obscure.

The availiability of hypo-γ-globulinemic experimental animals afforded us the opportunity to explore "natural immunity" to Group A streptococci in greater detail (1, 7). In the studies to be presented, it will be shown that germ-free mice are as resistant as conventional animals to infection with Group A streptococci lacking M protein and capsules, and that blood cells of newborn colostrum-deprived piglets phagocytize these strains and destroy them with the same efficiency as the blood cells of colostrum-fed piglets. Furthermore, it will be shown that natural opsonins to Group A organisms are present in serum of colostrum-deprived piglets but that the polymorphonuclear leucocytes of these newborn animals, even in the absence of serum opsonins, can recognize, phagocytize and destroy avirulent Group A organisms as efficiently as the blood phagocytes of colostrum-fed animals.

MATERIALS AND METHODS

Strains of Group A streptococci and other organisms

The strains of Group A streptococci employed in this study were selected for variation in M protein content and degree of encapsulation. Some of these were stock strains kindly supplied by Drs. Rebecca Lancefield, Armine T. Wilson and W. Barry Wood. The remainder were isolated from throat cultures of patients studied in the Northwestern University Clinics and The Children's Memorial Hospital Outpatient Department. The relative size of the capsule was estimated by growing the stock cultures overnight (approximately 18 hr) in Todd-Hewitt broth and then

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[2] Dr. Cohen participated in this study during the tenure of a predoctoral traineeship from Public Health Service Grant 2 T1 A1 57.

subculturing 1 ml into 9 ml of a 10% fresh normal rabbit serum-Todd-Hewitt broth mixture for 2 hr at 37°C. In later experiments it was found that 0.7% bovine serum albumen could be substituted for 10% rabbit serum. Wet India-ink preparations of the 2-hr cultures were examined microscopically (8), and the capsules were graded by comparing the diameter of the total organism (capsule plus coccus) to the coccus alone. A ratio greater than 1 was graded 1+; greater than 2, 2+; greater than 3, 3+, etc. The conditions selected for protein concentration and duration of incubation for maximal capsule production had been established by previous studies (9). The relative M protein content of a strain was estimated by determining the highest dilution of a hot acid extract (2) which precipitated with a standard lot of type-specific antiserum. The extracts were made from the cells harvested from 40 ml of an 18-hr Todd-Hewitt broth culture and were standardized to contain between 2×10^8 and 3×10^8 organisms/ml. The relative amount of M protein was recorded as the reciprocal of the highest dilution of antigen which gave a visible precipitate.

To insure the homogeneity of some encapsulated strains, particularly variants with large capsules and no apparent M protein, these strains were grown on solid media containing hyaluronidase, low glucose concentration and very little moisture to prevent capsule formation (8). Under these conditions variants whose colonies were either matt, glossy, transparent or opaque could be distinguished among some encapsulated strains of the same M protein type, notably the S23 variants of Type 14 organisms.[3]

Germ-free mice

The germ-free and conventional mice used in these experiments were 6- to 8-week-old males of the HaM/ICR strain of Swiss mice. In early experiments the germ-free animals were bred in our laboratories from stock supplied by the Lobund Institute[4], and in later experiments were supplied by the Charles River Laboratories, Brookline, Mass., from strains also derived originally from Lobund stock. The animals were maintained in plastic isolators with appropriate sterility controls. Conventional mice of the same strain were raised under clean but not sterile laboratory conditions. Both groups were fed fortified laboratory chow (Ralston formula 5010) *ad lib.* Challenge experiments with both germ-free and conventional mice were performed in the isolators under identical conditions. Analysis of the electrophoretic pattern of the serum proteins confirmed published reports of the relative hypo-γ-globulinemic state of the germ-free mice (5). The approximate amount of γ-globulin in pools of serum estimated from total protein determinations and paper electrophoresis was 0.13 g/100 ml for germ-free and 0.87 g/100 ml for conventional mice. The indirect bactericidal test (10) and the long chain test (11) were used to assay the sera of germ-free and conventional mice for anti-M antibody. Type-specific anti-M antibody to any of the strains studied was not detectable. Anti-streptolysin O, anti-hyaluronidase and anti-streptococcal diphosphopyridine nucleotidase were not present in the sera of either conventional or germ-free mice. The sera of both groups of mice also failed to agglutinate cell walls of Groups A, B, C, D and G streptococci.[5]

Challenge experiments

The cultures of the strains of organisms employed were grown overnight in fresh Todd-Hewitt broth and standardized by an optical density technique to a concentration of approximately 2×10^8 chains/ml of broth. The challenges were made by injecting 0.5 ml of serial 10-fold dilutions of the standard culture by the intraperitoneal route. The LD_{50} and its standard error were estimated by the probit graph method of Miller and Tainter (12). The methods of Reed and Muench (13) and of Karber (14) gave comparable LD_{50} values for each challenge. The standard error of the Reed and Muench LD_{50} was calculated by applying the formula of Pizzi (15).

Colostrum-deprived piglets (CD piglets)

Piglets were obtained in earlier studies by caesarian sections performed at term on sows of the Hampshire breed.[6] The newborn piglets were bled from the heart either immediately or after

[3] We were kindly assisted in the identification of these variants by Drs. Armine T. Wilson and Grove Wiley.

[4] Mr. P. T. Trexler of the University of Notre Dame aided us in establishing the germ free laboratory at Northwestern University in which these studies were made.

[5] Kindly supplied by Dr. H. D. Slade.

[6] Supplied by Mr. Harold Rosenwinkle, Plano, Illinois.

being maintained for several days under aseptic conditions in plastic isolators, deprived of colostrum and fed on boiled cow's milk and bread. Colostrum-fed piglets 1 to 2 days old, delivered normally and allowed to suckle, were bled as controls. In all experiments, comparisons between colostrum-fed (CF) and colostrum-deprived (CD) piglets were made with animals whose ages did not vary by more than 1 or 2 days. In later studies, piglets were delivered vaginally under aseptic technique into sterile plastic bags and transferred promptly to sterile plastic isolators. This was done to avoid gross contamination of the piglets and to reduce the risk of sepsis during experiments performed in their first few days of life.

Immunoelectrophoretic patterns were obtained by reacting colostrum-deprived piglet serum and whole sow serum with a rabbit antiserum prepared against whole sow serum. Pig γ-globulin was purified by ion-exchange column chromatography with diethylaminoethyl and carboxymethyl cellulose (16). The slow-moving band obtained when purified γ-globulin was reacted against rabbit anti-sow serum was not demonstrable with colostrum-deprived piglet serum (Fig. 1). This observation was consistent with the studies of Sterzl *et al.* (17), who were able to identify γ-globulin in minute amounts (10 to 40 μg/ml) by this method only in colostrum-deprived piglet sera concentrated 100 times. Additional studies in our laboratory confirmed reported observations (18) that the complement activity of the colostrum-deprived piglet serum was very low (approximately 1 to 2 50% hemolytic units/ml) and that undiluted colostrum-deprived piglet serum contained no antibody against *E. coli*, 01, 2, 4, 8, 75 and rough strains of *E. coli* as well as against *S. typhosa* by a hemagglutination method.[7]

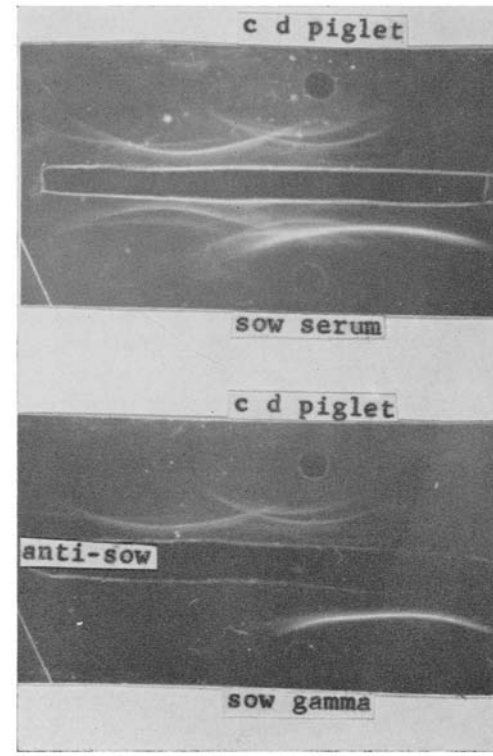

Figure 1. Immunoelectrophoretic analysis of colostrum-deprived (cd) piglet serum, sow serum and sow γ-globulin reacting with rabbit anti-sow serum.

Phagocytosis experiments

1. Direct method. The rate of phagocytosis in mouse and piglet bloods was studied by methods previously described in detail (4) and outlined only briefly here.

Aliquots of 0.3 ml of lightly heparinized whole blood (10 units/ml) were mixed with 0.1 ml of an 18-hr bacterial culture which was adjusted to a turbidity reading of 100 on a Klett-Summerson colorimeter and diluted 1:50 in Todd-Hewitt broth (final culture dilution contained approximately 4×10^6 cells/ml). The mixture was incubated at 37°C in an apparatus that rotated the tubes end over end at 8 rpm. Duplicate blood smears were made at 20, 40, 60 and 120 min. One hundred leucocytes were counted in each smear, and the percentages containing streptococci were recorded. The standard deviation of replicate determinations made by the same observer was approximately $\pm 5\%$ in the range of 10 to 90% phagocytosis.

2. Bactericidal method. Survival of intra- and extracellular organisms in piglet leukocyte-serum mixtures was studied by Cohn and Morse's modification (19) or the original method of Maaløe (20). A suspension of white blood cells was prepared by rapidly sedimenting red blood cells in a 6% dextran solution (1 part of 6% dextran to 2 parts of blood) for 30 to 40 min and washing the white blood cells harvested from the supernatant with

[7] The agglutination tests against these strains were kindly made by Dr. Calvin M. Kunin.

Tyrode's buffer. The washed cells were resuspended to a concentration of 20,000 polymorphonuclear leukocytes/mm^3 (2×10^7/ml) in Tyrode's buffer containing 10% of the fresh test serum. The bacterial culture to be studied was grown 18 hr at 37°C, washed in Tyrode's buffer and adjusted to contain approximately 2 to 3 $\times$ 10^8 organisms/ml. The bacterial suspensions were adjusted by appropriate dilution to result in a bacteria-to-leukocyte ratio of 1:1 in the final test mixture. A 1.5-ml amount of the leukocyte suspension was mixed with 0.5 ml of the adjusted bacterial suspension. The tubes were rotated at 37°C and aliquots of 0.2 ml were removed at 0, 30 and 60 min during the incubation period. Pour plates were prepared, and the number of surviving bacteria were determined by colony counts made after overnight incubation at 37°C. To determine the survival of bacteria within leukocytes and in the extracellular fluid, 1 ml of the mixture was removed at 0, 30 and 60 min, diluted 1:5 in Tyrode's buffer and spun at 500 rpm at 0°C in a No. 2 International centrifuge for 3 min. A 0.2-ml amount of the upper layer of the supernatant was removed, and pour plates of appropriate dilutions were made to determine the surviving number of organisms which escaped phagocytosis. The remaining supernatant was carefully removed from the packed cells. The sedimented cells were resuspended in 1 ml of Tyrode's buffer, and 0.2-ml aliquots of this suspension were removed and appropriate dilutions plated to determine the number of surviving organisms within the leukocytes.

3. Absorption method. In some experiments CD piglet and germ-free mouse plasmas were absorbed with strains of streptococci or staphylococci. The bacterial cells grown in 40 ml of Todd-Hewitt broth for 16 to 18 hr at 37°C were harvested, washed three times with Tyrode's buffer and sedimented by centrifugation. Four milliliters of plasma were added to the bacterial pellet and mixed. This resulted in a bacterial concentration which varied from 5×10^9 to 1×10^{10} cells. The plasma was allowed to react with the cells at 0°C for 16 to 18 hr with constant mechanical agitation. After this time the bacteria were separated by centrifugation at 10,000 rpm in a refrigerated Sorvall centrifuge, and the plasma was sterilized by passage through a millipore filter held in a Swinney syringe adapter.

RESULTS

Resistance of germ-free mice to direct challenge with Group A streptococci

Table I shows the results of experiments in which germ-free and conventional mice were challenged with nine strains of Group A streptococci of varying degrees of virulence. Strains lacking M protein and capsules (T2/44/19) were not virulent for either germ-free or conventional mice. Both groups of mice resisted large inocula of these strains without significant differences in

TABLE I

Resistance of germ-free (GF) and conventional mice (CM) to strains of Group A streptococci

Group A Strain	M Protein[a]	Capsule[a]	$-\text{Log } LD_{50}$[b]		P[c]
			GF	CM	
T2/44/19	0	0	1.1	0.9	>0.1
327W (T1)	0	±	0.7	0.9	>0.1
T3D58X	0	++	2.8	2.7	>0.1
T14 Latino	10	0	3.4	3.5	>0.1
T14 I.H.	10	±	4.5	3.0	>0.1
T14S23 (variant matt)	0	+++	3.5	2.5	>0.1
T14S23 (heterogeneous)	1	+++	5.0	3.1	0.05
T30D24	60	++	7.0	3.2	<0.01
T12SF42	120	++++	8.2	6.5	>0.05

[a] See "Materials and Methods."

[b] Reciprocal of the log of the dilution of standardized culture producing LD_{50}.

[c] Comparison of GF and CM LD_{50} values.

TABLE II

Determination of comparative virulence of the T30D24 strain (M, +++; capsule, ++) of Group A streptococcus in germ-free and conventional mice

Status of Host	Per Cent Mortality/Dose Dilution[a]							−Log LD_{50}	S.E.[b]	*P*
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}			
Germ-free			100	100	80	50	20	7.0	<0.31	
Conventional	90	50	40	10				3.2	±0.30	<0.01

[a] Undiluted culture was washed and adjusted in broth to contain 2 or 3 × 10^8 organisms/ml. Ten mice were injected in each group.

[b] S.E., standard error.

mortality. No difference in resistance could be demonstrated in comparative experiments with germ-free and conventional mice when these animals were challenged with an M-negative variant which produced capsules of moderate size (T3D58X). The encapsulated strain, however, was approximately 100 times more virulent than the unencapsulated strain in both groups of animals. Unencapsulated strains containing small amounts of M protein (T14 Latino) were 100 times more virulent than unencapsulated, M-negative strains. Despite this increased virulence, the LD_{50} responses of the germ-free and conventional mice were comparable.

Germ-free and conventional mice were both highly susceptible to well-encapsulated strains rich in M protein (T12SF42). At a dilution of the culture of 10^{-8}, the minimal challenge dose was reached (1 to 2 organisms), and the difference in LD_{50} between the two hosts could not be determined. A significant difference between the resistances of germ-free and conventional mice was clearly demonstrated only with a strain of streptococci containing moderate amounts of M protein and capsule (T30D24). Repeated studies with this strain showed it to be significantly more virulent for germ-free than for conventional mice. Table II shows the results of a typical challenge experiment with this strain in greater detail. A difference in LD_{50} of 3.8 logs was observed.

Effect of passively transferred serum globulin on resistance of germ-free mice to streptococci

It was considered of importance to determine whether or not the difference in resistance of the germ-free and conventional mice to infection with moderately virulent strains of Group A streptococci was due to a greater number and amount of acquired antibodies in the serum of the latter. Accordingly, a globulin fraction was prepared from conventional adult mouse serum by ammonium sulfate precipitation at 0.33 saturation (γ-globulin). Each of 25 germ-free mice was injected intraperitoneally with approximately 10 mg of γ-globulin in 1.0 ml of saline. The same number of germ-free mice were injected with 1.0 ml of saline. The mice were challenged 24 hr later with log dilutions of T30D24 18-hr Todd-Hewitt broth cultures diluted in saline. Five mice were used for each dilution.

No protection of the germ-free mice by this fraction of mouse globulin from conventional animals was demonstrated. When the experiment was repeated employing the globulin fraction precipitated from conventional mouse serum by 50% saturation with ammonium sulfate, however, a slight but significant degree of protection was observed. These results, shown in Table III, suggest that factors other than the antibodies

TABLE III

Effects of mouse serum globulins upon resistance of germ-free mice to Group A streptococci

Intraperitoneal Injection, 0.5 ml	Mortality after Challenging with T30 D24[a]	
	−Log LD_{50}	*P*
γ-Globulin	6.4	
		>0.1
Saline	7.0	
Whole globulin	3.9	
		<0.05
Saline	6.3	

[a] Group A streptococcal strain of intermediate virulence.

TABLE IV

Protection of germ-free (GF) and conventional (CM) mice by type-specific antiserum to challenge with virulent T12 streptococci

Mice	Intraperitoneal Injection, 0.5 ml	Mortality after T12 Challenge			
		Experiment I		Experiment II	
		$-\text{Log } LD_{50}$	P	$-\text{Log } LD_{50}$	P
GF	T12 antiserum	5.4	<0.1	6.2	<0.05
CM	T12 antiserum	3.6		3.8	
CM	Normal saline	>7.0		>7.0	

found in serum γ-globulins may be important in mouse resistance to virulent streptococcal infection. To test this hypothesis, the protective effect of hyperimmune, heated, rabbit type-specific antiserum to virulent T12 organisms was compared in germ-free and conventional mice. In each of two experiments, 25 germ-free and the same number of conventional mice were injected with the highest dilution of rabbit anti-serum which had previously been determined to confer significant protection upon conventional mice. In these experiments, the conventional mice were protected to a greater extent than were germ-free mice by the same dose of homologous type-specific rabbit antiserum (Table IV). It appeared, therefore, that factors other than specific antibodies could account for differences in the survival of germ-free and conventional mice challenged with virulent strains of Group A streptococci.

In vitro opsonization studies

In order to confirm and extend the results obtained by the direct *in vivo* challenge experiments described above, a series of experiments were carried out in which the *in vitro* opsonization of streptococci could be studied under more defined conditions and compared with the *in vivo* results.

These experiments were carried out by adding to minimally heparinized blood taken from conventional or germ-free mice an inoculum of bacteria calculated so that the ratio of bacteria to leukocytes was approximately 1:1. Several strains of streptococci of varying degrees of virulence were studied in this manner to determine the efficiency of phagocytosis under these conditions. The response of germ-free and conventional mouse bloods to four strains of streptococci studied was essentially identical, although the rates of phagocytosis of the more virulent strains (T30D24, T3D58X) was considerably less than those observed with the avirulent strains (T2/44/19, T1 327W), as would be expected.

In view of the efficient phagocytosis of avirulent streptococci by germ-free mouse blood, it was of interest to determine whether or not this was a specific opsonic effect which could be significantly reduced by absorption of antibodies at low temperature with homologous organisms. In these experiments, the blood cells were separated from the plasma by slow speed centrifugation, washed by resuspension and centrifugation in Tyrode's buffer and then resuspended in either unabsorbed, absorbed or heated homologous plasma. Absorptions were carried out at 0°C for 16 to 18 hr. The reconstituted bloods were then inoculated and handled as described above. Results of a typical experiment of this kind are shown in Figure 2. Absorption of the plasma with staphylococci at low temperature did not significantly alter the rate of phagocytosis of avirulent streptococci. Absorption with the homologous streptococcal strain reduced the percentage of phagocytosis to the same degree as that observed in the system using heated serum or Tyrode's buffer to resuspend the cells. Repeated absorptions did not

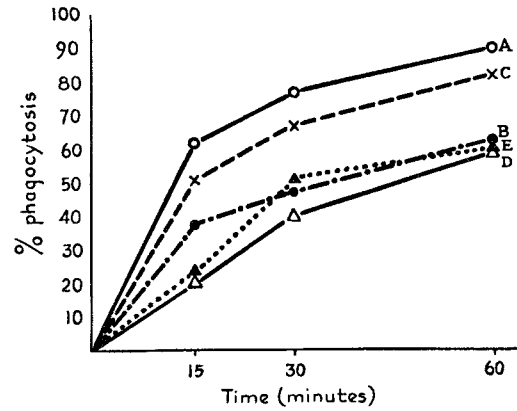

Figure 2. Phagocytosis of avirulent Group A streptococci by germ-free mouse leukocytes resuspended in germ-free mouse plasma treated as follows: A, O——O, unabsorbed; B, ●-·-·-●, absorbed with homologous streptococci; C, X- - -X, absorbed with staphylococci; D, △——△, heated plasma; E, ▲····▲, Tyrode's buffer (control).

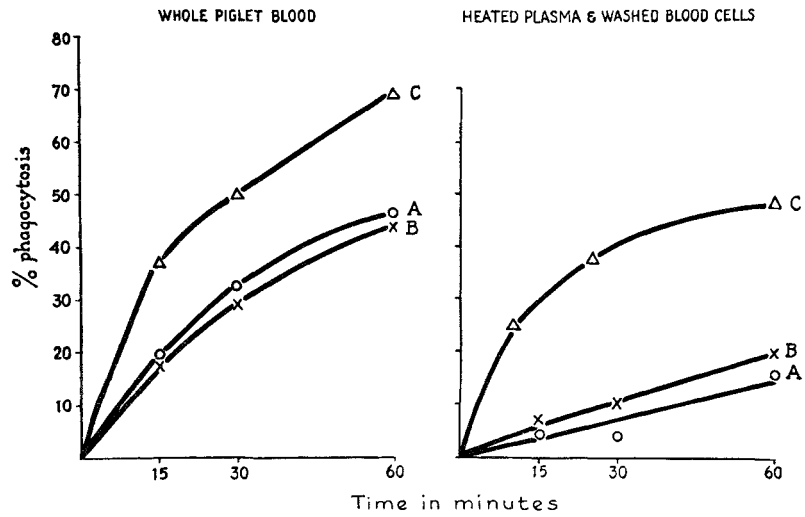

Figure 3. Phagocytosis of avirulent Group A streptococci by piglet blood. A, O——O, colostrum-deprived piglet blood; B, ×——×, colostrum-fed piglet blood; C, △——△, human blood.

further reduce the percentage of phagocytosis significantly. It appeared, therefore, that opsonins were present in germ-free mouse serum against avirulent Group A streptococci although, even in their absence, phagocytosis of these strains by the blood of germ-free mice was vigorous.

Opsonization of streptococci by piglet blood

Because it was difficult to interpret the origin and nature of the serum opsonins against Group A streptococci demonstrated in germ-free mouse blood, an animal with less acquired specific antibody was sought. Our attention was drawn, therefore, to the newborn piglet. Additional studies on the *in vitro* opsonization of streptococci by the blood of newborn, colostrum-deprived piglets were carried out in a system similar to that described for the germ-free mouse. The rate of phagocytosis of avirulent strains of Group A streptococci (those lacking M protein and capsules) appeared to be identical in colostrum-deprived (CD) and colostrum-fed (CF) piglet bloods (Fig 3). Two different avirulent strains were studied (T2/44/19 and T1 327W). Phagocytosis of these strains by piglet blood was somewhat slower than that observed with human blood. Virulent strains of Group A streptococci resisted phagocytosis similarly in CD and CF bloods.

The presence of thermolabile opsonins in the piglet bloods was demonstrated by separating blood cells and plasma from lightly heparinized samples, heating the plasma for 20 min at 56°C, and resuspending washed blood cells in heated and unheated homologous plasma. Human blood was treated similarly and included in the experiment to provide a comparison of the relative efficiency of piglet phagocytosis in this system. The reconstituted bloods were inoculated with the M-negative, capsule-negative T2/44/19 strain and the rate of phagocytosis was studied. Phagocytosis was markedly decreased to a similar degree in CD and CF piglet bloods by heating their plasmas. The effect on human blood included in the same experiment was similar (Fig. 3).

Attempts to absorb opsonins against Group A streptococci from CD piglet blood

To determine whether or not the thermolabile serum opsonins against avirulent Group A streptococci demonstrated in CD piglet blood required an intermediate "natural" antibody, the sera of these animals were absorbed overnight at 0°C with, respectively, large numbers of the homologous strain of streptococci, and a strain of coagulase-negative staphylococci. Absorption of the CD piglet serum with staphylococci at low temperature did not reduce the rate of phagocytosis of avirulent streptococci significantly, whereas absorption at low temperature with homologous streptococci reduced phagocytosis moderately but consistently in repeated experiments (Fig 4). The

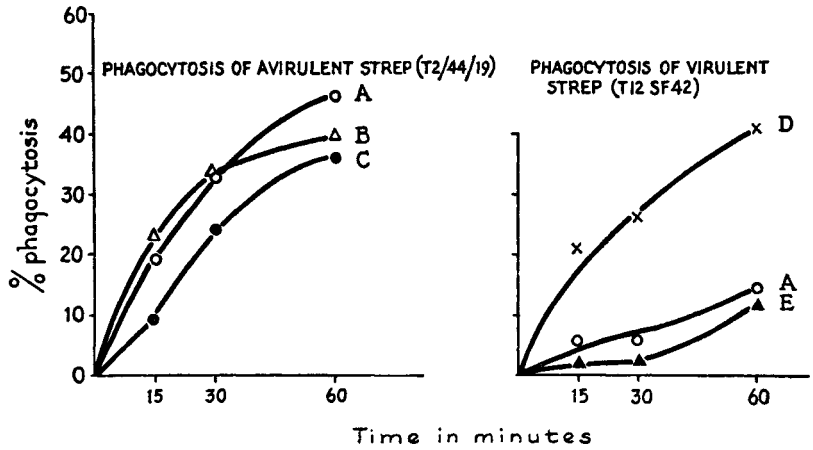

Figure 4. Phagocytosis of Group A streptococci by colostrum-deprived piglet leukocytes resuspended in colostrum-deprived piglet serum treated as follows: A, O——O, unabsorbed; B, △——△, absorbed with staphylococci; C, ●——●, absorbed with avirulent streptococci; D, ×——×, unabsorbed piglet serum plus heated rabbit anti-T12 serum; E, ▲——▲, serum D absorbed with T12 streptococci.

residual phagocytic activity following absorption by homologous organisms was considerable, however, and approximately equal to that of CD piglet blood reconstituted after heating the serum. Thus, although "natural opsonins" to Group A streptococci were demonstrated, phagocytosis of avirulent strains was quite active even after such opsonins were absorbed.

For comparison, the experiments with avirulent Group A streptococci were made simultaneously with experiments employing virulent streptococci (Fig. 4). In the latter, it was demonstrated that the addition of heated type-specific rabbit antiserum resulted in highly efficient phagocytosis of the virulent streptococci by CD piglet blood. Thus, the thermolabile opsonins and leukocytes of the colostrum-deprived piglet blood possessed full potential for phagocytosis of virulent streptococci in the presence of type-specific antibody. Absorption of the added rabbit type-specific antibody was shown to be complete under the same conditions employed for absorbing "natural" opsonins from the intact CD piglet serum (Fig. 4).

Bactericidal effect of phagocytosis by CD piglet blood

Although the preceding experiments demonstrated highly efficient phagocytosis of avirulent Group A streptococci and of staphylococci by colostrum-deprived piglet blood, the method of direct observation employed did not provide information concerning the efficiency with which peripheral blood phagocytes of these animals destroyed ingested organisms. Accordingly, experiments were carried out to study the survival of Group A streptococci in mixtures of piglet serum and washed leukocytes. At varying intervals after inoculation, the blood cells were separated from the serum and extracellular organisms by differential centrifugation. The method employed permitted a determination of the rate of phagocytosis by determining the decrease in number of bacteria which had not been phagocytized and remained in the supernatant. At the same time, it measured survival of bacteria in the sedimented blood phagocytes at varying periods after their ingestion. Mixtures of 10% piglet serum in Tyrode's buffer supported the growth of the strains of Group A streptococci studied and showed no direct serum bactericidal effect. Undiluted piglet serum was not used because it was variable in its effect on the growth of different strains of streptococci. Nevertheless, in experiments made with whole heparinized bloods, there was no consistent difference in bactericidal effect noted between CD and CF bloods.

The results of these experiments made it evident that colostrum-deprived piglet blood cells phagocytized and destroyed avirulent Group A streptococci at least as efficiently as those of colostrum-fed animals, and virulent streptococci were equally resistant to phagocytosis and destruction by CD and CF bloods.

DISCUSSION

The above studies provide some information relevant to a number of features of the host-parasite relation of mammals to Group A streptococci. Perhaps most striking is the importance of M protein and hyaluronic acid capsules as virulence factors. Lacking these, the Group A streptococcus is unable to infect 6- to 8-week-old germ-free mice and cannot resist phagocytosis and intracellular destruction by the blood cells of newborn colostrum-deprived piglets. Specific antibodies to cell wall constituents other than M protein seem to be of relatively little importance in the mouse's defense against M-negative, capsule-negative Group A organisms.

The resistance of these animals to infection with virulent strains possessing moderate amounts of both M protein and capsules does involve, however, factors which confer greater resistance upon the conventional, as compared with the germ-free, mouse. We were unable to show that this difference in resistance was due to globulins precipitated from mouse serum by 0.33-saturated ammonium sulfate. The difference in resistance of germ-free and conventional animals was minimized, however, by administration of whole globulin fractions from conventional mouse serum to germ-free mice. The nature of these serum factors has not yet been elucidated and the limited supply of germ-free mice available to conduct these experiments made it difficult to study further the factors involved. Because the difference in resistance of germ-free and conventional animals to challenge with partially virulent Group A variants is not very great (usually approximately 2 to 3 logs in LD_{50}), the protective effect of whole globulin upon the germ-free mouse was not easily demonstrated and required relatively large numbers of animals and repeated experiments for statistically significant results. Some inferences concerning the role of specific antibodies may be drawn from the fact that equal amounts of type-specific antiserum protected germ-free mice less efficiently than conventional mice against challenge by homologous-type virulent strains. This would suggest a greater competence of the reticuloendothelial system in the conventional mouse under these conditions. In the peripheral blood, however, no differences in the destruction of these organisms could be demonstrated between germ-free and conventional mice or between CD and CF piglets. The polymorphonuclear phagocytes primarily responsible for this result are quite capable of destroying Group A streptococci in animals with limited immunologic experience. There may be little need for the germ-free mouse to call upon all of the resources of the reticuloendothelial system to defend himself against avirulent Group A streptococci, whereas challenge with virulent strains may elicit more subtle differences in the efficiency of the entire phagocytic system, differences which could be mediated by serum factors not yet identified.

Our studies have demonstrated the presence of opsonins against avirulent Group A streptococci in CD piglet sera, which bears on the question whether or not "natural" opsonins against Group A streptococci exist which are not conventionally acquired specific antibodies. The opsonins absorbed at 0°C from CD piglet serum by avirulent Group A streptococci represent either minute amounts of induced maternal antibodies which managed to pass through the multilayered pig placenta, or they are constitutive opsonins which can bind to a variety of bacterial surfaces, except to those whose chemical structure is either identical with the host's tissues (e.g., hyaluronic acid) or entirely unique (e.g., M protein). In support of placental transmission is the evidence of Segre and Kaeberle (21, 22). These authors postulate that intrauterine transmission of minute amounts of antibody in the sow accounts for differences in the response of CD piglets to certain antigens.

The opsonic experiments presented in this study are comparable to the results of Sterzl and his co-workers (17, 18). Avirulent Group A streptococci bind a serum opsonin in CD piglets which in turn reacts with thermolabile opsonins. The opsonic factor for avirulent Group A streptococci, like Sterzl's bactericidal factor for R strains of *E. coli*, could represent extremely small amounts of induced antibodies. The activity of these opsonins against large inocula of organisms, and the difficulty in absorbing such opsonins completely unless large numbers of bacterial cells are employed, make it seem unlikely to us that minute amounts of placentally transferred, induced antibody are solely responsible for the opsonic effect of CD piglet serum against Group A streptococci. The monolithic concept that all serum factors which opsonize foreign particles are antigen-induced antibodies should not deter

the search for other serum factors which may also have affinity for a broadly reactive cell surface and which may enhance phagocytosis and contribute to natural immunity. The CD piglet may help to identify such factors, if, as these studies suggest, they are indeed present.

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SUMMARY

A study was made of the resistance of germ-free and conventional mice to direct challenge with Group A streptococci of graded virulence. Germ-free and conventional mice were highly, and equally, resistant to nonencapsulated strains of Group A streptococci lacking M protein. Greater susceptibility of germ-free mice could be demonstrated only with Group A strains containing both M protein and capsules. This difference could be abolished by a globulin fraction of normal mouse serum but not by normal mouse γ-globulin or by homologous type-specific antiserum.

In vitro studies with bloods of germ-free mice and colostrum-deprived piglets provided additional evidence that phagocytosis of avirulent Group A streptococci required little, if any, specific antibodies. Bloods from these animals phagocytized and destroyed streptococci as readily as did their conventional counterparts. Thermolabile opsonins against avirulent Group A streptococci were readily demonstrable in the blood of colostrum-deprived piglets, whose serum contains only minute amounts of γ-globulin. Absorption of the sera of these animals at low temperatures with large amounts of homologous streptococci resulted in some decrease in opsonization, but considerable residual phagocytosis was apparent. The leukocytes of colostrum-deprived, and of colostrum-fed, animals were equally capable of phagocytizing and destroying Group A streptococci in the presence or absence of specific opsonins.

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