Non-Type Specific Resistance to Group A Streptococci in Germ Free and Conventional Mice.* (28625)

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Resistance to infection with virulent strains of Group A streptococci has been shown to be type specific, that is, dependent upon the action of antibodies directed against the type of M protein produced by the invading organisms. This M protein antigen, of which 51 immunologically distinct types have been identified thus far, has been associated with the virulence and resistance to phagocytosis of Group A organisms(1). Antibodies to M protein have been found to be potent opsonins and to confer protection against streptococcal disease of homologous type in both laboratory animals(2) and in natural human infection (3).

The capsule of Group A streptococci, which consists for the most part of hyaluronic acid, has been implicated as an additional streptococcal product associated with virulence. Although its role in virulence has not been clearly defined, the capsule has been shown to impede phagocytosis(4).

Host resistance which is not dependent upon the M anti-M system was studied by challenging germ free and conventional mice with Group A variants of graded M protein and capsule characteristics. The results form the basis of this report.

Materials and methods. 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. The germ free mice were supplied by Charles River Laboratories (CR1) and were derived from Lobund Institute stock. The animals were maintained with appropriate sterility controls in our laboratory in plastic isolators. Challenge experiments with low germ free and conventional mice were performed in the isolators. The conventional mice were raised under clean but not sterile laboratory conditions for numerous generations. Analysis of the electrophoretic patterns of the serum proteins confirmed the published reports of the relative hypogammaglobulinemic state of the germ free mice(5).

The indirect bactericidal test(6,7) and the long chain test(8) were used to assay the sera of the germ free and conventional mice for anti-M antibody. Type specific anti-M antibody was not detectable. Anti-streptolysin O(9), antihyaluronidase(10), and anti-strepto DPNase(11) 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.‡

The challenges were carried out with Group A streptococcal strains which were chosen to exemplify varied hyaluronic acid capsule and M protein characteristics.§ The cultures were grown overnight in fresh Todd-Hewitt broth and standardized by an optical density technique to a concentration of approximately 2×10^8 chains per ml of broth. The challenges were made by injecting by intraperitoneal route 0.5 ml of serial log dilutions of the standard culture. The M protein and capsule of a variant were graded, usually from the same culture that was used to challenge the mice.

The relative size of a capsule was estimated by inspecting wet India ink capsule preparations. Optimal capsule formation was obtained by subculturing the organism for 2 hours in 10% fresh normal rabbit serum or

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TABLE I. Resistance of Germ Free (GF) and Conventional Mice (CM) to Strains of Group A Streptococci.

Group A strain	M* protein	Capsule*	-Log LD_{50}†		P‡	No. of mice challenged	
			GF	CM		GF	CM
T2/44/19	0	0	1.1	0.9	>.1	15	15
T3 D58X	0	++	2.8	2.7	>.1	39	39
T14 Latino	10	0	3.4	3.5	>.1	47	60
T30 D24	60	++	7.0	3.1	<.01	50	40
T12 SF42	120	++++	8.2	6.5	.05	20	20

* See materials and methods.
† Reciprocal of log dilution of standardized culture producing LD_{50}.
‡ Comparison of GF and CM LD_{50} values.

in 0.7% bovine serum albumen. Capsules were graded by comparing the diameter of the total organism (capsule plus coccus) to the coccus alone.

The M protein content of a strain was estimated by finding the highest dilution of a standardized culture extract which would give a noticeable precipitate with standard typing serum(12). The relative amount of M was graded as the reciprocal of the final titer of antigen. The LD_{50} and its standard error were estimated by the probit graph method of Miller and Tainter(13). The methods of Karber(14) and Reed and Muench(15) gave comparable LD_{50} values for each challenge.

Results. Germ free and conventional mice were challenged with 11 strains of Group A streptococci of varying degrees of virulence. The results obtained with 5 representative strains are shown in Table I.

Strain T2/44/19 previously had been typable (Type 2) but had lost its ability to produce both M protein and capsule. It was not virulent for either conventional or germ free mice. The two groups of mice resisted large inocula of this strain with insignificant difference in mortality.

Strain T3 D58X was an M negative variant that produced a capsule of moderate size (2 plus). The presence of this capsule was correlated with an increase in virulence of almost 2 log dilutions compared with the unencapsulated T2/44/19. T3 D58X did not differentiate, however, between conventional and germ free animals.

Strain T14 Latino produced M protein in relatively small amounts. A capsule was not detectable. M protein in the absence of a capsule was associated with increased virulence (about 100 times) compared with the avirulent strains. Despite this increased virulence the LD_{50} responses of the germ free and conventional mice were equal.

Strain T30 D24 combined a capsule of moderate size (2 plus) with large amounts of M protein. It was significantly more virulent for the germ free than for the conventional mice. A difference in LD_{50} of 3.9 logs was observed. The graphic statistical analysis of this difference is shown in Fig. 1.

Strain T12 SF42 was held in an optimal phase of virulence by frequent mouse passage. Germ free mice and conventional mice were both susceptible to challenge with this strain. At a dilution of the culture of 10^{-8} the minimal challenge dose was reached (1-2 organisms) and the difference in LD_{50} between the two hosts therefore was limited.

Discussion. Homologous M antibody was not detectable in the sera of either germ free or conventional mice. Resistance of these animals to challenge with Group A streptococcal strains therefore may be interpreted as due to factors other than type specific immunity.

The ability of the germ free mice to survive large inocula of strains lacking M protein and capsule (*e.g.,* T2/44/19) suggests that the kind of antibody available to a germ free mouse may be sufficient to opsonize the uncovered Group A cell surface. On the other hand, preliminary studies of *in vitro* phagocytosis in germ free mouse and colostrum deprived piglet bloods indicate that phagocytosis of such avirulent streptococci, although enhanced by the presence of serum opsonins,

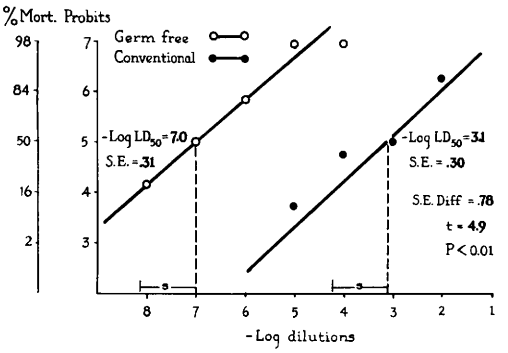

FIG. 1. Graphic analysis by Miller-Tainter method(13) of the LD_{50} and standard error (S.E.) of challenge experiments made on germ free and conventional mice with a strain of Group A streptococci of intermediate virulence (T30 D24, see text).

may proceed effectively in their absence(16). Hence antibody may not be critical in defending against challenge with these strains.

Strains producing either M protein or capsule were more virulent for both germ free and conventional animals. The resistance of the germ free appeared equal to that of the conventional mice in spite of the presence of either of these virulence factors alone.

The conventional mice, however, were significantly more resistant to moderately virulent strains which produced *both* M protein and capsule in moderate amounts (*e.g.*, T30 D24). The greater amount of gamma globulin and the presumably greater variety of cross reacting antibodies available to the conventional mouse(17) could account for this non-type specific resistance. On the other hand, it is possible that the increased susceptibility of the germ free mice to challenge with these strains was due to a relatively greater susceptibility to streptococcal toxins. The recent demonstration that the response of germ free mice to endotoxin is equal to that of conventional mice(18), however, tends to argue against this possibility.

Non-type specific resistance to challenge with Group A organisms appeared to be superseded by the requirement for type specific antibody only in the special case of the most virulent strains. When a strain is optimally equipped with M protein and capsule (*e.g.*, T12 SF42) cross reacting antibodies may be all but excluded from the bacterial cell surface so that non-type specific opsonins no longer may be able to promote phagocytosis of virulent organisms.

The germ free animal appears to be useful for the study of factors in the host parasite relationship other than those most obviously responsible for virulence and resistance. More subtle factors which may account for variation in host resistance may be revealed by the use of animals with limited antigenic exposure.

Summary. Non-type specific resistance to Group A streptococcal variants was demonstrated by *in vivo* challenges of germ free and conventional mice. The presence of type specific anti-M antibodies was excluded by long chain and indirect bactericidal tests on the animals' sera. Both germ free and conventional mice were equally resistant to Group A strains lacking both M protein and capsule. The presence of either M protein or capsule increased virulence equally in germ free and conventional mice. However, strains containing *both* M and capsule in moderate amounts were significantly more virulent for germ free than for conventional animals. Both germ free and conventional mice were highly susceptible to a strain with maximal M protein and capsule. Resistance due to cross reacting antibody was superseded by requirements for anti-M antibody only when the infecting organism contained very large amounts of M protein and capsule.

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Effect of Specific Anti-Sera on C^{14} Amino Acid Incorporation into Cellular Protein of Mouse Ascites Tumor Cells.* (28626)

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Goldberg *et al.* have described the effect of specific anti-sera on Erhlich ascites tumor cells(1). When observed by phase microscopy, numerous small clear pouches immediately form upon exposure of the cells to specific anti-serum and after one-half hour incubation at 37.5°C the cells become markedly enlarged. The nucleus, along with the intracellular particles, is restricted to one side of the cell and the remaining area of the cell (congealed pouches) is clear and apparently contained by a membrane. Green *et al.*(2) have reported that these cells lose a significant amount of total cell protein, amino acids, potassium and nucleotides. It has also been reported(3,4,5) that these cells have lost the capacity to oxidize exogenous glucose, but maintain the ability to oxidize succinate.

We have found that the uptake of amino acid by cells and subsequent incorporation of labeled amino acid into the gross protein is inhibited by anti-serum and that percent of inhibition correlates well with percent of cell rupture. This observation has yielded a rapid assay method for anti-sera and this method and results are reported below.

Materials and methods. L-valine-1-C^{14} (5.73 μc/μmole) was obtained from the New England Nuclear Corp. and made up in saline so that 0.1 ml contained 0.25 μc. The solution was frozen in small aliquots and stored at —20°C until required. NCTC-109 was obtained from Microbiological Associates. Male white rats between 180-200 g were obtained from Holtzman Co. The S-180 ascites tumor and lymphosarcoma 6C3HED was provided by Dr. T. McCoy, Noble Foundation, Ardmore, Okla., and maintained in Swiss mice and C3H mice, respectively. The C3H mice were purchased from the Texas Inbred Co., Houston, Texas, and the Swiss mice from A. R. Schmidt Co., Madison, Wis.

Preparation of antigen. All steps were carried out under sterile conditions. Tumor cells were removed from mice injected intraperitoneally with tumor one week previously, transferred to a graduated centrifuge tube and the tube sealed with a serum cap. The tube was centrifuged at 800 $\times$ *g* for 5 minutes and the supernatant fluid removed from the cell pellet by aspiration through the serum cap. Nine volumes of saline were injected into the tube with enough force to break the cell pellet. The tube was again centrifuged, the saline wash was removed by aspiration and the cells resuspended in 9 volumes of saline.

This preparation always contained varying amounts of red cells (1-5% of total cell volume). Rats weighing approximately 200 g

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