A LIVE STREPTOMYCIN-DEPENDENT PASTEURELLA MULTOCIDA VACCINE FOR THE PREVENTION OF HEMORRHAGIC SEPTICEMIA

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

BETTY DONG WEI

1977

ABSTRACT

A LIVE STREPTOMYCIN-DEPENDENT *PASTEURELLA MULTOCIDA* VACCINE FOR THE PREVENTION OF HEMORRHAGIC SEPTICEMIA

By

Betty Dong Wei

A type B *Pasteurella multocida* was used for the development of a streptomycin-dependent (Str^D) vaccine. *P. multocida* R-473, a hemorrhagic septicemia strain, was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine to increase the likelihood of encountering a streptomycin-dependent mutant and plated on agar containing 400 µg/ml of streptomycin. Replica plating was used to differentiate dependent from resistant colonies. Mice and rabbits were vaccinated with a Str^D mutant and challenged along with unvaccinated controls 21 days later with the wild type R-473. Protection of greater than 4 logs was shown for the vaccinated mice. All vaccinated rabbits were protected and all unvaccinated controls succumbed to a challenge of 500 or 1000 LD_{50}.

High mortality in clinical cases of hemorrhagic septicemia creates severe economic losses especially in southeast Asia and Africa. A live preparation might prove more immunogenic than some of the killed vaccines presently in use. Hemorrhagic septicemia is only one of a wide range of diseases caused by *P. multocida*. Str^D organisms may be of use as vaccines for other pasteurelloses such as rabbit "snuffles", fowl cholera, and pneumonic bovine pasteurellosis.

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By

Betty Dong Wei

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1977

Dedicated to my father and mother

Mr & Mrs Yen O. Dong

and to my husband

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ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to Dr. G. R. Carter, who started my interest in streptomycin-dependence, for his helpful suggestions and guidance throughout my graduate studies. I would especially like to thank Dr. R. R. Brubaker and Dr. L. Snyder for their critical suggestions in the course of my research work and to Drs. R. J. Moon and M. J. Patterson for kindly serving on the advisory committee. Grateful acknowledgement is also made to Mrs. Dorothy Boettger for her efforts in securing necessary supplies and media and to Drs. E. Sanders and C. H. Coy for their donations of experimental animals. Special thanks to Mr. A. Wayne Roberts for his assistance in the laboratory and for proofreading the manuscript. I would like to thank Dr. M. M. Chengappa and Mr. Amar Gupta for their assistance in the vaccination studies.

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HEMORRHAGIC SEPTICEMIA

A Review of the Literature

History

Pasteurellosis was first described in cattle by Bollinger in 1875 and the causative agent was isolated by Kitt in 1885. In 1880, Pasteur described the organism which caused fowl cholera. Gaffky found the organism responsible for rabbit septicemia in 1881 and Loeffler for swine plague in 1886. A German pathologist, Hueppe noting similarities between the diseases in various hosts and their causative organisms, collectively named the disease hemorrhagic septicemia and the organism *Bacillus septicemiae hemorrhagiae*. The disease was extended to buffalo when it was described by Oreste and Armanni in 1887. In 1896, Kruse introduced the binomial, *Bacillus bovisepticus*. In 1900, Ligniers described the organism and diseases more completely and used the name, *Pasteurella*, which had been previously suggested by Trevisan. Another name, *Bacterium multocidum*, came into use in 1899. Rosenbusch and Merchant's binomial of 1937, *Pasteurella multocida*, has wide but not universal usage. A review is due by the International Congress for Microbiology to clear questions about classification and nomenclature. The name, hemorrhagic septicemia was at one time extended to include bovine shipping fever, a respiratory disease of complex etiology with pasteurellas often secondary invaders to *Parainfluenza-3* virus (46). The current definition of hemorrhagic septicemia decided by the Food and Agriculture Organization of the United Nations in 1962 (7) states that it is an acute disease of cattle and buffalo caused by type B *P. multocida* with high mortality in clinical cases. A hemorrhagic

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septicemia isolant from Central Africa was found by Carter (12) to be a second serotype, (type E).

The Disease and Its Epidemiology

Hemorrhagic septicemia (H-S) occurs in southern Europe, the USSR, north, central and east Africa, the Near East, and southern Asia. It has not been reported in South America, Australia, south Africa or Japan, and its occurence in North America has been confirmed only a few times. Epizootics in American bison were reported in 1912 (27), in 1922 at Yellowstone National Park (22), and in 1965 at the National Bison Range, Moiese, Montana (26). Two groups of young Holstein-Friesian cattle were affected in Pennsylvania in 1969. Serotype 6:B, the same as in the bison, was isolated (32).

The greatest incidence of H-S is in the rainy season, when there is an increase in work demanded of draft animals. Cattle are likely to be in poor condition after the dry weather. Nutrition and management are often changed at this time. Stress, high humidity, changes in temperature, and various minor infections may precipitate H-S. Cattle in dry regions of Equatorial Africa show naturally acquired immunity but no clinical disease, so extraneous factors must be needed to precipitate the disease. Cattle and buffalo of all ages are affected.

The percent of animals infected in a particular country may be small but certain areas might sustain high losses, up to seventy percent. Usually, losses in a village amount to one to ten percent. Immediate vaccination with plain broth bacterin in the midst of an outbreak saves some animals. Thailand and India are countries with high

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yearly losses of 10,000 to 50,000 cattle and buffalo. The losses in most Asian countries remain the same year after year, indicating some kind of balance being reached despite present vaccinal methods of control, a balance that can only be changed with improved prophylaxis.

Because of the seasonal nature of the disease, parasitic vectors have been sought but never confirmed. From the pathology of the disease, it is reasonable to assume that the nasopharynx is the main route of entry of the organism, either by inhalation or ingestion. Baldrey (7) in 1911, sprayed fodder with a culture of *Pasteurella*, but only the debilitated became ill. Normal animals became immune, indicating a subclinical infection. Large numbers of bacteria (on the order of 10^8) from an aerosolized culture deposited in the nose can cause infection in cattle. The carrier animal is the source of the microorganism. Soil can harbor the organism for short periods and indirectly transmit the disease but there is no permanent soil reservoir (7). Greater than ten percent of cattle and buffalo in India and southeast Asia have naturally acquired immunity to hemorrhagic septicemia as shown by a rise in antibodies (7). The ten percent figure has been constant for the past fifty years. An outbreak begins with a decrease in resistance in an animal harboring *Pasteurella* types B or E. Once infected, the organism is spread in the feces, saliva, and nasal discharges which harbor large numbers of the organism. The size of the outbreak depends on the condition of the animals and the persistence of *Pasteurella* in the herd. When half of the herd is immunized, outbreaks can be prevented, possibly due to lessening the chance for a first case and decreasing the number of **susceptibles**. A village affected one year is usually spared the next since immunity presumably developed the previous year. The incidence in the district though remains the same.

The Microorganism

Pasteurella multocida is a small, gram negative, often pleomorphic rod ranging from cocco-bacillary to filamentous forms. Nikiphorova (7) demonstrated with the electron microscope a capsule on the type B organism that is 0.5 um thick, smaller than some other *Pasteurella* types. Tryptose agar with carbohydrates show iridescent colonies 1 mm in diameter at 24 hours at 37 C. Strains dissociate to duller, smaller "blue" variants that are non capsulated. Carter (11) proposed S (smooth), SR (intermediate), and R (rough) as the main colonial types. Type I (B) does not form mucoid colonies. Staining of the fresh isolates reveal bipolar staining with methylene blue.

Different strains of *P. multocida* show great variations in their ability to ferment carbohydrates. In general, glucose, sucrose, levulose, saccharose, mannose, galactose, mannitol and fructose are fermented by most strains. Lactose, maltose, trehalose, rhamnose, inositol, salicin, and inulin are usually not attacked. Some strains of *P. multocida* isolated from dogs and cats do ferment maltose and on occasion, lactose. Variable utilization is observed with xylose, arabinose, dulcitol, sorbitol, raffinose, dextrin, and glycerol (28,51). Organisms in the genus *Pasteurella* characteristically are catalase positive, reduce nitrates to nitrites, are oxidase positive and gelatinase negative. Ornithine decarboxylase is usually produced. Arginine dihydrolase and lysine and glutamic acid decarboxylases are absent. Malonate and citrate are not utilized. Motility, urease production, and growth on MacConkey Agar are negative. Indole production is positive. Urease activity has been reported in canine and feline strains. Results of the examination of 1,268 isolates from various animal hosts show no

correlation between host preference and biochemical reactions (28).

Clinical Signs and Lesions

The mortality rate, once the clinical disease is established is 100 percent. Cattle show more variable pathologic signs than buffalo because they tend to be affected less acutely and live longer. More pneumonic lungs are found in cattle than in buffalo. The symptoms are fever, salivation, nasal discharge, dullness, respiratory distress, and prostration. Bacteremia can be demonstarted in six to twelve hours after experimental infection and saliva often is positive culturally at this time. The initial bacteremia lasts a short time due to the filtering action of the liver and spleen where the organism continues to multiply. A second bacteremia occurs and counts of 1×10^6 organisms per ml have been found (17). Blood fibrinogen increases and venous pressure decreases. *Pasteurella* can be cultured from feces, urine, and milk. The terminal condition is reminiscent of and indeed may be endotoxic shock. Edema of the ventral neck and often the forelegs is observed. The tongue is swollen and protruding. In atypical cases, throat edema is not observed. After death, edema of the glottis, perilaryngeal, and peritracheal tissues is found. Punctate hemorrhages may be seen in those tissues but the edema fluid, which is loaded with *Pasteurella* remains clear or slightly tinged with blood. Petechial hemorrhages are found in the auricles and under the serous membranes throughout the body. Lungs are often congested with thickening of the interlobular septa. Lymph nodes of the thoracic and peritoneal regions are congested and sometimes hemorrhagic. Early signs of peritonitis occur and calves may show

hemorrhagic gastritis or gastro-enteritis. The spleen is often unchanged except for occasional small hemorrhages.

Intravenous injection of type B lipopolysaccharide gives severe blood stained diarrhea. The LPS is thought to be the cause of the intestinal lesions and signs. The toxic changes are usual for a septicemia (45). The hemorrhages, despite the name, are not spectacular.

The Antigens of *Pasteurella multocida*

The antigenic components of *Pasteurella multocida*, as with other gram negative bacteria, have been difficult to elucidate. There are difficulties in getting pure macro-molecular fractions to work with and risks of discarding important components in extraction and purification procedures. Very small quantities of antigens can stimulate an antibody response *in vivo*. The cell wall and capsule form a continuum and antigens are distributed without notion of clear cut boundaries between cellular layers. The idea of somatic versus capsular antigens is somewhat arbitrary and expresses quantitative differences in antigen derived from various extraction procedures. Successive extractions with saline yield mixtures of antigens. Amounts of bound protein-polysaccharide and polysaccharide decrease as the numbers of extractions increase while amounts of lipopolysaccharide remain relatively constant. Saline extracts (2.5 %) of optimally antigenic (Phase I) *Pasteurella multocida* yield 12 lines of precipitation on Ouchterlony plates (7,17). Other strains, especially if subcultured, yield less than 12 lines. It is possible that some of the precipitin lines are artifacts of fractionation or represent enzyme proteins. Of these 12 lines, 2 are thought to

be polysaccharide, 1 lipopolysaccharide, and 9 are proteins or protein complexes (7). Sodium chloride extraction techniques yielded polysaccharides of high nitrogen content thought to be mucopolysaccharide and was represented by 1 precipitin band. It is thought that the other band represents a polymer of fructose and other sugars. No single homogeneous protein could be found which could account for the protection conferred by active immunization.

The lipopolysaccharide fraction is constitutionally and biologically similar to that of other gram negative bacteria (5,6,35). It is presumed that the LPS makes up a large portion of the cell wall as in other gram negatives. Lipopolysaccharide can be isolated by the phenol-water method of Westphal, but can be found released at all steps of extraction with 2.5 % NaCl.

The administration of either whole culture or endotoxin fractions caused similar, widespread vascular lesions (45). Widely distributed hemorrhages, edema and general hyperemia were the most obvious changes, with pneumonia a constant finding. Toxins of *Pasteurella* have been known since Pasteur's time. Baldrey in 1907 (7) discovered that filtrates from old cultures were lethal for rabbits. Bain found that filtrates of infected sera produced fever in rabbits and mice suggesting the presence of lipopolysaccharide (7). Presently, there is controversy as to the presence of an exotoxin. Dhanda (15) isolated from type B Mukteswar 52 strain a toxic protein which comprised one percent of the cellular dry weight, and was inactivated at 56 C for 30 minutes. However, Bain has not been able to obtain such a toxin from the Mukteswar or other strains (7).

Two major antigenic components, capsular antigen and endotoxin have been identified in saline and phenol-water extracts (40,41). Indirect

evidence, obtained by adsorption with specific antigens of mouse protective antibodies from bovine anti-whole cell serum against type B indicate that the type B capsular antigen played a part in protection (41). Capsular antigen, free of endotoxin was prepared by fractional precipitation from aqueous solution by addition of polar organic solvents. Purity from endotoxin was determined by rabbit pyrogenicity and chick embryo lethality tests. The capsular antigen is a high molecular weight acidic polysaccharide. Polyacrylamide gel electrophoresis showed some molecular weight heterogeneity so criteria of purity, as distinct from freedom from endotoxin, was difficult to establish. The antigen was not protein since it was heat stable, resistant to pronase, unable to stain with protein stains in polyacrylamide gel and had low light absorption at 280 nm. Mouse protection tests showed that adsorption of anti-whole cell bovine serum by the type B capsular antigen eliminated protection. However, this does not eliminate the possibility that there exist other protective antigans, since the antiserum may not have contained sufficiently high levels of antibodies against these to confer protection. Carter and Annau (9), and Bain (2) found fresh encapsulated isolates to be better antigens. Other workers, Bain (3), Dhanda (16), and Knox and Bain (31) described capsular antigens associated with protein components as having a protective role. Cell walls have been found to induce more protection than cytoplasm or culture filtrates (53).

A heat stable, particulate lipopolysaccharide-protein complex isolated from encapsulated *P. multocida* by extraction with formalinized saline was found to be antigenic (1,25,44). Injection of milligram amounts into mice, rabbits, and calves produced toxic reactions and frequently death (44). Survivors demonstrated high immunity to challenge without toxicity. The LPS-protein antigen resembled endotoxin

preparations in chemical composition, heat stability, and toxicity but was more immunogenic than LPS isolated from the same strains by the Westphal procedure (44). It cannot be overlooked that the active immunity of the LPS-protein complex could be due to traces of nontoxic, specific, capsular antigen.

Typing of *Pasteurella multocida*

Various methods have been used in typing strains of *P. multocida*. The mouse protection test of Roberts (47) and the indirect hemagglutination test of Carter (13) have been most commonly used. Roberts' types I, II, III, and IV have been correlated with Carter's types B, A, C, and D. A fifth group, type E (12), has been confirmed as separate from B in reciprocal serum protection tests in mice (14). Type C, once thought to be prevalent in the nasopharynx of dogs and cats, has since been dropped. Types B and E are associated with hemorrhagic septicemia, type B occurring in southeast Asia and E occurring in central Africa. The antigen typed by Carter's method is the polysaccharide component of the capsule which is adsorbed to the red blood cells in the indirect hemagglutination test. The substance responsible for the specificity of the IHA test is thought to be carbohydrate or polysaccharide in nature because proteins are not adsorbed to unmodified erthrocytes (10).

It was observed by Carter that many type A strains possesed large capsules of hyaluronic acid. Bain noted that the type II, III, and IV of Roberts also had large hyaluronic acid capsules. These immunologic types of Roberts paralleled occurrences of differing lipopolysaccharide substances or somatic "O" antigens. There is now reason to believe that

Roberts' II, III, and IV are type A strains. Types A and D occur most widely geographically.

Namioka (37) found that some type A strains differed in pathogenicity for chickens in causing fowl cholera. Some strains killed three month old chickens in 48 hours in small doses while other strains did not kill even at doses of two million organisms. Therefore, as far as chicken pathogenicity was concerned, there were two types of group A. Namioka went on to investigate the somatic or "O" antigen (38). The somatic antigen was derived from 1 N HCl treatment. It is thought to be LPS combined with protein in nature. Namioka found 12 different "O" groups from studies of 50 strains of *P. multocida*. Along with Carter's capsular antigens, this represents 15 serotypes: 6 somatic antigens for type A, 2 for type B, 6 for type D, and 1 for type E.

The *P. multocida* group B responsible for the etiology of hemorrhagic septicemia has only one "O" group, somatic group 6. Bain (4) also reported an organism isolated from an Australian cattle wound that belongs to type B (Australian strain 989). This group B organism did not cause hemorrhagic septicemia in experimental cattle. It had no serological or biochemical differences in its capsule when compared with other type B strains. No protection was afforded in mice against type 6:B when the animals were vaccinated first with strain 989. Namioka found a new somatic antigen for 989, group 11. The central African H-S strain type E was determined to be "O" 6. Partial cross protection occurs in mice between type B and E. The Asian strain protects against challenge with the African strain but not vice versa.

Prince and Smith found 20 different antigens by means of gel precipitation and immunoelectrophoresis (42). They concluded that the most important antigens relating to serotype and protection were in

their terminology, the β fraction (capsule) and the γ antigen (LPS) which corresponded to Carter's capsular and Namioka's somatic antigens.

Mechanism of Immunity

The precise mechanism of immunity is unknown. Passive transfer studies indicate that immunity is largely humoral (7,60). The most significant antibodies are antibacterial. Bactericidal antibodies have not been conclusively demonstrated in H-S. The antibody role could be opsonization with subsequent phagocytosis. Phagocytosis is increased in the immune animal, but the fate of phagocytosed cells have not been investigated. In challenge experiments, survival is proportional to the presence of circulating antibodies as determined by IHA and mouse protection tests. Prince (42) observed that immune animals had circulating antibodies to α, β, or γ antigens. Dhanda (16) showed good correlation between circulating antibody and immunity.

Vaccines Presently Used Against Hemorrhagic Speticemia (7)

Plain Bacterin. Plain broth bacterin grown from solid or broth media initiate immunity quickly, in five days, so its use has merits in the face of an outbreak. However, immunity rarely lasts over six weeks. Immunity is low grade as demonstrated in an epizootic in Malaysia in cattle vaccinated just three weeks previously (55). The density of bacterin should be 0.15 grams dry weight per liter. A 10 ml dose contains 1.5 mg *Pasteurella*. Formalin is used as the killing agent.

Delpy's Vaccine. Delpy's vaccine is presently being used only in Iran. A suitably encapsulated strain is chosen that sufficiently lyses in distilled water and merthiolate. Saponin (0.5 %) is added after 50 % clearing. A 2 ml dose contains 2 mg of bacteria and produces an edematous swelling of 12 to 20 cm at the site of subcutaneous injection. The disadvantages of this vaccine is that it causes an unpopular swelling and must be agar grown, which makes harvesting difficult.

Alum Precipitated Vaccine. This vaccine has been used in the Philippines, southern India, central Africa, and the USSR. Ten percent or hot twenty percent potash alum is added to formalinized, aerated bacterial broth suspension to give 1 % alum in the vaccine. The pH is adjusted to 6.5 to obtain maximum flocculation. The route of vaccination is subcutaneous and dosage is the same as that for the plain broth bacterin. A 2 mg dry weight dose affords immunity for up to five months. However, some tissue reactions to the vaccine occur and the preparation is not free from causing shock.

Oil Adjuvant Vaccine. A 2 mg dose provides good immunity for one year. Liquid paraffin and lanolin are added as emulsifiers. Two mg of *Pasteurella* are added to a mineral oil emulsion. Lanolin was initially used as a stabilizing agent and to decrease the toxicity of the vaccine, but a decrease in adsorption was noted. The oil adjuvant depot at the site of inoculation serves as a prolonged source of antigen. In 1962, the Food and Agriculture Organization recommended the use of killed cultures with adjuvants.

Live Attenuated Vaccines. Oreste and Armani in 1887 attenuated cultures by growing them at 32 C and also by passages through pigeons. In 1954, Hudson used decapsulated "blue" variants to immunize cattle based on a decrease in pathogenicity in mice. A few immunization trials in

Thailand (55) showed that immunity in cattle lasted several months but no controlled studies were done. With the initiation or improvement of lyophilization techniques in countries where hemorrhagic septicemia is endemic, live vaccines may eventually be the vaccine of choice.

STREPTOMYCIN

A Review of the Literature

Streptomycin was discovered in the laboratory of Waksman in 1944 (48) as a fermentation product of *Streptomyces griseus*. Dihydrostreptomycin, a chemical derivative, is nearly as active as the natural antibiotic but is less toxic. The principle conclusion reached on streptomycin's mode of action is that it is a specific inhibitor of protein biosynthesis at the ribosome level (8,18,19,21,23,34,36,49,52,57). Spotts and Stanier (52) proposed a unitary hypothesis for the action of streptomycin, postulating that the ribosome is the site of action of the antibiotic and that it is also the site responsible for phenotypic expressions of susceptibility, resistance and dependence (23,29,57,59). In *E. coli*, susceptibility, resistance and dependence to streptomycin are expressions of multiple alleles of a single genetic locus (52). Streptomycin dependence (Str^D) is the third alternative state governed by a triple allelic gene (43).

Since ribosomes can be separated into their 30S and 50S subunits and reconstituted into active 70S particles, hybridization studies of subunits from streptomycin sensitive (Str^S) and resistant (Str^R) *E. coli* showed that the site of streptomycin action is on the 30S subunit (8,19, 21,33). Streptomycin binds specifically to the 30S subunit (18,43). The attachment site is generated during the association of the entire 30S particle (8). Equally bactericidal dihydrostreptomycin binds at the same site but with lower affinity. After the addition of streptomycin, the rate of protein biosynthesis declines to zero within 1/4 to 1/3 of the normal doubling time (23). In cell free systems, streptomycin

inhibits the incorporation of radioactive amino acids into proteins while amino-acyl transfer RNA accumulates (23). Sensitive or resistant *E. coli* are correspondingly sensitive or resistant to inhibitions of incorporation of phenylalanine (23). Phenylalanine incorporation studies using synthetic polynucleotides (poly-U) have shown that the 30S ribosomal subunit is involved (19,36). The binding of phenylalanine increases in the presence of streptomycin when streptomycin-dependent 30S subunits are reconstituted with 50S subunits from either Str^D or Str^S cells (19). Cell free extracts remain susceptible to streptomycin if 30S subunits from Str^S cells were used regardless of origin of the 50S subunits. Furthermore, the 30S subunit can be dissociated into its 16S ribonucleic acids and 20 proteins (23). One of these proteins P10 (nomenclature of Ozaki,39) or S12 (nomenclature of Wittman,59) determines an organism's response to streptomycin. S12 is responsible for streptomycin sensitivity . Only mutations in that specific protein account for resistance or dependence phenotypes (18,29,34). The StrA locus, mapping between argG and malA in *E. coli*, determines the protein product S12 (39,43). Chromosome mediated streptomycin resistance is governed by this gene. *In vitro* ribosome reconstitution studies with isolated ribosomal proteins showed S12 to be the determinant for the dependent phenotype (33). One Str^D *E. coli* differed from its wild type parent by one amino acid in protein S12. Lysine was replaced by glutamine in position 42 (20,30). Studies were done to determine whether the S12 alteration was always the same. Amino acid replacements in two mutants to streptomycin dependence in S12 were found to be different from each other and from a mutant studied by Funatsu and Wittman (20). Therefore, it is likely that many amino acid replacements can lead to mutants dependent on streptomycin (30). Mutations can arise at different but closely linked loci of the

same gene. Biochemical analysis of ribosomal protein S12 from three Str^R alleles showed that a single amino acid exchange correlated with each mutant type (20). A similar approach can be used with the streptomycin dependence. Different loci can be affected so dependent mutants may ultimately have a range of variation in phenotype including differing abilities to revert and differing concentration requirements for streptomycin (34).

Streptomycin and Conformation Changes in the Ribosome

It has been proposed that the effects of streptomycin are due to distortions of ribosomal binding sites for t-RNA (36). Ribosomes are inactivated by low Mg^{++} concentration and reactivated with heat and proper ionic conditions. Streptomycin hinders both inactivation and reactivation of ribosomes and there are indications that changes in ribosomal conformation are involved in these interconversions (36). The 30S subunit from Str^S *E. coli* is able to bind two molecules of streptomycin (8). Streptomycin resistant ribosomes only bind five percent of the amount of streptomycin as sensitive cells. When streptomycin is bound, the ribosome cannot bind phenylalanine-tRNA. This binding further changes the conformation of the ribosome so that amino-acyl tRNA binding is not efficient (36). It appears that different concentrations of streptomycin cause different conformational changes (49). Catalytic levels of streptomycin cause inhibition of phenylalanine incorporation with no effect on misreading of the ribonucleic acid code. Misreading is stimulated at the higher streptomycin to ribosome ratios. Different streptomycin concentrations can cause inhibitions of Phe-tRNA binding

or stabilization of this binding. The stabilizing effect may be the cause of misreading since it may allow mismatched tRNA-mRNA complexes which otherwise might be short-lived (36).

The Lethal Effect of Streptomycin

It has not been resolved whether the lethal effect of streptomycin is due to 1) inhibitions of chain elongation by immobilizing peptidyl tRNA on its ribosome binding site (23) or 2) inaccuracies in translation. The binding phenomenon itself does not kill (8). Streptomycin induced killing may not require tight binding to ribosomes. Protein synthesis need not completely stop before cell death occurs (34). A dual mechanism of action (34,57) may be in operation with killing separate from the initial lesion on protein synthesis. Dihydrostreptomycin and streptomycin have the same bactericidal potency but have different affinities for ribosomes. Different strains of bacteria bind differing amounts of streptomycin. It was once proposed that high levels of streptomycin exert an irreversible effect on ribosome function *in vivo* by forming aberrant initiation complexes which permanently inactivate ribosomes (23,43). Formation of initiation complexes apparently takes place (57,58). Studies using the poly-U system proved that ribosomes were slowed but not permanently inactivated (49). Only after the block on protein synthesis is partially released can stimulation of misreading begin. If all ribosomes were inactivated at initiation, there would be no chance for misreading to occur. Killing by streptomycin is antagonized by chloramphenicol and other substances that form a stable blockade of ribosomes in polysomes (58).

Streptomycin causes a cyclic blockade of ribosome initiation, blockage of chain elongation, gradual release, and reinitiation (57). This cyclical blockage accounts for the dominance of sensitivity in Str^S/Str^R heterozygotes. Streptomycin interferes with only a proportion of ribosomes which is enough to halt protein synthesis. Inactive Str^R ribosomes regain activity when the cells are lysed and excess mRNA is provided. Streptomycin is also known to cause a gradual release of ribosomes from polysomes (36). Once affected, inactive ribosomes do not resume activity in extracts. However, the ribosomes are not inert, participating in continuous attachment and release from polysomes. Although no longer able to synthesize protein, the ability to attach to mRNA is retained. Reinitiated affected ribosomes carrying fMet-tRNA do not proceed with chain elongation since pulse labelling studies show little valine is incorporated as compared to methionine (57). The Str^S phenotype in heterozygotes is dominant because Str^R ribosomes are deprived of the opportunity to initiate. Streptomycin susceptible ribosomes attach to mRNA for a longer time period (half life is five minutes vs 15 to 20 ribosomes per second in normal initiation) and successfully competes with Str^R ribosomes for binding sites.

Point Mutations as a Basis for Resistance and Dependence

Mutations to streptomycin resistance and dependence usually arise by base substitutions (50). N-methyl-N'-nitro-N-nitrosoguanidine (NTG) produces point mutations by substituting one base for another. Acridine half mustard ICR-191 induces the addition or deletion of bases causing frame shift mutations. ICR-191 proved a poor mutagen for genes

affecting streptomycin resistance while NTG was 250 to 1000 times more effective (50). NTG induced approximately equal numbers of dependent and resistant mutants. That ICR-191 was a poor mutagen indicates that frame shift mutations are not allowed or are lethal. A change in a single amino acid is allowed, whereas changes in a whole sequence of amino acids in an important organelle protein must produce non-viable cells.

Resistance to aminoglycoside antibiotics can be achieved in several ways, 1) R-factor genes for a variety of drug inactivating enzymes have been detected. Streptomycin and spectinomycin can be inactivated by adenylating enzymes (43,54). Phosphorylating enzymes have been discovered for streptomycin, paromamine, kanamycin, and neomycin (43,56). 2) An altered response to streptomycin may be due to permeability changes (34). 3) Mutations in the S12 protein genotypically alters response to streptomycin. Cell free ribosomal extracts have proven that the streptomycin-dependent phenotype is due to major genotypic and not permeability changes. Streptomycin-sensitive ribosomal extracts ceased synthesizing proteins in the presence of streptomycin while resistant cell free ribosomal extracts continued protein synthesis (52). Optimal protein synthesis was restored to cells or cell free extracts of dependent strains when streptomycin was added back to the medium (18).

Mutations That Mask the Dependent Phenotype

Genetic analysis of revertants from streptomycin-dependence (Str^D) to streptomycin-independence (Str^I) demonstrated that many have

mutations near but not coincident with the StrA locus. Using ribosomal reconstitution experiments, two dimensional gel electrophoresis, and phosphocellulose column chromatography, several laboratories have reported that a number of revertants have altered proteins S4 or S5 of the 30S subunit (24,29,30,33,34,59). Regardless of the Str^D S12 allele, Str^I mutants arise due to changes only in S4 or S5 (24). Revertants containing mutations in other than protein S12 support the suggestion that these are not true revertants but mutations that suppress the dependent phenotype. Streptomycin-dependence may be suppressed by alterations in either S4 or S5. Alterations in S4 give proteins which drastically differ in amino acid composition, molecular weight and amino acid sequence (30) which lends evidence for frame shift type mutations. Single amino acid replacements are found in S5 mutations as determined by two dimensional gel electrophoresis; base substitution is suspected as the mechanism of reversion here. A third alteration in S4 can compensate for an alteration in S5 in such a way that the original streptomycin-dependent phenotype produced by S12 is expressed (59). In 190 mutants studied (59), mutations to Str^D, Str^I, and Str^R were accounted for by alterations only in the three proteins S4, S5, and S12. A study by Hasenbank (24) showed that 40 of 100 revertants from Str^D had electrophoretically altered proteins; 24 were altered in S4, 16 altered in S5. Deusser (24) showed in a study that 5 of 14 revertants had changes in S4 or S5, and an additional 3 of 14 revertants were discovered by immunologic and not electrophoretic means since neutral amino acids were replaced by neutral amino acids. Conceivably, Hasenbank's study could have had as high as 60 to 65 out of 100 mutants with alterations only in S4 or S5 if undetected neutral substitutions were taken into account (24). In a study of Str^I revertants of Str^D *Bacillus stearothermophilus* (29), 2 of 13 and 4 of 13

had alterations on S4 and S5 respectively. Not all mutations of S4 and S5 occurred in the same position. The S5 proteins migrated to 5 different positions on two dimensional electrophoresis, and S4 proteins to 17 positions. The locations of S4, S5 and S12 in the assembled ribosome are quite different. S4 is an internal protein, S5, a fractional protein added late in the assembly and S12 is not well defined.

There was no direct correlation between the specific ribosomal protein altered and the ultimate phenotype. In addition to the elimination of dependence, many differed from the parent strain and from each other in their growth rate, response to streptomycin *in vivo*, ability of ribosomes to bind streptomycin, and activity of ribosomes in cell free systems. S5 could produce the same phenotypic change as a change in S4 but some changes in S4 could produce varying phenotypes (34). These results clearly support the concept of extensive, complex interaction among ribosomal proteins leading to vastly different structural conformations.

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ARTICLE

A LIVE STREPTOMYCIN-DEPENDENT *PASTEURELLA MULTOCIDA* VACCINE FOR THE PREVENTION OF HEMORRHAGIC SEPTICEMIA

By

B. D. Wei and G. R. Carter

(Manuscript to be submitted to the Journal
of the American Veterinary Medical Association)

SUMMARY

A type B *Pasteurella multocida* was used for the development of a streptomycin-dependent (Str^D) vaccine. *P. multocida* R-473, a hemorrhagic septicemia strain, was mutagenized with N-methyl-N'-nitro-N-nitrosoguanidine to increase the likelihood of encountering a streptomycin-dependent mutant and plated on agar containing 400 µg/ml of streptomycin. Replica plating was used to differentiate dependent from resistant colonies. Mice and rabbits were vaccinated with a Str^D mutant and challenged along with unvaccinated controls 21 days later with the wild type R-473. Protection of greater than 4 logs was shown for the vaccinated mice. All vaccinated rabbits were protected and all unvaccinated controls succumbed to a challenge of 500 or 1000 LD_{50}.

INTRODUCTION

The use of live attenuated bacteria in a vaccine against disease caused by *Pasteurella multocida* was first tried by Louis Pasteur. Presently, killed oil adjuvant preparations are the vaccines recommended for the prevention of hemorrhagic septicemia (H-S) (2). A live, attenuated *Pasteurella multocida*, the Clemson University or CU strain, is available as a commercial vaccine for fowl cholera and is distributed in lyophilized form (3). Killed bacterial preparations often protect against homologous but not heterologous challenge (11). Administration of antibiotics in the feed of turkeys vaccinated with the CU strain suppressed the immune response (5), proof that the organism in a vaccine must survive to some extent in the animal before optimal immunity is attained. Other workers have had success with an avirulent, high temperature mutant in the prevention of fowl cholera (15). The higher efficiency of live as opposed to killed bacteria as vaccines may be due to 1) the preservation of intact antigens of the virulent organism by obviating the use of harsh chemicals and physical procedures, 2) the formation of antigens produced *in vivo* that may have been lost after continuous subculturing, 3) the greater immunity produced by allowing some multiplication in the animal (5,12,14).

Vaccines that have shown much promise in inducing immunity, in innocuousness and in ease of administration are the live, streptomycin-dependent (Str^D) preparations. Streptomycin-dependent mutants have been developed for *Escherichia coli* (10,14), *Salmonella typhi* (7,22), *Salmonella enteritidis* (6,23,24), *Shigella flexneri* (16,17,18), and *Vibrio cholera* (9).

Because of asymptomatic carrier states and the acute nature of hemorrhagic septicemia, control of the disease depends on prophylaxis. With the improvement of culturing and lyophilization techniques in areas where H-S is endemic, live vaccines may be the vaccine of choice. A Str^D *P. multocida* vaccine was developed, found to be stable against reversion, and was protective for mice and rabbits.

MATERIALS AND METHODS

Bacterial Strain. *Pasteurella multocida* strain R-473 (type 6:B) was the parent strain from which the streptomycin-dependent vaccine was derived. This isolant originally came from a bovine hemorrhagic septicemia case in Egypt and had been maintained in lyophilized form in our laboratory. The culture was confirmed to be a type B by the indirect hemagglutination test (4).

N-methyl-N'-nitro-N-nitrosoguanidine[a] *(NTG) Mutagenesis.* *P. multocida* R-473, a streptomycin-sensitive strain was mutagenized by the modified methods of Adelberg (1), and Osechger and Berlyn (19) to obtain resistant and dependent colonies. Ten ml of a four hour logarithmic phase culture grown in tryptose broth with 0.3 % yeast extract[b] was centrifuged for 10 minutes at 20,000 x g. The pellet was resuspended in .05 M Tris-maleic acid buffer, pH 6. The cells in buffer were

a Aldrich Chemical Co., Milwaukee, Wisconsin
b Difco Laboratories, Detroit, Michigan

mutagenized with a final concentration of NTG at 0.1 mg/ml for 15 min at 37 C. The suspension was centrifuged, washed once with broth, centrifuged again and resuspended in 100 ml of tryptose broth with yeast extract and incubated overnight. The next day, the culture was centrifuged at 20,000 x g for 10 min and resuspended in only 1 ml of tryptose broth with yeast extract. Ten streptomycin[c] agar plates (tryptose agar (TA), 0.3 % yeast extract, 100 µg/ml streptomycin (Str)) were inoculated with a sterile, bent glass rod. The plates were incubated for 48 hours.

Selection of Str^D From Str^R Colonies. Each of the ten spread plates were replica plated by the method of Lederberg (13), first onto plain tryptose agar with yeast extract and then onto tryptose, yeast extract agar with 100 µg/ml of streptomycin. The replicated plates were incubated for 48 hours. Colonies that grew better on the TA + Str plates than on the plain TA were individually picked with a straight wire, one half of the colony put on another TA and the other half on TA + Str in order to check the dependency. Two Str^D colonies #34 and #37 which grew exceptionally well on TA + Str but not at all on plain TA were chosen for the production of vaccine. These colonies were maintained by weekly transfers on TA + Str and also lyophilized.

Reversion Studies. 1) Heavy loopfuls of the Str^D *Pasteurella* were periodically streaked onto plain TA plates in order to determine revertants to streptomycin independence. 2) Overnight broth cultures of Str^D R-473 were centrifuged and washed 3 times to rid the broth of

c Streptomycin-sulfate, Pfizer Laboratories, New York

streptomycin and plated heavily on plain TA along with a known number of streptomycin-sensitive wild type R-473. The same dilution of wild type R-473 was plated alone on another set of TA plates. A significant increase in the number of colonies growing on the combined Str^D and wild type plates would be interpreted as indicating the presence of revertants, yet assuring, by the growth of a known number of wild type colonies, that conditions allowed for revertants to be detected. Since the TA plates were devoid of streptomycin, no Str^D colonies would be found.

Vaccine Preparation. The chosen Str^D colonies were grown in 25 ml of tryptose broth with 0.3 % yeast extract and 400 µg/ml streptomycin for 24 hr at 37 C in a shaking water bath. The culture was centrifuged, washed once with saline, centrifuged again and resuspended in saline to the original volume. The vaccine was checked for purity by a gram stain, a culture on TA + Str agar, and by biochemical tests. For each vaccination study, the number of organisms in the washed broth was determined by viable cell counts of ten fold dilutions on spread plates. A non streptomycin-containing plate was always inoculated to assure that no streptomycin-independent organisms were present.

Viability of a Rehydrated Vaccine at Room Temperature. A lyophilized vial of the Str^D vaccine strain R-473 was rehydrated and grown in 25 ml of tryptose, yeast extract broth with 400 µg/ml of streptomycin. Its viability at room temperature was checked by plating appropriate dilutions in duplicate for two weeks.

Vaccination Studies. Swiss Webster ICR albino mice[d], females weighing 18 to 22 g were placed 6 in a cage and fed *ad libitum*. Groups of 36 were vaccinated intraperitoneally or subcutaneously with 0.1 ml of the Str^D vaccine. Groups of 6 were challenged 21 days later with 0.5 ml of ten fold dilutions of frequently mouse passaged wild type R-473 by the same route of inoculation as the vaccination. Mice that died subsequently to challenge were randomly selected and organs were cultured on plain and streptomycin incorporated tryptose agar plates. Organisms were identified as *Pasteurella multocida* when these reactions were observed: slight acid production in the triple sugar iron agar slant and butt with no production of gas or H_2S, oxidase and indole production, reduction of nitrates to nitrites, negative lactose and maltose fermentation, negative urease production, failure to grow on MacConkey Agar, and typical nonhemolytic, translucent colonies on bovine blood agar plates.

Sixteen New Zealand albino rabbits, nine months old, were vaccinated subcutaneously with 0.5 ml of vaccine. They were maintained in separate cages and fed *ad libitum*. Eight control rabbits remained unvaccinated. After 21 days, eight vaccinated and four control rabbits were challenged with approximately 500 LD_{50} of the wild type R-473. A similar group was challenged with 1000 LD_{50}. The LD_{50} was previously determined to be 34 organisms. Rabbits that succumbed to challenge were necropsied and cultured on plain TA and TA + Str.

Challenge of both mice and rabbits was done with a recently mouse passaged *P. multocida* R-473 grown for 8 to 10 hours in a shaking water bath at 37 C. Purity of the culture was assured by gram stain and biochemical tests. Viable counts were made by the spread plate technique.

d Spartan Research Animals, Haslett, Michigan

Seven Holstein calves, aged 4 to 16 weeks were subcutaneously vaccinated on the shoulder with 2 ml of an unwashed 24 hour Str^D culture. This vaccine represented 7.6×10^8 live Str^D *P. multocida*. The calves were kept in isolation and observed for three weeks.

RESULTS

Reversion Studies. No revertants to streptomycin-independence were found by heavily plating the Str^D colonies used for the vaccine on plain tryptose, yeast extract agar. Occasionally, a thin haze of growth could be detected at the point of heaviest inoculum. Subculturing some of this material on another TA plate failed to show any more growth and it was thought that this growth was due to residual streptomycin transferred from the original media. It is also possible that the dependent cells had enough of the antibiotic inside the cell to allow growth for one or two generations. Heavy inocula on TA + Str plates showed growth of colonies 1 mm in diameter in 48 hours.

A 1×10^{-6} dilution of wild type, streptomycin-sensitive R-473 was plated on 3 plain TA plates. This yielded 280, 225, and 275 colonies. The same volume (0.1 ml) of the 1×10^{-6} dilution of wild type was plated simultaneously with 4.7×10^7 Str^D cells on 3 other TA plates and growth of 242, 219, and 249 streptomycin-independent colonies was found. Thus, no streptomycin-independent colonies due to reversion could be detected.

Stability of the Str^D Vaccine at Room Temperature. After 24 hours incubation in 25 ml of tryptose, yeast extract broth with 400 µg

streptomycin, a rehydrated vial of lyophilized Str^D R-473 had a viable count of 2.5×10^9 cells/ml. No growth appeared on agar without streptomycin. The culture was kept on a shelf at room temperature and viable counts made 7 times in 16 days. An average of 2.5×10^8 cells were maintained in the vaccine from day 2 to day 16. On day 16, 4.0×10^8 cells remained. Parallel platings on plain agar showed no growth.

Vaccination Studies. The accumulated mortalities after challenge in the mouse vaccination studies are given in Tables 1, 2, and 3. No adverse effects due to the vaccine were observed. The intraperitoneal route of challenge infrequently caused convulsions and immediate death, 4 times in vaccinated and 3 times in unvaccinated control animals. A second administration of vaccine 14 days prior to challenge did not alter the degree of protection (Table 1). Subcutaneous vaccinations were not as effective as intraperitoneal vaccinations when comparable dilutions of the challenge strain were used. Subcutaneous trial 1 gave protection of 1.9 logs. Two logs protection is the minimal requirement for commercial *P. multocida* vaccines according to Ose and Muenster (20). It was found that more organisms were needed to overwhelm the controls when the subcutaneous route of injection was used. Perhaps the subcutis does not foster growth as well as the rich peritoneum or there is loss of some organisms through drainage of the 0.5 ml challenge inoculum out of the mouse. When lower dilutions of challenge culture were used, the subcutaneous vaccinations protected just as well as the intraperitoneal. The LD_{50} for each trial was calculated by the method of Reed and Muench (21). As few as 5 organisms were able to kill 50 % of unvaccinated controls while the vaccinated could survive up to 50,000 to 500,000

TABLE 1 -- INTRAPERITONEAL VACCINATION AND CHALLENGE

CHALLENGE DILUTIONS OF *PASTEURELLA MULTOCIDA* STRAIN R-473*	ACCUMULATED MORTALITY IN MICE 7 DAYS AFTER CHALLENGE				
	TRIAL 1		TRIAL 2		
	VACCINATED**	CONTROLS	VACCINATED	VACCINATED + BOOSTER	UNVACCINATED CONTROLS
10^{-4}	0/3	6/6	1/6	0/6	6/6
10^{-5}	1/4	6/6	1/6	0/6	6/6
10^{-6}	1/4	6/6	0/6	0/6	0/6
10^{-7}	1/4	6/6	0/6	1/6	6/6
10^{-8}	1/3	3/6	0/6	0/5	4/4
10^{-9}	0/3	4/6	0/2		4/5

* Undiluted challenge broth. Trial 1. 1.1 x 10^9 cells/ml, Trial 2. 3.6 x 10^9 cells/ml
** Vaccine dose of 0.1 ml. Trial 1. 5.9 x 10^8, Trial 2. 1.6 x 10^8 cells.

TABLE 2 -- SUBCUTANEOUS VACCINATION AND CHALLENGE

TRIAL 1

CHALLENGE DILUTIONS OF *PASTEURELLA MULTOCIDA* STRAINS R-473*	ACCUMULATED MORTALITY IN MICE 7 DAYS AFTER CHALLENGE	
	VACCINATED**	UNVACCINATED CONTROLS
10^{-4}	3/6	6/6
10^{-5}	1/6	0/6
10^{-6}	0/6	0/6
10^{-7}	5/6	6/6
10^{-8}	5/6	6/6
10^{-9}	1/6	5/6

* Undiluted challenge broth had 6.8×10^8 cells/ml
** Vaccine dose of 0.1 ml: 4.6×10^7 cells

TABLE 3 -- SUBCUTANEOUS VACCINATION AND CHALLENGE

TRIAL 2

CHALLENGE DILUTIONS OF *PASTEURELLA MULTOCIDA* STRAIN R-473*	ACCUMULATED MORTALITY IN MICE 7 DAYS AFTER CHALLENGE	
	VACCINATED**	UNVACCINATED CONTROLS
10^{-1}	3/6	ND
10^{-2}	1/6	6/6
10^{-3}	1/6	6/6
10^{-4}	1/6	6/6
10^{-5}	0/6	5/6
10^{-6}	0/6	6/6

* Undiluted broth had 9.9×10^7/ml wild type R-473.
** Vaccine contained 5.9×10^7 cells in 0.1 ml dose.

virulent organisms. Greater than 4 logs protection was consistently observed (Table 4). *Pasteurella multocida* was consistently isolated from organs of mice that died following the challenge dose. Parallel cultures on TA + Str revealed that no Str^D organisms were responsible for the deaths.

In the rabbit vaccination study, one control rabbit died 24 hr after challenge. The 7 remaining unvaccinated controls died between 48 and 72 hr after challenge. After 7 days, all 16 vaccinated rabbits were still healthy (Table 5). Four out of the 8 control rabbits that succumbed were randomly selected and necropsied, 2 from the 500 LD_{50} and 2 from the 1000 LD_{50} challenge. The heart, liver, and lung were cultured and any organisms isolated were identified biochemically. Moderate to heavy growth of *Pasteurella multocida* was found in the heart and liver of two of the animals in pure culture with the exception of a few coliform contaminants. Light to moderate growth of *P. multocida* was found in the other two rabbits. No bacteria could be cultured from any of the lungs.

Despite the large number of live *P. multocida* (7.6×10^8) injected, the seven calves showed no noticeable adverse reactions to the vaccine. No inflammation at the site of inoculation was observed. Bain (2) found the subcutaneous minimum lethal dose in buffalo to be 1×10^4 type B *Pasteurella multocida*.

TABLE 4 -- PROTECTION AFFORDED BY STR^{D} VACCINE IN MICE

ROUTE OF VACCINATION AND CHALLENGE	LD_{50} 7 DAYS AFTER CHALLENGE	DILUTION	LOGS PROTECTION	NUMBER OF ORGANISMS REPRESENTED
INTRAPERITONEAL	CONTROL	$10^{-8.8}$		< 5
TRIAL 1	VACCINATED	$10^{-4.4}$	4.4	5,000 - 50,000
INTRAPERITONEAL	CONTROL	$10^{-8.3}$		4 - 40
TRIAL 2	VACCINATED	$< 10^{-4.0}$	> 4.3	
	VACCINATED + BOOSTER	$< 10^{-4.0}$	> 4.3	$> 400,000$
SUBCUTANEOUS	CONTROL	$10^{-7.8}$		3 - 30
TRIAL 1	VACCINATED	$10^{-5.9}$	1.9	340 - 3400
SUBCUTANEOUS	CONTROL	$< 10^{-6.0}$		< 50
TRIAL 2	VACCINATED	$10^{-1.4}$	> 4.6	50,000 - 500,000

TABLE 5 -- SUBCUTANEOUS VACCINATION AND CHALLENGE OF RABBITS

CHALLENGE DOSE OF *P. MULTOCIDA* R-473	ACCUMULATED MORTALITY 7 DAYS AFTER CHALLENGE	
	VACCINATED	CONTROLS
500 LD_{50}	0/8	4/4
1000 LD_{50}	0/8	4/4

DISCUSSION

Live, streptomycin-dependent vaccines have been successfully developed and tested for enteric organisms. Several serotypes of enteropathogenic *Escherichia coli* have been made streptomycin-dependent and used to vaccinate young monkeys by the oral route. Protection against challenge with the virulent organism lasted one month (10). Human adults were treated orally with 1 x 10^{12} Str^D *E. coli* without side effects (14). Streptomycin-dependent *Salmonella typhi* was safely given to human volunteers in doses up to 1 x 10^{11} cells. Challenge with 1 x 10^5 virulent bacteria showed significant protection (7). In 1963, live, Str^D *Shigella flexneri* 2a was given to soldiers in bacillary dysentery endemic areas in five oral doses. Virulent *Shigella* challenge caused dysentery in 5.5 % of unvaccinated controls and in none of the vaccinated volunteers. There were no untoward reactions to the vaccine and the carrier rate remained unchanged. The Str^D *Shigella* vaccine conferred a significant degree of protection never before seen (17). Lyophilized Str^D *Shigella* vaccines were as effective as freshly prepared cultures and induced a lower incidence of post vaccinal reactions (18).

A Str^D *Pasteurella multocida* vaccine was developed and found to be protective for mice and rabbits against challenge from high numbers of wild type virulent organisms. Protection of 4 logs was observed after parenteral vaccination and challenge. Commercial *P. multocida* vaccines are considered adequately immunogenic when an efficiency of 2 logs protection is attained. Administration of the Str^D vaccine resulted in no detectable side effects or deaths in mice or rabbits. The Str^D phenotype remained stable from reversion to independence when large numbers

of cells were plated on non streptomycin media. Other workers have reported reversion rates from as many as 0.2 to 8 in 1 x 10^8 cells (24) to as low as 0.0 in 4 x 10^{12} (16). In an oral vaccine, even if a revertant appeared, it would not be able to compete with the large numbers of normal bacteria in the alimentary tract (14,16,24). The most practical method of vaccine administration for the prevention of hemorrhagic septicemia might be via the drinking water or by intranasal aerosol. Oral or intranasal administration of a Str^D vaccine for this disease would take advantage of the probable natural route of infection, through the nasopharynx (2).

High mortality in clinical cases of hemorrhagic septicemia creates severe economic losses especially in southeast Asia and Africa. A live preparation might prove more immunogenic than some of the killed vaccines presently in use. Hemorrhagic septicemia is only one of a wide range of diseases caused by *Pasteurella multocida*. Streptomycin-dependent organisms may be of use as vaccines for other pasteurelloses such as rabbit "snuffles", fowl cholera and pneumonic bovine pasteurellosis.

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