

THE PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES  
AGAINST K88 PILI FROM PORCINE ENTEROTOXIGENIC ESCHERICHIA COLI

by

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MASTER OF SCIENCE

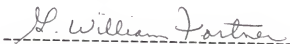
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## INTRODUCTION

### ENTEROTOXIGENIC *ESCHERICHIA COLI*

The ability of certain bacteria to adhere to eukaryotic cells via specific adhesive mechanisms is a fundamental characteristic enabling colonization of host tissue *in vivo*. This adherence is of key importance for pathogenic organisms which must compete with commensal microorganisms for successful colonization of host epithelial surfaces. It is this ability to adhere to specific receptors on the small intestines of young animals that differentiates enteropathogenic strains of *Escherichia coli* from the nonpathogenic strains that normally inhabit the intestinal tract.

These noninvasive enterotoxigenic strains are responsible for diarrheal illness in several animal species. The efficacy of these enteropathogens depends on two fundamental characteristics, both of which are encoded for by extrachromosomal DNA, or plasmids. These plasmids code for bacterial accessories which confer a certain advantage to the microorganism containing this information. One plasmid coded factor confers upon *E. coli* the ability to colonize the small intestine. In ligated segments of the intestines, both pathogens and nonpathogens multiply equally well due to the elimination of the effects of intestinal motility. However in unligated intestine of neonatal pigs

enteropathogenic strains of *E. coli* are at a selective advantage for survival (19). The enteropathogens adhere to the mucosa and are thus shielded from the flushing action of peristaltic movement which eliminates the nonpathogens that lack adherence factors. Once attached, rapid proliferation can occur. In natural cases of *E. coli* disease in piglets, the organisms are found in the anterior small intestines in 100 to 10,000 times their normal numbers (1,10,30).

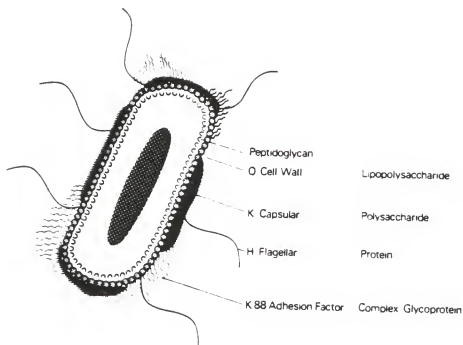
A second plasmid factor results in the production of enterotoxins, which causes the disease pathology. The enterotoxins act locally on the cells of the intestinal mucosa, causing derangement of normal gut functions, secretion of fluids, and other symptoms of *E. coli* disease. There are two forms of enterotoxins produced by these pathogenic *E. coli*. The first is known as the heat-stable enterotoxin (ST), which is a small molecule of about 4500-5000 daltons. Its effects are seen in a rapidly transmitted 'signal' across the membrane of the target cell producing a change in the guanylate cyclase activity resulting in an massive efflux of ions leading to the ultimate diarrheal expression. The other enterotoxin is designated as heat-labile (LT) and is considerably larger than ST with a molecular weight of 80,000 to 105,000 daltons. It resemble both the effects and the antigenicity of cholera toxin. The diarrheal symptoms are produced by the activation of adenylate cyclase activity followed by rapid  $\text{Na}^+$  and  $\text{Cl}^-$  ion transport into the

intestinal lumen resulting in extensive fluid loss (34). The specific adhesion to the small intestinal epithelium is of key importance in the development and persistence of the disease syndrome as that area is the most sensitive to enterotoxigenic activity with the gut becoming progressively refractory after the first few feet (31). Proliferation of these enterotoxigenic *E. coli* only in the colon is unlikely to have an adverse effect on the host animal. It is evident that these *E. coli* have become specially modified in a manner which allows them to remain in close proximity to the target cells which are most susceptible to the production of the disease effects.

Enteropathogenic *E. coli* must therefore possess specific surface characteristics that enable them to adhere to the gut mucosal cells (see drawing below). The surface of these cells is covered with substances capable of provoking immunological reactions, and it is these surface antigens that are commonly used for the serological classification of pathogenic and nonpathogenic strains. The most common types of antigens are divided into 3 classes: 1) Somatic O antigens which are lipopolysaccharide complexes and constitute part of the cell wall. They are thermostable and are not inactivated by temperatures of 100°C or 121°C; 2) The flagellar H antigens which are protein in nature and are inactivated at 100°C; 3) The K antigens which form an envelope or capsule round the cell wall.

All of the surface antigens which confer adhesive properties

on the enterotoxigenic strains have been grouped into the K class of antigens and have been identified as nonflagellar, filamentous, proteinaceous appendages on the bacterial cell surface. These appendages have been described as "fimbriae"



"pili", and "adhesins". They are thinner and more numerous than flagella and confer adhesive properties to the bacteria including hemagglutinating activity. The most common type of fimbriae are named Type 1 fimbriae. These fimbriae, or pili, enable *E. coli* to adhere to a wide variety of eucaryotic cells, i.e. erythrocytes of several animal species (6), leukocytes,

epithelial cells (22), and a host of other animal, plant, and fungal cells. All of these Type 1 fimbrial adhesive properties are inhibited by D-mannose. These Type 1 fimbriae are the most universal adhesins of *E. coli* which help to colonize epithelial surfaces and therefore contribute to the pathogenicity of enteropathogenic *E. coli* strains.

In contrast to Type 1 fimbriae there is another class of adhesins which occur exclusively on enterotoxigenic *E. coli* strains. They adhere to the intestinal epithelia of a very limited number of animal species and specifically agglutinate only certain species of erythrocytes. The hemagglutinating activity of these adhesins is not inhibited by mannose. Since the plasmids that code for the pili can be replicated independently of the chromosome they can be transferred from one strain of *E. coli* to another, or from *E. coli* to other enterobacteria.

The first of these types of pili, numbered K88, was first described in 1961 in a paper studying *E. coli* strains isolated from diseased swine. It was soon recognized that porcine neonatal diarrhea was characterized by the presence of *E. coli* bearing this K88 surface antigen proliferating in the small intestines of the piglets, thus causing the disease (12). Since then the surface antigenic characteristics of *E. coli* have received considerable attention. Along with K88 other adhesins have been found to be associated with the expression of the same



disease pathology. Pili designated as 987P and F41 have also been identified as virulent factors in porcine diarrheal illnesses. The same disease pathology occurring in calves is mediated by the presence of an adhesin denoted as K99, while the human counterpart, in the form of human neonatal diarrhea, is characterized by the presence of another pilus type, CFA/I or CFA/II (5). All of the enterotoxigenic strains of *E. coli* have been found to produce the ST enterotoxin in conjunction with all of the various pili forms, while only K88, CFA/I, and CFA/II have been associated with the LT enterotoxin form (17,20,21,32). It should be again noted that regardless of enterotoxin type without the presence of the specific pili the enteropathogen would be ineffectual even though it possessed the plasmid necessary for enterotoxin production.

#### THE K88 ADHESINS

As previously mentioned, the K88 antigen, discovered in 1961 by Orskov et. al. (24,25), was the first of a fairly small group of adhesins found to be an essential virulence factor of enterotoxigenic *Escherichia coli* (ETEC) in swine. The characterization of this pili was greatly advanced by an isolation procedure developed by Stirm et. al (35,36), which released the pili by heating a bacterial suspension to 60°C for 20 minutes, or by treating in a Waring blender. It was determined that the isoelectric point of the K88 antigen was

approximately pH 4.2 and that the pili were essentially insoluble at pH ranging from 3.5 to 5.5, while being readily soluble at pH 6.5 or above. By making use of this characteristic, along with the known sedimentation coefficient of 35S, purification was achieved by a series of isoelectric precipitations and preparative ultracentrifugation. The resulting purified K88 antigen was determined to be a protein in contrast to the polysaccharide nature of the other K antigens known at that time. Using electron microscopy, it was found that the K88 positive bacteria were covered by a filamentous material. An examination of the purified K88 antigen showed the same fimbrial structures and were visualized as thin flexible threads approximately 0.1 to 1.5  $\mu\text{m}$  in length and 2.1 to 7 nm in diameter (18).

Early work utilizing specific antisera revealed two distinct variants of the K88 antigen, denoted K88ab and K88ac. As implied by the nomenclature both strains share a common antigenic determinant site, as well as expressing a unique variation. Recently another new variant, K88ad, was described from porcine ETEC isolates in the Netherlands (7,8). There also appear to be similar minor variations between strains containing K88ac and K88ab adhesins as detected using homologous antisera in double diffusion assays. All strains of K88-positive bacteria, that were serologically tested, shared the common K88a determinant,

which infers a potential requirement of this epitope for the integrity of the pili on the bacteria. The serological variations in the K88 antigenic makeup may represent an effort of the bacterium to escape the immunological pressure imposed upon the K88-positive population by recent large scale use of vaccines containing K88 antigens. The new K88ad variant may be a prime example as it first appeared in 1973 after the introduction and use of such K88 containing vaccines. Another possible explanation could be natural selection of K88 variants caused by an alteration of the 'receptor' site on the intestinal epithelium itself. There have been cases of pigs resistant to the effects of K88-positive strains of bacteria. The occurrence of such 'resistant' pigs, due to potential alterations in the intestinal epithelial cells, may have led to selection of other naturally occurring K88 variants that are better suited for the new 'receptors'. This theory of adaptation to a new 'receptor' is supported by the fact that monospecific antisera directed against the altered antigenic epitope, whether K88b or K88d, can neutralize the ability to adhere to the intestinal brush border membranes (38).

In analyzing the structure and composition of the K88 variants Mooi and de Graaf (8,18) found that purified K88 pili moved to a single band on a sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) with an apparent molecular weight of between 23,500 to 27,500 depending on the K88 variant isolated. This

implies that the K88 pili is composed of identical subunits. They also found only trace amounts of either carbohydrates or lipid, which may be a contaminant from the bacterial cell wall (36), again indicating that the K88 antigen is not a glycoprotein. The amino acid composition of the K88 antigens showed the presence of all the common amino acids with little variation between the different K88 species. A major exception is the complete absence of cysteine, which implies that the subunits in the K88 pili are not connected by disulfide bridges, but by hydrophobic or charge interactions between adjacent subunits. It has been shown that the last 45 amino acids of the C-terminus of each subunit are hydrophobic containing only one charged residue. This finding supports the idea of hydrophobic interactions. It is of interest that in comparing the primary amino acid sequences of K88ab with the known partial sequence of the K88ad variant there are only 15 amino acid differences. These differences are spread along the peptide chain and not confined to any one particular region. Most of the amino acid changes involve charged amino acids, which most likely occur at the surface of the protein (11). The implications of these amino acid variations on the immunological activity of the pili is of primary importance, though as yet no adequate probes have been found to study these aspects of the K88 amino acid modulation.

#### IMMUNITY

Since the adhesion of these ETEC is a prerequisite for the

pathogenic expression of the illness, it is important to understand the mechanism by which this adhesion is mediated. There are several studies which report the absence of K88 binding. At present there is no clear understanding concerning the nature of the 'pili-receptor' interaction. It has been shown that certain phenotypes of pigs, designated "adhesive", are susceptible to the effects of these ETEC strains and show a great degree of enteropathogenic colonization of the small intestinal brush borders on autopsy. Other 'non-adhesive' pig phenotypes show no such bacterial colonization upon exposure to known K88-positive organisms, thus inferring the lack of a definitive 'receptor' site on their intestinal epithelial membranes. These phenotypes have been found to be inherited in a Mendelian manner(27,28).

It has also been established that, *in vitro*, the binding of K88 pili to brush border membranes could be inhibited by the addition of polyclonal antisera against these pilus antigens (38). It was found that antisera specific for either K88b, c, or d was capable of inhibition of binding of their homologous pili antigens only. This suggests that these epitopes (ie. b, c, and d) are directly involved in the binding of the pili. It is also possible that the evolution of these variants shows an adaptation by the ETEC to environmental pressures in order to avoid immune surveillance or to coadapt to changing 'receptor' sites in their hosts (8). It has long been known that an immune sow can confer

this immunity passively to their offspring through the colostrum (2,3,14,15). Both of these cases suggests an important role of antibody-mediated immunity, whether active or passive, in the therapy and potential control of this disease.

A better understanding of the mechanism of the enteropathogens interaction with the porcine intestinal epithelial cells would be greatly aided by the availability of highly specific reagents that react with high affinity to a series of epitopes on the ETEC pili. The availability of an unlimited source of these reagents would greatly facilitate the diagnosis and possible prophylaxis of these enteropathogenic diseases. It is to this end that the production of hybridomas, which secrete monoclonal antibodies against the K88 pili, was undertaken and is described herein.

MANUSCRIPT

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES  
AGAINST K88 PILI FROM PORCINE ENTEROTOXIGENIC ESCHERICHIA COLI

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Running Title: Anti-K88 Monoclonal Antibodies

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#### ABSTRACT

Hybridomas secreting monoclonal antibodies directed against the K88 pili from porcine enterotoxigenic *Escherichia coli* (ETEC) have been produced and characterized. The monoclonal antibodies demonstrate strong reactions against both purified K88 pili and K88-piliated ETEC in the enzyme-linked immunosorbent assay (ELISA). While a prevalence was shown for specificity to the *ab* antigenic variants and may be specific for the *b* determinant, one monoclonal bound all K88-positive strains and may be specific for the *a* determinant on all K88-positive ETEC. The *a* specific monoclonal was shown to be useful for diagnosis of porcine ETEC in a direct ELISA with sensitivity to 50 ng/ml pili protein.



## INTRODUCTION

The phenomenon of bacterial adhesion has considerable biological significance. Adhesive characteristics may benefit the bacteria by: 1) anchoring the microorganism in a favorable habitat, from which it might otherwise be swept away, and 2) by placing it in surface contact with a nutrient source. Noninvasive enterotoxigenic *Escherichia coli* strains isolated from porcine neonatal diarrheal diseases have been shown to possess surface pili designated K88, which enable bacterial adherence to the small intestinal mucosa (5,9,12,16,32). Once attached, these enteropathogens cause diarrhea by producing enterotoxins. These toxins increase the activity of adenylate cyclase in the susceptible epithelial cells of the small intestine causing a net efflux of fluid into the intestinal lumen (7). The correlation between the presence of K88 antigen, adhesion of the bacteria to the small intestines, and subsequent diarrheal pathology has been clearly demonstrated (2). The detection of the K88 antigen in porcine *E. coli* is indicative of the enteropathogenicity of the strain as K88+ strains are nearly always also enterotoxigenic (25).

K88+ but nonenterotoxigenic *E. coli*, as well as isolated K88 antigen, could compete with ETEC strains for binding to the intestinal brush border membranes, thus reducing the disease

effects (1,5). Anti-K88 antibodies also prevented K88+ ETEC attachment to small intestinal epithelial cells *in vitro* (16,32). In addition, transient passive immunity can be conferred on the neonatal suckling piglets through the colostrum from a sow immunized with vaccines containing the K88 antigen (26). The overall success of such programs has been highly variable with major difficulties arising from the inconsistency between various vaccination procedures and the varied individual responses to the vaccines.

Though the etiology of the disease is fairly well known great difficulties have arisen in attempts to elucidate the method of attachment to the brush border membranes, to define the actual 'receptor' site on the intestinal membranes, and to determine the actual antigenic characteristics of the K88 pili. At present there is a great need for more precise reagents for the analysis of these problems. This report describes a series of monoclonal antibodies specific for intact K88 pili.

## MATERIALS AND METHODS

**Bacteria.** Serotypes of *E. coli* used for pili production and characterization were: NADC 1260-G1108E 0141:K85:88ab; 80-2575 46.1 0157:K88ac:H43; Morris 08:K87:88ad (The gift of Dr. Richard Wilson, Department of Veterinary Science, Pennsylvania State University); 0263:K87:88ab and 0101:K88-:K99+. Cultures were maintained by passage on Trypticase soy agar (Difco Laboratories) plates at room temperature. Prior to use in assays K99+ bacteria were grown on E agar plates at 37°C overnight to insure pili production (8).

**Purification of K88 antigen.** The K88 antigen was purified using a modification of the method of Stirm et. al.(27). Strains of K88+ *E. coli* were incubated in Trypticase soy broth cultures at 37°C overnight. These cultures were used to heavily inoculate Blood agar plates (TSB + 5% defibrinated whole sheep blood) that were incubated at 37°C for 18-24 hours to stimulate pili production (8). Confluent surface growth was aseptically harvested in 0.015 M phosphate buffered saline (PBS), pH 7.2, and the resulting bacterial suspension was homogenized in a Waring blender for 2 minutes at high speed to release the K88 pili. Cellular debris was removed by centrifugation at 27,000 x g for 15 minutes. As described by Isaacson(15), an additional extraction was performed on the cell pellet resuspended in PBS containing 1.0M sodium chloride. The pooled supernatants were

stored at 4°C for three days in the presence of 0.2% sodium azide. The solution was then filtered through a type RA membrane filter (1.2u pore size; Millipore Filter Corp.) to remove sediment.

The K88 pili (pI 4.0-5.5) were then precipitated at pH 5.0 by the slow addition of 7% acetic acid with gentle stirring. Precipitated protein was collected by centrifugation at 3,000 x g for 10 minutes. Following centrifugation at 3,000 x g for 10 minutes, the pellet was washed twice in PBS at pH 5.0 and the supernatant was re-extracted at pH 5.0 in the presence of 15% saturated ammonium sulfate. The pooled extracts were incubated overnight at 4°C prior to centrifugation, washing and dissolution in PBS, pH 7.2. The material was subjected to two more isoelectric precipitations to obtain a pure pili preparation. The final pili preparation was analyzed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20), and stored at -20°C with 0.2% sodium azide in 0.15 M PBS, pH 7.

Immunization. Female BALB/c mice were injected intraperitoneally at 4 weekly intervals with 50ug of purified intact K88 pili in 0.2ml of Hank's balanced salt solution (HBSS; Gibco Laboratories). The mice were then bled via the tail vein and antisera clarified by centrifugation. The sera were tested for antibody titer and specificity against K88 pili by an enzyme-linked immunosorbant assay (ELISA). Animals with the

highest titers were allowed to rest for two weeks and then boosted with 10ug of purified K88 pili in 0.1ml HBSS intravenously.

Production of hybridomas. Female BALB/c mice, immunized as described above, were sacrificed by cervical dislocation four days after receiving the i.v. boost. The spleen cells were removed, washed, and viability estimates made by trypan blue exclusion. Spleen cells were then combined with P3X63Aq8.653 myeloma cells in log growth at ratios ranging from 2:1 to 4:1 splenocytes to myeloma cells. The fusion protocol of Fazekas de St. Groth and Scheidegger (6) was followed. Cell fusion was accomplished by exposing a loosely pelleted mixture of splenocytes and myeloma cells to a fusion solution containing 50% (wt/vol) polyethylene glycol (3000-3700M.W.; Baker Chemical Co.) in HBSS and 5% dimethyl sulfoxide (Sigma Chemical Co.).

Fused cells were gently resuspended in Dulbecco's modified Eagle's medium (Flow Laboratories) supplemented with essential amino acids and vitamins,  $5 \times 10^{-5}$  M 2-mercaptoethanol, 20% fetal calf serum,  $10^{-4}$  M hypoxanthine,  $4 \times 10^{-7}$  M aminopterin, and  $1.6 \times 10^{-5}$  M thymidine (HAT medium) (23). The cell suspension was aliquoted, dropwise, over 96-well cluster plates (Costar), that contained a feeder layer of mouse peritoneal macrophages ( $3-6 \times 10^3$  cells/well) in HAT medium. The medium was replenished on day 4 and every third day after that by replacing half the well volume with fresh HAT medium.

After 8-10 days, supernatants from hybridomas were screened for specific antibody production by ELISA. Hybridomas of interest were subcloned twice by limiting dilution techniques in media lacking aminopterin. Selected clones were expanded into 24 well culture plates and cultured in complete medium without the addition of hypoxanthine, aminopterin, and thymidine. Expanded monoclones were then used for antibody production in ascitic fluids. All selected monoclonal cell lines, as well as the cloning plates themselves, were frozen in the presence of 10% dimethyl sulfoxide and 40% fetal calf serum at -70°C (11,31).

Production of ascitic fluid. For production of ascitic fluid BALB/c mice were injected intraperitoneally with 0.5ml of Pristane (2,6,10,14-tetramethyl pentadecane) at 7 to 10 day intervals. Three days following Pristane injection primed mice were injected intraperitoneally with  $3-6 \times 10^6$  hybridoma cells, in 1ml HBSS. Ascites fluid was drawn 7 to 10 days later, using an 18-gauge needle. The ascites was then clarified by centrifugation and stored at 4°C as a precipitate in the presence of 45% saturated ammonium sulfate solution, pH 7.0.

Purification of monoclonal antibodies. Monoclonal antibodies were purified from ascitic fluid by either of two methods depending on their isotype. Monoclonals of immunoglobulin class G were purified by DEAE-Affigel Blue chromatography (4). Monoclonals of the IgM class were isolated by

gel filtration through Sephacryl S200 (Pharmacia) (3).

ELISA. The ELISA used was a modification of the procedure described by Stocker et. al. (28). For hybridoma screening and titer determination microtiter plate wells (96 well cluster plates; Nunc) were coated with 100ul of a solution of K88 pili (1.0 ug/ml) in 0.15 M PBS coating buffer, pH 9.6, per well. These plates were allowed to incubate for 2 hours at 37°C or overnight at room temperature. After blocking with bovine serum albumin (BSA) and washing, the plates were incubated for 2 hrs at 37°C with 50ul of supernatants from growing hybridomas at a 1:2 dilution in (0.15 M PBS-0.05% Triton X-100-1% BSA). Anti-K88 antibodies were identified by incubation with rabbit anti-mouse immunoglobulins-conjugated with horseradish peroxidase (Cappel Laboratories) and detected with O-phenylene diamine (Sigma Chemical Co.).

For monoclonal specificity, the ELISA procedure was performed as described except that aliquots of monoclonal antibodies were plated followed by incubation of serial dilutions of various pili preparations. The presence of bound pili was detected by using polyclonal rabbit anti-K88 antisera followed by incubation with goat anti-rabbit horseradish peroxidase conjugated antibodies and substrate.

Class determination of K88 monoclonal antibodies.  
Supernatants and ascitic fluid were used in the ELISA as

previously discussed using plated K88 pili. Dilutions (1:500) of rabbit antisera against mouse immunoglobulins M, G1, G2a, G2b, and G3 (Cappel Laboratories) were added to the washed plates. After a 30 minute incubation at room temperature, goat anti-rabbit horseradish peroxidase conjugate (1:2,000 dilution) was added and incubated for 30 minutes at room temperature. The assay was completed as described.

Immunoblot analysis. The SDS-PAGE was run as described by Laemmli et. al. (20) using 10% monomer. The gel was electroblotted to nitrocellulose paper as described by Towbin et.al. (30). The material was electrophoretically transferred anodally to the nitrocellulose sheet in 25mM Tris/192mM glycine/20% (v/v) methanol buffer system, pH 8.3, for 1 hour at 100 volts (1.2-1.5 amps).

An immunoperoxidase assay was used to detect antigen bands on the nitrocellulose sheet. The nitrocellulose was immersed and shaken overnight in blocking buffer (0.9% NaCl/10mM Tris-HCl, pH 7.4) with 1% BSA. It was then incubated with a 1:10 dilution of hybridoma supernatants or 1:100 dilution of ascitic fluid in blocking buffer with 3% BSA for 3 hours at room temperature. The paper was washed in 5 changes of blocking buffer for 10-15 minutes. The blots were then incubated with a 1:1,000 dilution of rabbit anti-mouse immunoglobulin-peroxidase conjugate (Cappel Laboratories, Inc.) in blocking buffer and shaken for 3 hours at room temperature, washed 5 times and visualized by the addition



of a substrate solution containing 4-chloro-1-naphthal. The substrate solution was prepared fresh from stock solutions of 60mg 4-chloro-1-naphthal in 20ml of methanol, and a hydrogen peroxide stock solution of 50ul 30% hydrogen peroxide in 10ml of blocking buffer. The working solution contained 3ml of the 4-chloro-1-naphthal stock, 1ml of the hydrogen peroxide stock in 25ml of 10mM Tris-base, pH 7.0. The color reaction was stopped after approximately 10 minutes by flushing with distilled water. No color development was noted without the use of K88 specific antisera during the first incubation.

Radioimmunoprecipitation. A crude K88 pili preparation was obtained by the method of Stirm et. al.(27) as described previously, except the preparation was used after the initial isoelectric precipitation. The K88 extract was labeled with <sup>125</sup>I using the chloramine T method (14) and dialyzed overnight against 0.15 M PBS, pH 7.2, at 4°C.

Immunoprecipitation was performed as outlined by Jones (17). The cell pellet was resuspended in SDS-PAGE reducing sample buffer, mixed and centrifuged. The supernatant was then applied to a 10% SDS-polyacrylamide gel. After the run the gel was fixed and dried onto 3MM Whatman Chromatography Paper (Whatman Ltd.). The dried gel was then sectioned and the radioactivity of each fraction was determined.

Enzyme conjugation of monoclonal antibodies. Monoclonal antibodies were precipitated from ascitic fluid as described. The precipitate was centrifuged at 10,000xg for 10 minutes and resuspended in 0.01 M sodium carbonate buffer, pH 9.5, and dialyzed overnight at 4°C against three 1 liter changes of the same buffer. The protein concentration was then determined by a Bio-Rad Protein Assay (Bio-Rad). The concentration was adjusted to 5 mg/ml. The procedure for enzyme conjugation of the monoclonal antibodies was a modification of Nakane and Kawaoi (21,22,29).

## RESULTS

### Monoclonal antibody production and characterization.

Hybridomas obtained from the fusion of BALB/C spleen cells, immune to purified K88 pili, and P3X63Aq8.653 myeloma cells were screened for specific antibody 8 to 10 days after the fusion. Of 600 culture supernatants tested 62 wells indicated antipili activity. Six positive clones that had consistently high ELISA values were expanded and cloned by limiting dilution methods. The resulting monoclonal antibodies were then analyzed for immunoglobulin isotype. The majority of the antibodies were found to be IgM or IgG2b isotypes, however IgG1 and IgG3 subclasses were also identified (Table 1).

**Specificity and titer of monoclonal antibodies.** Hybridoma cells were utilized for the production of ascitic fluid. Parallel ELISA analysis was made using serial dilutions of the ascitic fluid and a high-titer polyvalent rabbit anti-K88 antisera (Fig.1). All monoclonals tested demonstrated comparable dose response curves in assays utilizing plates coated with the K88 pili preparation originally used as the immunogen (0263:K88ab). It should be noted that plate wells coated with purified pili from other serological K88 variants (K88ac:ad) showed variable results in the initial ELISA screening. These inconsistencies were circumvented by coating the plate wells with

the monoclonal antibodies and detecting bound pili by utilizing rabbit polyvalent antiK88 antisera as described in the methods section. The remainder of the ELISA specificity screening was carried out in this manner.

The ELISA screening indicated that the majority of the monoclonal antibodies isolated were specific for the K88b epitope (Fig. 2 and Table 1). All of the tested monoclonals showed comparable ability to detect the K88b determinants regardless of the *E. coli* strain used, as seen in Fig. 2 a through e. The one exception was monoclonal 2C1.16 (Fig. 2f), which showed a capability of detecting any K88 variant, therefore demonstrating an affinity for the K88a antigenic site common to all K88 piliated *E. coli*. None of the monoclonals tested showed binding to K99 pili. Neither de-piliated *E. coli* nor *E. coli* bearing 987P or K99 pili could be detected in these screenings, when utilizing the appropriate homologous polyvalent antisera (data not shown).

#### Immunoblot analysis of monoclonal antibody activity.

Immunoblot assays were also performed to verify the antigen-antibody binding specificity. Of the monoclonal antibodies tested, all but one gave strong reactions to the free subunits of the pili which appeared as strong bands of approximately 26,000 molecular weight (Fig. 3, lanes A-F and Table 1). This corresponded to an identical band as detected by the rabbit polyvalent antisera (Fig. 3, lanes 1,2). It is of interest to note that the polyvalent antisera cross reacted with

several other protein bands when exposed to an immunoblot using a crude pili preparation (Fig.3, lane 1). These reactions were not seen when utilizing any of the purified pili preparations and the monoclonal antibodies.

Monoclonal 2C1.16 was tested for its ability to detect the subunits of other K88 variants. In all cases this monoclonal antibody was able to give a strong reaction with all K88 variants. Though these variants showed variable molecular weights (23,000-27,000) they share a common antigen-antibody binding site with 2C1.16. All other monoclonals tested for cross-reactive binding to K88 variants showed their single specificity for the K88b pili.

Radioimmunoprecipitation of K88 pili As indicated by immunoblot analysis monoclonal 2C7.81 showed no reactivity to the free subunits of the SDS-treated pili. In order to verify its specificity for the pili we ran a radioimmunoprecipitation assay. Both polyvalent antisera and monoclonal 2C7.81 were capable of immunoprecipitating the K88 antigen (Fig. 4). The polyvalent anti-K88 antisera again demonstrated extraneous protein peaks. The monoclonal antibodies of 2C7.81 demonstrated one major peak corresponding with the 26,000 molecular weight of the free K88ab subunit, thus verifying 2C7.81's reactivity in the ELISA and indicating a conformational specificity rather than specificity to the free subunits of K88ab. One other minor peak was noted for 2C7.81 and corresponded to a molecular weight range

of approximately 51,000 to 55,000, indicating the presence of a possible K88 subunit dimer.

Utilization of monoclonal antibodies in ELISA. To determine the monoclonal antibodies efficacy as a clinical tool an ELISA was performed utilizing monoclonal antibody coated plates as well as monoclonal-horseradish peroxidase conjugates (MAB-HRPO) to detect pili. Serial dilutions of the pili were incubated in the monoclonal antibody-coated plates after which they were incubated with the MAB-HRPO. The ELISA was then developed as previously described. Figure 5 demonstrates the typical titration curve for this ELISA, with all monoclonal antibodies tested showing a comparable detection capability. The sensitivity for this assay was from 50 to 80ng/ml for purified pili.

## DISCUSSION

We have described the production of hybridomas and characterization of a series of monoclonal antibodies demonstrating a strong affinity for the K88 pili of porcine ETEC. Though the spleen cells used for fusion were harvested from hyperimmunized animals, the majority of the monoclonal antibodies obtained were of the IgM class (10 of 16 studied, all data not shown). This may, in part, be a reflection of the nature of the pili itself. The intact K88 pili is composed of identical subunits, as evidenced by the appearance of a single band upon dissociation in SDS-PAGE. Coupled with this is the largely hydrophobic nature of the K88 subunit (24). The net result could be favorable to the stimulation of the production of IgM class antibodies without collateral lymphocyte help, as is the case for many lipopolysaccharide responses.

The ELISA was the primary method used in this study for the characterization of the monoclonal antibodies as to isotype and specificity. The original technique utilized pili-coated plates for the detection of monoclonal antibody binding; however, variability in the form of high backgrounds, as well as variation in repeated screenings lead to an alteration in the ELISA procedure. By plating the monoclonal antibodies, followed by incubation with the various forms of pili, the background was reduced by 2 to 4 fold (ie. from absorbances of 0.1 to 0.02-0.04

at 490nm) and the ELISA results showed excellent reproducibility. This may be due again to the overall hydrophobic nature of the K88 pili, which may be responsible for nonspecific hydrophobic interactions between not only the monoclonals being screened, but also between the conjugated antibodies used for their detection. It is conceivable that in the plating and blocking process antigenic binding sites or the overall native conformation of the pili may have been compromised or physically blocked. Both of these explanations may be valid as demonstrated by the fact that several marginally positive reactions were completely eliminated, while others were found to be quite strongly reactive upon subsequent testing with the altered ELISA technique. It is believed that the screening of the pili and piliated ETEC utilizing monoclonal antibody coated plates allows for the maintenance of the native pili structures and availability of antigenic binding sites while greatly limiting the hydrophobic interaction effect. Though hydrophobic interactions are still possible, the increased exposure of the pili during the repeated washings will eliminate the great majority of the nonspecific binding.

It has been reported that all K88 variants express the determinant K88a, as demonstrated by the use of polyclonal anti-K88 antisera (10). The other antigenic determinants (b, c, and d) seemed to be alterations in response to environmental pressures. These pressures could be either in response to an



altered 'receptor' site on the intestinal epithelial cells, or an effort to escape immunological pressures within an increasingly immune population. It is, therefore, possible to speculate that the a epitope is required for the structural integrity of the pili and is probably more internalized or less immunogenic, as evidenced by the difficulty in obtaining monoclonal antibodies to this epitope (2C1.16 was the only crossreactive monoclonal developed, thus far, after multiple fusions). It also infers the involvement of the b, c, and d epitopes in the potential 'receptor' binding of pili to brush border membranes. Inhibition of binding studies are now underway to determine the ability of each monoclonal to block the adhesion of the pili variants to the porcine intestinal epithelium.

Through the use of this battery of monoclonal antibodies it may now be possible to investigate the nature of the 'pili-receptor' interaction. It is known that specific polyclonal antisera to K88 can neutralize the adhesiveness of the pili *in vitro*; in addition, colostrum from immunized sows can confer passive immunity to suckling piglets *in vivo*. By using a battery of subunit specific monoclonal the possibility of specific 'receptor' binding may now be analyzed. Klemm has predicted as many as eight potential antigenic determinants along the K88 subunit (13,18,19). By utilizing tryptic and cyanogen bromide digests, studies of the pili antigenic nature and its potential physiological interactions can be carried out. The

results of this study could lead to the use of a synthetic peptide copy of a specific immunogenic site (ie. K88a) as a potent vaccine against K88-piliated porcine ETEC strains. Peptides of this nature, conjugated with BSA carriers, have already shown strong anti-peptide responses (33).

Presently, two serological variants of the K88 antigen, K88ab and K88ac, have been internationally accepted. A third K88 variant, K88ad, was recently described from porcine ETEC isolates (10). The current clinical assays rely on either polyclonal antisera or monospecific antisera derived from cross absorption methods. These reagents have proven to be extremely useful in the detection and characterization of ETEC isolates. Problems can, however, arise as the quality and the quantity of such antibodies can again be highly variable as to both specificity and affinity to the antigen. This variability can be seen from animal to animal as well as from individual bleedings from the same animal.

The monoclonal antibodies described here are being used in ELISA screening of clinical isolates. Monoclonone 2C1.16 has been utilized in a single-antibody ELISA (Fig. 6) and has given strong positive reactions to all K88 piliated ETEC, while showing no cross reactivity to K88-negative strains. It should be noted that the rabbit polyclonal K88 antisera often led to high background readings, not found with the monoclonal antibodies. This variability could be explained by possible crossreactivity

with other cellular components as seen in the immunoblot when using a crude pili preparation. Thus, these monoclonal antibodies represent a constant, unlimited source of high affinity anti-K88 antibodies for clinical application. Recent clinical studies of 277 porcine isolates of enterotoxigenic *E. coli* showed absolute correlation between pathology, toxin production and reactivity with the anti-K88a monoclonal antibody, 2C1.16, and no reactivity to K99+ or 987P+ bacteria (K.W.Mills, R.B.Westerman, R.M. Phillips, G.W. Fortner, and J.M. Greenwood, Submitted for publication).

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Table 1

## Properties of Anti-Pili Monoclonal Antibodies

Monoclonal	Antibody Isotype	Specificity for K88 Pili Subgroup	Epitope Type
3A5.70	IgM	ab	Subunit
1B1.59	IgM	ab	Subunit
2C7.81	IgG <sub>2b</sub>	ab	Conformational
3B9.46	IgG <sub>3</sub>	ab	Subunit
1C4.58	IgG <sub>1</sub>	ab	Subunit
2C1.16	IgG <sub>2b</sub>	ab,ac,ad	Subunit

## FIGURE LEGENDS

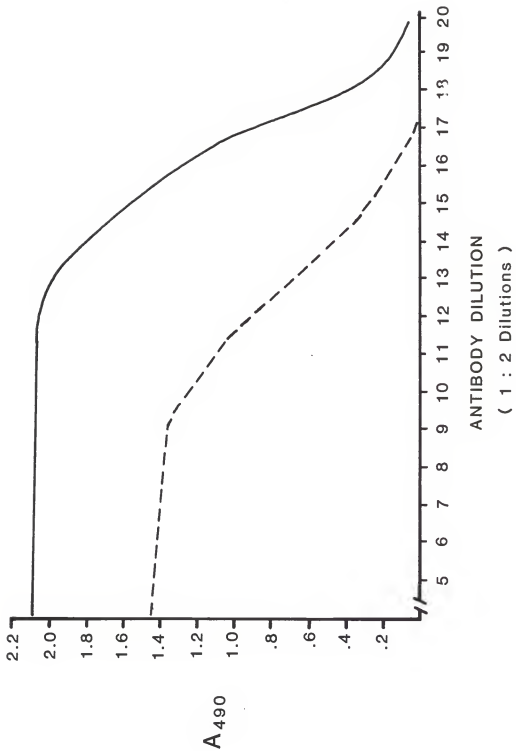
Figure 1. Titer of anti-K88 monoclonal antibodies as determined by ELISA. Microtiter wells were coated with 10 ug/ml K88ab pili. Serial dilutions of 3A5.70 (————) or rabbit polyclonal anti-K88 sera (-----).

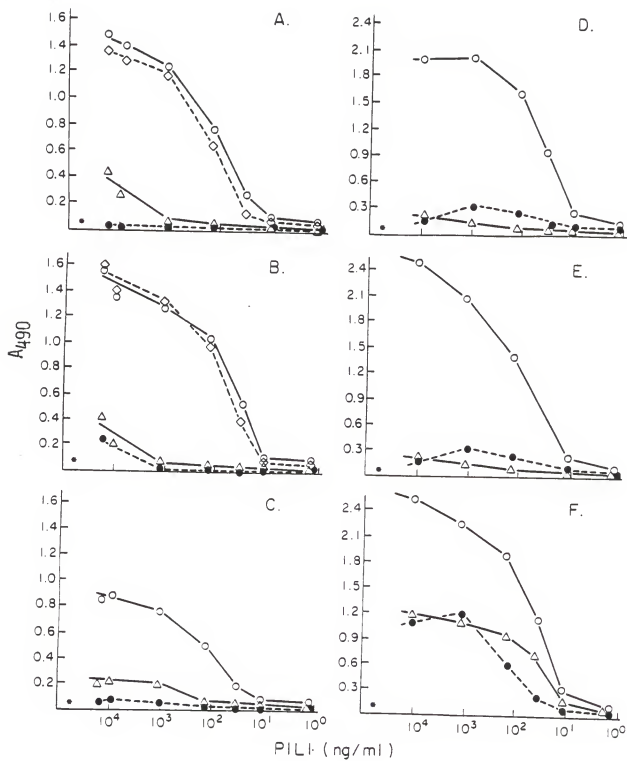
Figure 2. Determination of monoclonal antibody specificity by ELISA. Plates were coated with a 1:1,000 dilution of a specific monoclonal antibody preparation. (A) 3A5.70, (B) 1B1.59, (C) 2C7.81, (D) 3B9.46, (E) 1C4.58, (F) 2C1.16. Incubation was with serial dilutions of purified pili preparations and quantitation followed by detection with rabbit polyclonal K88 antisera and HRP0-goat anti-rabbit antisera with absorbance reading at 490nm. (●) =0263:K88ab; (◆) =0141:K88ab; (▲) =0157:K88ac; (●) =08:K88ad; (\* ) =0101:K99+.

Figure 3. Immunoblot analysis of monoclonal antibodies. PAGE of 20 ug of K88ab purified pili preparation, lanes 2 and A-F; or 20 ug of crude pili preparation, lane 1. Following electrophoretic transfer detection of bands was by polyclonal anti-K88 antisera, lanes 1 and 2; or by appropriate monoclonal antibodies A-F (see Figure 2 for designation of monoclonal antibodies).

Figure 4. Radioimmunoprecipitation of <sup>125</sup>I-labeled K88ab pili. Crude K88 pili preparations were radiolabeled with <sup>125</sup>I and incubated with 2C7.81 (—o—) or rabbit anti-K88 (—). Precipitation was with *S. aureus* cells. SDS-PAGE was followed by sectioning of the gel and counts of individual gel slices.

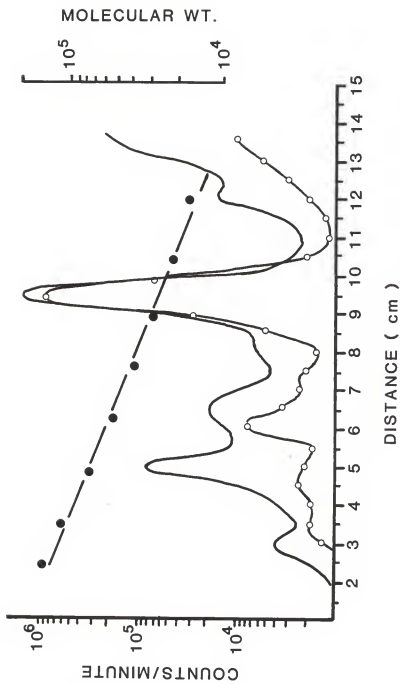
Figure 5. Titration of K88 pili by ELISA. Monoclonal 2C1.16 was plated at dilutions of 1:1,000 followed by incubation with serial dilutions of K88 pili. Bound pili were detected by incubation with a 1:2,000 dilution of 2C1.16-HRPO conjugate.

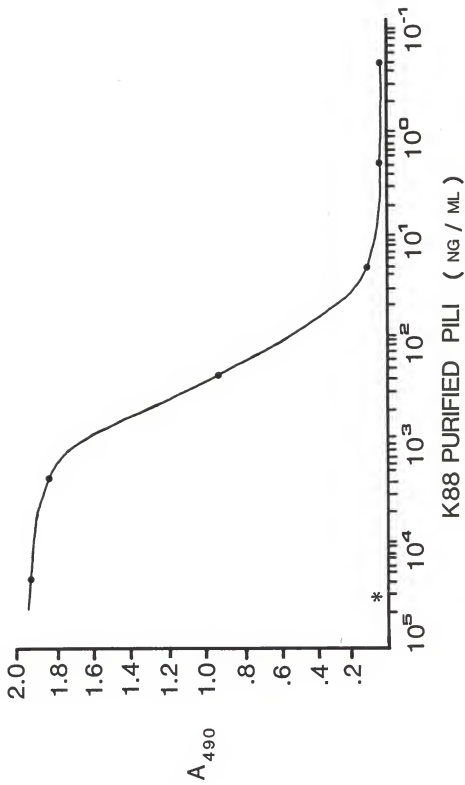












## FINAL DISCUSSION

In addressing the etiology of ETEC mediated diseases, the role of the adhesions, as virulence factors, is of utmost importance. Without the ability to adhere to the small intestinal mucosa, the most susceptible site for enterotoxin reactivity, the enteropathogens would be of little or no consequence. The virtue of these pili to the ETEC is beyond question, when following the disease pathology during the past two decades. It was discovered in the early 1960's that the presence of the K88 pili corresponded directly with the expression of porcine neonatal diarrhea. Since that time much attention has been directed to the study of these pili antigens. K88 variants have been discovered, the most recent (K88ad) being described in 1973. It is assumed that these variants reflect an adaptation of the ETEC to environmental pressures caused in part by widespread use of vaccines containing K88ab pili. This may also account for the gradual disappearance of the K88ab pili variant from the ETEC population. To date, the major prophylactic measures have insured only temporary and transient relief of these disease outbreaks.

The major problem lies in the understanding of the pili interaction with the brush border membranes of the porcine small intestines. Information accumulated so far infers a

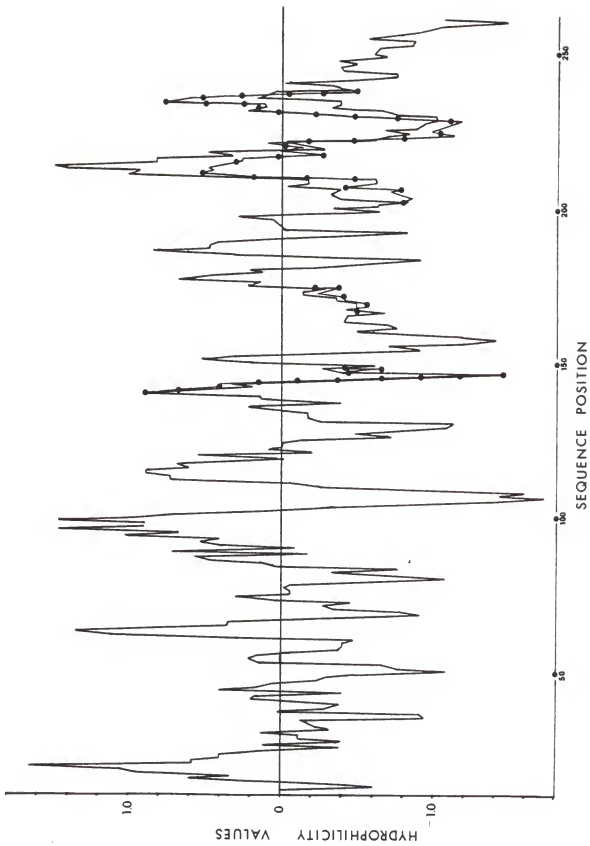
"pili-receptor" type of interaction. These data were obtained through the use of serological inhibition studies. It was demonstrated that polyclonal antisera directed against K88b, c or d epitopes could block the binding of the homologous pili to the intestinal epithelial cells (38). These reactions were not cross-reactive. It is also accepted that antisera directed against the common K88a epitope could inhibit the binding of all K88 piliated organisms. In these later studies, however, discrepancies are not uncommon. While some studies show inhibition of K88 pili binding using anti-K88a antisera, others failed to demonstrate these activities. For the most part, these discrepancies are mere reflections of a major weakness in the study of these enteropathogens, the availability of singly specific, high affinity reagents directed against each of the many antigenic epitopes present on these pili. This thesis has described the production of a battery of such reagents in the form of monoclonal antibodies.

With monoclonal antibodies the clinical diagnostic fields are presented with an unlimited source of highly specific, high affinity antibodies for detection of K88-piliated ETEC, as well as rapid karyotyping of the specific pili variant involved. This is a vast improvement over the present polyclonal anti-K88 antisera presently in use, which may show considerable variability between each animal used to derive the antisera. Variations in titer and specificity may also be demonstrated

between individual bleedings of the same animal. Since the antisera obtained is of a polyclonal nature, few if any definitive studies as to the antigenic makeup of the pili could be undertaken with any great success.

Perhaps the greatest asset the monoclonals represent is the potential capability of elucidating the antigenic nature of the K88 pili. In 1981 the complete amino acid sequence for the K88ab pili was determined by Klemm and Mikkelsen. The known sequence for the pili subunit provides a means of predicting possible antigenic determinant sites from hydrophilicity values. The result of this kind of analysis is shown in Figure 6. A hydrophilicity graph shows as many as 9 antigenic sites on the K88 subunit. These sites may also represent potential sites of interaction between the pili and the small intestinal epithelial cells of the infected animal. A partial amino acid sequence for the K88ad variant has also been determined by Gaastra. Of interest is the fact that in the C-terminal end of the subunit, the amino acid differences between variants greatly alters the potential antigenic profile, reducing one site while producing another. The meaning of these changes has been beyond investigation until pure reagents of single antigenic specificities were developed. The monoclonal antibodies may provide a source of such reagents. Through their use it may be possible to determine exact sites of interaction and to elucidate the nature of these reactions of pili to host.

Figure 6. Hydrophilicity chart of the ETEC K88ab pili amino acid sequence.



Finally, these monoclonal antibodies may prove to be an extremely valuable tool in the therapy and prophylaxis of K88-piliated ETEC diseases. By the aforementioned studies a single highly immunogenic site may be identified, or alternately, a single site shared by all K88 variants may be defined (i.e., K88a). Once determined such a site could be manufactured as a synthetic peptide which could be used as a vaccine for the stimulation of an active immunity against all K88 related diarrheal disorders. The monoclonals may also provide a source of antibodies for passive oral immunizations of piglets, as is the case for K99 monoclonal antibodies in the bovine system.

In summary, the monoclonal antibodies described in this thesis provide a rapid clinical diagnostic tool for identification of K88 mediated ETEC diarrheal diseases. They also represent a key to the analysis of the antigenic makeup of the K88 pili, a means to study the elusive nature of the K88 pili interactions with the porcine small intestinal epithelial cells, and a probable method of eliminating or inhibiting these interactions.



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## APPENDIX

### PRODUCTION OF MONOCLONAL ANTIBODIES

Since the inception of somatic cell hybridization for the production of monoclonal antibody producing hybridomas (16), a great deal of attention has been focused on optimizing the quick and effective generation of these cell lines. The diversity on techniques result from innumerable variations beginning with immunization protocol through to the ultimate screening and establishment of the final desired clonal line. Several groups have dedicated large amounts of time and effort in attempts to determine the basic parameters that will help insure successful production of the hybridoma cell lines (4,6,23,26,33).

The basic purpose of hybridoma technology is the production of an immortal cell line actively secreting antibodies of single specificity directed at a predefined antigen. The immune spleen cells, however, display a very limited mortality in tissue culture with most splenocytes dying within the first week or two of culturing. This difficulty was overcome by fusing the mortal spleen cells with a syngeneic immortal myeloma (or plasmacytoma) cell line. As the myeloma cells are of a B cell origin many of them retain the ability to synthesize and secrete a heavy and/or light immunoglobulin chain of their own. However, a few cell lines have been isolated which either fail to produce these

immunoglobulin chains or upon production are unable to secrete their chains. These cell lines are greatly desired as this eliminates the contamination of the desired monoclonal antibodies by the extraneous immunoglobulin chains.

The actual fusion process, first accomplished using Sendiae virus, is now commonly performed by exposing a mixture of spleen cells and myeloma cells to a solution of polyethylene glycol, which, like its viral counterpart, causes membrane fusion between cells resulting in multinucleated cells. As these cells divide the chromosomal complement is fairly unstable leading to the ultimate loss of chromosomes. Though this loss is not completely random, with the preferential loss of chromosomes from one cell or the other, a method of selecting the desired fusion product is essential.

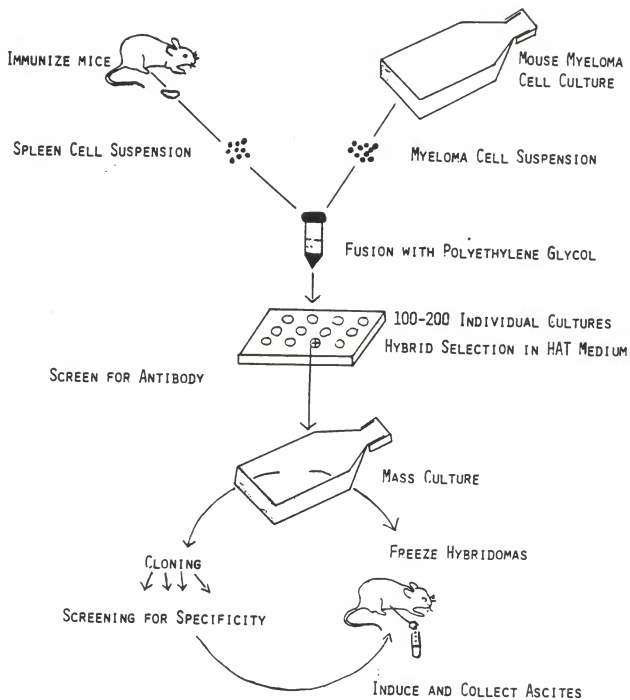
The basic selection procedure was made possible by the isolation of specific myeloma cell lines that can be selectively removed from the fusion culture by exposure to specific media. The myeloma cell lines commonly employed (P3-NS-1; P3-X63Ag8.653; Sp2/0-Ag14) are lines which, in addition to their lack of immunoglobulin chain production, also lack the enzyme hypoxanthine-guanine-phosphoribosyl transferase (HGPRTase). This enzyme is required in a salvage pathway for nucleotide biosynthesis. Therefore if the ~~de novo~~ nucleotide synthesis is blocked these cells are unable to continue cell division in culture. Utilizing the characteristics of each cell involved in



the fusion, selection of desired hybridomas can be accomplished. The cells after fusion can consist of products of spleen-spleen cell fusions, myeloma-spleen cell fusions, and myeloma-myeloma fusions (as well as individual cells of either species). By exposure of the fusion products to a selection media, known as HAT media (Hypoxanthine-Aminopterin-Thymidine), the desired fusion products may be obtained. Since aminopterin, a folic acid analog, blocks the *de novo* nucleotide biosynthesis, all myeloma-myeloma cell fusions and free myeloma cells soon die off. Though the spleen cells, as well as spleen-spleen cell fusions, can produce the required enzyme to overcome this selection, their own limited viability in cell culture results in their early passive selection and elimination. The resulting myeloma-spleen cell fusions survive by virtue of the myeloma's immortality complemented by the enzyme production potential of the spleen cell fusion partner.

The hybridomas are then screened for production of the desired antibody, cloned to obtain a pure cell line, and ultimately expanded for large scale antibody production in animals. The following protocol is one used for the derivation of the monoclonal antibody producing hybridomas described within this thesis. It represents a basic synopsis of many of the methods previously referenced.

METHOD



## PROTOCOL

### A) Materials:

#### Standard Media:

DMEM (Dulbecco's Modified Eagle's Media w/ L-Glutamine)  
10% Fetal calf serum  
10mM HEPES  
0.05mM 2-mercaptoethanol  
Supplemented with: MEM Essential & Nonessential Amino  
Acids; MEM Vitamins; Sodium Pyruvate and PSF (Penicillin,  
Streptomycin and Fungizone)

#### Select Media:

##### Stock Solutions:

100X HT: (10mM hypoxanthine; 1.6mM thymidine)  
Dissolve 68mg. hypoxanthine and 19.4mg. thymidine in  
50ml deionized distilled water (dH2O) warmed to  
70-80°C. Filter sterilize and store at -20°C.

1000X A: (0.4mM aminopterin)

Dissolve 8.8mg aminopterin in 40ml dH2O by dropwise  
addition of 1.0M NaOH. Bring the pH to 7.6, adjust  
volume to 50ml, and filter sterilize. Store in 5ml  
aliquots at -20°C.

(NOTE: Aminopterin is light sensitive. If  
deterioration occurs it will result in lack of  
selection pressure against the myeloma cells)

##### 50X HAT:

To 25ml of 100X HT add 2.5ml 1000X A in 22.5ml dH2O.

##### 1X HAT:

To 98ml of standard medium add 2ml of 50X HAT stock.

##### 1X HT:

To 99ml of standard medium add 1ml of 100X HT stock.

#### Fusion solutions:

##### SF-DMEM (Serum free media):

Prepare standard media without the addition of fetal  
calf serum.

##### PEG solution:

1g Polyethylene glycol (PEG: BAKers 3000-3700)  
1ml SF-DMEM  
0.1ml dimethyl sulfoxide (DMSO:Sigma Chemical Co.)

Dissolve the PEG and sterilize by autoclaving for 12-15 minutes at 120-130°C.

(NOTE: PEG should be odorless and colorless. It should be autoclaved with fast exhaust and removed from the heat as quickly as possible to avoid the formation of carbonyl compounds (aldehydes), which significantly reduce the colony survival rate of hybridomas (13)).

## B) Fusion Protocol:

- 1) Day -4: (Four days prior to fusion day)
  - a) Boost the hyperimmunized BALB/c mouse selected to be used for the fusion.
  - b) Prepare the myeloma cell line to insure  $2-5 \times 10^7$  cells in log phase growth of not more than 70% confluency on the day of the fusion.

(NOTE: As immunization protocols are so diverse they will not be directly addressed here, the final boost prior to fusion should be done 4-5 days prior to fusion day and may be administered either intraperitoneally or intravenously, the latter method being preferred for soluble antigens of limited availability as it requires lower antigenic doses.)

- 2) Day -2:
  - a) Prepare 500ml of SF-DMEM and 200ml of 1x HAT media.
- 3) Day -1:
  - a) Set up fusion plates (3 96well tissue culture clusters; Costar) with 1 drop (approximately 50ul) of 1x HAT media per well. Place the plates in an incubator with 10% carbon dioxide at 37°C.
  - b) Prepare feeder cells as follows:

Peritoneal cells are obtained by peritoneal lavage using from 6-8ml of SF-DMEM. These cells are washed and counted in SF-DMEM. A typical lavage will produce  $1-5 \times 10^6$  cells per mouse, half of which are lymphocytes while the remainder are macrophages. Macrophages are often preferable due to their adherent nature, which enables easy separation from resulting hybridomas, and their long term viability in tissue culture. These cells are resuspended in 1x HAT media at  $6 \times 10^4$  cells/ml and distributed dropwise (approximately 50ul) into each well of the 96 well plates, which are then returned to the 10% CO<sub>2</sub> incubator.

(NOTE: The value of feeder cells in conditioning the media and enhancement of hybridoma growth is universally accepted, though the origin of these feeders differs

with various researchers. Any of the following may be used as feeder cells with comparable efficacy: thymocytes (2-5x10<sup>5</sup> cells/well), splenocytes (1x10<sup>4</sup> cells/well), or peritoneal cells (2-5x10<sup>3</sup> cells/well).

- 4) Day 0: (Fusion day)
- Warm the SF-DMEM, HAT media, and prepare the PEG solution.
  - The hyperimmunized BALB/c mouse is sacrificed by cervical dislocation and its spleen aseptically removed. The splenocytes are teased into SF-DMEM, vortexed gently to create a single cell suspension, and finally allowed to set for 2-5 minutes in a 15ml centrifuge tube to allow large debris to settle out. The supernatant is then transferred to a 50ml centrifuge tube and washed twice with SF-DMEM. The cells are then counted by trypan blue exclusion and resuspended at 10<sup>7</sup> cells/ml in SF-DMEM.
  - In parallel, the flasks containing the Aq8 myeloma cells (P3-X63 Ag8.653 cell line) are flushed and the cells are washed twice with SF-DMEM, counted, and likewise resuspended at 10<sup>7</sup> cells/ml in SF-DMEM.
  - Splenocytes are then combined with myeloma cells at ratios ranging from 2:1 to 10:1 splenocytes to myeloma cells. These are then gently vortexed and centrifuged at 200xg for 8 minutes to form a loose pellet.

(NOTE: The exact ratio may be dictated by the availability of viable cells. As blast cells seem to fuse preferentially it is recommended that the splenocyte number be approximately 4 to 5 times that of the myeloma cells. A typical fusion may utilize between 2-4x10<sup>7</sup> to 10<sup>8</sup> total cells.)

- The pellet is further loosened by light tapping of the centrifuge tube. Place the tube into a holder that can be slowly shaken during the fusion process. Slowly add one milliliter of warm (37°C) PEG solution dropwise over a one minute period with constant gentle shaking of the tube. Slowly add 1ml of SF-DMEM over the next minute with gentle swirling. The fusion mixture should appear as a suspension of fine cell clusters. Over the next 2-3 minutes another 10ml of SF-DMEM is added with continuous swirling.

(NOTE: Though the fusion frequency increases with extended exposure of the cells to the PEG solution, so does the toxicity and cell death. The allowable duration of exposure is dictated by the percent of PEG in the

solution. A PEG solution of 30-35% may be tolerated for up to 7 minutes, while a 40-50% PEG solution becomes toxic after only 1-2 minutes. It is therefore important to steadily dilute the toxicity while maintaining the highest fusion efficiency. Dilutions should be done slowly at first as cell wall integrity is greatly compromised at high PEG concentrations.)

- f) Centrifuge the fusion mixture at 200xg for 5-8 minutes to again form a loose pellet. Resuspend the pellet in 15-20ml of 1x HAT media by very gentle pipetting of media to create a suspension of small cell clusters. Distribute the suspension dropwise (approximately 50ul) over the 3 96 well culture plates containing feeder cells and return the plates to the 10% CO incubator. It is useful to reserve a few wells, on one of the plates for the plating of myeloma cells only as a control to confirm the selectivity of the HAT media.

#### C) Feeding schedule:

- 1) Allow the plates to stand undisturbed for the first four days at which time they may be fed by addition of a drop (50ul) of HAT media to each well. The wells can be fed every 2-3 days thereafter, depending of cell need, by the aspiration of half of the media in each well and the addition of 100ul of fresh HAT media.
- 2) Hybridomas should be observable by days 7-10, with many fast growers appearing as early as day 5. Most hybridomas arise within the first two weeks after fusion and are easily observed as small white clusters when holding the plate up to the light and viewing the wells from below.

(NOTE: Though hybridomas may arise later than two weeks these are not only slow growers but are typically weak producers as well.)

- 3) Screening of the hybridomas for specific antibody production should be done as soon as possible. Desired hybridomas can be expanded to 24 well tissue culture cluster trays containing feeder cells in 1x HT media when they reach a cell number of from  $5 \times 10^5$  to  $10^6$ .

(NOTE: The hybridomas should be grown in HT media for several weeks after removal from HAT media in order to insure that no residual aminopterin is present. Aminopterin has an extremely high affinity for the enzyme it inhibits, therefore even the most minute amount can cause cell death in the absence of available hypoxanthine and thymidine. Removal can only be accomplished by dilution in culture. It has even been suggested that hybridomas be permanently cultured in

HT media to avoid this (1,2).

D) Cloning:

- 1) Cloning of hybridomas should be done as soon as a producer of the desired antibody has been identified to avoid possible overgrowth of the positive clone by other non-producing or nonspecific clones.
- 2) Limited dilution cloning is the most commonly used form of cloning and is based on statistical probability. Cells are diluted so that they contain 5, 1, and 0.5 cells per well when they are distributed over a 96 well culture plate in the presence of feeder cells (32 wells for each dilution). In approximately two weeks clones should again be evident. If only 30-40% of the wells of any of the respective dilution groups show growth, those cells of that group are statistically considered to be clones arising from a single cell. These clones should again be screened for retention of their production capability and cloned once again to guarantee the clonality of the final product.

E) Final Processing:

- 1) Cloned hybridomas should be expanded in large-scale cultures slowly in order to maintain a good log growth phase. Once

the cell numbers reach approximately  $10^7$  cells they should be frozen at  $-70^{\circ}\text{C}$  in standard DMEM in the presence of 10% DMSO (dimethyl sulfoxide; Sigma Chemical Co.) (9,37).

- 2) Though active hybridomas may produce from 10-60ug of antibody per milliliter of culture a 100-1000 fold increase in yield may be obtained by growth of these cells as ascites in animals. Syngeneic animals may be preconditioned by 0.5ml intraperitoneal injections of Pristane (2,6,10,14-tetra-

methyl pentadecane; Aldrich) at 10 and 3 days prior to cell injection. Approximately  $2-5 \times 10^6$  actively growing hybridomas injected intraperitoneally will usually result in an ascitic tumor within 7-14 days. From 4-8ml of ascitic fluid can be collected by the insertion of an 18-gauge needle into the peritoneum and drainage of the fluids into a centrifuge tube. Ascitic fluids may be drawn 2-3 times from one animal before the tumors become too solid and burdensome for the animal. The animal may then be sacrificed and bled to yield from 1-3 milliliters of serum containing up to 30mg per ml of the desired monoclonal antibody. The resulting yield from one animal may approach from 100-300mg of monoclonal antibodies.

THE PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES  
AGAINST K88 PILI FROM PORCINE ENTEROTOXIGENIC ESCHERICHIA COLI

by

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B. S., Kearney State College, 1975

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AN ABSTRACT OF A MASTER'S THESIS

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#### ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) strains are responsible for neonatal diarrheal diseases in animals and man. Their strains produce enterotoxins, which disrupt normal intestinal cell function and result in a massive efflux of fluids. This disease, however, is mediated by the presence of surface adhesins, called pili, which enable bacterial attachment and colonization of the susceptible areas of the small intestines. Without such adherence the expression of the disease pathology is eliminated. It has been demonstrated that antibodies, via colostrum from immune animals, can confer a passive immunity against these enteropathogens to their offspring.

Hybridomas secreting monoclonal antibodies directed against the K88 pili from porcine ETEC have been produced and characterized. Several of the monoclonal antibodies have been described and demonstrate strong reactions against both purified K88 pili and K88-piliated ETEC in the enzyme-linked immunosorbent assay (ELISA). These monoclonal antibodies are valuable reagents for the diagnosis of ETEC induced diarrheal diseases. They also represent a valuable key to the elucidation of the actual mechanism by which these enteropathogens actually interact with their host intestinal epithelial cells, as well as a potential therapeutic agent in the inhibition of such interactions.

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K88