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IMMUNOCHEMICAL AND ADHERENCE STUDIES ON EDP 208 AND CFA/I PILI

University — Université

ALBERTA

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

Ph.D. BIOCHEMISTRY

Year this degree conferred — Année d'obtention de ce grade

1985

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Immunochemical and Adherence Studies on EDP208 and CFA/1 Pili

by

Elizabeth A. Worobec

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Doctor of Philosophy

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

FALL 1985

THE UNIVERSITY OF ALBERTA

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DEGREE: Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Immunochemical and Adherence Studies on EDP208 and CFA/1 Pili submitted by Elizabeth A. Worobec in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The primary product after trypsin digestion of the EDP208 pilin monomer was the N-terminal dodecapeptide (N-acetyl-Thr-Asp-Leu-Leu-Ala-Gly-Gly-Lys-Asp-Val-Asp-Lys). This peptide was chemically synthesized, coupled to bovine serum albumin (BSA) using a photoreactive crosslinking agent, and used in immunoblot and enzyme linked immunosorbant assays (ELISA), to show that it was capable of interacting with anti-EDP208 pilus specific antibodies. This peptide was also shown to be immunogenic since the synthetic peptide-BSA conjugate was capable of producing antibodies in rabbits and these antibodies were found to interact with native EDP208 pili.

In order to examine the residues responsible for the antigenicity of the EDP208 N-terminal dodecapeptide, a series of synthetic peptide analogs were produced. The relative affinities of these peptides for EDP208 pilus specific antibodies was determined by a competitive ELISA using the Fab fragment of anti-EDP208 pilus immunoglobulin G (IgG). From these experiments it was established that the antigenic region resides in the N-terminal pentapeptide and the key residues responsible for the antibody-antigen interaction are the N-acetyl-Thr^1, Leu^3, and Leu^4.

Affinity chromatography of anti-EDP208 pilus IgG on a column having the N-terminal dodecapeptide-BSA conjugate as

the ligand, resulted in the separation of two distinct species of antibodies. One population was specific for the N-terminal dodecapeptide while the second population did not interact with the dodecapeptide but was capable of interacting with intact EDP208 pili. These results suggested that a second antigenic determinant exists in the remainder of the EDP208 pilus protein.

Monoclonal antibodies were produced against colonization factor antigen 1 (CFA/1) pili. Four monoclones were selected for further study. As determined by immunoblot and ELISA assays, three of the four monoclones were specific for only CFA/1 pili, whereas the remaining clone produced antibodies capable of interacting with CFA/1 and K99 pili. The latter type of pili are E.coli adhesins specific for bovine and ovine intestine. K99 pilus specific antibodies were able to inhibit the hemagglutination ability of E.coli possessing CFA/1 pili, as well as interact with CFA/1 pili in a competition ELISA. These results indicated that a common element may exist between these two pili types.

To further examine the specifity of CFA/1 pilus monoclonal and polyclonal antibodies, immunoblot and competition ELISA assays were performed using native CFA/1 pilin peptides. Only the polyclonal antibodies were capable of interacting with any of the fragments tested and these antibodies were found to bind a CNBr fragment encompassing residues 47-78. Several synthetic peptides, representing

different regions of this fragment were produced and tested in competition ELISAs using polyclonal and monoclonal antibodies. No interactions were detected between any synthetic peptide and any of the antibodies. Thus, it was concluded that the major epitope in the cyanogen bromide fragment may be conformational in nature, and not represented by the linear synthetic peptides.

CFA/1 pili were also studied in connection with their role in adherence to mammalian tissues. The ability of CFA/1 P^+ or CFA/1 P^- E.coli H-10407 to adhere to a variety of tissue culture cell lines, human and animal intestinal cells and human erythrocytes was examined by electron microscopy and viable adherent bacteria assays. Little or no adherance was detected of either P^+ or P^- bacteria to tissue culture or isolated intestinal cells. However, CFA/1 pilus mediated adherence was observed to human erythrocytes. This adherence was inhibited by increasing concentrations of CFA/1 pili, as well as by the pre-treatment of the erythrocytes with trypsin and neuraminidase. This suggested that the putative receptor for CFA/1 pili on human erythrocytes may be a glycoprotein possessing a key N-acetylneuraminic acid moiety.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Bill Paranchych for his guidance and support during my stay in his laboratory. Next I must thank my co-workers who have provided me with friendship and scientific stimulation. These include Drs. Laura Frost and Tania Watts, along with Brett Finlay, Kathy Volpel, Britt Pasloske and Dr. Sastry Parimi.

A special thanks is extended to the following people without whom this research could not have been possible: Dr. Bob Hodges and his people who provided me with synthetic peptides and peptide conjugates as well as guided me in my attempt at peptide synthesis; Drs. Bhaghrith Singh and Padma Shastry, along with Wally Smart, from the Department of Immunology, who provided guidance and assistance during the production of monoclonal antibodies; Dr. Glen Armstrong and his technical staff (Peter Pieroni and Elizabeth Fodor), from the Department of Medical Microbiology, for collaboration in the bacterial adherence project; Dr. Doug Scraba and Roger Bradley, in the Department of Biochemistry, and Dr. R. Whitehouse and Richard Sherburne, in the Department of Medical Microbiology, for their expertise in electron microscopy; Dr. Mark Peppler, from the Department of Medical Microbiology, who supervized me during my production of bacterial LPS; Dr. L. Smillie for the use of

his facilities and Mike Natriss for amino acid analyses.

Finally, I would like express my sincere thanks to my family, especially my mother Anne Worobec, who always wanted me to succeed, and David Huebert for continuous support and friendship during my years as a graduate student.

This work was supported by the Alberta Heritage Foundation for Medical Research in the form of a studentship and a research allowance.

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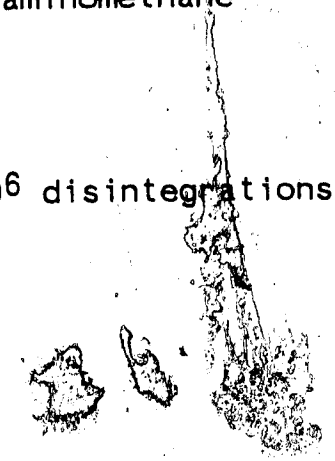
ABBREVIATIONS

A	absorbance
AB-ONSu	the N-hydroxysuccinimide ester of 4-azido-benzoic acid
Ac	Nα-acetyl
Boc	t-butyloxycarbonyl
BSA	bovine serum albumin
BSS	basic salt supplement
Bzl	benzyl
CFA	colonization factor antigen
cfu	colony forming units
2C1Z	2-chlorobenzyloxycarbonyl
CNBr	cyanogen bromide
CPase	carboxypeptidase
cpm	counts per minute
DCC	dicyclohexylcarbodiimide
DFP	diisopropyl fluorophosphate
DIEA	diisopropyl ethylamine
DMSO	dimethylsulfoxide
D_2O	deuterium water
DTT	dithiothreitol
EDTA	disodium(ethylenedinitrilo)tetraacetate
ELISA	enzyme linked immunosorbant assay
ER	C-terminal fragment released after tryptic digestion of EDP208 pilin

Fab	antigen binding fragment
Fc	crystallizable fragment
FCS	fetal calf serum
Folac	an Inc Folac conjugative plasmid carrying the *E. coli lac* operon
For	N$^{\alpha}$-formyl
HAT	hypoxanthine aminopterin thymidine
^{1}HNMR	proton magnetic resonance
HPLC	high pressure liquid chromatography
IgG	immunoglobulin G
IgM	immunoglobulin M
LPS	lipopolysaccharide
mA	milliAmp
mCi	milliCurie, 2.2×10^{9} disintegrations per minute
MEM	minimal essential medium
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PMSF	phenylmethyl sulfonyl flouride
POPOP	p-Bis[2-(5-phenyloxazoyl)]-benzene
PPO	2,5 diphenyloxazole
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
TCA	trichloroacetic acid

TEMED	N,N,N′,N′-tetramethylenediamine
TFA	triflouroacetic acid
TLC	thin layer chromatography
TPCK	L-1-tosylamido-2-phenylethyl(chloromethyl ketone)
tris	tris(hydroxymethyl) aminomethane
TSA	tryptic soy agar
TSB	tryptic soy broth
µCi	microCurie, 2.2 x 10^6 disintegrations per minute
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight



CHAPTER I

INTRODUCTION

A protein antigenic determinant, or epitope, is a term used to describe a region of a protein which is recognized by antibody molecules and/or T cells in the immune response to the whole protein (Atassi, 1984). This response can also be elicited by either a peptide or a peptide conjugate on a larger carrier protein, such as bovine serum albumin. In general, peptide conjugates elicit a better immune response. Anti-peptide antibodies can be generated to any region of a protein and these antibodies can in turn recognize the intact protein, even if the region is not part of an antigenic site (Atassi and Habeeb, 1977).

The study presented in this communication describes the attempt to delineate the antigenic structure of the bacterial pilin protein generated by two distinct strains of Escherichia coli. The schemes used to achieve this goal are similar to those which will be discussed in this section. A brief review of what has been done to date in the elucidation of the antigenic structure of proteins will be presented, followed by background information on the two proteins used in this particular study.

A. Antigenic Structure of Proteins

The ability to elicit an immune response by a protein is termed immunogenicity, with the protein as the immunogen. This is a complex response in which contributions are made by B cells (the antibody secreting cells), T helper and suppressor cells as well as the idiotypic network and major histocompatibilty complex (Atassi, 1984). A protein, or a fragment thereof, is called antigenic if it is recognized by the product of an immune response, such as the antibodies. Chemically synthesized or native peptides have been shown to elicit antibodies which interact with the native protein (Lerner, 1982). However, one must not confuse these regions to those which are immunogenic when the whole protein is used as the antigen.

Two distinct classes of antigenic determinants have been identified. One type can be made up of surface residues of a protein which come into close proximity with each other due to the folding of the polypeptide chain. These residues are not normally found as a string of adjacent amino acids. This type was once called conformational but since both types are sensitive to conformational changes this type has been more correctly called discontinuous (Atassi, 1978). The other form usually is comprised of a continuous region of the polypeptide chain and is often called a sequential determinant. It is generally considered

that all antigenic determinants are topographical in that they are structures found on the surface of the protein. They may be contained in one segment of the protein but not necessarily involve all the amino acid residues in the segment. Certain regions of the surface of the protein may be called immunodominant since it is to this region that the majority of the protein specific antibodies are produced during an immune response. This could be due to special structural features of this region or to the regulatory mechanism of the immune system (Benjamin et al., 1984).

Very few proteins have been thoroughly examined with regard to their antigenic structure. The best known examples include myoglobin, lysozyme, cytochrome c, serum albumin and hemoglobin. The two classical examples are myoglobin and lysozyme. These two proteins are discussed briefly in what follows.

1. Sperm Whale Myoglobin

Sperm whale myoglobin is a protein made up of 153 amino acids (Edmundson, 1965). The amino acid sequence is known as well as the three dimensional X-ray crystal structure (Takano, 1977). Atassi (1975) described the complete antigenic structure of this protein, which consisted of five different sites. Each of these sites, which include residues 15-22, 56-62, 94-99, 113-119, and 145-151, consists of consecutive amino acids located at bends or expos-

ed corners of the native protein. This is an example of a protein whose antigenic determinants are all sequential or continuous in nature. Atassi also reported (Twining et al., 1981) that 99.8% of the antibodies in various anti-myoglobin antisera could be removed by binding sequentially to these five sites. He also suggested that these sites are the only antigenic determinants on the protein as well as on myoglobins from other mammalian species. This was the first protein to be completely examined for antigenic determinants and provided the basis for the study of other proteins.

Although Atassi's study of the immunochemistry of myoglobin is historically important, results from other researchers have provided a different concept of the antigenicity of this protein. For example, Berzofsky et al. (1980) found that several monoclonal antibodies to sperm whale myoglobin were specific for topographic epitopes and did not interact with the five sequencial determinants described by Atassi (1972). This and other examples are documented by Benjamin et al. (1984) in their review of the antigenic structure of proteins.

For the study of myoglobin, Atassi developed what may be known as the classical approach to delineating antigenic sites of a protein (Atassi, 1972). He used five general procedures to elucidate the following parameters: a) effect of conformational changes on the immunochemistry of the

protein; b) the effect of specific chemical modifications of amino acids in the protein, e.g. citraconylation of lysine residues; c) isolation of a large number of overlapping peptides and examination of their ability to interact with anti-protein antibodies; d) the effect of specific chemical modifications on reactive peptides and e) verification of the other approaches using chemically synthesized peptides in the assays established with the native protein and peptides. He stressed that none of the approaches alone is sufficient for providing accurate information but results from one approach must be confirmed by at least one of the others.

2. Lysozyme

The antigenic structure of lysozyme was deduced using the forementioned strategies. Atassi et al. (1976) found that this protein had three antigenic sites, each consisting of sequentially distant but spacially adjacent residues brought into close proximity by the folding of the polypeptide chain. It represents a protein whose antigenic structure is discontinuous in nature. The precise delineation of these sites was accomplished by using a novel approach to those previously described. This approach is called surface-simulation synthesis. They chemically synthesized these determinants such that the spacially adjacent surface residues were directly linked via a peptide bond with the appropriate spacing between residues acheived by

the insertion of glycine residues. For example, the first site was constructed of the residues Arg-125, Arg-5, Glu-7, Arg-14 and Lys-13. The length of the region was calculated to be 3 nm and the site was satisfied by the synthetic peptide Arg-Gly-Gly-Arg-Gly-Glu-Gly-Gly-Arg-Lys. This peptide does not exist in the native lysozyme molecule but adequately represents the arrangement of residues on the surface of the protein and efficiently interacts with lysozyme specific antibodies.

However, as with myoglobin, recent studies on the antigenicity of lysozyme have revealed that epitopes other than the ones described by Atassi exist. One site in particular is contained in the disulfide loop (residues 64-80). Several groups have delineated this antigenic site (Arnon (1977); Smith-Gill et al., 1982) which was not found to be antigenic by Atassi et al. (1976).

On the basis of these and other studies, Atassi proposed several general conclusions relating to antigenic determinants of proteins (1984). Antigenic sites are usually small (5-10 key residues) and possess discrete boundaries. They are usually associated with the surface of the protein but one must be aware that not all exposed regions of a protein necessarily constitute antigenic sites. They are present in small numbers and most important, they are very sensitive to conformational changes as well as to changes in the environment of the region (e.g. amino acid substitu-

tion directly in the site or in areas influencing the conformation of the site).

Another feature of importance is the heterogeneity of the response to an antigenic site of a protein. Atassi (1984) described two theories for the basis of this heterogeneity. First, he suggested that a variety of antibodies exist which have identical specifities for a specific antigen, that is they interact with the same antigen but have different affinities or binding energies for the antigen. Secondly, in his study of myoglobin and lysozyme (Atassi, 1972), he found that a part of the antigenic site can bind some of the antibodies to the whole site. He postulated this could be due to the production of antibodies against various parts of the site. Thus, one may not have a population of antibodies possesing different specificities but rather antibodies with a difference in binding affinities for the same antigen.

Recently, new approaches to the analysis of antigenic determinants of proteins have been introduced. The first of these new approaches involves the ability to predict the location of antigenic regions just by knowing the amino acid sequence of the protein. Hopp and Woods (1981) based their prediction rules on the surmise that antigenic determinants are surface features which are frequently found in regions which are exposed to solvent. These rules also rely on the presence of charged, hydrophilic amino acids in or

near an antigenic region. Each amino acid in the sequence of the protein is assigned a hydrophilicity value and these values are repetitively averaged along the length of the polypeptide chain. According to the results presented by this group, a high point of hydrophilicity invariably lies thin or immeadiatly adjacent to an antigenic determinant. However, several cases exist where the antigenic sites of proteins are hydrophobic in nature. For example, Atassi (1984) found in the case of myoglobin that only two of the five antigenic sites represent hydrophilic maxima according to the Hopp and Woods criteria. Also, several hydrophilic regions of this protein are not antigenic. This reinforces the idea that although all antigenic sites are exposed to the surface of a protein, not all exposed regions are antigenic. In other cases, hydrophobic interactions quite often provide a major contribution to the antigen-antibody complex. For one, the immunodominant region of influenza virus hemagglutinin is entirely hydrophophobic (Atassi and Webster, 1983). It is possible that hydrophobic regions become exposed because of the three dimensional folding of the protein. Since the prediction rules do not take secondary structure into consideration, one must be aware of this limitation when using such rules. These rules, however, could be very useful in cases where one is unable to use classical immunochemistry. For example, these rules are often used for viral proteins which are extremely difficult

to purify in the quantities necessary to perform a thorough examination. They could also be used in cases where the DNA sequence is known of a protein whose function or location is not well characterized (e.g. foot and mouth virus, Pfaff et al., 1982; Epstein-Barr virus, Dillner et al., 1984).

Another approach to predicting the antigenic structure of proteins deals with the mobility of certain segments of the protein. Westhof et al. (1984) were the first to introduce this idea. They analyzed lysozyme, myoglobin, and tobacco mosaic virus protein (TMVP), whose X-ray crystalographic structures were well enough defined to determine atomic temperature factors for each amino acid. The calculated temperature factors, when plotted versus each residue, provides a graphic image of the degree of moblity existing along the polypeptide chain. These plots were used to examine the correlation between mobile segments of the protein and the location of their antigenic determinants. They found that their predictions were very accurate for myoglobin and TMVP which both have sequential antigenic determinants. The authors stated that their system is only useful for proteins with the sequential type of determinant but that it will probably be this type of epitope which will be selected for synthetic vaccines.

Tainer et al. (1984) also considered the importance of mobility for antigenic determinants of proteins. The protein they chose to study was myohemarythrin, whose crys-

tal structure is also well defined. A plot of temperature factors versus amino acid sequence produced regions of high and low mobilty. From this plot they selected several peptides for synthesis and subsequent antibody production. All antibodies raised against rigid peptides interacted only weakly or not at all with the native protein while those raised against mobile peptides reacted very well. These results thus suggested that mobilty can be correlated with antigenicity. Again, the polypeptides of choice contained sequential determinants. This method of prediction, like the others is limited in its usefulness for identifying discontinuous epitopes and has the disadvantage that it relies too heavily on information obtained from the three dimensional structure of the protein. Since the X-ray crystallographic structure of only a limited number of proteins has been established to the resolution necessary for this method, this correlation can not be applied to the majority of proteins whose antigenic structures are of importance.

The most reliable method for the delineation of antigenic determinants of proteins is probably that described by Atassi, primarily the use of fragments obtained from proteolytic or chemical cleavage of the intact protein. The prediction rules of Hopp and Woods appear equally useful, especially in cases where insufficient amounts of protein are available for classical immunochemistry, provided one is aware of the limitations regarding discontinuous and hy-

drophobic epitopes. The study of the contribution of mobility to antigenicity is only useful when the X-ray crystal structure of the protein in question is known.

B. Synthetic Peptide Vaccines

The elucidation of antigenic and immunogenic regions of viral and bacterial proteins is desirable if synthetic vaccines for these diseases are to be constructed. The use of synthetic peptides conjugated to carrier proteins is rapidly becoming a feasible agent for vaccination. The usual method of vaccination has involved the use of killed or live but attenuated (inactivated) organisms, purified viral proteins or inactivated bacterial toxins. However, problems have arisen with these vaccination schemes, predominantly due to the limited source of viral material and the continuous evolution of strains with serological differences. The synthetic approach offers a solution to these problems in that only the relevant antigenic moiety necessary for protective immunity is used (Arnon et al., 1983).

Bacterial synthetic peptide vaccines are still quite new as the use of inactivated toxins has been quite successful (e.g. cholera toxin B subunit vaccines, Holmgren, 1981). However, a few good examples do exist for peptide vaccines. For one, Audibert et al. (1981) have shown that active immunization against diptheria can be achieved using

a tetradecapeptide representing residues 188-201 of the diptheria toxin linked to a carrier protein. This peptide conjugate can elicit antibodies in guinea pigs which in turn attach to the toxin and neutralize its lethal effect. Another good example is found with the M protein of Streptococcus pyogenes. This surface protein confers resistance of these bacteria to phagocytosis by mammalian macrophages. Beachey et al. (1981) synthesized a 35 residue peptide which, when injected into rabbits engendered both a cellular and a humoral immune response to the intact protein. Passive immunization of mice with the resultant rabbit sera provided protection against a challenge with type 24 Streptococci. This appears to be an important step in the develoopement of a vaccine for acute rheumatic fever, glomerulonephritis and rheumatic heart disease which are all caused by this organism.

As for viral synthetic vaccines, the recent literature has shown an increase in the studies in this area. A 1983 review by Arnon et al. describes several examples where synthetic peptides from corresponding capsid and envelope proteins have been used to elicit antibodies capable of neutralizing foot and mouth, hepatitis B and influenza viruses. In the majority of the studies the peptides of choice were predicted from the amino acid sequence projected from the nucleotide sequence, since important viral proteins are often difficult to purify or isolate. The pre-

determined synthetic peptides were usually attached to carrier proteins and the resultant conjugate used as the immunogen. The resulting antibodies were assayed for the ability to neutralize the intact virus. Successful experiments have been noted by many groups (e.gs. feline leukemia virus, Nunberg et al., 1984; Epstein-Barr virus, Luka et al., 1983).

A significant problem with many vaccines involves the increasing appearance of antigenic variation from strain to strain. Strain specific regions must be avoided. Rowlands et al. (1983) found that several distinct serotypes of foot and mouth virus existed and antibodies elicited against the major immunodominant region of one poorly neutralized a different serotype. Studies are continuing for both viral and bacterial systems to identify a common element capable of neutralizing a broad range of strains. Hancock et al. (personal communication), for example, have raised monoclonal antibodies directed against the Pseudomonas aeruginosa outer membrane protein F which interacts with a variety of P. aeruginosa strains. These antibodies also conferred passive immunity to mice challenged with different strains of these bacteria. Thus, there is a high probability that a broad spectrum vaccine can be developed, with synthetic vaccines providing the basis of disease prevention in the future.

C. Antigenic Determinants and Vaccines of Bacterial Pili

Bacterial pili are thin, nonflagellar filamentous structures located on the surface of a variety of bacteria. They were first identified by Houwink (1949) and Anderson (1949) as thin filaments and later termed fimbria (Latin for thread or fibre; Duguid et al., 1955) and pili (Latin for hair-like, Brinton, 1965). For convention, the term pili will be used for several filaments, pilus for one filament and pilin for the protein subunit making up the filament. One pilus consists of approximately 100 identical pilin subunits arranged in a helical array (Brinton, 1965). Most pili can be placed into one of two groups. The first group includes conjugative pili. They are encoded by self-transmissible plasmids (Achtman et al., 1971). These pili are involved in bacteria-bacteria interactions during the mating process where the donor, or piliated bacterium, attaches to the recipient bacterium prior to transfer of genetic material (Helmuth and Achtmann, 1978). The second group include the colonization pili or adhesins. These structures are often found on pathogenic bacteria and enable these organisms to adhere to specific tissues of the host. This adherance property allows the bacteria to colonize the susceptible tissue, after which toxins or other pathogenic mechanisms lead to the disease process. These types of pili are often called colonization factors.

The characterization of antigenic and immunogenic determinants of pili from either one of the forementioned groups would provide useful information regarding the pilus structure and function. Generally, antigenic determinants are located on the surface of the protein (Atassi, 1984). For conjugative pili, these regions may be involved in the donor-recipient cell interaction or in bacteriophage-pilus attachment. In the case of colonization pili, immunodominant peptides could provide the basis for synthetic vaccines as well as provide information on the region of the pilus involved in the adhesion to mammalian tissues. To date, only a few pilus proteins have been examined with regards to antigenic determinants. These will be discussed along with the current state of knowledge regarding pilus vaccines.

1. Conjugative pili

The major antigenic determinants of three serologically distinct conjugative pili have been elucidated. All three pilus proteins contain N terminal acetylated amino acids and in all three instances these residues are major contributors to the antigenicity of the protein. In the EDP208 pilus system, the N-acetyl group and the first five amino acids contain the major epitope. The important contact points in this antigen-antibody interaction are the N-acetyl Thr^1, Leu^3 and Leu^4. This site was delineated using synthetic peptide analogs of the N-terminal reg-

ion in an immunological competition assay. The N-terminal octa- and dodecapeptides were also found to be highly immunogenic (Worobec et al., 1983 & 1985).

The F and ColB2 pilus proteins have been sequenced by DNA technology (Frost et al., 1984; Finlay et al., 1984) and were found to be identical except for minor changes in the N-terminus. Finlay et al. (1985) concluded that these N-terminal regions also harbour the major antigenic sites of these pilus proteins. They found that synthetic and native peptides comprizing the first 8 amino acids of the F protein and the first 6 amino acids of the ColB2 protein are capable of interacting with 60-80% of the homologous pilus specific antibodies, as detected by the same competition assay used for the EDP208 system (Worobec et al., 1985). However, these peptides did not interact with heterologous antisera and can be classified as type specific determinants.

There appears to be a pattern developing with the antigenic determinants of conjugative pili. The major epitopes are all located in the N-termini of the pilus proteins and all involve the blocked N-terminal amino acid. This region may be involved in the cell-cell interaction during the mating process. All three conjugative pilus types are also capable of attaching bacteriophages. They efficiently bind the DNA filamentous phage, f1, and each have specific phage which attaches only to one pilus type

(Willets and Skurray, 1980). The N-terminal region of the different pilus proteins may be involved in the type specific phage binding, while some part of the remainder of the protein could be important for the interaction with f1 phage. Studies into the different phage binding patterns of these and other conjugative pili is presently underway (Frost et al., personal communication).

2. Colonization Pili

Rigorous characterization of antigenic determinants in adhesive pili has been performed in very few instances, although many theorectical predictions have been reported. The two pilus proteins subjected to a thorough immunochemical characterization are those produced by Neisseria gonorrhoeae (Rothbard et al., 1984 and 1985) and Pseudomonas aeruginosa K (Sastry et al., 1985). Rothbard et al. (1984) produced native and synthetic peptides of gonoccocal pilin from N. gonorrhoeae strains MSII and R10. They found that antisera raised against the homologous pilus filaments and native peptides bound synthetic peptide which represented a 30 amino acid disulfide loop near the carboxy terminus of the protein. However, heterologous sera did not interact with these peptides, suggesting that each of the peptides contained strain specific epitopes. Peptides corresponding to homologous sequences in both pilus proteins were found to be only weakly immunogenic. The poor immuno-

genicity of these regions, explains at least in part, why pili from a single gonococcal strain are not suitable for use as a vaccine. They did find a region which was capable of eliciting antibodies which interacted with the pilus protein when prepared as a synthetic peptide-conjugate. This same region in the native protein was not immunogenic. Antibodies produced against this particular peptide were capable of interacting with pili from both strains. More recently, this group (1985), using synthetic peptide conjugates of gonococcal pilin, found that antisera directed against the residues 69-84 were the most efficient in binding pili from all stains tested. Sera against this peptide also inhibited a heterologous gonococcal strain from adhering to human target cells. Thus it is this type of cross reactive peptide which could be considered as a candidate for an effective vaccine against gonorrhea.

Sastry et al. (1985) determined that the major antigenic site of the P. aeruginosa K (PAK) pilus protein re-sides in the central portion of the protein (encompassing residues 82-110). This epitope apears to be conformational in nature. Watts et al. (1983) originally identified four regions of this protein which interacted with PAK pilus specific antisera. Sastry et al., using a more sensitive assay system, deduced which of these regions is the most immunogenic. More recently, Sastry et al. (1985) determined the amino acid sequence of the pilus pro-

tein produced by P. aeruginosa O (PAO). This protein and that from PAK are highly homologous for the first 54 residues, but there is no homology in the region encompassing residues 55-96, and there is limited homology in the remainder of the sequence. The major antigenic site of the PAK pilus protein resides in the heterologous, or strain specific region. The mapping of the antigenic sites of the PAO pilin protein is underway. By analogy to the gonococcal system, the immunodominant region in Pseudomonas pili will probably be found in the strain specific region of the protein. Thus, this system will also require the selection of a peptide which is common to all Pseudomonas strains but exposed on the surface of the intact pilus.

No definitive immunochemical studies have been performed on other colonization pilus types. The amino acid sequence of the K88 pilin subunit has been determined for three serological variants which include K88ab (Gaastra et al., 1981), K88ac (Josephsen et al., 1984) and K88ad (Gaastra et al., 1983). There are from 20 to 30 differences in the sequences of these proteins, all of which appear to occur in clusters. A hypervariable region was found between residues 162 and 175 (the proteins are 264 amino acids in length). Hopp and Woods (1981) prediction rules indicate that this region does not coincide with a region of high antigenic potential but the Gaastra group speculated that this strain specific area should contain a major epitope

(Josephsen et al., 1984). Antigenic studies are underway. One should note that promising results have been obtained in the prevention of neonatal diarrhea in pigs with the use of purified K88 pili as a vaccine (Nagy et al., 1978). Pregnant sows were inoculated with purified K88 which resulted in the passive protection of those newborn piglets who suckled on the immunized mothers. The immunity was mediated by anti-pilus antibodies in maternal colostrum. Unless antigenic variation from strain to strain becomes a problem, it appears that synthetic peptide vaccines may not be necessary. However, the elucidation of antigenic determinants on these pilus proteins is still useful in elucidating the structure of the pilus and the mode of attachment of these filaments to porcine intestinal epithelial mucosa.

The amino acid sequence has also been determined for the K99 pilin protein (Roosendaal et al., 1984). This group noted that many sequential and genetic similarities exist between this pilus and the pyelonephritis associated pilus (PAP) (de Graaf et al., 1984; Normark et al., 1983). The only functional similarity is that they both mediate attachment to epithel cells, PAP to uroepithelial cells of humans and K99 to calf and lamb intestinal cells. The hydrophilicity profile of the K99 protein indicates that five areas are possible candidates for antigenic sites. Roosen-

daal et al. mentioned that the antigenic properties of a synthetic peptide from one of these regions is under investigation. As with the K88 pilus, successful pilus vaccines have been developed. Morgan et al. (1978) vaccinated pregnant dams with purified K99 or 987P (another colonization pilus type involved in porcine neonatal diarrhea) pili. This vaccination conferred protection to neonatal suckling pigs against diarrhea caused by enterotoxigenic E. coli bearing the homologous type of pili. No strain associated variations have been identified with these two pilus proteins; thus it may not be necessary to pursue the search for antigenic determinants on these proteins solely for the intent of synthetic peptide vaccines. However, as with all other pilus types discussed, the antigenic properties of pili can provide valuable information regarding pilus structure and function.

The antigenic structures of colonization factors 1 and 2 (CFA/1 and CFA/2), likewise have not been examined. Klemm and Mikkelsen (1982) used the Hopp and Woods hydrophilicity rules to predict the location of five major candidates for antigenic sites located throughout the CFA/1 pilus protein but no further studies on the nature of these sites have been reported. However, several groups have shown, using animal models, that the administration of these colonization factors confers protection to challenge with the appropriate strains of enterotoxigenic E. coli

(CFA/1, de la Cabada et al., 1981; CFA/2, Boedeker et al., 1982). These two pilus types are found on bacteria causing diarrhea in humans. Whether or not synthetic vaccines will be necessary for human consumption will depend on the outcome of further trials.

Levine et al. (1983) have discussed the advantages of developing a polyvalent pilus vaccine for immunization against human diarrheal disease. They suggested that along with CFA/1 and CFA/2 pili one should include type 1 (mannose sensitive) pili. These types of pili are found on the majority of enterotoxigenic E. coli strains, and are involved in attachment of E. coli to intestinal mucosa (Issacson et al., 1978). However, Levine et al. (1982) found in human volunteer studies that no consistant evidence of protection with these pili was observed. Salit et al. (1983) isolated and characterized type 1 pili from several clinical sources and found that there was considerable homology in the N-terminal amino acid sequence of these pilus proteins. However strain specific regions may exist in these pilus proteins. This may be why Levine found no conclusive protection with type 1 pili in his immunization studies. If such is the case, the elucidation of the antigenic determinants of several of these isolates would confirm the suspicion of variation and provide further information for the production of a broad spectrum synthetic vaccine.

The antigenic structure of the majority of pilus pro-

teins has not yet been established. Since pili vaccines appear to be relatively efficacious, the need for synthetic peptide vaccines may not be necessary although the production of synthetic peptides may be more economically feasible when large amounts of the immunogen are difficult to obtain.

C. Nature of Pili used in this Study

Although many pilus types exist, only two were selected for immunochemical examination in this study. Two physiologically distinct pilus systems were chosen. One was the conjugative pilus EDP208 and the other was CFA/1, a representative from the colonization group. The aim was to identify and characterize the major antigenic determinant of each of these pilus proteins and in doing so gain some insight into their structural and functional characteristics.

1. EDP208 Conjugative Pili

Conjugative pili are encoded by self-transmissible plasmids and are necessary for transfer of genetic material from the donor to the recipient bacterium (Achtmann *et al.*, 1971). The EDP208 pilus is encoded by a derepressed derivative of the naturally occurring *lac* plasmid, Fo*lac* (incompatibility group Fo*lac*), which was originally isolated from *Salmonella typhi* (Falkow and Baron, 1962). This derepressed

EDP208 plasmid was shown by Bradley and Meynell (1978) to produce pili which are identical to those encoded by the original S.typhi strain. Since the EDP208 strain is multi-piliated, large amounts of purified pili are readily obtained from it. EDP208 pili are serologically unrelated to the best characterized conjugative pili, F pili, and are resistant to the F-specific RNA phage. However, both pilus types can attach the filamentous DNA phage, f1 (Datta, 1975) suggesting possible structural similarities between the two pili. These two types of pili are also similar on the basis of electron microscope and X-ray fibre diffraction studies (Folkhard et al., 1979). According to electron microscopy, both pili have a diameter of 8.5 nm and are up to 20 μM in length. X-ray fibre diffraction studies indicated that both are hollow cylinders with an outer diameter of 8 nm and inner diameter of 2 nm plus each consists of subunits in four coaxial helices of pitch 12.8 nm.

The EDP208 pilin subunit has an apparent molecular weight of 11,500 daltons (Armstrong et al., 1980) and is co-purified with tightly associated carbohydrate and phosphate. Armstrong et al. (1981), were able to remove all of the carbohydrate and phosphate from this pilus. Armstrong et al. (1980), noted that the overall amino acid compositions of F and EDP208 pilin were very similar. Recently, Frost et al. (1984) published the DNA sequence of the F pilin gene which represented a protein consisting of 70

amino acids, with a corresponding molecular weight of 7200 daltons. The N-terminal sequence of the EDP208 pilin protein was also deduced by Frost et al., (1983) and bears no resemblance to that of the F protein except for the N-acetyl group on the N-terminal amino acid, which is common to both.

Finlay et al. (1983) characterized the EDP208 plasmid and found that a 16.5 kilobase region was required for pilus production. The subcloning and DNA sequencing of the EDP208 pilin gene is underway (Finlay, unpublished) and the comparison of the resultant sequence with that obtained for F will be important to substantiate the amino acid homologies presented by Armstrong et al. (1980). Armstrong also found distinct structural similaries between the two. Circular dichroism revealed that both have an alpha helical content of 65-70%.

Tryptic digestion of EDP208 pilin resulted in the release of predominantly the N-terminal dodecapeptide (Frost et al., 1983). This dodecapeptide was chemically synthesized, covalently coupled to bovine serum albumin using a photoreactive crosslinking reagant and used as both an antigen and an immunogen. Worobec et al. (1983) found that this dodecapeptide contained a major antigenic determinant which was immunogenic as well as antigenic. This site was further delineated with the use of synthetic peptide analogs and the immunodominant region was found to reside in

the first five amino acids. The key contact points with pilus specific antibodies were the N-acetyl Thr^1 and the leucine residues in the third and fourth positions (Worobec et al., 1985). The role of this region in the function of the pilus has yet to be established.

2. Colonization Factor Antigen 1 (CFA/1) Pili

CFA/1 pili were first identified by Evans et al. (1975) as an antigen on the surface of enterotoxigenic E. coli strain H-10407 (078:H11) originally isolated from a human subject in Bangladesh. These pili were found to be necessary for the adherance and subsequent colonization of the human small intestine (Evans et al., 1977 and 1978). Once colonized, these bacteria secrete toxins responsible for inducing the excretion of water and salts from the intestinal mucosa, resulting in diarrhea. CFA/1 pili have been detected on a variety of ETEC strains isolated from patients experiencing acute diarrhea from various developing countries such as Thailand (Changchawalet et al., 1984), South Africa and Ethiopia (McConnell et al., 1981) and Mexico (Evans et al., 1978).

The gene encoding the structural protein of CFA/1 pili has been located on a 60 megadalton nonconjugative plasmid which also encodes the heat stable (ST) toxin and is mobilized by a second smaller plasmid encoding the heat labile (LT) toxin (Elwell and Shipely, 1980). The expres-

sion of CFA/1 pili is temperature dependent in that pilus production is stopped when the bacteria are grown at 18°C as opposed to 37°C (Klemm, 1982). Electron microscopy reveals the CFA/1 pilus as a rigid, thread-like structure which is 0.5-1.0 μM in length and 7 nm in diameter containing an apparent axial hole (Gaastra and de Graaf, 1982). A single CFA/1 pilus consists of approximately 100 identical pilin subunits, each with a molecular weight of 15,058 daltons. The complete amino acid sequence has been established by Klemm (1982).

Evans et al. (1977) correlated the presence of CFA/1 pili on H-10407 with the ability of these bacteria to agglutinate human type A erythrocytes in the presence of mannose. Faris et al. (1980) demonstrated that the glycoconjugate GM_2 was capable of inhibiting this hemagglutination reaction and proposed that this moiety may represent a component of the CFA/1 specific receptor on human erythrocytes. The nature of the CFA/1 pilus specific receptor on human intestinal cells has not been examined.

The antigenic characteristics of CFA/1 pili are of interest in relation to the development of synthetic vaccines as well as a means of describing the mode of attachment of these structures to human intestinal mucosa and erythrocytes. The CFA/1 pilus protein was examined for antigenic determinants using native and synthetic peptides in several immunoassays with CFA/1 pilus specific polyclon-

al and monoclonal antibodies. These studies, as well as those descibing the role of CFA/1 pili in the adherence of H-10407 bacteria to human erythrocytes and a variety of mammalian tissues, are presented in this treatise.

The study of the two forementioned pili involved the development of relevant immunochemical assays which have been applied to the elucidation of antigenic determinants on other pilus proteins (ColB2 and F pili, Finlay et al., 1985; PAK pili, Sastry et al., 1985). Useful information was obtained regarding the structure of both pilus types as well as their respective roles as mediators of adherance: EDP208 during the mating process and CFA/1 in pathogenic adhesion. The results from these studies are presented in what follows.

CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Bacteria

Enterotoxigenic Escherichia coli, strain H10407 (078:H11), containing the CFA/1 plasmid was used for CFA/1 pilus production and attachment studies. This was an original isolate from a subject in Bangladesh which was kindly donated by Margaret Finlayson, Provincial Laboratories, Edmonton, Alberta. Other bacteria used in attachment studies were E. coli H10407 CFA/1$^-$ (078:K80:H11), a non-piliated, non-pathogenic strain (obtained from Francis Jackson, University of Alberta, Edmonton, Alberta), and a non-piliated strain isolated from the E. coli H10407 wild type (obtained from R. Silver, Food and Drug Administration, U.S.A).

EDP208 conjugative pili were purified from the E. coli strain ED3873, which consisted of the EDP208 conjugative plasmid in the host strain JC6256 (K12 F$^-$ trp lac), kindly donated by N.S. Willets, Biotechnology Australia PTY Ltd., Roseville, New South Wales, Australia. The EDP208 plasmid is a naturally occuring lac plasmid, F$_o$lac, orig-

inally isolated from Salmonella typhi (Falkow, 1962).

E. coli VIDO1 (O101:K$^-$)K99$^+$ bacteria were used in specificity studies of CFA/1 monoclonal antibodies. This strain was obtained from J.F.C.A. Pantekoek, Alberta Agriculture, Edmonton, Alberta.

2. Animals

BALB/cCr (H-2^d) mice used in CFA/1 monoclonal antibody production were maintained at the University of Alberta Health Sciences Animal Center. Breeding stock was originally obtained from Jackson Laboratories, Bar Harbour, Maine. Mice used were 22 weeks of age.

Female New Zealand White rabbits, obtained from Ellerslie Animal Farms, University of Alberta, Edmonton, Alberta, were used for the production of polyclonal antisera. The rabbits were between 6 weeks and 2 years of age during the various injections and bleeds. This strain of rabbits was also used for attachment studies wherein rabbits ranging from neonates to 2 year old adults were sacrificed and small intestine removed for enterocyte and section production.

3. Tissue Culture Strains

Monoclonal antibodies directed against CFA/1 pili were secreted from hybridoma cells made from the fusion of BALB/cCR murine splenocytes with NS1/Sp2 myeloma cells (a

fusion of NS1, ATCC TIB 18, and Sp2, ATCC CRL 158,. cell lines obtained from B. Singh, University of Alberta, Edmonton, Alberta). NS1/Sp2 is an azoguanine resistant, non-secreting myeloma cell line derived from BALB/c mice.

The tissue culture cell lines used for the study of CFA/1 pilus specific adhesion of H10407 bacteria are listed in Table II-1. All lines were obtained from the American Type Culture Collection, Rockville, Maryland.

NS1/Sp2 cells were thawed prior to each fusion and passaged only to the desired density needed for fusion. Other cell lines were passaged no more than 20 times prior to thawing a new sample. All lines were frozen in 20% (v/v) dimethylsulfoxide in fetal calf serum and stored in liquid nitrogen.

4. Bacterial Culture Media

L-broth consisted of 1% (w/v) tryptone (DIFCO Laboratories, Detroit, Michigan) and 1% (w/v) NaCl, pH 7.2. TSB agar (DIFCO) was used for the growth of ED3873 bacteria for the production of EDP208 pili and for determining the number of colony forming units (CFU) when performing attachment assays.

CFA broth (Evans et al., 1977) consists of 1% (w/v) Casamino acids (DIFCO), 0.15% (w/v) yeast extract (DIFCO), 1 ml/liter of 5% (w/v) $MgSO_4$ and 0.1 ml/liter of 5% (w/v) $MnCl_2$, pH 7.4. CFA agar (CFA broth with 2% (w/v)

TABLE II-1

Tissue Culture Cell Lines Used in Adherence Assays

ATCC Number	Common Name	Comments
HTB 40	HuTu 80	Human duodenal adeno-carcinoma
CCL 241	FSH 74 Intest.	Normal human fetal
CCL 239	Human Colo.	Human colonic mucosal cells
CCL 6	Intestinal 407	Human embryonic origin contains HeLa markers
CCL 2.1	HeLa 229	Human cervix epith-elial carcinoma

agar, DIFCO) was used for the growth of all types of H10407 bacteria.

E. coli producing K99 pili were grown on improved Minca media (Guinee et al., 1977) which consists of 1.36 g KH_2PO_4, 10.1 g Na_2HPO_4-$2H_2O$, 1.0 g glucose, 1.0 ml trace salts solution 1.0 g Casamino acids, 12.0 g agar, 0.1 ml of 20 mg/ml biotin, 0.1 ml of 8 mg/ml tryptophan (Sigma Chemical CO., St.Louis, MO.), 1.0 ml of 5 mg/ml ampicillin (Sigma), 0.1 ml of 0.8 mg/ml thiamine, and distilled water to make it 1 liter, pH 7.5. The trace salts contained, per liter: $MgSO_4$-$7H_2O$, 10 g; $MnCl_2$-$4H_2O$, 1 g; $FeCl_3$-$6H_2O$, 0.135 g; and $CaCl_2$-$2H_2O$, 0.4 g.

All bacterial media were autoclaved for 20 minutes at 20 lb/in^2 at 126°C. Antibiotics were added after media were autoclaved and cooled to 50°C.

5. Tissue Culture Media

NS1/Sp2 myeloma cells and established CFA/1 monoclones were grown in RPMI 1640 (Gibco Canada Inc., Burlington, Ont.) containing 10% (v/v) heat inactivated (56°C for 30 min.) fetal calf serum (FCS, Gibco), 1% (v/v) 100 mM sodium pyruvate, 1% (v/v) 200 mM L-glutamine (Gibco), 1% (v/v) 50 mM oxaloacetic acid and 1% (v/v) penicillin/streptomycin (10,000 units/10,000 µg/ml, Gibco). New hybridomas were grown in HAT media until they reached the density indicative of subcloning. HA media was the fore-

mentioned supplemented RPMI media containing 2 ml/liter of 50 X HAT which is 0.0194 g thymine (Sigma), 0.068 g hypoxanthine (Sigma) and 5 ml of 1000 X aminopterin (0.0176 g aminopterin (Sigma) in 100 ml of 0.1 M NaOH) per 100 ml of distilled water. All cell types used during monoclonal antibody studies were grown at 37°C, 7% CO_2 and 95% humidity.

Tissue culture cell lines used in bacterial attachment studies were grown in minimal essential media (MEM) with nonessential amino acids and Earle's Basic Salt Suppliment (BSS, Gibco) with, 10% FCS at 37°C, 5% CO_2 and 100% humidity. The medium for CCL241 fetal intestinal cells was supplimented with 0.1 mM oxaloacetic acid (Sigma), 0.5 mM sodium pyruvate (Sigma), and 0.2 units/ml insulin (Sigma).

All tissue culture media were filter sterilized using a 0.2 μm Millipore filter (Millipore Corp., Bedford, Mass.).

6. Buffers

Standard saline citrate (SSC) is 0.15 M NaCl, 0.0015 M sodium citrate, pH 7.0. Phosphate buffered saline (PBS) contains 8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4-12 H_2O, 0.2 g KCl in 1 liter of distilled water, pH 7.4.

7. Chemicals, enzymes and reagents

All solutions were prepared from analytical grade reagents in double distilled water unless otherwise stated.

Acrylamide and N,N'-methylenebisacrylamide (electrophoresis grade) were obtained from BDH Chemicals Ltd., Poole, England. Guanidine hydrochloride and Ultra pure Tris-HCl were obtained from Canadian Scientific Products Ltd, London, Ont. Trypsin (TPCK), chymotrypsin, and carboxypeptidase A (DFP) were purchased from Worthinton Chemical Corp., Freehold N.J. Type IV papain was purchased from Sigma. CsCl was obtained from Terochem Laboratories, Edmonton, Alta. Bovine serum albumin (BSA) was purchased from Sigma.

Toluene scintillation fluid consists of 0.5 g POPOP (p-Bis{2-[5-phenyloxoyl]}-benzene) and 6.0 g of PPO (2,5 diphenyloxazole), both scintillation grade from Eastman Chemicals, in 1 liter of toluene. Aqueous Counting Scintillant (ACS) was purchased from Amersham Corp., Arlington Heights, Illinois.

All high pressure liquid chromatography (HPLC) solvents were HPLC grade obtained from BDH Chemicals. HPLC water was deionized by passing distilled water through a Millipore Milli-Q filter system. Triflouroacetic acid used for HPLC was obtained from Pierce Chemical CO., Rockford, Illinois.

Boc (t-butyloxycarbonyl) amino acids purchased from:

Protein Research Foundation, Japan; Bachem Fine Chemicals Inc., Marina del Rey, Calif.; Chemical Dynamics Corp., South Plainfield, N.J.; Spenco Division of Beckman Instruments Inc., Palo Alto, Calif.; and Vega-Fox Biochemicals, Tucson, Ariz., were used without further purification.

Methylene chloride (CH_2Cl_2) from Terochem Laboratories was distilled over $CaCO_3$ prior to use. 1-4 Diisopropyl-ethylamine (DIEA) was distilled first over NaH then over ninhydrin prior to use. Picric acid, from BDH Chemicals Ltd., was dissolved in methylene chloride and dried over $MgSO_4$ before use. Pyridine was distilled over ninhydrin.

Co-poly(styrene, 2% divinylbenzene) benzhydrylamine-HCl resin was purchased from Protein Research Foundation and 4-methyl benzhydrylamine resin was purchased from Vega Biochemicals.

8. Radioactive materials

Protein A ^{125}I, 25 µCi/ml in 0.03 M phosphate buffer containing 40% ethanol and acetic acid (pH 4.0), was obtained from New England Nuclear Corp., Dupont Canada, Lachine, Quebec. $Na^{125}I$, 1.1 mCi/60 µl in 1 N HCl, used for iodinations was obtained from Edmonton Pharmaceutical Center, Edmonton, Alberta. (1-^{14}C) Gly (0.25 mCi) was obtained from NEN.

B. Hemagglutination

The ability to detect the presence of CFA/1 pili on H10407 by hemagglutination was useful when growing bacteria for pilus purification and when using piliated and unpiliated bacteria for adhesion experiments. H10407 bacteria bearing CFA/1 pili are capable of agglutinating human type A erythrocytes. The assay was performed as follows: Colonies selected from CFA agar plates were suspended in 10 µl of 1% (w/v) mannose (in PBS) on glass slides. Bacteria can also be suspended in L-broth, as used in attachment assays, and 10 µl of this mixed with the mannose. To this, 10 µl of a 10% (v/v) erythrocyte suspension (in 0.9% NaCl) was added and hemagglutination observed. Hemagglutination occurred immeadiately for bacteria with CFA/1 pili and not at all for CFA/1 minus strains. To detect if any type I pili (often called mannose sensitive hemagglutinins) were present, the assay was done with human and guinea pig erythrocytes, using PBS lacking mannose.

Inhibition of hemagglutination by CFA/1 pilus specific monoclonal antibodies and K99 pilus specific polyclonal antibodies (obtained from J.F.C.A. Pantekoek) was performed as decribed above except that 10 µl of the undiluted antibody source was added to the bacterial-mannose suspension prior to the addition of erythrocytes. Inhibition was judged at 5 and 10 minutes after the addition of red blood

cells. The ability of CFA/1 monoclonal antibodies, CFA/1 polyclonal antibodies and K99 specific polyclonal antibodies to inhibit hemagglutination by VIDO1 K99$^+$ bacteria was done as described except that VIDO1 bacteria were suspended in PBS and a 10% (v/v) suspension of sheep erythrocytes were used.

C. Pili Purification

1. CFA/1 Pili

H10407 bacteria possessing CFA/1 pili were grown on a single 100 mm diameter by 15 mm petri dish (Fisher Scientific Co., Canada) containing CFA agar, at 37°C for 24 hours. The bacteria were removed from the plate by gentle scraping, resuspended in CFA broth and 0.2 ml per plate of this suspension was used to inoculate 14.4 cm diameter petri dishes containing CFA agar. The plates were incubated overnight at 37°C. Bacteria were again removed from the plates by scraping and mannose resistant hemagglutination performed to make sure the bacteria were still producing CFA/1 pili. Bacteria were suspended in approximately 300 ml of SSC (for a 40 plate preparation) and stirred for 1-3 hours, using a magnetic stirrer, at 4°C. Removal of pili from the bacterial surface was accomplished by blending with a Sorvall Omnimixer at 2000 rpm for 5 min. The cells were removed by centrifugation at 8,000 x g for 10 minutes

while pili remained in the supernatant. To remove any pili which may be trapped in the cell pellet, the pellets were washed with 200 ml of SSC and recentrifuged. The supernatants were pooled and subjected to an ammonium sulfate precipitation (25% w/v), at 4°C for 16 hours. The precipitated pili were removed by centrifugation at 8000 x g for 15 minutes. The pellet containing pili and any other proteins which were co-precipitated was resuspended in distilled water and layered on a preformed CsCl step gradient. The gradient consisted of 5 ml CsCl of density 1.50 g/ml, a 4 ml step containing 1.40 g/ml, followed by three 2.5 ml steps of density 1.30, 1.20, and 1.10 g/ml. 20 mls of pili were layered on to this gradient and samples centrifuged at 20,000 rpm for 16 hours in a Beckman SW27 rotor using a Beckman L2-65B ultracentrifuge. The pilus band (rho=1.307) was removed from the gradient and dialyzed against distilled water to remove the CsCl. The pili were tested for purity using sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and electron microscopy. The most common contaminant, flagellin, was usually removed using a second CsCl step gradient.

2. EDP208 pili

EDP208 pili were purified according to the specifications of Armstrong et al., 1980. Briefly, ED3873 bacteria were grown on TSB agar in aluminum trays (27 cm by 38 cm

by 1.25 cm) by incubation overnight, at 37°C. The cells were removed from the trays by gentle scraping, suspended in SSC, and stirred with a magnetic stirrer for 2 hours, at 4°C. This was sufficient to remove the majority of pili from the bacterial cell surface. Cells and debris were removed by centrifugation and the cell pellet was washed 2 or 3 times with SSC to ensure that the majority of the pili were removed. Supernatants containing the pili were pooled, adjusted to 2% (w/v) polyethylene glycol 6000, 0.5 M NaCl and soluble pili allowed to precipitate overnight at 4°C. Precipitated pili were removed from solution by centrifugation and the pili pellet was resuspended in distilled water. This solution was layered on preformed CsCl gradients, prepared as described in Section C.1 of this chapter, and centrifuged for 20 hours at 20,000 rpm in a Beckman SW27 rotor. The pilus band (rho = 1.2-1.3) was removed from the gradient and dialyzed against distilled water to remove the CsCl. Purity of the preparation was determined by SDS-PAGE and electron microscopy.

D. SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE of proteins and peptides was carried out by the method of Lugtenberg (1975), unless specified otherwise. A slab gel apparatus with 0.75 mm Teflon spacers and 20 well comb, purchased from Bethesda Research Laboratories

Inc., Gaithersberg, MD., was used. The running gels were 10-12 cm long and contained 15% acrylamide-0.27% methylene-bis-acrylamide, while the stacking gels were 1-2 cm long and contained 7% acrylamide-0.18% bis acrylamide.

1-5 µg of lyophilized protein or 5-10 µg of peptide were dissolved in 20 µl of sample buffer which consists of 1.25 ml Tris-HCl (0.25 M, pH 6.8), 1 ml of 10% SDS, 1 ml of 50% glycerol, 0.2 ml of beta-mercaptoethanol, 0.2 ml of 0.04% (w/v) bromphenol blue and 0.35 ml of water. Samples were boiled for 20 seconds and loaded on the gel. Gels were run at 30 mA constant current until the samples had entered the running gel, then run at 45 mA constant current until samples had travelled the desired distance. After electrophoresis, gels were stained overnight in a solution containing 0.5% (w/v) Coomassie Brilliant Blue R (Sigma), 25% (v/v) isopropanol and 10% (v/v) acetic acid, then destained using several changes of a 10% (v/v) acetic acid, 10% (v/v) methanol destaining solution.

SDS-PAGE of lipopolysaccharide (LPS) was carried out according to the procedure of Laemlli (1970). The BioRad Protean slab gel apparatus and 1.5 mm spacers and 20 well comb, obtained from BioRad Laboratories Canada Ltd., Mississauga, Ont., was used. The running gel had a final acrylamide concentration of 14% and the stacking gel had a final acrylamide concentration of 5%. All solutions used were made up in water deionized by the Milli-Q filtration sys-

tem.

For rapid diagnosis of the type of LPS on the surface of bacteria, 10 µl from a 60 ul digestion mixture containing 10^9 bacteria, proteinase K and Laemlli sample buffer (supplemented with 50 mM dithiothreitol, DTT) was boiled for 5 minutes and loaded on the gel. For purified LPS, 0.6-10 µg was resuspended in 10 µl of Laemlli sample buffer, boiled for 5 minutes and loaded on the gel. Laemlli sample buffer consisted of 2 ml of 10% SDS, 0.4 ml beta-mercaptoethanol, 1.0 ml glycerol, 0.625 ml 1.0 M Tris pH 6.8, 1.0 ml of water and enough bromphenol blue to ensure a blue colour. The gels were run at 10 watts constant power for 3.6 hours.

LPS gels were silver stained according to the procedure of Tsai and Frasch (1982). The gel was removed from the apparatus and placed in a chromic acid washed glass dish containing destain/fixer made up of 40% (v/v) ethanol: 5% (v/v) acetic acid and fixed overnight. The fixer was poured off and replaced with 200 ml of freshly prepared oxidizing reagent (1.4 g periodic acid in 200 ml of fixer) which was allowed to react for 5 minutes with constant mixing. The gel then was transferred, with the minimal amount of manipulation, to a second acid cleaned dish containing 500 ml of degassed, deionized water. The gel was washed for 15 minutes in this manner using three changes of water. The silver reagent was then added and allowed to react for 10

minutes. The silver stain reagent must be prepared fresh prior to use and was made by adding 2 ml of concentrated NH_4OH to 28 ml of 0.1 N NaOH (diluted from Fisher 1.0 N NaOH), followed by the slow addition of 5 ml of 20% (w/v) $AgNO_3$ (Fisher). It is important to have the solution stirring vigorously during the addition of the silver. To this, 115 ml of degassed, distilled water was added. The staining solution was poured off and gel washed as before with degassed, distilled water. After the final wash, 400 ml of developer (0.5 ml of formalin or formaldehyde and 50 mg of citric acid in 1 liter of water) was added and stained bands allowed to develop. Developement was stopped with the addition of 7% acetic acid in water. Gels were stored indefinitely in 7% acetic acid provided they were kept out of the light.

E. Electron Microscopy

Samples for electron microscopy were applied to copper grids which had been coated with parlodian and carbon. Grids were negatively stained with 1% (w/v) sodium phosphotungstate, pH 7-7.2. Samples were viewed and photographed in a Phillips EM 300 transmission electron microscope.

For thin sections, erythrocytes with bound bacteria were fixed overnight in 3.5% buffered (0.1 M cacodylate buffer, pH 7.2) glutaraldehyde, containing 0.075% ruthenium

red (Sigma Chemical Co., St. Louis, Mo.) when indicated. Ruthenium red interacts with polyanions and is often used for visualization of cell surface polysaccharides (Luft, 1971). The fixed suspension was then washed 3 times with 0.075% ruthenium red in 0.1 M cacodylate buffer, then post fixed for 1-2 hours with 1% OsO_4 in the same buffer. After briefly rinsing with buffer, the samples were dehydrated in ethanol as follows: 15 minutes in 25% ethanol, 15 minutes in 50% ethanol, 15 minutes in 75% ethanol, 15 minutes in 90% ethanol, 3 X 15 minutes in absolute ethanol. This was followed by two 15 minutes washes in propylene oxide. The sample was left overnight in an open vial containing a 1:1 mixture of propylene oxide:epon (LX112, Ladd Research Industries Inc., Burlington Vermont), then transferred to fresh epon in Bean capsules. Thin sections were cut with a glass knife (produced using a LKB 7801B Knifemaker) using a microtome (Reichert-Jung Ultra Cut). The sections were stained with 5% uranyl acetate in methanol for 10 minutes, washed thoroughly with water and placed in lead citrate for 2-6 minutes. The stained sections were washed again with water and dried prior to viewing in the electron microscope.

For experiments in which Protein A labelled gold beads were used to visualize pilus-antibody interactions, the following procedure was used. Bacteria were spread on TSA plates and incubated overnight, at 37°C. The resul-

ting bacterial lawn was gently scraped off the plate and resuspended in 1.0 ml of distilled, deionized water. A 1:10 dilution of this suspension was made and 10 ul placed on a carbon coated grid. The drop was left on the grid for 5-10 minutes, then the grid was washed twice with distilled, deionized water. The appropriate dilution of purified IgG was added to the grid and allowed to incubate for one hour, at room temperature. This was followed by four washes in 0.5% (w/v) BSA, in PBS, after which a drop containing a 1:100 dilution of Protein A-gold (Protein A G15, EM-Grade, SPI Supplies, Toronto, Ont.), in the BSA solution, was added for another hour, at room temperature. The grids were washed four times in 0.5% BSA, then stained with 1% phosphotungstate, as previously descibed prior to viewing in the electron microscope.

Thin sections and Protein A gold experiments were performed by P. Pieroni, Dept. of Medical Microbiology.

F. Protein and Peptide Chemistry

1. Amino Acid Analysis

Routine amino acid analyses were performed on a Durram D-500 amino acid analyzer. Samples were hydrolyzed for 24, 48 and 72 hours in 6 N constant boiling HCL containing 0.1% phenol, in evacuated, sealed tubes at 110°C. Quantities were calculated as the average value from these three

time periods of hydrolysis. For serine and threonine, values were estimated by extrapolation to zero time of hydrolysis and for isoleucine, leucine and valine, the 72 hour values were used. Methionine was determined as methionine sulfone by oxidizing the protein with performic acid (Moore, 1963). Peptide resins were hydrolyzed as above in a mixture of 2 ml of 12 N HCl, 1 ml of acetic acid and 1 ml of phenol, according to Gutte and Merrifield (1971).

2. N - terminal Analysis (Dansylation)

Dansylation of the amino terminal residue of various peptides and proteins was carried out according to Hartley (1970). 5-10 nmol of protein was suspended in 0.1 M sodium bicarbonate and lyophilized in a 5 mm glass tube. This was dissolved in 10 µl of double distilled water and 10 µl of dansyl chloride (1-2 mg/ml in acetone). Tubes were sealed with parafilm and incubated for 1 hour at 37°C, after which the solution changes colour from yellow to clear. The samples were lyophilized and hydrolyzed in 50 µl of 6 N constant boiling HCl at 110°C in evacuated, sealed tubes for 24 hours. The hydrolyzed samples were dried in vacuo and suspended in 8 µl of acetone:acetic acid (3:2, v/v) and spotted on 5 by 5 cm micropolyamide sheets (Schleicher and Schull, obtained from Pierce Chemical Co., Rockville, Ill.) for dansyl amino acid identification by thin layer chromatography. Thin layer chromatography was carried out as des-

cribed by Needleman (1975), using three solvent systems and two directions.

3. Preparation of pilin monomers

Pilin monomers of both EDP208 and CFA/1 pili were prepared as follows: To a suspension of pili in water, enough SDS was added to make 1% (w/v). This was boiled for 5 minutes and allowed to cool. 20-30 ml of acetone was added and pilin allowed to precipitate in an ice bath for 1 hour. The precipitate was pelleted by centrifugation at 10,000 x g for 20 minutes and resuspended in the desired volume of distilled water.

4. Enzymatic digestion

Lyophilized CFA/1 pili or pilin used for tryptic digestion was suspended in a minimal volume of 0.1 M ammonium bicarbonate, pH 8.2. To this, trypsin (TPCK) in 0.01 M HCl was added, initially as 1 in 50 (enzyme:substrate) molar ratio and allowed to digest for 4 hours at 37°C. A further aliquot of trypsin was added to a final concentration of 0.04 moles of trypsin per mole of pili/pilin and digestion continued overnight. Digestion was stopped by lyophilization of the digestion mixture.

Carboxypeptidase A (Cpase A) digestion of pilin was performed according to Needleman (1975). 25 µl of Cpase A stock (60 mg/ml) was mixed with 100 µl of freshly made 1.0%

(w/v) ammonium bicarbonate. To this suspension, enough 0.1 M NaOH was added to dissolve the enzyme, then the pH was adjusted by adding 0.1 M HCl dropwise and checked using pH paper. If too much HCl is added the enzyme becomes insoluble and the procedure must be repeated. The volume of the activated Cpase A solution was raised to 1.0 ml by the addition of 1% NH_4HCO_3. Approximately 100 nmol of pilin was precipitated with acetone. The pellet obtained by centrifugation was resuspended in 50 µl of 1% NH_4HCO_3 containing 1% SDS. Twenty ul of the Cpase A solution was added and digestion at 37°C was allowed to proceed for the desired length of time (usually 1, 3, 5, and 24 hours). The appropriate controls were treated identically and included all buffers without the enzyme or protein and all buffers plus the enzyme but without the protein. The reaction was stopped by the addition of 200 µl of 1 M acetic acid which also causes precipitation of the protein. The precipitate was pelleted by centrifugation and supernatants, which contained the free amino acids, lyophilized and resuspended in water several times to remove the volitile buffers. They were then subjected to amino acid analysis using the Durrum D-500 amino acid analyzer. All reactions were performed in 1.5 ml microcentrifuge tubes (Bio-Rad). Centrifugation was done in an Eppendorf 5414 Microcentrifuge.

5. Cyanogen bromide (CNBr) digestion and purification of

CNBr peptides

CNBr cleavage of CFA/1 pili was performed according to the procedure of Needleman (1975). 1 µmol of lyophilized pili was dissolved in a minimal volume of 70% formic acid (2-3 ml). To this, 500 molar excess of solid CNBr per methionine was added (since CFA/1 pilin has 3 methionines, 1500 umol of CNBr was used) and the reaction allowed to proceed for 24 hours at room temperature. To stop digestion 30 ml of water was added to the digestion mixture and the sample immediately shell frozen and lyophilized.

Partial purification of CFA/1 CNBr peptides was achieved following the procedure of Klemm (1982). The lyophilized sample was dissolved in 1 ml of 6 M guanidine HCl in 0.2 M NH_4HCO_3, pH 8.0 and loaded on a Pharmacia Sephadex G-75 column (118 by 1 cm) equilibrated with the same buffer. The column was run at a flow rate of 2 ml/hr. Fractions were collected using a Pharmacia Frac 300 fraction collector and absorbance at 280 nm measured using a Pharmacia UV-1 Single Path Monitor. The appropriate fractions were pooled, lyophilized then resuspended in a minimum volume of water and passed through a Pharmacia PD-10 column (10 by 1 cm) for desalting purposes. The contents of each pool were examined by SDS-PAGE, amino acid analysis and dansylation N-terminal analysis. Each of the three pools (called CNBr1, 2, or 3) was found to contain one predominant peptide along with low levels of the other two.

G. Peptide Synthesis

1. Basic Principles of solid phase peptide synthesis

All of the peptides in this study were synthesized by solid phase synthesis according to the technique of Erickson and Merrifield (1976). Amino acids, having their side chains and N-alpha amino group blocked by protecting groups, are sequentially coupled to an insoluble resin support. The alpha carboxyl group of a protected amino acid was activated and coupled to the deprotected alpha-amino terminal of a peptide on the resin, hence, achieving C to N synthesis. Briefly, the first amino acid is linked to a benzhydrylamine support in the presence of the coupling agent dicyclohexylcarbodiimide (DCC). The next amino acid was coupled after the Boc group of the peptide on the resin is removed by treatment with 50% triflouroacetic acid (TFA) in CH_2Cl_2. The deprotected peptide was neutralized with 5% DIEA in CH_2Cl_2. The next Boc-amino acid was mixed with the coupling agent DCC and added to the resin. This procedure was repeated to ensure the maximum coupling of each residue. Once the peptide had been completed, the peptide was acetylated, then detached from the resin using anhydrous hydrofluoric acid (HF). The extent of coupling after each difficult residue or routinely after every third residue, was done by picric acid monitoring. The entire

procedure was automated, with the exception of the coupling of the first amino acid, and performed on a Beckman Peptide Synthesizer Model 990.

2. Chemical synthesis of EDP208 and CFA/1 pili peptides

EDP208 pilin peptides were prepared by A. Taneja, B. Wu and R. Parker from the laboratory of R. Hodges. The CFA/1 pilin peptides were prepared following the same procedures used in that laboratory, which was described in detail by Worobec et al. (1985).

All peptides were synthesized on co-poly(styrene, 2% divinylbenzene) benzhydrylamine-HCl resin (hydrochloride salt from Protein Research Foundation, substitution = 0.8 nmol of free amino groups per gram of resin). The program used for the attachment of each amino acid was a modification of that described by Hodges et al (1981) and was reported by Worobec et al (1985). A radioactively labelled Boc(1-^{14}C) glycine residue prepared by the method of Itoh et al. (1975) was incorporated in the EDP208 pilin peptides at position 7 (Worobec et al., 1983). Monitoring, by the picric acid method, was carried out after the coupling of the first amino acid and at various places along the developing peptide chain. The program for picric acid monitoring was described by Worobec et al. (1985).

After synthesis was completed, the peptide was cleaved from the dried resin following the procedure of

Hodges and Merrifield (1975) by suspending the resin in 1 ml of 10% (v/v) anisole, followed by the addition of anhydrous HF and mixing at 4°C for 45 min. The solvents were removed under reduced pressure, resin washed with ether (to remove the anisole) and the peptide extracted with 4 x 5 ml of TFA. The combined TFA washes were evaporated and the residue redissolved in water and lyophilized. All peptides cleaved from the benzhydrylamine resin had C-terminal amides.

3. Purification of synthetic peptides

CFA/1 pilin peptides AcP(55-64), AcP(55-70), AcP(61-70), and AcP(91-99) were purified by reversed phase high pressure liquid chromatography (HPLC) using a Waters μBondpak analytical C18 column (obtained from Waters Associates, Milford, Mass.) with a linear AB gradient where solvent A was 0.1% TFA/water and solvent B was 0.05% TFA/acetonitrile.

EDP208 synthetic peptides were purified using either a Beckman Ultrapore C3 reverse phase HPLC column or a Whatman PXS-C8 column. An AB gradient was used where A and B were as descibed for the purification of CFA/1 peptides. All peptides were purified using a gradient of 1% B/min. and a flow rate of 1 ml/min. The only peptide not purified by this procedure was AcP(1-12) which was purified according to the specifications of Worobec et al. (1983), using

an AG1 X 4 column (1.8 X 50 cm) and eluted with 0.1 M pyridine acetate pH 5.0 at a flow rate of 60 ml/hour at 55°C. The purity of all peptides was examined by HPLC and amino acid analysis.

4. Preparation of formyl amino acids

To study the role of the naturally occuring acetylated amino terminus of EDP208 pilin, synthetic peptide analogs were made using N-formyl instead of N-acetyl as the N terminal blocking group. Formyl glycine and formyl-O-benzyl-L-threonine were prepared by J.M.R. Parker according to the procedure of Worobec et al. (1985). Both formyl derivatives were checked for purity by melting point determination and ^1H NMR (D_2O) as descibed in Worobec et al. (1985). These amino acids were coupled to the appropriate peptide while still on the resin, using the procedures outlined in Section G.2 of this chapter.

5. Preparation of covalently cross-linked peptide-BSA conjugates

Synthetic peptides containing lysine residues were covalently linked to BSA using the synthetic crosslinker N-hydroxysuccinimde ester of 4-azidobenzoic acid (AB-ONSu, kindly donated by P. Chong, prepared according to Chong et al., 1981). The peptides were derivatized with the photoaffinity probe as described by Worobec et al. (1983). Brief-

ly, ^{14}C labelled or unlabelled peptide, at a concentration of 1 µmol in 250 µl of aqueous $NaHCO_3$ (0.4 mg in 250 µl of water) was placed on ice and 10 µmol of cold or ^{14}C labelled AB-ONSu, (6100 cpm/nmol) in 250 µl, was added dropwise over a period of 10 minutes, with constant stirring. The reaction was allowed to proceed for 1 hour at 0°C then at room temperature for 24 hours. The modified peptides were generally purified by reverse phase HPLC, as described for the purification of EDP208 synthetic peptides, except for the original AcP(1-12) peptide which was purified using a 1.6 x 100 cm Sephadex G-25 column and eluted with 0.1 M NH_4HCO_3 at a flow rate of 10 ml/hour. In both cases, the products were monitored by radioactivity as well as UV absorbance.

A 5-10 fold molar excess of the modified peptide to BSA (Fraction IV) was used in the coupling procedure. One to two mg of lyophilized peptide-probe was dissolved in 75-150 µl of a BSA solution (made up by dissolving 125 mg of BSA in 1 ml of 0.1 M KH_2PO_4, pH 6.8). The mixture was irradiated for 1 hour using a RPR 208 preparative reactor equipped with RPR 3500 Angstrom lamps (obtained from Rayonet; The Southern New England Ultraviolet Co., Middleton, Conn.) which resulted in the crosslinking of peptide to BSA via the photoaffinity probe. For the most part, the peptide-BSA conjugate was purified by reverse phase HPLC on a Synchropak RP-8 column (250 X 10 mm; flow rate of 4 ml/

min., obtained from Synchrom Inc., Linden, Ind.) using a gradient of 2% B/min where the solvents A and B were the same as previously described. The peptide conjugate ET1-BSA (or AcP(1-12)-BSA) was the only exception and it was purified as described by Worobec et al (1983), using a 1.6 X 100 cm Sephadex G-50 column eluted with 1 mM HCl at a flow rate of 10 ml/hour. The ratio of peptide to BSA was usually 1-3 peptide:1 BSA but ET1-BSA was also obtained at a 72 peptide:1 BSA by combining 20 µmoles of modified peptide with 0.1 µmole of BSA (a 200:1 ratio) and photolyzing for 90 minutes. These ratios were calculated by the amount of radioactivity in each sample.

H. Immunological Methods

1. Antisera production

Anti-pilus polyclonal sera used in pilus antigenic structure determination was obtained from female New Zealand white rabbits. 100 µg of pure pili (in sterile 0.9% NaCl) and an equal volume of Freunds complete adjuvant (Difco) were thoroughly mixed by passing the solution back and forth through a double hubbed needle attached to two syringes. This mixture was injected subscapularly and in the gluteal region of the rabbit. The same procedure was done 2-4 weeks later except that Freunds incomplete adjuvant was used. The rabbits were bled from the ear vein 2

weeks later and sera titered using the ELISA described later in this section. Usually titres of 1/1-5 x 10^6 were obtained. If lower titres were obtained, a second injection of the protein was necessary and done as above using incomplete adjuvant. The rabbits were bled at two week intervals until the desired quantity of sera was obtained.

Anti-peptide BSA antisera, used in early studies of the immunogenicity of EDP208 synthetic peptides, were prepared by initially injecting the rabbits subcutaneously at several sites with a total of 600 µg of the peptide conjugates in complete adjuvant. This was followed with a booster injection one month later with the same amount of protein in incomplete adjuvant. The rabbits were bled from the ear vein at two week intervals after the final injection.

Pilus specific murine antisera, used to determine which mice to use for monoclonal antibody production, was made using an alternate procedure. Female BALB/cCR mice were injected intravenouly via the tail vein with 10 µg of purified CFA/1 pili in 0.2 ml of saline. One week later, a second injection was administered. The mice were bled from the tail vein one week after the final injection and the titre of the sera determined by ELISA. The mice with the best titres were selected for monoclonal antibody experiments.

In the case of both animal types, pre-bleeds were obtained prior to any injections and sera tested for any

reactivity in an ELISA to the protein in question. In the studies presented here, anti-pilus specificity was negligible prior to injection.

2. Immunoblot procedure

Proteins and peptides were transferred from SDS-polyacrylamide gels to nitrocellulose and reacted with anti-pilus or anti-peptide conjugate antibodies, followed by antibody-antigen complex detection using ^{125}I labelled Protein A. The procedure was similar to that of Towbin et al. (1979) except that ^{125}I labelled Protein A was used instead of ^{125}I labelled sheep immunoglobulin G (IgG).

After electrophoresis, proteins were transferred to nitrocellulose paper using an Electro-blot Apparatus (E-C Apparatus Corp., St. Petersburg, Fla.). Briefly, the gel was placed on a piece of filter paper, which was previously wetted with the transfer buffer, and placed on the cathode side of the electrophoresis grid. The gel was overlayed with a piece of nitrocellulose (0.45 µm, Schleicher and Scheull) which was pre-soaked with distilled water. All air bubbles which were present between the gel and the nitrocellulose were gently removed by rubbing, after which the entire complex was covered with a second piece of wet filter paper. This "sandwich" was held in place on the grid with several layers of Scotch Brite pads (3M Company), and the grid was closed and lowered into the electrophoresis

tank containing enough transfer buffer to cover the grid. The transfer buffer consisted of 0.025 M Tris-HCl, pH 8.3; 0.192 M glycine and 20% (v/v) methanol and was stored at 4°C until use. The transfer buffer was circulated through the tank with a pump during the procedure to enhance cooling. Electrophoresis was carried out for 2.5-3 hours at 4 mA/cm^2, at which point proteins up to 68,000 daltons were completely transferred as detected by Coomassie blue staining of the transferred gel. Samples were routinely run in duplicate during SDS-PAGE to ensure that one half of the gel could be stained while the other half was transferred.

Pilin, peptides or peptide conjugates bound to nitrocellulose were detected by the reaction of anti-pilus or peptide conjugate antibodies followed by radioactive Protein A (Protein A specifically binds to the Fc portion of IgG and some IgM's). After transfer, the nitrocellulose paper was placed in 25-50 ml of Buffer I containing an appropriate dilution of rabbit antisera or mouse monoclonal antibodies (1:25 to 1:250 dilutions were used in these studies) and incubated overnight at 37°C, with constant shaking. Buffer I consists of 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.25% (w/v) gelatin and 0.05% (v/v) Nonidet P40 (nonionic detergent P40, an octyl-phenol-ethylene-oxide condensate containing an average of 9 ethylene oxide per molecule, obtained from Sigma Chemical Co.). This was

followed by a 2 hour washing step in 50 ml of Buffer I, at 37°C with shaking. 1 µCi of ^{125}I Protein A in 50 ml of Buffer I was added and incubated at 37°C for 2 hours with constant shaking. The paper was then briefly rinsed with distilled water and washed with Buffer II for 1 hour at 37°C, with shaking. Buffer II consists of 1 M NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4, 0.25% gelatin and 0.4% (w/v) sarcosyl (97% n-lauryl-sarcosine, from Sigma). Since this buffer has a high salt and detergent content, limited exposure to the paper is recommended to ensure that the proteins are not removed from the paper. The paper was finally rinsed with distilled water, then thoroughly dried prior to autoradiography using Kodak X-OMAT AR film. The film was exposed to the paper at -70°C for 2-7 days and developed using a Kodak X-OMAT automatic developer.

3. Immunoglobulin G isolation

Pilus specific IgG was isolated from polyclonal rabbit sera or mouse ascites fluid using a Pharmacia Protein A Sepharose CL-4B column. One gram of freeze dried Sepharose was swollen for 15 minutes in 2.5 ml of PBS and placed in a 10 ml syringe, to act as column. The column was equilibrated with PBS. 1-2 ml of serum or ascites fluid was filtered with a 0.45 µm Millipore filter and loaded on the column. The sample was washed through the column with 3 volumes of PBS. One ml fractions were collected and the absorbance at

280 nm was measured for each fraction. This effluent contained all serum proteins excluding IgG. The IgG bound to the column was eluted with 10 ml of 1.0 M acetic acid. Again, 1 ml fractions were collected and the absorbance at 280 nm measured. A large peak was observed, fractions were pooled and dialyzed against PBS or distilled water. All fractions were tested for anti-pilus activity in an ELISA. The resulting IgG was stored in the presence of NaN_3 at -20°C until needed. The concentration of IgG was determined using the relationship A_{280} = 1.6 for a 1 mg/ml solution of IgG (Watters and Maeliche, 1983).

4. Preparation of Fab fragments from IgG

Pilus specific IgG, purified from rabbit antisera using Protein A sepharose CL-4B affinity chromatography (as descibed in Section H.3), was subjected to papain digestion to obtain Fab fragments according to the procedure of Porter (1959). 3-5 mg of lyophillized IgG was resuspended in 1 ml of digestion buffer (100 mM Na_2PO_4, pH 7.2; 10 mM cysteine; and 2 mM EDTA). A 1:100 (w/w) ratio of papain to IgG was added and digestion allowed to proceed for 16 hours at 37°C. Digestion was stopped by the addition of enough iodoacetamide to make a final concentration of 0.15 M and any insoluble material was removed by centrifugation in a microcentrifuge. The supernatant was loaded on a Sephadex G-100 column (1 X 37 cm) equilibrated with a buffer

made by combining 100 ml of 0.1 M Tris-HCl pH 7.2, 100 ml of 0.067 M Na_2PO_4 pH 7.4 and 800 ml of 0.14 M NaCl. The column was run at a flow rate of 20 mls/hr. Fractions were collected using a Pharmacia Frac-300 fraction collector and fractions monitored by a Pharmacia single path UV-1 monitor set at 280 nm. Two peaks were routinely observed. These fractions were pooled and checked for the presence of Fab by SDS-PAGE and by passing the pools through a Protein A Sepharose column. One pool usually contained undigested IgG and bound to the Protein A column. The other pool did not bind to the Protein A column and was found to be Fab. The Fc portion became insoluble and was removed by centrifugation prior to loading the sample on the G-100 column. Maximum yield of pure Fab was usually 25% of the input IgG (on a molar basis).

5. ELISA assays

The principles of the enzyme linked immunosorbant assay (ELISA), used for the detection of pilus specific antisera, IgG, and Fab were described by Voller et al. (1974). The assays used in this study were modifications of the original assay. Microtitre plates (Dynatech, Immulon I plates, Alexandria, Va.) were coated with 200 µl per well of a 5 µg/ml of purified pili, pilin or peptide conjugate in 0.5 M sodium bicarbonate coating buffer (pH 9.6), for 16 hours, at 4°C, in a moist chamber. This represented a

saturating concentration of protein. The antigen saturated wells were washed 3 X 3 minutes with PBS containing 0.05% (v/v) Tween 20 (PBS-T). After washing, the plates were incubated for 2 hours at room temperature with 200 µl per well of the appropriate dilution of antibody in PBS-T containing 1% (w/v) BSA (PBS-T-BSA). The presence of BSA prevented the non-specific attachment of antibody molecules to the microtitre wells, resulting in false A_{405} readings. Unbound or excess antibody was washed away with PBS-T and remaining antibody-antigen complexes incubated with goat-anti-rabbit (or mouse) IgG conjugated to alkaline phosphatase (obtained from Boehringer Mannheim Corp., or Sigma and used at a 1:1000 dilution for sera and IgG or at a 1:500 dilution for Fab) for 2.5 hours, at room temperature. The plates were then washed and the alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma 104 substrate tablets at 5 mg/tablet) was added as a 1 mg/ml solution in 10% (v/v) diethanolamine. After an appropriate time interval, the absorbance at 405 nm was determined with a Titertek Multiscan ELISA plate reader (Flow Laboratories Canada, Mississauga, Ont.). The titre of individual antisera was calculated using a linear regression program where X = (1/Antibody dilution) and Y = ($1/A_{405}$). The titre or end point was described as the antibody dilution required to obtain an A_{405} of 0.05 after a predetermined length of time (usually about 15 minutes).

To compare the ability of synthetic peptides, native peptides, peptide conjugates and whole pili to interact with pilus specific antibodies, a competition ELISA similar to that described by Jolivet et al. (1983) was used. Microtitre plates were coated with whole pili as described above. Various concentrations of competitors (synthetic peptide, peptide conjugate or native fragment) or whole pili were incubated with 25-50 pmol/ml of pilus specific IgG or Fab in PBS-T-BSA for 1 hour at 37°C, then overnight at 4°C. Aliquots (200 µl) of each competitor/antibody mixture were then transferred to the pilus coated wells and allowed to incubate for 2 hours, at room temperature. This allows any uncomplexed antibody to react with the pili attached to the wells. This reflects the ability of the competitor to inhibit the pilus antibody interaction. After washing, the antibody-pilus complexes were quantitated colorimetrically as previously described, using the alkaline phosphatase assay. The results were presented graphically, using semi-log graph paper, where the abscissa was expressed as competitor concentration and the ordinate as % inhibition. Percent inhibition was calculated as follows:

$$\% \text{ INHIBITION} = \left(\frac{A_{405} \text{ COMPETITION}}{A_{405} \text{ NO COMPETITION}} \times 100\right) - 100$$

The "no competition" values were obtained by performing the ELISA with the same concentration of antibody used in the

competition mixture, but without any competing protein. Each value represents an average of 3-5 readings. The results from this type of experiment were quantitated by the I-50, which is the concentration of the competitor required to give 50% inhibition. The I-50 was also used as the direct measurement of the affinity binding constant, aK, as described by Nieto et al. (1984).

I. Monoclonal Antibodies

1. Hybridoma production and propagation

Two female BALB/cCR mice, which were primed with CFA/1 pili as described in this chapter, Section H.1, were boosted by intravenous injection of 10 µg of pili. Four days later, the animals were sacrificed by cervical dislocation, spleens removed and spleen cells fused with NS1/Sp2 myeloma cells according to the proceure of Kohler and Milstein (1976). Spleen cells were isolated by gentle mashing of the spleen in a mesh sieve. This procedure allowed the spleen cells to effuse through the sieve, while the connective tissue remained behind. The spleen cells were resuspended in FCS free RPMI media and washed twice by pelleting the cells at 800 X g, for 10 minutes. The cells were counted and the viability checked by diluting in 0.4% trypan blue and counting in a hemocytometer. The final concentration of total spleen cells was [illegible] X 10^8. NS1/Sp2 cells

were washed and a final concentration of 1.0×10^7 total cells was used for the fusion (spleen:myeloma cell ratio of 20:1). The fusion was performed using 1 ml of 50% (w/v) PEG 1460 (Dow Chemicals, in RPMI media). The fused cells, suspended in RPMI plus 20% FCS, were plated out at a density of 1×10^7 cells/100 µl/well into Linbro 96 well microtitre plates and incubated at 37°C, with 7% carbon dioxide and 95% humidity. HAT media was added to each well on day 1 and fresh HAT added on days 2, 3, 5 and 8 after the fusion. On day 8, the resulting supernatant solutions were screened for anti-CFA/1 antibodies by ELISA (described below). The wells showing the highest antibody levels and having visible clones, were subcloned by diluting the well contents to 100 cells/ml in RPMI plus HT and adding 20 µl of this suspension to 180 µl of 10^7 normal mouse erythrocytes per well of a 96 well plate. Five original clones were subcloned with each clone occupying a separate plate. The final cell concentration for subcloning was 2 cells/well. After 6 days of growth, the subclones were screened by the ELISA and 10 clones with the highest antibody levels were selected for further growth. These 10 clones were grown in RPMI plus 20% FCS until they reached a density at which the cells could be frozen. Cells were usually frozen in the presence of 20% DMSO in FCS at density of 1×10^7 cells/ ml according to the procedure of Kohler and Milstein (1975). Five of these 10 monoclones were chosen on the bas-

is of antibody titre for antibody amplification in mouse ascites fluid.

2. Production of ascitic fluid

BALB/cCR mice (5 mice per monoclone) were injected intraperitoneally with 100-200 µl of pristane (2,6,10,14-tetramethyl pentradecane, Aldrich Chemicals) at least 2 weeks prior to use for ascites production. They were then irradiated with 500 rads. Twenty-four hours after irradiation, the mice were injected intraperitoneally with 3-6 x 10^6 hybridoma cells in 1 ml [illegible]. 7 to 10 days later, the mice appeared distended, at which point the ascitic fluid was removed using an 18 gauge needle. The fluid was centrifuged at 8,000 x g to remove cells and debris, then filter sterilized using a 0.45 µm Millipore filter. The monoclones treated in this fashion were called WPC-1, 3, 5 and 6 and were used for all further studies with CFA/1 monoclonal antibodies.

3. ELISA for hybidoma screening, titre determination and monoclonal specificity

The ELISA used for hybridoma screening was a modification of the procedure of Voller et al. (1974) and similar to that described in Section H.3 of this chapter. Briefly, microtitre plates were coated with 100 µl/well of a 4 µg/ml solution of CFA/1 pili for 6-8 hours at 4°C. After wash-

ing, the plates were incubated with 100 µl/well of supernatants from the growing hybridomas for 16 hours at 4°C. Anti-CFA/1 pilus specificity was detected colorimetrically using goat-anti mouse IgG conjugated to alkaline phosphatase (1:500 dilution) and the alkaline phosphatase substrate p-nitro-phenyl phosphate. Absorbance at 405 nm was determined as described previously.

For titre determination and specificity of antibody produced in ascites (WPC-1, 3, 5 and 6) and supernatants (all 10 monoclones), the ELISA was as described above except antigens were allowed to coat the wells for 16 hours and the antigen saturated wells were incubated for only 2 hours with various dilutions of the antibody source (in PBS-T).

4. Class determination of CFA/1 monoclonal antibodies

Ascites fluid from WPC-1, 3, 5 and 6 and the supernatants of the remaining 6 CFA/1 monoclones were used in the ELISA as described above in order to determine their immunoglobulin isotype. After coating microtitre wells with CFA/1 pili, the supernatants diluted 1:3 (starting with a 1:10 dilution) and ascitic fluid diluted 1:5 (starting with a 1:50 dilution) were added and plates incubated for 2 hours at room temperature. This was followed by incubation with goat-anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM conjugated with alkaline phosphatase (kindly donated by

B. Singh, University of Alberta, Edmonton, Alta.). The A_{405} determined after the addition of the substrate was used to distinguish the isotype of the particular monoclonal antibody. In all cases, only a single isotype was recognized for each antibody. This was also additional proof that the antibodies were monoclonal.

J. Iodination

Anti-pilus IgG was iodinated using 1,3,4,6-tetrachloro 3 alpha, 6 alpha-diphenylglycouril (Iodo-gen, obtained from Pierce Chemicals). 100 µg of purified, lyophilized IgG was dissolved in 0.33 M sodium phosphate pH 7.4 and transferred to a 12 x 75 mm tube which was precoated with 40 µg of Iodo-gen (20 µl of 2 mg/ml Iodo-gen in chloroform dried onto the bottom of the tube under nitrogen gas). 0.5 mCi of $Na^{125}I$ was added and the mixture incubated for 60 seconds. The reaction mixture was removed from the Iodo-gen tube and transferred to a second tube containing PBS with 0.1% BSA and 0.1 M KI. This stops the iodination process. 10 µl of this sample was removed, diluted in 2.5 ml PBS and 10 µl of the diluted material counted. This value was then used when calculating the specific activity of the labelled protein. The remaining reaction mixture was passed through a G-25 column (1 x 10 cm) which was equilibrated with 1% BSA in PBS. The iodinated IgG was eluted with

PBS. One ml fractions were collected and 10 µl aliquots were counted for 2 minutes using an LKB 1270 Rackgamma II counter. Peak fractions were pooled and subjected to TCA precipitation to determine which pool contained the protein. TCA precipitation was performed as follows: 25 µl of each sample was mixed with 1 ml of 1% BSA in PBS and 0.5 ml of ice cold 10% (v/v) TCA for TCA + samples or 0.5 ml of PBS for TCA - samples. Protein was allowed to precipitate for 10 minutes on ice, then the tubes were centrifuged at 500 x g to remove the precipitate. 25 µl of each supernatant was counted for radioactivity. Specific activity was calculated using the following formula:

$$\text{Specific Activity} = \frac{\text{Total cpm Added X TCA Precipitable Fraction}}{\text{µg Protein Added}}$$

$$\text{TCA Precipitable Fraction} = \frac{(\text{cpm in (TCA -)} - \text{cpm in (TCA +)})}{\text{cpm in (TCA -)}}$$

Total recovery, which represents the amount of protein removed from the column, was calculated using the following equation:

$$\text{Total Recovery} = \frac{\text{cpm in pool X TCA Precipitable Fraction}}{\text{Specific Activity}}$$

The iodinated protein was run on a SDS polyacrylamide gel. The gel was dried using a BioRad Model 224 Slab Gel Dryer

and set up for autoradiography using Kodak X-OMAT AR film. This was done to establish the amount of iodine in the IgG and in the BSA carrier protein. Usually the majority of counts were localized to the IgG molecules.

K. Affinity Chromatography

1. Preparation of Activated CH Sepharose 4B-ligand column

The general procedure for coupling ligands to Activated CH Sepharose 4B was as described by Pharmacia. Briefly, 0.5-1.0 g of freeze dried Activated Sepharose was placed in a sintered glass funnel. 20 ml of 1 mM HCl was added and resin allowed to swell for 15 minutes. The resin was then washed with 200 ml of ice cold 1 mM HCl while in the funnel and under vacuum. Six mg of synthetic peptide AcP(1-12) conjugated to BSA (^{14}C-gly at 400 cpm/mol) or 1 mg of synthetic peptide AcP(1-8) (also 400 cpm/nmol) was dissolved in 2.5-5.0 ml of 0.1 M $NaHCO_3$ and 0.5 M NaCl pH 8.0. These two species represent the N-terminal peptide of EDP208 pilin. Several aliquots were removed, spotted on Whatman 3 mm filter disks and 10 ml of toluene scintillation fluid was added to the dried disks. The disks were then counted in a Beckman LS 6800 scintillation counter. The washed resin and the protein solution were combined in a 10 ml disposable syringe (to act as the column apparatus), then mixed by inversion for one hour at room tempera-

ture. The effluent was collected and column washed with 2 column volumes of the reaction buffer to remove any unattached protein. The unreacted groups on the sepharose were blocked by incubating the column in 0.1 M Tris HCl, pH 8.0 for one hour at room temperature. The effluent was collected and the column washed with alternating column volumes of 0.1 M sodium acetate, 0.5 M NaCl pH 4.0 and 0.1 M Tris-HCl pH 8.0. This was done 5 times, then washed with a final 5 volumes of PBS. 2-3 ml fractions were collected at each stage. 500 µl aliquots from each fraction was mixed with 10 ml of ACS and counted in the scintillation counter. Generally, approximately 50% of the input protein or peptide remained covalently attached to the column.

2. Affinity purification of anti-[redacted]208 pilus specific antibodies

The general procedure for affinity purification of antibodies was the same whether using a 100 µl aliquot of the column or the whole column and was as follows: 1 ml of pure IgG in PBS was added to the resin and incubated with constant mixing for 2 hours at 37°C. Depending on the IgG preparation, 1 ml contained 0.35 to 0.79 mg. The fate of the input IgG was monitored either by following the absorbance at 280 nm or counting aliquots at each step in the gamma counter when ^{125}I labelled IgG was used. The effluent was collected, either by pelleting the resin in an Ep-

pendorf Centrifuge 5414 for 30 seconds when 100 µl of the resin was used, or by elution from the 10 ml syringe column when a large amount of IgG was used in the experiment. The resin was washed with several volumes of PBS and 1 ml fractions were collected. After the final wash, 1 ml of 6 M guanidine HCl in 0.2 M ammonium bicarbonate, pH 8.4 was added and the resin was incubated for 1 hour at 37°C. The effluent was collected, the resin washed with PBS, and the fractions collected as above. The unbound antibody in the first effluent was pooled and stored at - 20°C. The bound antibody, which was removed from the resin by treatment with 6 M guanidine-HCl, was pooled and dialyzed overnight against distilled water then stored at -20°C.

3. Characterization of affinity purified antibodies using ELISA

The specificity of the three species of anti-EDP208 pilus specific antibody obtained from affinty chromatography with Activated CH Sepharose-AcP(1-8) or AcP(1-12)-BSA were examined using both types of ELISA described in Section H.5 of this chapter. In the regular ELISA, microtitre wells were coated with 5 µg/ml of EDP208 pili and 5 ug/ml of AcP(1-12)-BSA. The three types of IgG (input, unbound and bound) were serially diluted, starting with 25 pmol/ml and used in the assay. Antibody-antigen complexes were detected colorimetrically using goat-anti-rabbit IgG

alkaline phosphatase followed by p-nitrophenyl phosphate.

L. Lipopolysaccharide (LPS) Purification and Analysis

1. Rapid identification of LPS type

Bacteria were grown overnight at 37°C on m[redacted] normally used for pilus purification. The bacteri[redacted]e gently scraped from agar plates and suspended in PBS to a density of 10^9 cells/ml as determined by adjusting t[redacted] O.D. at 5[redacted]0 nm to 0.12. One ml of the suspension was pelleted in an Eppendorf microcentrifuge Model 5414 for 5 minutes. The supernatant was removed and pellet suspended in 50 µl of Laemmli sample buffer containing 50 mM DTT (see Section D of this chapter for specifications). The solution was sonicated in a Branisonic 220 sonicating water bath and then boiled for 5 minutes. After cooling, 10 µl of proteinase K (Boeringer Mannheim Corp., 1 mg/ml in Laemmli sample buffer) was added. The samples were mixed well and incubated for 2 hours at 60°C with intermittent mixing. Aliquots were then reboiled for 5 minutes and 10 µl of each were loaded on a slab gel for SDS-PAGE. After electrophoresis, the gel was fixed overnight and silver stained. Both gel preparation and staining were discussed in Chapter II, Section D. The presence of a ladder indicates smooth LPS. The absence of a ladder (a series of silver stained bands representing the range of multiple repeating oligosaccha-

ide linkages on the LPS core) but presence of a low molecular weight band represents rough LPS.

2. Purification of LPS by Hot Phenol-Water extraction

LPS purification was according to Westphal and Jann (1965). Bacteria were grown overnight on six, 14.4 cm diameter petri dishes containing the appropriate agar. Bacteria were removed by gentle scraping and washed once in water and pelleted by centrifugation at 8000 x g. The pellet was weighed to get an estimate of the amount of bacteria. 0.7 - 1.5 g of pelleted bacteria was used in these experiments. 35 ml of 70°C double distilled water was added to suspend the pellet. The sample was reheated to 70°C and mixed with 35 ml of preheated 90% (v/v) aqueous phenol for 15 minutes at 70°C. The mixture was cooled and centrifuged at 5000 x g for 50 minutes after which three layers were observed: an insoluble residue in the bottom phenol phase, a white layer at the phenol-water interface and a slightly opalescant top aqueous layer. The top aqueous phase, containing the LPS, was carefully removed. The remaining phenol layer was re-extracted once more with water. The aqueous layers were pooled and the LPS precipitated by adding 1 mg/ml of dry sodium acetate and 3 volumes of acetone, and incubating overnight at -20°C. The precipitate was removed by centrifugation at 8000 x g for 10 minutes. The pellet was washed first with 70% (v/v) acetone, then 100% acetone.

The final pellet was dried under nitrogen. The pellet was resuspended in 10 ml of double distilled water and centrifuged at 45,000 rpm for 2 hours, in a Beckman L2-65B centrifuge, using a Beckman 50 Ti rotor. The supernatant was removed, and the pellet was resuspended in more water and recentrifuged. The final pellet was resuspended in a minimum volume of water and lyophilized. Yields ranged from 1-8 wt%. Purity was checked by SDS-PAGE. Amount of contaminating protein was checked by the Lowry procedure (1951) and found to be less than 1%, by weight.

3. Detection of LPS specific antibodies by ELISA

To check if any LPS was co-purified with various types of pili, both direct and competition ELISA's were done using anti-pilus antisera. The direct ELISA was a modification of that described in Chapter II, Section H.5. Microtitre wells were coated with 100 µl of the pili examined as well as with 500 µg/ml of purified LPS from the strain in question. The coated wells were then incubated with 1/10 dilutions of pre-immune sera, pilus specific sera or monoclonal antibodies. Antibody-antigen complexes were detected colorimetrically with the alkaline phophatase system as previously described.

Competition ELISAs were done to determine if LPS was contaminating the pili used in previous assays. Microtitre wells were coated with pili as before. The competition mix-

tures contained either pili or LPS as the competitor (with LPS at a starting concentration of 200,000 pmol/ml for rough LPS and 300 µg/ml for smooth LPS). Antibody-antigen complexes were detected colorimetrically using alkaline phosphatase. Percent inhibition was calculated and the results presented graphically by plotting % inhibition versus competitor concentration.

M. Bacterial Adherence Assays

1. Adherence of H10407 bacteria to tissue culture cells

Tissue culture cells were grown in MEM containing 10% FCS to confluency in 24-well tissue culture plates (0.5-2.0 x 10^6 cells/well). H10407 bacteria were grown overnight on CFA/1 agar. The bacteria were then scraped from the agar and gently resuspended in L-broth to limit shearing pili from the cells. This suspension was checked for the presence of CFA/1 and type 1 pili by hemagglutination (Chapter II, Section B) and by antibody agglutination using 10 µl of cells in 10 µl of CFA/1 specific antisera. Bacteria were then diluted to 2-4 x 10^{10} CFU/ml (A_{660} = 0.2-0.3) in L-broth. The adjusted suspensions were serially diluted in MEM. Tissue culture wells were washed with MEM, to remove unattached cells and FCS-containing growth medium. To the washed monolayers, 200 µl of a 10^{-6} (containing 2-4 x

10^4 CFU/ml) dilution of bacteria was added and incubated for 2-3 hours on ice. 100 µl of the 10^{-7} and 10^{-8} dilutions of the input bacteria were plated on TSA plates to determine the number of CFU added to the monolayer. After incubation, unattached bacteria were removed and wells were washed with three 0.5 ml aliquots of MEM. The washes were pooled and 100 µl plated on TSA plates, then incubated overnight at 37°C. All experiments were done in duplicate and the number of unbound bacteria was determined by counting the resultant colonies. The extent of bacterial adherence to FCS coated, cell free tissue culture plates was determined in the same manner.

2. Adherence of H10407 bacteria to rabbit intestinal cells

Rabbits ranging in age from neonates to 2 year old adults were used for the examination of attachment of H10407 P^+ and P^- bacteria. Adherence assays were performed using 0.5 x 0.5 cm intestinal pieces. Rabbits were sacrificed and the small intestines removed. The excised intestines were placed immeadiately in ice cold MEM tissue culture medium then cut open longitudinally. The intestinal sheets were gently spread open, luminal surface up, on a chilled glass plate. Mucous was removed with cotton swabs. The clean intestine was then cut into pieces with a razor blade and pieces placed in MEM medium in tissue culture

plates.

The adherence assay used was similar to that previously described. The bacteria were suspended in L-broth and diluted as before. Adherence to pieces was performed in 24 well tissue culture plates. Pieces were placed in 200 µl of MEM containing the final bacterial suspension (5-8 x 10^3 bacteria). After incubation for 2 hours on ice, the supernatant solutions were removed from the pieces which were then washed with three 0.5 ml aliquots of MEM. The wash solutions were pooled and 100 µl plated on TSA plates. The results were expressed as percent of input bacteria unbound.

Similar experiments were performed with pig, mouse, guinea pig and human intestinal pieces. The assays were identical and results expressed in the same fashion as that previously described.

3. Adherence of H10407 bacteria to human buccal cells

The basic adherence assay used was that described in the previous section. Buccal cells were harvested by scraping the inner lining of the mouth with cotton swabs. Cells were suspended in MEM and washed twice by centrifugation at 900 x g for 5 minutes. Cells were resuspended at approximately 2 x 10^6 cells/200 µl and added to 1.5 ml microcentrifuge tubes. Bacteria were grown, checked for piliation and diluted as described previously. 200 µl of bacteria (5-

8 x 10^3) was added to the buccal cells and incubated as before. Supernatant solutions were removed, buccal cells were washed with MEM, and 100 µl of the pooled supernatants solution were spread on McConkey agar (Difco) plates. As with the other experiments, bacterial binding to microfuge tubes in the absence of buccal cells was determined.

4. Adherence of H10407 bacteria to human type A erythrocytes

The presence of CFA/1 pili on H10407 bacteria was detected by the ability of these bacteria to agglutinate human type A erythrocytes (Evans et al., 1977). Hence, the ability of H10407 CFA/1 P+ and CFA/1 P- bacteria to adhere to erythrocytes was examined, using the same assay desribed previously in this section. Out dated human erythrocytes (obtained from the Canadian Red Cross, Edmonton, Alta.) were washed three times in PBS (containing 1 mM Mg^{+2} and [illegible] mM Ca^{+2}) by centrifugation at 120 x g. Bacteria were checked for piliation then adjusted to a density 2-4 x 10^{10} CFU/ml. 50 µl of a 10^{-3} dilution of the bacterial suspension was mixed with 200 µl of a 2 x 10^8 cell/ml erythrocyte suspension in sterile 15 ml capped, conical centrifuge tubes and incubated for 1 hour at 0°C. After incubation, the red blood cells were pelleted, the supernatants were collected and diluted appropriately. These dilutions were plated, on TSA plates, along with the appro-

priate dilutions of input bacteria. For the estimation of the number of bacteria bound to the erythrocytes, the final erythrocyte pellet was incubated for 20 minutes, at 37°C with 200 µl of 0.05% (w/v) trypsin. 100 µl of the trypsinized pellet was plated on TSA plates. The resultant colonies were enumerated and compared to the number of CFU added to the assay to determine the percent of bacteria which were associated with the erythrocytes (refered to as % input bacteria bound). To determine the effect of this trypsinization on bacterial viability and division, the appropriate strains of bacteria were plated on TSA plates before and after incubation for 20 minutes, at 37°C in 0.05% trypsin. The standard control experiments were performed in the absence of red blood cells and the results were expressed as % of input bacteria unbound. Incubation at 0°C was chosen as the standard temperature to minimize bacterial division during the assay. This assay was also performed using types O and B blood with no significant difference in results. Type A blood was chosen as the result of convention in keeping with the hemagglutination studies done by Evans et al. (1977).

To determine if the adherence of H10407 bacteria to erythrocytes was pilus specific, erythrocytes were preincubated with increasing amounts of CFA/1 pili for one hour at 0°C prior to performing the adherence assay described earlier. Results were expressed graphically by plotting %

bacteria bound versus concentration of competing pili.

To determine the effects of neuraminidase and trypsin on pilus receptors, human type A erythrocytes were pre-treated with these enzymes prior to performing the adherence assay. Two mg of trypsin (bovine pancreatic trypsin, type III, Sigma Chemical Co.) were dissolved in one ml of PBS containing 1 mM Ca^{+2} and 1 mM Mg^{+2}. A 1:50 dilution of washed, packed erythrocytes was incubated for one hour, at 37°C with a final concentration of 1 mg/ml trypsin. The reaction was stopped with PMSF (6.7 mg of PMSF was dissolved in 1.0 ml of DMSO the diluted 1:10 in PBS). The same procedure was used for the neuraminidase (Boehringer Mannheim, from Clostridium perfringes) treatment except a final concentration of 1.0 µg/ml was used. The binding assay described earlier in this section was then performed and the percent of input bacteria bound to the erythrocytes was calculated.

CHAPTER III

The Immunodominant Region of the EDP208 Pilus Protein

A. Introduction

EDP208 conjugative pili are encoded by a derepressed derivative of a naturally occuring lac plasmid, Folac, originally isolated from Salmonella typhi (Falkow and Baron, 1962). Armstrong et al. (1980) have shown that EDP208 pili contain a single repeating polypeptide subunit of 11,500 daltons, each with a blocked N-terminus. More recently, Frost et al. (1983) used ^1H NMR to identify the blocking group as an N-acetyl moiety. They also determined the amino acid sequence of the 12 residues at the N terminus of this protein. This dodecapeptide was the primary product released upon digestion of the pilin monomer with trypsin, without any further cleavage in the remaining C terminal region.

The wish to characterize the immunological properties of this EDP208 pilin N-terminal dodecapeptide brought about the studies discussed in this chapter. This provided a model system in which it was possible to precisely delineate a major antigenic determinant using synthetic peptides and peptide-BSA conjugates and sensitive immunological assays

such as immunoblotting and competitive ELISA. These experiments allowed us to identify a major antigenic site, to deduce which amino acids were necessary for antibody-antigen interactions and to determine whether the antigenic region was immunogenic. These studies could provide a general strategy for identifying antigenic sites on other pilus proteins, primarily those which are virulence factors on pathogenic bacteria. They also provide the basis for potential vaccine development through the use of synthetic peptides. Finally, the examination of the antigenic structure of such proteins provides a useful strategy for the surface mapping of proteins, by locating regions on the surface of the protein that are recognizable by the mammalian immune system.

B. Results and Discussion

1. Preliminary studies

As shown by Frost et al.(1983), treatment of the EDP208 pilin monomer with trypsin resulted in a cleavage at Lys^{12} to yield an N-terminal dodecapeptide, ET1 (Mr= 1500), and the remaining C terminal fragment, ER (Mr=10, 000). ET1 will also denote the equivalent synthetic peptide AcP(1-12), which has a C-terminal amide instead of the free alpha carboxyl group. Partial cleavage also occured at Lys^{8} to yield the smaller fragments ET2 (residues 1-8)

and ET3 (residues 9-12). The sequences of these peptides are shown in Table III.1. To determine whether any of these peptides were antigenic, all four were purified acccording to the method of Frost et al. (1983) and used in an ELISA with anti-EDP208 pilus specific antisera. ET1 and ER showed some reactivity with the antibody whereas ET2 and ET3 failed to show any significant reaction with the antibody. These results suggested that both the N-terminal and C-terminal regions may contain antigenic sites. However, it was difficult to determine whether the antigenicity of the EDP-208 peptides was being masked in any way by their attachment to a solid substrate in the microtitre wells. To overcome this problem, and the fact that the native fragments were difficult to purify in the quantities needed for immunological studies, the N-terminal dodecapeptide was synthesized and covalently coupled to a BSA carrier protein. The remaining studies were then performed with either synthetic peptides or their corresponding peptide-conjugates.

2. Specificity of anti-pilus antisera for EDP208 pili

Before proceeding with further experiments to localize antigenic sites on the EDP208 pilus protein, the antibody source was examined for pilus specificity. We were particularily concerned with the possibilty that the anti-pilus antiserum may contain antibodies against the highly immunogenic surface molecule, lipopolysaccharide (LPS),

TABLE III.1

Amino acid sequence of tryptic peptides from EDP208 pilin

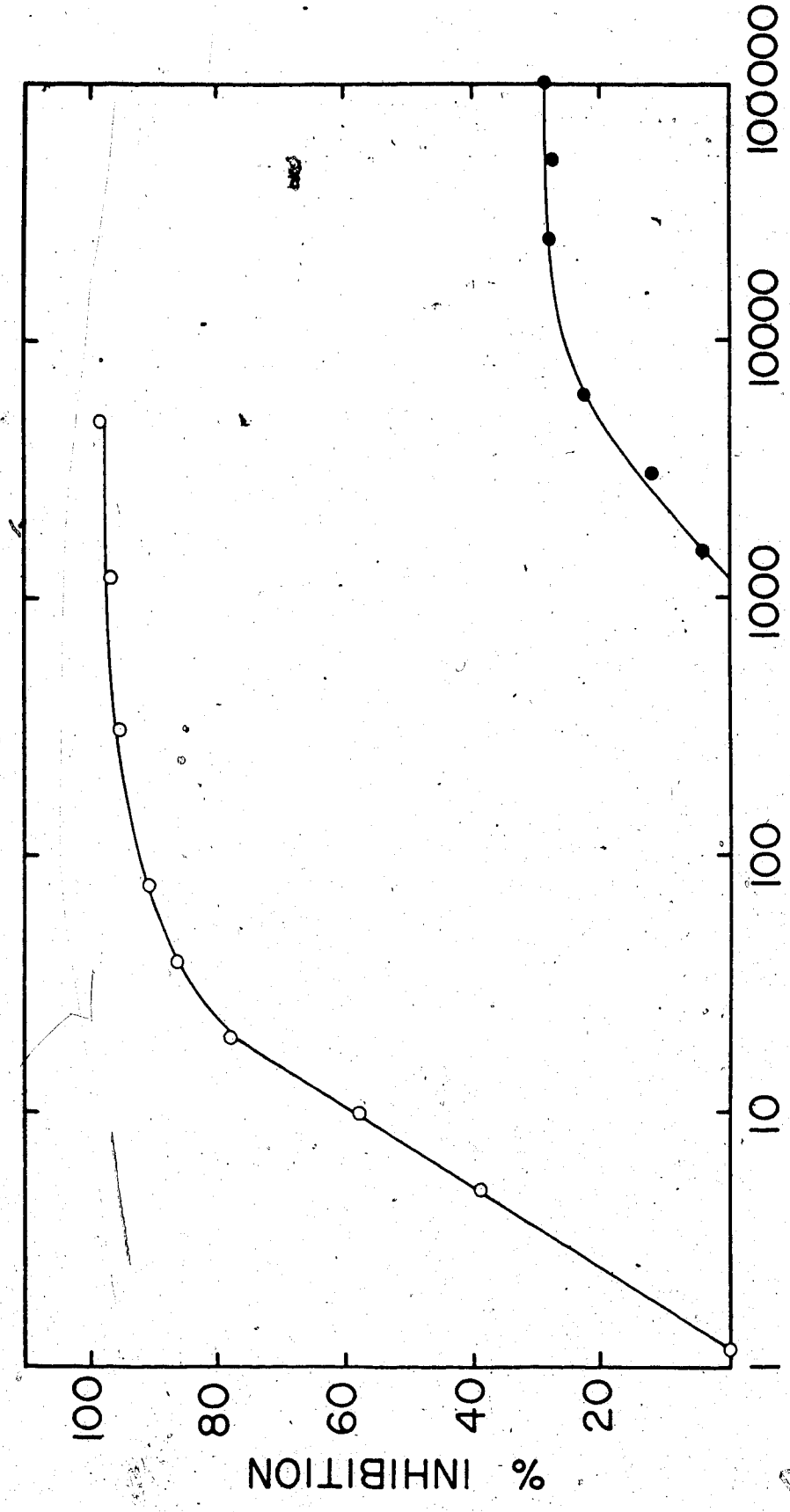
Peptide	No. of Residues	Amino Acid Sequence[a]
ET1	12	Ac-Thr-Asp-Leu-Leu-Ala-Gly-Gly-Lys-Asp-Val-Asp-Lys
ET2	8	Ac-Thr-Asp-Leu-Leu-Ala-Gly-Gly-Lys
ET3	4	-Asp-Val-Asp-Lys

a. Sequence from Frost et al. (1983).

which could have co-purified with the pili. To test for this possibility, LPS was purified from the E. coli strain JC6256 (the strain used for EDP208 pili preparation) and examined for its abilty to interact with anti-pilus antiserum.

LPS from JC6256 was determined to be the rough type, Ra, using the proteinase K digestion procedure described in Chapter II, Section L.1. The LPS was then purified on a large scale by the hot phenol water method (Chapter II, Section L.2) and checked for purity by SDS-PAGE followed by silver staining (Figure III.1). The purified LPS was then used in a direct ELISA (Chapter II, Section L.3) to test for the presence of anti-LPS antibodies in pre-immune and EDP208 pilus specific sera. In this thesis a direct ELISA refers to an assay where: 1) antigens are used to coat the microtitre wells, 2) antibody is added to the antigen coated wells, 3) a second antibody having alkaline phosphatase crosslinked to it is used to detect the extent of the antibody-antigen interaction in 1) and 2). In both cases anti-LPS antibodies were not detected. The same LPS was then used in the competition ELISA (Chapter II, Section L.3). The competition was performed using purified IgG from EDP-208 pilus specific antisera and results are shown graphically in Figure III.2. Contrary to results from the direct ELISA, one can see that purified LPS was capable of titrating out a small amount of anti-LPS IgG, but that the rela-

Figure III.2 Competitive ELISA using purified LPS from E.coli JC6256 as the competitor. Microtitre wells were coated with EDP208 pili were incubated with competition mixtures consisting of LPS, EDP208 pili and pilus specific IgG. IgG was present as 25 pmole/ml in each mixture. Details of the assay are found in Materials and Methods.
Symbols: ○ EDP208 pili as the competitor, ● JC6256 LPS as the competitor.


% INHIBITION
100
80
60
40
20
1
10
100
1000
10000
100000
COMPETITOR CONCENTRATION (pmoles / ml)

tive strength of the LPS-IgG interaction was approximately 1000 fold less than that of the pilus-IgG interaction. It is therefore unlikely that the anti-LPS antibodies would interfere in any significant way in immunological assays involving antigenic determinants of EDP208 pilin.

3. Antigenicity and immunogenicity of the N-terminal dodecapeptide, ET1

The N-terminal dodecapeptide was synthesized by solid phase synthesis, as described in Chapter II, Section G.2 and covalently coupled to BSA (ET1-BSA) as described in Section G.5 of the same chapter. The final peptide had the sequence N-alpha-Thr-Asp-Leu-Leu-Ala-Gly-Gly-Lys-Asp-Val-Asp-Lys-amide which was identical to the native sequence of this peptide. The peptide was coupled to BSA through the epsilon amino groups of Lys^8 and Lys^{12}, using the photoreactive coupling reagant, The N-hydroxysuccinimide ester of 4-azidobenzoic acid (ABONSu). The peptide conjugates had ET1:BSA ratios of 2.8:1 and 72:1. The 72:1 conjugate was used for immunizing rabbits to make anti-ET1-BSA antisera.

a) ELISA studies using anti pilus and anti ET1-BSA antisera

Antisera obtained from rabbits immunized with either purified pili or synthetic ET1-BSA were prepared as described in Chapter II, Section F.1. The antisera were used in direct ELISA's to determine the extent of interaction

with pili, ET1, ER and ET1-BSA. ER is the insoluble C terminal fragment obtained after trypsin digestion of the pilin monomer. The assays were performed as described in Section F.5 of Chapter II and involved coating microtitre wells with saturating amounts of antigen, and incubation with dilutions of the appropriate sera followed by detection of antibody-antigen complexes with goat-anti-rabbit conjugated to alkaline phosphatase. The results obtained are shown in Figure III.3.

The ET1-BSA conjugates interacted to a large extent with antipilus antisera, indicating that ET1 is an antigenic determinant. This was also reflected in the ability of free ET1 to interact with the antipilus antibodies. The A_{405}, or ELISA values are quite low for the free peptide which probably reflects the steric effects caused by the peptide binding to the plastic wells. That is, the region of the peptide containing the antigenic site may also be the site of attachment to the plastic. Alternately, ET1 may not be binding to the microtitre wells to the same extent as the corresponding peptide-BSA conjugate. With ET1-BSA, the peptide is coupled to BSA via lysine residues leaving the rest of the peptide free to interact with antibody. There was little difference in antibody binding to either the 2.8:1 or 72:1 conjugate. This could be explained by the extensive crosslinking of peptide on the 72:1 conjugate, resulting in masking all but a small fraction of the ET1

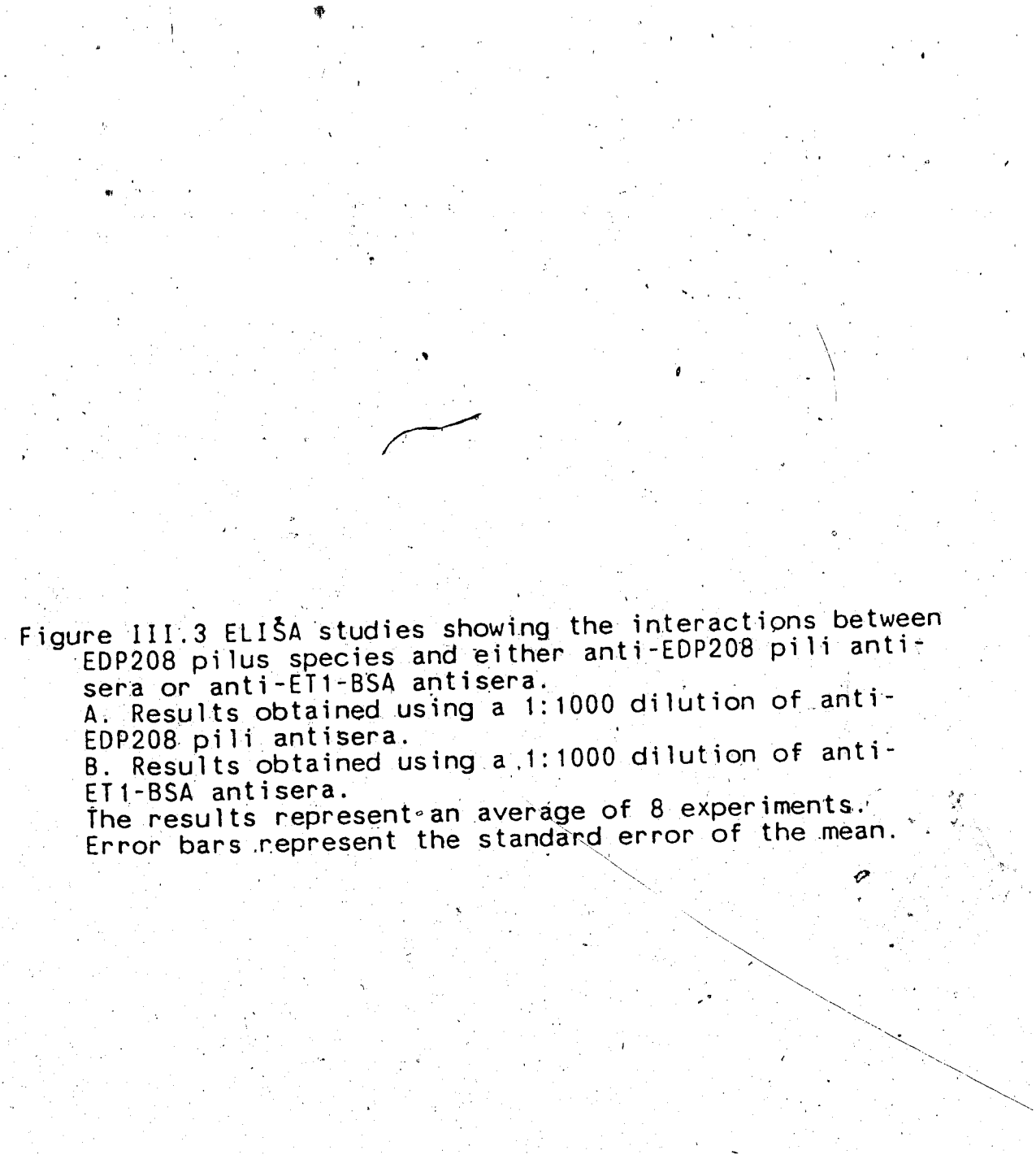
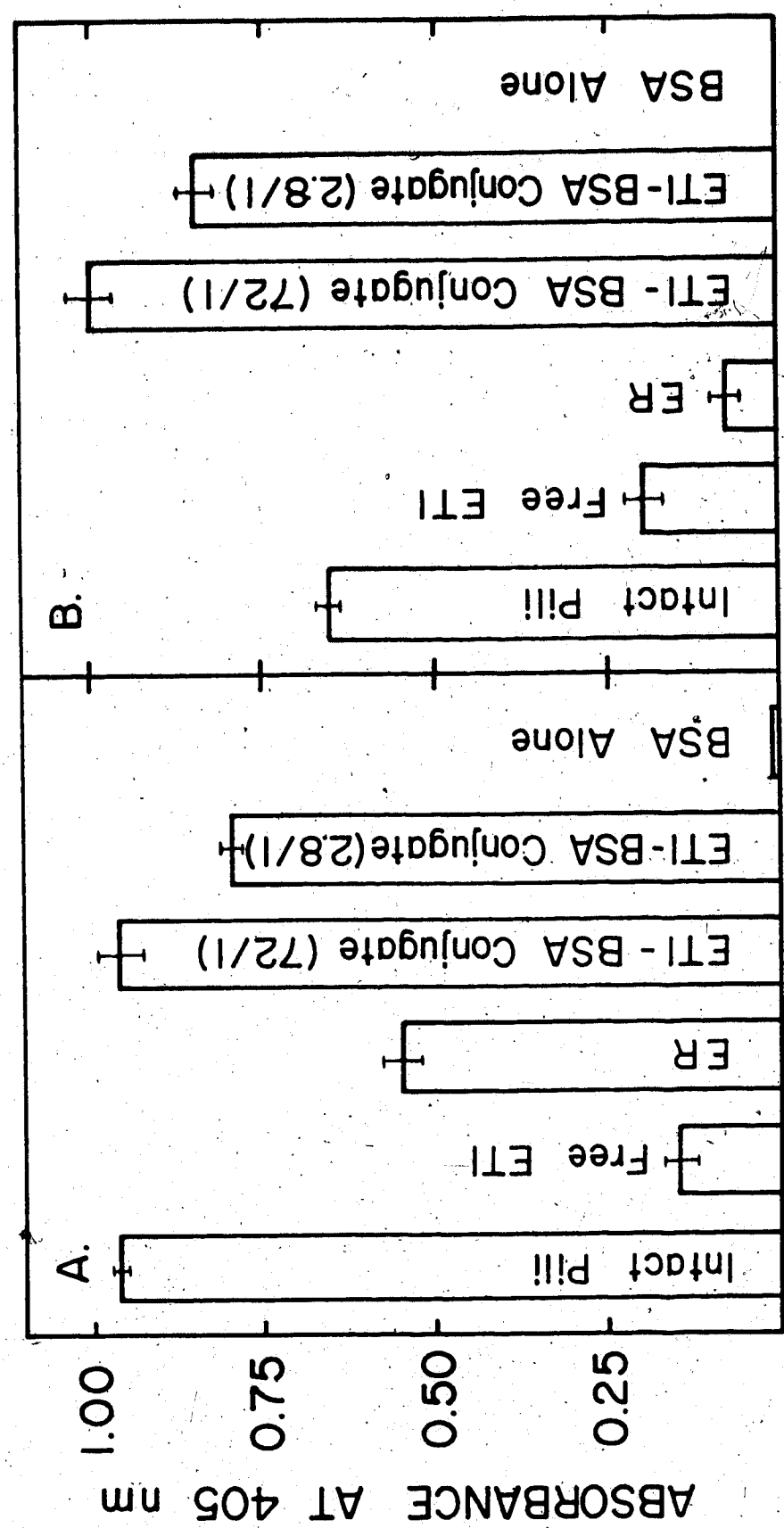


Figure III.3 ELISA studies showing the interactions between EDP208 pilus species and either anti-EDP208 pili antisera or anti-ET1-BSA antisera.
A. Results obtained using a 1:1000 dilution of anti-EDP208 pili antisera.
B. Results obtained using a 1:1000 dilution of anti-ET1-BSA antisera.
The results represent an average of 8 experiments.
Error bars represent the standard error of the mean.


ABSORBANCE AT 405 nm
1.00
0.75
0.50
0.25
A.
Intact PIII
Free ETI
ER
ETI - BSA Conjugate (72/1)
ETI-BSA Conjugate(2.8/1)
BSA Alone
B.
Intact PIII
Free ETI
ER
ETI - BSA Conjugate (72/1)
ETI-BSA Conjugate (2.8/1)
BSA Alone

molecules.

ET1-BSA was also immunogenic, as indicated by the results in panel B of Figure III.3. Anti-ET1-BSA antibodies were capable of interacting with intact pili, as well as the free peptide and peptide conjugate. There was a low level of interaction with the C terminal peptide ER but this may be explained as being due to a small amount of undigested pilin contaminating the ER preparation.

On the basis of these experiments it was concluded that at least two antigenic sites existed on the pilus protein. Antipilus antibodies reacted strongly with the N-terminus and ER in the ELISA. Even if one considers that a small amount of pilin may be contaminating the ER preparation, the level of interaction with ER suggests that this fragment probably contains one or more antigenic determinants. However, since further digestion of ER was difficult because of it's insolubility and high proportion of hydrophobic residues (see Table III.2 for amino acid compositions), the examination of this fragment for antigenic regions was postponed.

b) Immunoblot analysis of EDP208 pilus species

A second approach used to evaluate the antigenicity of ET1 and ER was that of immunoblotting. The procedure was described in Chapter II, Section F.2 and involved the electrophoretic transfer of protein from SDS-polyacrylamide

TABLE III.2

Amino acid compositon of EDP208 pilin and corresponding tryptic peptides[a]

Amino acid	Pilin	ER	ET1
Lys	7	5	2
His	0	0	0
Arg	2	2	0
Asp	10	7	3
Thr	12	11	1
Ser	2	1	0
Glu	2	1	0
Pro	0	0	0
Gly	11	9	2
Ala	9	8	1
1/2Cys	2	N/D[b]	0
Val	10	8	1
Met	5	5	0
Ile	10	8	0
Leu	12	10	2
Tyr	2	2	0
Phe	7	7	0
Trp	0	0	0
Total	103	84	12

a. Amino acid composition based on values obtained from amino acid analyses done by Frost et al. (1983).

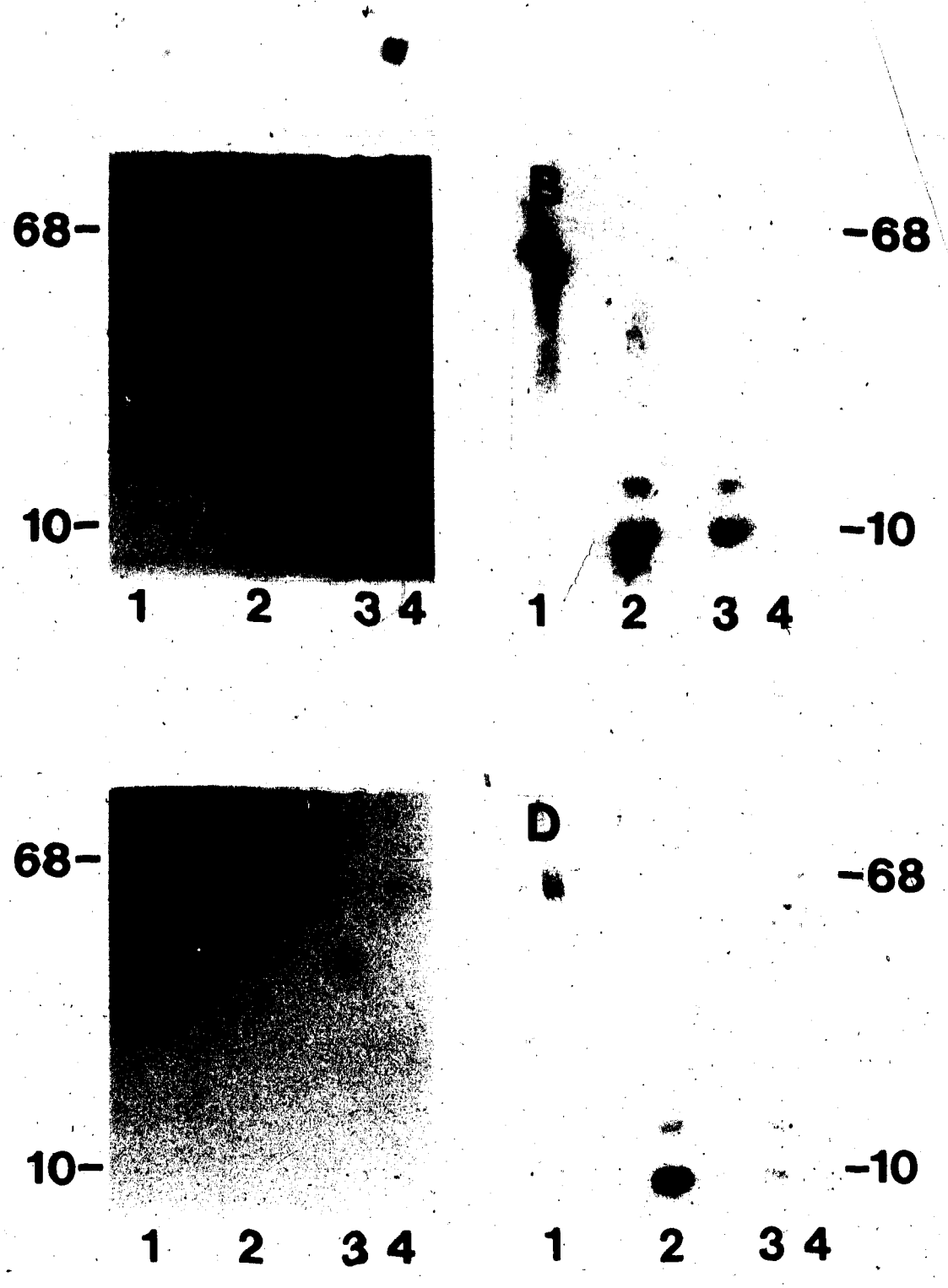
b. Not determined.

gels to nitrocellulose paper. The nitrocellulose was treated with pilus specific or ET1-BSA specific antisera and antibody-antigen complexes detected using ^{125}I labelled protein A (from Staphylococcus aureus) followed by autoradiography. Duplicate gels were run: one was used for the transfer of proteins to nitrocellulose, while the other was stained with Coomassie blue for direct visualization of the protein.

As shown in Figure III.4A, all proteins were easily detected by the staining procedure. Both pilin and ER usually migrated in the monomeric state but often ran as a dimer which was readily seen after immunoblotting and autoradiography (Figure III.4B). Extended boiling of these two species often led to aggregation as detected by SDS-PAGE (unpublished results). Only the 2.8:1 ET1-BSA conjugate was used as the 72:1 complex was too large to penetrate the gel. The ET1-BSA migrated differently than BSA which can be due to the modifications of BSA during the conjugation process. Figure III.4C shows that the transfer process was efficient as no protein bands were detected upon staining of the gel after transfer.

Figure III.4B and 4D represent autoradiograms obtained for the corresponding nitrocellulose sheets treated with anti-pilus and anti ET1-BSA antisera, respectively. As expected on the basis of the ELISA results in Figure III.3, anti-pilus antisera (Figure III.4B) reacted approximately

Figure III.4 SDS-polyacrylamide gel electrophoresis and transfer of EDP208 pilus proteins to nitrocellulose paper.
Lane 1, 5 µg of ET1-BSA (2.8:1); lane 2, 5 µg of EDP208 pili: lane 3, 5 µg of ER; lane 4, 5 µg of BSA.
A. Coomassie blue stained SDS polyacrylamide gel.
B. Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose and reacted with a 1:1000 dilution of anti-EDP208 pili antisera and ^{125}I labelled protein A.
C. Coomassie blue stained duplicate of gel A after the transfer process.
D. Autoradiograph of proteins transferred from gel A to nitrocellulose and reacted with a 1:50 dilution of anti-ET1-BSA antisera and ^{125}I labelled protein A.


68-
10-
1 2 3 4
1 2 3 4
-68
-10
D
68-
10-
1 2 3 4
1 2 3 4
-68
-10

twice as strong with pilin as with ER. The interaction with ET1-BSA was high, confirming that ET1 constitutes an antigenic region. Figure III.4D shows the expected interaction of anti-ET1-BSA antisera with ET1-BSA, as this was the immunogen, but more interesting is the interaction with pili. This re-enforces the observation that the N terminal dodecapeptide is an antigenic site and confirms the idea that this region in the intact pilus is immunogenic. This antiserum did not react with BSA in either immunoblot or ELISA experiments. This was not surprising since any anti-BSA antibodies were probably complexed by the large amounts of BSA used in the antibody dilution buffers. As mentioned earlier, the weak interaction of ER with the anti ET1-BSA sera was probably due to contaminating, uncleaved pilin.

Both types of antisera were capable of interacting with pili to a larger extent than the peptide conjugate in this assay. In the ELISA there appears to be an equivalent amount of interaction of the two species with the two sera. The reason for this was unclear but may reflect the different levels of accessibility of ET1 for antibody binding in the two assays. In the immunoblot, the proteins may be bound to the nitrocellulose by a different region of the molecule than in the ELISA and the antigenic site may be masked in the former assay.

On the basis of the results from the two experimental procedures discussed in this section, one can conclude that

the N-terminal dodecapeptide is one of the major antigenic determinants of the EDP208 pilus protein, and that one or more additional sites may exists in the C terminal portion of the protein. ET1 was also shown to be immunogenic, since antibodies raised to this peptide interacted with pili. Since this area was also available for trypsin digestion, it is likely that it is exposed on the surface of the protein, perhaps as a loop or hinge that is available for antibody binding and B-cell recognition.

4. The role of the acetylated N-terminus in the antigenicity of the N terminal dodecapeptide, AcP(1-12)

Antigenic prediction rules, such as those established by Hopp and Woods (1981), suggest that antigenic determinants are usually located in, or adjacent to, regions of greatest hydrophilicity. On this basis, one would predict that the C terminus of ET1 (residues 7-12, Gly-Lys-Asp-Val-Asp-Lys-amide) should contain the antibody combining site since this region was more hydrophilic than the N terminal part (residues 1-5, NAc-Thr-Asp-Leu-Leu-Ala). However, coupling of ET1 to BSA through residues Lys^{8} and Lys^{12} did not abolish the antigenicity of the peptide, suggesting that these residues may not be involved with antibody binding. Thus, it became necessary to consider the less hydrophilic N-terminal region of ET1 as the antigenic site and to ask if the unusual N-terminal blocked amino acid NAc-Thr

contributed to the antigenicity of this peptide. To attempt to answer this question, a series of analogs of ET1 were synthesized, coupled to BSA and used in immunoblot and ELISA assays. The list of peptides synthesized for all subsequent experiments are in Table III.3, with modified residues circled.

The first peptide analog to be synthesized was AcP (1-8). This peptide was covalently linked to BSA and tested for antigenicity using the same techniques as described for ET1. AcP(1-8) was found to be as antigenic and immunogenic as the dodecapeptide. Immunoblot analysis, as well as ELISA studies (data not shown), using anti-AcP(1-8)-BSA antibodies confirmed that this smaller peptide contained the antigenic site of ET1 and that the octapeptide was just as immunogenic as the dodecapeptide, in that these antibodies strongly interacted with intact EDP208 pili. These results support the suggestion that the antigenic determinant is located in the hydrophobic rather than the hydrophilic region of the N-terminal dodecapeptide.

The next question asked was how important is the N-acetyl moiety on the N terminal amino acid for antigenicity. To answer this question, a number of analogs of AcP (1-8) were made, covalently attached to BSA and tested for antigenicity in the direct ELISA. The results from the ELISA are shown in Figure III.5. Each analog was examined for its ability to interact with pilus specific (Figure III.5A) or

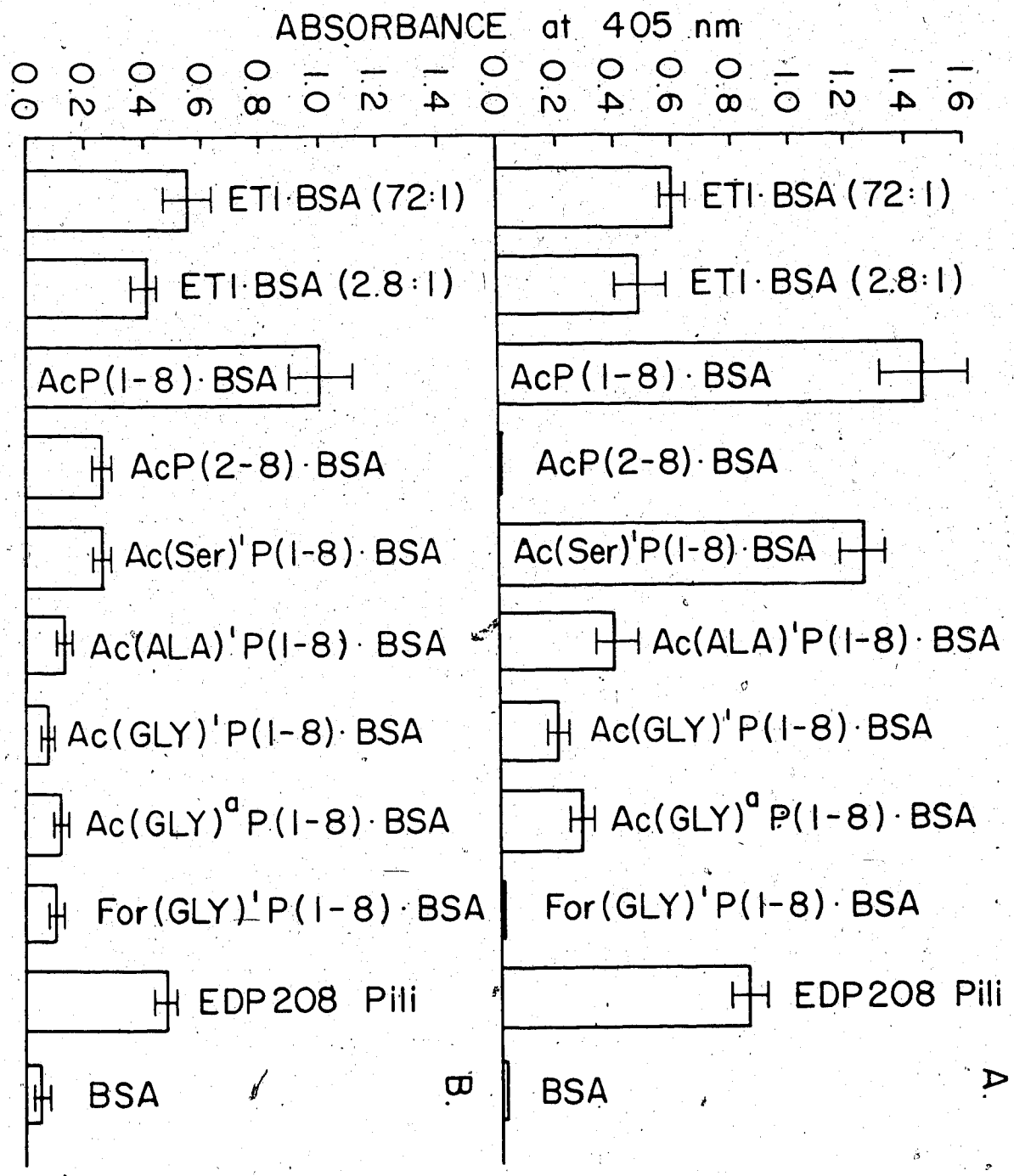
TABLE III.3

Amino acid sequences of synthetic peptide analogs of the N-terminal dodecapeptide of EDP208 pilin

Peptide[a]	Peptide Sequence
AcP(1-12)	Ac-Thr-Asp-Leu-Leu-Ala-Gly-Gly-Lys-Asp-Val-Asp-Lys-amide
AcP(1-8)	Ac-Thr-Asp-Leu-Leu-Ala-Gly-Gly-Lys-amide
Ac(Ser)^{1}P(1-8)	Ac-Ser-Asp-Leu-Leu-Ala-Gly-Gly-Lys-amide
Ac(Ala)^{1}P(1-8)	Ac-Ala-Asp-Leu-Leu-Ala-Gly-Gly-Lys-amide
Ac(Gly)^{1}P(1-8)	Ac-Gly-Asp-Leu-Leu-Ala-Gly-Gly-Lys-amide
Ac(Gly)^{a}P(1-8)	Ac-Gly-Thr-Asp-Leu-Leu-Ala-Gly-Gly-Lys-amide
For(Gly)^{1}P(1-8)	For-Gly-Asp-Leu-Leu-Ala-Gly-Gly-Lys-amide
AcP(2-8)	Ac-Asp-Leu-Leu-Ala-Gly-Gly-Lys-amide
Ac(Gly)^{5}P(1-8)	Ac-Thr-Asp-Leu-Leu-Gly-Gly-Gly-Lys-amide
Ac(Gly)^{4,5}P(1-8)	Ac-Thr-Asp-Leu-Gly-Gly-Gly-Gly-Lys-amide
Ac(Gly)^{3,4,5}P(1-8)	Ac-Thr-Asp-Gly-Gly-Gly-Gly-Gly-Lys-amide
Ac(Gly)^{2,3,4,5}P(1-8)	Ac-Thr-Gly-Gly-Gly-Gly-Gly-Gly-Lys-amide
AcP(1-5)	Ac-Thr-Asp-Leu-Leu-Ala-amide
Ac(Gly)^{1}P(1-5)	Ac-Gly-Asp-Leu-Leu-Ala-amide
Ac(Gly)^{2}P(1-5)	Ac-Thr-Gly-Leu-Leu-Ala-amide
Ac(Gly)^{3}P(1-5)	Ac-Thr-Asp-Gly-Leu-Ala-amide
Ac(Gly)^{4}P(1-5)	Ac-Thr-Asp-Leu-Gly-Ala-amide
Ac(Gly)^{5}P(1-5)	Ac-Thr-Asp-Leu-Leu-Gly-amide
ForP(1-5)	For-Thr-Asp-Leu-Leu-Ala-amide
AcP(1)	Ac-Thr-amide

a. Nomenclature example: Ac(Ser)^{1}P(1-8) is the synthetic N-terminal acetylated EDP208 pilin fragment, residues 1-8 with a C terminal amide and a serine substitution at position 1. (Gly)^{a} represents an additional gylcine on the N-terminal end of residue 1. The circled residues are the modifications from the native sequence.

Figure III.5 ELISA studies showing the ability of synthetic peptide conjugates to interact with anti-EDP208 pili or anti-ET1-BSA antisera.
A. Results obtained using a 1:1000 dilution of anti-EDP208 pili antisera.
B. Results obtained using a 1:1000 dilution of anti-ET1-BSA antisera.
Details of experiments are in Materials and Methods.
Error bars represent the standard error of the mean obtained from 3 individual experiments.


ABSORBANCE at 405 nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
ETI·BSA (72:1)
ETI·BSA (2.8:1)
AcP(1-8)·BSA
AcP(2-8)·BSA
Ac(Ser)'P(1-8)·BSA
Ac(ALA)'P(1-8)·BSA
Ac(GLY)'P(1-8)·BSA
Ac(GLY)ᵃP(1-8)·BSA
For(GLY)'P(1-8)·BSA
EDP208 Pili
BSA
B
A

ET1-BSA specific antisera (Figure III.5B). All of the peptides in this set of experiments were modified at the N-terminus. Dealing with panel A first, the most striking result was seen with AcP(2-8)-BSA, which did not react at all with the pilus specific antisera. The same result was observed in immunoblot experiments with AcP(1-8)-BSA specific antisera (data not shown) This suggested that the threonine at position 1 was important for antibody binding to this peptide. It was not certain if the threonine side chain was important or the spacing between the N-acetyl of Thr^1 and the carboxyl of Asp^2 was the feature necessary for antibody binding. The importance of the side chain functional groups of the first amino acid was examined by replacing the threonine in the first position with a serine, an alanine or a glycine. The conservative change from threonine to serine, Ac(Ser)^{1}P(1-8)-BSA, resulted in only a minor decrease in antibody binding. This suggests that the methyl of the threonine side chain is unimportant for the peptide-antibody interaction. When the threonine was replaced by either an alanine or a glycine, Ac(Ala)^{1}P(1-8)-BSA or Ac(Gly)^{1}P(1-8)-BSA, a significant decrease in antibody binding occurred, re-enforcing the hypothesis that the hydroxyl group of the threonine is important for peptide antibody interactions. The addition of an extra glycine to the N-terminus, Ac(Gly)^{a}P(1-8)-BSA was carried out to determine the importance of the length and spacing between func-

tional groups in the antigen antibody interaction. This peptide also showed a decreased ability to bind pilus specific antibodies, but not lower than that seen with Ac(Ala)^{1}P(1-8)-BSA or Ac(Gly)^{1}P(1-8)-BSA. At this stage, it is difficult to form any firm theories as to the actual contributions of the N-acetyl and the threonine hydroxyl to the peptide-antibody interaction.

The final modification in this set of experiments was the replacement of the NAc-Thr with an N-formyl glycine, For(Gly)^{1}P(1-8)-BSA. This peptide conjugate was unable to interact with anti-pilus antibodies, suggesting that both the acetyl and the hydroxyl moieties are important functional groups in the antibody-antigen interaction. To examine the contribution of each of the remaining amino acid side chains in this antigenic peptide, several additional analogs were synthesized and subjected to a competition ELISA. The results are discussed in the next section of this chapter.

Panel B of Figure III.5 represents the relative affinities of the forementioned synthetic peptide-conjugates for anti-ET1-BSA antibodies. The results are quite similar to those observed for anti-pilus antibodies. The octapeptide binds more antibody than any of the other conjugates or even pili. This may reflect the ability of the synthetic peptide to conform to the antigen binding site of the antibody more efficiently than same region in intact pilin. The

peptide probably has a greater flexibility than the pilus protein. Ac(Ser)^{1}P(1-8)-BSA did not interact with these antibodies to the same extent as observed with anti-EDP208 pilus specific antibodies. This may be due to a difference in specificity of the two antibody sources perhaps involving the importance of the methyl group of Thr^1. All of the other conjugates bound anti-ET1-BSA antibodies to some extent, even AcP(2-8)-BSA and For(Gly)^{1}P(1-8)-BSA which did not interact with pilus specific antisera. Since the antibody source is polyclonal, it probably contains antibodies which recognize overlapping determinants on a protein. For example, one antibody population may bind to residues 1-6, while another recognizes residues 2-7. This phenomema was observed by Atassi (1975) during his studies on the antigenic structure of myoglobin. However, AcP(2-8) does not interact with pilus specific antisera, suggesting that if there does exist a group of antibodies specific for this peptide, this population was too small to be detected by the assays used in these studies.

This series of experiments provided two additional pieces of information regarding the nature of the antigenic determinant at the N-terminus of the EDP208 pilus protein. First, the N-terminal octapeptide was found to be equally antigenic to the dodecapeptide, thus reducing the antigenic site from 12 to 8 residues. Second, the acetyl and hydroxyl moieties of the first amino acid, NAc-Thr, were found to

be key functional groups in the antigen-antibody interaction.

5. Identification of specific amino acids responsible for the antigenicity of the N terminal octapeptide, AcP(1-8)

The results presented in the previous sections showed that the major antigenic determinant of EDP208 pilin is in the N-terminal octapeptide, AcP(1-8). Moreover, studies with synthetic analogs of the octapeptide containing modifications in the N-terminal amino acid, NAc-Thr, revealed that this residue contributes significantly to the antigen antibody interaction in this system. To further deduce the importance of each residue in the octapeptide in this interaction, the unconjugated peptides presented in Table III.3 were synthesized and examined immunologically. However, in this series of experiments the peptides were subjected to a "competition" rather than a "direct" ELISA procedure.

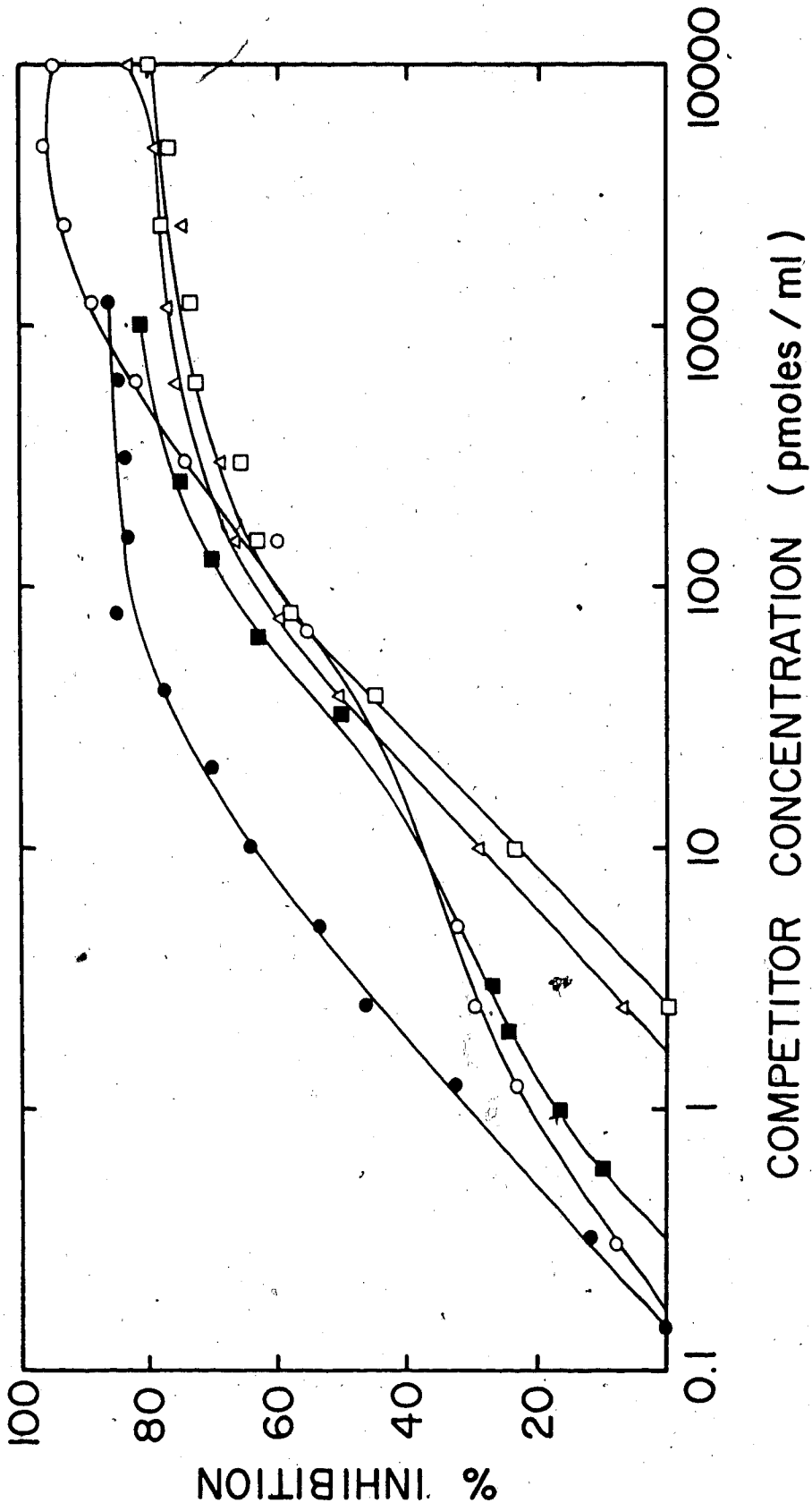
The competition ELISA is particularily useful for comparing the antigen-antibody interaction of a series of peptides. To avoid complications which may arise from the use of divalent IgG molecules (higher binding constants were obtained with intact pili than with peptides when IgG was used in this assay, due to the ability of IgG to cross-bridge on the pili but not with free peptides: data not shown), monovalent Fab fragments were prepared (Chapter II,

Section F.4) and their ability to interact with EDP208 pili and peptide antigens was compared as described in Chapter II Section F.5. This procedure provides a measure of the amount of anti-pilus antibody in the polyclonal Fab preparation which is directed against a specific antigenic determinant, as well as a quantitation of the ability of each peptide to inhibit the pilus-antibody interaction (eg. 50% inhibition). It should be noted that free peptides can be used in the competition ELISA whereas peptide-conjugates are preferred for use in the direct ELISA.

Figure III.6 shows the results of a competition ELISA in which the activity of free and BSA conjugated AcP(1-12) and AcP(1-8) were compared to that of whole EDP208 pili. It may be seen that at maximum inhibition whole pili titrated almost all of the pilus specific Fab, while the free peptides and their corresponding peptide conjugates titrated approximately 80-85% of the same pilus specific Fab. These results confirmed earlier observations that the major proportion of antibodies in the anti-EDP208 pilus sera are directed against an epitope in the N-terminal octapeptide region.

From Figure III.6, one can also observe that the I_{50} values, i.e., the concentration of competitor required to achieve 50% inhibition of antibody (in this case Fab) binding to the pilus coated microtitre wells, were essentially the same (approximately 50 pmol/ml) for the free

Figure III.6 Competition ELISA using synthetic peptides, peptide conjugates, and EDP208 pili as competitors with EDP208 pilus-specific Fab as the antibody source. Fab used at 25 pmole/ml in competition mixtures. See Materials and Methods for experimental detail.
Symbols: O EDP208 pili, ● AcP(1-8)-BSA, □ AcP(1-12), ■ AcP(1-12)-BSA, △ AcP(1-8).


% INHIBITION
100
80
60
40
20
0.1
1
10
100
1000
10000
COMPETITOR CONCENTRATION (pmoles / ml)

peptide and intact pili. The I_{50} value for the AcP(1-12)-BSA conjugate was also in the general range (25 pmol/ml), while AcP(1-8)-BSA yielded a value of 4 pmol/ml. Thus, it is evident that the relative affinity of EDP208 pilus specific antibodies for the N-terminal octapeptide is virtually the same as that for whole pili, suggesting that the competition ELISA is the method of choice for comparing the interaction of synthetic peptides with anti-pilus antibodies.

a. Calculation of apparent affinity constants (aK) from I_{50} values obtained from the competition ELISA

Nieto et al. (1984) discussed another aspect of the competition ELISA that is applicable to the studies described in this chapter. These authors described the relationship between the apparent affinity constant, aK, and the I_{50} values obtained from inhibition curves. They showed that aK can be defined as the reciprocal concentration of free hapten (or peptide) required for 50% inhibition of antibody binding to immobilized antigen (or pili which is coating the microtitre wells). The aK values calculated from their ELISA studies were found to be in good agreement with K values obtained from classical experiments used to determine affinity constants, such as the Farr assay (Stupp et al., 1969). The Farr assay involves the immunoprecipitation of several concentrations of radiolabel-

led antigen with a constant amount of antibody. The affinity constant is calculated from the Sips plot of log (b/Ab_t-b) versus log[Ag]: when $\log(b/Ab_t-b)=0$, then $K = 1/[Ag]$. b is the concentration of bound antigen and Ab_t is the total number of antibody binding sites. Nieto et al. (1984) cites several examples where the aK and K values are very similar. One example was that of an anti-hapten monoclonal antibody called 31-76. The aK value of 1.9×10^6 l/mole was in agreement with the K of 1.10×10^6 l/mole determined by Rothstein and Gefte (1983), using floures-cence quenching. Nieto et al. (1984) also performed both the Farr assay and competitive ELISA to determine the average affinity constant for an anti-p-azophenyl-arsenate antibody. Results from the Farr assay described an affinity constant of $K = 1.96 \times 10^5$ l/mole, whereas the inhibition assay produced an $aK = 1.0 \times 10^5$ l/mole. On the basis of these considerations, aK values were calculated from the I_{50} values for each of the peptide analogs examined in the present study. The I_{50} and corresponding aK values are presented in Table III.4.

A brief description of Nieto's logic for correlating the apparent affinty constant to the I_{50} values from competition experiments is as follows:

If one assumes that antibody reacts with free antigen (Ag_f) and the antigen attached to the solid surface (Ag_s) then the following reaction can occur:

TABLE III.4

Relative affinities of EDP208 pilus specific antibodies for synthetic peptide analogs of the N-terminal antigenic region

Peptide	I-50[a] (pmole/ml)	Group Altered	aK[b](1/mole)
EDP208 pili	45	none	2.2×10^7
AcP(1-12)	45	none	2.2×10^7
AcP(1-8)	35	none	2.9×10^7
Ac(Ser)^{1}P(1-8)	45	Thr^1	2.2×10^7
Ac(Ala)^{1}P(1-8)	8,000	Thr^1	1.3×10^5
Ac(Gly)^{1}P(1-8)	10,000	Thr^1	1.0×10^5
Ac(Gly)^{a}P(1-8)	5,000	Ac-Thr^1	2.0×10^5
For(Gly)^{1}P(1-8)	300,000	Ac-Thr^1	3.3×10^3
AcP(2-8)	no inhibition	Thr^1	none
Ac(Gly)^{5}P(1-8)	190	Ala^5	5.2×10^6
Ac(Gly)^{4,5}P(1-8)	62,000	Leu^4Ala^5	1.6×10^4
Ac(Gly)^{3,4,5}P(1-8)	100,000	Leu^3 - Ala^5	1.0×10^4
Ac(Gly)^{2,3,4,5}P(1-8)	>100,000	Asp^2 - Ala^5	$<1.0 \times 10^4$
AcP(1-5)	9	none	1.1×10^8
Ac(Gly)^{1}P(1-5)	5,000	Thr^1	2.0×10^5
Ac(Gly)^{2}P(1-5)	390	Asp^2	2.6×10^6
Ac(Gly)^{3}P(1-5)	58,000	Leu^3	1.7×10^4
Ac(Gly)^{4}P(1-5)	180,000	Leu^4	5.6×10^3
Ac(Gly)^{5}P(1-5)	170	Ala^5	5.9×10^6
ForP(1-5)	75,000	CH^3	1.3×10^4
AcP(1)	no inhibition	none	none

a. I-50 = competitor concentration required for 50% inhibition in the competitive ELISA

b. aK is the apparent affinity constant determined by the method of Nieto et al. (1984).
aK = 1/I-50

$$Ab + Ag_f = AbAg_f \quad K_f = (AbAg_f)/(Ab)(Ag_f) \qquad (1)$$

$$Ab + Ag_s = AbAg_s \quad K_s = (AbAg_s)/(Ab)(Ag_s) \qquad (2)$$

In our case the free antigen is the competitor, either pili or synthetic peptide, and the solid surface antigen is the pili coating the microtitre wells. Canatarero et al. (1980) found that there was no competition for binding to the solid surface in a mixture of proteins, provided that the total protein concentration was low. This means that using small concentrations of both antibody and competitor in the competition mixture will ensure that any exchange of bound or free antigen in the antigen binding pocket of the antibody will be minimal when the competition mixture is incubated in the pili coated microtitre wells.

They also assume that Ag_s bound to the antibody is almost negligible with respect to the total Ag_s, thus K' can be defined as:

$$K' = K/K_s(Ag_s) = (Ab)(Ag_f)/(Ab)(Ag_s)(Ag_f) \qquad (3)$$

When 50% binding is achieved, the antibody concentration bound to the solid phase $[(AbAg_s)_{50}]$ is half of the value found when no antigen is used in the competition ($[AbAg_s]_{100}$), which are the results obtained from the non-competitive data in our assay. Thus:

$$(AbAg_s)_{100} = 2(AbAg_s)_{50} \qquad (4)$$

and from equations (1) and (3):

$$(AbAg_s)_{100} = K/K'(Ab)_{100} \qquad (5)$$

where $(Ab)_{100}$ is the concentration of free antibody in

the absence of antigen. In our case we used from 2.5-5.0 x 10^{-8} mole/l of antibody in all competition experiments. From equation (3), (4) and (5):

$$K = 1/(Ag_f)_{50} \times 2(AbAg_f)_{50}/(Ab)_{100} \quad (6)$$

where $(Ag_f)_{50}$ is the concentration of free antigen and $(AbAg_f)_{50}$ is the concentration of soluble antibody-antigen complex that is in equilibrium with the solid phase antibody-antigen complex when 50% inhibition has occured. They assumed that $(AbAg_f)_{50}$ and $(Ab)_{100}$ concentrations were similar enough so that:

$$2(AbAg_f)_{50}/(Ab)_{100} = 2(AbAg_f)_{50}/(Ab)_{100} - 1 \quad (7)$$

and aK can be defined as:

$$aK = 1/(Ag_f)_{50} \times 1 \text{ or } aK = 1/(Ag_f)_{50} \quad (8)$$

They make the assumption that the amount of antibody at 50% inhibition is so small that it becomes negligible when calculating aK. The final relationship is similar to the final relationship between the K calculated by other methods, that is $K = 1/[Ag]$. Nisonoff and Pressman (1958) described the average association constant, K_o, for an antibody as the reciprocal of free hapten concentration when one half of the combining sites on the antibody are occupied. Nieto et al. (1984) assume that the association and dissociation rate of the antibody-antigen interaction are similar enough to be called equal, and thus can correlate the Nisonoff relationship to the aK.

Application of the Nieto relationship to some of the

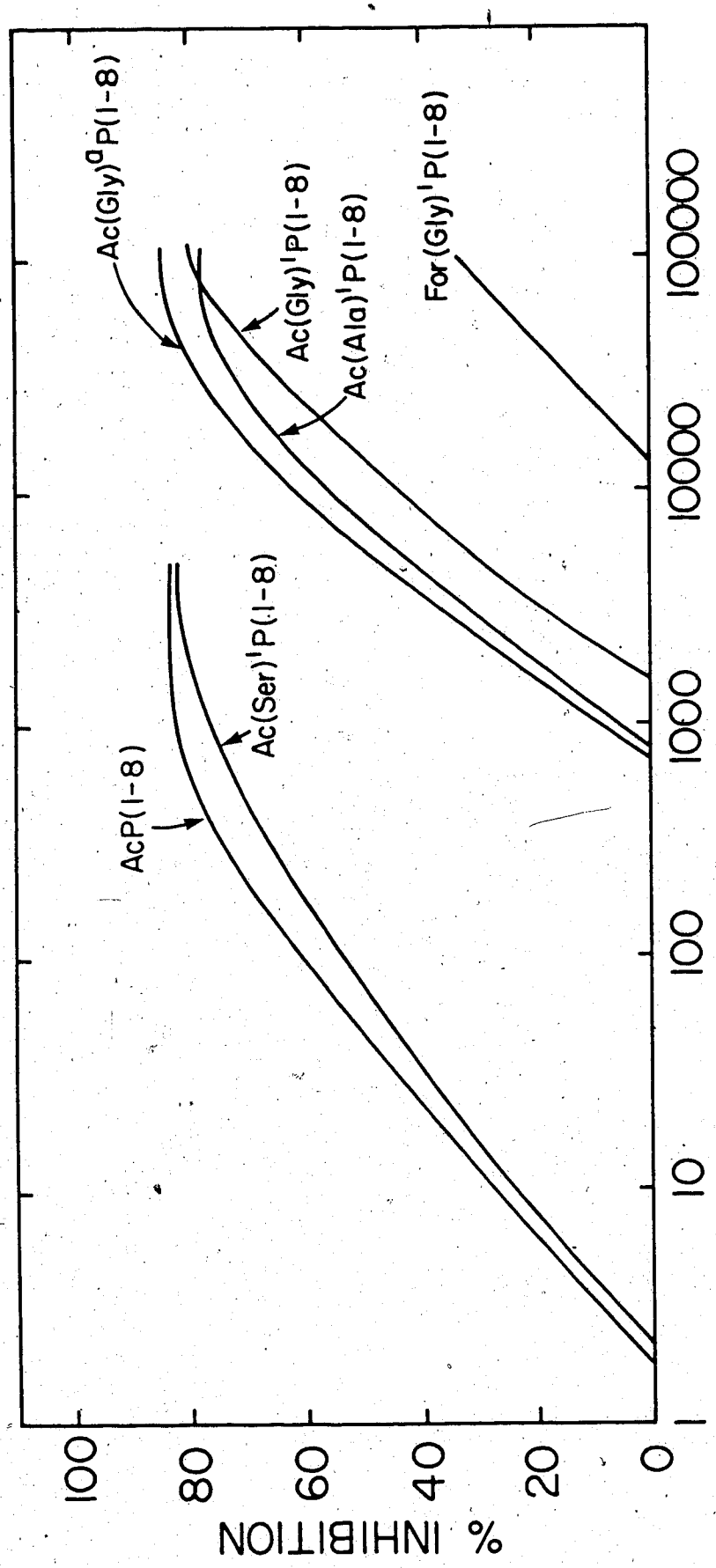
I_{50} values obtained from experiments described using EDP208 pilus specific antibody resulted in the list of affinity constants found in Table III.4, under the heading of aK. The aK values of AcP(1-5) and EDP208 pili, for example are 1.1×10^8 1/mole and 1.0×10^7 1/mole, respectively. These are in the range of K values determined by more conventional methods (Nimmo et al., 1984; Griswold and Nelson, 1984). The values for EDP208 pilus antigens are one log higher than most found in the literature, which may reflect the high affinity antibodies generated by our methods. The aK values for the peptides containing the major antigenic site are higher than that calculated for pili which again may be attributed to the flexibility of a peptide over a multimeric protein and their ability to assume a better fit in the antigen-binding pocket of the antibody. The only aspect not discussed by Nieto was the valency difference between Fab and IgG. Nisonoff (1960) found that the equilibrium constants for the association of antibody or Fab with a hapten are approximately the same, thus it probably makes no difference in the aK determination whether IgG or Fab was used.

b) Synthetic analogs of AcP(1-8)

The importance of the N terminal amino acid of EDP208 pilin, acetyl threonine, was examined using a series of peptide analogs made by replacing this residue with other

amino acids (Table III.3). The results of the competition ELISA with N-terminally modified analogs of AcP(1-8) are presented graphically in Figure III.7 (inhibition curves) and quantitatively in Table III.4 (I_{50}'s and aK). All peptides were compared to AcP(1-8), instead of whole pili, since this peptide was shown to contain the major antigenic determinant of the pilus protein and the results in Figure III.6 showed that this peptide has the same relative affinity for anti-pilus antibody as intact pili. When the threonine was replaced by serine, Ac(Ser)^{1}P(1-8), there was little detectable decrease in the peptide's ability to bind antibody, as compared to AcP(1-8). This suggests that the methyl group on the threonine side chain is unimportant for antibody binding. Replacing the threonine with an alanine, Ac(Ala)^{1}P(1-8), or a glycine, Ac(Gly)^{1}P(1-8), resulted in a decrease in binding affinity reflected in a 300 fold increase in the concentration of peptide needed for 50% inhibition (see Table III.4 for the I_{50} values). This suggests that the hydroxyl group on the threonine (or the serine) in the first position is important for antibody binding to this antigen. A further decrease in binding affinity was observed for the peptide For(Gly)^{1}P(1-8). These results indicate that both the acetyl and hydroxyl moieties of the N terminal threonine are necessary for optimum antibody binding. The insertion of an extra glycine between the acetyl group and the threonine (Ac(Gly)^a

Figure III.7 Competition ELISA using synthetic peptide analogs of AcP(1-8) having subtitutions for the N-terminal amino acid as competitors and EDP208 pilus specific Fab as the antibody. Fab used at 25 pmole/ml in competition mixtures.
Each curve is labelled appropriately.

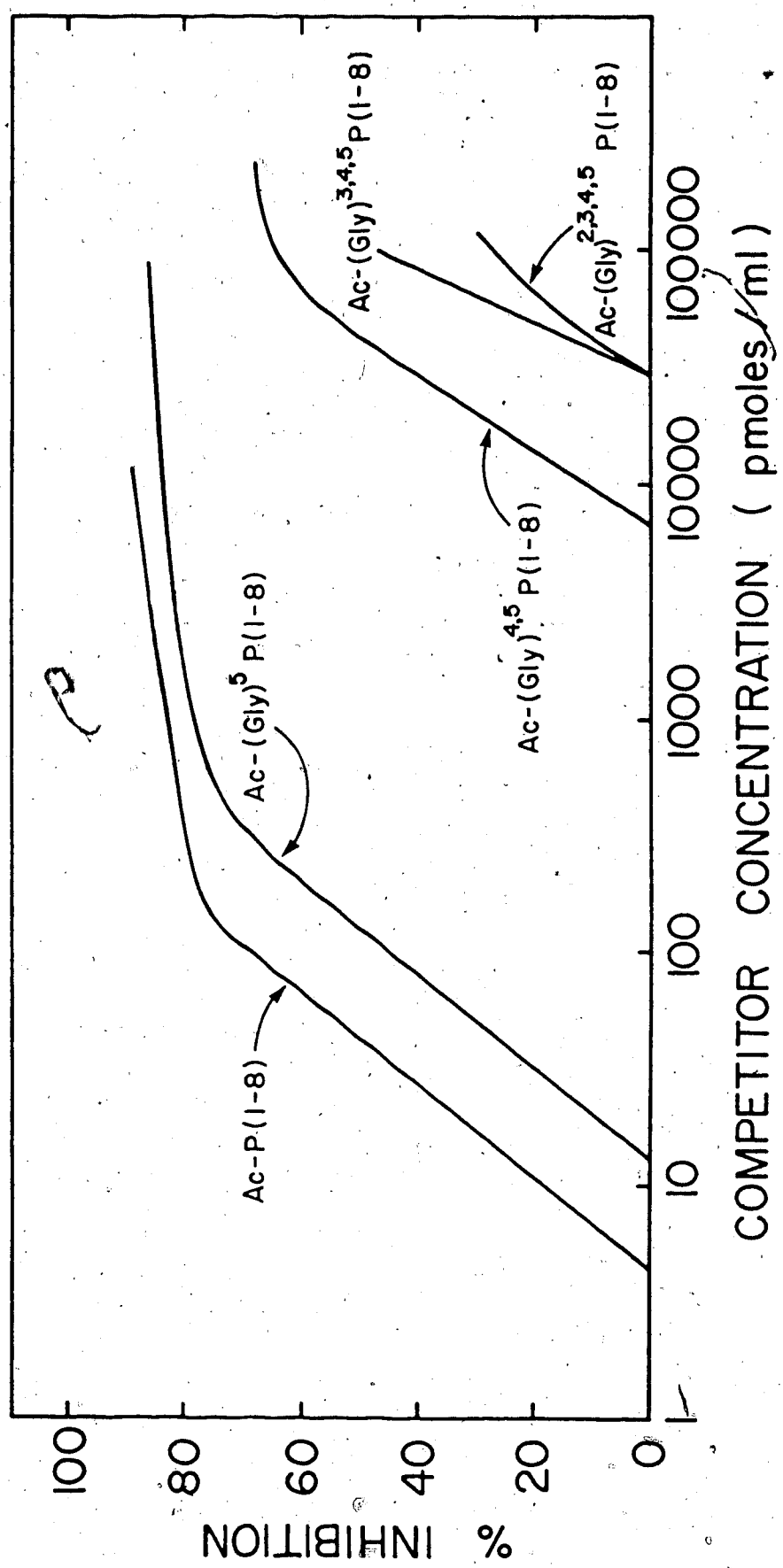

% INHIBITION
100
80
60
40
20
0
1
10
100
1000
10000
100000
COMPETITOR CONCENTRATION (pmoles / ml)
Ac(Gly)^{9}P(1-8)
Ac(Gly)^{1}P(1-8)
Ac(Ala)^{1}P(1-8)
For(Gly)^{1}P(1-8)
Ac(Ser)^{1}P(1-8)
AcP(1-8)

P(1-8)), resulted in a similar binding profile and affinity values as Ac(Gly)^{1}P(1-8), suggesting that the acetyl and hydroxyl groups must be in close proximity to have effecient antibody binding. The complete removal of the first amino acid, as with AcP(2-8), destroyed any detectable antibody-antigen interaction, confirming the importance of the N-acetyl threonine in the antibody-antigen interaction of this system.

In general, the results obtained from the competition ELISA were very similar to those found with the direct ELISA performed with the corresponding peptide conjugates and presented in Figure III.5. The only difference was seen with the peptide For(Gly)^{1}P(1-8), which did not react with anti-EDP208 pilus antibodies in the direct ELISA. The competition ELISA showed that this peptide did interact to a small extent with the antibody source.

The results found with the N-terminally modified peptides could be interpreted to mean that the acetyl threonine is the only residue responsible for the antigenicity of this region. For reasons discussed in the previous section, however, this did not seem likely. To determine the possible contribution of other residues in the peptide, another set of analogs of AcP(1-8) was synthesized, where each amino acid in the peptide was progressively replaced with a glycine. These peptides are listed in Table III.3. The results presented graphically in Figure III.8, while

Figure III.8 Competition ELISA using synthetic analogs of AcP(1-8) which have adjacent residues replaced with glycines as competitors. EDP208 pilus specific Fab was used at 25 pmole/ml in competition mixtures. Each curve is labelled appropriately


% INHIBITION
0
20
40
60
80
100
1
10
100
1000
10000
100000
COMPETITOR CONCENTRATION (pmoles/ml)
Ac-P(1-8)
Ac-(Gly)5 P(1-8)
Ac-(Gly)4,5 P(1-8)
Ac-(Gly)3,4,5 P(1-8)
Ac-(Gly)2,3,4,5 P(1-8)

I_{50} and aK values are listed in Table III.4.

Replacement of Ala^5 with a glycine, $Ac(Gly)^{5p}$ (1-8), resulted in a slight decrease in antibody binding, indicating that the alanine methyl side chain group contributes only slightly to the overall antibody binding. However, the accumulative effect of removing both the alanine and leucine-4 side groups in $Ac(Gly)^{4,5p}(1-8)$, resulted in approximately a 3 log decrease in the aK values, when compared to AcP(1-8). This suggests that the bulky leucine group in the fourth position is very important for the interaction with antibody. When leucine 3 was also replaced with a glycine, $Ac(Gly)^{3,4,5p}(1-8)$, there was a further decrease in the aK value. It is therefore thought that both leucine 3 and 4 are important in the pilus-antibody interaction. The final analog, in which Asp 2 was also replaced by a glycine residue, $Ac(Gly)^{2,3,4,5p}(1-8)$, provided a further, but not substantial, decrease in binding affinity. This suggests that the carboxyl of the second residue is only involved to a small extent in the binding process

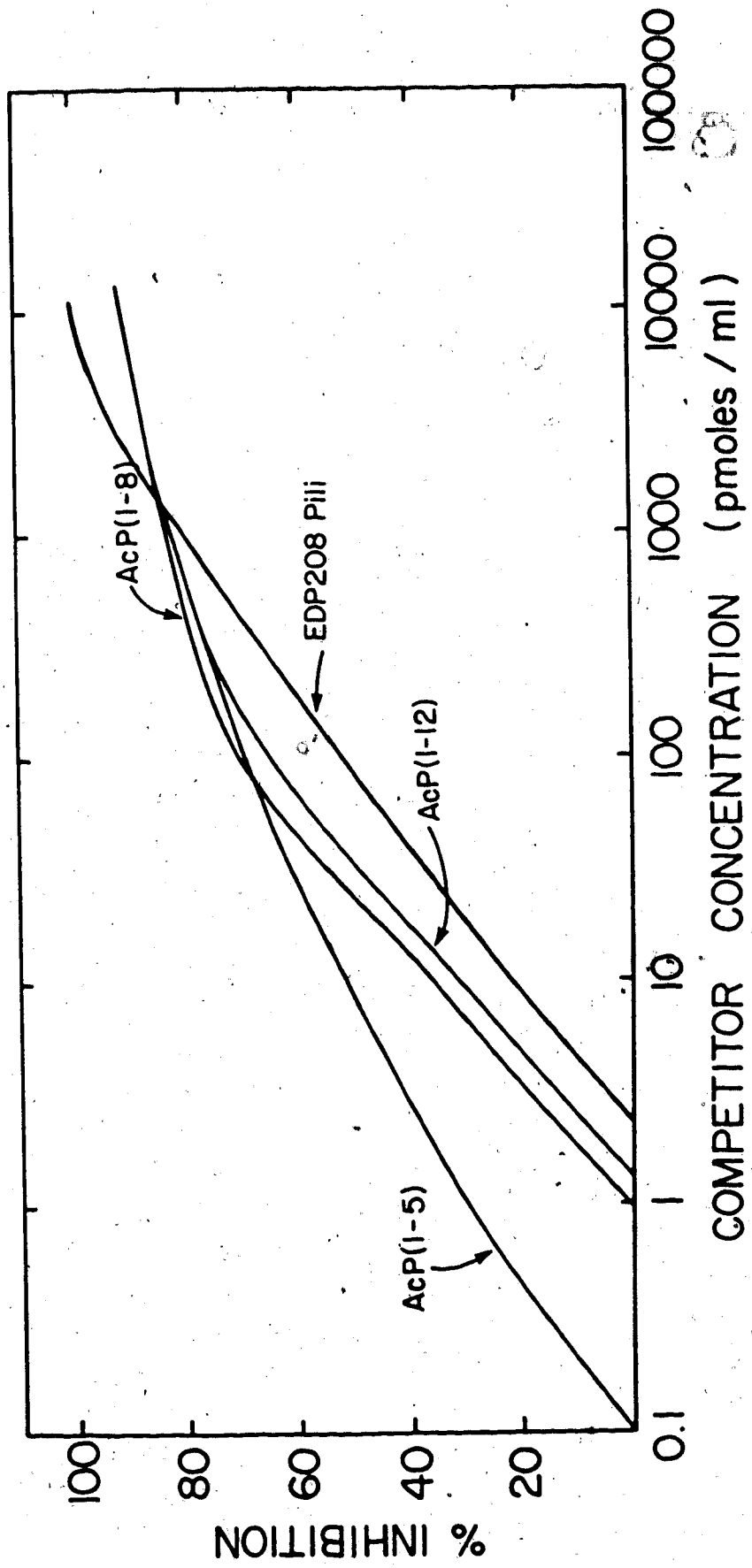
The results from this experiment indicate that two additional residues are involved in the antibody-antigen interaction of the N terminal octapeptide. Residues 3 and 4, both leucines, play a major role. Although preliminary experiments suggested that the acetyl threonine may be the only residue necessary for antibody binding, the present results show that the other groups are also involved. It is

particularily worth noting that peptide Ac(Gly)^{2,3,4,5} P(1-8) which is essentially the N terminal threonine followed by a string of glycines, has a very reduced antibody binding capacity, suggesting that the NAc-Thr is only one of several residues that contribute to the interaction of AcP(1-8) to pilus specific antibody. This was further substantiated when the analog, NAc-Thr-amide, AcP(1), was shown to have no detectable inhibition activity in the competition ELISA.

c) Synthetic AcP(1-5) and related synthetic analogs

Since the peptide Ac(Gly)^{5}P(1-8) was highly active in the competition ELISA (Figure III.8), it seemed possible that the entire antigenic N-terminal site could be localized within the N-terminal pentapeptide. The octapeptide has two glycines in the sixth and seventh positions which probably contribute little to the antibody interaction. The N-terminal pentapeptide, AcP(1-5), was therefore synthesized and used in the competitive ELISA to compare its antibody affinity with that of AcP(1-12), AcP(1-8) and whole pili. The results are presented in Figure III.9 (inhibition curves) and Table III.4 (I_{50} and aK values). All three peptides were capable of titrating more than 80% of the pilus specific antibodies, as compared to EDP208 pili, which titrated out 100%. This suggests that the pentapeptide contains the major antigenic determinant of the pilus

Figure III.9 Competition ELISA using synthetic analogs of the N-terminal region of EDP208 pilin and EDR208 pili as competitors. EDP208 pilus specific Fab, at 25 pmole/ml was used as the antibody source. Each curve is labelled appropriately

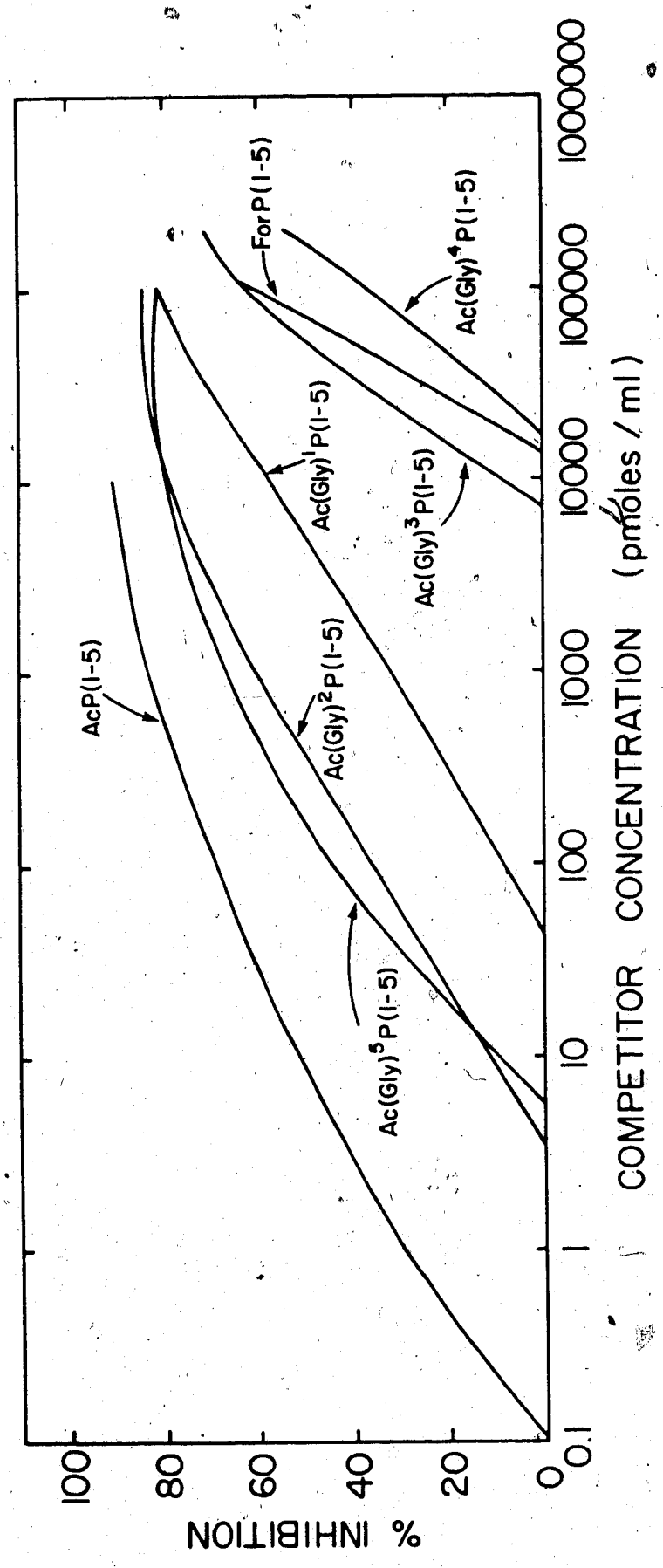

% INHIBITION
100
80
60
40
20
0.1
1
10
100
1000
10000
100000
1000000
COMPETITOR CONCENTRATION (pmoles / ml)
AcP(1-8)
EDP208 Pili
AcP(1-12)
AcP(1-5)

protein but alternate sites may occur in the native protein since whole pili are capable of titrating out 20% more antibody in this assay than the synthetic peptides. It is worth noting that as the peptide is shortened from the C terminus (Figure III.9), the I_{50} values decrease (Table III.4). This suggests that the shorter peptide containing only the antigenic determinant is more flexible in presenting a conformation that fits the antigen binding site of the anti-pilus antibody.

The results in Figure III.9 indicated that AcP(1-5) is a major antigenic determinant. To investigate the contribution of each of the five residues to the antigen-antibody interaction, several analogs of Acp(1-5) were synthesized. These peptides are listed in Table III.3 and represent modifications such that each amino acid in the native sequence was replaced by a glycine. These peptides were used as competitors in a competition ELISA and the results are presented graphically in Figure III.10. The relative affinities of each peptide are listed in Table III.4.

Replacement of alanine at position 5, $Ac(Gly)^5$-P(1-5), resulted in a small decrease in antibody binding, with respect to AcP(1-5). Replacement of aspartic acid at position 2, $Ac(Gly)^2P(1-5)$, also showed only a minor decrease in antibody binding when compared to AcP(1-5). These results indicate that the alanine and the aspartic acid residues make only weak contributions to the antigenicity

Figure III.10 Competition ELISA using synthetic analogs of AcP(1-5) as competitors. The analogs have the indicated residue replaced with a glycine. EDP208 pilus specific Fab, at 25 pmole/ml was used as the antibody source. Each curve is labelled appropriately.


% INHIBITION
0
20
40
60
80
100
0.1
1
10
100
1000
10000
100000
1000000
COMPETITOR CONCENTRATION (pmoles / ml)
AcP(1-5)
Ac(Gly)1P(1-5)
Ac(Gly)2P(1-5)
Ac(Gly)3P(1-5)
Ac(Gly)4P(1-5)
Ac(Gly)5P(1-5)
ForP(1-5)

of the pentapeptide. These conclusions were also obtained from experiments performed with the AcP(1-8) analogs Ac $(Gly)^{5}$P(1-8) and Ac$(Gly)^{2,3,4,5}$P(1-8) (Figure III.8 and Table III.4). Substantial decreases in antibody binding, as reflected by I_{50} and aK values in Table III.4 were observed for peptides Ac$(Gly)^{1}$P(1-5), Ac$(Gly)^{3}$P(1-5), and Ac$(Gly)^{4}$P(1-5), in which the threonine residue and each of the two leucines were replaced with glycines. There was a 550 to 20,000 fold increase in the I_{50} values of these peptides as compared to AcP(1-5). A substantial (8300 fold) increase in the I_{50} value was also observed when the acetyl moiety was replaced by a formyl group, ForP(1-5). This re-enforces the importance of the acetyl methyl group in the antigenicity of this peptide.

The importance of the two leucine residues was initially noted with the peptides Ac$(Gly)^{4,5}$P(1-8) and Ac $(Gly)^{3,4,5}$P(1-8) (Figure III.8) which had I_{50} values of 62,000 amd 100,000 pmol/ml, respectively. All of these observations indicate that two areas of the peptide are contributing to the antigen-antibody interaction: the N-terminal acetyl threonine and the two leucines at position 3 and 4.

The chemical structure of AcP(1-5) is schematically presented in Figure III.11, with the key reactive groups circled. The important contact points with the antibody for acetyl threonine are the side chain hydroxyl, via hydrogen

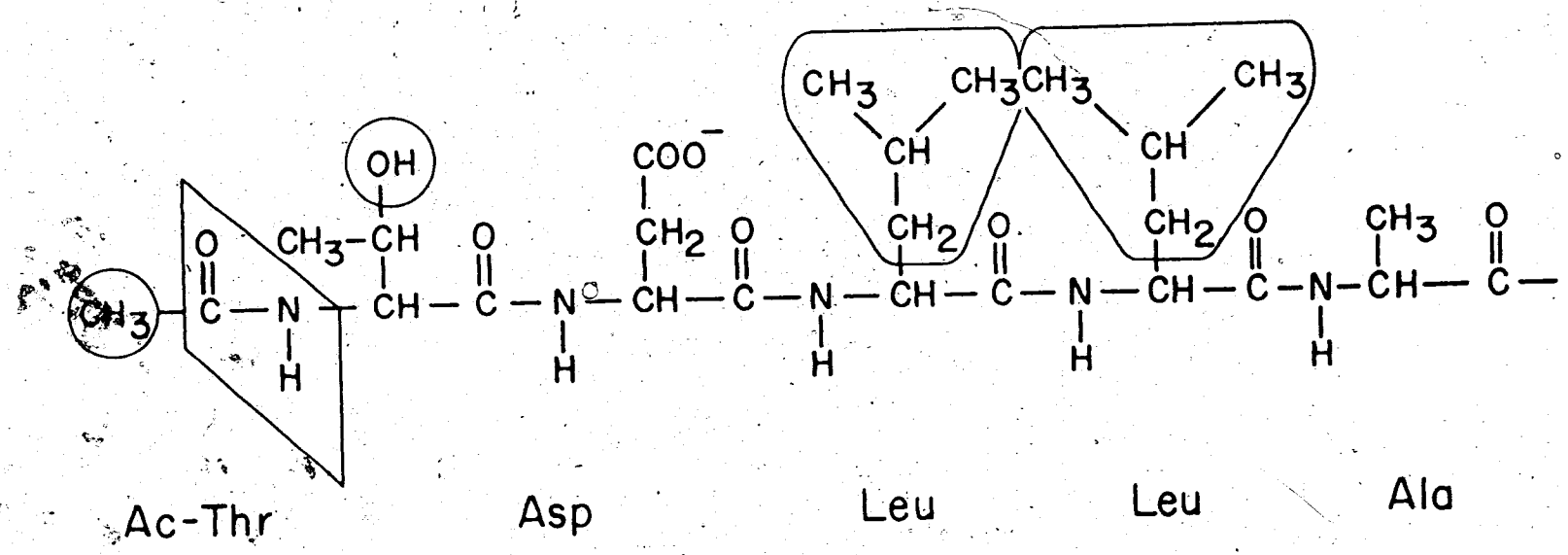


Figure III.11 Chemical structure of AcP(1-5).
The key groups for antibody interaction are circled.

bonding and the acetyl methyl by hydrophobic interaction. The two leucine side chains are probably involved in hydrophobic interactions with the antibody. None of these interactions, individually, are sufficient for antibody binding. Neither AcP(1), which is only acetyl threonine, nor AcP (2-8), which is missing the threonine but has both leucines, were capable of showing detectable inhibition in the competition ELISA (Table III.4).

One could speculate that the acetyl threonine is the first residue to interact with the antibody and initiates the binding of the remainder of the antigenic region. This is similar to a proposal made by Lemieux et al. (1983) to describe the forces involved in the binding of blood group substances to antibodies. They postulated that the main driving force was initiated by the establishment of polar interactions at the periphery of the binding site. On the other hand, it is equally possible that the free energies of the contact points are additive (Schecter, 1974) but a certain minmum free energy must be present to achieve initial binding (Eisen, 1980) or competition in the ELISA.

6. Affinity purification of two distinct species of anti-EDP208 pilus antibodies

The final step in the characterization of the antigenic structure of EDP208 pili was the estimation of the amount of pilus specific antibody present in the total

polyclonal population. Affinity columns were made using a synthetic peptide representing the N-terminal antigenic site as the ligand. Purified IgG from the pilus specific antisera was passed through the column and the amount of IgG binding to the column determined.

The column support chosen for this affinity column was Pharmacia's Activated CH Sepharose. The ligand binding group was a six carbon spacer followed by the active ester of carbodiimide. It specifically binds to free amino groups on the ligand to form a peptide linkage. The resulting ligand-support bond was stable to ligand leaching even after extended use. Details of column preparation are found in Chapter II, Section K.1.

The first affinity column was prepared using the synthetic peptide AcP(1-8) which had a single free amino group at Lys 8 available for attachment to the activated column. This column was used as a model for the development of the proper affinity chromatography methodology. The actual column used in the experiments described in this section was made using the peptide conjugate AcP(1-12)-BSA. The rationale for using the peptide conjugate was the expectation that there would be an increase in the efficiency of attachment to the column because the BSA molecule has more free amino groups than the peptide alone. In both cases the peptide/conjugate was intrinsically radiolabelled so that the amount of ligand attached to the column

could be determined. Approximately 50% of the input of either the peptide or the conjugate was found to bind to the column, indicating that either the peptide or the conjugate could be used as the ligand since both attached with equal efficiency to the resin. The column used in the present studies contained 84 nmoles of peptide per one gram of resin.

To determine how much anti-EDP208 pilus specific IgG bound to the AcP(1-12)-BSA column, two procedures were examined for feasiblity in future experiments. First, purified IgG was radiolabelled with ^{125}I (Chapter II, Section J) and mixed with cold IgG to make up the input IgG to pass through the column. The fate of the IgG was traced following the ^{125}I label. Second, cold IgG was used as the input antibody and the fate of this source followed by measuring the A_{280} of each sample during the procedure. The details of the basic affinity assay are described in Chapter II, Section K.2 and involved collection of unbound IgG from the column effluent and removal of bound (AcP(1-12) specific) IgG using guanidine hydrochloride. Regardless of the assay method less than 0.1% of the input IgG bound to the column. This appeared to be a reasonable percentage since the source of the IgG was polyclonal.

The bound IgG was classified as monospecific with regards to the N-terminal antigenic site of the pilus protein. The IgG that did not bind to the affinity column

was also saved for further characterization. Direct ELISAs were performed on these two species and the results are presented in Figure III.12. The ELISA was performed as described in Chapter II, Section K.3. Microtitre wells were coated either with EDP208 pili or AcP(1-12)-BSA and each antibody species was allowed to interact with these antigens. All species interacted with pili but the unbound fraction of IgG did not interact to the same extent as the others. Since the antipilus antibodies in the unbound fraction did not interact with the N-terminal peptide (Figure III.12 B), it probably contains antibodies which are specific for an additional antigenic site in the pilus protein. As expected, the bound IgG reacted with AcP(1-12)-BSA.

To determine whether the second antigenic determinant may be associated with the C-terminus of the protein (the second epitope on F and ColB2 pilus proteins was thought to reside in the C-terminus, Frost, personal communication), the EDP208 pilin monomer was made (Chapter II, Section F.3) and subjected to carboxypeptidase A digestion (Chapter II, Section F.4). Digestion with CPase A was done for 1, 3, 5 and 24 hours. Table III.5 shows that similar amounts of amino acids were released whether the digestion was performed for 1 or 24 hours. Whole pili, pilin, and pilin digested with CPase A for 24 hours were used as competitors in a competition ELISA using the input and unbound IgG fractions as the antibody source. The results are presented

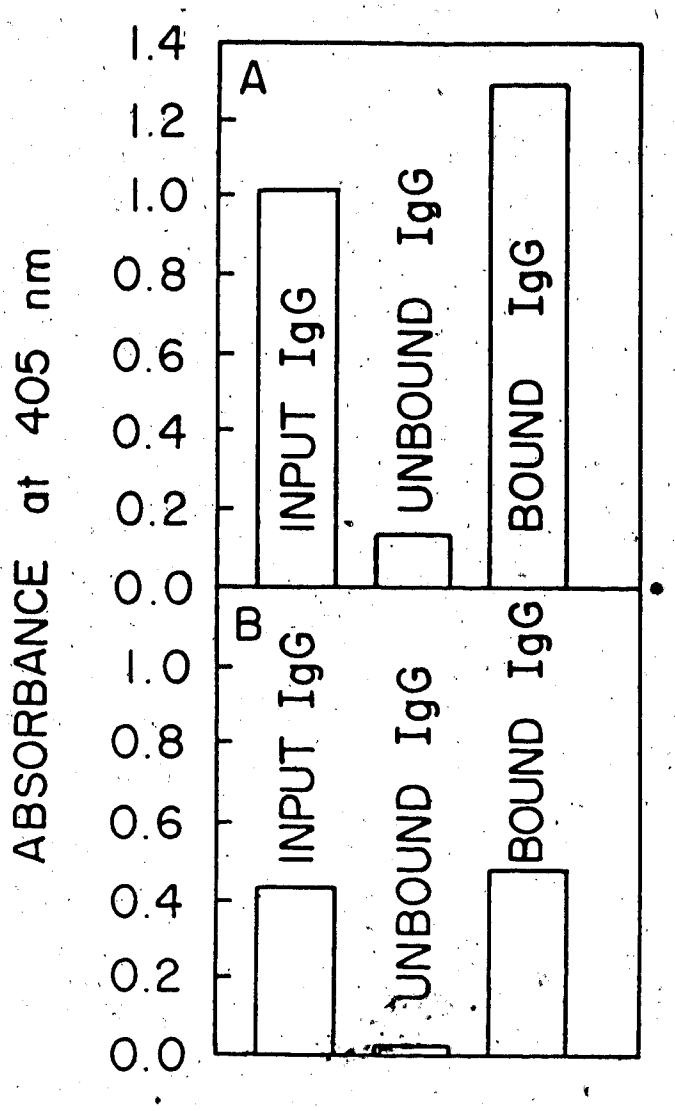


Figure III.12 ELISA studies using affinity purified EDP208 pilus specific IgG. Input IgG is the polyclonal IgG prior to affinity purification using the Activated CH Sepharose - AcP(1-12)-BSA affinity column. Unbound IgG is the fraction of total IgG which was not retained on the affinity column. Bound IgG refers to the IgG which did remain on the affinity column and was removed using guanidine hydrochloride. Experimental details are described in Materials and Methods.
A. Results obtained when microtitre wells were coated with 5 µg/ml of EDP208 pili.
B. Results obtained when microtitre wells were coated with 5 µg/ml of AcP(1-12)-BSA.
All IgG was used at 12.5 pmole/ml.

TABLE III.5

Amino acids released after carboxypeptidase A digestion of EDP208 pilin

1 Hour Digestion		24 Hour Digestion	
Residue	nmoles released[a]	Residue	nmoles released[a]
Thr	12.3	Thr	17.4
Val	9.4	Val	12.2
Ile	10.9	Ile	16.0
Leu	12.0	Leu	16.8
Phe	7.8	Phe	10.9
Lys	9.8	Lys	13.6

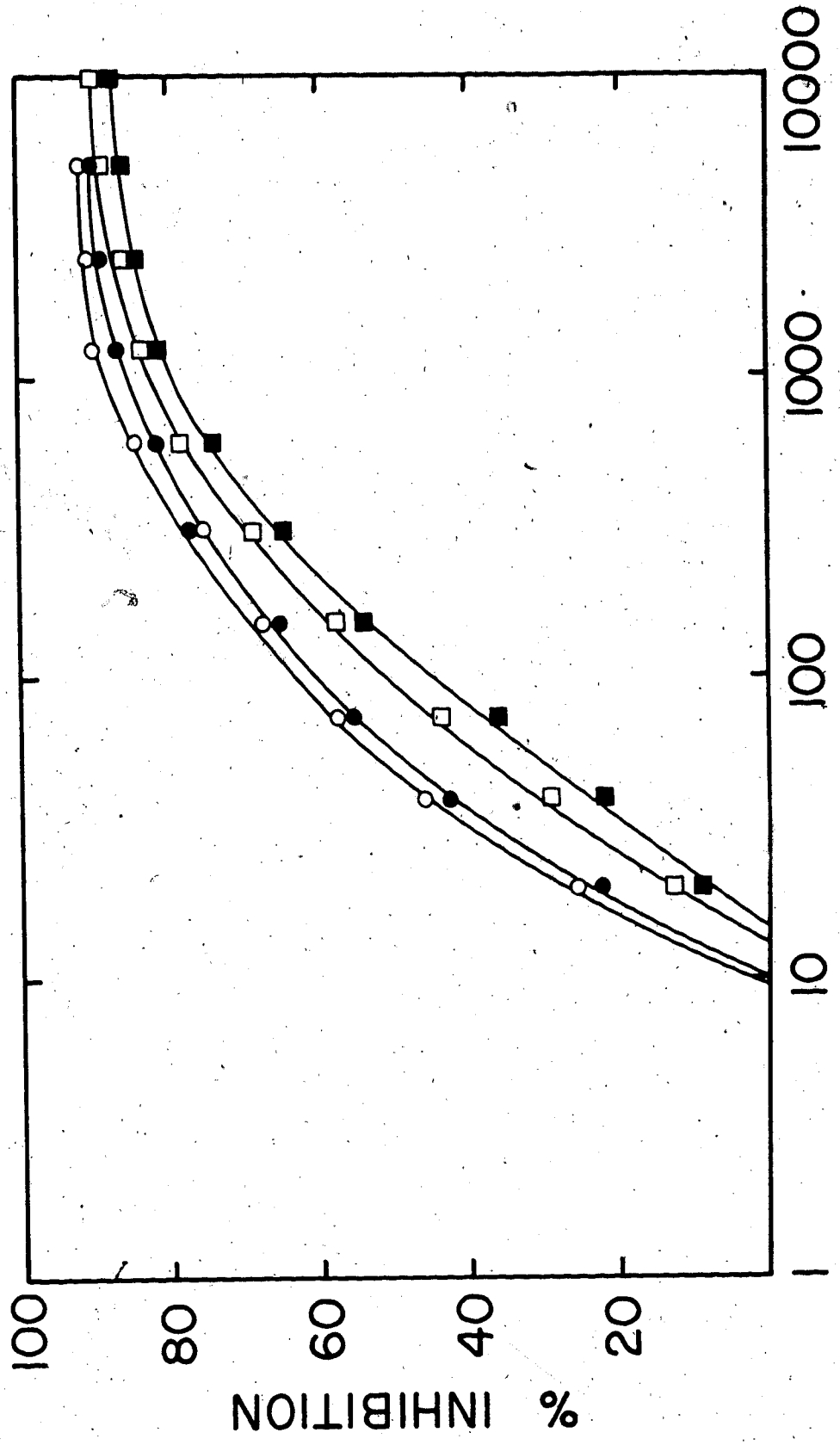
a. Amino acids and concentrations released were determined from amino acid analysis of digestion supernatants.

as inhibition curves in Figure III.13. One can see that there is very little difference in the affinities of either antibody for any of the competitors. From this one can conclude that the second population of antibodies is not directed at a determinant involving the immeadiate C terminus of the EDP208 pilus protein.

7. Electron microscopy of affinity purified antibodies

Electron microscopy experiments were also performed to deduce the regions of the intact pilus to which each of the affinity purified antibodies are interacting. Intact EDP208 pili were incubated with the unfractionated IgG, the unbound fraction of IgG and the N-terminal specific anti-Ac P(1-8)-BSA IgG (previously passed through a BSA-sepharose column to remove any anti-BSA IgG). This was followed by the addition of Protein A linked to gold particles, used for the detection of IgG when viewed in the electron microscope (see Chapter II, Section E for experimental details). The pilus-IgG-Protein A gold complexes were viewed in the electron microscope and the resulting micrographs are presented in Figure III.14. Panel A represents the interaction of EDP208 pili with the unfractionated pilus specific IgG, while Panels B and C show the results obtained with the unbound and N-terminal specific AcP(1-8)-BSA IgG, respectively. Panel D shows EDP208 pili after incubation with non-immune IgG. This is the control which represents the extent

Figure III.13 Competition ELISA using EDP208 pilin and carboxypeptidase treated pilin as the competitors with unfractionated and the unbound, uncharacterized, EDP208 pilus specific IgG as the antibody sources. IgG was used at 25 pmole/ml.
Symbols: O Input IgG with EDP208 pilin as the competitor, ● Input IgG with 24 hour carboxypeptidase digested EDP208 pilin as the competitor, □ Unbound IgG with EDP208 pilin as the competitor, ■ Unbound IgG with 24 hour carboxypeptidase digested pilin as the competitor. IgG terminology is the same as described for Figure III.12.


% INHIBITION
100
80
60
40
20
1
10
100
1000
10000
COMPETITOR CONCENTRATION (pmoles / ml)

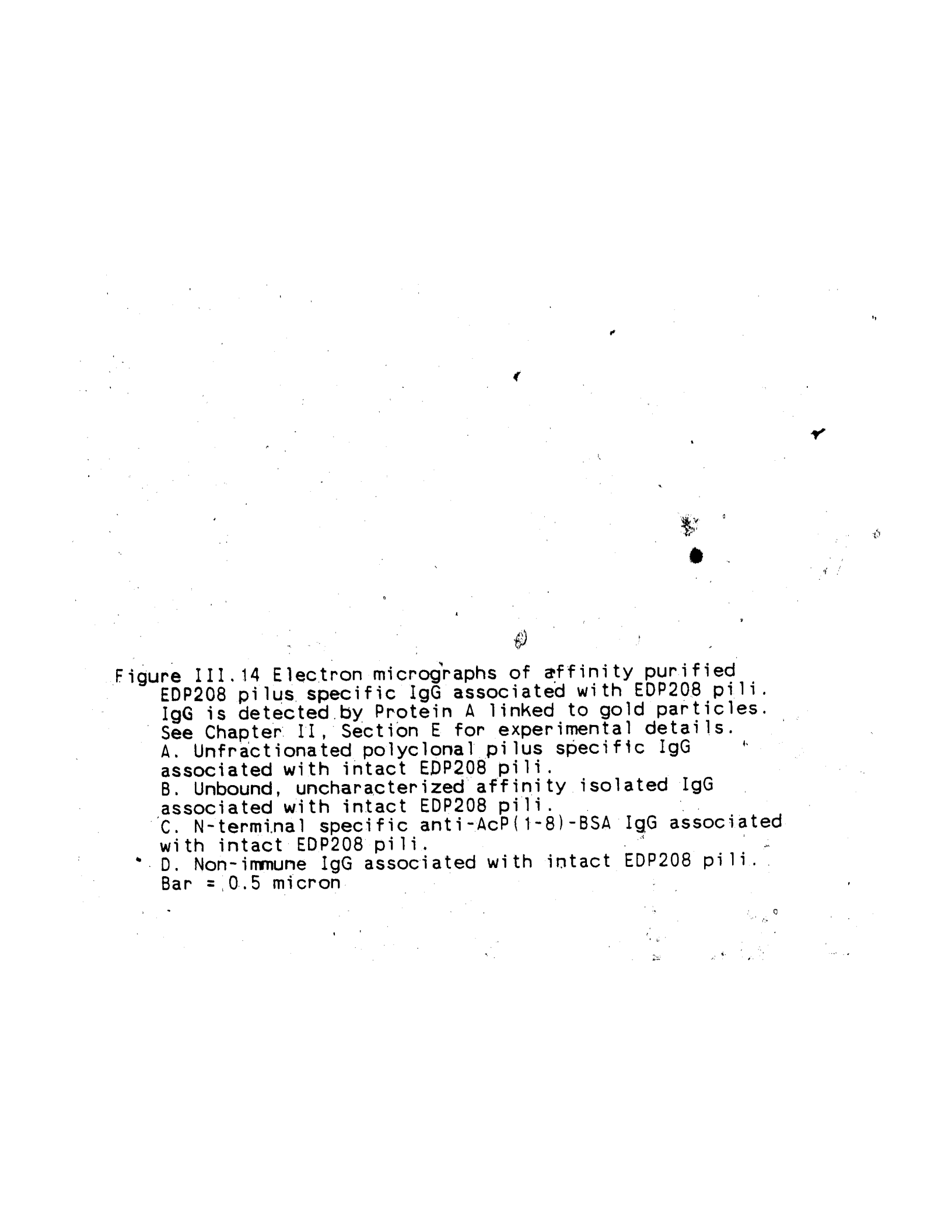
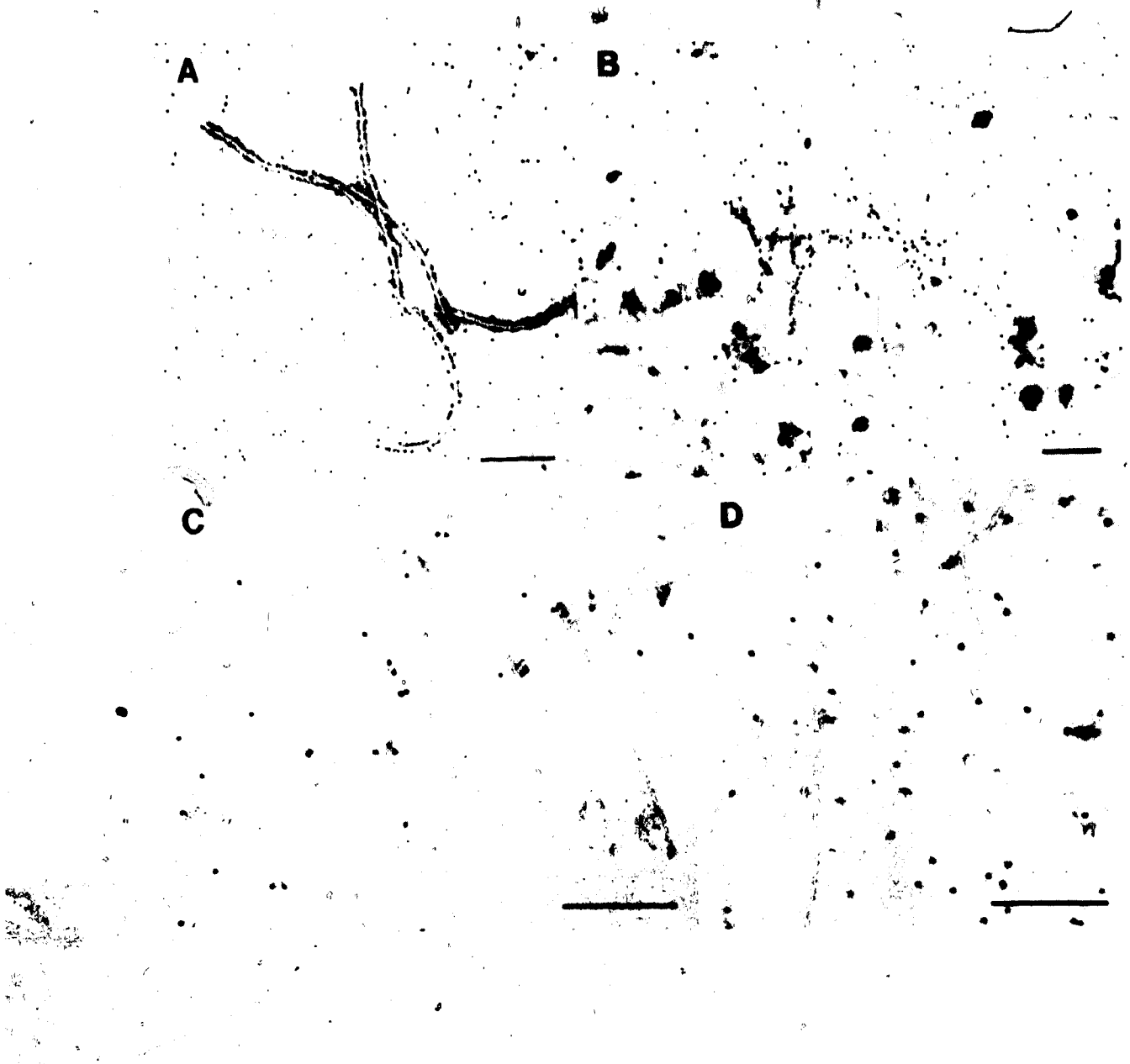


Figure III.14 Electron micrographs of affinity purified EDP208 pilus specific IgG associated with EDP208 pili. IgG is detected by Protein A linked to gold particles. See Chapter II, Section E for experimental details.
A. Unfractionated polyclonal pilus specific IgG associated with intact EDP208 pili.
B. Unbound, uncharacterized affinity isolated IgG associated with intact EDP208 pili.
C. N-terminal specific anti-AcP(1-8)-BSA IgG associated with intact EDP208 pili.
D. Non-immune IgG associated with intact EDP208 pili.
Bar = 0.5 micron



of non-specific pilus-IgG-gold interactions.

It may be seen that both the unfractionated and the unbound fraction of IgG become attached to the lateral surface of the pilus (Panels A and B). The results obtained using the N-terminal specific anti-AcP(1-8)-BSA antibodies are found in Panel C. These antibodies did not bind to the side of the pilus but appear to attach to the pilus tip. Similar results were obtained when these antibodies were concentrated 10 fold (data not shown). Frost et al. (personal communication) also observed attachment of an F pilus specific monoclonal antibody to the tips of the intact F pilus in a similar manner to that seen with the EDP208 N-terminal specific antibody.

The foregoing observations suggest that two distinct epitopes are present on the EDP208 pilus, one exposed on the lateral surface of the pilus which is recognized by the unbound, uncharacterized population of IgG and one exposed on the pilus tip which is recognized by IgG specific for the N-terminal octapeptide. Both are on the surface of the pilus, thus available for B-cell recognition during the immune response.

Armstrong et al. (1980) found that the EDP208 pilin monomer was very resistant to either chemical or proteolytic digestion. Trypsin cleavage resulted in the release of the N-terminal dodecapeptide, leaving the remaining region insoluble and resistant to further cleavages. Thus, it may

be difficult to determine what constitutes the other epitope(s) of this pilus protein. The results presented in this section, however, have indicated that the second, uncharacterized fraction of EDP208 pilus specific antibodies do not recognize an epitope at the immeadiate C-terminus of the protein, nor do they recognize a conformational determinant as these antibodies interact equally with pili or the denatured monomer in the competition ELISA.

C. Conclusion

The EDP208 pilus appears to be an ideal system for studying antibody-antigen interactions. Preliminary experiments indicated that the location of a major antigenic site was the N-terminal dodecapeptide. This peptide was found to be immunogenic as well, since animals immunized with either the dodecapeptide or the corresponding N-terminal octapeptide conjugated to BSA, produced antibodies capable of interacting with the whole pilus. The subsequent studies using analogs of either the octapeptide, AcP(1-8), or the pentapeptide, AcP(1-5), revealed that this epitope consists of only five residues. According to Lerner et al. (1981), a minimum of seven residues are necessary for antigenicity, but Schecter (1974), using poly-L-alanyl residues and tetra-alanine analogs, found that antibodies were capable of recognizing a 3 residue linear sequence.

Experiments carried out with synthetic peptide analogs of AcP(1-8) and AcP(1-5) (Figure III.7, 8 and 10) showed that major recognition sites for pilus specific antibodies include the NAc-Thr and the leucine side groups at positions 3 and 4. The acetyl methyl and the two leucine side chains may be involved in hydrophobic interactions with the antigen binding cleft of the antibody, while, the hydroxyl group of the threonine is probably involved in hydrogen bonding.

X-ray diffraction studies of immunoglobulins have indicated that the antigen combining site is a groove with the approximate dimensions of 1.5 x 0.6 nm and 0.6 nm deep (Polzak et al., 1976). This groove can accomodate a peptide of five amino acids in the extended, beta sheet conformation, to ten amino acids in an alpha helical conformation. The major antigenic determinant of EDP208 pili is consistant with this structure. Since it is a pentapeptide it is probably in the extended conformation. However, elucidation of the precise configuration of the bound peptide in the antibody binding site must await X-ray crystallographic studies on co-crystallized antigen-antibody complexes (Amit et al., 1985).

Prediction rules for determining antigenic sites on proteins were descibed by Hopp and Woods (1981). These rules are extensively used for deducing antigenic regions without using the classical but tedious methods of Atassi

(1975). These rules state that antigenic determinants lie in or adjacent to areas of high hydrophilicity. Based on this premise, one would predict that the C terminal end of the dodecapeptide AcP(1-12), which has two lysines at positions 8 and 12, should contain the antigenic site. However, this study has shown that it is the hydrophobic groups which provide the N terminal site with the most stable contact points with the antibody. This is not a new discovery, as both Lemieux and Atassi have reported this phenomenon. Lemieux et al. (1983) showed that hydrophobic interactions were important in the interaction of carbohydrates with antibodies. Atassi and Webster (1983) and Atassi (1975) found the same was true for some of the antigenic sites of myoglobin and influenza virus hemagglutinin. For the EDP208 N terminal determinant, the hydrophilic amino acids are not major contributors to the antibody-antigen interaction and may serve to position the highly hydrophobic region on the surface of the protein.

Recently, Finlay et al. (1985) identified the major antigenic determinants of two other conjugative pilus proteins. These two pili, F and ColB2, are related in sequence, structure and function. Both proteins have an N-acetylated alanine as the N terminal amino acid. As with the EDP208 pilus protein, the major antigenic site of these two proteins is located at the N terminus, with the acetyl group as an essential component. There are no similarities

between the two F pilus types and EDP208 except, perhaps in their role in conjugation. Helmuth and Achtman (1978) showed that during bacterial mating, the pilus of the donor cell attaches to the surface of the recipient cell, perhaps via a specific receptor, after which the two cells are brought together. It is believed that the two cell membranes then fuse to permit genetic material to pass from the donor to the recipient. Perhaps it is the N-terminal region of these pilus proteins which is involved in this cell to cell contact. No studies have been initiated to examine this relationship. As for the comparison of the EDP-208 antigenic site with those from other pilus types, no homologies have been identified. Klemm (1981) suggested that the K88 major antigenic determinant was located in the central portion of the protein. Rothbard et al. (1984), found that the immunodominant region of the gonococcal pilus protein was in the C terminal region, while Watts et al. (1983) found that there were four antigenic regions which were localized at the N and C termini of the Pseudomonas aeruginosa K pilus protein. Thus one cannot predict from other pilus proteins, where an antigenic determinant may reside.

Finally, the studies discussed in Section B.6 of this chapter revealed the presence of an alternate antigenic, and immunogenic region on the EDP208 pilus protein. Preliminary studies, using the native C terminal tryptic frag-

ment, ER (Figure III.3 and 4), suggested that a second determinant existed in this peptide. This idea was further supported by the demonstration that a unique class of pilus specific antibodies does not interact with the N terminal antigenic site but does interact with whole pili. To date, the nature of this region is uncharacterized, although preliminary studies reported in this thesis suggest that it is not associated with the immeadiate C-terminus of EDP208 pilin.

Chapter IV

Monoclonal Antibodies Against CFA/1 Pili

A. Introduction

Kohler and Milstein (1976) developed the technology to produce immortal cell lines which continuously and reproducibly produce unique antibody molecules. These cell lines are prepared by the *in vitro* chemically mediated fusion of splenocytes (B cells) from mice immunized with a specific immunogen and cells from a mouse tumor (myeloma cells). The resulting hybrid cell (hybridoma) has the antibody producing capacity acquired from the mouse B cell and the ability to grow permanently in culture (a characteristic acquired from the myeloma cell). The antibodies produced by these hybrid cells are called "monoclonal antibodies" as each hybrid stems from a single B cell and when grown in culture produces a single clone of identical cells (Nowinski *et al.*, 1983).

This chapter deals with monoclonal antibodies produced against colonization factor antigen 1 (CFA/1) from enterotoxigenic *E.coli*. CFA/1 are filamentous structures, often called pili, found on the surface of enterotoxigenic bacteria. CFA/1 pili are thought to be necessary for the

adherence and subsequent colonization of these bacteria to the human intestinal mucosa. Once colonized, the bacteria secrete toxins which are the causative agent of diarrhea (Evans et al., 1978). Monoclonal antibodies were prepared against this pilus for use in the identification of antigenic determinants on the pilus protein and in the identification of mammalian cell receptors for these pili. Such antibodies may eventually be useful in the preparation of synthetic vaccines for diarrheal disease. This chapter deals with the production and specificity of these monoclonal antibodies and their use in the study of CFA/1 pilus antigenic structure.

B. Results and Discussion

1. Monoclonal antibody production

A detailed description of how monoclonal antibodies to CFA/1 pili were produced is provided in Chapter II, Section I.1 and I.2. Female BALB/cCr mice were immunized with whole pili and spleen cells fused with NS1/Sp2 myeloma cells. 250 putative CFA/1 hybridomas were screened by the direct ELISA (Chapter II, Section I.3) for anti CFA/1 activity. From these, the 5 having the highest antibody titre were selected for subcloning. Four monoclones were further selected from the five 96 well plates of subclones, on the basis of high ELISA titres and used to produce mouse asci-

tic fluid. The four monoclones were named WPC-1, WPC-3, WPC-5, and WPC-6.

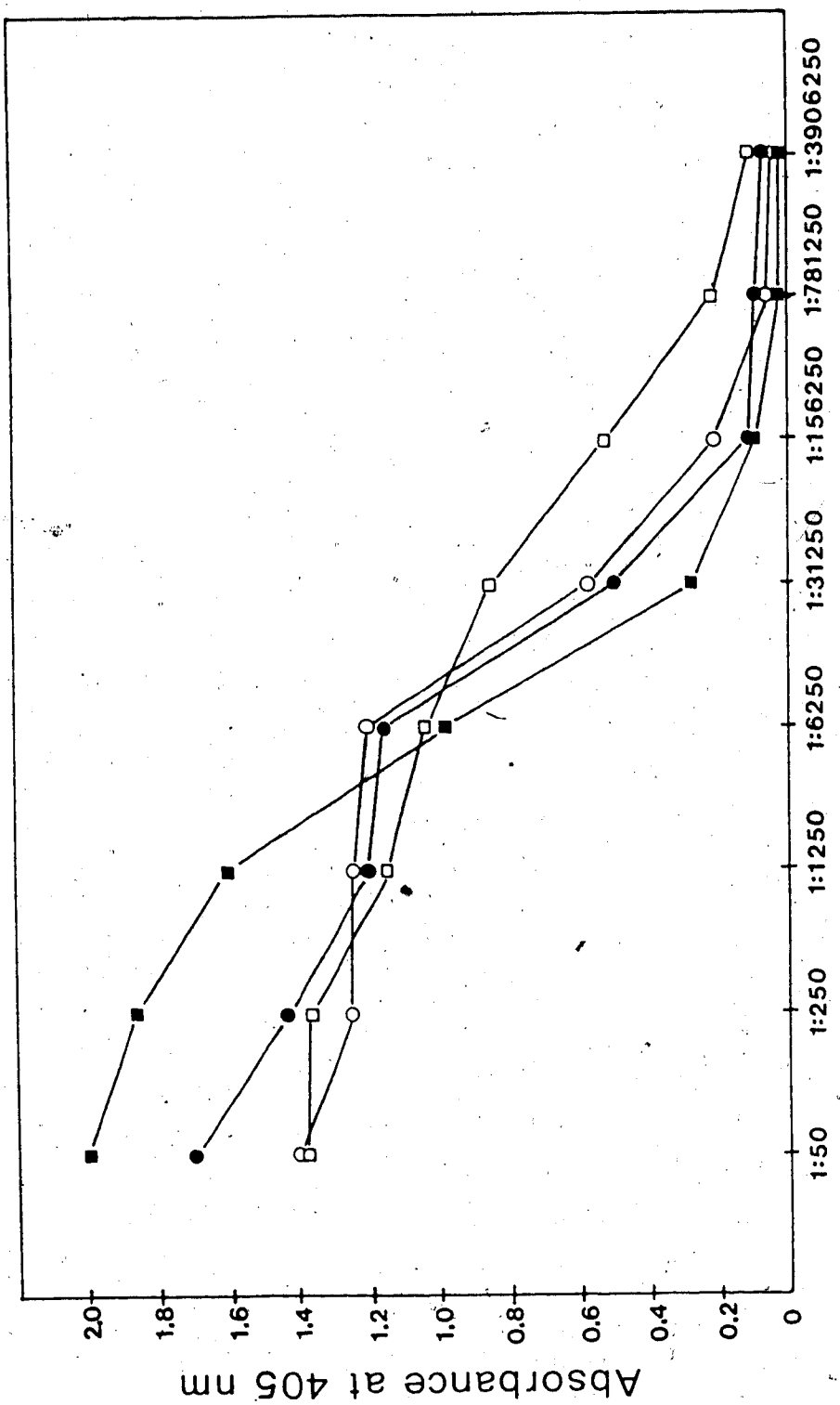
The isotypes of the four CFA/1 monoclonal antibodies chosen were found to belong to the IgG_{2a} immunoglobulin sub-class. Since all antibodies were of the same isotype, it may be suggested that they are derived from the same original clone. However, as shown below, all four antibodies have different properties. Also, since hyperimmune mice were used for the fusion, it is possible that the cells available for the fusion were the high affinity IgG_{2a} producing B cells.

2. Monoclonal specificity as determined by ELISA

Antibodies produced by monoclones WPC-1, WPC-3, WPC-5, and WPC-6 were examined for activity towards CFA/1 pili using the direct ELISA. As shown in Figure IV.1, all four have similar profiles and endpoints (from $1/5 \times 10^5$ for WPC-6 to $1/1.3 \times 10^7$ for WPC-5, endpoints were calculated using a linear regression program, as described in Chapter II, Section H.5).

To test for the possibility that small amounts of lipopolysaccharide (LPS) may have been present in the pili used to immunize the mice, LPS was purified from H10407 $CFA/1^+$ bacteria (Chapter II, Section L.2) and used as the antigen coating microtitre wells in the direct ELISA. Crude ascites, induced by the monoclones WPC-1, WPC-3, WPC-5, and

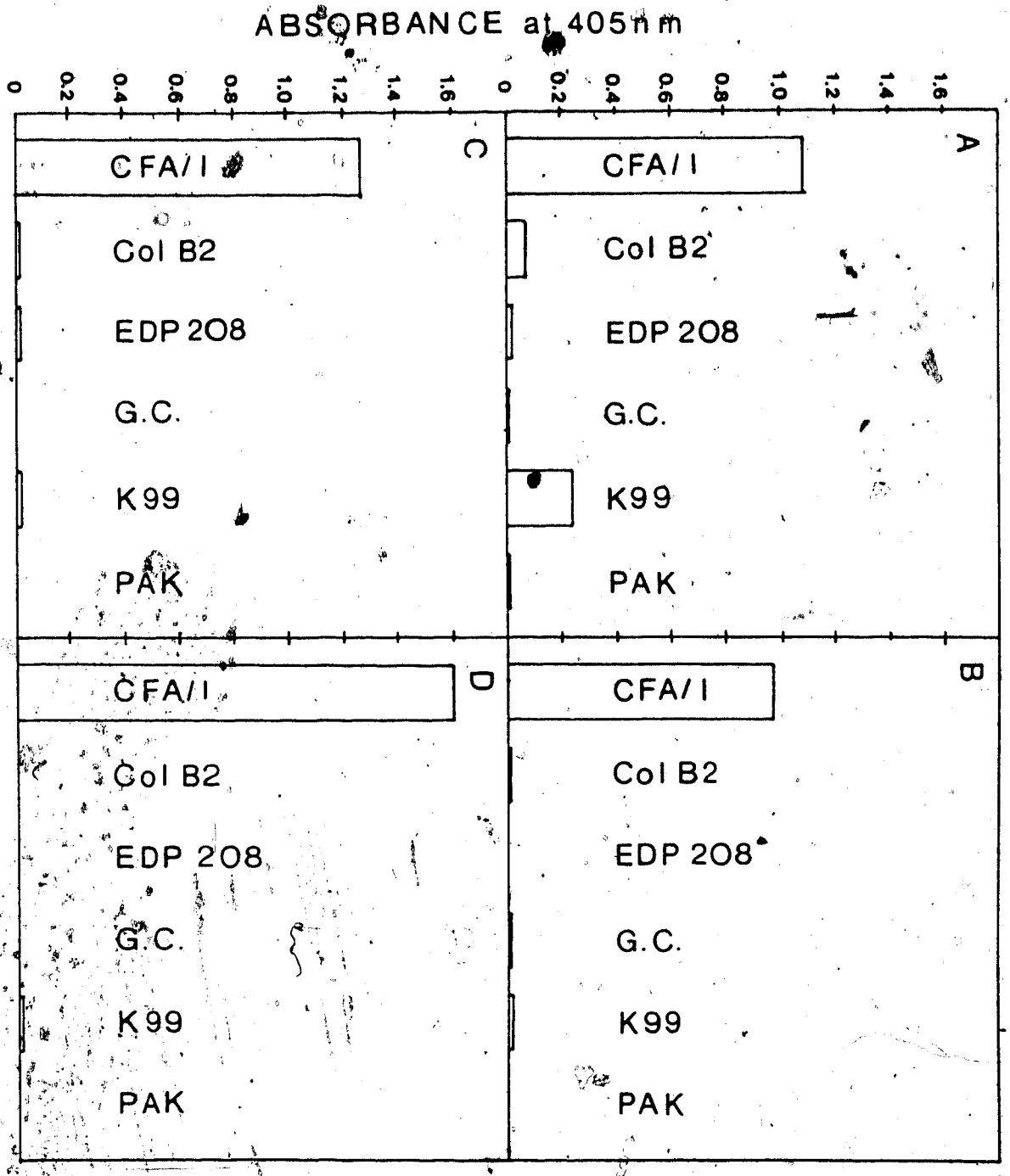
Figure IV.1 Titre of CFA/1 monoclonal antibodies, as determined by the direct ELISA. See Materials and Methods, Section I.3 for experimental details.
Symbols: O WPC-1, ● WPC-3, □ WPC-5, ■ WPC-6.


Absorbance at 405 nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1:50
1:250
1:1250
1:6250
1:31250
1:156250
1:781250
1:3906250
Antibody Dilution

WPC-6, was diluted 100 and 1000 times and used as the antibody source in this assay. There was no detectable interaction between the monoclonal antibodies and LPS. These monoclones are therefore specific for CFA/1 pili.

Each of the four monoclonal antibodies were examined for interactions with other types of pili, using the direct ELISA. Any homologies between the CFA/1 and the other pilus types can be detected by this method. In the assay, microtitre wells were coated with a saturating concentration of CFA/1 pili and 5 other pilus types. These pili included two conjugative, plasmid encoded types from E.coli, EDP208 and ColB2; two adhesive types, K99 from E.coli and gonococcal (G.C.) from Neisseria gonorrheae (kindly donated by G. Schoolnik, Stanford University, Palo Alto, Calif.), and PAK pili from Pseudomonas aeruginosa K. Various dilutions of the four monoclonal antibodies were used and antigen-antibody complexes were detected using goat anti-mouse IgG labelled with alkaline phosphatase. Figure IV..2 shows the results of this experiment. Panels B, C, and D represent the results obtained using WPC-3, WPC-5, and WPC-6, respectively. There was little or no interaction between these antibodies and the non CFA/1 pili. The results in Panel A reveal a distinct reaction of WPC-1 with K99 pili and a minimal reaction with ColB2 conjugative pili. The interaction with K99 may represent a similar region in both pili types. Since the amino acid sequence of K99 (Roosendaal et

Figure IV.2 ELISA assay showing interactions between CFA/1 monoclonal antibodies and various types of pili. Details of the assay are described in Chapter II, Section I.3. Microtitre wells were coated with the indicated pili, followed by incubation with a 1:1250 dilution of ascitic fluid from each of the monoclones and antigen-antibody complexes quantitated at 405 nm, using the goat anti rabbit IgG alkaline phosphatase enzyme reaction.
A. Results obtained using antibodies from WPC-1.
B. Results obtained using antibodies from WPC-3.
C. Results obtained using antibodies from WPC-5.
D. Results obtained using antibodies from WPC-6.
G.C. is gonococcal pili from Neisseria gonorrhoeae.
PAK is pili from Pseudomonas aeruginosa K.


ABSORBANCE at 405nm
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
A
CFA/I
Col B2
EDP 208
G.C.
K99
PAK
B
CFA/I
Col B2
EDP 208
G.C.
K99
PAK
C
CFA/I
Col B2
EDP 208
G.C.
K99
PAK
D
CFA/I
Col B2
EDP 208
G.C.
K99
PAK

al., 1984) pili, bears no resemblance to that of CFA/1 pili (Klemm, 1982), it is not a sequence homology which is recognized by this monoclonal antibody. However, both pilus types are virulence factors on enterotoxigenic E.coli, and both are involved in bacterial adherence to intestinal mucosa. One may speculate that a common functional determinant is present in CFA/1 and K99 pili. K99 pili are responsible for the adherence of bacteria to the intestinal mucosa of calves and lambs, which eventually results in neonatal diarrhea (Runnells et al., 1980). Faris et al. (1980) located what they believed was a common receptor for CFA/1 and K99 pili on erythrocytes. This receptor was the ganglioside GM_2 glycoconjugate. Recently, Smit et al. (1984) found that the actual erythrocyte receptor for K99 pili was a glycolipid which was unrelated to the ganglioside GM_2. They did not test the ability of CFA/1 pili to interact with this receptor. If the receptor for CFA/1 pili does turn out to be the same as that for K99, one would predict that these two structures may have a common conformational epitope, which is recognized by the WPC-1 antibodies.

The interaction between WPC-1 and the conjugative pilus, ColB2 is more difficult to explain. The amino acid sequence of each protein has been determined (CFA/1 by Klemm, 1982, and ColB2 by Finlay et al., 1984), but no recognizable homologies are identifiable. The two pili types, moreover, have different functions. One could argue that

both pili types are involved in cell-cell contact, CFA/1 with human intestinal cells and ColB2 with other bacterial cells during the mating process. Thus, structural similarities involving the adherence function may exist. However, the cross reaction is low when compared to that with K99 and any similarities between the two pilus types may not be significant.

3. Monoclonal specificity as determined by immunoblot analysis

An alternate method for examining the specificities of monoclonal antibodies from WPC-1, WPC-3, WPC-5, and WPC-6 involves the immunoblot procedure (Chapter II, Section H.2). The six pili types used in the direct ELISA were run on SDS-polyacrylamide gels and electrophoretically transferred to nitrocellulose paper. The papers were then incubated with antibodies from one of the four monoclones, then antibody-antigen complexes were detected using ^{125}I labelled protein A from Staphylococcus aureus followed by autoradiography. Duplicate gels were stained with Coomassie blue for direct visualization of the proteins. The results are shown in Figure IV.3. The stained gel is shown in panel A, while panel B is a gel stained after the transfer process to show that all proteins have been efficiently transferred. Panels C, D, E, and F represent the autoradiographs obtained from nitrocellulose papers treated with antibodies

Figure IV.3 Immunoblot analysis of CFA/1 monoclonal antibodies.
Lane 1, 5µg of prestained protein standards (BRL) ovalbumin (43,000 daltons), alpha-chymotrypsinogen (25,700 daltons), beta-lactoglobin (18,000 daltons), cytochrome c (12,300 daltons), bovine trypsin inhibitor (6,200 daltons), and insulin B chain (3,000 daltons); lane 2, 5 µg of CFA/1 pili; lane 3, 5 ug of K99 pili; lane 4, 5 µg of P pili; lane 5, 5 ug of EDP208 pili; lane 6, 5 µg of PAK pili; lane 7, 5 ug of gonococcal pili.
A. Coomassie blue stained proteins after SDS-PAGE.
B. Coomassie blue stained duplicate of gel A after the transfer process.
C. Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose and treated with a 1:25 dilution of WPC-1 antibodies and ^{125}I protein A.
D. Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose and treated with a 1:50 dilution of WPC-3 antibodies and ^{125}I protein A.
E. Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose and treated with a 1:50 dilution of WPC-5 antibodies and ^{125}I protein A
F. Autoradiograph of proteins transferred from a duplicate of gel A to nitrocellulose and treated with a 1:25 dilution of WPC-6 antibodies and ^{125}I protein A.

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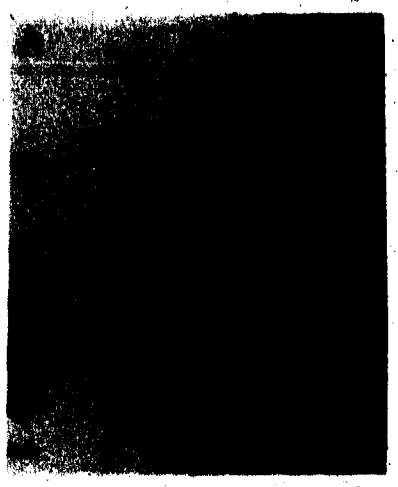
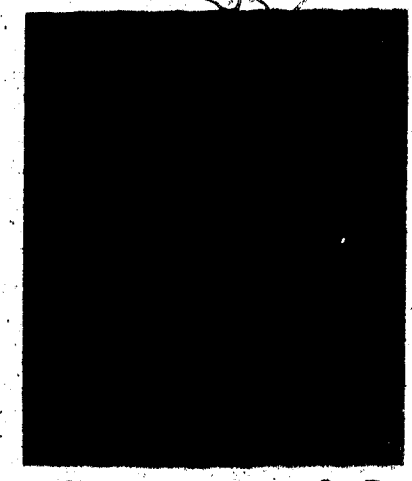


1 2 3 4 5 6 7

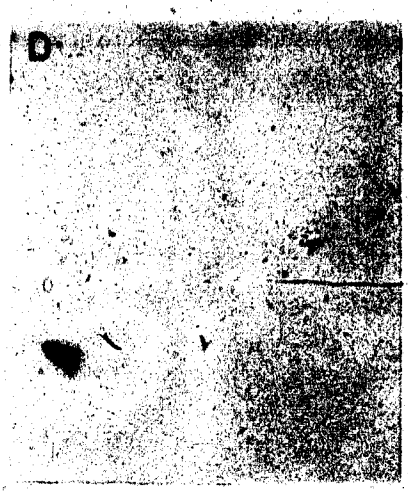


1 2 3 4 5 6 7

C

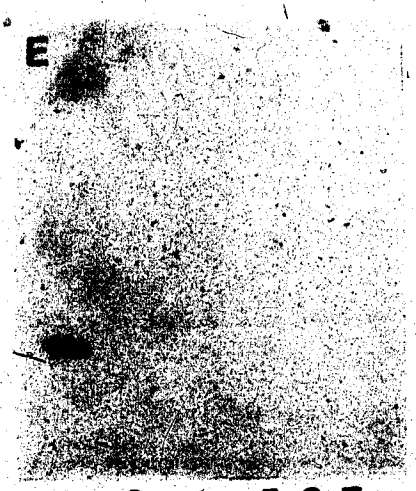
1 2 3 4 5 6 7

D



1 2 3 4 5 6 7

E



1 2 3 4 5 6 7

F

1 2 3 4 5 6 7

from WPC-1, WPC-3, WPC-5 and WPC-6, respectively. It may be seen that there was no detectable interaction between any of the antibodies and the pilus types other than CFA/1. This confirms the results obtained with WPC-3, WPC-5, and WPC-6 from the ELISA. However, unlike the results shown in Figure IV.2, WPC-1 antibodies only recognized CFA/1 pili in this assay. The putative common determinant on the K99 pilus may be conformation dependent and unrecognizible after denaturation of the protein in SDS gels.

4. Inhibition of hemagglutination by monoclonal antibodies

Evans et al. (1977) described a correlation between the presence of CFA/1 pili on enterotoxigenic E.coli and the ability of these bacteria to agglutinate human type A erythrocytes in the presence or absence of mannose. Since the presence of mannose does not interfere in the hemagglutination, CFA/1 pili are often called mannose resistant hemagglutinins (MRHA) unlike type 1 pili which will not hemagglutinate in the presence of mannose and are called mannose sensitive hemagglutinins (MSHA, Salit and Gotschlich, 1977).

To study the interaction of bacteria bearing CFA/1 pili and human type A erythrocytes, the effect of the CFA/1 monoclonal antibodies on hemagglutination was examined. H10407 CFA/1$^+$ bacteria were preincubated with undiluted

antibody from WPC-1, WPC-3, WPC-5 and WPC-6, then used in the hemagglutination assay (Chapter II, Section B). All four were capable of inhibiting hemagglutination. This suggests that the antibodies are either binding to the region on the pilus responsible for erythrocyte interaction or binding to an unrelated area and causing a steric inhibition of the cell-cell contact.

Since WPC-1 was capable of interacting with K99 pili in the direct ELISA, it was of interest to determine whether this antibody could inhibit the hemagglutination of bacteria possessing K99 pili, or whether K99 specific antibodies could inhibit CFA/1 induced hemagglutination.

First, H10407 bacteria possessing CFA/1 pili were preincubated with K99 pili specific polyclonal antiserum and tested for mannose resistant hemagglutination. Interestingly, the antiserum was capable of inhibiting the hemagglutination, thus, lending support to the earlier observation (Figure IV.2) that CFA/1 and K99 pili possess a common determinant. However, the attempt to inhibit agglutination of sheep erythrocytes by the K99 containing E.coli strain VIDO1 failed with all four monoclonal antibodies. Thus, although there does appear to be a region of similarity between the two pili, this region may not be important for the interaction of K99 pili with erythrocytes.

5. Electron microscopy of monoclonal antibody-pilus interactions

Immunoelectron microscopy was used to examine where CFA/1 monoclonal antibodies attach to the intact pilus. Purified IgG from monoclones WPC-1, WPC-3, WPC-5, and WPC-6 was incubated with intact CFA/1 pili. Each mixture was placed on carbon coated grids, negatively stained and vejwed in a Philips EM300 electron microscope. Similar results were obtained for all four antibodies. The results using antibodies from WPC-5 are shown in Figure IV.4. Panel A shows intact pili without the antibody treatment and panel B shows CFA/1 pili treated with the monoclonal antibody. The antibodies are binding along the entire surface of the pilus in what appears to be a helical pattern. Pili are believed to be made up of hundreds of repeating subunits which are arranged in a helical array (Brinton, 1965; Folkhard et al., 1981). It is therefore possible that the antibodies bind to each subunit, giving rise to the observed helical pattern. Abraham et al. (1983) found a similar pattern of monoclonal antibodies binding to type 1 pili. They also noted that the disruption of the pilus structure with denaturants abolished the binding of these monoclonal antibodies. They concluded that their antibody was directed against a quaternary determinant which was at the subunit-subunit interface. Since the monoclonal antibodies directed against CFA/1 were capable of interacting with the denatur-

Figure IV.4 Electron micrographs of CFA/1 pili before and after treatment with monoclonal antibodies.
A. CFA/1 pili without the presence of antibodies.
B. CFA/1 pili treated with monoclonal antibodies from WPC-5.

ed pilus protein, they were probably interacting with a sequential epitope in the pilin subunit. However, since WPC-1 was unable to interact with the K99 pilus after exposure to denaturants (SDS-PAGE), WPC-1 may bind to a conformation dependent epitope involving quaternary structure in the K99 pilus.

6. Relative affinities of monoclonal and polyclonal antibodies for CFA/1 pili

The final experiment in the study of CFA/1 monoclonal antibodies was designed to determine their relative binding affinities for CFA/1 pili using competitive ELISA's. The experimental details of this assay are found in Chapter II, Section H.5 and the theory behind the assay and the calculation of affinity constants are found in Chapter III, Sections B.5 and B.6, respectively. The microtitre wells were coated with CFA/1 pili. A suitable range of concentrations of free CFA/1 pili were incubated with 25 pmole/ml of IgG from monoclones WPC-1, WPC-3, WPC-5, and WPC-6 as well as IgG from CFA/1 and K99 pilus specific polyclonal antisera. The pili-IgG mixtures were allowed to interact with the pili coated wells and the extent of inhibition of this interaction calculated for each pili concentration. The results are presented graphically as inhibition curves in Figure IV.5. I_{50} values (the amount of competitor required for 50% inhibition of the antibody-pilus coated well in-

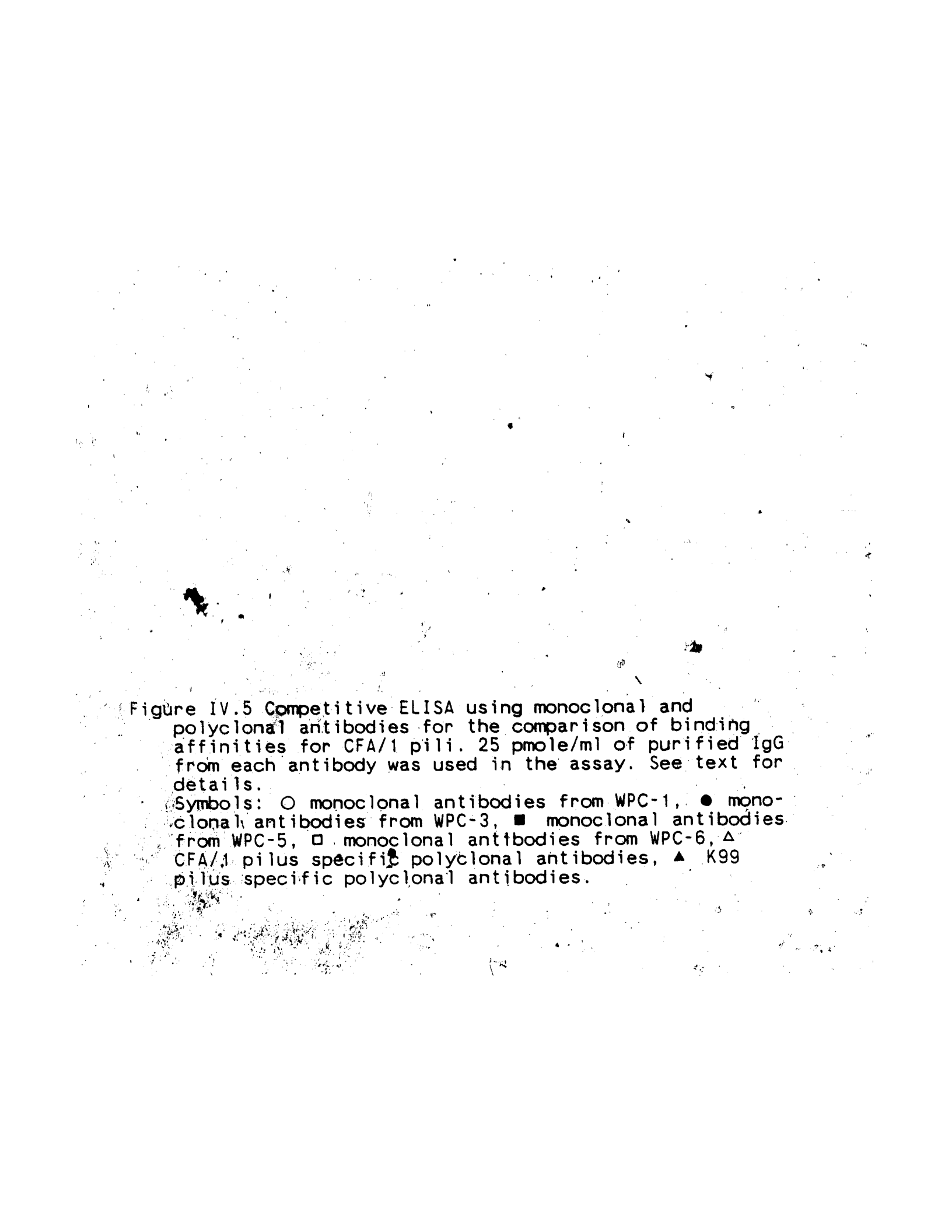
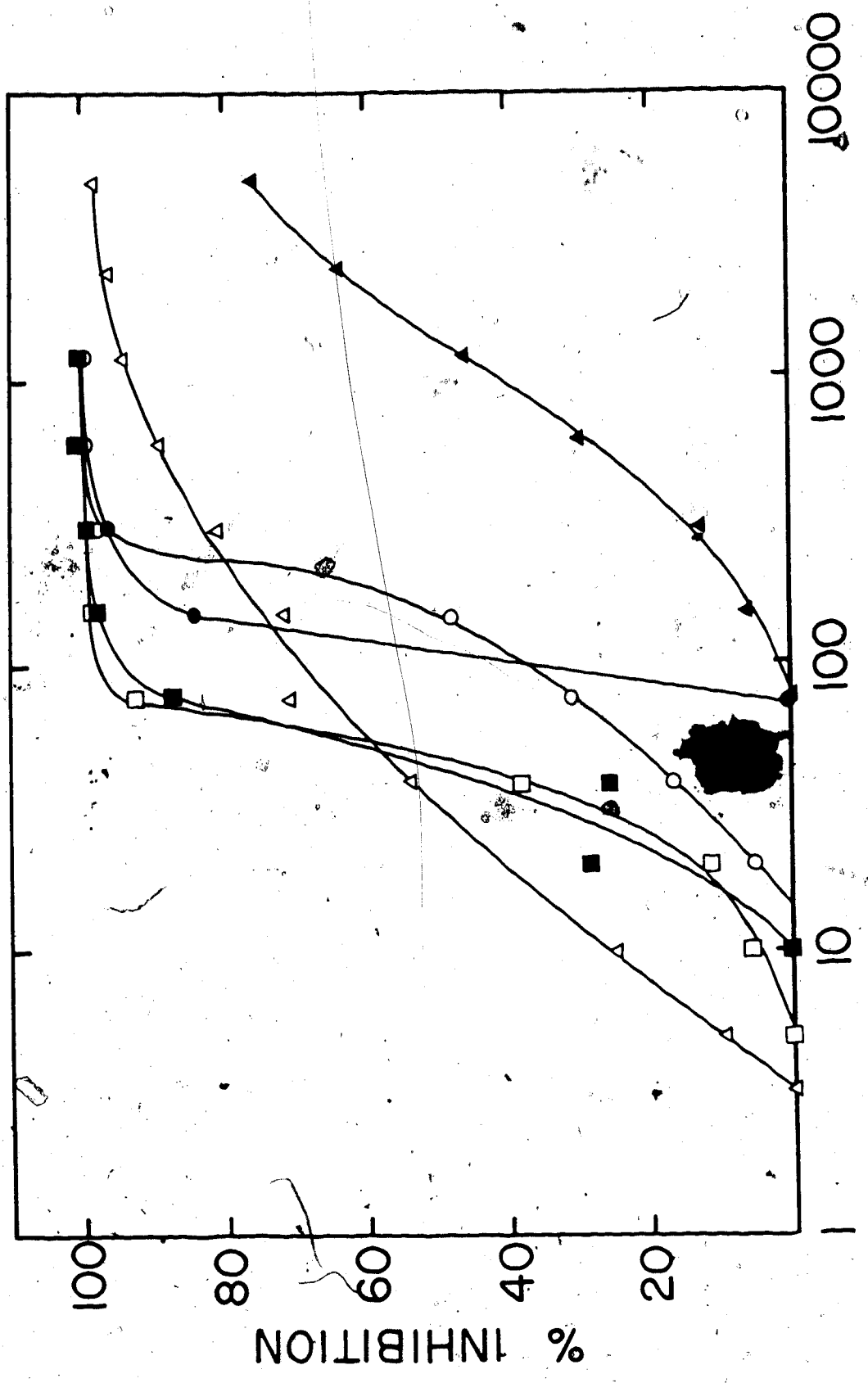


Figure IV.5 Competitive ELISA using monoclonal and polyclonal antibodies for the comparison of binding affinities for CFA/1 pili. 25 pmole/ml of purified IgG from each antibody was used in the assay. See text for details.
Symbols: ○ monoclonal antibodies from WPC-1, ● monoclonal antibodies from WPC-3, ■ monoclonal antibodies from WPC-5, □ monoclonal antibodies from WPC-6, △ CFA/1 pilus specific polyclonal antibodies, ▲ K99 pilus specific polyclonal antibodies.


% INHIBITION
100
80
60
40
20
1
10
100
1000
10000
COMPETITOR CONCENTRATION (pmoles/ml)

teraction) and aK's (the apparent affinity constants calculated as described by Nieto et al., 1984) are listed for each antibody in Table IV.1. One can see from Figure IV.5 that all four monoclonal antibodies have a similar inhibition curve. Different binding affinities were observed for each monoclonal antibody (Table IV.1), suggesting that they originated from separate hybridomas. The inhibition curves of the polyclonal IgG have a different shape than those of the monoclonal antibodies. This may be explained by the fact that monoclonal antibodies are specific for one determinant on the pilus, whereas antibodies from a polyclonal source represent a mixture of antibody types to all the antigenic regions on the pilus, including overlapping areas of the same determinant. This heterogeneity results in a shallow slope in the inhibition curve in comparison to the sharper slope observed for the monoclonal antibodies.

A distinct crossreactivity was again found to exist between CFA/1 and K99 pili (Figure IV.5). The affinity of K99 antibodies for CFA/1 pili was lower than that seen for CFA/1 pilus specific antibodies (Table IV.1). The reason for the weaker interaction of K99 antibodies for CFA/1 pili is not clear. Chapter III describes the examination of modifications in antigenic sites and the effect of changes in antigen-antibody interactions with modifications in antigenic sites for another pilus protein. Substitutions in the sequence of a major antigenic determinant resulted in a

Table IV.1

Comparison of affinities of monoclonal and polyclonal antibodies for CFA/1 pili[a]

Antibody source	I-50[b] (pmole/ml)	aK[c] (1/mole)
WPC-1	150	6.7×10^6
WPC-3	110	9.1×10^6
WPC-5	45	2.2×10^7
WPC-6	40	2.5×10^7
CFA/1 pilus specific polyclonal antisera	33	3.0×10^7
K99 pilus specific polyclonal antisera	1400	7.1×10^5

a. 25 pmole/ml of purified IgG from each antibody source was used in a competition ELISA with CFA/1 pili as the competitor. Details of the experiment are found in the Materials and Methods and results presented graphically in Figure IV.5.

b. I-50 is the concentration of competitor required for 50% inhibition of antibody binding to the antigen coated microtitre wells.

c. aK is the apparent affinity constant for the antibody. aK = 1/I-50, according to Nieto et al. (1984).

shift in binding affinities as reflected by inhibition curves similar to that seen with the K99 antibodies. (See Figure III.8). However, since no direct sequencial homology was found between the K99 and CFA/1 pilus proteins, the common determinant may be conformational in nature.

7. Antigenic Determinants on CFA/1 pili

An attempt was made to characterize the specificities of the CFA/1 specific monoclonal antibodies described in the foregoing. Native and synthetic peptides were used in immunoblot and competition ELISA experiments along with CFA/1 pilus specific monoclonal and polyclonal antibodies. The results are presented in what follows.

a) CFA/1 native peptides

Tryptic digestion of CFA/1 pili and pilin was performed as described in Chapter II, Section F.4. Both intact pili and the pilin monomer were relatively resistant to this digestion as determined by SDS-PAGE. Cyanogen bromide chemical cleavage of the pilus protein was more successful in that three large fragments were released, as predicted on the basis of the amino acid sequence of the protein (Figure IV.6). These fragments were partially purified by conventional gel exclusion chromatography as described in Chapter II, Section F.5. Further attempts to purify these fragments to homogeneity failed, thus precluding any immun-

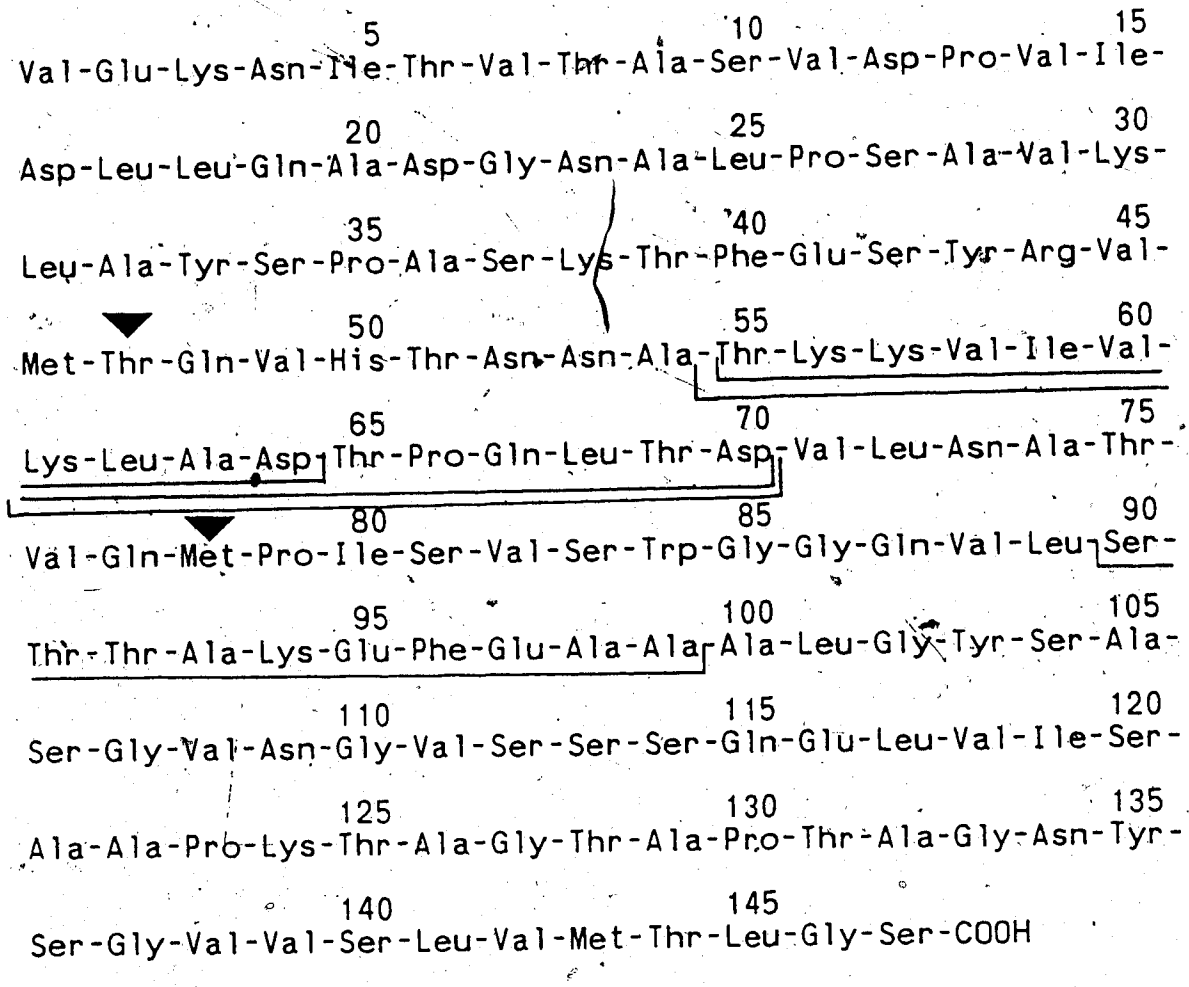


Figure IV.6 Primary structure of CFA/1 pilin (Klemm, 1982). The beginning and end of CNBr3 are indicated by ▼. Synthetic peptides used in subsequent immunoassays are underlined.

ochemical analysis of each individual fragment. However, the three fragments were readily separated by SDS polyacrylamide gel electrophoresis (Figure IV.7), thus providing the possibility of immunoblot analysis of these peptides. Hence, the CNBr peptides, (called CNBr1 for the higher molecular weight peptide, CNBr2 for the middle band and CNBr3 for the lower molecular weight peptide) were subjected to SDS-PAGE, electrophorectically transferred to nitrocellulose and the blotted peptides were reacted with either CFA/1 pilus specific polyclonal antisera or antibodies from the CFA/1 specific monoclones WPC-1, WPC-3, WPC-5 and WPC-6. Details of the immunoblot are as desribed in Chapter II, Section H.2. None of the monoclonal antibodies reacted with any of the CNBr peptides, in the immunoblot assay (data not shown). This suggests that the four monoclonal antibodies may be specific for a conformationally dependent epitope which is disrupted by the denaturing conditions of the SDS-PAGE. However, the polyclonal antibodies did interact with the lower molecular weight peptide, CNBr3 (residues 47-78, Figure IV.8), suggesting that an antigenic determinant is localized in this peptide fragment.

b) CFA/1 Synthetic Peptides

The CNBr peptide identified as antigenic by the previous experiments was difficult to purify in sufficient quantities to produce subfragments for competition ELISA's.

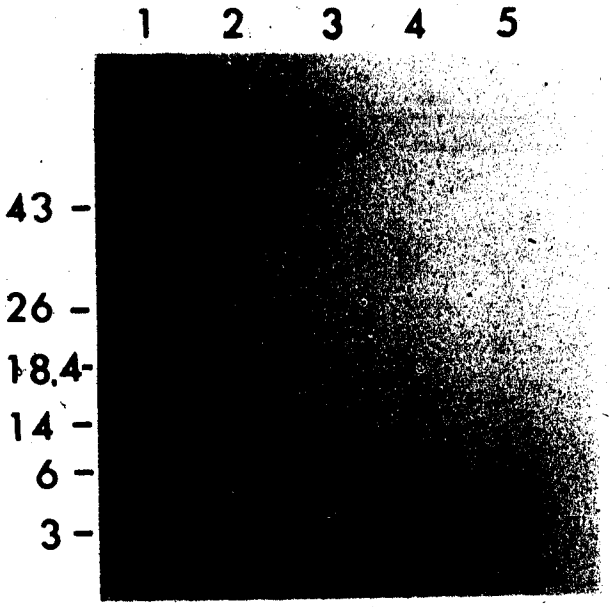


Figure IV.7 SDS-polyacrylamide gel electrophoresis of CFA/1 pili and corresponding cyanogen bromide peptides. 1 = from to to bottom, ovalbumin, alpha chymotrypsinogen, beta lactoglobulin, lysozyme and cytochrome c (doublet), bovine pancreatic trypsin inhibitor (BPTI), and the A and B chains of insulin, molecular weights of the standards are indicated by the thousands; 2 = CFA/1 pili; 3 = CNBr1 (residues 79-147); 4 = CNBr2 (residues 1-46); 5 = CNBr3 (residues 47-78).

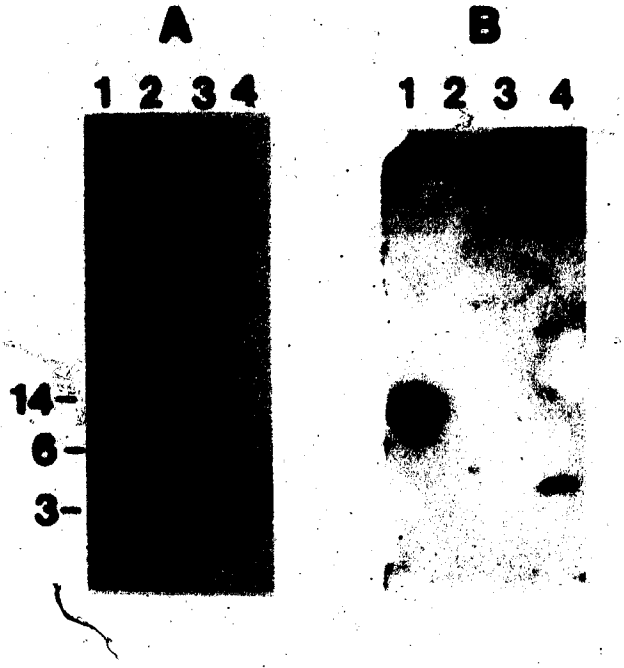


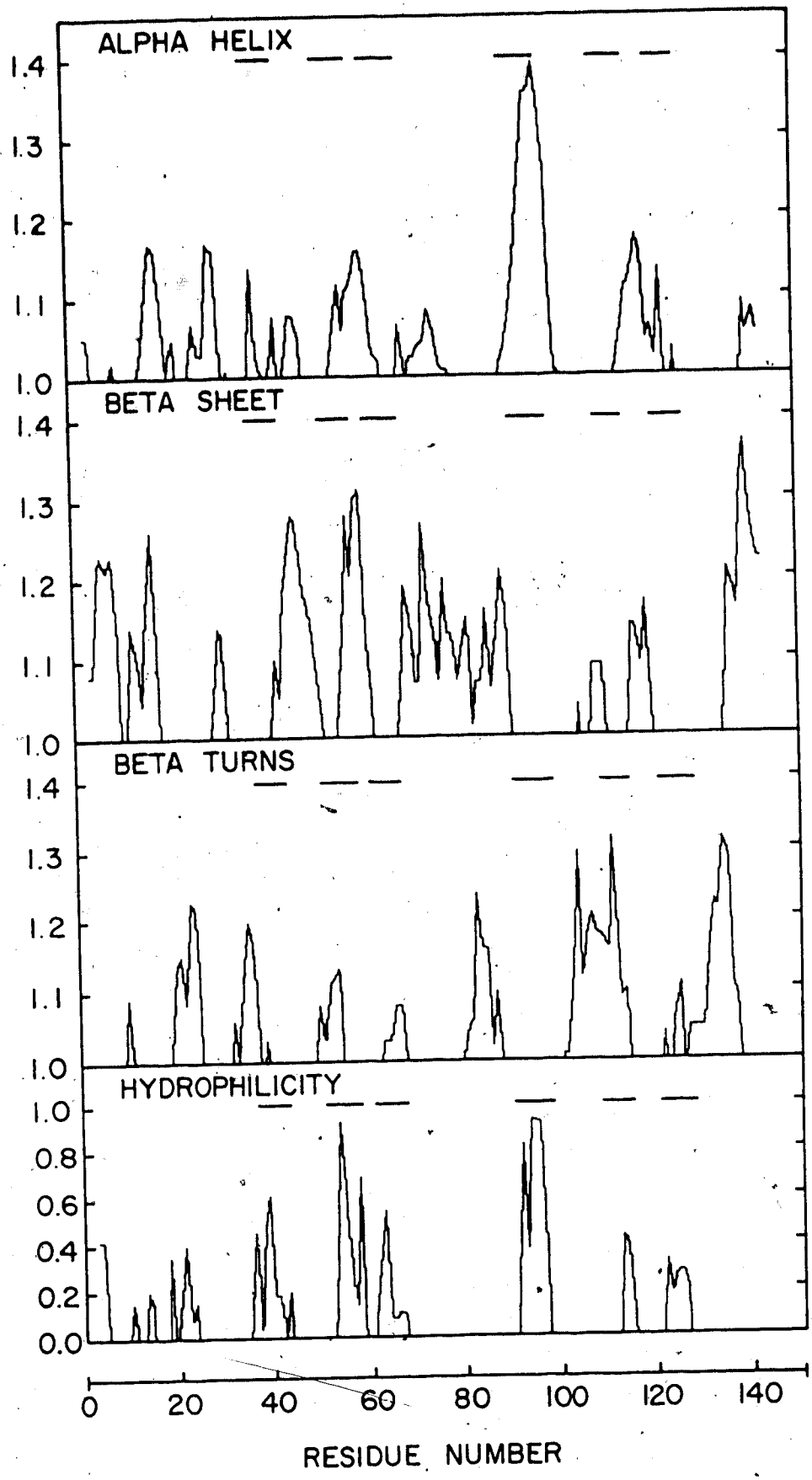
Figure IV.8 Immunoblot analysis of CFA/1 pilin and CNBr peptides.
A. SDS-PAGE of proteins prior to electrophoretic transfer to nitrocellulose.
1 = CFA/1 pili; 2 = CNBr1; 3 = CNBr2; 4 = CNBr3.
B. Autoradiograph of gel A after transfer to nitrocellulose and treatment with a 1:250 dilution of CFA/1 specific polyclonal antisera followed by ^{125}I labelled protein A.
See materials and Methods for experimental details.
Key molecular weights are noted on the left side, in the thousands

Attempts were therefore made to further delineate the CFA/1 epitope in CNBr3 through the use of synthetic peptides.

Secondary structure and hydrophilicity prediction studies were performed on the CFA/1 pilin sequence in order to identify peptides possessing strong antigenic characteristics. The resulting analyses are presented in Figure IV.9. Panels A, B, and C are alpha helix, beta sheet and beta turn profiles, respectively, as determined from the parameters of Chou and Fasman (1978). Panel D represents the Hopp and Woods (1981) hydrophilicity profile for the same protein. Each point represents an average of six consecutive amino acids. The regions considered to be potential candidates for antigenic sites are indicated by bold, horizontal lines. One can see from Panel D, that two large hydrophilic maxima exist. The region centered on residues 91-98 also coincide with a strong alpha helix flanked on either side by beta turns and appears to be the best candidate. The second hydrophilicity maxima is broader and spans the residues 52-68. It appears to be predominantly beta in structure and does posses a turn centered on the proline in position 66. It was these two regions which were initially selected for synthesis and examination.

The sequence of the two synthetic peptides called AcP(55-64) and AcP(90-99) are found in Table IV.2. AcP(55-64) was chosen because it overlaps the two adjacent putative antigenic regions 52-59 and 61-68, as well as the fact

Figure IV.9 Antigenicity profile and secondary structure predictions of the CFA/1 pilus protein. Each point is an average of 6 amino acids. Horizontal lines signify predicted antigenic determinants.
A. alpha helix content as determined by Chou and Fasman (1978).
B. beta sheet content as determined by Chou and Fasman.
C. beta turn content as determined by Chou and Fasman.
D. hydrophilicity content as determined by Hopp and Woods (1981).


ALPHA HELIX
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1.3
1.2
1.1
1.0
BETA SHEET
1.4
1.3
1.2
1.1
1.0
BETA TURNS
1.4
1.3
1.2
1.1
1.0
HYDROPHILICITY
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
120
140
RESIDUE NUMBER

Table IV.2

CFA/1 Synthetic Peptides

Name	Sequence
AcP(55-64)	Ac-Thr-Lys-Lys-Val-Ile-Val-Lys-Leu-Ala-Asp-amide
AcP(61-70)	Ac-Lys-Leu-Ala-Asp-Thr-Pro-Gln-Leu-Thr-Asp-amide
AcP(55-70)	Ac-Thr-Lys-Lys-Val-Ile-Val-Lys-Leu-Ala-Asp-Thr-Pro-Gln-Leu-Thr-Asp-amide
AcP(90-99)	Ac-Ser-Thr-Thr-Ala-Lys-Glu-Phe-Glu-Ala-Ala-amide

that it resides in the CNBr fragment which interacted with CFA/1 pilus specific polyclonal antisera. AcP(90-99) was chosen because it was highly hydrophilic, suggesting a strong antigenicity potential. Competition ELISAs were performed using the two synthetic peptides as competitors along with antibodies from both polyclonal and monoclonal sources. Both purified IgG and Fab were used as the antibody in the assays. Neither peptide was capable of interacting with any type of antibody as detected by the competition assay (data not shown).

Two other peptides, AcP(61-70) and AcP(55-70) (see Table IV.2 for sequence and Figure IV.6 for location in protein) were synthesized under the premise that peptide AcP(55-64) did not encompass the entire antigenic region and the presence of the proline residue at position 66 may be a key contact point with the antibody. Again neither peptide was able to show any reactivity with any of the monoclonal or polyclonal CFA/1 pilus specific antibodies. It is possible that the antigenic region in CNBr3 is located in the regions not examined with synthetic peptides (e.g. residues 46-55 or 70-78) but these peptides did not represent good candidates for antigenic sites in terms of the parameters presented in Figure IV.9. However, as discussed in Chapter I, prediction rules are not always reliable when searching for antigenic determinants. It is also possible that the antigenic site in CNBr3 is discontinuous

in nature, that is comprized of amino acids distant in the protein sequence but brought into close proximity due to the folding of the polypeptide chain. If this were the case, the systematic use of overlapping synthetic peptides would not be sufficient to identify this type of epitope. As for the specificity of the four monoclonal antibodies WPC-1, WPC-3, WPC-5 and WPC-6, one can also assume that they recognize a conformational entity, not present in the three CNBr peptides, or a region destroyed by the cyanogen cleavage of the CFA/1 pilus protein.

C. Conclusions

Four monoclonal antibodies were successfully made against CFA/1 pili. Of the four, three (WPC-3, WPC-5 and WPC-6) were highly specific for only CFA/1 pili, as detected by ELISA and immunoblot analyses using six different pili types. One showed specificity towards K99 and ColB2 pili. All four had similar titres (Figure IV.1) and binding affinities (Table IV.1) and the apparent affinity constants for the antibodies were in the same range as those calculated for other monoclonal antibodies (Nimmo et al., 1984). Electron microscopy showed that all four antibodies bound to determinants in the pilin monomer, as each bound to the lateral surface of the intact pilus in an apparently helical array (Figure IV.4). The actual specificity of these

antibodies was not elucidated.

Monoclonal antibodies from WPC-1 were capable of interacting with K99 pili in the direct ELISA. This suggested that the two proteins may possess a common antigenic region. These two pili types have very similar biological functions. They are both thought to be necessary for bacterial attachment to intestinal cells, CFA/1 to human small intestine (Evans et al., 1978) and K99 to calf and lamb intestine (Runnells et al., 1980). This adherence leads to bacterial colonization and subsequent diarrhea. Faris et al. (1980) indicated that the ganglioside GM_2 may serve as the receptor on erythrocytes for both CFA/1 and K99 pili. However, Smit et al. recently found that the erythrocyte receptor for K99 pili was actually a glycolipid which had little resemblance to GM_2. These authors did not include CFA/1 pili in their studies.

Norgren et al. (1984) found that for bacteria possessing the Pap pili (pyelonephritis associated pili), pilus formation was not sufficient for bacterial adhesion to uroepithelial cells. Other gene products, in conjunction with the pilus, were necessary for adherence. Labigne-Roussel et al. (1984) reported a similar observation with a strain of uropathogenic E.coli which expressed a protein which promoted hemagglutination in the absence of pili. These two observations indicate that different binding domains exist, one for erythrocytes and one for epithelial cells. It is

possible that K99 and CFA/1 pili share one of these domains and that WPC-1 is directed against the common region.

K99 pilus specific polyclonal antibodies were capable of inhibiting the hemagglutination of human erythrocytes by bacteria possessing CFA/1 pili, as was the case with the CFA/1 monoclonal antibodies. However, the reverse was not true; CFA/1 antibodies did not interfere with K99 specific hemagglutination. This diminishes the possibility of a common erythrocyte binding domain between K99 and CFA/1 pili. The inhibition of hemagglutination by K99 specific antibodies may reflect a steric inhibition by antibodies binding to a region close to the receptor binding domain. One can still not rule out the existance of a common region important in epithelial cell adhesion nor a region of general homology which has nothing to do with pilus function.

Competition ELISA's using K99 pilus specific antibodies resulted in a definite interaction between these antibodies and CFA/1 pili. The apparent affinity of the K99 antibody-CFA/1 pili interaction was about 50 fold lower than that involving CFA/1 monoclonal or polyclonal IgG. This was probably due to the presence of minor differences in the two determinants.

The existance of crossreaction between pili from different bacterial strains is not a new discovery. Watts et al. (1983) found that polyclonal antisera specific for one pilus from a strain of Pseudomonas aeruginosa was capable

of interacting with pili from a related Pseudomonas strain as well as with pili from Neisseria gonorrheae. All three pili types had a region of sequential homology which was the basis of this cross reaction. The possibility of a sequential homology between CFA/1 and K99 pili does not exist, according to the published sequences of the two proteins but one can not exclude a conformational homology.

Immunoblot analysis using the four monoclonal antibodies failed to detect any interactions with pili other than CFA/1. Monoclonal WPC-1 did not react with K99 in this assay, although a distinct interaction was detected with ELISA assays. Abraham et al. (1983) found a similar result with one of their monoclonal antibodies directed against type 1 pili. They found that after denaturation the pilus protein was unable to bind a particular antibody. They stated that this antibody may bind to a region spanning two pilin subunits, perhaps involving a subunit-subunit interface. WPC-1 may recognize a similar epitope on the K99 pilus which would be destroyed during SDS-PAGE. The region on the CFA/1 pilus is probably within the pilin monomer since antibody interacts with the denatured pilin.

The actual specificity of the CFA/1 monoclonal antibodies has yet to elucidated. Studies with CNBr peptides from the CFA/1 pilus protein indicated that polyclonal antibodies recognize a peptide encompassing residues 47-78. Attempts to identify the epitope within this fragment fail-

ed suggesting that the epitope may be conformational or discontinuous in nature. Since the monoclonal antibodies failed to interact with any of the peptides, it is likely that they are also specific for a conformational epitope. However, they may also be directed at a region on the protein destroyed by chemical cleavage.

In conclusion, four CFA/1 specific monoclonal antibodies were isolated and partially characterized. ELISA and immunoblot studies with intact pili and peptides showed that these antibodies are probably directed against conformationally dependent epitopes on the CFA/1 pilus protein. If it is this region of the pilus which is responsible for interacting with receptors on human intestinal cells one could propose the use of these antibodies as the basis for a passive, orally administered preventative treatment against diarrhea, similar to that described by Sherman et al. (1983) using monoclonal antibodies to K99 pili for the prevention of diarrhea in calves.

CHAPTER V

CFA/1 Pilus Mediated Adherence to Mammalian Cells

A. Introduction

CFA/1 pili have also been studied in connection with their role in adherence to mammalian tissues. Evans et al. have found that E.coli bearing CFA/1 pili bind to intestinal epithelial cells of neonatal rabbits (1975). Moreover, the presence of CFA/1 pili coincided with the production of diarrhea when enterotoxigenic E.coli was administered to healthy human volunteers (1977). These studies were generally accepted even though the Evans group did not examine the molecular basis of this bacterial-intestinal cell interaction. Thus the experiments presented in this chapter were performed to establish the basis of this interaction with the idea of elucidating the nature of a receptor for CFA/1 pili on intestinal cells.

Faris et al. (1980) determined that the ganglioside GM_2 was capable of inhibiting the interaction of H-10407 bacteria possessing CFA/1 pili with human type A erythrocytes. The hemagglutination of human erythrocytes by these bacteria was established by experiments performed by Evans et al. (1977) and has been routinelly used as a rapid bio-

logical assay for the presence of CFA/1 pili on enterotoxigenic bacteria. This chapter also deals with the interaction of H-10407 bacteria with red blood cells and preliminary efforts to establish the nature of the receptor for CFA/1 pili.

B. Results and Discussion

1. Adherence of H-10407 bacteria to tissue culture cells

Initial studies of the adhesive qualities of H10407 bacteria were performed using a variety of human tissue culture cell lines. The basic assay used in these experiments is described in Chapter II, Section M.1. The ideal assay for assessment of adherence is to radiolabel the bacteria, and determine the number of bacteria attached to the mammalian cell by tracing the radioactivity. However, CFA/1 pili are only produced when bacteria are grown on solid media, thus making efficient incorporation of radioactive label difficult (data not shown). The next best assay was the one chosen for these studies, that is, the viable bacterial cell assay (Mackowiak and Marling-Cason, 1984). The presence of CFA/1 pili on the bacteria used in the assay was monitored by testing the ability of these bacteria to hemagglutinate human type A erythrocytes in the presence of mannose. The hemagglutination of guinea pig erythrocytes was also performed in the absence of mannose

to ensure that the bacteria did not possess type 1, or mannose sensitive pili. The presence and/or absence of CFA/1 pili was also determined by the extent to which each type of bacteria was able to agglutinate CFA/1 pilus specific antisera. The P^- or $CFA/1^-$, control bacteria were selected from $CFA/1^+$ H-10407, by screening several colonies for the absence of CFA/1 pili, as determined by the lack of hemagglutination, antibody agglutination, and by electron microscopy. The terminology used throughout this chapter will be H-10407 P^+ for bacteria possessing CFA/1 pili and H-10407 P^- for those without CFA/1 pili.

The tissue culture cells were grown to monolayers in 24 well tissue culture plates. The monolayers were thoroughly washed, placed on ice and 200 µl containing 5-8 x 10^3 colony forming units of P^+ or P^- H-10407 was added. Binding was allowed to proceed for 3 hours, after which the supernatant solutions were plated to establish the number of unbound bacteria. The percent of input bacteria which did not bind to the tissue culture cells was calculated and the results are presented in Table V.1. These results represent the averages of several determinations and their standard deviations. One can see that on the average very little of the input bacteria adhered with no significant difference observed between the binding of piliated and non-piliated bacteria (as determined by the Student t test). Since the tissue culture cells used repre-

Table V.1

Adherence of H-10407 Bacteria to Tissue Culture Cells

Cell Line (origin)	H-10407 Strain	% Input Bacteria Unbound[a] +/- S.D.[b]	N[c]
HeLa (cervix)	P^+	98.0 +/- 2.8	3
	P^-	91.8 +/- 8.5	3
Colo (colon)[d]	P^+	93.5 +/- 6.5	2
	P^-	75.0 +/- 25.0	2
HuTu (duodenum)[d]	P^+	74.5 +/- 10.0	3
	P^-	79.8 +/- 9.3	3
CCL241 (fetal)	P^+	84.2 +/- 11.5	3
	P^-	90.5 +/- 13.5	3
INT. 407 (intestine)	P^+	82.4 +/- 7.2	4
	P^-	84.5 +/- 4.0	4
None	P^+	74.7 +/- 18.5	4
	P^-	85.8 +/- 12.6	4

a. 5-8 x 10^3 colony forming units of H-10407 P^+ or P^- bacteria were initially added to the tissue culture monolayers and bacterial adherence assayed as descibed in the text. The percent of input bacteria unbound represents the amount of bacteria initially added to the monolayer which were recovered from the supernantant and wash solutions after incubation of bacteria and tissue culture cells. This was determined from plating these solutions on TSA plates and enumerating the resultant colony forming units. For all tissue culture cell lines the difference between P^+ and P^- attachment was found to be statistically insignificant ($p > 0.2$, as determined by the Student t test).

b. standard deviation

c. N = the number of experiments used in calculation of bound and unbound bacteria, each experiment was performed in duplicate.

d. originating from an adenocarcinoma.

sent many areas of the human intestine, these results appear to contradict the observations of Evans et al. (1975) and Knutton et al. (1984a). As previously mentioned, Evans found that the presence of CFA/1 pili on bacteria fed to humans correlated with the production of diarrhea, suggesting that CFA/1 pili mediate bacterial colonization of the gut prior to causing the disease. Knutton showed, using light and electron microscopy techniques that H-10407 P^+ bacteria adhered to human duodenal enterocytes and brush borders at a mean of 4.0 bacteria per cell. They theorized that this adherence resulted in colonization which eventually leads to severe diarrhea. In contrast, pilus mediated adherence was not observed in our tissue culture assay system. This could be explained by the fact that the majority of these cells are from carcinomas and the appropriate receptor for CFA/1 pili has been altered or lost. However, the fetal intestinal cells (CCL241) were normal, but pilus mediated binding was still not observed. To resolve this contradiction, the assay was performed on freshly isolated intestinal pieces from a variety of mammalian sources.

2. Adherence of H-10407 bacteria to mammalian intestinal cells

The objective of this study was to corroborate the results presented by Evans et al. (1975) regarding adherence to neonatal rabbit intestinal cells and to determine

if the same bacteria will also adhere to a number of other intestinal types.

Evans et al. (1975) injected H-10407 P^+ and P^- bacteria into the intestinal lumen of the duodenum of four day old rabbits. The rabbits were sacrificed 18 hours later and assessed for diarrhea and H-10407 colonization of the small intestine. They observed an increase in colony forming units of the piliated bacteria injected into the rabbits with a concomitant diarrheal response. The experiments presented in this section examine the interaction of the same strains of bacteria with intestines using the adhesion assay.

Neonatal rabbits were sacrificed and their small intestines removed. Pieces of the duodenal region were prepared and assayed for binding of H-10407 P^+ and H-10407 P^- bacteria as described in Chapter II, Section M.2. The results are presented in Table V.2 and represent the percent of input bacteria (5-8 x 10^4 colony forming units) which did not bind to the intestinal pieces. As observed with the tissue culture cells, only a minimal amount of adherence occurred, with no statistical difference between the adherence of piliated or non-piliated bacteria. This is in direct contrast with the observations of Evans et al. and the discrepancy may be attributable to several factors. First, in our assay the mucous layer on the intestine was carefully removed before proceeding with the assay. It

Table V.2

Adherence of H-10407 Bacteria to Neonatal Rabbit Intestines

H-10407 Strain	% Input Bacteria Unbound[a] +/- S.D.[b]
p^+	97.8 +/- 11.7
p^-	95.5 +/- 3.5

a. 5-8 x 10^4 colony forming units of H-10407 P^+ or P^- bacteria were added to the intestinal piece and bacterial adherence assayed as described in the text. Percent of unbound bacteria represents the percent of the bacteria initially added to the assay which was recovered in the supernatant and wash solutions. Supernatant and washes were combined and plated on TSA plates. The resultant colony forming units were compared to those obtained from plating the initial or input bacterial suspension. The data represents the averages of four experiments, each performed in duplicate. The difference between P^+ and P^- attachment was found to be statistically insignificant ($p > 0.2$, as determined by the Student t test).

b. standard deviation

could be possible that the bacteria initially interact with the carbohydrate rich mucous layer followed by actual attachment to the enterocytes. However, adherence should still be observed in our assay even if this theory was correct. Also, it is believed that the duodenum is virtually sterile due partly to the mucous layer. Bacteria are quickly removed from this area by the mucous which acts as conveyor belt (Finegold et al., 1983). Only bacteria which possess mechanisms to penetrate the mucous and firmly attach to the underlying brush borders remain pathogenic. Interactions of this type should have been detected in our assay. The second factor which may have resulted in a difference between this study and that of Evans et al. may be related to the temperature at which the assay was performed. The results presented in Table V.2 were obtained from experiments performed at 0°C. However, previous experiments which were also performed at 37°C and at room temperature, yielded similar adherence values (results not shown). Therefore the assays were performed at 0°C to ensure that bacterial division was limited. Finally, the possibility exists that the strain used in this study was different from that used by Evans. It is possible that over the years the strain has lost an intestinal adherence factor which is unrelated to CFA/1 pili. This factor may be another component associated with the pilus, similar to the afimbrial hemagglutinin found in uropathogenic E. coli des-

cribed by Labigne-Roussel et al. (1984). It is also worth noting that Dean and Issacson (1982), in their studies of the role of various pilus types in adherence to rabbit intestinal villous epithelium, observed similar results to those presented in Table V.2. They found that 987P and K99 piliated E.coli adhered to the rabbit intestinal cells in an in vitro assay but CFA/1 positive E.coli failed to do so. More recently, Laux et al. (1984) demonstrated that E. coli strains positive for K88 or K99 pili readily adhered to murine intestinal epithelial cells but CFA/1 piliated bacteria did not. These results confirm our observations but provide no explanation for the differences between these results and those of Evans et al.

The same adherence assay was performed using intestinal pieces isolated from 3 month old pigs, adult rabbits and guinea pigs. The results were similar to those obtained with the infant rabbit. Human buccal epithelial cells were also harvested and assayed (Chapter II, Section M.3) but no appreciable adherence to these cells of either piliated or non-piliated H-10407 was detected. The final experiment in this series was perfomed with intestinal pieces obtained from human ileal biopsies. Again, no detectable binding of either type of bactera was observed. The interaction of both types of bacteria to all of the intestinal cells was observed by phase contrast light microscopy but no obvious CFA/1 pili-mediated bacterial-intestinal cell interactions

were detected. The only intestinal type not examined was the duodenum, due to the difficulty in obtaining biopsies in this region. Since it was to duodenum that Knutton et al. (1984a) observed marked H-10407 P^+ attachment, it would be advantageous to study this intestinal region. However, until an appropriate source of human duodenum can be obtained no further examination of pilus mediated adherence to intestinal cells will be performed.

3. Adherence of H-10407 bacteria to human erythrocytes

As discussed previously, Evans' group (1977) devised a rapid method for the identification of bacteria possessing CFA/1 pili by the ability of such bacteria to hemagglutinate human type A erythrocytes in the presence of mannose. This suggests the presence of a specific interaction between the two cell types. Faris et al. (1980) deduced that the component on the red blood cell membrane which is important in the pilus-red cell interaction contains the ganglioside GM_2. The experiment of choice by this group was that of inhibition of hemagglutination by this glycoconjugate. The studies presented in this section approach the problem in a more direct fashion. The actual binding to the red cell was quantitated and the effect of several agents on this interaction was examined with a view to characterize the putative red cell receptor for CFA/1 pili.

The first stage in this study was directed at estab-

lishing that a pilus specific interaction with the erythrocyte does occur. The binding assays used in this section were similar to those in previous sections of this chapter and are described in detail in Chapter II, Section M.4. 4 x 10^7 erythrocytes were incubated, at 0°C, with 3-5 x 10^6 colony forming units of H-10407 P^+ or P^- bacteria for 60 minutes. The number of adherent and non-adherent colony forming units was determined as described before. The results are shown in Table V.3. Two different categories of results are presented. The first set is the total amount of bacteria removed from the bacterial suspension during the attachment assay. This value was determined by plating the suspension, before and after incubation with erythrocytes, on TSA plates and assaying for the number of colony forming units (CFU) which occur after incubation of the plates overnight, at 37°C. The percent of the originally added bacteria (input bacteria) remaining in the supernatant was subtracted from 100 to obtain the value in Table V.3.

The second category refects the number of bacteria obtained after the treatment of the final erythrocyte pellet with 0.05% (w/v) trypsin. Trypsinization resulted in the partial lysis of the erythrocytes, and was thought to release the major proportion of attached bacteria. This suspension was plated on TSA plates to quantitate the resultant CFUs. The percent of the input bacteria located by

Table V.3

Adherence of H-10407 Bacteria to Human Erythrocytes

	% of Input Bacteria[a] +/- S.D.[b] H-10407 P+	H-10407 P-
Total Bacteria Removed from Supernatant[c]	78.4 +/- 1.0	84.9 +/- 10.2
Total Bacteria Recovered[d]	34.3 +/- 8.2	13.2 +/- 3.3

a. 3-5 x 10^6 colony forming units of H-10407 P+ or P- bacteria were mixed with 4 x 10^7 erythrocytes and bacterial adherence assayed as described in the text. Each value represents the mean of 4 experiments, each performed in duplicate.

b. standard deviation

c. Bacteria remaining in the supernatant after incubation of bacteria and erythrocytes, then centrifugation of erythrocytes was determined by spreading the supernatant solution on TSA plates and counting the number of colonies formed after incubation at 37°C. The percent of the input bacteria found in the supernatant was calculated and subtracted from 100% to obtain the amount of bacteria assumed to be attached to the erythrocytes.

d. After the incubation of the erythrocytes with bacteria, the erythrocytes were centrifuged, then treated with trypsin. The trypsinated erythrocytes were spread on TSA plates and incubated at 37°C to determine the number of colony forming units of bacteria which may have originated from cell-associated bacteria.

this method was called the number of bound bacteria but are more aptly called the "percent of input bacteria recovered". Previous experiments to assess the effect of trypsin on bacterial viability showed little or no inactivation (data not shown).

The major points to note from the information presented in Table V.3 are that both P^+ and P^- bacteria appear to be attaching to the erythrocytes. When looking at values obtained for P^+ and P^- bacteria removed from the supernatant, there appears to be little difference in the adherence of the two strains to the erythrocytes. However, when considering the values obtained for bacteria removed from the erythrocyte pellet (i.e. bound bacteria), a significant difference is evident between the adherence of P^+ and P^- bacteria ($p < 0.05$, as determined by the Student t test), with more P^+ bacteria adhering than P^-. These results suggest the possible involvement of CFA/1 pili in the adherence of P^+ but not P^- bacteria to human erythrocytes. Similar results were obtained when the experiment was performed at room temperature. Neither hemagglutination nor adherence occured when the assay was performed at 37°C (data not shown). The assay was performed at 0°C to prevent bacterial division during the duration of the experiment.

The fact that only H-10407 P^+ bacteria were capable of hemagglutinating human erythrocytes implicated the in-

volvement of CFA/1 pili in the adherence results presented in Table V.3. It was hypothesized that two different modes of attachment may exist, distinguishing the attachment properties of P^+ and P^- bacteria to erythrocytes. An additional experiment was therefore carried out to determine the effect of purified CFA/1 pili on the adherence of H-10407 P^+ bacteria to erythrocytes. The first experiment to establish if CFA/1 pili were involved in attachment was to determine if the observed adherence to erythrocytes was pilus specific. Human type A erythrocytes were pre-treated with 2.1 mg/ml of purified CFA/1 pili prior to performing the previously described adherence assay. The results are shown in Table V.4. As one can see there is a significant decrease in the adherence of P^+ bacteria to pilus-treated erythrocytes. However, the pre-treatment of erythrocytes with pili had no significant effect on the attachment of P^- bacteria. Thus, one can conclude that the attachment of CFA/1 piliated bacteria to erythrocytes is primarily pilus mediated. A second adherence mechanism also exists, but it is not inhibited by CFA/1 pili. The nature of this mechanism was not persued.

To establish if the inhibition of H-10407 P^+ adherence to erythrocytes by purified CFA/1 pili was dose related, erythrocytes were pre-teated with various concentrations of CFA/1 pili prior to performing the previously described adherence assay. The results are presented in Figure

Table V.4

Adherence of H-10407 Bacteria to Erythrocytes in the Presence or Absence of CFA/1 Pili[a]

H-10407 Strain	Treatment	% Input Bacteria Bound[b] +/- S.D.[c]	% Difference +/- S.D.[c]
P^+	- CFA/1 pili	24.0 +/- 5.7	15.5 +/- 9.6[d]
P^+	+ CFA/1 pili	8.5 +/- 3.9	
P^-	- CFA/1 pili	6.8 +/- 1.9	0.9 +/- 4.2[e]
P^-	+ CFA/1 pili	5.9 +/- 2.3	

a. Adherence experiments were performed either without pretreatment of the erythrocytes or with treatment of the erythrocytes with 2.1 mg/ml of purified CFA/1 pili prior to performing the assay.

b. 3-5 x 10^6 colony forming units of H-10407 P^+ or P^- bacteria were added to 4 x 10^7 erythrocytes and bacterial adherence assayed as described in the text. The number of bacterial colony forming units determined by plating the erythrocyte pellet (obtained after incubation with bacteria, followed by centrifugation and trypsinization) on TSA plates was used to calculate the the percent of input bacteria associated with the erythrocytes. Each value represents the mean of 4 experiments, each performed in duplicate.

c. standard deviation.

d. The difference in the adherence of P^+ bacteria in the presence and absence of CFA/1 pili was found to be statistically significant ($p < 0.05$, as determined by the Student t test).

e. The difference in the adherence of P^- bacteria in the presence and absence of CFA/1 pili was found to be statistically insignificant ($p > 0.2$, as determined by the Student t test)

V.1 as the percent of the input bacteria bound to the erythrocytes versus pili concentration. The percent of input bacteria bound was determined by plating the final erythrocyte pellet (obtained after incubation with bacteria, followed by gentle centrifugation) on TSA plates and comparing the number of CFUs associated with this pellet and the actual number of CFUs initially added to the assay mixture. Each experiment was done in duplicate and 3-6 experiments were done at each pilus concentration. There was a definite decrease in attachment of the piliated bacteria with the increase in concentration of pili. There was a large standard deviation found for many of the adherence values. This can be attributed to the difference in degree of piliation of the bacteria from experiment to experiment as each experiment was performed on a separate day. However, the general trend of inhibition of bacterial binding in the presence of increasing amounts of free pili is readily observed. Saturation appears to occur at 19-1960 ug/ml. This range of concentrations is in keeping with those used by Issacson et al. (1978) when establishing the 987P pilus specific adherence of bacteria to porcine intestinal epithelial cells. From these and the previous experiments it can be assumed that a pilus specific adherence of H-10407 P^+ bacteria to erythrocytes exists.

Faris' group also examined the effect of pretreatment of red blood cells with two enzymes, trypsin and neuramin-

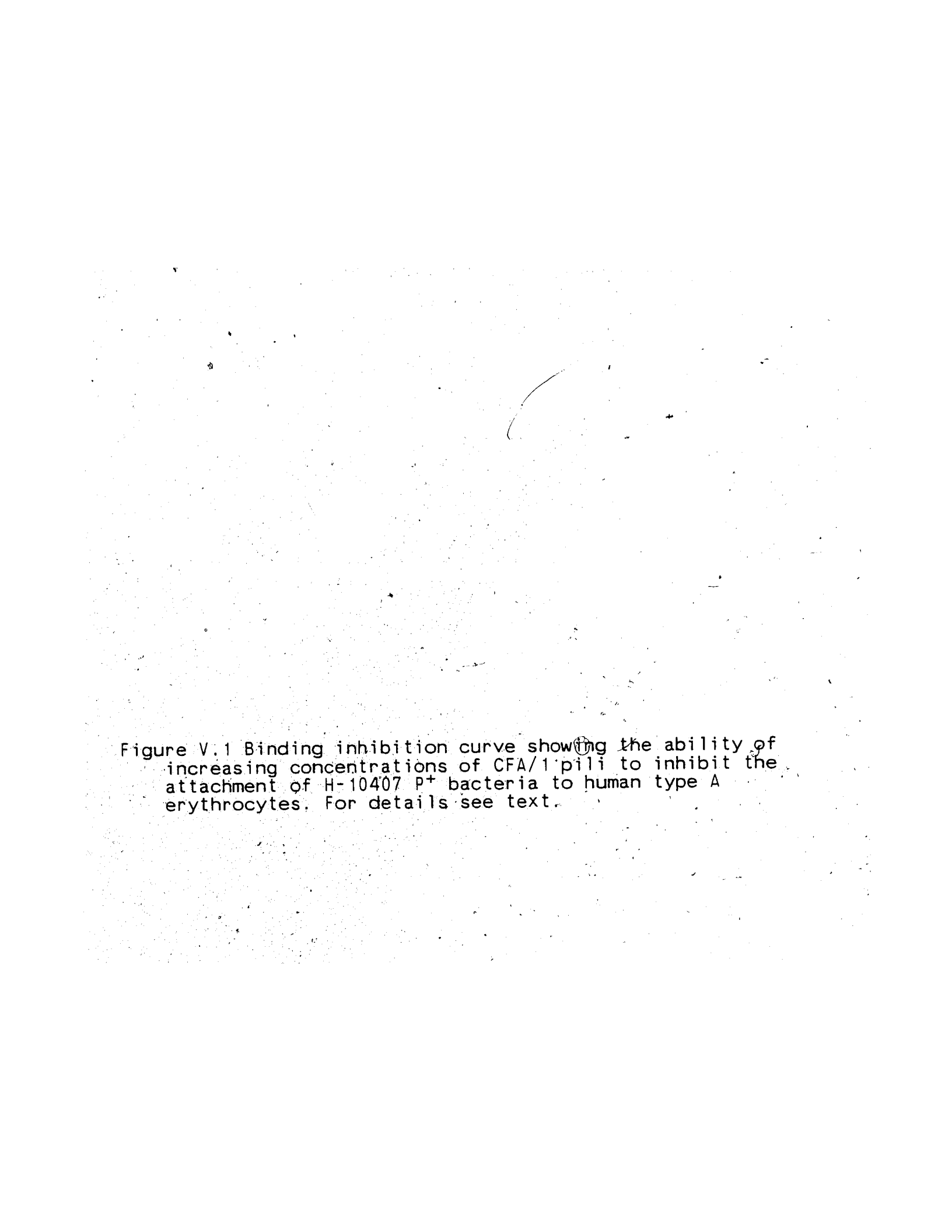
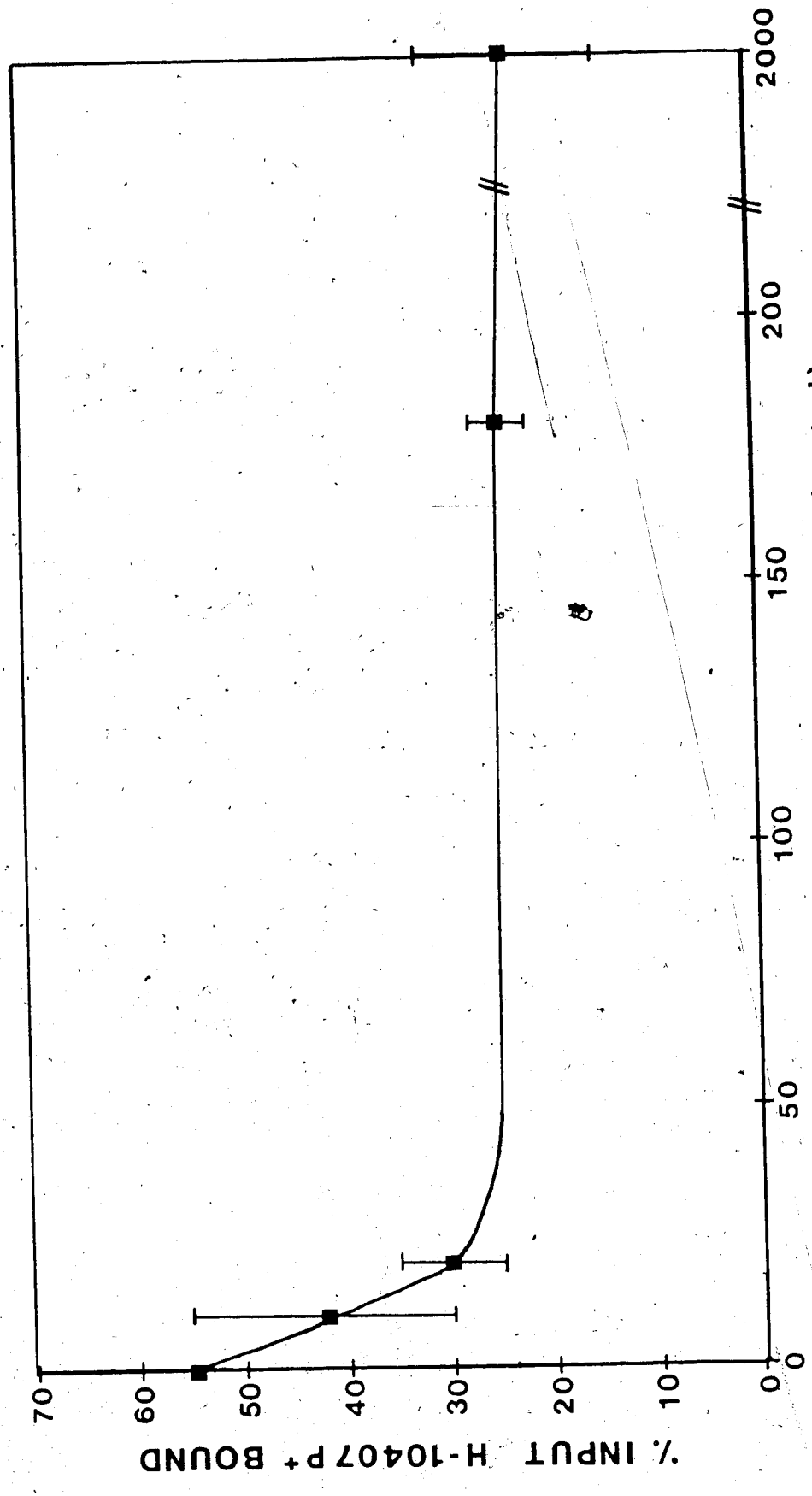


Figure V.1 Binding inhibition curve showing the ability of increasing concentrations of CFA/1 pili to inhibit the attachment of H-10407 P^+ bacteria to human type A erythrocytes. For details see text.


% INPUT H-10407P+ BOUND
0
10
20
30
40
50
60
70
0
50
100
150
200
2000
CFA/1 PILI CONCENTRATION (µg/ml)

idase on hemagglutination by H-10407 bacteria. They found that red blood cells treated with neuraminidase but not trypsin lost the ability to interact with the bacteria in question. The effect of these two enzymes was examined in our system. Human erythrocytes were pretreated for one hour with both of these enzymes (method described in Chapter II, Section M.4) prior to performing the adherence assay. The results are presented as the percent of input bacteria bound and are found in Table V.5. A statistically significant decrease in the binding of piliated bacteria was observed with both enzymes ($p < 0.05$) whereas the treatment had no significant effect on the binding of unpiliated bacteria ($p > 0.2$). Since there was no effect on the unpiliated bacteria, one can assume that the components disrupted by the enzyme treatment were pilus specific. One should note, however, that the results obtained with trypsin are in contrast to those obtained by Faris, although our results with neuraminidase are similar to those of Faris. Our findings suggest that the receptor for CFA/1 pili may be a glycoprotein.

The final approach to assessing the role of CFA/1 pili in the interaction between H-10407 P^+ bacteria and human erythrocytes involved electron microscopy. The erythrocytes were mixed with P^+ and P^- bacteria, the suspension was washed and fixed, then stained with ruthenium red. The stained material was imbedded in epon and thin

Table V.5

Adherence of H-10407 Bacteria to Trypsin or Neuraminidase Treated Human Erythrocytes

Treatment	H-10407 Strain	% Input Bacteria Bound[a] +/- S.D.[b]
None	P^+	33.7 +/- 10.0
	P^-	13.9 +/- 4.2
Trypsin	P^+	18.3 +/- 4.3[c]
	P^-	15.1 +/- 4.6[d]
Neuraminidase	P^+	19.4 +/- 3.8[c]
	P^-	12.1 +/- 1.0[d]

a. 3-5 x 10^6 colony forming units of H-10407 P^+ or P^- bacteria were mixed with 4 x 10^7 pretreated erythrocytes and bacterial adherence assayed as described in the text. Each value represents the amount of the bacteria initially added to the assay which was recovered from the final erythrocyte pellet. After incubation of erythrocytes and bacteria, the erythrocytes were pelleted by gentle centrifugation and plated on TSA plates. The resultant colony forming units were compared to those obtained from the plating of the initial bacteria suspension. Two to ten experiments were performed, each in duplicate.

b. standard deviation.

c. The difference between the attachment of H-10407 P^+ bacteria to either trypsin or neuraminidase treated erythrocytes and untreated erythrocytes was found to be statistically significant ($p < 0.05$, as determined by the Student t test).

d. The difference between the attachment of H-10407 P^- bacteria to either trypsin or neuraminidase treated erythrocytes and untreated erythrocytes was found to be statistically insignificant ($p > 0.2$, as determined by the Student t test).

sectioned for microscopic examination. The entire procedure is described in Chapter II, Section E. Figure V.2 shows electron micrographs of of P^+ bacteria with erythrocytes (Panel A), of P^- bacteria with erythrocytes (Panel B) and equivalent samples which were not treated with ruthenium red (Panels C and D). The piliated bacteria, regardless of the treatment employed, appeared to be suspended from the surface of the red cell membrane by thin filaments, which could be considered to be pili. Knutton et al. (1984b) made the same observations when they performed similar electron microscopic experiments. It should be noted that ruthenium red is thought to bind to negatively charged surface polysaccharides (Luft et al., 1971). However, CFA/1 pilus proteins have little or no carbohydrates covalently associated with them (Gaastra and de Graaf, 1982), thus the basis for ruthenium red staining remains to be elucidated. Unpiliated bacteria also appear to be adhering to the erythrocytes. This binding was also detected by the adherence assay results described in Tables V.3, V.4 and V.5. As seen in Figure V.2, panels B and D, unpiliated bacteria associate much closer to the erythrocyte membranes than do piliated bacteria. The nature of this adherence is presently not understood.

In summary, the adherence of H-10407 bacteria to human erythrocytes during the hemagglutination reaction may be pilus specific, although adherence also occurs with un-

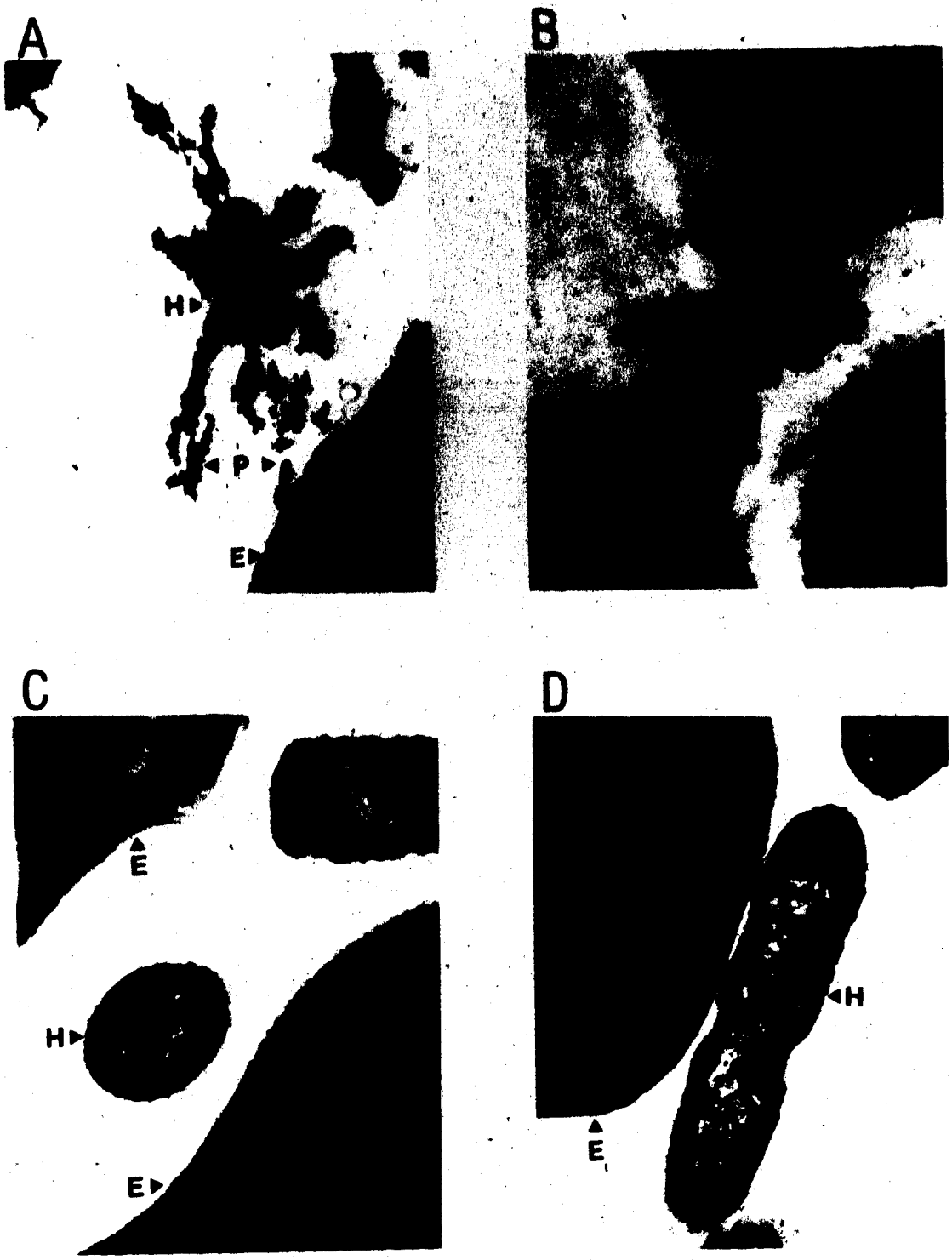
Figure V.2 Electron micrographs of thin sections of H-10407 bacteria associated with human erythrocytes. Details of sample preparation are described in Materials and Methods. Each photograph represents a 52,000 X magnification. P = CFA/1 pili, H = H-10407 bacterium, E = erythrocyte

A. Ruthenium red stained interaction of H-10407 P^+ bacteria with erythrocytes.

B. Ruthenium red stained interaction of H-10407 P^- bacteria with erythrocytes.

C. H-10407 P^+ bacteria interacting with erythrocyte, without ruthenium red stain.

D. H-10407 P^- bacteria interacting with erythrocytes without ruthenium red stain.


A
H
P
E
B
C
E
H
E
D
H
E

piliated bacteria. The receptor for CFA/1 pili on the erythrocyte was found to be sensitive to trypsin and neuraminidase, suggesting a glycoprotein with an N-acetyl neuraminic acid moiety may contribute to this receptor.

4. Interaction of H-10407 bacteria with synthetic carbohydrate conjugates

Another approach to the determination of a receptor for CFA/1 pili involved the ability of H-10407 P^+ bacteria to agglutinate latex beads coated with synthetic carbohydrate moieties. CHEMBIOMED Ltd. (Edmonton, Alberta) prepared the carbohydrate linked beads listed in Table V.6. H-10407 P^+ and P^- bacteria were suspended in PBS, mixed with the beads and assessed for agglutination. Neither the P^+ nor the P^- bacteria were capable of agglutinating any of the carbohydrate conjugates. Unfortunately, the ganglioside GM_2 was unavailable so we could not determine if Faris' conclusion that this glycoconjugate is the receptor was correct.

C. Conclusions

The initial aim of this project was to identify a tissue culture cell line which possessed the appropriate receptor for CFA/1 pili. This would provide a convenient supply of receptor material for biochemical characteriza-

Table V.6

Synthetic Carbohydrates[a] Used in H-10407 Agglutination Assays

Structure	Trivial Name
βGal(1→3)βGlcNAc ↑(1→4) αFuc	Lewis A
αFuc(1→2)βGal(1→3)βGlcNAc ↑(1→4) αFuc	Lewis B
αGalNAc(1→3)βGal	
βGal(1→4)βGlc	β Lactose
βGal(1→3)βGlcNAc	Lewis C
βGal	
βGal(1→3)αGalNAc	T
βGal(1→3)βGlcNAc(1→6)βGal	
βGal(1→4)βGlcNAc(1→3)βGal	
αGal	
αGalNAc(1→3)βGalNAc	Forssman-Disac
βGal(1→4)βGlcNAc	β LacNAc
αMan	
αGalNAc(1→3)βGalNAc(1→3)αGal	Forssman-Trisac
αGal(1→4)βGal	P_1
βGal(1→3)βGalNAc	GM_1
αMan(1→2)αMan	
βGlc(1→2)αMan	
βGalNAc(1→2)βGal	
βGal(1→4)βGlcNAc ↑(1→3) αFuc	
βGlcNAc(1→4)βGal	
βGalNAc(1→4)βGal	
α-L-Fuc(1→4)βGal	
αNANA(2→6)βGal(1→4)βGlcNAc	
αNANA(2→3)βGal	
αNANA(2→6)βGal(1→4)βGlc	

a. carbohydrates were synthesized and covalently coupled to Latex beads by CHEMBIOMED Ltd., Edmonton, Alberta.

tion. However, none of the human cell lines tested in our assay appear to interact with H-10407 P^+ bacteria. The same results were found with a variety of freshly isolated mammalian mucosal epithelial cells. The only definitive adherence of CFA/1 piliated bacteria was found using freshly isolated human duodenal cells (Knutton et al., 1984a). Our study of the HuTu cell line, of duodenal adenocarcinoma origin, resulted in little attachment of either piliated or non-piliated H-10407. This could have been attributed to the origin of the line, in that being from a carcinoma the cells may have either lost the normal cell surface receptor for CFA/1 pili or the receptor was altered in some way. Once normal human duodenum becomes available the appropriate experiments will be performed to determine in CFA/1 pili do have a role in attachment to this tissue.

The results obtained using intestinal pieces from infant rabbits are contradictory to those observed by Evans et al. (1975). They found that CFA/1 piliated H-10407 bacteria but not the unpiliated equivalent colonized the gut of neonatal rabbits and resulted in diarrhea. We found, in our assay system, that there was little attachment of either P^+ or P^- H-10407. Dean and Issacson, (1982), also found little or no adherence of H-10407 P^+ or P^- bacteria to rabbit intestinal epithelium. Further, Deneke et al., initially observed that CFA/1 positive H-10407 bacteria were capable of adhering to human duodenal epithelial

cells (1984). More recently, Deneke reported that a substantial decrease in the adherence of CFA/1 positive E.coli to the same intestinal cells occurred when the assay was performed in the presence of sulfhydryl-modifying agents (1985). Since CFA/1 pili do not contain any sulfur containing amino acids (Klemm, 1982), this data suggests that CFA/1 pili are not involved in attachment but adherence is mediated by another cell surface component which does possess an essential sulfhydryl moiety. It is also interesting to note recent work published by Evans et al. (1983) which describes in vitro binding of many strains of E.coli bearing either CFA/1 or CFA/2 pili to trypsinized fetal intestinal epithelial tissue culture cells. Careful scrutinization of their data revealed very inconclusive correlation between the presence of CFA/1 pili and adherence. In fact, H-10407 P^+ bacteria were far less efficient in binding than other strains. A reasonable explanation for this discrepancy may involve a change in the bacteria during successive subculturing in the laboratory. The bacteria we used possess CFA/1 pili, as detected by electron microscopy and antibody agglutination but may have lost other surface components which may be neccesary for adherence to intestinal cells. The presence of non-fimbrial hemagglutinins and adhesins on uropathogenic E.coli have been demonstrated (hemagglutinin by Labigne-Roussel et al., 1984, and adhesins by Norgren et al., 1984). In both cases pili were also

expressed but other proteins were necessary for either hemagglutination or adherence to epithelial cells. One can speculate that there is a second protein, perhaps at the bacterial cell surface or incorporated with the pilus, that is required for adherence to either human or rabbit intestinal cells. This also suggests that two distinct receptors may exist on the two tissue types. We found that hemagglutination of red blood cells by H-10407 P^+ bacteria does not occur at 37°C but in vivo adherence of these bacteria to intestinal cells should occur at this physiological temperature for these bacteria to be considered as pathogens. Since our bacteria are capable of hemagglutinating erythrocytes and have been shown to interact with the red cells in a pilus specific manner one can assume that it is the pilus which mediates the hemagglutination reaction but it is the intestinal adhesin which has been lost. Thus, if our bacteria are indeed deficient in this component they should not adhere to either infant rabbit or human intestinal cells. This theory will be persued upon receipt of human duodenal epithelium.

CFA/1 pilus mediated adherence to human erythrocytes was effectively demonstrated. Adherence of H-10407 P^+ bacteria was inhibited by pretreatment of the erythrocytes with intact, purified CFA/1 pili. This pilus mediated adherence was greatly reduced after treatment of the red cells with trypsin or neuraminidase, suggesting that the

putative receptor may be a glycoprotein possessing an important N-acetyl neuraminic acid moiety. The effects of these two enzymes on intact human erythrocytes is well documented. Steck et al. (1971) found that only the sialoglycoproteins are digested to any extent when erythrocytes are treated with trypsin. Anderson et al. (1981) reported that trypsinization of erythrocytes resulted in the release of 40 amino acids and 80% by weight of the carbohydrate moieties of the major erythrocyte glycoprotein, glycophorin. Fairbanks et al. (1971) found that treatment of erythrocyte ghosts with neuraminidase primarily effected the glycoproteins resulting in their increased mobilities on SDS-PAGE. One could speculate that one of these glycoproteins, perhaps glycophorin, could contain the putative erythrocyte receptor for CFA/1 pili.

CFA/1 pilus mediated adherence to human erythrocytes was also demonstrated by electron microscopy. In photographs the piliated bacteria appeared to be attaching to the red cell membrane by long filaments, presumably via the pilus tip. One could speculate that a specific structural feature is present at the pilus tip to interact with the putative red cell receptor. A similar effect has been demonstrated in phage binding to the F conjugative pilus. Filamentous DNA phage bind exclusively to the tips of this pilus (Marvin and Hohn, 1969) whereas icosohedral RNA phage such as R17 attach along the sides of the pilus (Crawford

and Gesteland, 1964). Recently, Frost et al. (unpublished) produced a monoclonal antibody specific for the tip of the F pilus. It is possible that the same effect is occurring with the CFA/1 attachment to erythrocytes. One could speculate that steric factors determine that only the tip of pilus binds to the red cell membrane but the observation that purified CFA/1 pili are not capable of agglutinating human erythrocytes strongly suggests that these pili are monovalent. One should also note that in all experiments performed using erythrocytes, P^- bacteria were capable of attachment. This adherence was not altered by the treatment of the red cells with enzymes and was not inhibited by intact CFA/1 pili. This could reflect a nonspecific adherence, perhaps mediated by bacterial LPS or some abundant cell surface protein. One could speculate that two types of interactions are occurring, a highly specific pilus mediated one and a nonspecific interaction. The nature of the second adherence mechanism was not examined.

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Appendix

Publications Arising from this Thesis

Watts, T. H., Worobec, E.A., & Paranchych, W. (1982) "Identification of pilin pools in the membrane of Pseudomonas aeruginosa." J. Bacteriol. 152: 687-691.

Worobec, E.A., Taneja, A.K., Hodges, R.S., & Paranchych, W. (1983) "Localization of the major antigenic determinant of EDP208 pili at the N-terminus of the pilin protein" J. Bacteriol. 153: 955-961.

Worobec, E.A., Shastry, P., Smart, W., Bradley, R., Singh, B., & Paranchych, W. (1983) "Monoclonal antibodies against colonization factor antigen 1 pili from enterotoxigenic Escherichia coli." Infect. Immun. 41:1296-1301.

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Armstrong, G.D., Worobec, E.A., Pieroni, P., & Paranchych, W. (1985) "Comparison studies on the attachment of H10407 CFA/I^+ enterotoxigenic Escherichia coli to erythrocytes, tissue culture cells and isolated pieces of intestine." Can. J. Micro. submitted.

Worobec, E.A., Pieroni, P., Frost, L.S., Armstrong, G.D., & Paranchych, W. (1985) "Location of major antigenic determinants on conjugative pili." manuscript in preparation.