ABSTRACT

Jeffrey Scott Miller. A STUDY OF THE SALMONELLA FLAGELLAR ANTIGENS THAT ARE BOUND BY A MURINE MYELOMA PROTEIN, MOPC 467. (Under the direction of A. Mason Smith) Department of Biology, April, 1978.

It has been previously reported that a murine IgA myeloma protein, MOPC 467, from BALB/c mice binds flagellar protein (flagellin) from several serotypically distinct species of Salmonella. In this study, affinity chromatography has been used to purify cyanogen bromide digest polypeptide from flagellin which reacts with purified MOPC-467 myeloma protein (M467).

M467 purified by immunoadsorption on a flagellin-CH-Sepharose 4B column was coupled to CNBr activated Sepharose 4B. CNBr digests of purified flagellin from various species of Salmonella were passed over the M467-S4B columns and fractions reacting with M467 were eluted in an acid buffer. The eluted fractions were further chromatographed on a G-50 Sephadex column.

Various species of Salmonella flagellin, CNBr digests of flagellin, and G-50 fractions of affinity elutions were analyzed by polyacrylamide gel electroporesis in Sodium Dodecyl Sulphate (SDS) including gradient gels and double diffusion in agar gel (Ouchterlony). An 18,000 MW polypeptide was found which was common in all

the species studied suggesting an antigenic determinant common to all Salmonella flagella.

Passive hemagglutination inhibition was used to detect quantitative differences in binding capacities of flagellin from seven species of Salmonella. It was found that the homogeneous myeloma protein M467 bound with varying avidity flagellin from the different species.

A STUDY OF THE SALMONELLA FLAGELLAR ANTIGENS THAT ARE BOUND BY A MURINE MYELOMA PROTEIN, MOPC 467

A Thesis

Presented to

The Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Biology

by

Jeffrey Scott Miller

April 1978

A STUDY OF THE *SALMONELLA* FLAGELLAR ANTIGENS THAT ARE BOUND BY A MURINE MYELOMA PROTEIN, MOPC 467

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ACKNOWLEDGEMENT

This thesis serves as a symbol of my gratitude and respect for A. Mason Smith, whose continual instruction and efforts as my thesis advisor made this manuscript possible.

I also wish to thank the members of my reading committee for their helpful comments.

Special thanks to Carolyn Griffin for her technical assistance and Mary Rout for her clerical skills.

Dedicated

to

My Parents and My Wife, Daria

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Introduction

Immunologists are continually seeking experimental models that contribute to the elucidation of the binding mechanisms between antigens and the specific antibodies made against them. The predominant requisite in such a model is the homogeneity of the antibody population that is used by the experimentor. Adherence to this requisite enables the experimentor to molecularly define the model system in exact immunochemical terms. Monoclonal plasmacytomas found in inbred mice produce an ascitic fluid that contains a high concentration of homogenous antibody(1). Plasmacytomas are of particular interest since they consist of immunoglobulin-secreting cells derived from the formation of tumors in plasma cells specialized to produce immunoglobulin molecules of single light and heavy chain classes(1). Therefore, it is accepted that the immunoglobulin found in the peritoneal fluid is a product of the normal developmental process.

The plasmacytomas found in mice, dogs, cats, and man can develop spontaneously, but usually with a low incidence (2,3,4). A method for inducing plasma cell tumors was originally discovered by Merwin and Algire who were conducting experiments using implanted diffusion chambers within the peritoneal cavity of BALB/c mice(5). Through the course of their experiments they had discovered a hemmorrhagic ascites accompanied with a plasma cell tumor. Subsequent

studies had revealed that these experimental myelomas could be induced by intraperitoneal injections of mineral oil and other compounds, ie., pristane (6,7,8). Additional investigations had indicated that there was a unique genetic susceptibility of the BALB/c and NZB strains for induction of plasmacytomas, as the incidence in other strains was considerably lower (9,10).

Potter et al have demonstrated, by extensive screening procedures, that approximately 5% of the myeloma proteins found in mice bind with specific antigens (11,12). The ability of the myeloma immunoglobulins to precipitate, agglutinate, or bind any of a variety of antigens was used to screen for their specificity. Recently, an IgA myeloma protein M467 from BALB/c mice, carrying the murine plasmacytoma MOPC 467, was found to have specific binding activity for flagellar proteins (flagellin) from over twelve species of Salmonella, Pasteurella, and Herellea(13). Since many of the species of Salmonella had distinct flagellar H antigens, as serotypically classified by the Kauffman-White scheme(14), the evidence suggested that the homogeneous myeloma protein might be reacting with a common antigenic determinant as yet undescribed. Studies have suggested that a common H antigen exists among several species, much as a common K antigen is found among several Enterobacteriacaea (14). There have been isolated instances of cross reactivity between two distinct flagellar serotypes reported, suggesting common antigenicity. Investigations by Langman

et al. have indicated, using passive hemagglutination inhibition, that five Salmonella flagellins, hitherto regarded as antigenically distinct, were shown to share common determinants (15). Langman further proposed that flagellar antigens be designated variable (Hv) or common (Hc); in these terms the Kauffman-White scheme described only the Hv determinants. The M467-binding antigen would then be categorized by the Hc classification. Joys has also reported weak cross reactions between S.adelaide, S.derby, and S.enteriditis(16).

The M467 myeloma is unprecedented in its significance to the field of immunology since it is, so far, the only plasma cell tumor that elaborated a homogeneous antibody that specifically binds a protein antigen. All other antigens bound by myeloma proteins have been non-proteins (1). Therefore, the purpose of this present study was twofold; (1) to develop a technique which could be used to isolate polypeptides from enzymatic digests of purified Salmonella flagellin which were reactive with M467, and (2) to determine the antigenic region of the flagellin molecule that binds with M467.

Bacterial flagella provides an intriguing source of antigens for the chemical characterization of antigenic determinants and studies of the specificity of antibodies against these determinants. There are few such sources of allelic variability within a single gene locus as is found in Salmonella flagellar genes. Superimposed on this

molecular variability is the antigenic specificity found among flagellin (the protein monomer which polymerizes to form intact flagellum) from the Salmonella group, and the ability of these antigens to initiate an immune response in mammalian systems(14,17,18).

The presence of flagella-specific antigens was first recognized in S.choleraesuis(19). Since then, antigen types have been extensively categorized by the Kauffman-White scheme using specific serotyping techniques(14). This scheme has provided the standard for classification of Salmonella species based on their antigenic specificities. The letters "O" and "H" have been used to designate the cell wall antigens and the flagellar antigens respectively(20).

Many Salmonella species exhibit two alternative "sets" of flagellar H antigens designated phase I and phase II (21). There exists two allelic structural genes, Hl and H2, each at individual loci and each specifying the primary structures of the flagellin of the respective phases(22). The Hi operon is composed of the operator gene ahl and the structural gene Hl, together they possess the genetic information for synthesis of phase I antigens(23,24). Synthesis of phase II antigens is controlled by an operon including the operator gene ah2, a regulator gene RGHl, and a structural gene H2(25). Salmonella phase I antigens occur by the transcription of the Hl operon while the H2 operon remains inactive. During phase II, the regulator gene RGHl, transcribes a repressor against the Hl locus.

In this phase, only the structural gene H2, is expressed. Epistasis of H2 to H1 is believed to result from an operator-negative mutation allowing derepression of the H1 gene(25).

The multiple allelic arrangement of the structural genes has been shown to be quite complex, contributing to the variable antigenicities found in the Salmonella group. The high frequency of point mutations and the short generation times found in Salmonella are probably the major factors for such multiplicity(26). The H antigenic type of a particular species of flagellin is a complex consisting of a number of subunits (antigen type determinants) which, for descriptive convenience, are often indicated by a simplified symbol(27). For example, the "g" serotype is essentially a complex of several antigenic determinants found within the flagellin structure. The antigenic determinants have been shown to be, in many cases, a complex of several distinct antigens mapped as separate units and found as a linear array within the structural gene(28). Transductional analysis of the structural gene has shown that intragenic recombination can occur between donor and recipient bacterial strains. Five mutant sites within H1-i of S.typhimurium were mapped with at least 13 antigenic factors(29,30,31). The "g" complex antigens were also shown by transductional analysis to contain at least nine seperate antigenic specificity factors mapped as individual units(28). The transductional analysis studies have indicated

Table I. Flagellar H antigens used in this study.

Species	PHA inhibition (467)	Kauffman-White Antigens Phase I	Phase II
S.milwaukee	Hi	f,g	Monophasic
S.typhimurium	Lo	i	1,2
S.adelaide	Hi	f,g	Monophasic
S.mississippi	Hi	b	1,5
S.anatum	Lo	e,h	1,6
S.donna	Lo	1,v	1,5
S.greenside	Lo	z	e,n,x

that the H structural gene, of any particular Salmonella species studied, was composed of several discrete regions that individually code for a single antigenic determinant, and that each region on the structural gene may be multi-allelic.

The discussion, thus far, serves to elucidate the antigenic mechanisms found among the genus Salmonella. This antigenic variability gives rise to a heterogeneous population of antibodies induced by the many antigenic determinants present in a single challenging species. In this regard, the specificity of M467 presents a rare and fortuitous experimental situation dealing with antigen-antibody reactions. The homogeneity of M467 antibody and other myeloma proteins has been shown by many investigators (1). The implications of the present study stress the significance of investigating a monospecific antigen-antibody reaction, using M467, without the added complexity of a heterogeneous population of antibodies against several of the many determinants present in the flagellin molecule. The M467-binding antigen is of interest due to its common occurence in many bacterial species as evidenced by its cross-reactive binding with M467(13,32). Therefore, the importance of developing specific isolation methods for proteolytic digests of the flagellin antigens was intuitively obvious and emphasized that the affinity chromatography technique used in the present study could be a prototype for further studies dealing with monospecific antigen-antibody

reactions. The S4B-467 affinity column profile indicated that the M467 antibody is "choosing" the antigenic component of the digest based on antigenicity. Work relevant to the results reported here has been accomplished by Parish et al. (33,34).

Parish et al. had characterized the fractions resulting from a CNBr digest of S.adelaide flagellin using molecular sieving and polyacrylamide gel electrophoresis(33).

Parish reported that four CNBr fragments were formed from S.adelaide digests that correlated with the three susceptible methionine residues found by amino acid analysis (33,35,36). Starting from the amino terminal end, the molecular weights of the polypeptides were 12,000(B); 18,000(A); 4,500(C); and 5,500(D), respectively(33,36). Further studies by this group showed that antigenicity resided primarily in the 18,000MW fragment(35). Fraction A contained at least three antigenic determinants of which at least two were suggested to be different(18). Genetically this could be expected as evidenced by the many sites shown by intragenic recombination within the H1 locus(29,31).

More refined studies investigating the tryptic peptides of the i antigen from S.typhimurium flagellin have shown that the antigenicity resided in a relatively small region, found to be a pentapeptide(16,37). These results inferred that it is the primary structure of the flagellin that directly dictates antigenicity, and not the tertiary folding of the molecule to form a composite binding site(26).

This assumption correlates well with the genetic mechanisms known to control synthesis of bacterial flagella(28). In the present study, a CNBr digest of S.milwaukee was accomplished with subsequent isolation of an antigenically active fragment using affinity chromatography. Affinity chromatography is believed superior to the molecular sieving methods used by Parish and his co-workers since the antigenically active fragment is isolated specifically based on its antigenicity. Isolating and defining the antigen in its smallest active entity, the haptenic region, would lead to further studies which could provide insight into the active binding site within the antibody molecule. Knowledge of this type could lead to a better understanding of the mammalian defense mechanisms against protein antigens.

Materials and Methods

Preparation of Flagellar Protein (Polymer)

Salmonella species were cultured selecting for greatest motility. Motility test medium (Motility Test Medium; Difco Laboratories, Detroit, Mich.) was used by innoculating the medium within a 50 x 5 mm open end tube that rested in the center of the test media contained in a 125 x 15 mm tube. The end of the smaller tube extended 0.5 cm above the media. The innoculated tubes were incubated at 37 C for 24 hours. On day two, cultures that had migrated to the outside of the smaller tubes were used to innoculate a second motility tube, in a likewise manner. On day three, motile cultures from the second motility tube were used to innoculate 250 ml of Tripticase Soy Broth (TSB; Baltimore Biological Laboratories, Cockeyville, Md.) and were incubated at 37 C for 24 hours. On day four, 6-7 ml of the TSB innoculum was added to each 125 ml aliquot of Brain Heart Infusion Agar (BHIA; Difco, Detroit, Mich.) slants in Blake bottles. These were cultured at 37 C for 24 hours. Harvesting was accomplished using 10 ml of 0.4% formalin and a bent glass rod used for "raking" the formalin killed cells. The harvest was centrifuged at 14,000 x G for 15 minutes and the resulting pellet resuspended in 0.4% formalin and spun again. The pellet was resuspended in the formalin solution at a concentration of 30 grams

of cells/liter and subjected to shearing in a Waring Blender for 10 minutes at low speed. The sheared flagella was centrifuged at 8,000 x G for 20 minutes to remove the cellular fraction. The supernatant, containing flagella, was centrifuged in a Beckman L5-50 preparative centrifuge at 114,000 x G for 20 minutes. The pellet was resuspended in deionized water and acidified with 1N HCl to a pH of 2.0. The solution remained at this pH for one hour before neutralizing with 0.1 N NaOH. The resulting solution was centrifuged at 100,000 x G for one hour. The supernatant was added to six equivalent volumes of saturated ammonium sulfate and allowed to polymerize at 4 C overnight. The resulting polymer (POL) was centrifuged at 25,000 x G and the pellet resuspended in 50-100 ml of deionized water. This suspension was dialyzed exhaustively against distilled water with several changes. The dialyzed solution was then lyophilyzed and weighed.

Purification of MOPC 467 anti-flagellin (M467)

The initial step in purification of M467 antibody was done by ammonium sulfate (NH_4SO_4) precipitation. Fifty ml of saturated ammonium sulfate was added, in a dropwise manner, to fifty ml of ascites fluid obtained from MOPC 467 tumor bearing BALB/c mice. The solution remained unstirred at room temperature for two hours. The precipitate was centrifuged 20,000 x G. The resulting precipitate was dissolved to one-half the original volume

in saline and the solution was again precipitated and washed in a similar manner using final concentrations of 47% ammonium sulphate. The precipitate was dissolved in saline and dialyzed exhaustively against saline.

Affinity Chromatography-Coupling of flagellin monomer (MON) to CH-S4B (CH-MON). Fifteen mg of CH-Sepharose 4B (Pharmacia, Uppsala, Sweden) was swelled overnight in 60 ml of 0.5M NaCl. To further remove lactose and dextran preservatives, the gel was washed with 200 ml of 0.5M NaCl per gram of Sepharose on a sintered glass filter. Distilled water was used as a final wash to remove the NaCl. One hundred and fifty mg of S.milwaukee POL was dissolved in 100 ml of distilled water and acidified with 0.1N HCl to pH 2.0. The solution remained at this pH for one-half hour before raising the pH to 5.0 with 0.1N NaOH. The gel was combined with the protein solution and the mixture gently swirled. Carbodiimide (Eastman Chemicals, Rochester, N.Y.) was added to obtain a final concentration of 0.1M carbodiimide. The pH was maintained at 4.5-6.0 for one hour after which the solution was allowed to react for 24 hours at room temperature in an incubator shaker. On day 2 the suspension was washed on a sintered glass funnel and the optical density (280 mm) of the supernatant determined. Coupling was represented by the following formula:

$$\% \text{ Coupling} = \frac{\text{tot.O.D. of reactant sol. -O.D. of supernatant}}{\text{tot.O.D. of reactant solution}} \times 100$$

The gel was then washed with alternating solutions of .02M Tris (hydroxymethyl) aminomethane (Tris)/HCl, pH 8.0,.15M NaCl (starting buffer) and .02M Glycine/HCl, pH 2.5, 1.0M NaCl (eluting buffer). The gel was finally resuspended in the starting buffer and poured into a 1.6 x 20 cm column. The bed volume was approximately 25 ml. The column was prerun overnight at 10 ml/hr in an upward flow with starting buffer. Five ml of the 47% ammonium sulfate cut M467 antibody was applied to the column and the optical density (280 nm) profile monitored. When the O.D. reached baseline the buffer was changed to the eluting buffer and the elution optical density monitored. All column effluents were collected in 4 ml aliquots. The elution peak was pooled and concentrated using an Amicon XM 100A molecular filtration membrane. The concentrate was then dialyzed against saline.

Purity of Purified Antibody

Molecular Gel Filtration. Five ml of the purified M467 from the fraction eluted from the CH-MON affinity column was dialyzed against starting buffer and applied to a Sephadex G200 column. The column dimensions were 2.6 x 100 cm (bed volume approximately 350 ml). A reverse flow rate of 12ml/hr was applied to the column and the $O.D._{280}$ of the effluent monitored.

Reduction and Alkylation. CH-MON Column purified M467 (20-30mg/ml) was dialyzed against starting buffer containing 2mM EDTA. The protein solution was placed in a screw cap

vial and 1.5 mg of Dithiothreitol (DTT) (Calbiochem) was added per milliliter of protein solution. The mixture was allowed to react in the dark for two hours at room temperature. Alkylation was achieved by adding 4.0 mg of iodoacetamide per milliliter of protein solution and allowing the reaction to proceed for 15 minutes at room temperature. The solution was then dialyzed against .15M NaCl.

Immunoelectrophoresis. The purity of the CH-MON column purified M467, the reduced and alkylated form, and the 47% ammonium sulphate cut of the ascites fluid were compared using agar gel immunoelectrophoresis. The antisera used was rabbit anti-mouse IgA, rabbit anti-whole mouse serum, and rabbit anti-467. Three-by-four inch glass plates were coated with 0.1% agarose (L'industrie Biologique Francais, Gennevilliers, France) and allowed to dry in a incubator at 37 C. One Oxoid I.D. Agar Tablet (Colab Industries, Glenwood, Illinois) was dissolved in 50 ml of .05M barbitone acetate buffer and placed in a boiling water bath. When the agar was completely dissolved 15 ml of the solution was applied to the base coated glass plate which rested on a leveling table. The plate was allowed to gel and was then stored at 4 C. A plastic template was used to punch the wells and troughs for the immunoelectrophoresis assay. The gel filling the wells was removed from the plate by aspiration using a pasteur pipette. The samples were applied to the wells

and a constant 38 volts applied across the plate for 50 minutes using the barbitone acetate buffer in a Gelman electrophoresis chamber. Following electrophoresis, the gel within the troughs was then removed and various antisera applied to each trough and allowed to diffuse overnight at 4 C. Presipitin lines were observable without staining and could be photographed directly. For staining, the plates were placed in .15M NaCl and distilled water, respectively, with several changes to remove all non-specific protein. Wet filter paper was applied to the top of the plates and incubated at 37 C until dry. The dried plates were placed in Courcon stain for two hours. Destaining was accomplished by diffusion in 10% acetic acid. The plates were then rinsed with distilled water and air dried.

Affinity Chromatography-Couplin of Purified M467 to Sepharose 4B (S4B-467)

Pre-swelled Sepharose 4B (Pharmacia, Uppsala, Sweden) was washed with 40 volumes of distilled water. The suspension was gently stirred on a magnetic stirrer under a ventillated hood. Three normal NaOH was added dropwise to obtain a pH of 11.0. Solid cyanogen bromide (CNBr) (Eastman Chemicals, Rochester, New York) was added at a ratio of 10:1 (S4B:CNBr) as the solution was continuously stirred. The pH was maintained within the range of 10.8-11.2 throughout the reaction using 3 NaOH. When the pH was stable (10-15 mins) at 11.0 the suspension was

washed with ice cold distilled water and filtered on a sintered glass filter. The activated Sepharose was added to the protein solution containing purified M467 at a concentration of 10mg/ml in 0.1M $NaHCO_3$. The final coupling ratio was approximately 60:1 (S4B:protein). Coupling was achieved at 4 C, overnight. On day two, the coupled S4B-467 was filtered on a sintered glass filter and the filtrate assayed for protein by reading the $O.D._{280}$. Percentage of coupling was calculated in a manner similar to the coupling of the CH-MON column. The S4B was sequentially washed with starting buffer, eluting buffer, starting buffer containing .05M ethanolamine, and finally starting buffer. The gel was resuspended in starting buffer and poured into a 1.6 x 20cm column. After packing, the column was washed overnight with starting buffer.

Polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulphate (SDS)

7.5% Homogeneous Gel Slabs. Eight by eight cm homogeneous gel slabs were constructed using a Pharmacia Gel Casting Apparatus. The gels were cast using the following solutions:*

(A) 15 g Acrylamide and .47 g of Bis-acrylamide in 50 ml of deionized water and filtered on Whatman No. 1 filter paper

* All reagents were reagent grade or electrophoresis grade (Eastman Chemicals, Rochester, New York).

(B) 100 ml of 1.5 Tris/HCl, pH 8.8

(C) 10% Sodium Dodecyl Sulphate (SDS) in deionized water at room temperature

(D) 10% ammonium persulfate. Prepared fresh daily.

(E) Tetramethylethylenediamine (TEMED)

The gel solution was prepared by combining 50 ml of solution A, 50 ml of solution B, 2 ml of solution C and diluting the mixture to 200 ml. The solution was deagsed for 1 minute, then .2 ml of this solution was added to .4 ml solution D and the mixture quickly poured into the casting tower containing the glass casettes. A water layer was gently applied to the top of the solution that fills the casettes. The casettes, themselves, were constructed using the Pharmacia Casting Kit which includes glass plates, plastic spacers, and solvent-resistant adhesive tape. After pouring, the gels were allowed to polymerize overnight at room temperature.

Electrophoresis. A Pharmacia electrophoresis chamber power supply and destainer were used for electrophoresis, staining and destaining of samples using a Tris/Acetate Buffer. The following solutions were prepared:

(A) Electrophoresis buffer: 48.46 g of Tris, 27.22 g sodium acetate, and 7.44 grams of EDTA were added to deionized water and adjusted to pH 7.4 with acetic acid and diluted to one liter. This comprised the stock solution which was diluted 10X for use in the electrophoresis system. 2g of SDS was added per liter of diluted buffer. A total of 3 liters was used for each run.

(B) 10 mM Tris/HCl, pH 8.0
1 mM EDTA
1% SDS
10% glycerol

(C) 1 g Bromphenol Blue in 100 ml of 7% acetic acid was mixed and filtered

Gels were prerun for one hour at 70 volts, constant voltage. Lyophilyzed samples of antigens were dissolved in solution B at a concentration of 5 mg/ml. Sample applicators were applied to the top of each gel between the glass plates and a pasteur pipette was used to remove any trapped air. Four to five μl of sample was mixed with 0.5 μl of solution C by drawing the plunger in and out from a 10 microliter Hamilton syringe. The sample was then applied to the well and a 70 volt constant voltage source applied until the tracking dye entered the gel. The voltage was then increased to 100 volts for 2 hours.

Protein Fixing and Removal of SDS. After each run the gels were immediately placed in a Pharmacia GD4 destaining tank and the gels electrophoretically stained with a constant 36 volts. Staining was accomplished in two 15 min intervals, turning the gel between intervals.

Electrophoretic Destaining. Gels were destained in the GD4 tank (Pharmacia) with 25% isopropanol and 10% acetic acid at 36 volts for 20-30 minutes. Gels were stored at 4 C in distilled water with .01% sodium azide.

Page with 4/30 Gradient Gels. Gels containing a gradient of 4% to 30% acrylamide were purchased from Pharmacia. All

procedures were identical to the homogeneous gels except the duration of electrophoresis. The gradient gels were allowed to electrophorese for 3 hrs before fixing.

Purification of the Antigenically Active Polypeptide from CNBr Digest

CNBr Digestion of Flagellin. A slightly modified procedure from that of Ada et al.(33) was used for digestion of flagellin with CNBr. Lyophilyzed POL was directly dissolved in 70% formic acid (10mg/ml) and agitated to insure solubility. CNBr was added at a ratio of 4:1 (CNBr:protein) and the contents agitated. Digestion continued for 18 hrs at room temperature without stirring. The final digest was then separated into two aliquots, one of which was immediately frozen in liquid nitrogen and lyophilyzed from the CNBr/formic acid solution for use in polyacrylamide gel electrophoresis (PAGE).

The other aliquot was dialyzed extensively against distilled water with a final dialysis against starting buffer. The dialysate was concentrated for 5 - 10 mg/ml using an Amicon UM2 molecular filtration membrane system. Three to four ml of the concentrated solution was applied to the S4B-467 affinity column and washed into the gel with a 2 ml/hr flow rate. The flow was then stopped for 10 minutes before resuming an upward flow rate of 10 ml/hr. Four ml fractions of effluent were collected and the O.D. monitored for each tube. When the absorption reached baseline the eluting buffer was then applied to the column

and the fractions collected and monitored in a similar manner. The eluted fraction was divided into two aliquots, one of which was dialyzed against distilled water, lyophilyzed and examined using PAGE in SDS. The other aliquot was concentrated using a Amicon UM2 molecular membrane filtration system and applied to a G50 Sephadex molecular sieving column.

G50 Sephadex Chromatography. Twenty grams of G50 Sephadex were added to 250 ml of distilled water and placed in a boiling water bath for one hour. The gel was allowed to cool overnight before degasing. The swelled Sephadex was then packed in a 1.5 x 90 cm column (170 ml bed volume) and equilibrated with .02M Gly/HCl, .15M NaCl, pH 2.5. The eluted sample from the S4B-467 affinity column was then applied to the column in an upward flow rate of 10ml/hr. All fractions were collected in 2 ml aliquots and read at an O.D. of 215 nm. Individual peaks were pooled and concentrated by molecular filtration membranes and dialyzed against distilled water. The peaks were then lyophilyzed for analysis by PAGE in SDS.

Passive Hemagglutination

Coupling of Sheep Red Blood Cells (SRBC) with MON. Defibrinated SRBC were centrifuged and washed 3 x in unbuffered saline. The cells were resuspended to 10% (v/v) in saline. Lyophilyzed POL was dissolved in .02M Tris/HCl, pH 8.0, .15M NaCl and acidified to pH 2 for 30 minutes. The monomerized solution was then neutralized

with .1N NaOH for a total concentration of 1 mg/ml. Two-tenths ml, .4 ml, and .6 ml of this MON solution were added to separate conical centrifuge tubes containing 2 ml of the 10% SRBC suspension. The tubes were then gently mixed and .2 ml of 3.75 mM chromic chloride was added to each tube and mixed again. The mixtures were allowed to stand at room temperature for 20 minutes. Tris buffered saline (TBS) with 5% fetal calf serum (FCS) and balanced salt solution (BSS) were used, respectively, for washing the coupled cells. The cells were resuspended in BSS with 5% FCS to a final concentration of .5% (v/v). The coupled cells were then tested for coupling using M467 or rabbit anti-flagellin serum in a standard microtiter system.

Limiting dilutions of M467 anti-flagellin. Titer analysis was performed to determine the limiting dilutions of M467 in the indicator system; where the SRBC-MON coupled cells were agglutinated to two serial dilutions of M467 antibody. Twenty five microliters of diluent (BSS with 5% FCS) were delivered to each well of the microtiter plate (Cooke Engineering, Arlington, Virginia). Column purified M467 was diluted 1:10 in small culture tubes. The 1/10 dilutions of M467 were placed in the first well of each row, respectively, of the microtiter plate by use of a twenty-five microliter microtitrator. Each microtitrator was then moved to the next successive well in the row, swirled, and moved again. This was

continued for 12 wells. Each well corresponded to a 1:2 dilution of the contents of the previous well. Twenty five microliters of 0.5% SRBC suspension were added to each well, including the negative control wells containing only diluent. A positive control was also used containing rabbit anti-MON. The entire plate was then covered with transparent tape to avoid evaporation. Titers were read after three hours at room temperature. Sera producing agglutination titered to 2 wells was used for the inhibition assay.

Inhibition of Passive Hemagglutination with MON from Various Species

Five mg of POL was acidified to form MON using identical volumes to insure equal concentrations among species. The optical density (215 nm) was used as a criteria to determine any dissimilarities in concentration between the various preparations. Twenty-five microliters of diluent was applied to each well of the microtiter plate. The MON inhibitors were then delivered to the first well in each row via a twenty-five microliter microtitrator, and serially diluted 1:2 along the row to 12 wells. Using a calibrated micro-pipette, twenty-five microliters of titered M467 (limiting agglutination to two wells) was applied to each well, except the positive control well. The plate was sealed with tape and incubated at room temperature for twenty minutes. Twenty-five microliters of 0.5% SRBC-MON suspension was then added to

each well. The plates were incubated at room temperature and read after three hours. In this assay the wells containing no M467 were positive controls. The wells containing M467, but no inhibitor, were the negative controls.

Double diffusion in agar gel (Ouchterlony)

One by three inch glass slides were base coated with 0.1% agarose and allowed to dry overnight at 37 C. Eight hundred and fifty mg of Ionagar no. 2 (Colab Industries, Glenwood, Illinois) were dissolved in 100 ml of 0.02M Tris/HCl, saline, at pH 8.0 and heated in a boiling water bath until thoroughly dissolved. Three and one-half ml of warm agar was applied to a base coated slide with a 10 ml disposable pipette. The slides were allowed to gel on a leveling table before placing them in humidity boxes. The slides were stored overnight at 4 C before using. Wells were punched with a brass template and the gel removed by aspiration. Pasteur pipettes were used to deliver samples to the wells. After filling, the slides were placed in a humidity box for 6-8 hours at 37 C. Precipitin lines were observed with diffused light.

Results

Purification of MOPC 467 Anti-flagellin protein

An immunoabsorbent column (CH-MON) comprised of monomerized S.milwaukee flagellin (MON), was used for purification of anti-flagellin present in 47% ammonium sulphate precipitated MOPC-467 ascites fluid. Figure 1 shows the optical density (280 nm) profile of the various CH-MON column fractions. Peaks A and B possibly indicate some molecular sieving occurring in the excluded (non-binding) fraction. The arrow in Figure 1 indicates the addition of eluting buffer which resulted in peak C. Peak C contained the antigen-binding fraction.

The eluted fraction, peak C, was subjected to molecular sieving on a Sephadex G200 column as described in Materials and Methods. The results shown in Figure 2 demonstrate the presence of two components, peaks A and B, indicating different molecular weight fractions that bound flagellin. Further analysis of the CH-MON eluted peak C was accomplished by reduction and alkylation followed by agar gel immunoelectrophoresis. Figure 3 shows the two samples used in immunoelectrophoresis and the appropriate antisera as described in the legend.

Well #1 contained the CH-MON column purified M467 which precipitated in two bands that are in agreement with the two peaks found in the G200 column results. Both precipitin lines have similar electrophoretic

Figure 1. CH-MON Affinity Column. Graph represents optical density at 280 nm vs column fractions resulting from 47% ammonium sulphate precipitated ascites. The arrow indicates the addition of eluting buffer (.02M Glycine/HCl, pH 2.5, 1.0M NaCl)

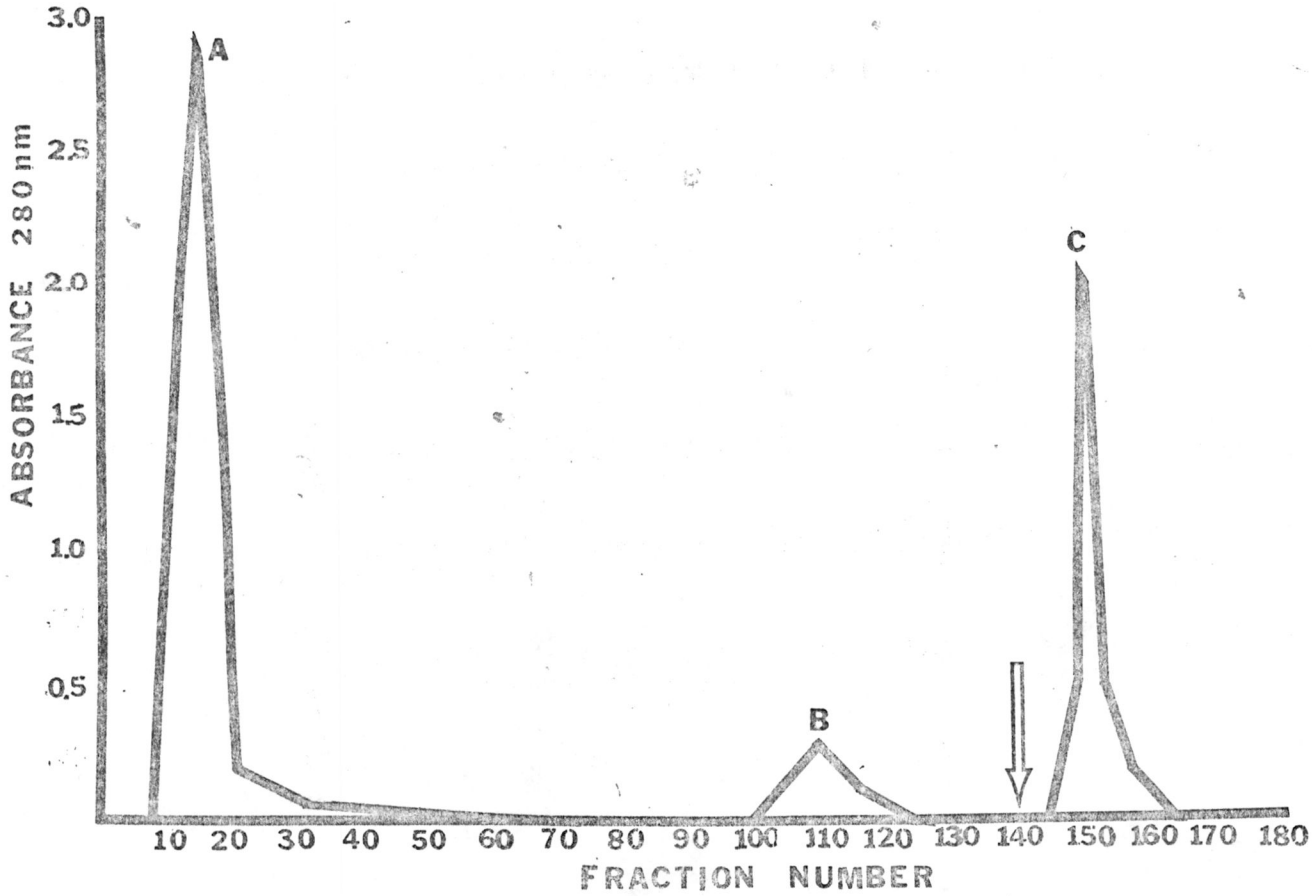

ABSORBANCE 280 nm
3.0
2.5
2.0
1.5
1.0
0.5
A
B
C
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
FRACTION NUMBER

Figure 2. Sephadex G200 Column. Optical density at 280 nm vs column fractions resulting from eluted sample from CH-MON column.

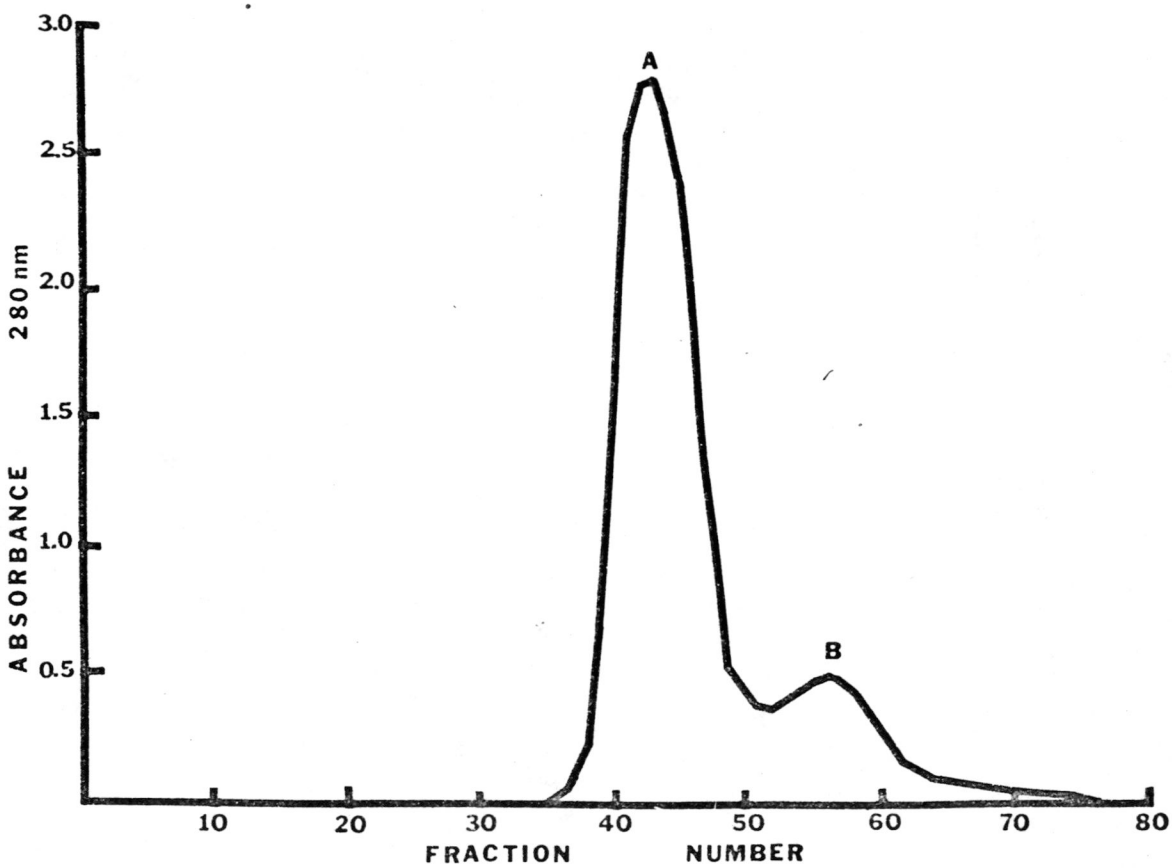
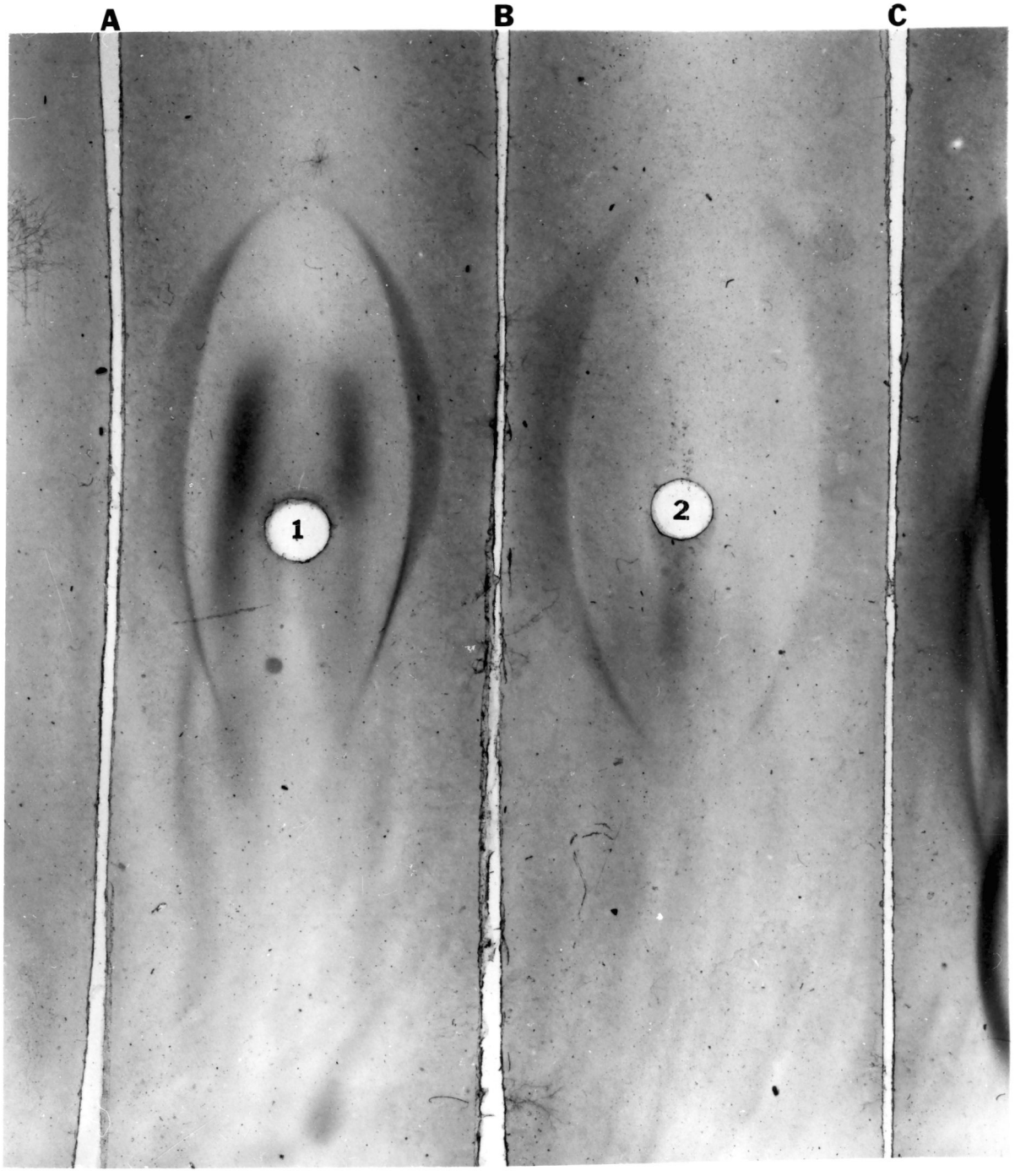

3.0
2.5
2.0
1.5
1.0
0.5
ABSORBANCE
280 nm
A
B
10
20
30
40
50
60
70
80
FRACTION NUMBER

Figure 3. Immunoelectrophoresis in agar gel of CH-MON column purified MOPC 467 anti-flagellin.
Wells: (1) CH-MON column purified M467
(2) reduced and alkylated CH-MON column purified M467
Troughs: (A) goat anti-mouse IgA (B) rabbit anti-mouse M467 ascites (C) rabbit anti-mouse whole serum


A
B
C
1
2

mobility, indicating molecularly similar components. In addition to this similarity, both lines precipitated with rabbit anti-M467 and goat anti-mouse IgA. It was concluded that the affinity column purified fraction found in M467 ascites is an IgA antibody present in monomer and dimer forms as previously described(13).

Purity of MON preparations

Purity of the flagellin (MON) preps for the seven species used in this study were assayed using PAGE in SDS with 7.5% gel slabs. The results shown in Figure 4 indicated that in each SDS preparation the presumed dimer and trimer forms were present along with the MON form. Samples 2, 4 and 5 in Figure 4 show a few additional bands which appear as faint bands. These additional bands may be due to slight acid hydrolysis in the preparation of flagellin. Alternatively, these additional bands may represent a small degree of protein contamination.

Purification of an antigenically active fragment from a CNBr digest of flagellin

The results of the separation of the CNBr digest fractions eluted from an S4B-467 affinity column are shown in the column profile in Figure 5. Peak A represents the portion of the digest which is not bound by M467 antibody. The lack of binding was confirmed by double diffusion in agar gel using purified M467 versus Peak A. Peak B did contain the binding activity

Figure 4. 7.5% Polyacrylamide Gel Electrophoresis in SDS. Various MONs from: (1) *S.milwaukee* (2) *S.adelaide* (3) *S.mississippi* (3) *S.typhimurium* (5) *S.greenside* (6) *S.anatum* (7) *S.donna*. A,B, and C refer to trimer, dimer, and monomer forms of flagellin, respectively.

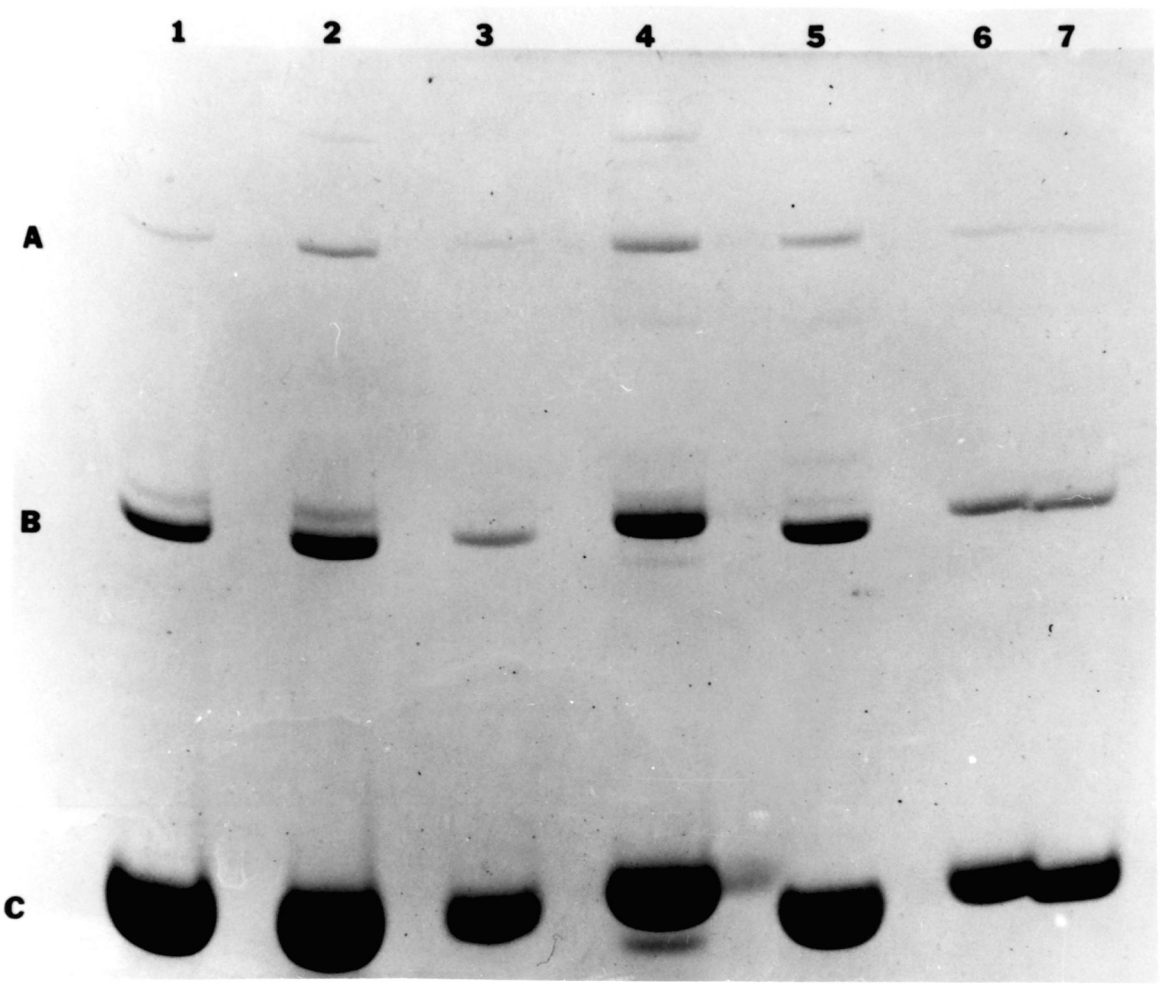
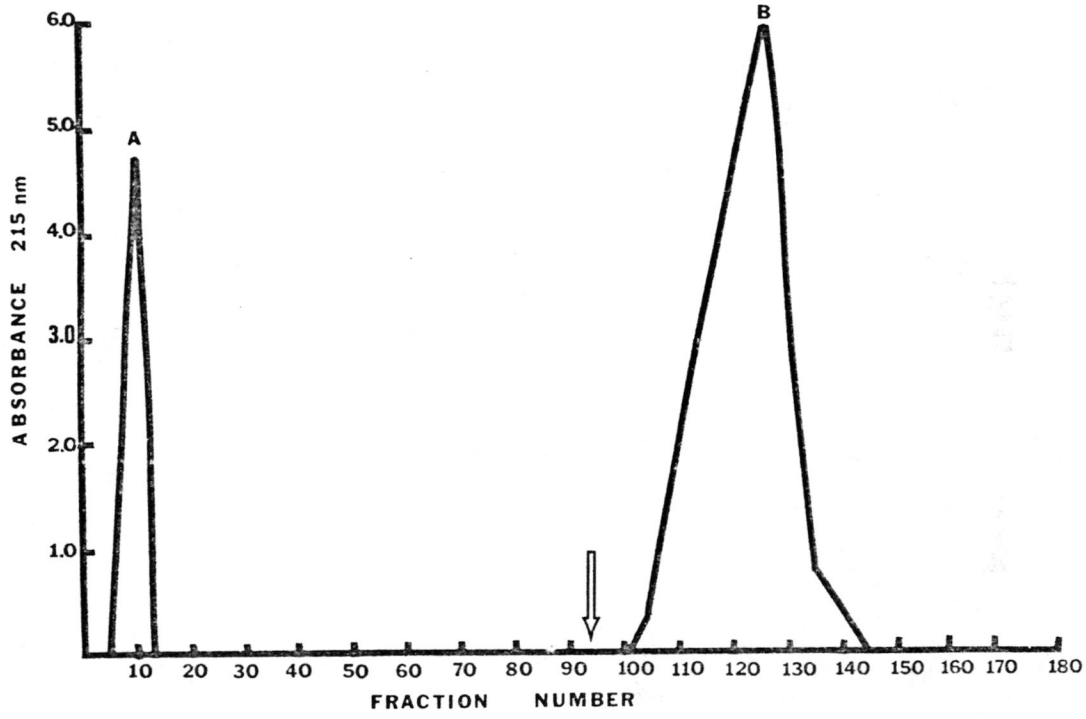

1
2
3
4
5
6
7
A
B
C

Figure 5. Results of the use of an S4B-467 affinity column. Optical density at 215 nm vs column fractions resulting from application of CNBr digested S.milwaukee flagellin to a S4B-467 affinity column. The arrow indicates the addition of eluting buffer (.02M glycine/HCl, pH 2.5, 1.0M NaCl)


ABSORBANCE 215 nm
6.0
5.0
4.0
3.0
2.0
1.0
A
B
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180
FRACTION NUMBER

and precipitated with M467 in agar gel. The arrow in figure 5 indicated the addition of eluting buffer. The eluted fraction was subjected to further separation by molecular sieving on G50 Sephadex. From the G50 results shown in Figure 6, it was evident that four identifiable peaks were present, representing four antigenically active CNBr fragments. Peaks A and B were shown to be relatively complete separations while peaks C and D were less separable which was possibly due to the peaks containing similar molecular size fractions.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate SDS

Analysis of the CNBr digest, the S4B-467 column fractions, and fractions from the G50 Sephadex column were performed using electrophoretic techniques as described in the Materials and Methods section. Sample 1, in Figure 7, consisted of three distinct bands. Band (a) in this figure corresponded to the monomer form (molecular weight approximately 40,000 - 49,000 daltons). The two higher molecular weight bands are thought to be aggregates of flagellin resulting in the formation of dimer and trimer forms. Purified MON has been shown in other electrophoretic systems to form similar bands(33).

Sample 4 in Figure 7 corresponded to the complete CNBr digest of S.milwaukee. This preparation was lyophilyzed directly from the formic acid/CNBr digest solution and applied to the electrophoresis protein

Figure 6. Results of the use of a Sephadex G50 column. Optical density at 215 nm vs column fractions resulting from application of the S4B-467 eluted fraction. Peak A (10-30), Peak B (40-50), Peak C (57-72), Peak D (73-85).

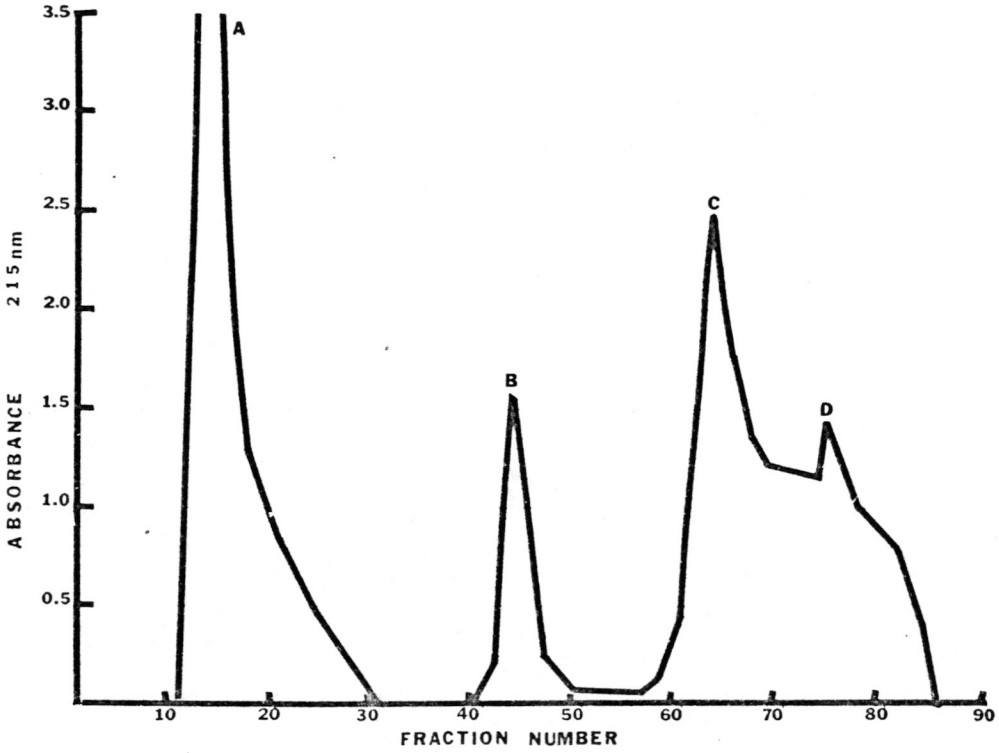
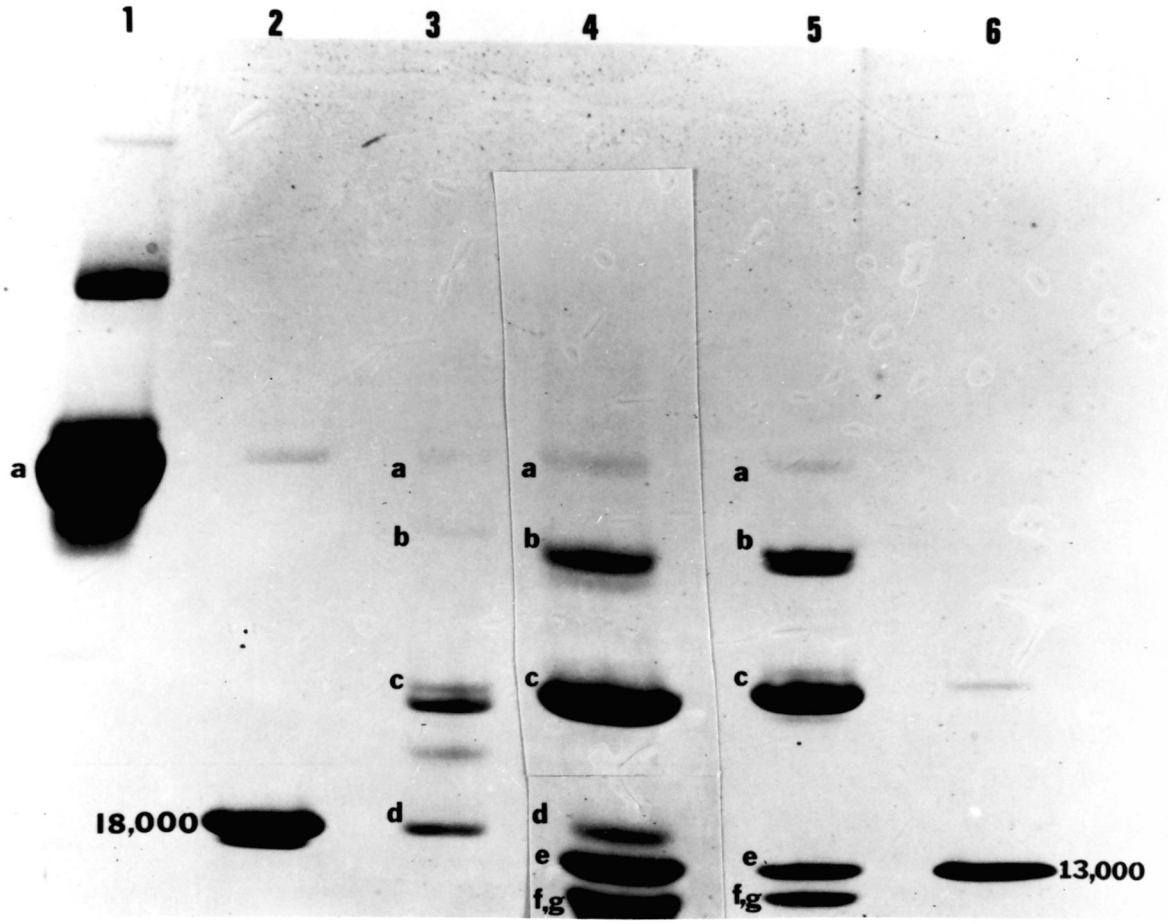

ABSORBANCE 215 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
A
B
C
D
10
20
30
40
50
60
70
80
90
FRACTION NUMBER

Figure 7. 4-30% Gradient Polyacrylamide Gel Electrophoresis in SDS. (1) S.milwaukee MON (2) 18,000 MW marker protein (3) eluted fraction from an S4B-467 column (4) a complete digest of S.milwaukee flagellin (lyophilized directly from CNBr/formic acid mixture) (5) complete CNBr digest of S.milwaukee flagellin (dialyzed against distilled water and then lyophilized) (6) 13,000 MW marker protein


1
2
3
4
5
6
a
18,000
a
b
c
d
a
b
c
d
e
f,g
a
b
c
e
f,g
13,000

solution containing SDS. As seen in the figure there were 7 bands present. Band (a) corresponded to undigested MON. Bands (d) and (e) were in the molecular weight ranges of the marker proteins in sample 2 and 6, respectively. Sample 5 contained the same CNBr digest which was exhaustively dialyzed against distilled water prior to lyophilization.

Noticeable in Figure 7 was the absence of band (d) in sample 5. The reassociation of CNBr fragments has been observed by Parish et al. (33). This is believed to be due to the removal of the homoserine lactone entity with subsequent reformation of the peptide bond. Parish and others have also reported solubility problems with the lyophilized 18,000 molecular weight fragment (band d) (33). It is presumed that the CNBr/formic acid mixture aids in preventing aggregation of the lyophilized fragment.

Sample 3 in Figure 7 contained the eluted (binding) fractions from the S4B-467 affinity column. There were four bands present in this sample that were also evident in the whole digest. The unidentified band was possibly due to some aggregation. Undigested MON (band a) is present, as might be expected, due to the presence of intact antigenic determinant. Band (d) was the smallest fragment present which was antigenically active against M467 antibody. The 18,000 MW band, band (d), was consistent with previous work describing a CNBr fragment

from S.adelaide which was bound by induced anti-flagellin antibodies. Therefore, bands (b) and (c) are most likely digestion fractions of flagellin containing the active fragment (d).

Fractions obtained from the G50 Sephadex column were analyzed on 7.5% acrylamide gel slabs as seen in Figure 8. Sample 1 contained the complete S4B-467 eluted fraction containing all four antigenically active fragments. Samples 2,3,4, and 5 represent peaks A,B,C, and D, respectively, from a G50 column described in Figure 6. Peak A corresponded to band (a) in the complete digest, which was known to be the undigested MON. Peak B is analogous to band (b). Peak C is a mixture of bands (c) and (d). Peak D represents purified band (d), or 18,000 MW fraction.

Inhibition of passive hemagglutination using MON species as inhibitors

The titers listed in Table I represent the last detectable serial dilution of various MONs used to inhibit the agglutination of SRBC-MON coupled cells by M467 antibody. Five of the MON species were coupled to erythrocytes and used in the assay. The results show that the ability of flagellin from seven different species of Salmonella to inhibit agglutination can be classed as either "Hi" or "Lo" inhibiting species. There was no substantial quantitative difference in the ability of any particular inhibitor to inhibit a "Lo" MON-coupled erythrocyte

Figure 8. 7.5% Polyacrylamide Gel Electrophoresis in SDS. (1) eluted fraction from an S4B-467 column (2) G50 Peak A (3) G50 Peak B (4) G50 Peak C (5) G50 Peak D

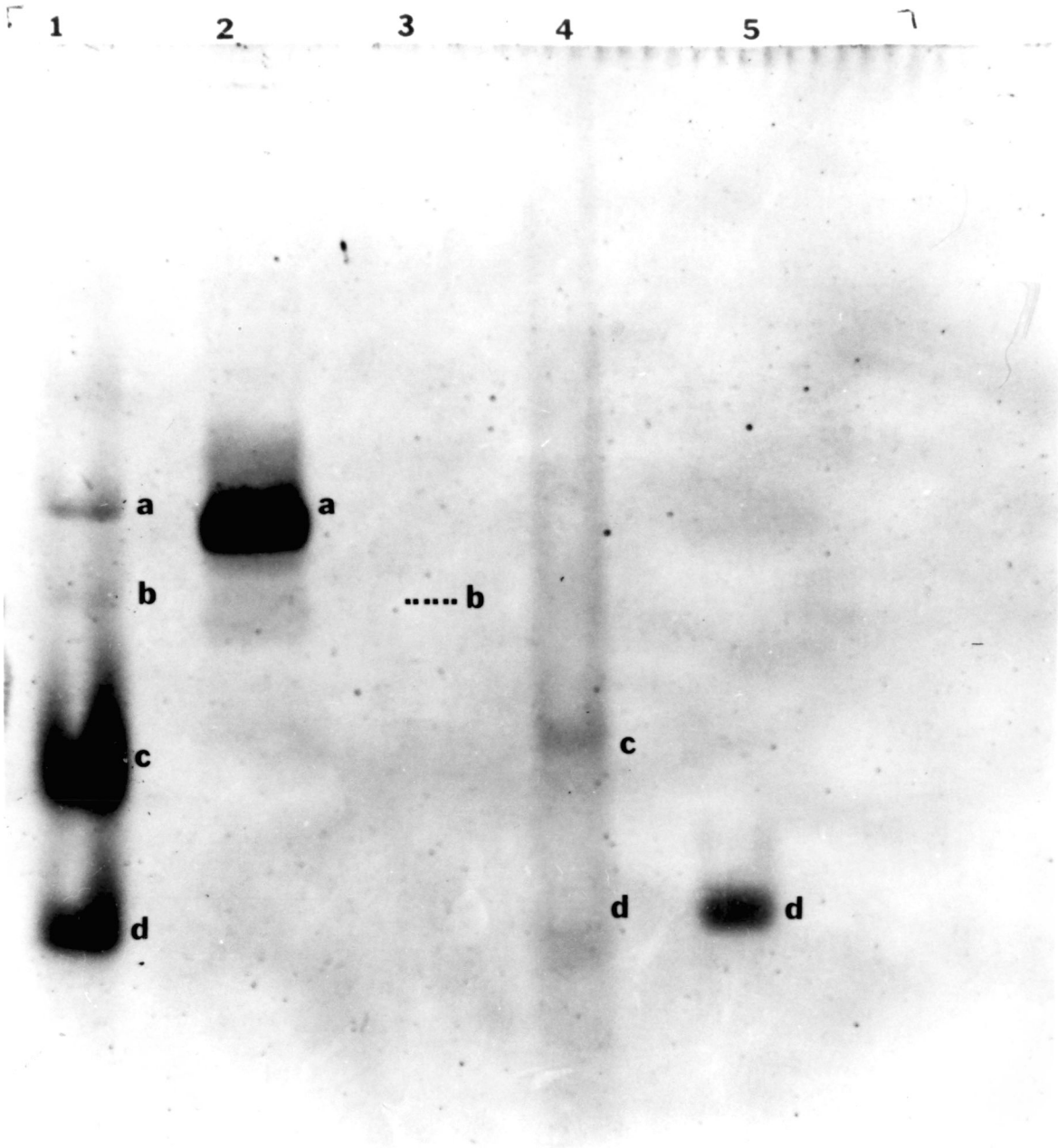

1
2
3
4
5
a
b
c
d
a
..... b
c
d
d

Table II. Inhibition titers of various MONs. The figures represent the last dilution which inhibits the agglutination reaction between the indicated SRBC-MON cell and M467 antibody.

INHIBITION TITERS ($-\log_2$)

Inhibiting MONS

SRBC-MONS	milwaukee	typhimurium	greenside	adelaide	mississippi	donna	anatum
milwaukee	6	2	1	8	7	2	2
typhimurium	7	4	4	8	9	-	-
greenside	7	1	1	8	7	3	2
adelaide	7	2	1	7	5	-	-
mississippi	6	3	3	6	6	2	1
Averages	6.6	2.4	2.0	7.4	6.8	2.3	1.7

versus a "Hi" MON-coupled erythrocyte. For example, S.milwaukee MON inhibited erythrocytes coupled to S. typhimurium, S.greenside, and S.adelaide with the same titer endpoint. If there was an affinity or avidity difference among species of MON to bind with M467 then it would be expected that the S.adelaide titer would be significantly less. The results presented here suggest there was a quantitative difference in the absolute amount of antigenically active material; whether this reflects number of antigenic determinants or numbers of flagellin molecules remains to be tested. Taking the averages of the last detectable inhibition titers for each inhibitor a histogram can be drawn to visualize these two groups as shown in Figure 9.

PAGE-CNBr digests of various MONs. A common fragment

To identify a common fragment which reacted with M467, and to demonstrate a molecular basis for classifying various flagellin into "Lo" or "Hi" inhibiting categories, the digests of each MON species was subjected to PAGE in SDS using 4-30% gradient gels. Figure 10 demonstrates the correlation of digest patterns and the inhibition titers of the various MONs. "Hi" and "Lo" (see legend) inhibiting titers had distinctly different protein bands. Noticeable in the "Hi" samples is the presence of the 12,000 molecular weight band and the two additional bands (f and g in Figure 7) with lower molecular weights. The "Lo" inhibiting samples are comprised of several distinct

Figure 9. Average hemagglutination titers of various Salmonella flagellin. Data from Table I in histogram form. The "H" and "L" letters refer to "Hi" and "Lo" inhibiting species based on relative graph heights.

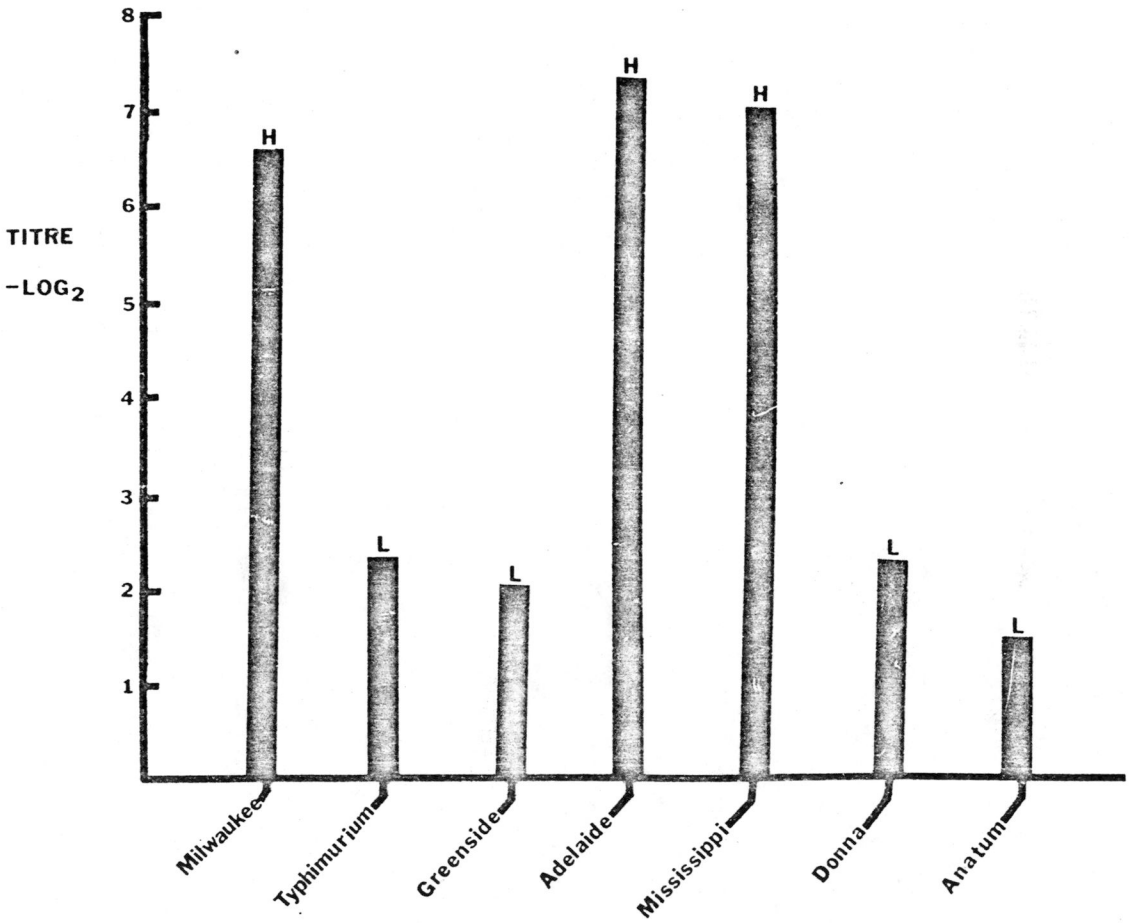
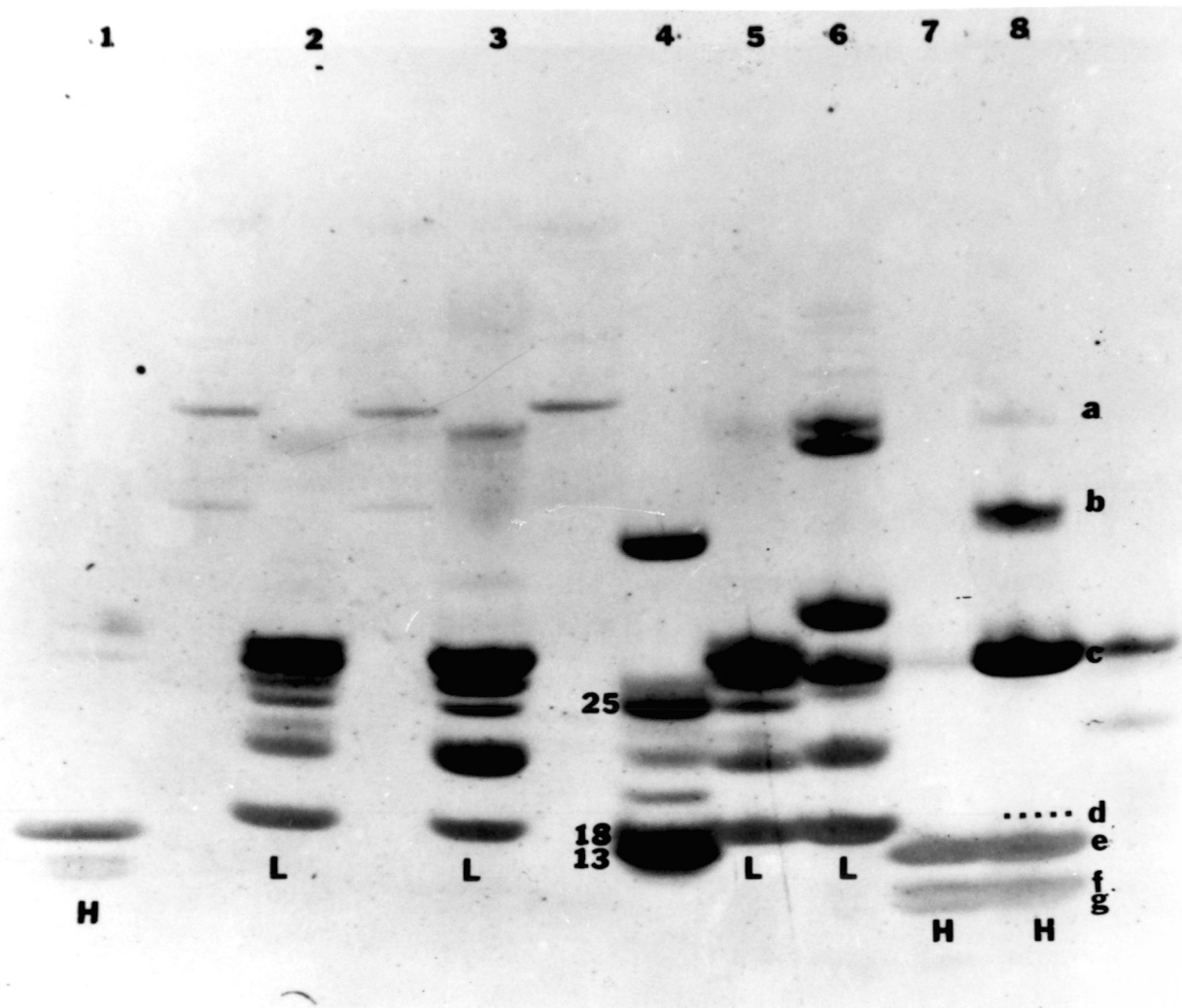

TITRE
-LOG2
8
7
6
5
4
3
2
1
H
H
H
L
L
L
L
Milwaukee
Typhimurium
Greenside
Adelaide
Mississippi
Donna
Anatum

Figure 10. 4-30% Gradient Polyacrylamide Gel Electrophoresis in SDS. CNBr digests of flagellin from: (1) S.adelaide (2) S.greenside (3) S. typhimurium (5) S.donna (6) S.anatum (7) S.mississippi (8) S.milwaukee. Sample(4) represents a mixture of marker proteins of 13,000, 18,000, and 25,000 daltons.


1
2
3
4
5
6
7
8
a
b
c
25
d
18
13
e
f
g
L
L
L
L
H
H
H

bands, none of which are lower than 18,000 daltons. An 18,000 MW fraction is present in each sample. This was found in the affinity column studies, using S.milwaukee, to be the antigenically active fraction.

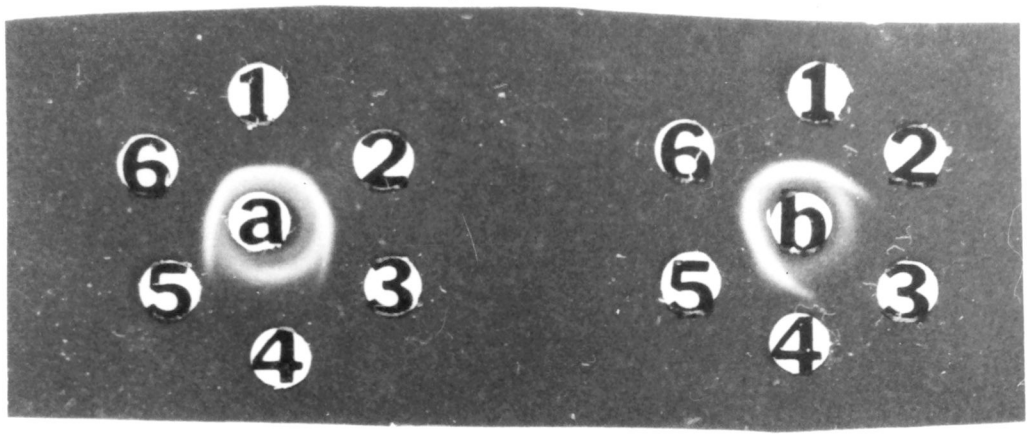
Double diffusion in agar gel

The precipitin patterns shown in Figure 11 demonstrate a partial identity between "Lo" and "Hi" inhibiting MON species in relation to their binding ability with M467. This is evidenced by the spur formation present between the two inhibitors.

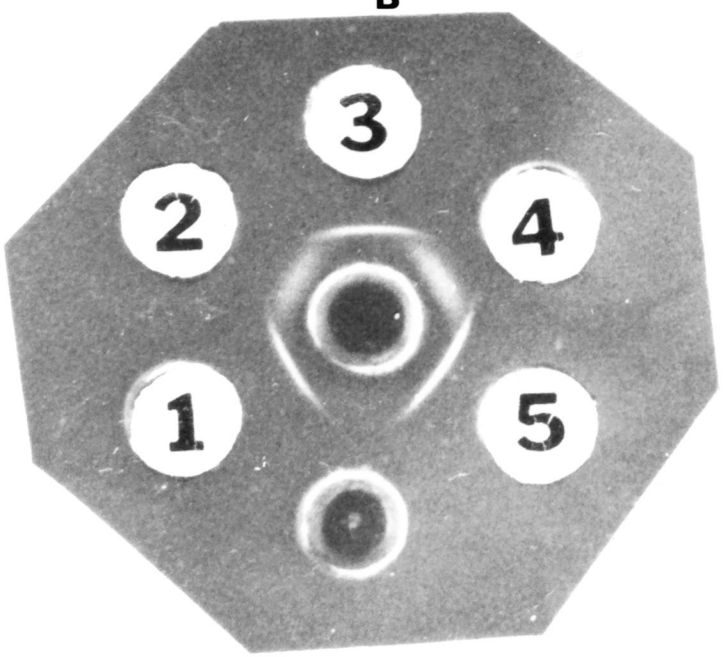
Figure 11A. Double diffusion in agar gel.
a Center well: CH-MON purified M467
High inhibitors - (1) S.milwaukee (2) S.adelaide (6) S. mississippi
Low inhibitors - (3) S.typhimurium (4) S.anatum (5) S.donna
b Center well: CH-MON purified M467
(1) S.milwaukee (2) S.anatum (3) S.donna (4) S.greenside (5) S.adelaide (6) S.typhimurium

Figure 11B. Double diffusion in agar gel.
Center well: CH-MON purified M467
(1) S.milwaukee (2) S.typhimurium (3) S. adelaide (4) S.greenside (5) S.mississippi

A



B



Discussion

In our laboratory, S.milwaukee was used for routine analysis. Serotypically, S.milwaukee and S.adelaide are identical, both being monophasic and both expressing the Kauffman-White f,g serotypes. With the assumption that these two species were genetically similar by virtue of their similar serotypes, one would expect the amino acid analysis to yield similar results(26). This rationale was used to compare the results found in Parish's group using S.adelaide and the results presented here using S.milwaukee. It was assumed that the digest fragments would be comparable in molecular weight due to the same three methionine residues. Figure 7 indicated that there were four major fragments D, E, F and G formed from cleavages at the three methionine residues. The additional bands A,B and C are suspected to be incomplete digests as mentioned in the results. It may be concluded from these results that only the 18,000 MW fragment bound to the S4B-467 affinity column, due to its antigenic specificity. Since bands (e), (f) or (g) were not bound to the affinity columns, it may be concluded, by exclusion alone, that the antigenic activity resided in the 18,000 MW fragment. This evidence was demonstrated in Figure 7 which showed that the S4B-467 eluted fraction (sample 3) was composed of bands a, b, c and d. Additionally, peak d, from the G50 column profile

shown in Figure 6, corresponded to the S4B-467 eluted peak d (18,000 MW), in purified form as shown in Figure 8. The fragments A,B, and C, bound to the affinity column and separated by G50 Sephadex molecular sieving (Figure 8), were presumably the incomplete digests containing the antigenically active fragment.

The conclusion proposed in this study, stating that the M467-binding antigen resided in the 18,000 MW CNBr fragment, agreed with the investigations by Parish and his co-workers. The distinguishing feature of the study presented here was the monospecificity of the myeloma protein versus the heterogeneity of the induced antibodies such as studied by Parish's group(35). It is probable that one of the many specific determinants discovered in previous investigations with Salmonella flagellin is the same determinant that binds with MOPC 467 antibody.

The ability of MON to precipitate with M467 in agar gel (Figure 11) indicated an adherence to classical lattice theory precipitation, hence, polyantigenic valency of the flagellin molecule in relation to M467. The number of binding sites per flagellin molecule is unknown, however, the binding of the homogeneous M467 antibody suggests that these sites are molecularly similar, if not identical, in amino acid residues. The possibility also exists that M467 cross reacts, with varying affinities, with several serotypic determinants present on the flagellin molecule itself, and among

flagellin from various species. A further purpose of my study was to determine if molecular heterogeneity existed among isolated determinants which were bound by M467.

Molecular and structural evidence indicating the presence of a CNBr fragment, common to those Salmonella species studied, is substantiated in Figure 9. In the "Lo" inhibiting species the 18,000 MW fragment is clearly observable. The "Hi" inhibiting digests are believed to possess solubility problems, hence, the 18,000 MW band is not evident in the gel run shown in Figure 9. In Figure 7 band D is evident and is most likely present in the other "Hi" inhibiting digests from S.mississippi and S.adelaide (demonstrated by Parish et al.). Since the 18,000 MW fragment in S.milwaukee has been shown by this study to possess the antigenic determinant binding M467, it is reasonable to assume that this fragment also contains the antigenicity in the other strains studied.

The functional role of the flagellin molecule in the region of the common H determinant warrants consideration because it is possible that M467 is binding with a region of the polypeptide chain that is functionally responsible for (1) flagellar motility or (2) structural integrity, such as alpha-helix retention(38, 39, 40). The functional regions would not be susceptible to the extensive genetic mutational changes found in bacteria, without producing a functionally deficient molecule. Wu and Kabat have

suggested that regions of unusual flexibility, called hinge positions, exist in light and heavy chains of mouse immunoglobulins(41). Flexibility in polypeptide chains has been shown to result from adjacent glycine residues(42, 43, 44). These adjacent glycine residues have also been observed in four of the tryptic peptides from S.typhimurium flagellin, aiding flexibility in the flagellar mechanism which is believed necessary for allowing wave propagation along the flagellum(37). Conformational changes in the flagellin molecule have been observed when the molecule polymerizes from the monomer forms(40). The need for flexibility during conformational changes is obvious. Therefore, it may be hypothesized that M467 anti-flagellin is binding with a common H antigenic determinant residing in a functional region of bacterial flagella; a region that has persisted through evolution due to its functional role.

These theoretical considerations bring to light a fundamental question dealing with the murine immune response to flagellin. If the MOPC 467 antibody is presumed to be a product of a 'normal' cell line present in BALB/c mice(1), and the 18,000 MW antigenic fragment appears to be common to several species of Salmonella, then why aren't cross reactions observed consistently between induced antisera to various species of flagellin? Using passive hemagglutination inhibition, Langman produced some significant findings dealing with the

response produced by a common H antigen(15). Langman showed, for example, anti-d antiserum had a titer of 8 against d sensitized SRBC and 9 against SRBC carrying phase II antigens 1 and 2, then after addition of POL-d the titer of d was reduced to 5, whereas the titer to 1,2 was reduced to 2. This finding suggested that the anti-common H antibody was present at a relatively lower serum concentration as compared to the serotypically specific antibody. This assumption is reinforced by the finding that antibodies detected by agglutination of SRBC sensitized with the same POL as was used for immunization could be absorbed by that POL only. In Langman's study only POL-d inhibited the anti-d agglutination of POL-d sensitized SRBC. It can, therefore, be theorized that in the normal immune response to flagellar antigens the strain specific antigens (Hv) initiate a stronger immune response than the common (Hc) determinants, as evidenced by agglutination techniques. The nature of this differential response is yet undetermined and might be resolved by further studies dealing with affinity differences between anti-Hc antisera binding with their respective antigens.

It is worth noting that if M467 anti-flagella is analagous with the anti-Hc antibody found in normal BALB/c sera (ie. both immunoglobulins derived from the same plasma cell line), then it would be reasonable to assume that the induced antisera reacting with Hc antigen is of the

IgA class. It is therefore possible that the serum concentration differences between anti-Hc and anti-Hv antibodies may be class related. This theory may be further supported by the fact that IgA has been known to be a secretory antibody and is the predominant immunoglobulin in the external secretions found in the mucous membranes(44). Perhaps the anti-Hc antibody is of the IgA class and acts as a first-line defense system in the murine gastrointestimal tract. If the flagellin antigens induced an immune response via the gut associated lymphoid tissue, most of the anti-Hc IgA could be present on the mucousal surfaces, relatively undetected in the sera. Alternatively, intravenous injections of MON may produce anti-Hv antibodies of the IgG class and anti-Hc antibodies of the IgA class. The IgA serum titer may be lower due to three factors; (1) a majority of the IgA being secreted as secretory IgA, (2) antigenic stimulation is more effective in the gut since a "seeding" of the secondary lymphoid tissue by a specific lymphocyte clone has occurred in this anatomical location, or (3) serum IgA has been known to possess a short half-life(44).

Differences in Passive Hemagglutination Inhibition among Flagellin from Various Species

A part of this study was to attempt to determine if any quantitative binding differences existed between M467 and the flagellin from various Salmonella species. Table I and Figure 10 show the quantitative abilities of each MON

to inhibit the binding reaction between SRBC-MON cells from various species and M467 antibody. There are insignificant differences between some species and significant differences between others. These two groups were arbitrarily labeled as "Hi" inhibiting species and "Lo" inhibiting species, as reported in the results section. Since the absolute amount of flagellin protein used for inhibition was relatively constant (± 0.3%), direct experimental error could not be attributed to these differences. There are several theoretical factors that may be responsible for this finding, as follow.

If M467 is not monospecific in binding with a single determinant, but rather cross-reacts with two chemically unique determinants on the same molecule, then this could account for these inhibition differences. This theory suggests there are two molecularly distinct determinants on the "Hi" inhibiting MONs and one specific determinant on the "Lo" inhibiting MONs, however, both groups are polyvalent, as previously discussed. The assumption that two separate determinants are present is strengthened by the partial identity (spur formation) precipitin patterns between "Lo" and "Hi" inhibiting species shown in Figure 11. An alternative to this theory is that the determinants are molecularly identical but there is a difference in the number of determinants per flagellin molecule, the "Hi" inhibiting species possessing more.

An interesting hypothesis that could describe these inhibition differences states that M467 was binding with a phase specific antigen. As previously discussed, the serotype of a particular Salmonella species changes when alternating between phase I and phase II forms. If, for example, M467 bound phase I antigen only, then in a harvest of a species expressing phase I and phase II antigens only the phase I portion of the MON yield would possess the common H determinant. In Table II, it was observed that S.adelaide and S.milwaukee were both monophasic, thereby theoretically assuring only the presence of phase I specific antigens. Both S.adelaide and S.milwaukee were found to be "Hi" inhibitors. All other strains studied were diphasic and "Lo" inhibitors, with the exception of S.mississippi. It is possible that S.mississippi did not phase shift before harvesting and the MON yield was exclusively phase I. Frequency of phase shifts has been shown to vary among differing species(45). A test of this hypothesis would involve the use of phase specific antisera incorporated into the culture growth medium. The use of phase specific antisera would allow for the subsequent harvest of only one phase(14).

The theoretical implications of a common, phase specific antibody may be consistent with the phylogeny of the immune response. Smith et al. have suggested that an M467 light chain idiotype is present in several strains as shown

by the banding patterns from isoelectric focusing techniques(46). It is unlikely that this complex polypeptide chain had evolved independently in each cell line, suggesting that M467 is a molecule which occurred early in the course of immunological evolution in the mouse. As the previous discussion has indicated, the bacterial flagellar system has an extremely complex genetic mechanism regulating flagellar synthesis. It would be safe to assume that the ancestoral gene would be the H1 locus due to its elementary translation in flagellar synthesis. Presumably, H1 and H2 loci have arisen by duplication of an ancestoral gene(26). It is a short step to surmise that MOPC 467 IgA antibody is directed against a phase I antigen found early in the evolutionary sequence of murine immunological development. This theory suggests that the genetic selection pressures, resulting from the mammalian immune response against the H1 antigens present on bacterial flagella, enhanced the evolution of an entirely new flagellar structural gene, the H2 locus. It was stated earlier that M467 might have been binding with an antigenic determinant in a functional region of the flagellin molecule which was not advantageously susceptible to point mutations. This assumption emphasized the need for a second structural gene that could allow mutations in this region.

Murine tumor immunobiology

The results presented in this investigation deal

with analysis of the binding characteristics of MOPC 467 antibody and isolation of the Salmonella common H antigen. Although it is assumed that this experimental system is not unlike 'normal' immune mechanisms(1), it is reasonable to consider how these findings relate to the neoplastic phenomenon. The theoretical implications presented here provoke some general questions in the field of tumor immunobiology.

Mice are able to develop plasmacytomas spontaneously but usually with a low incidence(2). Experimental plasmacytomas can be induced by intraperitoneal injections of mineral oils in BALB/c mice(6,7,8). Curiously, the BALB/c strain has a genetic predisposition to plasmacytoma induction by this method as evidenced by a relatively high yield as compared to other strains. The exact nature of this predisposition has not been elucidated, although studies have shown that BALB/c mice are exceptional responders to antigenic challenge using BSA(47). Potter states that neoplastic development appears to have three components: (1) an initiating factor that changes the activity and expression of genes in a somatic cell line; (2) an amplification mechanism that propogates "mutant" cells; and (3) a favorable or even permissable environment for growth of the abnormal cell (selection)(2).

It has been suggested that antigenic stimulation is an essential factor for induction of plasmacytomas since germfree mice have been shown to have a lower incidence

(48). If it is assumed that the antigen is an essential factor for induction, is there an initiating mechanism which selects a particular immunoglobulin-producing cell line based on molecular characteristics of the antigen, or is the selection entirely random? The former case presents some highly theoretical possibilities suggesting that plasma cells producing immunoglobulins directed against "common" antigens are significant in plasmacytoma induction. A general definition of "common" antigen, therefore, is in order. This investigator is using this descriptive term to classify any antigen that is not phylogenetically specific, but appears within the molecular constituttion of varying organisms (without reference to species, genera, etc.). Development of myeloma proteins with similar hapten binding activity has been observed in many different BALB/c hosts, especially with the phosphorylcholine-binding proteins and those directed against 6 beta 1-6D-galactan(2). These haptens are ubiquitous in nature and have been isolated from natural sources such as the BALB/c microbial flora, in the diet, or in cage bedding used to maintain the mice(2). The present findings, indicating that MOPC 467 is directed against a common H antigen found among various bacterial flagella, supports this theoretical consideration.

Antigens that are ubiquitous in nature can contribute to the induction process either by a selective mechanism, or by random selection. The latter mechanism merely

adheres to a probability formula implying that the antigen in question is a more probable "target" for its neoplastic plasma cell couterpart, based on its relatively high concentration. The former mechanism introduces a molecular factor within the antigenic complex, suggesting that "common" antigens are molecularly more suitable for involvement in the neoplastic process. Perhaps the immunoglobulin counterparts of these antigens possess relatively high affinities for these antigens, indicating that the B lymphocytes containing these globulin receptors are more susceptible. Paradoxically, it was stated earlier that the common H flagellar antigen induces a relatively weak response in a normal host, compared to the response to specific (Hv) antigens.

Another possibility lies in the phylogenetics of a particular plasma cell line. It was also stated earlier that the MOPC 467 plasmacytoma may be an idiotype present early in the evolution of the murine immunological response to flagellar antigens. Does this indicate an effete or non-effective cell line that has become obsolete?

The suppositions presented in this last discussion are merely questions which indicate the need for more investigations in order to resolve the importance of the antigenic factor in plasmacytoma induction. Affinity studies characterizing these antigen-antibody reactions will perhaps shed some light on these mechanisms.

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