DEMONSTRATION OF TYPE SPECIFIC PROTEINS IN EXTRACTS OF FUSOBACTERIA*

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(Received for publication, September 28, 1937)

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On the basis of their morphological, cultural, and biochemical characteristics, Slanetz and Rettger (1) identified four varieties of fusobacteria and designated them as types I, II, III, and IV. Since attempts to isolate type specific carbohydrates have thus far been unsuccessful in our hands as well as in those of Spaulding and Rettger (2), we shall report herewith the demonstration of type specific proteins in extracts of three of these types.

Strains of Fusobacteria Studied

Four strains were investigated (Table I). Strains A and B were isolated from the lungs of human cases of pulmonary abscess, while strains S and R came from an abscess in the gums of a patient with pyorrhea.

Fusobacterium A is a long, slender, pointed, non-motile, Gram-negative rod which, in stained smears, resembles bundles of twigs or crisscross figures and shows no evidence of granules. The individual bacilli are fairly uniform in size, being 0.5 μ thick and 3 to 7.5 μ long, with an occasional variant 20 to 30 μ long. The strain is strictly anaerobic and was isolated on plates of serum-extract-agar in Varney's phosphorus jar (5). The colonies are ground glass in appearance, fairly soft, friable, and granular, with serrated edges. When examined by transmitted light, they have a coarse, rosette pattern with slightly raised, yellowish centers. Several biochemical characteristics are given in Table I.

Fusobacterium B is also a strictly anaerobic, non-motile rod, without granules, but with a tendency to form interlacing filaments and U forms. Its measurements are similar to A. When viewed by reflected light, the colonies have a somewhat coarser pattern than type A. By transmitted light they appear as flat, round colonies with undulating or serrated edges and slightly raised, yellowish centers from which emanate delicate, linear markings. The fermentation reactions which

* Presented before the American Association for Experimental Pathology, Memphis, Tennessee, April 22, 1937.

TABLE I

Biochemical Reactions of Fusobacteria

Microorganisms	Source	Fermentation of carbohydrates													pH in 1 per cent glucose broth		Hydrolysis of starch (2 wks.)						Growth in presence of bile†								Proteolysis						
		Arabinose	Dextrin	Dextrose	Dulcite	Glycerol	Lactose	Levulose	Maltose	Mannite	Rhamnose	Salicin	Saccharose	Xylose	2 days	14 days	Hydrolysis*	Final pH	Benedict test for glucose	Hydrolysis of sodium hippurate (2 wks.)	Gelatin liquefaction	Odor	10 per cent	40 per cent	Utilization of citrate	Production of indol‡	Reduction of nitrates§	Production of H_2S‖	Action on milk**	Gas from peptone water	Defibrinated blood††	Coagulated blood‡‡	Fresh kidney§§	Brain	Beef heart‖‖	Coagulated egg white	Fibrinolysin***
Fusobacterium (S) type I	Pyorrhea	−	−	A	−	−	−	A	−	−	−	−	−	−	6.4	5.9	+++	7.1	−	−	−	Foul	++	−±	−	+	−	−	−	−	−	−	−	−	−	−	−
Fusobacterium (MiB; A) type II	Pulmonary abscess	−	−	A	−	−	−	A	−	−	−	−	−	−	6.5	5.9	++	7.1	−	−	−	"	++	−	−	+	−	+	−	−	−	−	−	−	−	−	−
Fusobacterium (MiB; B) type II	" "	−	−	A	−	−	−	A	−	−	−	−	−	−	6.5	5.9	++	7.1	−	−	−	"	Few colonies	−	−	+	−	−	Slight A coag.	−	−	−	−	−	−	−	−
Fusobacterium (R) type III	Pyorrhea	−	−	A	−	−	−	A	−	−	−	−	A	−	6.6	6.0	+++	7.1	−	−	−	"	" "	−	−	+−	Ft. tr.	−	−	−	−	−	−	−	−	−	−

* Eckford's method (3) employing iodine as indicator; ++++ complete disappearance of blue color; +++ and ++ partial hydrolysis, reddish-brown color; −no hydrolysis, blue color.

† Bile was incorporated in blood or serum agar plates.

‡ Tested with vanillin and Ehrlich's reagents.

§ Tested for nitrites only.

‖ Cultures grown on lead acetate agar.

** Brom-thymol blue added as indicator.

†† Hemoglobin digested; broth clear; flaky sediment at bottom of tube.

‡‡ Fibrin digested; same appearance as in defibrinated blood.

§§ No digestion; black flakes on surface of kidney; tested after 1 month's incubation.

‖‖ Ground and cooked.

*** Tested with human fibrin and thrombin; method of Tillett and Garner (4).

Types refer to classification of Slanetz and Rettger (1).

A, acid.

Ft. tr., faint trace.

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are given in Table I are similar to type A. Both of these strains belong to type II of Slanetz and Rettger.

A third strain, fusobacterium S, likewise strictly anaerobic, was isolated from the gums of a patient suffering with pyorrhea alveolaris. These Gram-negative bacilli are usually very short, measuring 0.5 μ in thickness and 1.5 to 5 μ in length, the average being 3 to 3.5 μ. The rods are non-motile, occasionally pointed, and grouped in parallel or V formations, without evidence of curves, waves, long filaments, or granules. When viewed by reflected light, the colonies have a fine, ground glass appearance, slightly conical, without surface markings, but with serrated edges and slightly darker centers. When tested with the loop, they are friable, but not mucoid. The biochemical reactions which are given in Table I warrant its recognition as type I (Slanetz and Rettger). The organisms grow well in an infusion-brain broth containing 0.1 per cent dextrose and 30 per cent ascitic fluid and also in hormone broth to which had been added 0.1 to 0.5 per cent agar, enriched with 20 per cent rabbit serum. Another suitable medium is semi-solid, hormone agar containing 10 per cent rabbit serum and a little hemoglobin.

A fourth strain, fusobacterium R, isolated from the same case of pyorrhea, corresponds to type III of Slanetz and Rettger. The pointed rods are 0.5 μ thick and vary from 3 to 100 μ in length, the average being from 6 to 12 μ. Characteristic granules, waves, and chains were observed, and in older cultures very long filaments were abundant. The colonies, when viewed by reflected light on serum agar plates, are finely granular with slightly raised, glistening surfaces and ragged edges of grayish white, mottled appearance with opaque centers from which radiate white striations. In transmitted light, the colonies seem yellowish in color with coarsely granular patterns and smooth edges.

All these types of fusobacteria proved to be non-pathogenic when injected subcutaneously into guinea pigs, rabbits, mice, or rats or intrabronchially into monkeys and dogs. They did not possess any fibrinolysin when tested with human fibrin and thrombin[1] by the method of Tillett and Garner (4). It will be noted that fusobacterium III is the only one which failed to ferment galactose. None of the strains are proteolytic, hydrolyze sodium hippurate (6), or utilize sodium citrate.

Preparation of Mass Cultures

In order to accustom the bacteria to cultivation in large volumes of liquid media, the growth from several agar slants was washed off and inoculated into tubes of extract broth. The latter were then transferred to 100 cc. Florence

[1] Dr. R. R. Madison of the Department of Bacteriology, Stanford University, carried out the tests for fibrinolysin in our laboratory.

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flasks containing 0.1 per cent cysteine hydrochloride in extract broth. Several pieces of filter paper were introduced in order to provide an adsorbing surface for the bacteria. Immediately before inoculation, the flasks were boiled to drive out oxygen and quickly cooled to 37°C. by means of an ice water bath. The volumes were adjusted to reach the middle of the neck of the flask and covered with a layer of 2 cm. of sterile yellow vaseline. Four or five flasks, containing 24 to 48 hour cultures, were used as seed material with which to inoculate 10 or more flasks of 1.5 to 2.0 liter capacity. The latter were also boiled, cooled, and covered with vaseline. After 2 or 3 days' growth the purity of each mass culture was checked by plating anaerobically and aerobically. The bacteria were thrown down in a Sharples centrifuge and used for the extraction of the protein fractions.

Preparation and Chemical Properties of Bacterial Proteins

The bacterial bodies from about 40 liters of broth were suspended in physiological saline, washed by centrifugation, dehydrated and defatted by means of acetone, ether, and chloroform, dried overnight in a vacuum desiccator, and ground in a Krueger (7) grinding apparatus for 1 or more days until the Gram stain showed complete absence of intact cells. The procedure followed was similar to that described by Heidelberger and Kendall (8) for the preparation of fraction D of *Streptococcus hemolyticus* and included the removal of the unbound carbohydrate and extraction of the bacterial proteins with a phosphate buffer solution at pH 6.5. A brine centrifuge was not available.

Table II summarizes the results of the chemical analyses of four bacterial proteins. The total Kjeldahl nitrogen, after correction for ash, varies from 11.06 to 16.25 per cent. The phosphorus content is close to 1 per cent and varies from 0.87 to 1.33 per cent. The usual color reaction for proteins, the biuret, xanthoproteic, Millon, Hopkins-Cole, and ninhydrin, were positive in all cases. The tests for carbohydrates were as follows: fusobacterium type II gave a positive Molisch reaction; type I was negative; and type III was doubtful. The Bial-Tollens test for pentose was negative for type III, strongly positive for type II, and weakly positive for the type I proteins. Whether or not these carbohydrates are chemically combined with the bacterial proteins and have antigenic or haptene properties, as is the case with the scarlatinal streptococcus studied by Heidelberger and Kendall (9), remains to be determined.

The proteins were digested slowly by pepsin at pH 3.0 and trypsin at pH 8.0 (Table III), when tested by the methods of Anson and Mirsky (10, 11). After digestion they no longer functioned as precipitinogens when mixed with either antibacterial or antiprotein

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TABLE II

Chemical Analyses of Proteins Extracted from Fusobacteria Types I, II, and III

Microorganisms	Total nitrogen*	P†	Biuret‡	Xantho-proteic	Hopkins-Cole	Ninhyd-rin	Millon	Molisch	Bial-Tollens (pentose)
Fusobacterium (S) type I	12.94	1.33	++	+++	+++	+++	++	−	+
Fusobacterium (A) type II	11.08	0.87	+++	+++	+++	+++	+++	+ (green)	++++
Fusobacterium (B) type II	11.06	1.18	+++	+++	+++	+++	+++	+++	++
Fusobacterium (R) type III	16.25	0.90	+++	+++	+++	+++	+++	+−	−

* Results of micro Kjeldahl determinations, corrected on basis of ash analyses.

† Microchemical analyses of P and ash were performed by the methods of Pregl by Mr. H. C. Johnson, Department of Biochemistry, University of California, Berkeley, under the direction of Professor Paul L. Kirk.

‡ All color reactions were performed with 1:1000 solutions.

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TABLE III

Disappearance of Precipitin Titers After 6 Days' Digestion of Bacterial Proteins with Purified Pepsin and Trypsin

Immune sera		Precipitin tests: Before proteolysis	Precipitin tests: After proteolysis	Controls: boiled* enzyme	Tyrosine liberated during proteolysis of 5 cc. of 2 per cent solution of protein†
Protein: fusiformis Type I; Enzyme: trypsin‡					
Fusiformis type I (homologous tests)	Antiprotein	1:32,000 ++ 1:16,000 +++	Neg.	8,000 ++	40γ
	Antibacterial	1:8,000 +++	"	8,000 ++	
Fusiformis type II (heterologous tests)	Antiprotein	Neg.	"	Neg.	
	Antibacterial	"	"	"	
Protein: fusiformis Type II; Enzyme: pepsin					
Fusiformis type II (homologous tests)	Antiprotein	1:32,000 ++++ 1:64,000 ++	Neg.	32,000 ++	39γ
	Antibacterial	1:8,000 +++ 1:16,000 ++	"	Neg.	
Fusiformis type I (heterologous tests)	Antiprotein	Neg.	"	"	
	Antibacterial	"	"	"	

* Boiling did not always serve to inactivate these purified enzymes.

† Methods of Anson and Mirsky (10, 11).

‡ Enzymes were purified by the method of Anson and Mirsky and Northrop (12).

TABLE IV

Precipitin Titers of Various Antiprotein Sera Tested with Homologous and Heterologous Fusobacterial Proteins

Antiprotein sera	Bacterial proteins: Fusobacterium I (S)	Fusobacterium II	Fusobacterium III
Fusobacterium (S) type I	++ 32,000	–	–
Fusobacterium type II	–	++ 64,000	–
Streptococcus hemolyticus (group A) (Mic)	–	–	–
Pneumococcus III	–	–	–
Staphylococcus aureus (Mic)	–	–	–

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sera. No new (heterologous) antigens appeared as a result of hydrolysis by proteolytic enzymes, tending to prove that the extracts are proteins and not carbohydrate haptenes. Repeated injection into the blood stream of rabbits yielded sera of high precipitin titer, thus demonstrating antigenicity, excepting in the case of type III which was unfortunately denatured in the process of grinding in the Krueger apparatus and had a high ash content (Table IV).

Preparation and Testing of Immune Sera

Rabbits were immunized with increasing doses of standardized suspensions of dead or living bacteria, injections being made daily for 3 days followed by 4 days of rest. Four or more weekly series were given and trial bleedings were made at frequent intervals to determine the precipitin titers. The animals were exsanguinated about 5 days after the last injection and the sera bottled under sterile precautions, without the use of preservatives.

The precipitability of the bacterial proteins was tested in the presence of homologous and heterologous immune rabbit sera, employing the technic of Lancefield (14–16). A 1:1000 dilution of the proteins was prepared in sterile, buffered saline at pH 7.8, with the aid of a few drops of N/10 NaOH. Sera were diluted with saline in the ratio of 2:3 (that is, 2 parts of serum plus 3 parts of salt solution) and the tests were incubated for 2 hours in the water bath at 37°C., followed by an overnight period in the refrigerator. It was found advantageous, in order to increase the volume of the precipitates, to prolong the period of ice box incubation up to 48, and occasionally to 72 hours. The tubes were then centrifuged at low speed for 10 minutes and examined with the aid of a Zeissler stereoscopic, wide field binocular colony microscope (Zeiss), employing a magnification of 25× and transmitted light. The precipitates, when viewed through a narrow slit in a black cardboard, were of a flaky or granular consistency, occasionally forming compact masses which could be easily broken up. They did not resemble the firm, glassy disks characteristic of bacterial carbohydrates.

As will be seen by reference to Tables IV and V, proteins extracted from fusobacterium types I, II, and III are immunologically type specific. No cross precipitin reactions were obtained between these proteins and heterologous type sera prepared by immunization with fusobacterial suspensions. There were, however, some cross reactions between the protein of fusobacterium type I and sera obtained by immunizing rabbits with cultures of an anaerobic streptococcus, *Streptococcus evolutus*, or with *Streptococcus hemolyticus* (group A). After adsorption of these sera with the corresponding bacterial suspensions, these secondary precipitins were readily removed. When

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TABLE V

Precipitation of Fusobacterial Proteins in the Presence of Unabsorbed Homologous and Heterologous Antibacterial Rabbit Sera

Proteins	Sera										
	Fusobacterium type I	Fusobacterium ((A)-MiB) type II	Fusobacterium ((B)-MiB) type II	Fusobacterium type III	*Strep. evolutus* (NiB)*	*Strep. hemolyticus* (Mic) group A	*Strep. hemolyticus* group A (Lancefield)	*Strep. micros* (Mic N1)*	*Bact. melaninogenicum* (py)*	*Bact. melaninogenicum* (M 19)	*Staph. aureus* (Mic)
Fusobacterium (S) type I	†136,000 ++ 68,000 +++	–	–	–	8,000 ++	34,000 ++++	–	–	–	–	–
Fusobacterium (A (MiB)) type II	–	8,000 ++	16,000 ++	–	–	–	–	–	–	–	–
Fusobacterium (B (MiB)) type II	–	4,000 ++	34,000 ++	–	–	–	–	–	–	–	–
Fusobacterium (R) type III	–	–	–	32,000 ++ 16,000 ++++	32,000 ++	–	–	–	–	–	–

* For additional studies on these bacteria see Weiss and Mercado (13 *a*, *b*).

† Figures refer to maximum dilutions of proteins at which strong precipitation was obtained (++ to ++++). All protein antigen dilutions were analyzed for N and corrections made for undissolved residues.

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precipitation tests were done with sera obtained by immunizing rabbits against bacterial proteins (instead of bacterial vaccines or suspensions of living bacteria) there was likewise evidence of type specificity. It was observed that bacterial suspensions were superior to solutions of proteins in adsorption tests.

When assayed for toxicity by intraperitoneal injection of white mice, the dosage being up to 2 cc. of 1:1000 solution, none of the three types of bacterial proteins was found to be toxic.

DISCUSSION

In the present communication, we have confirmed the observations of Slanetz and Rettger (1) on the cultural and biochemical characteristics of fusobacteria types I, II, and III. The following new data are presented: the behavior in a number of carbohydrate media not employed by these authors; absence of pathogenicity for various animals; final pH in 1 per cent glucose broth; hydrolysis of starch; failure to decompose sodium hippurate and citrates; absence of fibrinolysins and proteolytic enzymes.

By the use of special methods, it has been possible to grow the organisms in mass cultures and to extract from them proteins which are immunologically type specific. This discovery is of interest not only because there is at present very little, if any data in the literature on the proteins and carbohydrates of anaerobic bacteria in general, but also because it represents the second instance of a bacterial protein functioning in the capacity of a determinant of bacterial type. The first instance of this character was discovered by Lancefield (14), who found that in group A of the hemolytic streptococci, type specificity is determined by a denatured protein or M substance, while the groups are differentiated by means of a group specific polysaccharide or C substance. Among the other groups of the hemolytic streptococci (B, C, D, E, etc.), there is a group specific C substance and a type specific polysaccharide or S substance (15, 16).

It will be recalled in this connection that Tomcsik and Szongott (17) found in the capsule of the anthrax bacillus a substance containing 10 per cent nitrogen and free from phosphorus and sulfur, which they characterized as a protein, in spite of the fact that it did not give the usual color reactions. Ivánovics and Erdös (18), who con-

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tinued this work, concluded that this so called P substance was really a colloidal salt of an inorganic acid and not a protein, since it was resistant to proteolytic enzymes, including pepsin and trypsin. Subsequent investigations by Ivánovics and Bruckner (19) demonstrated that the haptene of the anthrax bacillus and of *Bacillus mesentericus* was in the nature of a polypeptide containing *l*-glutamic acid.

Our preliminary data indicate that there is also a group specific carbohydrate among the fusobacteria, which is extractable by the methods of Heidelberger and Avery (20). The presence of foul and poisonous gases in mass cultures of the fusobacteria has, however, retarded this work which is still in progress.

The demonstration of type specific proteins among the fusobacteria will, it is hoped, make possible further work on the serological classification of this interesting group of organisms, permitting precipitin tests to take the place of the uncertain and irregular agglutination tests. As shown by Varney (21), and Slanetz and Rettger (1), spontaneous agglutination occurs frequently with these organisms. These data may also aid in the study of the problem of the pathogenicity of the various fusobacteria and their possible rôle in the etiology of pulmonary abscess, bronchiectasis, Vincent's angina, pyorrhea alveolaris, etc. (22, 23).

SUMMARY AND CONCLUSIONS

1. The classification of fusobacteria into types, as suggested by Slanetz and Rettger on the basis of their cultural and biochemical characteristics, was confirmed. The following additional data are presented: the behavior of types I, II, and III in several previously untested carbohydrates; the final pH in 1 per cent glucose broth; the hydrolysis of starch; the failure to decompose sodium hippurate and citrates; the absence of pathogenicity for several types of experimental animals; and the absence of fibrinolysin and of proteolytic enzymes.

2. By the use of a technic described in the text, it was possible to grow the anaerobic fusobacteria in mass cultures.

3. With the methods of Heidelberger and Kendall, immunologically type specific proteins were extracted from fusobacteria types I, II, and III. This observation, which is in a sense an extension of the

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work of Lancefield on *Streptococcus hemolyticus*, group A, represents the second instance of a bacterial protein functioning as a determinant of bacterial types.

4. Preliminary data indicate that group specificity depends upon a carbohydrate which is extractable by the methods of Heidelberger and Avery.

Assistance was rendered by Dr. E. J. Czarnetzky and Dr. Clarence E. Larson in the chemical studies and by Mrs. M. C. Shevky in the serological investigations.

The interest and clinical cooperation of Dr. Harold Brunn, Chief of Staff of the Mount Zion Hospital, made this work possible. Thanks are also due to Dr. Rebecca Lancefield of The Rockefeller Institute and Dr. Michael Heidelberger of Columbia University who gave helpful criticisms and suggestions.

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