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Autor: Chen, T.H.

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From the George Williams Hooper Foundation, University of California School of Medicine, San Francisco, California.

The Antigenic Structure of *Pasteurella pestis* and its Relationship to Virulence and Immunity.[1]

By T. H. CHEN.

CONTENTS.

Introduction.

The study of the antigenic complex of *Pasteurella pestis* is extensive. The study of the antigens which are important with respect to virulence and immunogenicity is likewise extensive but our cumulative knowledge of these antigens is yet incomplete and might therefore be subjected to a critical review.

First important findings came from SCHÜTZE. His studies of the antigenic structure of *P. pestis* (42, 43) revealed 2 antigenic components of this organism, an envelope and a somatic antigen. A third antigen was postulated to account for virulence (43). Russian investigators, FADEEVA, GHELTENKOFF and KOROBKOVA, cited by JAWETZ and MEYER (29), likewise proposed that virulent plague bacilli possessed an antigen similar to the Vi antigen in Salmonella. This claim was supported although not confirmed by JAWETZ and MEYER in 1943 (29) because some property or chemical group must be responsible for virulence in *P. pestis* since avirulent organisms seemed identical with virulent ones in all respects except for the complete absence of virulence.

[1] The review is based on a seminar given at Hooper Foundation Monthly Staff Meeting, on April 5, 1963. These studies were sponsored by the Commission on Immunization, Armed Forces Epidemiological Board, and supported in part by the Office of the Surgeon General, Department of the Army.

Based on immunological experiments, SCHÜTZE (43) found that mice were readily immunized with a strain possessing an appreciable amount of envelope material, while bacilli with little envelope material were usually better immunizing agents for guinea pigs. This finding was attributed by OTTEN (40) to the presence of 2 immunizing antigens in the different strains, "rat antigen" and "guinea pig antigen". On the other hand, an alternate schema of *P. pestis* antigenic make-up was proposed by JAWETZ and MEYER in 1943 (29) when they confirmed SCHÜTZE's immunological data with their immunological and serological data. In their studies they used 3 symbols to indicate properties of the *P. pestis* organism rather than designate them as antigenic entities. The symbol Vi indicated the quality of virulence of the organism, Avi indicated a hypothetical quality constituting the main part of a "guinea pig antigen", and Soma designated a strain devoid of virulence and immunogenic power but nevertheless toxic. Using these 3 symbols they formulated an alternate schema of dissociative changes which may occur with the plague bacillus. In the virulent organism, Vi and Avi properties may be lost completely, and the amount of envelope may vary due to either "natural" or "forced" (growth under unfavorable conditions) dissociation. An avirulent variant maintaining both Avi, Soma and large amounts of envelope antigen would be an excellent vaccine for mice but mediocre for guinea pigs. A strain possessing the same properties but only a smaller amount of envelope antigen would be a better vaccine for guinea pigs than for mice.

The aforementioned findings by different investigators were verified by the recent finding of SPIVACK and associates in 1958 (44). They found that the envelope antigen, immunologically potent in the mouse, rat, and monkey, was also the protective antigen for the guinea pig. The failure of large amounts ($> 50\ \mu g$) of envelope antigen to protect guinea pigs was due to "immunoparalysis". In brief, the purified envelope antigen causes immunoparalysis in guinea pigs when large quantities are injected, but is highly antigenic in guinea pigs if very small quantities are injected with adjuvant. This finding eliminated not only the older equivocation of the "guinea pig antigen" and "rat or mouse antigen" in *P. pestis,* but also provided strong evidence that envelope antigen is the main protective antigen for all species.

However whether envelope antigen may or may not be identified as the virulence antigen remained open to inquiry. In 1954, ENGLESBERG and associates (27) demonstrated that one difference between virulent and avirulent strains was the quantity of envelope and toxin antigens. However the fact that many avirulent strains can also synthesize the envelope antigen in appreciable amounts indicated that full virulence required the presence of another determinant. BURROWS and BACON (10, 12) discovered the antiphagocytic VW complex and contended that this component was responsible for the virulence of a strain and that its inclusion in a vaccine gave a higher degree of immunity.

A review of recent studies on the chemically and serologically identified constituents of *P. pestis* responsible for virulence and immunogenic activity is the subject of this paper. Comprehensive treatments of *P. pestis*—antigenic structure, virulence, and immunity—may be found in the reviews of MEYER (36), POLLITZER (41), and GIRARD (28).

Morphology of P. pestis Studied by Light and Electron Microscopy.

The plague bacillus was discovered independently by KITASATO and YERSIN during the Hong Kong epidemic of 1894. The bacilli are

small, oval pleomorphic rods with rounded ends, somewhat shorter than the typhoid bacillus, but of the same thickness. Their size may vary considerably from the average of $1.5 \times 0.7\ \mu$. They stain readily with the basic aniline dyes, and are Gram negative. In stained preparations the central part of the bacillus is often left uncolored, giving the so-called bipolar staining, an important feature in identification. In cultures grown at 37°C an "envelope" can be demonstrated by means of a wet India ink preparation illuminated by a dark field condenser (2). The envelope appears as dark areas surrounding the bacterial cells. When the organisms are grown under favorable conditions the width of the envelope may exceed that of the cell itself. Sometimes several cells may be seen embedded in a single mass of envelope substance. Under conditions optimal for the production of envelope material, the yield is approximately 7% of the dry weight of the organism. This envelope material, the surface antigen of the plague bacillus, is of paramount importance in immunization against plague. It has been isolated in a highly purified form at the Hooper Foundation by BAKER and associates (4, 5) in 1947 and was renamed Fraction I. Its importance will be further discussed.

The characteristics of this extracellular envelope antigen were studied by CROCKER and associates (22). They found that virulent plague bacilli, grown on collodion film over hormone agar for 24 hours, appeared in electron micrographs as sharply outlined bodies of high electron density embedded in material of low electron density. The cells were separated in this material by the width of one bacillus. This low electron density material, the envelope antigen, was homogeneous throughout in contrast to the appearance of the pneumococcus capsule. For this reason mainly, the nonsomatic antigen is referred to as "envelope" instead of capsule. It is this envelope antigen which gives surface colonies of plague bacilli a stringy consistency when tested with a loop. More of it is found in virulent than in avirulent strains.

Antigenic Structure of P. pestis Demonstrated by Double Diffusion in Gel (Ouchterlony Technique).

The antigenic structure of plague bacilli, studied by the gel diffusion technique in conjunction with antigens from the closely related *P. pseudotuberculosis,* is of importance in the understanding of the relationship between virulence and immunogenicity in plague.

CHEN and MEYER (17) in 1955 first demonstrated the antigenic

complexity of this organism by the Ouchterlony test. Fraction I antigen, *P. pestis* strains 195/P and TRU and *P. pseudotuberculosis* strain 19 were tested against the anti-avirulent *P. pestis* gamma globulin. Four major diffusible antigens were discernible in *P. pestis* strain 195/P, a virulent strain. Two of these were common to TRU, an avirulent *P. pestis* strain, and *P. pseudotuberculosis* strain 19. Of the remaining two, one was identified as Fraction I, the other, the toxin, was found only in *P. pestis* strain 195/P. The precipitation lines appearing in order of their proximity to the *P. pestis* strain 195/P culture have been identified as Fraction I, Soma I, Fraction II (murine toxin) and Soma II. *P. pestis* TRU is an avirulent, essentially atoxic strain containing a negligible amount of Fraction I. Therefore it only produced the 2 somatic reaction lines. *P. pestis* strain TRU and *P. pseudotuberculosis* were indistinguishable by this test. However, the 2 organisms are easily distinguishable by biochemical methods, such as the urea test (47).

In the Ouchterlony test the antigens used were living cultures with the exception of the purified Fraction I antigen. Only 4 precipitation lines were revealed. However it was suspected that small amounts of other antigens may have remained undetected due to an excess of antibodies in the antiserum used. It was also learned that additional antigens do become evident if the culture is first concentrated by fractionation. Using the concentrated extract from *P. pestis* or the supernatant from a fully grown shake culture as antigen in the Oudin test, 3 additional antigens were revealed.

The antigenic complex of this organisms was further studied by BHAGAVAN and associates (8). Using anti-*P. pestis* A1122 gamma globulin and *P. pseudotuberculosis* antiserum in the Oudin test, 7 antigens were detected in *P. pestis* strains, of which 5 were common to *P. pseudotuberculosis*. Of these 5, three were thermolabile and 2 thermostable. Of the 7 *P. pestis* antigens 4 were thermolabile and 2 thermostable. The remaining antigen was haptenized Fraction I.

Subsequent work by CRUMPTON and DAVIES (23), who tested the Tjiwidej strain against its homologous antiserum in the Ouchterlony gel diffusion plate, in conjunction with animal experiments, revealed at least 3 more antigens. They confirmed the Fraction I and Fraction II (murine toxin) lines. Besides these, 2 additional important antigens, antigen 4 (24) and the specific polysaccharide (25) were characterized by them. Some details of these 2 antigenic components will be discussed later.

In 1960, LAWTON and associates (32) derived 18 monovalent antisera from polyvalent antisera by absorption with different antigenic components. Using the 18 monovalent antisera to detect

TABLE 1.

Antigens detected in Pasteurella pestis and Pasteurella pseudotuberculosis

Strains *	Virulence+	Antigens																	
		B	C	D	E	F	G	H	I	J	K	L	M	N	O	Q	T	V	W.
Alexander	Vi	+	+	+	+	+	+	±	+	+	+	+	0	0	+	+	+	+	+.
M 41	Vi	+	±	+	+	+	+	±	±	+	+	+	0	0	+	+	+	+	+.
Saka	Vi	+	+	+	+	+	+	±	+	+	+	+	0	0	+	+	+	+	+.
Yokohama	Vi	+	+	+	+	+	+	±	+	+	+	+	0	0	+	+	+	+	+.
M 23	Vi	+	+	+	+	0	+	±	+	+	+	+	0	0	+	+	+	+	+.
EV 76	Avi?	+	+	+	+	+	+	±	±	+	+	+	0	0	+	+	+	+	+.
Al224	Avi	+	+	+	+	+	+	±	+	+	+	+	0	0	+	+	+	0	0 .
Tjiwidej	Avi	+	+	+	+	+	+	±	+	+	+	+	0	0	+	+	+	0	0 .
A 12	Avi	+	+	+	+	+	+	+	+	+	+	+	0	0	+	+	+	0	0 .
TRU	Avi	+	+	0	+	0	+	+	±	+	+	+	0	0	+	+	0	0	0 .
P. pseudo-tuberculosis																			
3 E	Avi?	+	+	0	+	0	+	±	±	+	+	+	+	+	+	+	0	0	0 .
4 C	Avi	+	+	0	+	0	+	+	+	+	+	+	+	+	+	+	0	0	0 .

* Grown in heart infusion broth at 37° C.

+ Vi = Intraperitoneal LD_{50} in mice of less than 100 cells; Avi = $LD_{50} > 10^6$ cells; Avi? = LD_{50} approximately 10^4 cells (virulence of *P. pseudotuberculosis* tested in guinea pigs).

Data from LAWTON, FUKUI and SURGALLA (J. Immunol. *84*, 1960, 475).

the antigens specific to plague strains and *P. pseudotuberculosis* strains, they found 5 antigens specific for *P. pestis,* 2 antigens specific for *P. pseudotuberculosis* and 11 antigens common to both species (Table 1). However, later studies by BURROWS and BACON (14) in 1960 showed V and W antigens to be produced also in virulent strains of *P. pseudotuberculosis* recently isolated from natural infections. This finding demonstrated 16 antigens distinguishable in *P. pestis,* 3 of which have not been found in *P. pseudotuberculosis.* Thirteen are in common with *P. pseudotuberculosis* (46).

Some of these antigens have been purified and/or identified as being important to virulence and immunogenicity. They will be discussed subsequently.

Antigenic Components of P. pestis Isolated in Purified Form or Identified by Specific Serologic Technique and Their Connection with Virulence and Immunity.

Envelope Antigen—Fraction I (I A and I B).

Fraction I or the envelope antigen of BAKER and associates (4, 5) consists of 2 protein fractions, I A and I B, which exhibit chemical and physical differences. Fraction I A contains a carbohydrate and is soluble in a 0.25 saturated solution of ammonium sulfate at pH 7.0 to 7.5, while Fraction I B contains no carbohydrate, is soluble in a 0.3 saturated solution of ammonium sulfate, and is precipitated out of solution when the concentration of ammonium sulfate is raised to 0.33 saturation. Fraction I A and I B have high immunogenicity for mice, rats, guinea pigs, rabbits, monkeys and probably man.

ENGLESBERG and associates (27) have claimed the protective antigen to be responsible for the virulence of a strain. They also suggested that the Fraction I production must reach a certain level before it can overcome the host's defenses. Their supporting data (Table 2) show none of the avirulent strains to contain more envelope antigen than the virulent strains. On the other hand, the toxin content of some of the avirulent strains (e.g. strains EV-76 and 14) may be the same or more than that of the virulent strains. Generally, it can be seen that virulent strains do produce more toxin than avirulent ones. Although death in plague is due to processes initiated by the toxin, the data of Table 2 indicate that high toxicity alone is not sufficient to render an organism virulent.

The envelope substance was also found to protect *P. pestis* from phagocytosis (16). This is exemplified in Table 3, which shows the

TABLE 2.

Comparison of the quantity of toxin and envelope antigen (Fraction I) produced by several virulent and avirulent strains of *P. pestis*.

Strains	Envelope antigen (μg/mg. dry wt.)	Toxicity (No. LD_{50}/mg. dry wt.)
Virulent		
B741	150	29
195/P	140	20
New Mexico	140	32
Yreka	138	45
Shasta	137	42
Alexander	127	23
Avirulent		
B1456 No. 4	121	25
A1122	110	9
B868 No. 8	104	22
B2764 No. 6	94	23
E.V. 76	62	33
F7793 No. 10	52	13
14	30	30
TRU	25	0

Data from ENGLESBERG, CHEN, LEVY, FOSTER and MEYER (Science *119,* 1954, 413).

relative phagocytosis of virulent and avirulent organisms having varying amounts of Fraction I content by normal defibrinated guinea pig blood, and compares inhibition of phagocytosis after the addition of purified Fraction I B, or a relatively purified polysaccharide, "the 0.25 residue", of *P. pestis* to the mixture. Addition of the 0.25 residue to the normal blood and culture mixture altered the phagocytic index very little, since the 0.25 residue contained only a small amount of Fraction I. Addition of the highly purified protein Fraction I B to the normal blood and culture mixture markedly reduced the phagocytic index in all cases. These results indicate that phagocytosis of plague bacilli by normal guinea pig blood is interfered with or inhibited by the protein fraction, but not by the polysaccharide fraction of *P. pestis.*

That Fraction I is the main protective antigen against plague in animals as well as in humans has been established by work done at the Hooper Foundation. This aspect will not be covered in this review. Reviews on the general subject of plague immunization by MEYER (36, 37, 38), and papers by SCHÜTZE (42); BAKER et al. (5); CHEN (19); WALKER et al. (48); CHEN and MEYER (21); CHEN

TABLE 3.

Phagocytosis of virulent or avirulent P. pestis by normal defibrinated guinea pig blood and inhibition of phagocytosis by fractions of P. pestis

Constituents of the system	Amount, ml	Phagocytic index								
		Virulent strains				Avirulent strains				
		195/P		Yreka		A 1122			14	EV
Defibrinated guinea pig blood Saline Culture	0.7 0.1 0.1	3.02	3.44	4.20	4.10	7.70	8.20	12.80	42.20	5.92
Defibrinated guinea pig blood 0.25 residue Culture	0.7 0.1 0.1	3.34	1.70	4.30	3.60	6.60	5.40	8.00	36.00	2.40
Defibrinated guinea pig blood Fraction IB Culture	0.7 0.1 0.1	1.00	0.20	0.40	1.20	2.12	1.84	0.56	20.20	1.60

Data from CHEN and MEYER (J. Immunol., *72*, 1954, 282).

et al. (18) may be consulted. Highly purified Fraction I was found to be stable and highly protective. This was concluded from the positive correlation between Fraction I antibody level and the immunity derived from the administration of different vaccine preparations. Fraction I antibody levels as determined by complement fixation (20) and hemagglutination (16) tests using purified Fraction I as the antigen, were correlated with results of passive transfer protection tests in mice (35). The effectiveness of any antigen preparation was finally ascertained by challenge.

A direct relationship exists between Fraction I antibody titers and mouse protection indices (MPI), particularly in those animals given whole organism vaccine incorporated in Freund's incomplete adjuvant. Studies have shown that those animals, immunized with suitable plague vaccine and developing high CF and HA titers against Fraction I with low mouse protection indices, always survived the challenge infection. On the contrary, animals with no Fraction I antibody titers rarely survived the challenge infection. Moreover, data from CF, HA, and immunoelectrophoretic tests, performed on sera from vaccinated primates and human volunteers and patients recovered from infection, have confirmed these observations. The results presented in Table 4 reveal the correlation

TABLE 4.

Comparison of the opsonocytophagic indices and CF and HA titers of primates (Langurs) that recovered from infection *.

Animal No.	Opsonophagocytic Index		Anti-Fraction I Antibody Titers	
	Neutrophiles	Macrophages	CF	HA
731	10/100/15 **	13/100/19	0	0
733	53/100/137	41/100/31	16	256
734	18/100/21	12/100/21	0	0
739	61/100/156	72/100/83	128	8194
740	21/100/42	32/100/39	8	512
Normal serum	8/100/13	15/100/21	0	0

* Serum samples collected 6 weeks after infection.

** Number of cells containing bacilli/Number of cells counted/Total number of ingested organisms.

Procedure was recommended by Dr. M. J. Surgalla, Chief, Bacteriology Branch, Fort Detrick (reference see BURMEISTER and associates, Ann. Int. Med. *56,* 1962, 789-800).

TABLE 5.

Antibody response of patients who recovered from infection.

Patient	Date	CF Titers	HA Titers	Precipitation Lines ***
DLS *	3/9	8	2048	Fraction I, E
	3/18	4	2048	Fraction I, toxin, E
JOJ *	3/9	2	128	Not tested
	3/18	4	512	E
X **		32	8192	Fraction I, toxin, E, 1 unidentified

* Serum from 2 cases of plague in 1960. The patients were both treated before the samples were taken, 3 to 4 weeks after the onset of illness.

** A woman (Madagascar) 28 years old, was hospitalized 3 days after onset, and blood sample was taken 11 days later.

*** Immunoelectrophoretic test.

The second sample (3/18) from patient DLS and JOJ were kindly tested by Dr. W. D. Lawton in Maryland. DLS had antibodies only to E and L, and there were no lines with serum from JOJ.

between Fraction I antibody (CF and HA titers) to opsonocytophagic index. As noted, phagocytosis by neutrophiles and macrophages of resistant *P. pestis* directly paralleled Fraction I antibodies.

Serological tests on sera from patients who naturally contracted plague show an antibody response to Fraction I (Table 5). Immunoelectrophoretic analysis of these sera revealed precipitation lines

corresponding to Fraction I, toxin and antigen E (LAWTON [32]). Specific antitoxin was present in only some of the sera tested. The serological reaction patterns are generally similar to those of human volunteers vaccinated with our $Al(OH)_3$ adsorbed, formol-killed vaccine (MEYER, unpublished data). It is therefore suggested that by giving this killed vaccine, prepared in adjuvant, in 2 basic immunization doses and a later booster, a level of immunity may be obtained approaching that of persons recovered from natural infection.

V and W Antigens.

A method to study the behavior of virulent and avirulent strains of *P. pestis* was devised by BURROWS and BACON (10). They showed that virulent strains of *P. pestis* can be differentiated from avirulent strains by the ability of the former to develop marked resistance to phagocytosis by mouse polymorphonuclear leukocytes and that this property was due to the ability of virulent strains to produce not only Fraction I antigen but also antigens designated V and W. In their approach to these findings they first demonstrated that virulent strains of plague bacilli, in contrast to avirulent strains, can exist as three different types: 1) phagocytosis-sensitive S type: virulent strains grown on tryptic meat digest agar slants for 17 hours at 28°C, initially sensitive to phagocytosis and destroyed by mouse polymorphonuclear leukocytes both *in vitro* and *in vivo*. 2) Phagocytosis-resistant R type: from the incubation of an S type culture for 3 additional hours in tryptic meat digest broth at 37°C, an interval insufficient for the development of an envelope, but rendering them highly resistant to phagocytosis. 3) Phagocytosis-resistant M type: virulent organism grown *in vivo* in mice or in guinea pigs for 9 to 16 hours, at this time visibly well enveloped (encapsulated), rich in Fraction I, and highly resistant to phagocytosis. By contrast, avirulent plague bacilli treated identically retained their high sensitivity to phagocytosis. From these observations they concluded that there must be one or more additional antigenic components responsible for the virulence of a strain. The existence of 2 virulence antigens, designated V and W, were demonstrated by the Ouchterlony gel diffusion technique. The V and W antigens were claimed to be the major factors in determining the virulence of *P. pestis*. In their procedure, hyperimmune anti-R type or M type rabbit serum, heavily absorbed with acetone dried avirulent *P. pestis* grown at 37°C, was used as the standard serum. The absorption served to remove all antibodies corresponding to avirulent antigens thus leaving only antibodies against the virulent antigens

whatever they may be. When this absorbed serum was diffused against the virulent strain, 2 well defined lines of precipitation appeared, which were not seen when most avirulent strains were used. Such strains did not possess virulent factors except for the 3 strains, EV-76, Harbin and Elizabethville (11).

Crude preparations of W and V antigens have been precipitated respectively by 0.3 to 0.35 and 0.35 to 0.4 saturated solutions of ammonium sulfate by BURROWS (13). In solution these antigens are stable in the cold (2 to 4°C) and are nondialyzable. More highly purified preparations are not stable, becoming undetectable by diffusion methods, on prolonged storage. The V and W antigens are dissociation products of a larger entity, and are characterized as proteins.

BURROWS and BACON (12) demonstrated the protective effect of the V and W antigens by studying the antibody responses in mice, guinea pigs, and rabbits immunized with live, purine-dependent mutant strains. These mutant strains, derived from a fully virulent plague strain, were of different antigenic composition. The antibody response to the different antigenic components (Fraction I, V and W) as determined by the gel diffusion technique shows that rabbits, mice, and guinea pigs immunized with Fraction I positive strains all produced Fraction I antibodies; however, only rabbits responded immunologically in all instances to both V and W antigens. Guinea pigs responded immunologically only to the W antigen, while mice responded only to the V antigen with the exception of one instance when an antibody response was elicited against both the V and W antigens of the mutant strain M26A. The effectiveness of these antigens for protection of mice against plague infection was tested by challenging vaccinated animals with a wild strain MP6 and a mutant strain M23. The enhancement of resistance by the presence of the V and W antigens in the vaccines was demonstrated, particularly in the case of vaccinated animals challenged with an antigenically homologous strain.

The results of guinea pig experiment demonstrate that only those animals challenged with the wild strain MP6 gave a typical active immunization response. No conclusions can be drawn from the data on those animals challenged with the Fraction I negative, mutant strain M23.

Further studies on the isolation and purification of V and W antigens from strain M23 were done by LAWTON and associates (33) in 1963 using ammonium sulfate precipitation and DEAE cellulose chromatography. They identified the V antigen as a protein with a molecular weight of 90,000 and the W antigen as a lipoprotein with a molecular weight of 145,000. Both antigens were

reduced in titer by prolonged storage at 5°C or by lyophilization, but not by storage at —20°C.

The effectiveness of the purified V and W preparations was tested by challenging actively immunized guinea pigs and passively immunized mice with a normal fully virulent strain (Alexander). In guinea pigs V, but not W, was protective when sodium alginate was used as an adjuvant. Two equal doses of V antigen (total 100 μg) given intramuscularly 2 weeks apart protected all the guinea pigs. Anti-V, but not Anti-W serum (two 0.5 ml doses of serum) protected mice against subsequent challenge with the virulent Alexander strain.

An attempt to understand the relationship of Fraction I and VW antigens to virulence of *P. pestis* was aided by the studies of DONOVAN and associates (26). They studied the infectivity and lethality of the 2 antigenically different strains, enveloped MP6 (wild strain) and nonenveloped M23 (mutant strain), injected intradermally into guinea pigs. The fully virulent, enveloped plague bacilli established infection in the animals after intradermal doses approaching a single bacterium. All infections were lethal. Genetic loss of the ability to form envelope material resulted in loss of virulence but essentially no loss in the ability to establish infection. The envelope appeared to be much more essential for lethality than for infectivity in guinea pigs. These observations re-emphasized the importance of envelope antigen as a factor for full virulence in *P. pestis* (wild strain).

In more recent studies, JANSSEN and associates (30) found that avirulent strains which produced Fraction I, but no V and W antigens were more resistant to ingestion by free phagocytes than noncapsulated atypical virulent strains which produced V and W, but no Fraction I. They claimed that there was no strict correlation between virulence and the ability to resist phagocytosis. Rather the major factors in determining the virulence of *P. pestis* were the V and W antigens which permit survival and multiplication of organisms within phagocytic cells of the host. It appears that the factors determining virulence are Fraction I, associated with phagocytosis resistance and the VW complex associated with the ability to multiply intracellularly.

L Antigen.

Using a refinement of the gel diffusion technique, LAWTON and associates (32) demonstrated the presence of 11 antigens common to *P. pestis* and *P. pseudotuberculosis* (Table 1). It has long been

TABLE 6.

Correlation of the protective value of four vaccines against pneumonic plague in guinea pigs with the circulating antibodies detected prior to challenge.

Vaccine	No. Dead/Total	No. Sera Containing Anti-Fraction I	No. Sera Containing Anti-L	No. of Sera Containing Antibody Other than Anti-Fraction I or Anti-L
Cutter	0/12	12	0	1
Fraction I	1/12	12	0	0
Live EV76	0/12	10	12	3
Killed *P. pseudotuberculosis*	2/12	0	11	6
Controls	12/12	0	0	0

Data from LAWTON, FUKUI and SURGALLA (J. Immunol. *84*, 1960, 475).

recognized that *P. pseudotuberculosis* can be used successfully to immunize experimental animals, particularly guinea pigs against plague infection. One or more of the common antigens must be responsible for this cross-immunity. The L antigen (Table 1) was implicated as the protective antigen against plague since anti-L antibodies were always found in animals immunized with *P. pseudotuberculosis*. Moreover, sera containing anti-L antibody, with other precipitating antibodies either absent or present in minor amounts, have conveyed passive protection to mice. In the work of LAWTON and associates (32), the following four vaccines in Freund's adjuvant were administered intramuscularly: 1) 0.5 ml. Cutter vaccine (2×10^9 formol-killed virulent organisms/ml). 2) 5 micrograms of Fraction I (Hooper). 3) 1×10^6 live avirulent EV-76 grown on Difco blood agar base slants at 37°C. 4) 5 mg acetone killed and dried *P. pseudotuberculosis* (grown in Difco beef heart infusion broth at 37°C). Four weeks later the same doses of the vaccines were given without adjuvant. The animals were bled for serological assay and challenged via the respiratory route with 52,000 cells (10 LD_{50}) of the virulent strain Alexander on the 12th and 14th day respectively after the second injection. Their results (Table 6) indirectly demonstrate the protective value of the L antigen. In a subsequent test of the partially purified L antigen, no protection against plague infection was demonstrated. They thought it quite possible that fractionation of the L antigen had altered its immunogenicity. These experimental results warrant further investigation of the L antigen.

PF Antigen.

The fact that purified L antigen was of no protective value against plague infection led LAWTON and SURGALLA (34) to re-investigate the cross-protective antigen(s) of *P. pseudotuberculosis* and *P. pestis*. A partially purified antigen from virulent and avirulent strains of *P. pseudotuberculosis* and *P. pestis* was isolated by a method similar to the one which KEPPIE and associates (31) used for their atoxic immunizing complex plague vaccine. The rationale was that this procedure would extract the same antigen from *P. pestis* and *P. pseudotuberculosis*. The antigen so derived, called PF (protective factor) antigen was shown to protect guinea pigs against plague as early as the first day and up to 5 weeks after vaccination. However, this immunity could not be associated with any specific antibody obtained from immunized animals. Moreover anti-PF serum conferred no passive protection when injected into normal animals. The PF antigen was found to be a protein-lipo-polysaccharide complex, analogous in its action to endotoxin by increasing nonspecific resistance to infection. However, this antigen is relatively nontoxic in mice (LD_{50}: 5 mg i.p.) and guinea pigs (LD_{50}: 20 mg i.p.). It seemed to have no relation with antigen L, but the antibody co-precipitating the PF antigen was the same as the antibody directed against the specific polysaccharide antigen of DAVIES (25), which will be discussed later. The importance of incorporating the PF antigen in plague vaccine has been emphasized by them; since the avirulent *P. pseudotuberculosis* strain used to obtain PF cannot produce any of the antigens implicated in plague immunity, the nonspecific resistance to plague must be due to an antigen other than Fraction I, V, W, or plague toxin.

Antigen 4.

In 1957, CRUMPTON and DAVIES (24) isolated a protein from saline extracts of smooth *P. pestis* by precipitation with ammonium sulfate (0.15 to 0.25 saturation) and called it antigen 4. The conditions for the production of this antigen 4 are quite critical. It is produced only in well aerated broth cultures at pH 7.0, incubated at 37°C. It is first detected after approximately 16 hours of growth. Its production reaches a maximum at the end of the log phase. The addition of purified antigen 4 to a suspension of rough organisms produces a stabilizing effect on them and thus prevents them from settling faster than smooth organisms. This antigen occurs only in smooth forms of virulent and avirulent *P. pestis*.

It is also found in all virulent *P. pseudotuberculosis* strains, but not in smooth or rough avirulent forms. Therefore, it was suggested by these investigators that antigen 4 may represent a "virulence" factor in *P. pseudotuberculosis* but not in *P. pestis.* Its relationship to plague immunity was evaluated in mice. The killed smooth, killed rough, and live rough strains of vaccine did not differ significantly from one another in that they were of little protective value for mice. Only the live smooth strain of vaccine was protective for mice. These results confirm the previous finding of OTTEN (39) and SCHÜTZE (43) that live vaccines made from a smooth avirulent strain gave better protection than from a rough avirulent strain. It was concluded that antigen 4 contributed to the survival of smooth organisms *in vivo,* thereby prolonging the stimulus for protective antibody production. This antigen 4 by itself protects against neither plague nor pseudotuberculosis.

pH 6 Antigen.

In 1960, ARONSON and associates (3) reported that *P. pestis* cells suspended in a medium with the pH below 6.7 and incubated at a temperature of about 35°C underwent a change in their surface charge demonstrable by a decreased electrophoretic mobility. In later studies by BEN-EFRAIM and associates (6) this decrease in mobility was found to be associated with the formation of a specific antigenic surface component. The specificity of this antigen was demonstrated by diffusing it against hyperimmune anti-pH 6 antigen serum previously adsorbed with cells of a strain incubated at 37°C and pH 7. This pH 6 antigen was found in all virulent and avirulent strains, rough and smooth forms, but not in strains incubated at 37°C and pH 7, 28°C and pH 6, and pH 7. The pH 6 antigen differed from other *P. pestis* antigens in that the conditions suitable for production of the pH 6 antigen are not suitable for production of Fraction I, V, W and antigen 4. The suggestion was made that this antigen is one of the inherent properties of all strains of *P. pestis.* The pH 6 antigen is not stable in the chemically killed cells which were grown at 37°C and pH 6. Its presence was demonstratable by gel diffusion technique only on the first day after chemical treatment and not after prolonged storage. Anti-pH 6 antibodies may be found in mice infected with *P. pestis* even though the bacilli were previously grown under other (temperature or pH) conditions. Moreover, pH 6 antigen may be found in the homogenates of liver and spleen of the infected mice sacrificed 4 days after infection. The pH 6 antigen is formed *in vivo* because

the intracellular pH of the lymphatic tissue, monocytes and other sites of proliferation of *P. pestis* is close to that required for the *in vitro* formation of pH 6 antigen.

Further studies of the synthesis, purification, and biological activities of the pH 6 antigen were made by BICHOWSKY-SLOMNICKI and BEN-EFRAIM (9). They purified it with the aid of ammonium sulfate precipitation, preparative electrophoresis and calcium phosphate gel adsorption methods and identified it as a protein. The biological properties of the crude extract, cytotoxicity for monocytes, agglutination of red cells and induction of primary inflammatory lesions of the skin, were lost upon heating for 30 minutes at 56°C.

While this antigen is not a virulence factor like Fraction I, toxin, V and W antigens, the mortality rate in mice was significantly higher during the first 4 days following infection with 37°C and pH 6 grown cells than with 37°C and pH 7 grown cells. Beyond this 4-day period, the mortality rates in the 2 groups appeared similar. These observations taken in conjunction with the biological activities of the antigen lead BICHOWSKY-SLOMNICKI and BEN-EFRAIM to consider the synthesis of the surface pH 6 antigen as playing an important role in the pathogenesis of plague. However, because this antigen was synthesized by all the avirulent strains of *P. pestis* (TRU, TS, TSR and 14) investigated by them, it is difficult to explain its importance to the virulence of plague bacilli. The antibody response to this antigen was strong in mice and rabbits, but its protective value against infection has not yet been evaluated.

In recent studies, BEN-EFRAIM and BICHOWSKY-SLOMNICKI (7) demonstrated production of the pH 6 antigen by some *P. pseudotuberculosis* strains (3 out of 12 strains). The purified material was isolated from strain 27/C, and exhibited the same biological activities as the pH 6 antigen of *P. pestis*.

Toxin Antigen (murine toxin).

The toxic antigen or endotoxin has been purified by such investigators as SPIVACK and KARLER (45) at the Hooper Foundation, and AJL and associates (1) at the Army Service Graduate School. Purification was accomplished by various chemical procedures followed by continuous flow paper electrophoresis. It was characterized as a protein with a molecular weight of 74,000. Its LD_{50} is about 1 μg as tested in mice by intravenous route. The toxin is lethal to mice and rats and is therefore referred to as "murine

toxin". There is general agreement that death in plague is due to processes initiated by the action of the endotoxin. However, endotoxin is not considered essential for active immunization. The prevention of pathogenicity depends mainly on the control of multiplication of the organisms in host tissues during the crucial first stage of infection. In near terminal cases where antibiotic therapy has caused lysis of many bacilli antitoxin is needed to neutralize the toxin so released.

Specific Polysaccharide Antigen.

The purified polysaccharide described by DAVIES (25) is of minor significance in relation to plague virulence and immunity. LAWTON and SURGALLA (34) have demonstrated similarities to it in their protective PF antigen by the double diffusion in gel technique (the antibody co-precipitating PF was shown to be identical to the one directed against the polysaccharide). The polysaccharide is a hapten, and can be made antigenic only when conjugated with a protein. It is non-protective, is moderately toxic, and strongly pyrogenic.

Summary and Conclusions.

The recent knowledge of the antigenic constituents of *P. pestis* and their relationship to virulence and immunity has originated from the gel diffusion techniques of Oudin and Ouchterlony, modern methods of purification, and the use of Freund's adjuvant. Studies employing these techniques have led to the elucidation of the antigenic complexity, close antigenic relationship and cross-immunity between *P. pestis* and *P. pseudotuberculosis,* and the relationship of the identified antigens to virulence and immunity in plague. The defined antigens of *P. pestis* are Fraction I, Fraction II (murine toxin), V, W, L, PF, antigen 4, pH 6 antigen and the specific polysaccharide. The antigens mainly responsible for virulence of a plague strain are Fraction I, Fraction II, V and W antigens. Fraction I is much more essential for lethality than for infectivity as tested by intradermal injection of guinea pigs (26). The ability to produce Fraction I, does not alone determine the virulence of a strain. More important is the existence of V and W antigens which, as discovered by CAVANAUGH and RANDALL (15), enable an organism to survive and multiply within monocytes. Purified antigens known to elicit a protective reaction are Fraction I, Fraction II, V and PF. Fraction I is the main protective antigen against infection induced by wild plague strains. It may be expected that the addition of V and PF antigens, which protect

experimental animals, should give more adequate immunity in a vaccine preparation. However, there is no evidence of V antigens in the vaccine preparations now in use, and in addition no anti-V antibodies are demonstrable in the vaccinated host, in recovered patients and in experimentally infected animals. An effective method to enhance V production and evaluate the presence and protective effect of PF antigen in a vaccine preparation would be an important future task.

Recent experiments with an $Al(OH)_3$ adsorbed plague vaccine prepared with the fully virulent strain 195/P, rich in Fraction I, have indicated that the antibody response of the immunized experimental animals was similar to that of human subjects following inoculation of the same preparation (MEYER, unpublished data). In addition, the effectiveness of the vaccine was also determined by challenging the vaccinated animals with a wild strain of plague. The results obtained (CHEN et al., to be published), which indicated a heightened immunity produced by the vaccination, have practical implications for human use.

Purified Fraction I is stable and incites a strong antibody response. V antigen is reduced in titer if it is stored for longer than 1 to 2 weeks at 5°C or if it is lyophilized; it is stable when stored at —20°C. It is a weaker antibody inducer than Fraction I. The effectiveness of PF antigen prepared from *P. pseudotuberculosis* Type IV in protecting guinea pigs against plague have been shown from 1 day to 5 weeks after vaccination (34). More studies on this antigen undoubtedly will give a better understanding of this type of resistance to infection.

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Zusammenfassung.

Der Autor gibt eine Übersicht über die Anwendung der Geldiffusionsmethoden, moderne Reinigungsmethoden, und über die Verwendung von Freundschem Adjuvans für die Aufklärung der komplexen Antigenität, der nahen Verwandtschaft der Antigene und der Kreuz-Immunität zwischen *P. pestis* und *P. pseudotuberculosis* und den Zusammenhang zwischen den identifizierten Antigenen und Virulenz und Immunität bei einer Seuche.

Die Antigene aus *P. pestis,* die bestimmt wurden, waren Fraktion I, Fraktion II, V, W, L, PF, Antigen 4, pH 6 Antigen und die spezifischen Polysaccharide. Für die Virulenz eines Seuchebakterienstammes hauptsächlich verantwortlich sind Fraktion I, Fraktion II, V und W. Fraktion I ist wesentlich wichtiger für die Lethalität als für das Eintreten der Infektion. Die Fähigkeit, Fraktion I zu bilden, ist nicht allein bestimmend für die Virulenz eines Stammes, er muß auch V- und W-Antigene enthalten. Die gereinigten Antigene Fraktion I, Fraktion II, V und PF können zu Schutzwirkung führen. Fraktion I ist der wichtigste schützende Faktor gegen Infektionen, die durch wilde Stämme hervorgerufen werden. Man kann erwarten, daß Antigen V und PF, die in Versuchstieren Schutzwirkung hervorrufen, als Impfstoffpräparat bessere Immunisierung geben werden.

Résumé.

L'auteur a réexaminé l'emploi de la méthode de précipitation en gélose d'Oudin et d'Ouchterlony, les méthodes modernes de purification, l'emploi de l'adjuvant de Freund dans le but d'élucider la complexité et la proche parenté antigéniques de *P. pestis* et de *P. pseudotuberculosis,* le croisement d'immunité entre ces deux espèces, ainsi que, dans le cas de la peste, l'importance des antigènes connus à l'égard de la virulence et de l'immunité.

Les antigènes décrits de *P. pestis* sont les Fractions I, II, V, W, L, PF, l'antigène 4, l'antigène pH 6 et le polysaccharide spécifique. Les antigènes qui sont surtout responsables de la virulence d'une souche bactérienne sont les Fractions I, II, V et W. La Fraction I est beaucoup plus essentielle à la léthalité qu'à l'infectibilité. Le pouvoir de production de la Fraction I ne détermine pas par soi-même la virulence d'une souche ; les antigènes purifiés possédant un pouvoir vaccinant sont les Fractions I, II, V et PF. La Fraction I est l'antigène vaccinant principal dans les infections par souches sauvages. On peut s'attendre à ce que l'incorporation dans les vaccins des antigènes V et PF, antigènes ayant un pouvoir vaccinant envers les animaux de laboratoire, fournisse une immunité plus complète.