STUDIES ON THE SPECIFIC SUBSTANCE
OF *B. MALLEI**

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Since the valuable reports had been published by Heidelberger and Avery⁽¹⁾, many investigators studied the same problem concerning specific substances of various microorganisms, and most authors came to the same conclusion as the first workers, that the type-specific reaction depends upon a soluble substance of a carbohydrate nature which does not give rise to immunity when injected into experimental animals, while the non-specific reaction is due to species-specific protein which induces immunity by injection into animals. In contrast to those early works, Schiemann and Casper⁽²⁾⁽³⁾ suggested the existence of another type-specific substance which was identical in chemical nature with that of Heidelberger and his co-workers but it has such a different property in immunological activity as it gives rise to specific immunity by its injection into mice. From the immunological point of view, it must be considered an important one that those two type-specific substances have a different property in their antigenic activity. Enders⁽⁴⁾, also, recently isolated from autolysates of *Pneumococcus* a type-specific substance distinct from the so-called specific carbohydrate of the first workers. This new substance showed a specific reaction by precipitating the homologous antiserum after absorption with the specific carbohydrate described by Heidelberger and Avery, and also it caused fatal anaphylactic shock in guinea-pigs sensitized passively with homologous rabbit serum. From these observations it is without question that there are two specific substances of a carbohydrate nature which, however, are not the same in their antigenic activity

* A detailed description of the whole experiments was given in a previous paper⁽⁵⁾.

in vivo. The significance of those substances is not yet thoroughly interpreted, so that further study is necessary to be performed. For this reason, the isolation not only of the carbohydrate fraction but also of the fatty and protein fractions of the *Pneumococcus* was undertaken by Wadsworth and his collaborators. And they succeeded in the separation of the specific antigenic substance similar to those of Schiemann and Casper, and of Enders, in its ability of immunizing mice, but not the same as the so-called soluble specific substance reported by Heidelberger and Avery. The chemical nature of this specific substance was not determined, as they could not obtain a sufficient amount of this material for further chemical investigation.

The separation of specific and non-specific substances of glanders bacillus was first undertaken by the writer and the work on the practical diagnosis of glanders in infected horses by the precipitation method, in which the soluble specific substance was used as precipitinogen, was published in a previous paper⁽⁷⁾⁽⁸⁾. Since that publication, chemical, immunological and allergic studies have been carried out on the two substances derived from about 70 liters of broth culture of glanders bacillus. Only a brief record of the experimental results is given as follows.

1. PREPARATION

Preparation of soluble specific and non-specific substances was described in previous works⁽⁷⁾⁽⁸⁾⁽⁹⁾, and repeated here again for the sake of convenience:

One liter of two-week-glycerin-broth cultures of a virulent strain of glanders bacillus was heated at 100°C for one hour and after examining for purity by microscope, glacial acetic acid was added in a final strength of 1 per cent of acetic acid. The whole was placed in an ice-box overnight, and then was passed through a bacterial filter of the Mandler type. The filtrate was neutralized with *N*/5 NaOH. This neutralized solution was concentrated on a water-bath to one-tenth of the original volume, i. e., 100 c.c., to which 2 volumes of 95 per cent alcohol were added. After thorough mixing, the whole was placed again in the ice-box for two or three hours. An abundant precipitate which appeared was collected by centrifuging, and dissolved in 40 c.c. of distilled water.

After four or more repetitions of this acid-alcohol precipitation, no precipitate was caused by further addition of acid.

The following two preparations were used in the experiments described below: The precipitate which was produced by the second and third acid precipitation was used as a non-specific substance, while the precipitate of the fourth alcohol precipitation was used as a soluble specific substance of glanders bacillus.

2. CHEMICAL PROPERTIES

A. Specific Substance:

1. It is precipitated by alcohol, but not by glacial acetic acid.
2. It is soluble in water, and much more soluble in weak alkaline solution.
3. Total nitrogen of this substance is 3.73 per cent by Kjeldahl's method.
4. Dextrin reaction was positive when Lugol's solution was added.
5. Protein and sugar tests were performed before and after hydrolysis with sulphuric acid and hydrochloric acid as shown in Table 2, and the concentration of the material used in this experiment was 0.15 to 0.3 per cent of aqueous solution. The hydrolysis was done in a boiling water-bath for two hours.

Table I.

	Protein reaction										Sugar reaction				
	Precipitation reaction						Colour reaction								
	Trichloric acetic acid reaction	Phosphorowolfram acid reaction	Sulphosalicylic acid reaction	Picric acid reaction	Tannic acid reaction	Heller's test	Ninhydrin reaction	Biuret reaction	Millon's test	Molisch's test	Xanthoproteic reaction	Nylander's test	Fehling's test	Tommer's test	Benedict's test
Before hydrolysis	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
After hydrolysis (test material 10c.c. + $\frac{N}{10}$ HCl 2 c.c.)	+	+	*	*	+	*	±	+	+	±	*	*	*	*	*

After hydrolysis (test material 10c.c. + $\frac{N}{1}$ HCl 3c.c.)	-	+	-	+	+	*	-	-	-	+	*	+	+	+	*
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N. B. In this table, as in the next one, — indicates negative reaction, $\pm$, +, +, indicate degrees of positive reaction, * indicates that no experiment was done.

6. Serological activity of this specific substance was destroyed by hydrolysis and digestion of trypsin and pepsin.

B. Non-specific substance :

1. It is precipitated by alcohol and acetic acid, insoluble in water.

2. Total nitrogen of this substance is 13.51 to 12.76 per cent by Kjeldahl's method.

3. Protein, sugar, phosphoric acid and purine tests were carried out before and after hydrolysis with alkali and acid as shown in Table 2, and concentration of the test material used in this experiment was 0.5 per cent of aqueous solution. The hydrolysis was done in a boiling water-bath for two hours.

Table 2.

			After hydrolysis		
			(test material 10 c.c. + $\frac{N}{10}$ HaOH 0.3 c.c.)	(test material 10c.c. + $\frac{N}{1}$ HCl3c.c.)	(test material 10 c.c. + 1% HCl 10 cc.) (boiled for 3 hours)
Protein reaction	Precipitation reaction	Trichloric acetic acid reaction	—	—	+
		Phosphorouswolfram acid reaction	—	±	+
		Sulphosalicylic acid reaction	—	=	+
		Picric acid reaction	—	—	+
		Tannic acid reaction	—	+	+
	Colour reaction	Ninhydrin reaction	—	+	+
		Biuret reaction	±	+	+
		Millon's test	—	+	+
		Molisch's test	+	+	+

Sugar reaction	Nylander's test	—	—	—	+
	Fehling's test	—	—	—	+
	Trommer's test	—	—	—	*
	Benedict's test	—	—	—	*
Phosphoric acid reaction	Molybdic-acid-ammon reaction	—	—	—	+
	Ferric chloride reaction	—	—	—	+
Purine reaction	Murexide reaction	—	—	—	-(?)
	Xanthine base reaction	*	—	—	+

From these data, conclusions could be drawn as to their chemical nature as follows:

1. So-called soluble specific substance isolated from broth culture of glanders bacillus is glycoproteid in chemical nature.
2. The one portion, at least, of non-specific substance of glanders bacillus is nucleoprotein.

3. IMMUNOLOGICAL PROPERTIES

Both specific and non-specific substances had toxicity to laboratory animals; mice and guinea-pigs were killed in a few days by subcutaneous injection with a small dose, i. e., 0.001 g. of dried powder. Rabbits were given single injection intraperitoneally or intravenously to be tested for antigenic activity of two respective substances. The experiments were repeated three times in 33 rabbits in all. The dosage varied from 0.3 to 0.001 g. Twelve rabbits died before being bled to separate sera for experiments. Twenty one rabbits, consequently, were tested for demonstration of antibodies produced by the injection with two substances. On the fourteenth or nineteenth day after the injection, animals were bled and their sera were examined for antibodies.

Precipitative, agglutinative and complement-fixing activities were demonstrated in those sera of rabbits injected with the two substances respectively. But the degree of immunity in rabbits induced by the two substances was not same. The specific substance induced less immunity in rabbits than the non-specific one. And even the antigenic activity of the non-specific substance was also less active than that of the whole body of heat-killed

bacteria used as control in order to compare the immunizing activity of these three antigens.

From these evidences, therefore, the soluble specific substance of glanders bacillus is not the same as the soluble specific substance of Heidelberger and Avery; it differs from the latter not only in its chemical nature but also in its antigenic activity in vivo.

4. ALLERGIC REACTION

A. Subcutaneous injection test

Both specific and non-specific substances which were preserved in a desiccator in dried powder were weighed respectively and a dilution was prepared 2:600 with normal salt solution. Each amount of 1 c.c., 3 c.c. and 5 c.c. of the diluted solution was injected subcutaneously into experimental horses, i. e., 13 glandered horses and 9 normal ones, of which 9 infected horses and 3 normal ones were injected with the specific substance and the others were all injected with the non-specific substance. The diagnosis of these animals was made by the ophthalmic mallein test and complement-fixation test, and finally by autopsy-findings which were observed after the examinations were all finished.

1. Specific substance

After subcutaneous injection with each test dose of 1 c.c., 3 c.c. and 5 c.c. of the specific substance, 9 glandered horses showed a characteristic rise of temperature beginning usually in four hours. From this time on to the twelfth hour or later, the increase of temperature continued and reached its maximum, and then gradually subsided. The rise in temperature was accompanied by organic symptoms, such as quickened pulse, increased respiration, dullness, depression, shivering and anorexia. The local reaction that appeared at the point of inoculation was characterized by an inflamed, oedematous but not suppurative, circumscribed, painful, often flat and very extensive swelling. While in 3 normal horses the injection of the test material caused a very slight reaction or none; a little rise of temperature and a small local oedematous tumour, if appeared, soon subsided.

A description of each case was omitted in order to save space. Two cases are shown in Figs. 1 and 2.

Fig. 1. No. 13, glandered horse
(injected with the specific substance 5 cc.)

Fig. 2. No. 15, normal horse

Fig. 3. No. 8, glandered horse
(injected with the non-specific substance 1 cc.)

Fig. 4. No. 18, normal horse
(injected with the non-specific substance 1 cc.)

2. Non-specific substance

Non-specific substance was injected into 4 glandered horses and 6 normal ones in the same manner as that of the specific substance described above. The results obtained from the experiments on infected animals were also the same as in the cases of the specific substance. In normal horses, however, the injection of a non-specific substance produced a thermo-reaction and local swelling of a higher grade than those of the specific substance. From this evidence, it is clear that the specificity of the so-called specific substance is higher than that of the non-specific substance as shown in Figs. 3 and 4.

B. Ophthalmic test

1. Observation in horses

The dilution of 1 : 60 was prepared with normal salt solution from the dried test materials of the specific and non-specific substances respectively. The diluted solution was introduced into the conjunctival sac of the right eye of the experimental horses which were the same as those used in the subcutaneous test mentioned before. Standard mallein used as a control was introduced into the left eye of the horse. The test materials and mallein were rubbed on the inner surface of the lids with the aid of a soft writing brush. The observation lasted for 21 hours. In 13 glandered horses and also in 9 normal ones, no difference was found out between each reaction of the two test substances. The reactions of two substances in 13 glandered horses appeared from 2 or 3 hours after application; somewhat earlier and yet more intensely than the typical reaction of standard mallein. After reaching a maximum in 8 to 12 hours it began to disappear gradually. The reaction was characterized by a conjunctivitis which consisted of redness of the membrane, palpebral swelling, often very intense, with a purulent yellow secretion which collected at the inner canthus, then overflowed and ran down the face. In contrast to this evidence, in 9 normal horses, the two test substances produced nothing of a characteristic reaction, although the technique in the application of the test materials was quite the same as in the cases of glandered ones.

From the results obtained by this ophthalmic reaction, the

difference was not found out in their specificity of the two test substances.

2. Observation in rabbits

Five rabbits were given intravenous injection with specific and non-specific substances respectively, in various doses of 0.001 to 0.005 g. On the fourteenth day after injection, the ophthalmic reaction was examined. The specific substance was introduced into the left eye of the rabbit and the non-specific one was introduced into the right. A dilution 1:60 of these test materials was prepared with normal salt solution. The technique was the same as in the case of the horses stated before. The observation took place for 24 hours. The results were summarized as follows:

The two materials reacted to the rabbits injected with non-specific substance, and also non-specific substance reacted to the rabbits injected with specific substance, although it was a very weak reaction. The immunizing activity of the two test materials was recognized in different grades, that is, the non-specific substance was more active than the specific one which was almost inactive.

5. COMPLEMENT-FIXATION TEST

An antigen should be claimed to be as specific as possible in any serological tests. Furthermore, it is most desirable to have a handy and durable antigen prepared in Manchuria, where glanders is always breaking out in the equines and often even in human beings. The specific substance of the glanders bacillus described above is advisable to be tested for an antigen in the complement-fixation test which is extensively carried out in many countries as a most reliable method.

This dried powder, a readily soluble and specific substance stated above, should be found to be a highly satisfactory one as an antigen.

The experiments were carried out in about 200 kinds of sera from normal and glandered horses and in the sera from rabbits immunized against glanders bacillus⁽¹⁰⁾. The complement-fixation test with the specific substance used as an antigen furnished a very satisfactory basis for conclusions regarding the reliability and accuracy of the specific substance. It was also confirmed that the specific substance was preserved, at least, for 19 months without

loss of its activity in a dry condition in room temperature.

SUMMARY

1. Two substances could be isolated from broth culture of *B. Mallei*, of which one called the specific substance seems to be glycoproteid and the other the non-specific substance may be nucleoprotein in chemical nature.

2. The nucleoprotein gave rise to non-specific reaction not only in vitro but also in vivo.

3. The non-specific substance had an antigenic property in laboratory animals more active than that of the specific one.

4. The specific substance caused specific reaction in vitro, also produced a specific allergic reaction in vivo.

5. The purified specific substance is the most reasonable and convenient antigen in the practical diagnosis of glanders by means not only of allergic reaction but also of a complement-fixation test.

6. The true form of specific reactive substance of *B. mallei*, at least one portion of it, may belong to glycoproteid.

CONCLUSION

From broth culture of *B. mallei*, it is possible to isolate a specific substance not identical with that of Heidelberger and his co-workers in its antigenic activity and chemical properties. This substance is applicable as a reliable antigen in the diagnosis of glanders by methods of precipitine reaction⁽⁷⁾⁽⁸⁾, complement-fixation test and allergic reaction.

It is reasonable that this purified specific substance (or glycoproteid) is named "*Neomalleinum siccum*".

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鼻疽菌特異性物質ノ研究

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鼻疽菌ノぐりせりん肉汁培養ヨリ2種類ノ物質ヲ分離シ得タリ其1ハ非特異性物質ニシテ沈降反應ノミナラズあるぎー反應ニ於テモ非特異性ニ反應スルモノナリ之レヲ化學的性狀ヨリ觀ル時核蛋白 (Nucleoprotein) ニ屬スルヲ知リタリ他ノ1ハ所謂特異性物質ニシテ既ニ報告セル沈降反應ニ於テノミナラス補體結合反應及あるぎー反應ニ於テモ極メテ特異的ニ反應スベキ物質ニシテ化學的性狀ヨリ觀テ糖蛋白 (Glycoprotein) ニ屬ス前者ハ注射動物ニ對シ免疫賦與性後者ヨリモ強度ナリ後者即チ特異性物質ガ弱度ナガラ注射動物ニ對シ抗原性ヲ有スルノ事實ハ從來種々ナル細菌ニ就キ報告セラレタル種々ナル特異性物質ト免疫學的比較考察ヲ行フ上ニ重要ナリ多數ノ學者ハ種々ナル細菌ヨリ分離セル特異性特質中ニ含有セラル、窒素ヲ純化不十分ナル爲メ殘存セルモノト解釋セリ余ノ實驗ニヨレバ該特異性物質ニ含マレタル蛋白窒素ハ含水炭素ト化學的ニ結合ノ狀態ニアリテ之ヲ破壊スル時ハ其特異性反應ノ性能ニ影響スルヲ知リタリ該特異性物質ハ實際的方面ヨリ觀ルニ沈降反應、補體結合應及ビあるぎーノ抗原トシテ實地的鼻疽診斷ニ應用シ得ルノミナラズ乾燥粉末ノ狀態ニ保存スル時ハ室温ニ於テ19箇月間以上其ノ反應能力ヲ保有スルヲ觀察セリ

夫故化學的ニ糖蛋白ノ性質ヲ有スル鼻疽ノ純化特異性該物質ニ對シ Foth 氏ノ乾燥まれいんニ倣ヒ乾燥ねおまれいん (Neomalleinum siccum) ナル名稱ヲ附セントスルモノナリ (詳細ハ南滿獸醫畜產學會雜誌第14卷第2號昭和7年4月號ニアリ)

(自抄)