PRODUCTION OF HEMORRHAGIC NECROTIC SKIN LESIONS IN THE RABBIT BY MEANS OF HEMOPHILUS INFLUENZAE AND HEMOPHILUS PERTUSSIS*

By ERNEST WITEBSKY, M.D., AND HEINZ SALM, M.D.

(From the Bacteriological and Pediatric Divisions of Beth Israel Hospital, New York)

(Received for publication, July 10, 1936)

The skin biological differentiation of *Hemophilus influenzae* and *Hemophilus pertussis* is known in the Japanese literature through the work of Takagi and Okutani, according to a report of Kasahara (1). In the European literature, Gundel and Schlueter (2) first called attention to a possible differentiation between *H. influenzae* and *H. pertussis* by means of the different reactions following inoculation into the skin of a rabbit.

H. influenzae, when injected intracutaneously in the abdominal wall of rabbits, causes inflammation and erythema. The color of this lesion is pinkish red; sometimes a tiny yellowish pustule develops in the center. *H. pertussis*, on the other hand, produces a bluish discoloration in the injected area which sometimes is transformed into a hemorrhagic necrotic lesion of indistinct outline within 2 to 3 days. Occasionally one or the other strain may display a somewhat different picture, especially if examined only once.

This report will describe the experimental conditions under which *H. influenzae* can be made to induce severe hemorrhagic necrotic lesions, and the differences in behavior between *H. pertussis* and *H. influenzae* in this respect.

* Aided by a Grant of the Colonel Friedsam Foundation Fund.

Material

The following strains of *H. pertussis* and *H. influenzae* were at our disposal:

Strains of H. pertussis

(*a*) Strain A[1]
(*b*) " B[1]
(*c*) " G 51[2]
(*d*) " R 55[2]
(*e*) Strain H 56[2]
(*f*) " R 59[2]
(*g*) " L 62[2]
(*h*) " S 63[2]

Strains of H. influenzae

(*a*) Strain 158[1]
(*b*) " S 3[1]
(*c*) " 2621—Postmortem lung puncture[3]
(*d*) " 3607—Spinal fluid[3]
(*e*) Strain 3743—Eye culture[3]
(*f*) " 3878—Sputum[3]
(*g*) " 79—Sputum[3]
(*h*) " 385—Chest fluid[3]

For the cultivation of *H. pertussis*, a Bordet-Gengou medium was used. It was prepared in the following way.

500 gm. of peeled sliced potatoes are boiled until soft, in 1000 cc. of distilled water and 40 cc. of glycerine. The original amount of fluid is restored and strained through gauze. To 500 cc. of the filtrate, 1500 cc. of 0.6 per cent saline solution and 60 gm. of agar are added. After the agar has been dissolved by heat (avoid scorching), water is added to restore the original volume and the medium is bottled in 120 cc. amounts, autoclaved and refrigerated. If used, it is melted, cooled to 45°C. and 40 cc. of sterile, fresh, citrated or defibrinated rabbit blood warmed to 40–45°C. is added. Blood and medium are mixed gently without air bubble formation, poured into sterile culture tubes (5 x 5/8 inches), and allowed to congeal to slants. The surface should be smooth and moist, the color a rich cherry red. Since the pH is approximately 6.8, no adjustment of pH was necessary.

For the cultivation of *H. influenzae*, a Levinthal medium was used, prepared as follows:

2 per cent agar of pH 7.5 is melted and cooled to 60–70°C., and 5 to 10 per cent rabbit blood added. It is mixed well and put into a boiling water bath for 8 minutes if 1 litre is prepared; if 2 litres, for 12 minutes. Directly afterwards, the blood agar mixture is filtered through sterilized cotton which has been kept warm at about 60°C. The cotton may be moistened before sterilizing. Tubes (6 x 3/4 inches) are filled with the clear agar and allowed to congeal. All media are kept in the refrigerator until used. Chocolate slants also have sometimes been used for the cultivation of *H. influenzae*. 48 hour growth of *H. pertussis* and 24 or 48 hour growth of *H. influenzae* were used in the experiments.

---

[1] Obtained through the courtesy of the Department of Health, New York.

[2] Obtained through the courtesy of Dr. Shwartzman, The Mount Sinai Hospital, New York.

[3] From the Bacteriological Laboratory of Beth Israel Hospital.

Suspensions of *H. pertussis* were obtained by taking off the growth from the Bordet-Gengou slants by means of a platinum loop or sterile swab, and put into a few cubic centimeters of normal saline. Suspensions of *H. influenzae* were obtained by adding 3 to 4 cc. of saline to a Levinthal slant. The growth on the surface was washed off by means of a platinum loop. The suspension was taken off with a sterile pipette and put into another Levinthal slant to increase the density of the bacterial suspension. Equal distribution was obtained by squeezing the suspension several times with a sterile pipette. We also had good results by taking off the bacterial growth from the Levinthal slant by means of a sterile swab and putting the swab into saline. The final bacterial content of the suspension (after being washed one to three times) used in our experiments, varied between 2 to 5 billion per cc.

For the preparation of *B. typhosus* agar washing filtrates, the technic of Shwartzman (3) has been employed.

For the preparation of agar washing filtrates of *H. influenzae*, *H. influenzae* has been cultivated on Levinthal-Kolle flasks which had been seeded with 3 to 4 cc. of a saline suspension of *H. influenzae* washed off Levinthal slants. The bacterial growth was washed off the Kolle flasks with 3 to 4 cc. of 0.85 per cent saline suspension with the addition of 0.4 per cent phenol. The suspensions were pooled after being controlled for purity, then centrifuged. The supernatant fluid was filtered through a Berkefeld V candle. The bacterial sediment was suspended in saline and washed three times.

Method

The rabbits (weighing between 4 and 5 pounds) were shaved on the abdomen and injected intradermally with 0.25 cc. of a bacterial suspension or bacterial filtrate. 24 hours later, 1 to 3 cc. of a bacterial suspension of filtrate were given intravenously. Special care has to be taken that the intradermal injection is really intradermal and not subcutaneous.

It must be mentioned here that in our experiments inflammation and infiltration without hemorrhagic necrotic changes were called negative, even if they were pronounced. Hemorrhagic necrotic lesions only were considered as positive. For the purpose of abbreviation the lesions are marked + to ++++, according to the size of the lesion. Thus the difference between a negative and a one plus reaction is more important than the quantitative differences indicated by the number of plus signs. While it is very easy to read reactions caused by *H. influenzae*, readings of areas prepared with *H. pertussis* are somewhat more difficult. They show, almost from the beginning, a tendency to bluish violet discoloration which is followed by necrosis after a lapse of several days.

Influence of Intravenous Injection of Living H. influenzae on Areas Inoculated Intradermally with H. influenzae and H. pertussis

Experiment 1.—Two rabbits, 3-85 and 3-86, were injected intradermally with 0.25 cc. each of a suspension of

(*a*) *H. pertussis* strain R 55 right upper quadrant
(*b*) " " " H 56 " lower "
(*c*) *H. influenzae* " " 158 left upper "
(*d*) " " " 2621 " lower "

24 hours later the reaction of the areas prepared with *H. pertussis* (*a*) and (*b*) read in both rabbits as follows: A central bluish spot is surrounded by a white zone which again is surrounded by bluish violet areas of indistinct outline. The skin areas prepared with *H. influenzae* (*c*) and (*d*) show a quite different picture. They display erythema, infiltration and swelling; the color is pinkish red. There is a small pustule in the center.

TABLE I

Production of Hemorrhagic Necrotic Lesions in Rabbit Skin Intradermally Injected with H. influenzae and H. pertussis by Means of Intravenous Reinjection of Living H. influenzae

Rabbit No.	Strain No.			
	R 55 *H. pertussis*	H 56 *H. pertussis*	158 *H. influenzae*	2621 *H. influenzae*
3-85	±	±	++++	++++
3-86	±	±	++++	+++

Read 5 hours after intravenous reinjection.
± = doubtful reaction.
+ to ++++ = different degrees of hemorrhagic necrotic lesions.

The rabbits were now injected intravenously with 1.5 cc. of a suspension of living *H. influenzae* (about 3 billion organisms per cc.). The results obtained are shown in Table I.

It can be seen from Table I that the areas prepared intradermally with *H. influenzae* were transformed into hemorrhagic necrotic lesions a few hours following the intravenous injection. The intensity of the reaction in the area prepared with strain 158 was somewhat stronger in rabbit 3-86 than that with strain 2621. The aspect of the areas prepared intradermally with *H. pertussis* was scarcely, or not at all, changed.

During the observation time of 24 hours, the bluish violet discoloration of the skin areas prepared intradermally with *H. pertussis* increased in size and intensity.

The intravenous injection of *H. influenzae* had, however, no apparent influence upon the natural development of this lesion as proved in control rabbits which were not reinjected intravenously. In those rabbits, areas prepared with *H. influenzae* did not show any sign of hemorrhagic necrosis.

Influence of Intravenous Injection of Living H. pertussis on Areas Inoculated Intradermally with H. influenzae and H. pertussis

Experiment 2.—Three series, consisting of four rabbits each, were examined in the same order of experiment as in Experiment 1. But, instead of reinjecting intravenously suspensions of *H. influenzae*, suspensions of *H. pertussis* were given. In two of these series no reaction occurred whatsoever. In a third series in which double the amount of organisms were reinjected intravenously, hemorrhagic necrotic changes occurred in two out of four rabbits. Here, also, areas prepared intracutaneously with *H. influenzae*, only, became positive. There was no reaction in the areas prepared intradermally with *H. pertussis*.

Effectiveness of Heat-Killed H. influenzae upon Skin Areas Previously Inoculated with Different Strains of H. influenzae

Experiment 3.—0.25 cc. of a suspension of three strains of *H. influenzae*, 3607, 3743 and 3878, were injected intracutaneously into three different areas of the abdominal skin of three rabbits (2-29 to 2-31). 24 hours later, 1.5 cc. of a suspension of *H. influenzae*, strain 3607, was injected intravenously. The bacterial suspension employed for the intravenous reinjection had been put in a water bath at 60°C. for 1 hour. The fact that the bacilli had been killed by this procedure was controlled by culture. The results obtained are given in Table II.

Table II shows that the intravenous injection of heat-killed *H. influenzae* may also produce hemorrhagic necrotic lesions in skin areas of rabbits previously prepared intracutaneously with living *H. influenzae*. At the same time it can be seen that different rabbit individuals vary in their reactions towards different strains of *H. influenzae* if injected simultaneously. It cannot yet be decided whether or not the homologous strain has a somewhat more powerful reactivating potency.

Production of Hemorrhagic Necrotic Lesions in Skin Areas Injected Intradermally with Living and Killed H. influenzae by Means of Intravenous Reinjection of Living H. influenzae

Experiment 4.—0.25 cc. of a suspension of *H. influenzae*, strain 158, (*a*) living, (*b*) killed—in water bath, 1 hour, 60°C.; strain 3607 (*c*) living, (*d*) killed—in water

TABLE II

Production of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with Different Strains of H. influenzae by Means of Intravenous Reinjection of Heat-Killed H. influenzae

Strain No.	Before intravenous injection			5 hrs. after intravenous injection			24 hrs. after intravenous injection		
	Rabbit No.			Rabbit No.			Rabbit No.		
	2-29	2-30	2-31	2-39	2-30	2-31	2-29	2-30	2-31
(*a*) 3607, *H. influenzae*	−	−	−	++	+++	++	+	++	++
(*b*) 3743, *H. influenzae*	−	−	−	−	±	++	−	±	++
(*c*) 3878, *H. influenzae*	−	−	−	±	++++	−	−	+++	−

− = no hemorrhagic necrotic lesion.
± = doubtful reaction.
+ to ++++ = different degrees of hemorrhagic necrotic lesions.

TABLE III

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Intradermally Prepared with Living and Killed H. Influenzae Following Intravenous Reinjection of a Suspension of Living H. influenzae

Strain No.	Before intravenous injection		3 hrs. after intravenous injection		5 hrs. after intravenous injection		24 hrs. after intravenous injection	
	Rabbit No.		Rabbit No.		Rabbit No.		Rabbit No.	
	2-39	2-40	2-39	2-40	2-39	2-40	2-39	2-40
158, *H. influenzae*								
(*a*) living	−	−	+++	+++	++++	++++	*	*
(*b*) dead	−	−	+	++	++++	++++	*	*
3607								
(*a*) living	−	−	+	++++	++++	++++	*	*
(*b*) dead	−	−	+++	+++	++++	++++	*	*

− = no hemorrhagic necrotic lesion.
+ to ++++ = different degrees of hemorrhagic necrotic lesions.
* Rabbit dead.

bath, 1 hour, 60°C., was injected intradermally into two rabbits (2-39 and 2-40). 24 hours later, 1.5 cc. of a suspension of living *H. influenzae*, strain 3607, was injected intravenously. The result of this experiment is given in Table III.

According to the experiment shown in Table III, the intravenous injection of living *H. influenzae* may induce hemorrhagic necrotic lesions in areas prepared 24 hours previously with living as well as with killed *H. influenzae.*

It may be added that the intravenous injection of heat-killed *H. influenzae* was able to elicit hemorrhagic necrotic lesions in areas prepared with killed *H. influenzae* also. We have the impression, however, that the intensity of the hemorrhagic lesions obtained under those conditions is weaker than that obtained with living bacilli.

Influence of Intravenous Injection of B. typhosus Agar Washing Filtrate on Skin Areas Prepared Intradermally with H. influenzae and H. pertussis

Experiment 5.—The following experiment was made to decide the question whether the intravenous injection of *B. typhosus* agar washing filtrate (Shwartzman toxin) can induce hemorrhagic necrotic lesions in the rabbit skin 24 hours previously prepared with *H. influenzae* and *H. pertussis.* For this purpose, 0.25 cc. of a suspension of living bacilli of

(*a*) *H. pertussis* strain R 55
(*b*) " " " H 56
(*c*) *H. influenzae* " 158
(*d*) " " " 2621 and
(*e*) *B. typhosus* agar washing filtrate diluted 1:2

were injected intracutaneously into the skin of rabbit 3-87. 24 hours later, 1 cc. of a 1:3 dilution of *B. typhosus* agar washing filtrate was injected intravenously. The result of this experiment is summarized in Table IV.

As can be seen from Table IV, characteristic hemorrhagic necrotic lesions occurred in the areas prepared with *H. influenzae* as well as in the areas prepared as a positive control with *B. typhosus* agar washing filtrate following the intravenous injection of the latter. The areas prepared with *H. pertussis,* however, did not display any essential changes during the corresponding observation period.

Out of nine rabbits examined in this order, five gave identical or similar results. Three rabbits proved to be refractory, and one rabbit died shortly after the intravenous injection of *B. typhosus* agar washing filtrate.

Nineteen rabbits were injected intradermally with *B. typhosus* agar washing filtrate as well as with *H. influenzae*, and reinjected intravenously with *B. typhosus* agar washing filtrate. Of these rabbits, nine showed positive reactions in both areas and four were entirely negative. Six rabbits were positive in one area only, three of them in the area prepared with *H. influenzae*, but negative in the area prepared with *B. typhosus* agar washing filtrate; and conversely, three rabbits were positive in the area prepared with *B. typhosus* agar washing filtrate but negative in the area prepared with *H. influenzae*.

TABLE IV

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas of Rabbit 3-87 Previously Inoculated with H. influenzae and H. pertussis Following the Intravenous Injection of B. typhosus Agar Washing Filtrate

Strain No.	Before intravenous injection	3 hrs. after intravenous injection	7 hrs. after intravenous injection	24 hrs. after intravenous injection
(*a*) R 55, *H. pertussis*	±	±	+	+
(*b*) H 56, *H. pertussis*	±	±	+	+
(*c*) 158, *H. influenzae*	−	+++	++++	++++
(*d*) 2621, *H. influenzae*	−	++	+++	+++
(*e*) 1:2 *B. typhosus* agar washing filtrate.	−	++	++	++

− = no hemorrhagic necrotic lesion.

± = doubtful reaction.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with H. influenzae, as Well as with B. typhosus Agar Washing Filtrate, Following the Intravenous Reinjection of Living H. influenzae

Experiment 6.—To determine the effectiveness of the intravenous reinjection of living *H. influenzae* on areas prepared with *H. influenzae* as well as *B. typhosus* agar washing filtrate was the object of this experiment. Nine rabbits received intradermal injections of 0.25 cc. of suspensions of two to three different strains of *H. influenzae* and one intradermal injection of *B. typhosus* agar washing filtrate into the skin of the abdominal wall. 24 hours later, suspensions of living *H. influenzae* were given intravenously. Six of these rabbits developed marked hemorrhagic necrosis in one or more areas prepared with *H. influenzae* as well as with *B. typhosus* agar washing filtrate. The reactions obtained were of about the same strength. In one rabbit the lesion of the skin area prepared with *B. typhosus* agar washing filtrate was distinctly weaker than the area prepared with *H. influenzae*; in a second rabbit, just the opposite took place. One rabbit was refractory.

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with H. influenzae, as Well as with B. typhosus Agar Washing Filtrate, Following the Intravenous Reinjection of Killed H. influenzae

Experiment 7.—Eight rabbits have been examined in the same order as the preceding ones, the only difference being that the intravenous injection was performed with heat-killed *H. influenzae* (1 hour, 60°C.), instead of living ones. All eight animals reinjected intravenously with heat-killed *H. influenzae* developed hemorrhagic necrotic lesions in the areas prepared intracutaneously 24 hours previously with *B. typhosus* agar washing filtrate. Hemorrhagic necrotic lesions in areas prepared with living *H. influenzae* appeared in six out of the eight animals used.

TABLE V

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with Suspensions of Living H. influenzae and Their Corresponding Wash Waters Following the Intravenous Reinjection of B. typhosus Agar Washing Filtrate

Rabbit No.	Suspension of living *H. influenzae*		First wash water	Third wash water	*B. typhosus* agar washing filtrate
	Not washed	Washed three times			
5-63	+	+	−	−	−
5-64	++	++	−	−	++
5-65	++++	++++	±	−	++

Read 5 hours after intravenous reinjection.

− = no hemorrhagic necrotic lesion.

± = doubtful reaction.

+ to ++++ = different degrees of hemorrhagic necrotic lesions.

Comparison of the Effectiveness of Suspensions of Living H. influenzae with Their Corresponding Wash Waters

Experiment 8.—This experiment was made to decide whether the hemorrhagic necrotic lesions caused by *H. influenzae* are due to an exotoxin. Four rabbits, 5-62 to 5-65, were injected with 0.25 cc. of

(*a*) Suspension of *H. influenzae* strain 3878 not washed
(*b*) " " " " " " washed 3 times
(*c*) First wash water of " " " " " "
(*d*) Third " " " " " " " " "

24 hours later the rabbits were reinjected with 1 cc. of *B. typhosus* agar washing filtrate in a dilution of 1:10. Rabbit 5-62 died soon after the intravenous reinjection. Table V shows the result in the remaining three rabbits.

It can be seen from Table V that the intravenous injection of *B. typhosus* agar washing filtrate induced hemorrhagic necrotic lesions in the areas prepared with the bacilli themselves, whether not washed at all or washed three times. In one rabbit, 5-65, a doubtful reaction developed in the skin area prepared with the first wash water, while the third wash water was entirely negative in all three rabbits. Berkefeld filtration was not applied in this experiment to avoid possible loss of reactivity through the procedure of filtration.

Comparison of the Effectiveness of Suspensions of Killed H. influenzae with Their Corresponding Wash Waters

Experiment 9.—This experiment was performed in the same order as the preceding one, but heat-killed suspensions of *H. influenzae* were used for the intradermal

TABLE VI

Appearance of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with Suspensions of Heat-Killed H. influenzae and the Corresponding Wash Waters Following the Intravenous Reinjection of B. typhosus Agar Washing Filtrate

Rabbit No.	Suspension of killed *H. influenzae*		First wash water	Third wash water
	Not washed	Washed three times		
7-40	++	+++	−	−
7-41	++++	++++	+	−

Read 5 hours after intravenous reinjection.
— = no hemorrhagic necrotic lesion.
+ to ++++ = different degrees of hemorrhagic necrotic lesions.

injection instead of living ones. Three rabbits, 7-40 to 7-42, were injected with 0.25 cc. of

(*a*) Suspension of *H. influenzae* strain 79 killed, not washed
(*b*) " " " " " " " washed three times
(*c*) First wash water of suspension of *H. influenzae* strain 79 killed
(*d*) Third " " " " " " " " " " "

24 hours later the rabbits were reinjected with 1 cc. of *B. typhosus* agar washing filtrate in a dilution of 1:10. Rabbit 7-42 died soon after this injection. The result in the remaining two rabbits is given in Table VI.

Hemorrhagic necrotic lesions occurred in both rabbits in the areas prepared with washed, as well as with unwashed bacilli. The wash waters proved to be completely ineffective in one rabbit, while the other showed a one plus reaction with the first wash water.

Examination of a Suspension of H. influenzae Cultured in Kolle Flasks and Its Corresponding Agar Washing Filtrate Produced According to the Technic of Shwartzman

Experiment 10.—Since Shwartzman and Frisch (4) obtained an effective agar washing filtrate from *H. influenzae*, Shwartzman's technic has been employed to compare their results with those obtained with the technic used in our experiments reported thus far. For this purpose, *H. influenzae* has been cultivated in Kolle flasks, and bacilli and wash waters examined. Three rabbits, 5-51 to 5-53, were injected intradermally with 0.25 cc. of

(*a*) Suspension of *H. influenzae* strain 3878
(*b*) First wash water of " " " " " "
(*c*) Berkefeld filtrate of (*b*) = *H. influenzae* Shwartzman toxin

24 hours later, the rabbits were reinjected intravenously with *H. influenzae* Berkefeld filtrate. Table VII shows the result of this experiment.

TABLE VII

Hemorrhagic Necrotic Lesions in Areas Prepared Intradermally with a Suspension of H. influenzae and the Corresponding Agar Washing Filtrate Following the Intravenous Reinjection of the Latter

Rabbit No.	Suspension of *H. influenzae*	First wash water	Berkefeld filtrate of first wash water
5-51	±	++++	++
5-52	—	—	—
5-53	—	—	—

Read 5 hours after intravenous reinjection.
— = no hemorrhagic necrotic lesion.
± = doubtful reaction.
+ to ++++ = different degrees of hemorrhagic necrotic lesions.

As can be seen from Table VII, an effective agar washing filtrate was obtained from *H. influenzae* grown in Kolle flasks. The intravenous reinjection of this filtrate induced hemorrhagic necrosis in one rabbit in the area prepared with both first wash water and its Berkefeld filtrate, while the area prepared with the bacilli gave only a doubtful reaction. The reaction in the area prepared with *H. influenzae* Berkefeld filtrate is somewhat weaker than that in the area prepared with the first wash water, probably due to a loss of reactivity through the process of filtration.

Comparison of the Influence of the Technic Employed in Cultivation of H. influenzae on the Occurrence of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with Suspensions of the Bacilli and Their Corresponding Berkefeld Filtrates

Experiment 11.—This experiment deals with the comparative study of *H. influenzae* when cultured on slants (inoculated with platinum loop) and in Kolle flasks (inoculated with several cubic centimeters of suspensions of *H. influenzae* in saline). Six different areas of the abdominal skin of rabbits 6-08 to 6-13 were prepared intradermally with 0.25 cc. of

(*a*) Suspension of *H. influenzae* strain 385
(*b*) Berkefeld filtrate of " " " " " "
(*c*) " " from third washing " " " "
} Obtained from Kolle flasks

(*d*) Suspension of *H. influenzae* " "
(*e*) Berkefeld filtrate of " " " " " "
(*f*) *B. typhosus* agar washing filtrate
} Obtained from agar slants

24 hours later they were divided into two series of three rabbits each. The first series was reinjected intravenously with suspensions of *H. influenzae* grown in Kolle flasks and washed three times; the second series with the first Berkefeld filtrate (*b*). The result of this experiment is summarized in Table VIII.

TABLE VIII

Comparison of the Influence of the Technic Employed in Cultivation, on the Occurrence of Hemorrhagic Necrotic Lesions in Skin Areas Prepared with H. Influenzae and Their Corresponding Berkefeld Filtrates, Following the Intravenous Reinjection of Suspensions of H. Influenzae and Their Berkefeld Filtrates

Rabbit No.	From Kolle flasks			From slants		*B. typhosus* agar washing filtrate
	Suspension of *H. influenzae*	Berkefeld filtrate of first wash water	Berkefeld filtrate of third wash water	Suspension of *H. influenzae*	Berkefeld filtrate of first wash water	
6-08	++	+++	±	+	±	++++
6-09	+++	+	−	++	−	++++
6-10	+++	+++	−	+++	−	++++
6-11	±	−	−	±	−	+++
6-12	−	−	−	−	−	+++
6-13	−	−	−	−	−	−

Read 5 hours after intravenous reinjection.
− = no hemorrhagic necrotic lesion.
± = doubtful reaction.
+ to ++++ = different degrees of hemorrhagic necrotic lesions.

Table VIII shows that all three rabbits reinjected with suspensions of *H. influenzae* displayed hemorrhagic necrotic lesions in the areas prepared with *H. influenzae* from Kolle flasks as well as from agar slants. The Berkefeld filtrate prepared from growths in Kolle flasks (*b*) also gave a positive reaction, in contrast to the Berkefeld filtrate prepared from *H. influenzae* grown on agar slants (*e*). The Berkefeld filtrate prepared from the third washing gave a doubtful reaction in one rabbit only. Nevertheless, the washed bacilli, if injected intravenously, were able to produce hemorrhagic necrotic lesions. The three rabbits of the second series, reinjected with *H. influenzae* Berkefeld filtrate containing skin-sensitizing properties, did not induce any positive reactions in this experiment, except in the control areas prepared with *B. typhosus* agar washing filtrate. One rabbit showed a doubtful reaction in the area prepared with the bacilli themselves.

DISCUSSION

The experiments reported in this paper point to further differences in the skin biological behavior of *H. influenzae* and *H. pertussis*. They show that, under certain experimental conditions, it is possible to induce severe hemorrhagic necrotic lesions in the rabbit skin by means of *H. influenzae*. Preliminary experiments seem to prove the significance of the reported order of experiment for studies on involvement of the lung, if the first injection of *H. influenzae* is applied intranasally instead of intradermally. Shope demonstrated the rôle of *H. influenzae* in the pathogenesis of swine influenzae particularly. He showed that *H. influenzae* is able to activate the "virus disease" in swine. Concerning the production of hemorrhagic necrotic lesions in the skin of rabbits, the experiments of Shwartzman and his coworkers (5, 6) are of special interest. According to these authors, it is possible to obtain effective exotoxins from a large number of microorganisms, including *H. influenzae* and *H. pertussis*. We, too, could obtain a moderately effective exotoxin from *H. influenzae*, provided Shwartzman's method of cultivation was used. As far as it is possible to draw any conclusions from the experiments reported, it seems to be unlikely that exotoxins are a decisive factor in the pathogenesis of hemorrhagic necrotic lesions caused by *H. influenzae* under the given experimental conditions. This statement is upheld by the ability of

washed living and heat-killed *H. influenzae* to exhibit reactions, and the lack of this ability of the supernatant fluids and wash waters. The special attention given to the production of exotoxins and their undoubted importance may be the reason why factors have been overlooked so far, which may play an important rôle in the course of bacterial infections as such.

CONCLUSIONS

1. The intradermal injection of *H. influenzae* in the abdominal wall of rabbits induces inflammation, frequently combined with a central pustule. The corresponding injection of *H. pertussis* causes a bluish violet discoloration of the skin area involved which undergoes slight hemorrhagic necrotic changes within a few days.

2. The intravenous injection of living *H. influenzae*, 24 hours after the intradermal inoculation with living *H. influenzae*, is able to transform the respective skin areas into severe hemorrhagic necrotic lesions within 3 to 5 hours.

3. Heat-killed *H. influenzae*, if injected intravenously, may produce hemorrhagic-necrotic lesions in areas previously prepared with living or heat-killed *H. influenzae*.

4. *H. pertussis*, if injected intravenously, may cause, perhaps to a lesser extent, hemorrhagic necrotic lesions in skin areas 24 hours previously injected with *H. influenzae*.

5. The normal course of the infection of rabbit skin with *H. pertussis* is not, or not essentially, influenced by intravenous reinjection of living or killed *H. influenzae* or *H. pertussis*.

6. The agar washing filtrate of *B. typhosus*, if injected intravenously, can produce hemorrhagic necrotic lesions in rabbit skin prepared intracutaneously with living as well as with heat-killed *H. influenzae*. The intravenous injection of *B. typhosus* agar washing filtrate has no influence on areas prepared with *H. pertussis*.

7. Conversely, *H. influenzae* as well as *H. pertussis*, if injected intravenously, are able to produce hemorrhagic necrotic lesions in rabbit skin prepared 24 hours previously with *B. typhosus* agar washing filtrate.

8. The effectiveness of suspensions of *H. influenzae* apparently is confined to the bacteria themselves rather than to the supernatant

fluids. This does not exclude the possibility of producing effective exotoxins under special experimental conditions.

BIBLIOGRAPHY

1. Kasahara, M., *Klin. Woch.*, 1933, **2,** 1609.
2. Gundel, M., and Schlueter, W., *Zentr. Bact., 2. Abt., Orig.*, 1933, **129,** 461.
3. Shwartzman, G., *Proc. Soc. Exp. Biol. and Med.*, 1929, **26,** 843; *J. Infect. Dis.*, 1929, **45,** 232.
4. Shwartzman, G., *Klin. Woch.*, 1930, **41,** 1925.
5. Shwartzman, G., *J. Exp. Med.*, 1930, **51,** 571.
6. Koplik, L. H., *Proc. Soc. Exp. Biol. and Med.*, 1934, **32,** 309.
7. Witebsky, E., and Salm, H., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34,** 351.