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IMMUNITY STUDIES OF ROCKY MOUNTAIN SPOTTED FEVER.

II. Prophylactic Inoculation in Animals.

By HIDEYO NOGUCHI, M.D.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

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Of the many urgent and interesting problems regarding Rocky Mountain spotted fever, that of personal prophylaxis seems to be the most important. The experiments reported here were carried out in an attempt to devise a practical method by which a non-immune population might be rendered immune to the bites of infected ticks.

Ricketts and Gomez[1] early recognized the possibility of developing a satisfactory method of preventive inoculation, and carried out a few experiments in this connection but no adequate quantitative experimental analysis of the factors concerned in the production of artificial immunity. Since we have no pure culture of the etiological agent and cannot recognize it microscopically, we must rely solely upon animal inoculations for quantitative as well as qualitative estimation of the presence of the spotted fever organism. Since the number of causative organisms must bear a definite relationship to the amount of antibodies or the degree of immunity which is produced, a knowledge of the number and virulence of the organisms used is a prerequisite to an understanding of the phenomena of immunization. For example, 1 cc. of the citrated blood of guinea pigs with all the symptoms of spotted fever may contain only 1 or as many as 1,000 minimum lethal doses, but this fact can be ascertained only by careful titration of the material by animal inoculations, and unless the number of minimum lethal doses is determined in each instance, the immunization experiments carried out become meaningless or even misleading. Moreover, since the virus dies out in citrated or defibrinated blood

[1] Ricketts, H. T., and Gomez, L., Studies on immunity in Rocky Mountain spotted fever, *J. Infect. Dis.*, 1908, v, 221.

or serum, even at 4°C., it must be titrated simultaneously with the vaccination or subsequent immunity tests in which it is being used.

The selection of experimental animals is another important factor, and since our aim is to develop methods of protection for the human being, we must choose an animal in which the morbidity and mortality with respect to spotted fever infection approach as closely as possible those of man. The guinea pig excels other animals in this respect. The rabbit is far less susceptible than the guinea pig, and monkeys (*Macacus rhesus*) are extremely susceptible, those of a series of eighteen having shown 100 per cent morbidity and mortality. The age and size of the animal are very important. Full grown males, weighing 500 to 600 gm., are to be recommended; first, because of their comparatively greater resistance to banal laboratory infections and their undiminished and remarkably constant susceptibility to the spotted fever infection, and second, because the characteristic genital lesions are best observed in male animals. The consideration of such factors as just outlined is absolutely essential for formulating a practicable method of prophylactic vaccination.

The ordinary immunological procedures, known to be effective in other diseases, have been subjected to experimental determination of their possible application in the case of spotted fever: (1) the effect of injection of non-infective quantities of live spotted fever virus; (2) the effect of injection of killed virus; (3) the effect of injection of dead virus; (4) the effect of injection of freshly prepared neutral or superneutral mixtures of the live virus with immune serum; (5) the effect of heating and of age upon the immunizing properties of neutral mixtures of the virus with immune serum.

The two strains of spotted fever virus used in the present series of experiments were obtained through the kind cooperation of Dr. R. R. Parker, and we continue to use his designation of them as "Cooper" and "Barlow." The guinea pigs were almost always adult males weighing 500 to 600 gm. The virus was used in the form of citrated blood (equal parts of blood and citrate solution), usually drawn from the heart of the infected guinea pig on the 4th to 6th day of fever, preferably when the scrotal lesions became noticeable. Blood drawn on the 2nd or 3rd day of fever contains, in my experience, far less of the virus. Every specimen of blood was tested for bacterial contami-

nation on ordinary broth and slant agar and was titrated simultaneously with each experiment to determine the number of killing doses which it contained. All the injections of virus, or mixtures containing virus, were made intraperitoneally. The temperatures of the animals were taken every morning during the period of observation; that is, until death or recovery, or, in case no infection developed, for at least 2 weeks. The immune serums used in this work were chiefly from

TABLE I.

Effect of Non-Infectious Quantities of Virus.

Date of inoculation.	Material.	Amount.	Result.	Immunity test.
1922		*cc.*		
May 10.	Citrated guinea pig blood, Cooper strain (1 M.L.D. = 0.01 cc.).	0.001	No reaction.	June 13, 1922 (33 days), Cooper strain 1 cc. (1,000 M.L.D.); typical infection but recovered.
		0.0001	" "	Similarly tested; typical infection; death in 11 days.
		0.00001	" "	Similarly tested; typical infection; death in 11 days.
Jan. 25.	Citrated guinea pig blood, Barlow strain (1 M.L.D. = 1 cc.).	0.1	" "	Feb. 17, 1922 (23 days), Barlow virus 0.5 cc. (5 M.L.D.); typical infection; death in 10 days.
		0.01	" "	Similarly tested; typical infection; death in 19 days.

rabbits which had passed through a typical but non-fatal infection and had been bled 2 to 3 weeks after the temperature returned to normal.[2] Scrotal lesions were frequently observed in the infected rabbits. In some instances the immune serums came from rabbits which had received a second dose of virus (4 cc. citrated blood of the guinea pig) during convalescence; these serums are designated as "reinforced," and their titers are somewhat higher than those of the convalescent serums. All the immune serums were kept in the refrigerator at 4°C. except at time of use.

[2] Noguchi, H., Immunity studies of Rocky Mountain spotted fever. I. Usefulness of immune serum in suppressing an impending infection, *J. Exp. Med.*, 1923, xxxvii, 383.

TABLE II.

Effect of Injection of Killed Virus.

Date of inoculation, December 8, 1922.

Material.	Killed by.	Amount.	Result of inoculation.	Result of immunity test, 28 days later, against Barlow virus 0.1 cc. (1 M. L. D.).
		cc.		
Citrated guinea pig blood (Barlow + Cooper strain),* 100 M.L.D. in 1 cc.	Chloroform.	1	No reaction.	Typical febrile reaction; recovery.
		0.3	" "	Severe infection with typical scrotal lesions; recovery.
Same.	Ether.	1	" "	Severe infection with typical lesions; recovery.
		0.3	" "	Typical infection; died in 9 days.
		0.1	" "	Severe infection; necrotic skin and ear lesions; recovery.
"	Xylene.	1	Slight fever.	Severe infecton with necrotic lesions of scrotum and ear lobes; recovery.
		0.3	No reaction.	Typical severe infection; recovery.
		0.1	Slight fever.	Severe infection; died in 19 days.
"	Heating to 56°C. for 10 minutes.	0.75	No reaction.	Typical severe infection; recovery.
		0.3	" "	Typical infection; died in 14 days.
		0.1	Irregular fever (secondary infection?).	Typical infection.
Same virus, 1 cc. + immune serum, 1 cc.		1	No reaction.	No infection.
Same virus, 1 cc. + immune serum, 1 cc.		0.3	" "	Slight fever; no symptoms of spotted fever.
Same virus, 1 cc. + immune serum, 1 cc.		0.1	" "	Mild fever; no lesions; recovery.

Titration of virus used for testing immunity. January 5, 1923.

Barlow virus (guinea pig citrated blood).	Result of inoculation.
cc.	
1	Typical infection; killed for virus in 5 days.
0.1	" " died in 14 days.
0.01	Suspicious fever; recovery.
0.001	" " "

* Kept in refrigerator for 20 hours.

Effect of Non-Infective Quantities of Live Spotted Fever Virus.

That guinea pigs which survive a non-fatal but typical spotted fever infection resist subsequent infection has long been known, but whether or not animals receiving too small a quantity of the virus to become infected would acquire increased resistance remained to be determined. Ricketts and Gomez published some observations on this point,[1] stating that, although they had made no systematic attempt to determine the vaccinating properties of minute doses of virus, they had reason to believe that prophylactic vaccination could not be based on this principle. The present experiment shows (Table I) that no appreciable immunity develops under these conditions. Three guinea pigs received non-infective doses of one strain and two of another, and all succumbed to subsequent inoculation 33 and 23 days later, respectively.

Effect of Injection of Virus Killed Either by Chemicals or by Heating.

Chloroform, ether, and xylene were allowed to act upon the virus by shaking with it for 5 minutes. After the mixtures had been in the refrigerator (4°C.) for 20 hours, the clear portion in each case was used for inoculation. Guinea pigs were given the sterilized virus in doses of 1, 0.3, and 0.1 cc., and the animals were tested for immunity 28 days later by inoculation of a single minimum lethal dose of Barlow strain (0.1 cc.). There was no evidence of any increased resistance to the infection.

Another portion of the same virus was heated to 56°C. for 10 minutes in a water bath. Three guinea pigs were then inoculated with 0.75, 0.3, and 0.1 cc., respectively. No infection followed, and 28 days later each animal was given one minimum lethal dose of the virus (Barlow strain, 0.1 cc.). All had severe and typical infections, one dying in 14 days (Table II).

The failure of the animals to derive any immunity from the introduction of the killed virus was apparently due to modification of the immunizing substances by the chemical or physical agents, since the same quantities of virus (100, 30, and 10 M.L.D.) when mixed with immune rabbit serum, proved ample, as will be shown later, to render animals immune to the inoculation of the same test material.

Effect of Injection of Dead Virus.

Although the foregoing experiment had shown that the virus could not be killed without impairment of its immunizing properties, the question remained whether it might not retain its power to confer immunity if allowed to die out under such unfavorable conditions as, for example, low temperature for a long period. Hence several animals, which had been inoculated with specimens of virus kept in

TABLE III.

Effect of Injection of Dead Virus.

Strain.	Kept at 4°C. for a period of.	Amount.	Result of inoculation.	Result of immunity test, 40 days after inoculation, against Barlow virus 1 cc. (100 M. L. D.).
	days	*cc.*		
Cooper Guinea pig 11	69	2	No reaction; died of secondary infection in 9 days.	
Same	69	4 (of 1:6 solution in citrate).	No reaction.	Typical infection; died in 16 days.
Cooper Guinea pig 19	57	2	" "	Typical infection; died in 12 days.
Cooper Guinea pig 30	31	2	Typical infection; killed for material.	
Cooper Guinea pig 31	28	2	No reaction.	Typical infection; finally recovered.
Cooper Guinea pig 47	21	1	" "	Typical infection; died in 15 days.
Barlow Guinea pig 24	38	2	" "	Typical infection; died in 12 days.

the refrigerator (4°C.) for periods of 28 to 69 days and had had no infection, were tested 40 days later for immunity by the injection of 100 M.L.D. of virus (Barlow strain, 1 cc.). The animals all developed typical and, in most instances, fatal infections (Table III. Compare also Table IX, under the heading "Controls (heated virus alone).").

TABLE IV.

Effect of Injection of Neutral or Subneutral Mixtures of Virus and Immune Serum.

May 10, 1922. Virus: citrated guinea pig blood (Cooper strain), 1 cc. (100 M.L.D.).
Immune serum: rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.			
11	0.1	Mild fever; recovery.	33 days.	Complete against 1,000 M.L.D.
			10 mos.	" " 30 "
	0.01	" " "	33 days.	" " 1,000 "
23	0.1	" " "	33 "	" " 1,000 "
	0.01	Typical infection; recovery.	33 "	" " 1,000 "
			8 mos.	" " 1 "
			9 "	" " 5 "
25	0.1	Mild fever; recovery.	33 days.	" " 1,000 "
	0.01	Moderate fever; recovery.		
186	0.1	Mild fever; recovery.	10 mos.	" " 30 "
	0.01	Moderate fever; recovery.		
307	0.1	Mild fever; recovery.	10 mos.	" " 30 "
	0.01	Typical infection; recovery.		
314	0.1	Mild fever; recovery.	3 mos.	" " 1,000 "
	0.01	Moderate fever; recovery.	3 "	" " 1,000 "
316	0.1	Mild fever; recovery.	8 "	" " 1 "
	0.01	Moderate fever; recovery.		
326	0.1	Mild fever; recovery.	6 mos.	" " 100 "
	0.01	Typical infection; recovery.		
327	0.1	No reaction.	9 mos.	Complete against 5 M.L.D.
(reinforced).	0.01	Moderately severe infection.		

330 (reinforced).	0.1	No reaction.	33 days.	Complete against 1,000 M.L.D.
			10 mos.	" " 30 "
	0.01	Mild fever; died of intercurrent infection.		
341 (reinforced).	0.1	No reaction.	33 days	" " 1,000 "
			10 mos.	" " 30 "
	0.01	Moderate fever; recovery.		
350 (reinforced).	0.1	No reaction.	33 days.	" " 1,000 "
			10 mos.	" " 30 "
	0.01	Moderate fever; recovery.		

Titration of virus used for testing immunity. June 13, 1922.

Cooper strain virus.	Result of inoculation.
cc.	
0.1	Typical infection; death in 9 days.
0.01	" " " " 13 "
0.001	" " " " 11 "
0.0001	Mild fever; recovery.

Effect of Injection of Freshly Prepared Neutral or Superneutral Mixtures of Live Virus with Immune Serum.

It had been noticed early in these studies of immunity in spotted fever that guinea pigs which had received suitable mixtures of virus and immune serum remained well throughout several weeks of observation. The mixtures were innocuous, irrespective of the amount of virus introduced, provided the amount of immune serum were correspondingly adjusted. For example, 1 cc. of virus containing 100 or 1,000 M.L.D. might be injected without causing any infection when mixed with 0.1 cc. or more of immune serum, and animals so inoculated were found to be completely refractory to subsequent inoculation with as many as 100 or 1,000 M.L.D. The degree of immunity acquired was altogether comparable with that of animals which had received a subneutral mixture and had passed through a mild course of fever or a moderately severe infection with scrotal lesions. The immunity produced by subneutral mixtures is apparently the same as that following convalescence in animals which survive a typical severe infection caused by virus alone.

Table IV shows the neutralizing power of the serum of eight convalescent rabbits and of four hyperimmunized immune rabbits (*i.e.* which had received a second injection of virus during convalescence) in 0.1 and 0.01 cc. against 100 M.L.D. (Cooper strain, 1 cc.) and the degree of immunity developed in the guinea pigs. 0.1 cc. of the convalescent serum was an almost, but not completely, neutralizing dose, while the same quantity of the reinforced immune serums was sufficient to neutralize all infectivity. The guinea pigs were tested for immunity on several different occasions and with virus of various degrees of virulence. Of the eight animals tested 33 days later with 1,000 M.L.D., none became infected, and no change in temperature was observed. Two tested 3 months afterwards against 1,000 M.L.D. resisted infection. One animal was tested after 6 months with 100 M.L.D. and proved resistant. Several others were tested after longer periods—8, 9, and 10 months—against 1, 5, and 30 M.L.D. of virus and likewise proved immune.

Superneutral Mixtures.—In the case of human vaccination the neutral mixtures would leave too narrow a margin between the

neutral and the subneutral, and no margin can be too great to insure safety for the persons vaccinated. Since, however, a great excess of the immune serum in a mixture might reduce or even nullify the immunizing properties of the virus, it was necessary to determine this point. Hence a series of experiments was carried out in which a

TABLE V.

Effect of Injection of Superneutral Mixtures of Virus and Immune Serum.

May 25, 1922. Virus: citrated guinea pig blood (mixed Cooper and Barlow strains), 1 cc. (100 M.L.D.). Immune serum: rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.			
11	1	Mild fever; recovery.	7 mos.	Complete against 1 M.L.D.
23	1	No reaction.	7 "	" " 1 "
25	1	" "	18 days.	Died of intercurrent infection; no lesions of spotted fever after injection of 1,000 M.L.D. virus.
186	1	" "	1 mo.	Complete against 100 M.L.D.
			4 mos.	" " 1,000 "
307	1	" "	1 mo.	" " 100 "
314	1	" "	4 mos.	" " 1,000 "
316	1	" "	4 "	" " 1,000 "
326	1	" "	4 "	" " 1,000 "
327 329	1	" "	Reserved for later test.	
330	1	" "	4 mos.	Complete against 1,000 M.L.D.
			7 "	" " 1 "
			7½ "	" " 5 "
332	1	" "	7½ "	" " 5 "
349	1	" "	7½ "	" " 5 "
350	1	" "	Reserved for later test.	

considerable excess of the serum was assured by using 1 cc. against 100 M.L.D. of virus. Fourteen different samples of immune serum, twelve of which been used also in the previous series of experiments, were used (Table V), all but one of which (No. 11) completely neutralized the virus. The animals were tested for immunity after 1, 4, 7, and 7½ months, respectively, and found to be completely

TABLE VI.

Effect of Various Quantities of Immune Serum on the Immunizing Power of the Virus.

June 24, 1922. Virus: citrated guinea pig blood (Barlow strain), 1 cc. (100 M.L.D. or 1,000 M.I.D.).
Immune serum: rabbit.

Immune serum No.	Amount.	Vaccination reaction.	Immunity test.	
			Interval after vaccination.	Degree of protection.
	cc.		*mos.*	
23	2	No reaction.	5½	Complete against 100 M.L.D.
			6	" " 1 "
			6½	" " 5 "
	2	" "	3	" " 1,000 "
	1	" "	3	" " 1,000 "
			7	" " 1 "
			7½	" " 5 "
	1	" "	3	" " 1,000 "
			7	" " 1 "
			7½	" " 5 "
	0.5	" "	7	" " 1 "
			7½	" " 5 "
	0.5	" "	Lost by intercurrent infection after 19 days.	
	0.25	Slight fever; recovery.	Reserved for later test.	
	0.25	Definite febrile reaction; recovery.	3	Complete against 1,000 M.L.D.
	0.1	Mild fever; recovery.	3	" " 1,000 "
	0.1	Severe fever; no scrotal lesions; recovery.	Reserved for later test.	
25	2	No reaction.	3	Complete against 1,000 M.L.D.
	2	" "	Reserved for later test.	
	1	" "	3	Complete against 1,000 M.L.D.
	1	" "	5½	" " 100 "

0.25	Some irregular rises of temperature, no spotted fever symptoms; recovery.	7½	Complete against	5 M.L.D.
0.25	No spotted fever symptoms.	Died of intercurrent infection in 23 days.		
0.1	Mild fever; recovery.	7	Complete against	1 M.L.D.
0.1	" " "	Reserved for later test.		

Titration of mixed virus (Cooper-Barlow) used for testing immunity. June 24, 1922.

Citrated guinea pig blood.	Results of inoculations.
cc.	
1	Typical severe infection with very marked scrotal lesions; died in 9 days.
0.1	Typical severe infection with very marked scrotal lesions; died in 10 days.
0.01	Typical severe infection with very marked scrotal lesions; died in 16 days.
0.001	Severe infection with extensive necrosis of scrotal skin, soles, and ear lobes; recovered.
0.0001	No infection.

resistant to infection with virus containing in some instances as many as 1,000 minimum lethal doses.

The results showed that the immune serum may be added to the virus in great excess without unfavorably influencing the development of immunity.

TABLE VII.

Duration of Passive Immunity.

June 24, 1922.

Immune serum (rabbit) No.	Amount.	Result.	Immunity test.
	cc.		
23	1	No reaction.	Tested 12 days later against Cooper virus 1 cc.;* typical infection; died in 9 days.
25	1	" "	Tested 12 days later against Cooper virus, 1 cc.;* mild infection; recovery.
			Tested 2 months later against Cooper virus, 1 cc. (1,000 M.L.D.); no infection.
	1	" "	Tested 2 months later against Cooper virus, 1 cc. (1,000 M.L.D.); typical severe infection; died in 11 days.

Titration of Cooper strain virus used for immunity tests. September 25, 1922.

Guinea pig citrate blood.	Result of inoculation.
cc.	
0.5	Severe infection with scrotal lesions; killed for virus in 10 days.
0.1	Severe infection with scrotal lesions; killed for virus in 10 days.
0.01	Severe infection; died in 13 days.
0.001	" " " " 13 "
0.0001	No infection.

* Control died in 14 days.

To confirm and supplement the above experiments, another series of animals was inoculated with mixtures containing the same amount of virus with varying amounts of immune serum. Two immune serums were separately used, and for each dose two animals were inoculated. 2, 1, 0.5, 0.25, and 0.1 cc. of serum, respectively, were mixed with 1 cc. of virus containing 100 minimum lethal doses or about 1,000 minimum infecting doses. No reaction followed the inoculation in the

animals which received the mixture containing 0.5 cc. or more of serum, but animals which received 0.25 cc. or less all had some degree of febrile reaction, although no scrotal lesions were noted. Irrespective of whether the quantity of serum was subneutral, neutral, or superneutral, all the animals proved to be equally resistant to several subsequent attempts to infect them after intervals of 3 months (with 1,000 M.L.D. of virus), 5, 5½, 6, and 7 months (with 1 to 5 M.L.D.

TABLE VIII.

Protective Power of the Blood of Vaccinated Guinea Pig.

Date of vaccination, May 25, 1922.

Material: virus (Cooper and Barlow strains mixed), 1 cc. + immune rabbit serum, 1 cc.

This animal belongs to the series reported in Table V.

Interval after vaccination.	Test of blood for protective power of citrate plasma.*	Result of inoculation.
days		
30	1 cc. + virus, 0.1 cc. (10 M.L.D. or 100 M.I.D.)	Definite febrile reaction; recovered.
	0.2 cc. + same virus, 0.1 cc.	Typical severe infection, with scrotal and ear lesions; recovered.
	0.1 " + " " 0.1 "	Typical severe infection; died in 10 days.

* 10 cc. heart blood were mixed with 10 cc. citrate solution; plasma collected after centrifugation.

Of the fourteen other guinea pigs vaccinated on the same day with the same material, two were tested after the same interval (30 days) by injection of virus; both resisted infection with 100 M.L.D. of the same virus as used in the above experiment. The remaining twelve animals were tested at intervals of 4 to 7½ months with 1 to 1,000 M.L.D. of virus, and all resisted infection (see Table V).

of virus) (Table VI). It is evident, therefore, that the quantity of immune serum may be liberally increased if the mixture is to be used for vaccination.

The question arose whether the immunity which developed after the inoculations of neutral or superneutral mixtures might not be merely a passive immunity, due to the introduction of the immune serum. This point was decided by the experiment recorded in Table VII which showed that the inoculation of 1 cc. of a powerful

immune serum does not protect a guinea pig from subsequent infection 12 days or later; hence the immunity observed in guinea pigs 2 weeks or longer after the inoculation of neutral or superneutral mixtures cannot be ascribed to a passive immunity brought about by the antiserum introduced with the virus.

Protective Power of the Blood of Vaccinated Guinea Pigs.

From the theoretical point of view it was interesting to determine the neutralizing power of the blood of a guinea pig which had previously been inoculated with a neutral mixture of virus (Cooper-Barlow strains, 1 cc.) and immune serum (No. 28, 1 cc.). Blood was withdrawn from the heart 30 days after the time of vaccination. The citrated plasma was separated, and 1, 0.2, and 0.1 cc. were tested against 10 M.L.D. of the virus (Cooper strain, 0.1 cc.). The largest quantity, 1 cc., failed to neutralize the virus completely, but the animal recovered, while 0.2 and 0.1 cc. had no protective effect (Table VIII).

Fourteen other guinea pigs, vaccinated at the same time, and tested after the same period by injection of 100 M.L.D. of virus, all resisted the attempt to infect them. Hence the vaccinated guinea pigs are able to neutralize a far greater quantity of virus *in vivo* than the low neutralizing titer of their blood would lead us to expect.

Effect of Heating and of Age upon the Immunizing Properties of Neutral Mixtures of Virus and Immune Serum.

The strikingly effective protective value of a neutral or superneutral mixture of virus and immune serum against experimental spotted fever in guinea pigs suggested the possibility of applying the principle to human beings. The prospect of sero-vaccination, under the most rigid quantitative supervision of the virus and immune serum, is promising; nevertheless, it seems important, for the time being at least, to devise some method in which the virus in the mixture is killed, but which still gives a vaccine of definite protective value.

In Tables IX and X are recorded the results of experiments in which guinea pigs were inoculated with neutral or superneutral mixtures of virus and immune serum which had either been heated to 60°C. for

20 minutes in a water bath or preserved in the refrigerator (4°C.) for 32 days. One set of animals was inoculated with portions of the

TABLE IX.

Effect of Heating upon the Neutral Mixtures of Virus and Immune Serum.

October 5, 1922. Virus: citrated guinea pig blood, Cooper and Barlow strains (1 cc. contained 100 M.L.D.), 1 part. Immune serum: mixture of serum from several immune rabbits, 1 part.

Amount.	Result of vaccination.	Immunity test.		
		Interval after vaccination.	Tested against.	Degree of protection.
		Unheated.		
cc.				
2	No reaction.	18 days.	10,000 M.L.D.	Complete.
2	" "	18 "	10,000 "	"
2	" "	2 mos.	100 "	Died of secondary infection.
Controls (normal rabbit serum + virus).				
2	Typical infection; died in 11 days.			
2	Typical infection; died in 11 days.			
		Heated to 60° C. for 20 minutes.		
2	No reaction.	18 days.	10,000 M.L.D.	Mild fever; recovery.
2	" "	18 "	10,000 "	" " "
2	" "	2 mos.	100 "	Died of secondary infection.
		Controls (heated virus alone).		
2	No reaction.	18 days.	10,000 M.L.D.	Typical infection; died in 15 days.
1	" "	18 "	10,000 "	Typical infection; died in 12 days.
1	" "	2 mos.	100 "	Typical infection; died in 13 days.

freshly prepared mixture; another set received a portion of the same mixture, which had been previously heated; the remainder of the mixture was placed in the refrigerator and inoculated 32 days later.

Suitable controls (virus mixed with normal rabbit serum, and virus alone) accompanied each of the three sets of experiments.

TABLE X.

Effect of Age upon the Neutral Mixtures of Virus and Immune Serum.

Portions of the mixtures of virus and immune serum used on Oct. 5, 1922 (Table IX) were kept in the refrigerator at 4°C. until Nov. 6, 1922 (32 days).

Amount.	Result of vaccination.	Immunity test.		
		Interval after vaccination.	Tested against.	Degree of protection.
cc.				
2	No reaction.	16 days.	100 M.L.D.	Typical infection; died in 15 days.
2	" "	1 mo.	100 "	Irregular fever (secondary infection).
1	" "	16 days.	100 "	Suspicious febrile reaction but no lesions; died in 22 days of secondary infection.
1	" "	5 mos.	30 "	No infection.
	Controls (normal rabbit serum + virus).			
2	Secondary infection.			
1	No reaction.	16 days.	100 M.L.D.	Typical infection; died in 13 days.
	Controls (virus alone).			
2	No reaction.	1 mo.	100 M.L.D.	Typical infection; died in 19 days.
2	" "	16 days.	100 "	Typical infection; died in 14 days.
2	Suspicious febrile reaction; died in 16 days; spleen enlarged.			
1	No reaction.	1 mo.	100 "	Typical infection; died in 15 days.
1	" "	16 days.	100 "	Typical infection; died in 10 days.

Examination of the results shows, first that the mixture was superneutral, since there was no evidence of infection in any of the animals inoculated with the unheated fresh mixture. Two of these animals

were tested 18 days later against 10,000 M.L.D. of virus, the other 2 months later against 100 M.L.D., and both resisted infection. The latter animal died of intercurrent infection 3 weeks after the date of the immunity test. Normal rabbit serum had, of course, no neutralizing effect on the virus, and the two guinea pigs receiving the mixture of normal serum and virus died after 11 days.

Of the three animals which received the heated mixture of virus and immune serum, two, which were tested 18 days later against 10,000 M.L.D. of virus, had a mild febrile reaction but showed no lesions; the other, which was tested 2 months later against 100 M.L.D., died of intercurrent infection after 12 days; there were no specific pathological findings, and if the animal had a spotted fever infection, it must have been very mild. There is a great contrast between these results and those in the guinea pigs inoculated with the heated mixture of virus and normal rabbit serum, all of which died of typical infection.

Of the animals inoculated with the aged mixture, two died when tested 16 days later; one had a typical spotted fever infection, the other probably succumbed to secondary infection while recovering from mild spotted fever. The third, tested 1 month later, had irregular fever, but no symptoms of spotted fever. Of the animals which received the aged mixture of virus and normal rabbit serum, one died in 13 days of typical infection, the other was lost by intercurrent infection before the time of test. The aged virus alone produced neither infection nor immunity in the four animals receiving it; the two tested 16 days later died in 10 and 14 days, respectively, and the two tested after 1 month died in 19 and 15 days.

The contrast between the effect of fresh unheated and heated mixtures is again strikingly brought out in Table XI, in which are presented the results of the inoculation of smaller quantities of the superneutral mixture of virus and immune serum; one series of animals receiving respectively 1, 0.1, 0.01, and 0.001 cc. of the unheated, another the same quantities of the heated mixture. The injection of the heated mixture definitely modified the course of a subsequent test infection, although complete immunity was not induced even in a dose as large as 1 cc.; the unheated mixture conferred protection in a dose of 0.1 cc., and even in the animal receiving 0.01 cc. the infection was not fatal.

These findings confirm the earlier experiments establishing the effectiveness of fresh unheated neutral mixtures of virus and immune serum in producing immunity in guinea pigs. The uselessness of heated or aged virus, alone, or with normal rabbit serum, is definitely determined. The presence of a definite immunizing property in the

TABLE XI.

Effect of Superneutral Mixtures of Virus and Immune Serum in Various Amounts.

Virus: guinea pig serum, 1 cc., representing 1,000 M.L.D.

Immune serum: rabbit, 1 cc., representing ten times the quantity necessary to neutralize 1,000 M.L.D.

Amount.	Result of vaccination.	Result of immunity test, 34 days later, against 10 M. L. D. of virus.
	Unheated.	
cc. 1 {virus 500 M.L.D. serum 0.5 cc.	No reaction.	No infection.
0.1 {virus 50 M.L.D. serum 0.05 cc.	" "	" "
0.01 {virus 5 M.L.D. serum 0.005 cc.	" "	Severe infection; recovery.
0.001 {virus 0.5 M.L.D. serum 0.0005 cc.	Definite mild febrile reaction.	No infection.
	Heated.	
1	No reaction.	Mild infection; no lesions; recovery.
0.1	" "	Moderately severe infection; recovery.
0.01	" "	Severe infection; recovery.
0.001	" "	Typical infection; died after 14 days.

neutral mixture which had been heated to 56–60°C., and also, though in less degree, in the neutral mixture kept at 4°C. for 32 days, indicates the probable effectiveness of mixtures treated in this way for prophylactic vaccination in man, notwithstanding that these preparations fall much below the fresh mixtures in immunizing titer.

GENERAL CONSIDERATIONS.

From the findings outlined, it appears that the immunizing properties of the Rocky Mountain spotted fever virus are almost, if not totally, removed by heating to 60°C. for 20 minutes or by prolonged preservation at a low temperature. On the other hand, the immunizing power remains fully preserved when the virus is mixed with immune serum in neutral or superneutral proportions, notwithstanding the fact that such mixtures are completely devoid of infecting power. Moreover, heating of such mixtures to 56–60°C. for 20 minutes, or prolonged preservation at refrigerator temperature, does not altogether destroy, though it markedly reduces, the immunizing power. Since normal rabbit serum does not in any way influence or retard the rapid disappearance of immunizing properties from the virus under similar conditions (heating or aging), the persistence of immunizing power in the neutral or superneutral mixtures appears to be due to a specific reaction which takes place between the virus (antigen) and the immune serum (antibody).

For the purpose of inducing artificial immunity in man, the heated neutral mixtures of spotted fever virus and a highly potent immune serum may be used. The serum of infected guinea pigs is preferable to that of rabbits as the source of the virus, a high degree of virulence being more readily and uniformly maintained in the guinea pig. Immune serum from hyperimmunized rabbits is readily prepared and is capable of attaining a high degree of potency.[2] These two factors, highly virulent strains of the virus, and a powerful immune serum, are essential to the production of an effective sero-vaccine. The superneutral mixture is prepared as follows:

Blood is drawn from infected guinea pigs at the height of fever (4th to 5th day) and the clear serum separated from the clot.[3] The titer should be such that 1 cc. contains 100 M.L.D. of virus. By pooling a number of serums (after first making sterility tests of the individual serums to insure absence of secondary infection), virus of uniform

[3] It is imperative to determine by culture methods that the blood is free from any secondary bacterial infection, especially that caused by members of the paratyphoid group of bacilli, which induce febrile and splenic reactions strikingly similar to the symptoms shown by guinea pigs on the 4th and 5th days of spotted fever infection.

virulence can be obtained in large quantity. Immune rabbit serum, of a titer such that 0.1 cc. will neutralize 100 M.L.D., is mixed with the virus in ten times the neutralizing dose. A less potent immune serum is not suitable, because an inconveniently large quantity would be required. The mixture is heated to 56°C. for 20 minutes in a water bath and preserved in the refrigerator until used. Two or more injections of 1 cc. of the mixture may be given subcutaneously.

The procedure just outlined is a preliminary one only. The ultimate object of these experiments is a method in which the active (unheated) sero-vaccine may be safely applied to human vaccination.

SUMMARY.

Freshly prepared mixtures of spotted fever virus and immune rabbit serum in neutral or superneutral proportions confer complete immunity on guinea pigs.

The mixtures undergo a considerable loss in immunizing power when heated to 60°C. for 20 minutes, but are still capable, if used in sufficient quantity, of conferring a degree of immunity on the vaccinated animal such that a subsequent experimental infection is rendered less severe and non-fatal.

Unheated mixtures which had been preserved in the refrigerator at 4°C. for a period of 32 days still retained a certain degree of immunizing property.

The virus alone, or mixed with normal rabbit serum, when allowed to die out by prolonged preservation at refrigerator temperature, or when killed either by heating at 60°C. for 20 minutes or by chemicals (chloroform, ether, xylene) does not induce immunity in guinea pigs.