IMMUNOLOGICAL REACTIONS WITH A VIRUS CAUSING PAPILLOMAS IN RABBITS

III. Antigenicity and Pathogenicity of Extracts of the Growths of Wild and Domestic Species: General Discussion

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The Shope virus engenders papillomas when inoculated into domestic rabbits as well as cottontails, yet only rarely can it be recovered from these (1), and occasionally the virus-induced growths of cottontails fail to yield it. Yet serological studies have indicated that the virus exists in the growths from which it cannot be got, causing their continued proliferation, and eliciting antibodies roughly in proportion to the amount of papillomatosis present on the individual (2). As further evidence of the presence of the virus, Shope has recently found that suspensions of the virus-induced papillomas of wild and domestic rabbits will elicit virus-neutralizing antibodies when inoculated intraperitoneally into rabbits of homologous species, even though they cause no lesions when inoculated into the skin of susceptible animals (3).

The experiments now to be reported were done to learn more about the state of the virus, and of the complement-binding antigen, in the papillomas that yield no infectious virus on extraction. After they have been reported, the findings of all three papers will be discussed.

Complement Fixation Tests with Extracts of Non-Infectious Papillomas of Cottontails

A number of instances have been encountered in our laboratory in which large, flourishing papillomas, brought about by the experimental inoculation of Shope virus into highly susceptible cottontails, failed to yield virus on extraction. The experiment that follows was done to determine whether extracts of such growths will bind

complement when tested with suitable antisera. 6 extracts of growths that were known from previous tests to yield little or no active virus were compared as to complement-binding capacity and pathogenicity with 6 others containing virus in high titer.

Experiment 18.—1:20 saline extracts of the growths, which had been in 50 per cent glycerol for periods up to 30 weeks,[1] were prepared as usual, all in the same way, and centrifugalized twice at 3500 R.P.M. in the angle head centrifuge. The supernatant fluids were then tested for complement-binding capacity according to the method adopted as standard (Paper I), and for pathogenicity by inoculation into scarified areas on the skin of 3 normal domestic rabbits, according to a routine method (2).

Table XVIII, giving the results of the experiment, shows that the extracts that contained active virus in large amount bound complement notably well, while those containing little or no virus fixed it poorly, or not at all.

The Yield of Virus from the Natural and Experimental Papillomas of Cottontails

How does it happen that the virus-induced growths of some cottontails yield the virus and the complement-binding antigen in high titer, while others furnish neither in significant amount? The different amounts of virus obtained from growths of various duration, size, and character provide data bearing upon this point.

The naturally occurring papillomas are generally small, discrete, conical or discoid, superficial growths, 1 to 2.5 cm. across at the base, with horny peaks 1 to 3 cm. high. They are frequent, being present on one of every dozen or so cottontails trapped in certain Western states; and, though often multiple, are not usually numerous on the individual. Only occasionally can more than a few grams of papilloma tissue be obtained from a single animal, our maximum being 24.5 gm., although Shope, with wider experience, has sometimes obtained more.[2]

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[1] The preservation of papillomas in glycerol for many months does not harm the virus perceptibly, as Shope first noted; and materials preserved in this way in our laboratory for more than 2 years have retained their pathogenicity without any notable loss in titer. Indeed an apparent increase in the yield of virus has been observed in some cases when the growths had been kept long in glycerol at refrigerator temperature.

[2] Personal communication from Dr. Shope.

TABLE XVIII

Complement Fixation Tests with Extracts of Papillomas Yielding Much Virus and of Others Yielding Little or None

Source of papilloma extract	Pathogenicity tests*			Complement fixation tests								
W.R. No.	Test rabbits			Immune sera				Hyperimmune sera		Normal sera		Antigen controls (no serum)
	A	B	C	53	56	15	19	8-S	12-S	34	35	
35	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	0
36	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	0
4S	++++	+++±	+++±	++++	++++	++++	++++	++++	++++	0	0	0
1-30	++++	++++	++++	++++	++++	++++	++++	++++	++++	0	0	0
27	+++	+++±	+++±	+++±	+++±	+++±	+++±	+++±	+++±	0	0	0
20	+++	+++±	+++	±	0	+++	+++±	++++	+++	0	0	0
1-15	0	0	0	0	0	0	++++	++++	±	0	0	0
1-26	0	0	0	0	0	0	+±	++++	0	0	0	0
14	0	0	0	0	0	0	0	±	0	0	0	0
19	0	±	0	0	0	0	0	±	0	0	0	0
15	±	±	0	0	0	0	0	+	0	0	0	0
37	0	0	0	0	0	0	0	0	0	0	0	0
Serum controls (no antigen).....				0	0	0	0	0	0	0	0	0

Complement, 2 units in all tubes.

Antigen extracts diluted 1:20, sera 1:4.

* Readings made on 42nd day after inoculation of 5 per cent suspensions into test rabbits, according to standard scale,—see Table XIII.

TABLE XIX

The Yield of Virus from the Naturally Occurring and Experimentally Induced Papillomas of Cottontail Rabbits

Source of material					Pathogenicity tests with extracts of the growths								
					15 days			25 days			42 days		
Cottontail rabbit No.	Type of growths	Character and size of growths	Day growths procured, after virus inoculation	Weight of papilloma tissue carried by the animal	A	B	C	A	B	C	A	B	C
				gm.									
W.R. 24	Experimental	Large, fleshy, confluent, 6 x 4 cm. on both flanks, 3 cm. high, with underlying extensions	83rd	87.5	0	0	0	0	0	0	±	±	0
25	"	Large, fleshy, confluent, 6 x 4 cm. on both flanks, 3½ cm. high, with "satellite pearls"	111th	107.5	0	0	0	0	0	0	0	±	0
60	"	Large, vigorous, confluent and semiconfluent growths on abdomen	74th	43.5	0	0	0	0	±	0	0	±	0
57	"	Large, vigorous, confluent growth on abdomen, 6 x 4 cm., 1 cm. high	60th	23.0	0	0	0	0	±	0	0	±	0
20-S	"	Large, confluent and semiconfluent growths 6 x 5 cm. on both flanks, 1.5 cm. high	80th	71.2	0	+	0	++	++±	+++	+++	+++±	+++
7-S	"	Large, confluent, fleshy, 6 x 5 cm. on both flanks, 0.8 cm. high	28th	43.2	0	+	+	++	++±	++	++±	+++	+++

18-S	"	Large, confluent, fleshy, 6 x 4 cm. on both flanks, 1.4 cm. high	28th	47.5	+	++	0	+++	+++	++±	+++±	+++±	+++
4-S	"	Large, confluent, fleshy, 6 x 5 cm. on both flanks, 1.0 cm. high	20th	34.8	++	++	++	+++	+++	+++	+++±	+++±	+++±
10	Natural	8 discrete, fleshy, discoid papillomas 2 to 3 cm. across and 1 to 2 cm. high	Unknown	17.4	+++	+++	+±	++++	++++	+++	++++	++++	++++
9	"	9 discrete, fleshy, discoid growths 2 to 3 cm. across and 1 cm. high	"	14.4	++++	++++	+++	++++	++++	+++±	++++	++++	++++
8	"	7 discrete, fleshy, discoid growths 1 to 3 cm. across and 1 to 2 cm. high	"	10.5	++++	++++	++	++++	++++	+++	++++	++++	++++

Readings made according to the standard scale,—see Table XIII.
A, B, C = test rabbits.
Dilution of extracts, 1:20, except W.R. 60 and 57, 1:10.

The size of experimentally induced growths depends on the area inoculated. As a rule, we have rubbed large amounts of potent virus into broad areas of scarified skin, and the resulting massive growths have frequently weighed from 75 to 100 gm. or more.

Table XIX gives a synopsis of the findings with extracts of the growths of 3 cottontails with naturally occurring papillomas and 8 others bearing experimentally induced growths. All of the growths had been procured some months previously while the rabbits were alive. They were at once cut up and put into 50 per cent glycerol-Locke's solution in the refrigerator. Tests for pathogenicity were made on three separate occasions, each with 3 test rabbits. 5 per cent extracts of the experimentally induced papillomas of W.R. 24, 25, 20-S, 7-S, and 18-S were tested on one series of rabbits, 10 per cent extracts of the growths of W.R. 60 and 57 on a second series, while 5 per cent extracts of the naturally occurring growths of W.R. 8, 9, and 10 were tested in a third experiment. The susceptibility of the test animals varied little in the three experiments, and hence the readings have been brought together in the table. All of the materials, except those of W.R. 7-S and 18-S, were later tested in other experiments using other test animals, and the results agreed closely with those set down in Table XIX.

Extracts of the glycerolated materials from 4 of the experimentally inoculated rabbits (W.R. 24, 25, 60, and 57), which had carried large, confluent growths over periods up to 111 days, contained little if any active virus (Table XIX); while similar extracts of the growths of individuals that had carried experimentally induced papillomas for shorter periods (W.R. 20, 7-S, 18-S, and 4-S) contained virus of moderate pathogenicity. Extracts of the small, discrete, naturally occurring papillomas of W.R. 8, 9, and 10, on the other hand, contained virus of high titer, the materials all giving rise early to confluent papillomas. Individual host differences have an evident rôle in the findings, however: the experimental growths of W.R. 20-S yielded much more virus than those of W.R. 57 and 60, though their total mass was greater, and they had been present for a longer period.

The findings set down in Table XIX provide a fair sample of our general experience, which is to the effect that the small, discrete, naturally occurring papillomas of cottontail rabbits usually furnish virus of high titer; while the larger, confluent growths, produced with it experimentally in highly susceptible cottontails, yield it in moderate or small amounts or not at all.[3]

In tests already recorded (Tables III and X of Papers I and II), the sera of cottontail rabbits with large, experimentally induced growths have had high antibody titers. It is precisely such growths

[3] Shope has also noted these facts, incidentally to the routine titration of papilloma materials (personal communication).

that yield the least virus. Have the two findings any relation to one another? To learn about this, the growths of 2 cottontails with different antibody titers were compared as to their yield of virus and complement-binding antigen.

Experiment 19.—2 normal cottontail rabbits were inoculated by rubbing a 10 per cent extract of the growths of W.R. 7-N into scarified abdominal areas about 8 x 10 cm. across. The growths of one of the inoculated animals (W.R. 27) appeared sooner than those of the other (W.R. 26) and they became much larger, fleshier, and more numerous. On the 87th day after inoculation both rabbits were bled from the heart to secure serum, and the growths of each were pulled away and stored in 50 per cent glycerol in the refrigerator. Those of W.R. 27 were large, confluent and semiconfluent, fleshy, compact and onion-like, and they covered most of the broad, inoculated area. Their greatest height was 1.5 cm., and beneath them were a number of rounded "pearls" like those often formed by the deep extension of vigorous papillomas in cottontails. The growths of W.R. 26, although caused by the same strain of virus, were by contrast much smaller and fewer, discrete but fleshy "onions," scattered thinly over the inoculated site, and raised only 1 cm. or less above the skin level. The mass of papilloma tissue obtained from W.R. 27 was 49.8 gm., and from W.R. 26, 9.9 gm.

Representative portions of the glycerolated papilloma tissues of the 2 animals were extracted as usual (dilution 1:20), and the extracts tested for pathogenicity and complement-binding capacity. The complement fixation and virus neutralization titers of their sera were also determined.

The findings are shown in Table XX. The extract of the papillomas of W.R. 26 contained virus in high titer, confluent and semiconfluent growths appearing before the 15th day where it had been inoculated into all 3 test rabbits; but no growths ever appeared where the extract of the papillomas of W.R. 27 was inoculated. The extract of the growths of W.R. 26, containing virus in high titer, bound complement with all of the immune sera; while that of W.R. 27, devoid of pathogenicity, failed to bind complement under the same conditions. The sera of the 2 cottontails showed wide differences, that of W.R. 26 neutralizing a potent 5 per cent virus extract in low titer and binding complement poorly on test with 2 antigens, while the serum of W.R. 27 neutralized the virus in very high titer and fixed complement correspondingly.

This experiment proved highly informative. The large, confluent, fleshy papillomas of one animal yielded no active virus whatever. and the serum of this individual had great power to neutralize virus and to bind complement. Precisely the opposite state of affairs existed in the other rabbit, the growths being small, scattered, and slow growing, though engendered by the same inoculum, and the

TABLE XX

The Yield of Virus and of the Complement-Binding Antigen from the Papillomas of Two Cottontail Rabbits with Different Serum Antibody Titers

Cottontail rabbit No.	Day serum and growths procured	Number and character of papillomas	Weight of papilloma tissue carried by the animal	Tests with extracts of the papillomas					Tests with the sera							
				Pathogenicity tests*			Complement fixation tests†		Neutralization tests‡			Complement fixation tests§				
							Immune sera		5 per cent virus W.R. 56+			Dilutions of serum				
				15th day	24th day	42nd day	53	56	Whole serum	Serum 1:4	Serum 1:16	1:4	1:8	1:16	1:32	1:64
			gm.													
W.R. 26	87	Few, small, scattered, discrete, fleshy, less than 1 cm. high	9.9	+++	+++±	++++	++++	++++	+++	±	0	±	0	0	0	0
27	87	Many large, confluent and semiconfluent, fleshy, compact, 1.5 cm. high	49.8	0	0	0	0	0	++++	++++	+++±	++++	++++	++++	++++	+±

* 5 per cent extracts. +++ = semiconfluent papillomatosis; ++++ = confluent papillomatosis.

† 5 per cent extracts. Immune sera diluted 1:4. ++++ = complete fixation of 2 units of complement.

‡ Serum-virus mixtures incubated 2 hours at 37°C. and inoculated in 3 test rabbits. The readings given are those of one rabbit on the 20th day. ++++ = complete neutralization.

§ Antigen = 1:10 Berkefeld V filtrate of papillomas of W.R. 35. ++++ = complete fixation. The antigen and sera were not anticomplementary when tested in double amounts.

serum having little neutralizing power and none to bind complement.

When considered together, the findings in this experiment and in those of Tables XVIII and XIX suggest strongly that the failure of large, confluent papillomas to yield virus and complement-binding antigen may be related to the fact that the sera of animals carrying the large growths develop marked ability to neutralize the virus; but further tests must be made to settle the point definitely. However this may be, it is certain that the yield of the complement-binding antigen parallels the yield of virus from the papillomas of cottontails,—some growths providing much of the one and furnishing much of the other also, while others yield little or none of either.

Complement Fixation Tests with Extracts of Virus-Induced Papillomas of Domestic Rabbits, Snowshoe Hares, and Jack Rabbits

As already stated, the papilloma virus gives rise to growths of exceptional vigor when inoculated into the skin of domestic rabbits, yet only exceptionally can it be recovered from them, and then in attenuated state.[4] Many tests have been made in the course of the present work to determine whether extracts of virus-induced papillomas of domestic rabbits have the capacity to bind complement. The results make it clear that they are far inferior to cottontail materials in this respect, having little or no capacity of the sort. An illustrative experiment will be given.

Experiment 20.—Extracts were made in saline as usual of the glycerolated papillomas of 4 cottontail rabbits, known to yield the virus in high titer, and from the glycerolated growths of 4 domestic rabbits known from previous tests to yield virus in very small amount or not at all. After standing overnight in the refrigerator the extracts were centrifugalized twice as usual at 3500 R.P.M., and tested in dilutions of 1:5, 1:15, and 1:45 for capacity to bind complement in the presence of various dilutions of the hyperimmune serum of W.R. 8-S.

Table XXI shows the results of the experiment. Most of the antigens proved anticomplementary in a dilution of 1:5 when tested in

[4] Shope has procured several strains of virus that can be passed serially in domestic rabbits; but all of them are slightly or moderately pathogenic at best, giving only scattered or semiconfluent growths that appear long after inoculation (1).

double amount, and hence the findings with these dilutions have been omitted from the table. 3 of the 4 extracts from the cottontail materials bound complement notably well, while the fourth (W.R. 30-N)

TABLE XXI

Complement Fixation Tests with Extracts of Papillomas from Domestic and Cottontail Rabbits

Source of antigens	Rabbit No.	Dilutions of antigen	Complement fixation tests			
			Dilutions of serum			Antigen controls (no serum)
			1:5	1:20	1:80	
Cottontail rabbits (Papillomas yielding active virus)	10	1:15	++++	++++	++++	0
		1:45	++++	++++	++++	
	8-N	1:15	++++	++++	+++	0
		1:45	++++	++++	+++	
	55	1:15	++++	++++	++	0
		1:45	++++	++++	0	
	30-N	1:15	0	0	0	0
		1:45	0	0	0	
Domestic rabbits (Papillomas yielding little or no active virus)	K-22	1:15	++++	+±	0	0
		1:45	0	0	0	
	55-N	1:15	0	0	0	0
		1:45	0	0	0	
	12-85	1:15	0	0	0	0
		1:45	0	0	0	
	30-N	1:15	0	0	0	0
		1:45	0	0	0	
Serum controls (no antigen)			0	0	0	

Serum W.R. 8-S (hyperimmune).
Complement, 2 units in all tubes.

failed to do so in the dilutions shown, although it gave complete, specific fixation at a dilution of 1:5, and on the other occasions at 1:10. Only one of the materials from the domestic rabbits bound complement, and this was derived from growths that had yielded a small

amount of infectious virus on a number of previous trials. The other 3 extracts failed to show any specific complement-binding capacity at all. One of these (D.R. 12-85) had yielded virus in small amount on previous extractions, while the others (D.R. 55-N and 30-N) had proven non-infectious in all tests.

The papilloma virus will produce growths when inoculated into snowshoe hares and jack rabbits (4), and from them it can often be recovered, but in low titer. In the next test extracts of papillomas experimentally induced in 2 snowshoe hares and a jack rabbit were tested for capacity to bind complement in the presence of a known immune serum. Extracts of the papillomas of a domestic rabbit and of a cottontail were used for comparison.

Experiment 21.—The glycerolated papilloma material procured from the 2 snowshoe hares and from the jack rabbit had yielded infectious virus in small amount on several previous tests, while extracts of the warts of the domestic rabbit had proven non-infectious. The papillomas of the cottontail (W.R. 1240) had furnished large quantities of virus in many previous tests. For present purposes extracts of all 4 glycerolated materials were prepared as usual and tested with various dilutions of a potent immune serum.

The only extract that bound complement notably well in the test (Table XXII) was that of the cottontail growth, a material containing active virus in large amount. The extracts of the jack rabbit papillomas, although somewhat anticomplementary, exhibited some specific fixation, as did the extracts of the growths from the snowshoe hares and from the domestic rabbit, though none gave complete fixation.

The findings should be considered with those of Tables XIX and XXI. Together they show that extracts of the virus-induced papillomas of domestic rabbits, snowshoe hares, and jack rabbits, which contain infectious virus in small amount or not at all, have little or no complement-binding capacity.

Does the Complement-Binding Antigen Exist in Masked Form in the Non-Infectious Growths of Domestic Rabbits?

Shope has found that extracts of the "non-infectious" growths of domestic and cottontail rabbits elicit virus-neutralizing antibodies when injected intraperitoneally into rabbits of homologous species

(3). He has generously provided for complement fixation tests a number of virus-neutralizing sera obtained from rabbits immunized by intraperitoneal injections of papilloma extracts, some of which contained active virus while others did not. The sera bind complement in the presence of suitable antigens, as is shown by the tests that follow.

TABLE XXII

Complement Fixation Tests with Extracts of Papillomas from Various Species of Rabbits

Source of antigen	Rabbit No.	Dilutions of antigen	Complement fixation tests			
			Immune serum D.R. 9			Normal serum D.R. 1
			1:2	1:4	1:8	1:2
Cottontail rabbit	1240	1:15	++++	++++	++++	0
		1:45	++++	++++	+++±	
Jack rabbit	6	1:15	+++±	+++±	++±	+±
		1:45	++	±	±	
Snowshoe hare	1	1:15	++	±	0	0
		1:45	±	0	0	
Snowshoe hare	2	1:15	++±	+++	++±	±
		1:45	+	±	±	
Domestic rabbit	55	1:15	++±	+±	+	0
		1:45	+	0	0	
Serum controls (no antigen)			0	0	0	0

2 units of complement in all tubes.

Experiment 22.—3 of the sera (14-04, 14-05, and 14-06) were from domestic rabbits immunized by intraperitoneal injections of suspensions of the non-infectious virus-induced papillomas of a domestic rabbit, 3 (14-07, 14-08, and 14-09) from rabbits similarly inoculated with suspensions of the slightly infectious papillomas of a 2nd domestic rabbit, and 3 (14-10, 14-11, and 14-12) from rabbits immunized with suspensions of the highly infectious papillomas of a cottontail rabbit. All were tested in a dilution of 1:4 with a potent antigen according to the routine technique, the sera of 3 of the rabbits procured before the immunizing injections being used as controls (Table XXIII).

In a quantitative test with the sera of 6 of these rabbits (Table XXIV) it was found that those immunized with suspensions of cottontail papillomas, which contained much virus, bound complement in much higher titer than those immunized with suspensions of the domestic rabbit growths, which contained no active virus. The differences

TABLE XXIII

Complement-Binding Capacity of the Sera of Domestic Rabbits Immunized by Shope by Means of Intraperitoneal Injections of Suspensions of Papilloma Tissue

Source of serum			Complement fixation tests	
Rabbit No.	Immunization (2 intraperitoneal injections of 5 per cent suspensions of glycerolated papillomas)		Antigen W.R. 8-N 1:40	Serum controls (no antigen)
	Source of immunizing material	Amount of active virus in immunizing material		
14-04	Before injection	—	0	0
14-05	"	—	0	0
14-06	"	—	0	0
14-04	Domestic 12-86	None	±	0
14-05	"	"	++++	0
14-06	"	"	++++	0
14-07	Domestic 12-70	Little	++++	0
14-08	"	"	0	0
14-09	"	"	+++	0
14-10	Cottontail 12-34	Much	++++	0
14-11	"	"	++++	0
14-12	"	"	++++	0
Antigen control (no serum).....			0	

Complement, 2 units in all tubes.
Sera diluted 1:4.
Antigen, 1:40 extracts of glycerolated papillomas (W.R. 8-N).

correspond closely with those obtained by Shope in neutralization tests with the same sera.

The findings set down in Tables XXIII and XXIV show that an antigen capable of eliciting complement-binding antibodies is present in crude extracts of the "non-infectious" papillomas of domestic

rabbits, though in much smaller amount than in extracts of growths containing the virus. How has it happened, then, that extracts of the "non-infectious" growths have proved ineffective as complement-binding antigens in the tests *in vitro*? Can it be that the antigen, which Shope readily demonstrated in crude suspensions of the "non-infectious" growths, is eliminated by the centrifugation or filtration procedures used as routine in the preparation of antigens for the complement fixation tests? It could be so, for a preliminary effort to

TABLE XXIV

Complement Fixation Tests with the Sera of Domestic Rabbits Immunized by Intraperitoneal Injections of Crude Extracts of Domestic and Cottontail Papillomas

Source of serum		Complement fixation tests			
Domestic rabbits	D.R. No.	Dilutions of sera			Serum controls (no antigen)
		1:4	1:6	1:8	
Immunized with suspensions of D.R. papillomas containing no active virus	14-07	+	0	0	0
	14-08	0	0	0	0
	14-09	+	0	0	0
Immunized with suspensions of W.R. papillomas containing much virus	14-10	++++	++++	++++	0
	14-11	++++	++++	++++	0
	14-12	++++	++++	++++	0
Antigen control (no serum)			0		

Complement, 2 units in all tubes.

Antigen, 1:20 extract of cottontail papillomas (W.R. 8-N).

The sera were generously provided by Dr. Shope. His experiments showed that the sera of the rabbits immunized with suspensions of wild rabbit papillomas neutralized the virus considerably better than those immunized with suspensions of domestic rabbit growths (3).

stimulate the formation of complement-binding antibodies by repeated intravenous injections of large quantities of Berkefeld V filtrates of the "non-infectious" papillomas of domestic rabbits yielded negative results. Consequently resort was had to the intraperitoneal injection of crude suspensions as well as Berkefeld V filtrates of the same domestic rabbit papillomas to learn whether the immunizing antigen in the "non-infectious" extracts is retained by Berkefeld filters.

Experiment 23.—10 gm. of the papillomas of 3 domestic rabbits, which had been preserved in glycerol for many months in the refrigerator and had failed to yield infectious virus in a number of previous tests, was ground as usual and made up to 100 cc. with saline. The crude suspension was allowed to settle for 4 hours in the refrigerator, after which the densely turbid supernatant fluid was removed, stirred, and a part set aside for injection as such. The remainder was centrifugalized at 3500 R.P.M. in the angle head for 5 minutes, and the supernatant fluid was spun again at 3500 R.P.M., for 15 minutes. The second supernatant was barely opalescent. It was filtered through a new Berkefeld V 5 candle, coming through rapidly and water-clear. 6 cc. of the crude suspension was injected into the peritoneal cavity of each of 3 normal Dutch rabbits, and 6 cc. of the filtrate into 3 blood-related animals.

Eleven days later the procedure was repeated, using a fresh crude suspension and a V filtrate of the same materials, and injecting the rabbits as before. None developed growths at the sites where the skin had been punctured, or elsewhere. On the 9th day after the second injection the rabbits were bled 20 cc. from the heart, and the sera were tested for capacity to bind complement and to neutralize the virus. Sera from 3 normal Dutch rabbits, and from 3 others bearing large virus-induced papillomas, were tested concurrently for comparison.

As Table XXV shows, the rabbits immunized with the crude extracts of the non-infectious papillomas yielded sera that neutralized completely or partially an active 1 per cent virus filtrate when incubated for 2 hours with it in equal amounts prior to inoculation into the test rabbits. The sera compared favorably in this respect with the specimens procured from animals bearing large papillomas and tested concurrently. The sera of the rabbits injected with filtrates, on the other hand, had no neutralizing capacity whatever. The sera of the animals injected with crude extract failed to bind complement despite their neutralizing power, whereas the sera of the rabbits bearing the growths did so. It will be recalled in this connection that the neutralizing power of the serum of immunized rabbits regularly transcends the complement-binding power, whence one may infer that in the present instance the animals receiving crude extract had developed less immunity than had those bearing growths.

In this experiment (Table XXV) an antigen, present in the crude extracts of "non-infectious" papillomas and capable of eliciting virus-neutralizing antibodies, was removed by centrifugation and filtration, procedures that do not notably affect the content of infectious virus in potent extracts. Even the crude suspensions, though, evoked a relatively low grade of immunity, the sera of rabbits immunized with them neutralizing the virus in low titer, and not binding complement at all.

The next step manifestly was to find out whether the "masked"

antigen, present in crude suspensions of non-infectious growths might not act as a complement-binding antigen *in vitro* if the conditions were rendered suitable. The previous complement fixation tests with

TABLE XXV

Neutralization and Complement Fixation Tests with the Sera of Domestic Rabbits Injected Intraperitoneally with Crude Extracts and Berkefeld V Filtrates of Non-Infectious Domestic Rabbit Papillomas

Source of serum	D.R. No.	Neutralization tests*			Complement fixation tests		
		Serum 0.5 + 1 per cent virus 0.5			Antigens		Serum controls (no antigen)
		Test rabbits			W.R. 4-S	W.R. Tx	
		A	B	C			
Domestic rabbits injected with crude extracts of the non-infectious papillomas	2-58	+±	++±	++	0	0	0
	2-59	++++	+++±	++++	0	0	0
	2-60	++++	++++	++++	0	0	0
Domestic rabbits injected with Berkefeld V filtrates of the non-infectious papillomas	2-63	0	0	0	0	0	0
	2-64	0	0	0	0	0	0
	2-65	0	0	0	0	0	0
Domestic rabbits bearing virus-induced papillomas	2-54	++++	++++	++++	++++	++++	0
	2-74	++++	++++	++++	++++	++++	0
	2-10	++++	++++	++++	++++	++++	0
Normal domestic rabbits	3-04	0	0	0	0	0	0
	3-05	0	0	0	0	0	0
	3-06	0	0	0	0	0	0
Antigen controls (no serum)					0	0	

++++ = complete neutralization or complete fixation.
Complement, 2 units in all tests.
Serum 1:2 in all complement fixation tests.
Antigens, 1:20 extracts of glycerolated cottontail papillomas.
* Readings on the 25th day, according to the scale of Table X.

extracts of "non-infectious" papillomas had been done with specimens centrifugalized as usual, and these had bound complement poorly if at all (Tables XXI and XXII). In the next experiment crude suspensions were used, as well as centrifugalized.

Experiment 24.—5 per cent suspensions in saline were made of the highly infectious glycerolated papillomas of 2 cottontails (W.R. 53 and 54), and also of the glycerolated warts of 2 cottontail and 2 domestic rabbits that had failed to yield virus on a number of previous tests. They were left overnight in the refrigerator, then mixed by gentle shaking and allowed to settle for 30 minutes,

TABLE XXVI

Complement Fixation Tests with Crude and Centrifugalized Extracts of Infectious and Non-Infectious Papillomas

Source of antigens			Source of sera					
			Immune		Hyper-immune 8-S	Normal		Antigen controls (no serum)
Papillomas	Rabbit No.	Preparation	D.R. 7	W.R. 10		W.R. 31	D.R. 49	
Yielding much virus	W.R. 53	Crude	+*	++++	++++	0	0	0
		Centrif.	++++	++++	++++	0	0	0
	W.R. 54	Crude	+*	++++	++++	0	0	0
		Centrif.	++++	++++	++++	0	0	0
Yielding little or no virus	W.R. 19	Crude	0	0	0	0	0	0
		Centrif.	0	0	0	0	0	0
	W.R. 37	Crude	0	0	0	0	0	0
		Centrif.	0	0	0	0	0	0
	D.R. 2-08	Crude	0	0	0	0	0	0
		Centrif.	0	0	0	0	0	0
	D.R. 2-11	Crude	0	0	0	0	0	0
		Centrif.	0	0	0	0	0	0
Serum controls (no antigen).....			0	0	0	0	0	0

Complement, 2 units in all tubes.
Sera diluted 1:4.
Antigens, dilution 1:20.
* Prozone.

after which 3 cc. of the turbid supernatant fluids were removed for use. The remaining suspensions were now centrifugalized at 3500 R.P.M. for 5 minutes, and the supernatant fluids spun again for 15 minutes. The final fluids thus procured were practically water-clear. They were tested for capacity to bind complement along with the crude extracts, using several immune and normal sera (Table XXVI).

It will be seen that the crude suspensions of "non-infectious" domestic and wild rabbit papillomas failed to bind complement even under the conditions of the test, which were optimal (Table XXVI). If "masked" antigen existed in the crude suspensions, it was evidently present in amount too small or in a form unsuited to the reaction, possibilities that will be considered in the discussion.

GENERAL DISCUSSION

The findings described in the three papers will now be considered under the divisions into which they fall. A recapitulation of the results is essential in this connection.

To begin with a method was worked out that demonstrated the occurrence of complement fixation when the sera of rabbits carrying virus-induced papillomas were mixed with antigens consisting of saline extracts of the growths. The test followed classical lines and yielded consistent results. It proved highly specific. Most, but not all, of the sera of rabbits carrying the virus-induced papillomas fixed complement in the tests, their ability to do so varying roughly in proportion to the total mass of the growth borne by the individual. The sera of normal rabbits, on the other hand, and of rabbits with papillomas elicited by tarring, and of those with various other diseases, including vaccinia and syphilis, gave no complement fixation when mixed with effective antigens derived from the Shope papilloma.

Relation of the Virus-Neutralizing and Complement-Binding Antibodies

In previous work by Shope and ourselves (1, 2) it had been noted that the sera from animals carrying papillomas varied greatly in their virus-neutralizing capacity, some sera having almost none though obtained from rabbits that had borne growths for many weeks. It was of interest, therefore, to find whether the virus-neutralizing capacity of a given serum was proportional to its complement-binding capacity. This proved to be the case. Those sera that neutralized large amounts of the virus fixed complement readily, and often in high titer, their capacity to do so varying directly with their virus-neutralizing capacity; a few sera were encountered, however, that neutralized small amounts of the virus, yet failed to bind complement under the most favorable circumstances.

"Hyperimmunizing" injections were not necessary in order to secure good complement-binding antisera. These were readily obtained from animals that had borne natural or experimentally induced papillomas for some time; and it is reasonable to suppose that growths of long standing elicited "hyperimmune" responses under natural conditions, by liberating antigen more or less constantly over prolonged periods. Complement-binding antibodies could be stimulated in normal rabbits, however, by repeated intraperitoneal injections of extracts of the papillomas, according to the method employed by Shope to elicit virus-neutralizing antibodies (3).

From the findings already discussed it is evident that the complement-binding antibodies are evoked under the same, highly specific conditions as the virus-neutralizing antibodies, and in proportional amounts; and the evidence indicates that they are elicited by the same antigen. The view has been adopted, therefore, that complement fixation and virus neutralization with these sera are probably different manifestations of the action of a single antibody. The neutralization test appears to have a "lower threshold" than the complement fixation test, and smaller amounts of antibody may be detected by means of it; but it is not more sensitive than the complement fixation test in the effective range of the latter, as many experiments have shown.

Relation of the Complement-Binding Antigen to the Virus

A detailed study was next made of the relation of the complement-binding antigen to the virus. It was learned first that the complement-binding antigen is not present in extracts of the normal skin of rabbits, and not in effective amounts in extracts of the papillomas that contain little or no virus. The antigen is always found, on the other hand, in extracts of the growths that contain much virus,—and it varies in amount proportionally to the latter. On further comparison, the virus and the complement-binding antigen appear to have the same particle size; both pass readily through Berkefeld V candles, but are retained in Berkefeld W and Seitz filters. They are also thrown down together in the centrifuge. Furthermore, they are similarly affected by certain physical agents, being inactivated concurrently by heat and, generally speaking, by changes in pH. By irradiation with ultraviolet light, however, it was possible to render

extracts of the growths non-pathogenic without reducing their capacity to bind complement; and mild treatment with alkali attained the same effect,—results that find a parallel in similar tests with other virus materials (5).

The findings provide no hint of the presence of a "soluble antigen" (6), or of any material distinguishable from the virus itself, to account for the immunological activity of extracts of the papillomas. They make it evident that the virus and the complement-binding antigen are closely associated, if not identical; and in so doing they support the view that a single antigen (the virus) elicits both neutralizing and complement-fixing antibodies.

The Phenomenon of "Masking"

In the virus-induced papillomas of most domestic rabbits a singular state exists as concerns the virus. Although produced experimentally with it, and having the same histological character as the infectious growths of cottontails, the papillomas of domestic rabbits usually fail to yield the virus on extraction. The virus manifestly exists in them,—causing the continued proliferation of the papilloma cells, and stimulating the formation of virus-neutralizing and complement-binding antibodies in rising titer as the growths enlarge,—but its pathogenicity and its antigenicity are "masked" (3), even crude extracts failing, as a rule, to induce growths when inoculated into susceptible animals, or to bind complement when mixed with immune serum. The "masking" is not always completely effective though: the growths of some domestic rabbits yield a little virus and a little of the complement-binding antigen on extraction; and crude extracts of the non-infectious growths will usually evoke a slight antibody response when injected intraperitoneally into normal rabbits, though Berkefeld filtrates of the same extracts failed to do so in our experience. It is possible that the "masking" depends in some measure upon an aggregation of the active material, or upon its adherence to "insoluble" cellular constituents; but more extensive tests are necessary before this question can be decided.

"Masking" of the virus is not wholly confined to the papillomas of domestic rabbits. The large, confluent growths produced experimentally in wild rabbits often fail to yield the virus on extraction,

though the discrete, naturally occurring papillomas of cottontails usually furnish it in large amounts. Whether the "masking" of the virus in the large, confluent growths of some cottontails is related to the high antibody titer of the individuals bearing them, or to some other cause, is a question that will be taken up in a subsequent study.

As bearing on the state of the virus in the papillomas of cottontail and domestic rabbits it may be recalled that the sera of cottontails generally have much higher antibody titers than the sera of domestic rabbits with comparable growths. This finding may be adequately explained by assuming that the antigen provided by the growths of cottontail rabbits is more potent or more plentiful than that supplied by the growths of domestic animals. Two facts justify this assumption: the virus and the complement-binding antigen can be readily extracted in large amount from the growths of most cottontails, as not from those of domestic rabbits; and extracts of cottontail papillomas evoke a much greater antibody response than do those of the growths of domestic rabbits when injected intraperitoneally into comparable animals.

General Implications

From the findings as a whole it appears likely that the papilloma virus acts as the antigen that evokes virus-neutralizing and complement-binding antibodies *in vivo*; and that it is closely associated, if not identical, with the antigen that reacts with immune serum to bind complement *in vitro*. It manifestly causes the continued proliferation of the papilloma cells, but without bringing about their death, which takes place through maturation. The virus is evidently liberated in the host over long periods, eliciting "hyperimmune" responses under natural conditions; but there is no evidence that it elaborates "soluble antigens," or that it regularly produces changes in the cells it infects profound enough to render their constituents auto-antigenic, as may be the case in some virus diseases characterized by acute necrosis of the infected cells (7).

When viewed in the large, the immunological reactions of the papilloma virus show a remarkable simplicity, and a striking similarity to

those of the classical antigens of immunology. It seems probable that the papilloma virus has a homogeneous antigenic structure, or at any rate, one less diverse than that of some of the larger viruses,—those which evoke complex immunological reactions, vaccinia (8), psittacosis (9), and myxomatosis (10), for example. Yet it possesses all the distinguishing properties of a virus, as Shope demonstrated (1).

Have the findings of the present work any implications as regards the tumor problem? In answer to this question it may be pointed out that the immunological study of a virus-induced tumor has led to the finding of a complement-binding antigen in extracts of the growth which is closely associated, if not identical, with the virus causing it. Whether similar substances are present in tumors of unknown cause is a matter now under investigation. It may be stated already that extracts of the transplantable Brown-Pearce tumor of rabbits also contain a complement-binding antigen which is similar, in some of its general traits, to that derived from the virus-induced papilloma (11). Later papers will report the findings of a detailed study.

SUMMARY

A study has been made of the yield of virus and of the complement-binding antigen from the virus-induced papillomas of cottontail and domestic rabbits. Extracts of the discrete, naturally occurring papillomas of cottontail rabbits usually contain virus in large amount; and, as a rule, they also contain the complement-binding antigen in high titer. The confluent growths produced experimentally with the virus in some cottontails, on the other hand, often fail to yield the virus, or furnish it in small amount; and extracts of them have little if any complement-binding capacity. The sera of cottontails with massive papillomas from which the virus cannot be recovered often have high antibody titers.

Many extracts were tested of the virus-induced papillomas of domestic rabbits. None contained the virus in large amount, and the majority of them failed to manifest it on sensitive test. A few fixed complement in low titer when mixed with immune sera, but most failed to do so. Crude extracts of the "non-infectious," virus-induced papillomas of domestic rabbits stimulated the formation of virus-neutralizing and complement-binding antibodies in low titer when

injected intraperitoneally into normal rabbits of the same breed, but Berkefeld filtrates of the same materials proved devoid of this immunizing effect.

The significance of the findings described in the three papers is discussed. The evidence as a whole favors the view that the virus stimulates the formation of the virus-neutralizing and complement-binding antibodies *in vivo*; and many facts indicate that it is closely associated, and in all probability identical, with the antigen that reacts with immune serum to fix complement *in vitro*.

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