IMMUNOLOGICAL REACTIONS WITH A VIRUS CAUSING PAPILLOMAS IN RABBITS*

I. Demonstration of a Complement Fixation Reaction: Relation of Virus-Neutralizing and Complement-Binding Antibodies

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The immunological study of the Shope papilloma virus (1) has exceptional interest, for the virus causes enduring, proliferative growths that have the character of tumors (2). In the work now to be reported a complement fixation reaction has been used in addition to the neutralization test previously employed (3). Special attention has been paid to the relation of the virus and the complement-binding antigen in extracts of the growths, and to the relation of the virus-neutralizing and complement-binding antibodies in the sera of animals carrying the tumors.

In the present paper the complement fixation test will be described, and the results presented of experiments to determine its specificity. Tests will also be reported that deal with the relation of the complement-binding and virus-neutralizing antibodies, and with the amount of antibody in the sera of cottontail and domestic rabbits bearing the virus-induced papillomas. In two papers that follow, the properties of the complement-binding antigen will be considered, as also its relation to the virus; and some observations will be reported on the yield of virus, and of the complement-binding antigen, from the papillomas of domestic and cottontail rabbits.

Methods

The procedure adopted was essentially an adaptation of that worked out by Bedson and Bland (4). Briefly it consisted in mixing serum,

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complement, and tissue extract (antigen) in proper proportion, and allowing these to stand for 2 hours at room temperature to give time for complement fixation to occur. After this period the sensitized cells were added and the tubes incubated at 37°C. for 30 minutes. Readings were then made immediately, and again after the mixtures had stood overnight in the refrigerator.

Serum was obtained by bleeding from an ear vein or by cardiac puncture under ether anesthesia. The blood was allowed to clot in horizontally placed tubes previously coated on one side with sterile paraffin. After the clot had contracted overnight in the refrigerator, the serum was removed and cleared of cells by centrifugation. The sera were stored without added preservatives in the refrigerator at a temperature of about 4°C. Aseptic technique was invariably used in procuring and handling them, but in spite of this a few became contaminated with bacteria, and these were discarded. Immediately prior to use the sera were diluted as required with 0.9 per cent saline and heated at 56° for 30 minutes. In our experience a small percentage of domestic rabbit sera were found to be anticomplementary when tested in the maximum amounts used in these experiments (1:4, occasionally 1:2), and others became anticomplementary after standing for many months in the refrigerator. Only one anticomplementary wild rabbit serum was encountered out of a great number tested. The results with the anticomplementary sera were discarded. A large number of stored sera from domestic and wild rabbits have been used repeatedly over periods up to 15 months, without notable diminution in complement-binding capacity, or the development of anticomplementary properties.

Complement was obtained by bleeding 3 large guinea pigs 5 cc. from the heart on the afternoon before an experiment. The complement titer of the pooled serum was determined accurately just before each experiment, under conditions such as were to be employed in it: 9 tubes were set up with 0.4 cc. of 0.9 per cent saline in each, to render a volume comparable to that of the tests. Then 0.2 cc. of various dilutions of the pooled serum from 1:12 to 1:48 were run in, and finally 0.4 cc. of sensitized cells. The tubes were put into the water bath at 37°C. for 30 minutes and read immediately thereafter. The highest dilution of serum showing complete hemolysis was taken to represent one hemolytic unit of complement. 2 units of complement or slightly more were used in all of the tests reported here, but in other tests as much as 16 units of complement (the maximum amount tested) has been fixed when a potent antigen and antiserum were mixed. The pooled guinea pig sera were usually found to give complete hemolysis at a dilution between 1:20 and 1:40, and the serum was therefore used in the tests at a dilution of 1:10 to 1:20. Rarely the complement titer was found to be less than 1:20. In these cases the material was deemed unsuitable and therefore not used.

Preparation of the Antigens.—The papilloma virus can be readily recovered

from growths preserved for many months in 50 per cent glycerol; and so can the complement-binding antigen, as will appear. For this reason, and because our supply was great, we have used glycerolated tissues almost wholly as a source of antigen, though extracts of papillomas freshly procured and extracts of growths preserved by drying while frozen have also been found effective as complement-binding antigens.

Extracts of the preserved rabbit papillomas and other rabbit tissues were made by grinding the tissues thoroughly with sand in a mortar and suspending them in the required volume of 0.9 per cent saline (dilutions usually 1:10 or 1:20, sometimes 1:5 to 1:320). The crude suspensions were left overnight (occasionally longer) in the ice box. Just before use they were centrifugalized at 3500 R.P.M. for 5 minutes in the International centrifuge with angle head (51°), and the supernatant fluids removed with pipettes. These were again thrown down at 3500 R.P.M. for 15 minutes and the supernatant fluids, free from gross particles and almost water-clear, were carefully removed. Often the extracts were passed through Berkefeld filters in addition. A few papilloma extracts were slightly anticomplementary in the dilutions used, but it was learned early that the anticomplementary effect could be abolished or reduced by further dilution, or by heating at 56°C. for 30 minutes. All antigens were therefore heated immediately before use as routine, occasionally becoming very slightly opalescent in consequence.

The antigens were generally used on the day following their preparation, but occasionally 3 days or longer elapsed prior to their use. It can be stated, however, that a potent extract tested after standing 4 months in the refrigerator was found to have retained its complement-binding capacity.

Small portions of the glycerolated papillomas of a number of individual wild rabbits have been used repeatedly as sources of antigen with consistent results. Occasionally, however, as a given material became almost exhausted, with only a few remnants of tissue remaining, its effectiveness as a complement-binding antigen became somewhat less, as did its demonstrable content of virus.

Hemolytic System.—A 5 per cent suspension of thrice washed sheep cells in 0.9 per cent saline was made for each experiment. Rabbit amboceptor from 2 sources was used; one constantly throughout the early course of the work, and, when it was exhausted, the second for the remainder. Each was titrated at intervals of one week during the period of its use. The hemolytic titer of the first was constant at a dilution of 1:800, of the second at 1:3200. The 5 per cent sheep cell suspension was mixed in equal parts with the amboceptor (diluted to contain 2 hemolytic units in a volume of 0.2 cc.) and incubated at 37°C. for 10 minutes before it was added to the tubes of the test.

Procedure.—Quantities of 0.2 cc. were used in order to conserve materials. This made it expedient to use small test tubes (pyrex, lipped, round bottom, length 9 cm., internal diameter 9 mm.) and standardized pipettes, and to exercise precision in measurement.

The sera to be tested were first diluted with saline as required, and heated at 56°C. for 30 minutes, then 0.2 cc. of each was placed in the bottom of the appropriate tube. 0.2 cc. of complement (titrated immediately beforehand and diluted to contain 2 hemolytic units in 0.2 cc.) was then run in carefully near the bottom of each tube, and next 0.2 cc. of the antigen. Occasionally antigen was run into the tubes first and serum last, but complement was always added second. The tubes were gently shaken and allowed to stand at room temperature for 2 hours. 10 minutes before this time had elapsed the 5 per cent suspension of washed sheep cells was mixed in equal parts with the amboceptor (containing 2 units in 0.2 cc.) and put into the water bath at 37°C. for 10 minutes. 0.4 cc. of the sensitized cells were then added to all tubes and these put into the water bath at 37°C. for 30 minutes. Readings were recorded immediately thereafter, in terms of fixation:

++++	=	complete		fixation	of	complement	(no		hemolysis)	
+++	=	about	75%	"	"	"	(about	25%	"	)
++	=	"	50%	"	"	"	("	50%	"	)
+	=	"	25%	"	"	"	("	75%	"	)
±	=	"	10%	"	"	"	("	90%	"	)

The tubes were kept overnight in the refrigerator and read a second time according to the same scale the following morning. Occasionally the two readings varied slightly, and when this happened the second reading was regarded as representing the real end point of the reaction and was thus recorded finally in the protocols.

Tests for non-specific anticomplementary effect of each serum and antigen were set up concurrently in every experiment. Unless specifically stated in the protocols these contained twice the maximum amount of the material used in the experiment. In the absence of anticomplementary effect of either serum or antigen when tested in double amount, any reading of + or more was considered significant.

Sterile glassware and instruments were regularly employed.

Complement Fixation Tests with the Sera of Cottontail Rabbits

It was necessary to learn at the start whether complement fixation would occur if the serum of rabbits bearing the papillomas was mixed with antigens made from the growths. In a first experiment to test the point, the sera of 6 cottontail rabbits bearing naturally occurring papillomas were tested for capacity to bind complement when mixed with virus-containing extracts of glycerolated papillomas from 2 cottontails. The sera of 5 normal cottontails obtained from the same locality in Kansas were tested concurrently.

Experiment 1.—The 5 normal cottontails as well as the 6 bearing naturally occurring papillomas were kept under observation about 4 months before they were bled. At the time of bleeding one of the latter (W.R. 6) had only 3 tiny growths, the largest 1 x 0.4 cm. across and 8 mm. high. Another (W.R. 7) bore a single fleshy papilloma, 2.4 x 2.0 cm. across and 1 cm. high; while each of the 4 remaining animals (W.R. 8, 9, 10, 11) had 7 to 9 large, fleshy, onion-like or discoid papillomas, 2 to 4 cm. across, with peaks 2 to 4 cm. high. The growths were removed from all and put separately into 50 per cent glycerol-Locke's solution. Later the tissues were weighed, after the glycerol had been allowed to drain away and the papillomas dried on sterile gauze.

Two extracts of wild rabbit papillomas were used as antigens. The naturally occurring papillomas of W.R. 1240 had been in 50 per cent glycerol for 33 months. Many previous tests had shown that extracts of these contained virus in high titer. The experimentally induced papillomas of W.R. 42-N had been in glycerol for 16 months. Extracts of these papillomas were known to be infectious also, but much less so than were those of W.R. 1240. 1 gm. of the glycerolated tissue from each animal was ground separately and made up to a volume of 20 cc. with 0.9 per cent saline. These crude suspensions were left overnight in the refrigerator, then centrifugalized twice at 3500 R.P.M. for 5 and 15 minutes, with removal of the second, water-clear supernatant fluids for use. The tests were set up in the routine way previously described.

The findings set down in Table I make it evident that complement fixation does occur when saline extracts of the virus-induced papillomas are mixed with the sera of rabbits bearing the growths: all of the sera of the 6 cottontail rabbits bearing naturally occurring papillomas bound complement in the test, while the sera of the 5 normal cottontails did not do so. It will be noted further that the serum of W.R. 6, a rabbit with 3 tiny papillomas weighing only 300 mg. all told, fixed complement hardly at all; and the serum of W.R. 7, an animal bearing a single, good sized, fleshy growth, bound it only partly. The remaining 4 sera, from cottontails with several large, fleshy growths, gave practically complete fixation. These findings suggest a certain relation between the amount of papilloma tissue borne by an animal and the capacity of its serum to bind complement, a point which will receive further consideration later on.

In a second experiment the sera of 5 cottontails, which had borne experimentally induced papillomas for a considerable time, were similarly tested for complement-binding capacity. The sera of only 3 normal cottontails were available for comparison.

Experiment 2.—The 5 cottontails had been inoculated with 2 potent strains of virus into small areas of shaved skin on each flank with the tattoo machine. Growths appeared in all at every inoculated site after 15 to 25 days. In only one animal were the growths notably vigorous, but all were kept until 136 days had elapsed. When bled on this day one animal (W.R. 16) had 3 fleshy growths 2 to 3 cm. across and 1 cm. high; whereas complete retrogression of 2 growths had

TABLE I

Complement Fixation Tests with the Sera of Normal Cottontail Rabbits and of Others with Naturally Occurring Papillomas

Source of serum	Cottontail rabbit No.	Weight of the papillomas carried by the rabbit	Complement fixation tests		
			Antigen W.R. 1240	Antigen W.R. 42-N	Serum controls (no antigen)
		gm.			
Normal cottontail rabbits	W.R. 1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
Cottontail rabbits bearing naturally occurring papillomas	6	0.3	±	++	0
	7	3.2	++	+++	0
	8	10.5	++++	+++±	0
	9	14.4	+++±	+++	0
	10	17.4	++++	+++	0
	11	22.9	++++	+++±	0
Antigen controls (no serum).....			0	0	

Complement, 2 units in all tubes.

Sera diluted 1:4 in the tests with antigens, 1:2 in control tests.

Antigens, 1:20 saline extracts of glycerolated papillomas from 2 cottontail rabbits.

++++ = complete fixation (no hemolysis).
+++ = about 75% "
++ = " 50% "
+ = " 25% "
± = " 10% "
0 = no fixation (complete hemolysis).
W.R. = wild rabbit.
D.R. = domestic rabbit.

occurred in another (W.R. 17). The remaining 3 each bore one or two small, dry papillomas.

None of the 3 normal cottontail sera fixed complement in this test (Table II), while the sera of all of the 5 rabbits bearing the experimentally induced growths bound it completely.

In a third experiment the sera of 6 other cottontail rabbits were tested under broader conditions, to see if a relation exists between the amount of papilloma tissue borne by an animal and the complement-binding capacity of its serum.

TABLE II

Complement Fixation Tests with the Sera of Normal Cottontail Rabbits and of Others Bearing Experimentally Induced Papillomas

Source of serum	Cottontail rabbit No.	Complement fixation tests	
		Antigen W.R. 33-N	Serum controls (no antigen)
Normal cottontail rabbits	W.R. 12	0	0
	13	0	0
	14	0	0
Cottontail rabbits bearing experimentally induced papillomas	15	++++	0
	16	++++	0
	17	++++	0
	18	++++	0
	19	++++	0
Antigen control (no serum).....		0	

Complement, 2 units in all tubes.
Sera diluted 1:4 in tests with antigen, 1:2 in control tests.
Antigen, 1:10 saline extract of glycerolated cottontail papillomas.

Experiment 3.—To produce papilloma material for various purposes, a 10 per cent extract of the glycerolated growths of W.R. 7-N was rubbed into scarified areas on both sides of 20 normal cottontail rabbits. After 14 days confluent and semiconfluent growths had appeared in many of the animals, and soon thereafter in the remainder. A number of the rabbits were killed early, to procure their papillomas, and their sera were not taken. 8 individuals were kept, however, and these were bled from the heart at various times between the 28th and 116th days, records being made from time to time of the size and character of their growths. The sera were stored in the refrigerator for 4 months, during which time 2 specimens became cloudy owing to growth of bacteria, and hence were discarded. The 6 remaining sera were tested according to the routine procedure in dilutions of 1:4, 1:8, 1:16, and 1:32, with a 1:20 extract of W.R. 1240 papillomas as antigen.

TABLE III

Complement Fixation Tests with the Sera of Cottontail Rabbits Bearing Experimentally Induced Papillomas

Cottontail rabbit No.	Character and size of the growths when serum was procured	Day after virus inoculation when serum was procured	Complement fixation tests				
			Serum dilutions				Serum controls (no antigen)
			1:4	1:8	1:16	1:32	
W.R. 20	A few tiny, scattered, dry, discrete papillomas on both sides, none over 1 mm. high	28th	0	0	0	0	0
21	A few discrete, semiconfluent, dry papillomas on both sides, 2 mm. high. All had retrogressed by the 83rd day	83rd	0	0	0	0	0
22	9–10 discrete and semiconfluent growths on both sides, 1 cm. high	83rd	++++	++++	0	0	0
23	Semiconfluent growths over areas 6 x 4 cm. on both sides 1 cm. high	69th	++++	++++	++++	±	0
24	Confluent, fleshy papillomatous masses over areas 6 x 4 cm. on both sides, with peaks 3 cm. high	69th	++++	++++	++++	+++±	0
25	Confluent, fleshy papillomatous masses over areas 6 x 4 cm. on both sides with peaks 3½ cm. high	113th	++++	++++	++++	++++	0
Antigen control (no serum).....			0				

Complement, 2 units in all tubes.

Antigen, 1:20 saline extract of glycerolated papillomas (W.R. 1240).

Two of the sera (from W.R. 20 and 21) did not fix complement under the conditions of the test (Table III). These rabbits had borne only a few small growths over comparatively short periods. (W.R. 21 had been bled on the 83rd day, but its small growths had retrogressed completely several weeks previously.)

The serum of W.R. 22, withdrawn on the 83rd day, fixed complement completely in a dilution of 1:8, but not at all in a dilution of 1:16; while the serum of W.R. 24, secured a fortnight previously, gave complete or almost complete fixation in all dilutions up to 1:32. A reason for the difference in serum-antibody titer is at once apparent in the different course of the growths in these 2 animals, the papillomas of W.R. 24 being much more numerous and much larger on the 68th day than were those of W.R. 22 on the 83rd day. W.R. 25 had borne large, confluent growths for a long period, and its serum fixed complement in all dilutions tested.

The findings in this experiment (Table III) confirm and extend those already presented. It is evident that the sera of cottontail rabbits bearing the virus-induced papillomas, whether naturally occurring or experimentally induced, will fix complement in the presence of extracts of the growths, while the sera of normal cottontails have exhibited no such capacity. There is, furthermore, an evident relation between the total mass of papilloma tissue borne by an animal and the complement-binding capacity of its serum: in general, the sera of rabbits that have borne large growths over long periods fix complement in higher titer than the sera of others with small growths of shorter duration.

Tests with the Sera of Domestic Rabbits

The Shope virus causes characteristic papillomas when inoculated experimentally into the skin of domestic rabbits, and most of these animals develop virus-neutralizing antibodies after carrying the growths for a while (1, 3). Will their sera fix complement in the presence of suitable antigens, as the sera of cottontails with virus-induced papillomas have just been shown to do? To test the point, the sera were tested of 5 normal domestic rabbits and of 5 others that had borne large virus-induced papillomas for a considerable period.

Experiment 4.—5 normal domestic rabbits (Belted Dutch) had been inoculated with a 5 per cent suspension of W.R. 1240 virus by rubbing it into large scarified areas (about 9 x 10 cm.) on each flank. 8 days later small papillomas had appeared, scattered rather thickly over every inoculated site. These rapidly enlarged into large, confluent papillomatous expanses in every animal; but in 2 of them (D.R. 5 and 6) the growths were somewhat larger, fleshier, and more vigorous than in the remainder. By the 52nd day all bore huge, confluent papillomatous masses on both sides, 2 to 3 cm. high. All were bled 50 cc. from the

heart on this day, as were 5 normal Dutch rabbits from the same breeding colony, which has been kept in an isolated room for control purposes. After storage in the ice box for approximately 5 weeks the sera were tested for ability to bind complement in the presence of an antigen consisting of a freshly prepared 10 per cent Berkefeld V filtrate of the glycerolated papillomas of W.R. Tx, known from previous tests to contain virus in considerable amount.

TABLE IV

Complement Fixation Tests with the Sera of Normal Domestic Rabbits and of Others Bearing Large, Experimentally Induced Papillomas

Source of serum	Domestic rabbit No.	Character and size of growths on both sides	Complement fixation tests			
			Serum dilutions			Serum controls (no antigen)
			1:2	1:4	1:6	
Normal domestic rabbits	1	Nil, control	0	0	0	0
	2	" "	0	0	0	0
	3	" "	0	0	0	0
	4	" "	0	0	0	0
	5	" "	0	0	0	0
Domestic rabbits bearing large, experimentally induced papillomas	6	Confluent, 12 x 11 cm.	++++	++++	++++	0
	7	" 12 x 10 "	++++	++++	++++	0
	8	" 9 x 9 "	++++	++++	++++	0
	9	" 10 x 8 "	++++	+++±	+++	0
	10	" 10 x 10 "	+++±	++±	++	0
Antigen control (no serum).....				0		

Complement, 2 units in all tubes.
Sera diluted as indicated. All were procured on the 52nd day after inoculation.
Antigen, 1:10 Berkefeld V filtrate of cottontail papillomas (W.R. Tx).

None of the normal sera fixed complement, while the sera from the 5 papillomatous rabbits gave fixation in the 3 dilutions tested (Table IV).

In the following experiment the findings were extended by testing the sera of domestic rabbits that had borne somewhat smaller growths but over a longer period of time. 2 normal sera were included for comparison.

Experiment 5.—10 Dutch rabbits that had carried papillomas for long periods were bled. 2 of these (D.R. 11 and 12) had borne for 11 months 18 discrete

papillomas, the outcome of tattoo inoculation of virus. When the serum was procured the growths were large and discoid, averaging 3.5 cm. in diameter. 3 other animals (D.R. 13, 14, and 15) had been inoculated 4 months previously by tattooing virus into 6 small areas (2 mm. in diameter) on each side. When bled, these carried 12 discoid papillomas 2 to 4 cm. across. The remaining 5 rabbits bore 4, 5, or 6 discrete, semiconfluent or confluent growths on each side, which had been brought about by rubbing 4, 5, or 6 strains of virus into as many scarified rectangles on each side 6 months previously.

TABLE V

Complement Fixation Tests with the Sera of Normal Domestic Rabbits and of Others Bearing Experimentally Induced Papillomas

Source of serum	Domestic rabbit No.	Antigen W.R. 33-N	Serum controls (no antigen)
Normal domestic rabbits	1	0	0
	2	0	0
Domestic rabbits bearing large experimentally induced papillomas	11	++++	0
	12	++++	0
	13	++++	0
	14	++++	0
	15	++++	0
	16	++++	0
	17	++++	0
	18	++±	0
	19	++	0
	20	+	0
Antigen control (no serum).....		0	

Complement, 2 units in all tubes.
Sera diluted 1:4 in the tests with antigen, 1:2 in control tests.
Antigen, 1:10 Berkefeld V filtrate of glycerolated cottontail papillomas.

The sera were tested according to the routine procedure, with an antigen consisting of a 10 per cent extract of the glycerolated infectious papillomas of W.R. 33-N prepared as usual on the preceding day.

The results of the experiment are summarized in Table V. 7 of the 10 sera gave complete fixation, one (D.R. 20) gave almost none, while 2 others (D.R. 18 and 19) showed only moderate capacity to bind complement. The results with these latter 3 sera evidently represented variations in host reactions, for the papillomas of these

rabbits were as large and numerous as in some of the others, and they had been present for 6 months.

Experiments 4 and 5 show that the sera of domestic rabbits bearing experimentally induced papillomas generally possess the capacity to bind complement when mixed with antigens containing the virus, while the sera of normal domestic rabbits have no such ability.

Tests with Sera from Animals Carrying Tar Papillomas

It is conceivable that the results of the foregoing tests depend upon some constituent present in papillomas generally. For this reason an experiment was done next to see whether or not the sera of rabbits bearing papillomas elicited by tarring would bind complement under similar conditions.

Experiment 6.—Sera were procured from 6 gray-brown domestic rabbits bearing papillomas elicited by repeated applications of tar to the inner surfaces of their ears. All of these animals had been kept in isolation. One (D.R. 21) had been tarred twice weekly for 11 months. It bore 19 tar papillomas up to 7 mm. across and 1 cm. high. Another (D.R. 22) had been tarred similarly for 5 months: it had 4 large growths, one 2 cm. in diameter. The remainder had been tarred for 3½ months: all had 8 to 12 tar papillomas up to 1 cm. in diameter.

The sera were tested as usual with an antigen consisting of a Berkefeld V filtrate of the glycerolated papillomas of W.R. Tx.

In this test (Table VI) none of the sera from the rabbits bearing tar papillomas fixed complement in the presence of a potent antigen made from the virus-induced papillomas, whereas specimens from 2 domestic rabbits with growths of the latter sort bound it completely.

Tests with Sera from Animals Immune to the Shope Fibroma Virus

To test the specificity of the reaction further, sera were now employed from domestic rabbits immune to the fibroma virus as result of previous infection with it, and from others immune both to the fibroma virus and to myxoma virus.

Experiment 7.—The sera were generously provided by Dr. Shope. 2 were from domestic rabbits immune to the inflammatory strain of the fibroma virus. 7 others were from domestic rabbits in which fibromas had retrogressed and in which test inoculations with the myxoma virus had proven negative. All of the sera had been kept from 15 to 27 months in the refrigerator yet all were clear. They were tested in the routine way with an antigen consisting of a

freshly prepared 10 per cent Berkefeld V filtrate of the infectious papilloma of W.R. Tx.

In this experiment (Table VII) none of the sera from 2 rabbits immune to the fibroma virus and from 7 rabbits immune to the fibroma and myxoma viruses fixed complement in the presence of a potent papilloma antigen. The results are open to some question, however, because the sera had been kept for a long period before testing. In this connection it may be pointed out, though, that we have recently

TABLE VI

Complement Fixation Tests with the Sera of Domestic Rabbits Bearing Virus-Induced Papillomas and of Others with Papillomas Elicited by Tarring

Source of serum	Domestic rabbit No.	Antigen W.R. Tx	Serum controls (no antigen)
Domestic rabbits bearing papillomas elicited by tarring	21	0	0
	22	0	0
	23	0	0
	24	0	0
	25	0	0
	26	0	0
Domestic rabbits bearing virus-induced papillomas	7	++++	0
	8	++++	0
Antigen control (no serum)		0	

Complement, 2 units in all tubes.
Sera diluted 1:4 in tests with antigen, 1:2 in control tests.
Antigen, 1:10 Berkefeld V filtrate of glycerolated cottontail papillomas.

tested 3 sera that had been kept for 12 to 15 months in the refrigerator. None had become anticomplementary and all had retained their capacity to bind complement without perceptible loss in titer.

Further Control Tests on Specificity

In further elucidation of the specificity of the complement fixation reaction a test was done with the sera of 3 rabbits immune to vaccine virus, 4 with experimental syphilis, and one hyperimmunized against herpes virus.

Experiment 8.—The serum of the domestic rabbit hyperimmunized against herpes virus and of the 3 rabbits immune to vaccinia were generously provided by Drs. Rivers and Smadel. Dr. T. B. Turner kindly sent sera from 4 rabbits with experimental syphilis. The serum of one of these latter (D.R. 40) had been found markedly positive when tested according to the routine Kolmer-Wassermann technique. The others had not been tested. None had been kept longer than 4 months. In the present experiment, sera from 2 domestic rabbits with experimental papillomas were used for comparison: these had been kept 5 months

TABLE VII

Complement Fixation Tests with the Sera of Normal Domestic Rabbits and of Those Immune to the Fibroma and Myxoma Viruses

Source of serum	Domestic rabbit No.	Antigen W.R. Tx	Serum controls (no antigen)
Domestic rabbits immune to fibroma virus	27	0	0
	28	0	0
Domestic rabbits immune to both fibroma and myxoma viruses	29	0	0
	30	0	0
	31	0	0
	32	0	0
	33	0	0
	34	0	0
	35	0	0
Domestic rabbits bearing virus-induced papillomas	7	++++	0
	8	++++	0
Antigen control (no serum)		0	

Complement, 2 units in all tubes.
Sera diluted 1:4 in tests with antigen, 1:2 in control tests.
Antigen, 1:10 Berkefeld V filtrate of glycerolated cottontail papillomas.

in the refrigerator. All the specimens were tested in a dilution of 1:2 with 2 potent antigens. One antigen consisted of a 1:40 suspension of the glycerolated papillomas of W.R. 8-N, a notably infectious material; while the second was made by extracting the infectious papillomas of W.R. 33-N in a dilution of 1:10.

In this experiment (Table VIII) none of the sera from domestic rabbits immune to vaccinia or herpes viruses, or with experimental syphilis, fixed complement specifically with either of the 2 antigens, while both of the sera from papilloma-bearing rabbits bound it com-

pletely. It will be noted that there was a slight amount of fixation by 3 of the sera (D.R. 38, 41, and 43) in the control tests as well as with the antigens. This amount of fixation was deemed insignificant, being attributable to non-specific anticomplementary effect, perhaps consequent on the fact that the sera were used in a dilution of 1:2 and had stood for some months before being utilized.

TABLE VIII

Complement Fixation Tests with the Sera of Domestic Rabbits Immune to Vaccine Virus, and of Others with Experimental Syphilis

Source of serum	Domestic rabbit No.	Antigens		Serum controls (no antigen)
		W.R. 8-N 1:40	W.R. 33-N 1:10	
Rabbits immune to vaccine virus	36	0	0	0
	37	0	0	0
	38	0	±	±
Rabbit hyperimmunized against herpes virus	39	0	0	0
Rabbits with experimental syphilis	40	0	0	0
	41	±	±	±
	42	0	0	0
	43	+	+	±
Rabbits bearing virus-induced papillomas	6	++++	++++	0
	7	++++	++++	0
Antigen controls (no serum).....		0	0	

Complement, 2 units in all tubes.
All sera diluted 1:2.
Antigens, saline extracts of glycerolated cottontail papillomas from 2 rabbits.

The findings indicate that the complement fixation test has a specificity comparable to that of the neutralization test involving the same virus, for it is known that sera from rabbits bearing tar papillomas, or from rabbits immune to the fibroma, myxoma, or vaccine viruses, do not neutralize the papilloma virus (3). Manifestly the humoral principles responsible for complement fixation are engendered by a specific antigen liberated from the virus-induced papillomas.

Comparative Titers of the Sera of Cottontail and Domestic Rabbits

The sera of the cottontail rabbits thus far tested (Tables I, II, and III) fixed complement in higher titer than those of domestic rabbits bearing comparable growths (Tables IV and V). In order to learn whether this relation holds true generally, a number of sera were tested from rabbits of both species bearing virus-induced papillomas of different size and duration.

Experiment 9.—5 belted-Dutch domestic rabbits, each bearing 8 large papillomas, were bled 5 cc. from an ear vein. These had been selected at random from a group of 26 related rabbits, all of which had been inoculated 100 days before by rubbing 4 potent strains of virus into as many scarified rectangles on each side. All of the growths on the selected rabbits were large, fleshy and vigorous, averaging 3.5 x 5 cm. in area, with keratinized peaks rising 2 to 3 cm. above the skin level; and there was little difference from rabbit to rabbit, the papillomas of D.R. 48 being only slightly larger and fleshier than those of its fellows. 3 sera previously used from cottontails with large papillomas were again tested, and 2 others. These latter had been recently procured from 2 animals inoculated 86 days before by rubbing a potent 10 per cent virus fluid (W.R. 7-N) into scarified areas about 6 x 8 cm. on their abdomens. The growths of W.R. 27 had been confluent and very vigorous from the start, with a number of rounded subcutaneous pearls beneath them; while those of its fellow (W.R. 26) had always been discrete and much smaller.

All of the sera were tested in various dilutions with 2 antigens consisting of freshly prepared 10 per cent Berkefeld V filtrates of glycerolated materials from two sources, both containing virus in high titer.

The findings are summarized in Table IX, from which it will be seen that the sera of only one of the domestic rabbits (D.R. 48) bound complement in significant titer, whereas 4 of the 5 cottontail sera bound it in much higher titer. The serum of W.R. 26, the cottontail with comparatively small growths, gave practically no fixation with either antigen. Before these findings are appraised it should be pointed out again that both antigens consisted of 1:10 filtrates of notably pathogenic materials with great complement-binding capacity. Some of the results may have been due to inhibition of fixation in the presence of excess of antigen (prozone phenomenon[1]).

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[1] This phenomenon has been encountered a number of times throughout the work, with inhibition of fixation when there was a large excess of either antigen or antibody; and in an experiment which need not be given in detail the phenomenon was purposely demonstrated. Precipitation reactions have also been observed a number of times in collateral tests, when potent antigens and antisera were mixed and incubated at 37°C. for several hours.

TABLE IX

Complement Fixation Tests with the Sera of Domestic and Cottontail Rabbits Bearing Virus-Induced Papillomas

Source of serum	Rabbit No.	Complement fixation tests										
		Antigen W.R. 35					Antigen W.R. 1240					Serum controls (no antigen)
		Dilutions of sera					Dilutions of sera					
		1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	
Domestic rabbits bearing virus-induced papillomas	44	0	0	0	0	0	0	0	0	0	0	0
	45	0	0	0	0	0	±	0	0	0	0	0
	46	0	0	0	0	0	0	0	0	0	0	0
	47	+±	0	0	0	0	0	0	0	0	0	0
	48	++++	++++	+±	0	0	++++	++++	0	0	0	0
Cottontail rabbits bearing virus-induced papillomas	26	±	0	0	0	0	0	0	0	0	0	0
	22	++++	+++±	0	0	0	++++	+++±	0	0	0	0
	24	++++	++++	++++	+++	±	++++	++++	++++	+++±	0	0
	25	++++	++++	++++	+++	0	++++	++++	++++	+++	0	0
	27	++++	++++	++++	++++	+++	++++	++++	++++	++++	+±	0
Antigen controls (no serum).....			0				0					

Complement, 2 units in all tubes.

Antigens, 1:10 Berkefeld V filtrates of glycerolated papillomas.

The cottontail serum that failed to bind complement in significant titer came from a rabbit bearing relatively small growths, as compared with its fellows; but no such obvious difference exists to account for the very wide variations in the titer of the domestic rabbit sera. The growths in these animals were produced with the same inoculum, and they differed but little from animal to animal,—all being large, fleshy, and vigorous, and of the same duration. Manifestly individual differences played a large part in the results, as in the case of virus neutralization (1, 3).

In this experiment the sera of cottontail rabbits bearing large papillomas bound complement in much higher titer than the sera of domestic rabbits with comparable growths. Many subsequent tests have shown this to be a general rule.

Relation of the Complement-Binding and Virus-Neutralizing Antibodies

The foregoing experiments have shown that the sera of cottontail and domestic rabbits bearing virus-induced papillomas will bind complement when mixed with antigens consisting of extracts of the growths. From previous work (3) it was known that the sera of rabbits with papillomas will generally neutralize the virus when mixed with it *in vitro*. What relation do these humoral principles bear to one another? Do the complement-binding and virus-neutralizing antibodies exist in the same relative proportions in the blood?

In the first of two experiments to decide this question 6 domestic rabbit sera, known to differ in their capacity to bind complement, were tested for ability to neutralize the virus.

Experiment 10.—The complement-binding titers were determined by testing the sera in dilutions of 1:2, 1:4, 1:6, 1:8, and 1:10, with an antigen consisting of a freshly prepared 5 per cent suspension of W.R. 33-N papillomas. On the same day neutralization tests were made with the same serum specimens by incubating mixtures of the serum in equal parts with a virus filtrate (containing approximately 400 infective units of virus per inoculation dose) and inoculating these into scarified rectangles on the skin of 3 normal domestic rabbits, according to a standard procedure (3). The mixtures were incubated for 2 hours at 37°C. before inoculation. The lesions on the test animals were recorded at intervals of 3 to 4 days between the 14th and 33rd days. In order to conserve space the readings on but one test rabbit, on the 29th day, are given in the table. These express accurately the differences in neutralizing capacity of the sera as manifested by the entire course of the lesions on all 3 test animals.

It will be seen (Table X) that the serum of D.R. 6 bound complement com-

pletely in dilutions of 1:2 and 1:4, and almost completely when diluted 1:8 and 1:10; while the serum of D.R. 8 bound it almost as well. These 2 sera neutralized the 5 per cent virus filtrate (about 2000 minimal infective doses) completely, and did so almost completely when diluted 1:2. The sera of D.R. 18 and 19 fixed the complement only partially in dilutions of 1:2 and 1:4; and these neutralized the 5 per cent virus only partially, although neutralizing 1 per cent virus com-

TABLE X

Neutralization and Complement Fixation Tests with the Sera of Six Domestic Rabbits Bearing Virus-Induced Papillomas

Serum	Neutralization tests*			Complement fixation tests					
Domestic rabbit No.	Whole serum + 1 per cent virus	Whole serum + 5 per cent virus	Serum 1:2 + 5 per cent virus	Dilutions of sera 1:2	1:4	1:6	1:8	1:10	Serum controls (no antigen)
6	++++	++++	+++±	++++	++++	+++	+++	+++	0
8	++++	++++	+++	++++	++++	+++	++	+	0
18	++++	++±	++	+++	++	+	0	0	0
19	++++	++	++	++	±	0	0	0	0
44	+++	++	n.t.	+	0	0	0	0	0
20	+++	±	n.t.	±	0	0	0	0	0
Antigen control (no serum).....				0					

Complement, 2 units in all tubes.
Antigen, 1:20 saline extract of glycerolated papillomas (W.R. 33-N).
* ++++ = complete neutralization (no growth at the inoculation site).
+++ = about 75% neutralization.
++ = " 50% "
+ = " 25% "
± = " 10% "
0 = no neutralization (*i.e.*, confluent growths as from the virus-saline control mixtures).
n.t. = not tested.

pletely. The sera of D.R. 44 and 20 showed little capacity to bind complement, even in dilutions of 1:2; but they neutralized the 1 per cent virus almost completely, and a 0.2 per cent dilution of the virus completely, as was shown in other tests not recorded in the table.

It is evident that the complement fixation titers of these sera varied in parallel with their virus neutralization titers. Furthermore it is plain that sera can possess a considerable virus-neutralizing capacity

without being able to bind complement to a noticeable degree, at least under the circumstances of the test. The findings pose the question whether the neutralization test is more sensitive than the complement fixation reaction, a matter that will be taken up after the next experiment.

In order to extend the findings use was now made of a fact learned in Experiment 9,—namely that the sera of many cottontail rabbits with virus-induced papillomas will bind complement in much higher titer than sera from domestic rabbits with comparable growths. Acting on this knowledge, domestic and cottontail sera known to vary widely in complement fixation titers were compared as to content of virus-neutralizing and complement-binding antibodies.

Experiment 11.—The serum of D.R. 46, which had not bound complement in the test of Experiment 10, and that of D.R. 48, which had bound it completely at dilutions of 1:4 and 1:8, and partially at 1:16, were selected for the neutralization tests; as were also the sera of W.R. 26 and 27, the former having shown practically no capacity to bind complement (Experiment 9), whereas the latter fixed it completely in all dilutions up to 1:32, and partially at 1:64. A normal domestic rabbit serum (D.R. 49) and a normal cottontail serum (W.R. 2) were included for comparison. Neither of these had bound complement in many tests with various potent antigens.

The neutralization tests were done in the standard way (3), with several dilutions of the sera mixed in equal parts with a centrifugalized, almost water-clear, 5 per cent suspension of glycerolated papillomas from cottontail 56, this material being slightly more potent than a 5 per cent filtrate of W.R. 1240 virus. The mixtures were put into the water bath for 2 hours at 37°C. before they were rubbed into scarified skin areas on the 3 test animals.

Table XI shows the results of the experiment. The neutralization readings are those with one test animal on the 20th day, according to the scale of Experiment 10. The 2 normal sera failed to bind complement, and they did not neutralize the virus. Mixtures of these sera in equal parts with the 5 per cent virus fluid produced confluent areas of papillomatosis covering the entire inoculation site, and wholly similar to those produced by the virus-saline control mixtures. The serum of D.R. 46, which did not fix complement at all, neutralized the virus partially when undiluted, but had no great effect on it when diluted 1:4 or 1:16. The serum of W.R. 26 neutralized the virus (more than 2000 infective doses) almost completely when undiluted, and partially when diluted 1:4, but only slightly at 1:16. This serum had bound complement slightly. The serum of D.R. 48, and that of W.R. 27, neutralized the virus in high titer, and bound complement likewise.

Nine of the 10 sera of these two experiments had parallel complement fixation and neutralization titers, the range of which varied greatly. One of them neutralized the virus in low titer, yet failed to bind complement in the tests; and several neutralized the virus in greater or less amount, but fixed complement only slightly or not at all. It is plain, therefore, that the complement-binding and virus-neutralizing antibodies exist in the same relative proportions in the

TABLE XI

Neutralization Tests with Cottontail and Domestic Rabbit Sera with Markedly Different Complement Fixation Titers

Serum	Neutralization tests			Complement fixation tests				
Rabbit No.	5 per cent virus (W.R. 56) +			Dilutions of sera				
	Whole serum	Serum 1:4	Serum 1:16	1:4	1:8	1:16	1:32	1:64
D.R. 49	0	0	0	0	0	0	0	0
46	++	+	0	0	0	0	0	0
48	++++	+++	+±	++++	++++	+±	0	0
W.R. 2	0	0	0	0	0	0	0	0
26	+++±	+	±	±	0	0	0	0
27	++++	++++	+++±	++++	++++	++++	++++	+++

++++ = complete neutralization or complete fixation.

Complement, 2 units in all tubes.

Antigen, 1:10 Berkefeld V filtrate of glycerolated papillomas of W.R. 35.

Control tests of antigen and of all sera in double amount showed no anti-complementary effect.

sera of rabbits bearing the papillomas; but the neutralization test appears to have a "lower threshold" than the complement fixation reaction, although it is not notably more sensitive in the effective range of the latter.

SUMMARY

The sera of rabbits bearing virus-induced papillomas have been found to bind complement when mixed with antigens consisting of extracts or filtrates of the growths containing the virus. The sera of normal rabbits, of those immune to other viruses (vaccinia, herpes, fibroma, myxoma), of rabbits with syphilis, or of those with papillomas

consequent on tarring, did not fix complement upon admixture with the papilloma antigens.

The complement-binding antibody was present in the serum specimens in the same relative proportions as the virus-neutralizing antibody, and both were present in greatest amount in the sera of rabbits that had borne large papillomas over considerable periods of time. A few sera were come upon that neutralized small amounts of the virus yet failed to bind complement to any noteworthy degree in the tests.

The sera of cottontail rabbits fixed complement and neutralized the virus in much higher titer than the sera of domestic rabbits with comparable growths.

The implications of the findings will be discussed in a subsequent paper, after the properties of the complement-binding antigen have been scrutinized.

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