A NATURAL ANTIBODY THAT REACTS IN VITRO WITH A SEDIMENTABLE CONSTITUENT OF NORMAL TISSUE CELLS

II. Specificity of the Phenomenon: General Discussion

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In the preceding paper experiments are described which demonstrate the presence of a natural antibody in the blood of normal adult rabbits which reacts with a sedimentable constituent of normal rabbit tissue cells. Observations will now be reported which deal further with the distribution and affinities of antigen and antibody and with their characters.[1]

Reaction of the Natural Antibody and Various Rabbit Tissue Extracts

In the foregoing experiments, saline extracts of various rabbit tissues nearly all reacted with sera containing the natural antibody, though there were noteworthy and fairly regular differences in their capacity to do so (Table VI). It seemed possible that the quantitative differences might be due either to a single substance present in various amounts in the different tissue cells which reacts with a single natural antibody, or to a variety of organ- or cell-specific substances each reacting with its own natural antibody. In an experiment to learn more about this, the sera of 8 normal rabbits of various breeds were tested in mixture with antigens made from the freshly procured kidney, liver, spleen, brain, and heart muscle tissues of a single rabbit. Table X shows the results of the tests, which confirm and extend those of the preceding experiments. As in the tests of Table VI, the various sera manifested regular differences in their abilities to react with the several antigens, in general fixing complement best in mixture with the kidney antigen, next best with the one made from liver, and then with those derived from spleen, brain, and heart muscle, in the order named. The serum with the highest titer of the natural tissue antibody, as shown by its ability to react with the kidney and liver antigens—that of rabbit 69—also reacted best with the spleen, brain, and heart muscle antigens. And so too, in diminishing titers, did the sera of rabbits 63, 65, and 66. The rest of the sera (70, 74, 77, and 71) exhibited progressively weaker reactions in mixture with the kidney antigen. They failed to react perceptibly with the heart antigen and gave weak fixation or none at all in mixture with the brain, spleen, and liver antigens.

[1] To facilitate discussion, the tables and references in the two papers are numbered consecutively.

The regularity of the findings just described would seem to suggest that the natural tissue antibody has a specific affinity for a single substance, which is present in various amounts in different tissues. Further evidence that this is the case is provided by the findings of the next experiment, in which sedimented substances derived from normal rabbit kidney, brain, and liver, respectively, were used in absorption tests with sera known to contain the natural antibody.

The sera were first heated at 56°C. for 15 minutes to inactivate complement, and then were absorbed with washed sheep erythrocytes (2.0 cc. of packed cells plus 6.0 cc. of each serum, incubated 15 minutes at 37°C.) to remove any natural sheep hemolysin.

TAB

Complement Fixation Tests with

Source of serum											
Rabbit No.	Breed of rabbit	Kidney					Liver				
		Serum dilution					Serum dilution				
		1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32
69	New Zealand	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
63	Agouti hybrid	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++±
65	" "	++++	++++	++++	++++	++±	++++	++++	++++	+++	+
66	New Zealand	++++	++++	++++	+++±	+±	++++	++++	++++	+	0
70	Chinchilla	++++	++++	++++	++	0	++++	+	0	0	0
74	Blue Cross hybrid	++++	++±	+±	0	0	++	0	0	0	0
77	" " "	++++	++±	0	0	0	0	0	0	0	0
71	Chinchilla	+++	+±	0	0	0	0	0	0	0	0

The antigens were 1:30 saline extracts made as usual from the fresh normal tissues of a single agouti rabbit

The kidney, brain, and liver particles to be used for absorption were prepared by grinding the fresh tissues of a single rabbit, suspending these 1:10 in saline and centrifuging at 4400 R.P.M. for 10 minutes. The supernatant fluids were then spun at 25,000 R.P.M. for 1 hour in the air-driven machine, and the sedimented materials resuspended in one-third the original volume of dilute phosphate buffer, pH 7.3. The suspensions of kidney and liver particles were densely opalescent, that of the brain particles much less so. Just before use all of the suspensions were rendered isotonic by the addition of suitable amounts of 18 per cent NaCl solution. Mixtures of the four sera were made with 3 volumes of saline, and with 3 volumes of the 1:10 suspensions of kidney, brain, and liver particles, respectively. The absorption mixtures were incubated 2 hours at 37°C., then kept overnight in the refrigerator. None developed visible floccules. They were all spun at 25,000 R.P.M. for 1 hour and the supernatant liquids removed for test with antigens consisting of 1:30 saline extracts made as usual from the frozen kidney, brain, and liver tissues of another rabbit.

Table XI shows the results of the tests. All of the control mixtures of sera and saline reacted with the three antigens in varying degrees, whereas the specimens that

had been absorbed with the kidney substance failed to do so. The sera that had been absorbed with the brain material had no capacity to react with the brain and liver antigens and their ability to react with the kidney antigen was markedly reduced. The liver substance had absorbed completely the capacity to react with liver antigen and largely that to react with brain antigen, and had brought about a marked reduction in the reactions with the kidney antigen.

Manifestly some antibody remained in the mixtures absorbed once with the brain and liver materials, for these retained in some part their capacity to react with the kidney antigen. To learn whether this residual antibody could be removed, the specimens of rabbits 15-88 and 15-90 that had been absorbed once with brain substance were again absorbed as before, this time with an equal volume of a 1:10 suspension of

LE X

arious Rabbit Sera and Antigens

ANTIGENS															
	Spleen					Brain					Heart muscle				
	Serum dilution					Serum dilution					Serum dilution				
	1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32	1:2	1:4	1:8	1:16	1:32
	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++	++
	++++	++++	++++	++++	++±	++++	++++	++++	++++	+	++++	++++	++++	++++	±
	++++	++++	++++	+++	0	++++	++++	+++±	++	0	++++	++++	++	±	0
	++++	++++	+++	±	0	++++	+++	++	0	0	+++	+	0	0	0
	±	0	0	0	0	±	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

t (D.R. 19-55).

rabbit liver substance prepared as previously from the frozen tissues of the same animal; and likewise the specimens of rabbits 5-74 and 15-84 that had been absorbed first with liver were reabsorbed with a fresh preparation of brain. Subsidiary tests were then made to see whether the twice absorbed sera, along with appropriate saline controls, would still react with a normal rabbit kidney antigen. The controls did so, precisely as in the table; but the reabsorbed sera gave no reactions whatever.

In sum, the various tissue substances differed notably in their capacity to absorb the natural antibody and to fix complement in mixture with it, but there is no suggestion that these differences were more than quantitative (Table XI). On the contrary, the kidney substance (which was the most potent) absorbed completely the capacity of the sera to react with all of the antigens, and repeated absorption with either brain or liver substance (which were less potent) had the same effect. The findings have been repeatedly confirmed in other similar experiments.

TABLE XI

Absorption of the Natural Antibody with Suspensions of Sedimented Substances from Rabbit Kidney, Brain, and Liver

Normal rabbit (sera)	Absorbed with*	Complement fixation tests														
		Kidney antigen					Brain antigen					Liver antigen				
		Serum dilution					Serum dilution					Serum dilution				
		1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64
5-74	Nil, saline control	++++	++++	++++	++	0	+++±	++±	0	0	0	+++	++	+	0	0
15-84	" " "	++++	++++	++++	+++±	0	++++	++++	++	0	0	+++	++±	+±	0	0
15-88	" " "	++++	++++	++++	+++	0	++++	++++	++	0	0	+++	+±	0	0	0
15-90	" " "	++++	++++	++++	+++	0	++++	+++	+±	0	0	++	+±	0	0	0
5-74	Kidney substance	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15-84	" "	0	0	0	0	0	±	0	0	0	0	0	0	0	0	0
15-88	" "	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15-90	" "	0	0	0	0	0	±	0	0	0	0	0	0	0	0	0
5-74	Brain substance	+++	++	0	0	0	0	0	0	0	0	0	0	0	0	0
15-84	" "	++++	++++	++	0	0	0	0	0	0	0	0	0	0	0	0
15-88	" " ‡	++++	+++	+±	0	0	0	0	0	0	0	0	0	0	0	0
15-90	" " §	++++	+++	±	0	0	0	0	0	0	0	0	0	0	0	0
5-74	Liver substance §	++++	+++	±	0	0	±	0	0	0	0	0	0	0	0	0
15-84	" " ‡	++++	++++	++±	+	0	++±	+±	0	0	0	0	0	0	0	0
15-88	" "	++++	++++	++±	0	0	++±	0	0	0	0	0	0	0	0	0
15-90	" "	++++	++++	++±	0	0	++±	±	0	0	0	0	0	0	0	0

Antigens, 1:30 saline extracts of frozen normal rabbit tissues from a single normal rabbit.

* See text for description of method and materials.

‡ Negative after reabsorption with brain substance,—see text.

§ Negative after reabsorption with liver substance,—see text.

Reaction of the Natural Rabbit Antibody with Extracts of Tissues of Alien Species

The fact that the substance with which the natural antibody reacts can be extracted from many rabbit tissues (Tables VI and X) brings up the possibility that it may be present also in the tissues of other species. This proved to be the case. Tests were made using rabbit sera known to contain the natural antibody in mixture with saline extracts of the liver, kidney, and brain of the mouse, guinea pig, chicken, and rat. Fixation was invariably got, and the reactions were about as potent as those with extracts of corresponding rabbit organs. As a further step towards identifying the affinities of the natural antibody, it seemed important to learn whether absorption of rabbit sera with sedimented substances from rabbit tissues would remove their capacity to react with extracts of the tissues of alien species.

The results of an experiment to test the question are set down in Table XII, in which the sera of two domestic rabbits were absorbed precisely as in the experiment of Table XI (*q.v.*) with sedimented substances derived from rabbit kidney and liver. The absorbed sera, along with control, unabsorbed specimens, were tested with antigens made from the frozen kidney, liver, and brain tissues of various normal animals (rabbit, mouse, guinea pig, chicken, and rat). Both of the unabsorbed specimens gave fixation in mixture with all of the tested antigens (the chicken brain antigen was omitted through an oversight). As was expected from the findings already mentioned, fixation was about as potent with antigens procured from the alien species as with those from the rabbit, though the rat kidney antigen was notably more potent in this experiment for some reason not understood. Absorption with the sedimented substance from normal rabbit kidney rendered both sera devoid of capacity to react with any of the antigens, except for slight reactions in the mixtures containing rat and mouse kidney antigens. Absorption with the liver substance, which, as in the preceding experiment, was less potent as an absorbing material than the kidney substance, also removed largely or completely the capacity of the sera to react with all of the antigens, whether derived from rabbit tissues or from those of the other species.

Absorption with sedimented substances from normal rabbit tissues removed not only the capacity of rabbit sera to react with extracts of homologous tissues, but also their ability to react with extracts of the tissues of alien species (Table XII). It follows that the tissue substance with which the natural antibody reacts is probably the same, or much the same, whether derived from rabbit tissues or from those of other species.

The Natural Tissue Antibody As Distinct from Those Directed Specifically against the Rabbit Papilloma Virus and the Distinctive Substance of the Brown-Pearce Tumor, Respectively

In previous serological studies with the rabbit papilloma virus (6) and the distinctive substance of the Brown-Pearce tumor (1), the test antigens were prepared as routine from glycerolated (or occasionally frozen) tissues. These

TABLE XII

Complement Fixation Reactions with Normal Rabbit Sera and Tissue Antigens from Other Species

Normal rabbit sera	Absorbed with	Source of antigen	Kidney antigens					Liver antigens					Brain antigens				
			Serum dilution					Serum dilution					Serum dilution				
			1:8	1:16	1:32	1:64	1:128	1:8	1:16	1:32	1:64	1:128	1:8	1:16	1:32	1:64	1:128
15-88	Nil, saline control	Rabbit	++++	++++	++±	0	0	++±	+	0	0	0	+++	0	0	0	0
		Mouse	++++	+++±	+	0	0	++	0	0	0	0	+++±	+±	0	0	0
		Guinea pig	++++	+++±	+	0	0	+±	0	0	0	0	+++±	±	0	0	0
		Chicken	++++	+++±	0	0	0	+++±	++	0	0	0					
		Rat	++++	++++	++++	+++±	0	+++±	+	0	0	0	++++	±	0	0	0
	Sedimented substance from rabbit kidney	Rabbit	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Mouse	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Guinea pig	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Chicken	0	0	0	0	0	0	0	0	0	0					
		Rat	+	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sedimented substance from rabbit liver	Rabbit	+++	±	0	0	0	0	0	0	0	0	0	0	0	0	0
		Mouse	+++	0	0	0	0	0	0	0	0	0	+±	0	0	0	0
		Guinea pig	+++	0	0	0	0	0	0	0	0	0	±	0	0	0	0
		Chicken	+±	0	0	0	0	0	0	0	0	0					
		Rat	++++	+++±	+++	0	0	±	0	0	0	0	+±	0	0	0	0
15-90	Nil, saline control	Rabbit	++++	++++	+++	0	0	++	0	0	0	0	+±	0	0	0	0
		Mouse	++++	++++	++±	0	0	++	0	0	0	0	++++	+++	0	0	0
		Guinea pig	++++	+++±	++	0	0	++	0	0	0	0	+++	±	0	0	0
		Chicken	++++	+++	±	0	0	+++±	++±	0	0	0					
		Rat	++++	++++	++++	+++	0	++++	+	0	0	0	+++±	+±	0	0	0
	Sedimented substance from rabbit kidney	Rabbit	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Mouse	+±	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Guinea pig	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		Chicken	0	0	0	0	0	0	0	0	0	0					
		Rat	++±	±	0	0	0	0	0	0	0	0	0	0	0	0	0
	Sedimented substance from rabbit liver	Rabbit	+++	+	0	0	0	0	0	0	0	0	0	0	0	0	0
		Mouse	+++	++	0	0	0	0	0	0	0	0	+	0	0	0	0
		Guinea pig	+++	±	0	0	0	0	0	0	0	0	0	0	0	0	0
		Chicken	+±	0	0	0	0	0	0	0	0	0					
		Rat	++++	++++	++±	0	0	+	0	0	0	0	±	0	0	0	0

Both sera had first of all been heated at 56°C. for 30 minutes and absorbed with sheep erythrocytes (to remove complement and natural sheep hemolysin). The sedimented substances used for absorption were prepared precisely as in the experiment of Table XI, and the absorption were carried out in the same way.

were extracted overnight in the refrigerator, then spun clear, and heated at 56°C. for 30 minutes immediately prior to use,—thus following the time-honored, empirical procedures to avoid or diminish "non-specific" complement fixation. The findings of the present work have shown that the substance with which the natural tissue antibody reacts is adversely affected by glycerol, that it is largely inactivated upon heating at 56°C. for 30 minutes, and that it deteriorates rapidly when left in the refrigerator in suspension in isotonic saline. The procedures employed empirically in the preceding work were as though designed to destroy the tissue constituent with which the natural antibody reacts. Hence it is not surprising that the natural antibody remained undisclosed in the control tests of those studies.

It is interesting to note in this connection that the reactions of the natural tissue antibody and the sedimentable constituent of normal tissues were not observed by Hoyle (7) or by Cheever (8), who partially repeated the serological studies with the papilloma virus and the Brown-Pearce tumor antigen, respectively. Jacobs and Houghton (9), on the other hand, while attempting to repeat the serological experiments with the Brown-Pearce tumor, observed that some normal sera had the capacity to fix complement under the conditions of their tests, and that prolonged centrifugation or filtration through a Mandler filter or simply passage of time would reduce the complement-fixing capacity of their antigens. It seems unlikely that they encountered any sera containing the antibody that reacts specifically with the distinctive Brown-Pearce tumor antigen, for the reactions they observed were weak at best. The interpretation of their findings, which are not given in detail, must remain in doubt; but it is possible that reactions of the natural antibody and the normal tissue substance may have been responsible for the results.

With a view to learning whether the natural tissue antibody may have entered into the reactions already reported as involving the papilloma virus (6) and the Brown-Pearce tumor antigen (1), an experiment was set up in which specimens of the sera of normal rabbits, of rabbits carrying virus papillomas, and of rabbits implanted with the Brown-Pearce tumor were heated at 56°C. and at 65°C. and tested for capacity to fix complement in mixture with various antigens.

The extracts containing the antigens were made fresh from frozen tissues, as in the experiments of the present paper. Table XIII shows the results of the experiment; the details follow:—

The normal rabbit sera (5-65 and 5-69) were known from previous tests to contain the natural tissue antibody in high titer. Rabbits 14-71 and 14-72 carried four large pancake papillomas as result of the inoculation 42 days before of highly pathogenic W.R. (wild cottontail rabbit) 1-28 virus. The sera of rabbits 5-01 and 5-08 had been procured 31 days following multiple implantations with the Brown-Pearce tumor; they had been much used in the Brown-Pearce work already reported and were known to contain the specific antibody in high titer (1). Specimens of the sera were heated

TABLE XIII

Tests for Natural Tissue Antibody and for the Specific Antibodies Elicited by Virus Papillomas and the Brown-Pearce Carcinoma

SERA			ANTIGENS														
			Normal rabbit kidney					Virus papilloma—W.R.					Brown-Pearce carcinoma				
Heated (30 min.)	Procured from	Rabbit No.	Serum dilution					Serum dilution					Serum dilution				
			1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64	1:4	1:8	1:16	1:32	1:64
56°C.	(*a*) Normal rabbits	5-65	++++	++++	++++	++++	++	0	0	0	0	0	+++±	+++	++±	0	0
		5-69	++++	++++	++++	++++	+++	±	0	0	0	0	++++	++++	+++±	+	0
	(*b*) Rabbits carrying virus papillomas	14-71	++++	++++	++++	+++±	+±	++++	++++	++++	++++	++	++±	+±	0	0	0
		14-72	++++	++++	++++	+++	±	++++	++++	++++	++++	++±	++	±	0	0	0
	(*c*) Rabbits implanted with the Brown-Pearce carcinoma	5-01	++++	+++±	++	0	0	0	0	0	0	0	++++	++++	++++	++++	++++
		5-08	++++	+++±	++	0	0	0	0	0	0	0	++++	++++	++++	++++	++++
65°C.	(*a*) Normal rabbits (as above)	5-65	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		5-69	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	(*b*) Rabbits carrying virus papillomas	14-71	0	0	0	0	0	++++	++++	++++	+++±	±	0	0	0	0	0
		14-72	0	0	0	0	0	++++	++++	++++	+++±	+	0	0	0	0	0
	(*c*) Rabbits implanted with the Brown-Pearce carcinoma	5-01	0	0	0	0	0	0	0	0	0	0	++++	++++	++++	++++	++++
		5-08	0	0	0	0	0	0	0	0	0	0	++++	++++	++++	++++	++++

Antigens, 1:40 saline extracts of frozen tissues.

at 56°C. and at 65°C. and tested in mixture with antigens made precisely as in the other experiments of this paper: fresh, 1:40 saline extracts of frozen tissues from (*a*) normal rabbit kidney (D.R. 4-64), (*b*) a naturally occurring virus papilloma (W.R. K-1), and (*c*) a vigorously growing Brown-Pearce tumor from the leg muscles of D.R. 14-04.

From Table XIII it will be seen that all of the six sera contained the natural tissue antibody; for, when heated at 56°C., all fixed complement in mixture with the normal rabbit kidney antigen, but when heated at 65°C., all failed to do so. As already stated, the two normal rabbit sera had been chosen because they were known from previous tests to contain the natural tissue antibody in high titer. The titer of this was lower in the sera of the rabbits carrying virus papillomas and still lower in the sera of the rabbits carrying Brown-Pearce carcinomas, as the table shows, but this would seem to be merely fortuitous. It is interesting that the sera of the normal rabbits and those of the Brown-Pearce rabbits gave no fixation or practically none in mixture with the virus papilloma antigen. Manifestly, the tissue substance with which the natural antibody reacts was not present in effective amount in the 1:40 saline extract of the virus papilloma. The normal rabbit sera heated at 56°C. gave positive reactions in dilutions as high as 1:16 in mixture with the frozen Brown-Pearce tumor antigen; and so too, in lesser dilutions, did the sera of the rabbits carrying virus papillomas. But the reactions were of an entirely different order in the mixtures containing the sera of rabbits implanted with the Brown-Pearce carcinoma (5-01 and 5-08); in these fixation was complete in all dilutions tested.

The real and striking specificity of the serological reactions is brought out in the results with the mixtures containing the six serum specimens heated at 65°C.; for the natural tissue antibody was thus destroyed. The sera of the normal rabbits gave no reaction with any of the three antigens; those of the rabbits carrying virus papillomas (14-71 and 14-72) reacted to considerable dilutions with the virus papilloma antigen but failed to fix complement in mixture with the normal kidney or Brown-Pearce tumor antigens; and the sera of the rabbits implanted with the Brown-Pearce tumor (5-01 and 5-08) reacted with the Brown-Pearce tumor antigen in all of the dilutions tested, but gave no fixation in mixture with either of the other two antigens. There were no cross-reactions whatever.

From the findings just given (Table XIII) and those already recorded it becomes plain that the natural antibody and the normal tissue constituent with which this reacts can be largely disregarded in complement fixation tests with the papilloma virus and its antibody. For the normal tissue substance cannot be detected in the dilutions of papilloma extracts employed as routine, while furthermore, as already stated, glycerol inactivates the normal tissue substance, and glycerolated papillomas have been regularly employed as a source of antigen in complement fixation tests with the papilloma virus and its antibody. In addition, an extensive experience has now shown that the complement-fixing capacity of any given papilloma virus-immune serum invariably parallels its virus-neutralizing capacity; and many findings indicate that the antiviral antibody reacts with the virus itself or with an integral part thereof, and that it

has no affinity for other constituents of the papilloma cells nor any for normal tissue components (6).

The circumstances differ, however, with respect to serological experiments with extracts of the Brown-Pearce tumor. For, as Table XIII shows, normal rabbit sera that contain the natural antibody will react with freshly made extracts of frozen Brown-Pearce tumors, which would seem to contain a small quantity of the "normal" tissue constituent in addition to the tumor-specific one.[2] True, this reaction is not notably strong, even when sera containing high titers of the natural antibody are employed; and it can be avoided if the sera are heated at 65°C. for 30 minutes,—an amount of heating that has no significant effect upon the antibody that reacts specifically with the Brown-Pearce tumor antigen (Table XIII). Repeated experiments have shown, furthermore, that the reactions with normal sera can also be avoided if the Brown-Pearce tumor antigens are prepared as in the original work (1),—*i.e.*, by allowing the saline extracts to stand overnight in the refrigerator, then centrifuging and heating at 56°C. for 30 minutes prior to use. For in this way, as already mentioned, the tissue constituent with which the natural antibody reacts is inactivated. Even so, it has seemed essential to resurvey the question of the specificity of the serological reactions with extracts of the Brown-Pearce tumor. This has recently been done and the results will soon be published in detail.[3]

Strict Affinity of the Natural Antibody for the Sedimentable Tissue Constituent

In order to learn more about the specificity of the reaction between the natural antibody and the sedimentable constituent of normal tissues, an experiment was next set up in which rabbit sera known to contain various specific antibodies and presumably the natural antibody as well were tested for capacity to fix complement in mixture with antigens made from normal rabbit liver,

[2] More will be said about the presence of the "normal" tissue constituent in extracts of various diseased tissues in connection with the next experiment and in later communications.

[3] Briefly, the findings support the conclusions reached heretofore (1): The antibody that reacts specifically with the distinctive constituent of the Brown-Pearce tumor develops infrequently in ordinary hybrid rabbits implanted with the growth, as Cheever noted (8); yet it often reaches high titer in specially favorable hosts, as in the sera of rabbits 5-01 and 5-08 of Table XIII. It has never been found in the serum of normal rabbits, in that of rabbits carrying transplanted cancers of other sorts, or in the blood of control animals with syphilis or other laboratory infections. The distinctive substance with which it reacts is regularly present in large amounts in the Brown-Pearce tumor, but cannot be detected in extracts of the normal tissues of rabbits or in other rabbit neoplasms.

from the Brown-Pearce tumor, and from virus-induced fibromas, vaccinial lesions, and virus papillomas respectively.

Five sera were used. That of normal rabbit 6-72 was known from previous tests to contain a high titer of the natural antibody. Rabbit 5-52 had been implanted in six muscle situations with the Brown-Pearce tumor 46 days before bleeding: tumors appeared at all of the implanted situations and reached a size of 3.0 to 5.0 cm. across before the 18th day but regressed abruptly between the 18th and the 28th days. The serum of this rabbit had been found on previous tests to contain a high titer of the specific antibody for the distinctive substance of the Brown-Pearce tumor; it had not been tested for the natural antibody. Rabbit 1-74 had been used to titrate the fibroma virus; this had been inoculated into the skin of the rabbit at 24 situations, with result in as many large fibroma lesions which had healed when the animal was bled 38 days after the inoculations. The serum of rabbit 16-01 had come from an animal originally infected with vaccine virus, which had later received repeated injections of suspensions of the elementary bodies of vaccinia. It was generously made available by Dr. Joseph E. Smadel. Rabbit 16-41, which provided the final serum, had received three intraperitoneal injections of a filtrate containing large amounts of the papilloma virus. Its serum was known to contain the specific papilloma virus antibody in high titer.

The five antigens came from normal rabbit liver, the Brown-Pearce tumor, and from rabbit tissues infected respectively with the fibroma virus, vaccine virus, and the papilloma virus. The swollen testicles of a rabbit infected 3 days previously with fibroma virus had been saved frozen, as had also the normal liver and Brown-Pearce tumors of other animals. The glycerolated natural papillomas of W.R. 2-95 provided an antigen known to be rich in papilloma virus. The frozen liver, Brown-Pearce tumor and fibroma tissues, and the glycerolated W.R. papilloma tissue were ground as usual and suspended 1:10 in dilute phosphate buffer. The suspensions were spun at 4400 R.P.M. for 10 minutes to remove coarse tissue fragments and the supernatant fluids then spun at 25,000 R.P.M. for 1 hour in the high-speed centrifuge. The clear supernatant liquids were poured off and the sedimented pellets resuspended in the original volume of dilute buffer for use in the tests. Dr. Smadel generously furnished the fifth antigen: a potent suspension of the elementary bodies of vaccinia, freshly prepared by a standard method (10).

Table XIV shows the results of the tests. All of the sera except one (1-74) contained the natural antibody in considerable titer, as shown by the reactions with normal liver antigen of the specimens heated at 56°C., which were abolished when the sera were heated at 65°C. Normal serum 6-72 also reacted with the Brown-Pearce tumor antigen and with the fibroma antigen, but much less well with these than with the normal liver antigen; and so too with the three other sera that contained the natural antibody (5-52, 16-01, 16-41). It is especially noteworthy that none of the sera reacted with the purified suspension of vaccinia elementary bodies except that of D.R.16-01, which contained specific, heat-stable antivaccinial antibodies; and likewise, that none of the sera reacted with the antigen containing the papilloma virus except that of D.R. 16-41, which contained the specific papilloma virus antibody. As already mentioned, the reactions of the natural antibody were abolished when the sera were heated at

65°C. for 30 minutes, whereas the specific antibodies,—those directed respectively against the distinctive constituent of the Brown-Pearce carcinoma (serum 5-52), the fibroma virus or an associated soluble antigen (serum 1-74), the L-S complex of vaccinia (serum 16-01), and the papilloma virus (serum 16-41),—were but little affected.

TAI

Tests for Natural and Specific

SERA			ANTIGENS—S											
			Normal rabbit liver						Brown-Pearce tumor					
Heated (30 min.)	Rabbits	Rabbit No.	Antigen dilution						Antigen dilution					
			1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
56°C.	Normal	6-72	++++	++++	++++	++++	++++	+++±	++++	++++	++±	0	0	0
	With Brown-Pearce tumor	5-52	++++	++++	++++	+++	±	0	++++	++++	++++	++++	++++	+++
	Immune to fibroma virus	1-74	0	0	0	0	0	0	0	0	0	0	0	0
	Hyperimmune to vaccinia	16-01	++++	++++	++++	++±	+	0	++++	+++	+±	0	0	0
	Hyperimmune to pap. virus	16-41	++++	++++	++++	++++	++++	+++±	++++	++++	++++	+++±	++	0
65°C.	Normal	6-72	0	0	0	0	0	0	0	0	0	0	0	0
	With Brown-Pearce tumor	5-52	0	0	0	0	0	0	++++	++++	++++	++++	+++±	0
	Immune to fibroma virus	1-74	0	0	0	0	0	0	0	0	0	0	0	0
	Hyperimmune to vaccinia	16-01	0	0	0	0	0	0	0	0	0	0	0	0
	Hyperimmune to pap. virus	16-41	0	0	0	0	0	0	0	0	0	0	0	0

Antigens: All spun at 4400 R.P.M. for 10 minutes to remove coarse material, then at 25,000 R.P.M. for 1 hr vaccinia antigen, which was a suspension of elementary bodies prepared according to a standardized method
Sera: All 1:8.

The results of the experiment (Table XIV) show clearly that the heat-labile natural antibody, which was present in substantial amount in four of the five sera (all except 1-74), failed to react with antigens containing large amounts of vaccine virus and papilloma virus respectively, though it reacted notably well with the antigen made from normal rabbit liver and to a lesser extent with those derived from the Brown-Pearce tumor and the fibroma lesions. Manifestly, the natural antibody has a strict affinity for the sedimentable tissue constituent, which is present in extracts of certain diseased as well as normal tissues, and none for the two viruses mentioned.

GENERAL DISCUSSION

The experiments here described took origin from the observation that the blood serum of normal adult rabbits will fix complement when mixed with

LE XIV

Antibodies in Various Rabbit Sera

dimented materials from

Fibroma					Vaccinia lesion						Virus papilloma—W.R.					
Antigen dilution					Antigen dilution						Antigen dilution					
1:10	1:20	1:40	1:80	1:160	1:10	1:20	1:40	1:80	1:160	1:320	1:10	1:20	1:40	1:80	1:160	1:320
++++	++++	+++	0	0	0	0	0	0	0	0	0	0	0	0	0	0
++++	++++	+++±	+±	0	0	0	0	0	0	0	0	0	0	0	0	0
++++	++++	+++±	0	0	0	0	0	0	0	0	0	0	0	0	0	0
++++	++++	+++	±	0	++++	++++	++++	+++±	±	0	0	0	0	0	0	0
++++	++++	++++	++±	0	0	0	0	0	0	0	++++	++++	++++	++++	++++	++++
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
++++	++++	+±	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0	0	0	0	0	++++	++++	++++	+++	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0	0	0	++++	++++	++++	++++	++++	++++

ur with resuspension of the sedimented pellets in the original volume of dilute phosphate buffer,—except the and generously made available by Dr. Smadel.

saline extracts of normal tissues. Facts were procured which show that the reaction is due to a naturally occurring serum principle which combines specifically *in vitro* with a sedimentable constituent of many normal tissue cells. Both the serum principle and the substance with which it reacts have implications for discussion.

The serum principle would seem to be a natural antibody. For, as the experiments have shown, it appears naturally in the blood of almost all normal adult rabbits, and, like the generality of antibodies, can be precipitated from serum with ammonium sulfate, fixes complement in mixture with a single sub-

stance (or class of similar substances), and can be specifically absorbed thereby in the absence of complement. Furthermore it resembles other natural antibodies of normal rabbit's blood in being more labile to heat than are induced antibodies. Yet to state that the serum principle would seem to be a natural antibody is not to define it precisely, for the character of these is but poorly understood.

Origin and Character of the Natural Tissue Antibody

The natural tissue antibody manifestly originates under physiological conditions. It could be an autoantibody, meaning thereby that it might appear in response to constituents of normal tissue cells that act as antigens following liberation from injured or dying cells. Or it might be formed in a simpler way: the synthesis of globulin might normally take place—as implied in the theories of Haurowitz, Mudd, and others (12)—in such a way that some of the molecules would become specifically oriented to conform with a certain part of the sedimentable cell constituents, with result in a fraction of "normal" globulin having the special affinity that distinguishes what has here been termed the natural tissue antibody. Rabbit globulin as such does not manifest its properties, however, for the quantity of normal globulin differs but little from one adult rabbit serum to the next, as is well known, whereas the titer of the natural antibody varies quite widely. In this connection, the fact may be mentioned that a number of rabbit sera were recently tested for capacity to flocculate mastic—a reaction presumably brought about by normal globulin (11)—and, at the same time, for capacity to fix complement in mixture with normal tissue extracts. The various sera proved almost precisely alike in their ability to flocculate the gum but differed markedly in their reactions with extracts of normal tissue.

The natural tissue antibody would seem to be in a different category from certain other so called natural antibodies,—the ones effective respectively against herpes, yellow fever, and influenza viruses, *E. coli*, the toxins of scarlet fever and diphtheria, for example. For these latter are hardly "natural" in a strict sense but appear to represent responses to chance contacts with the viruses or with the specific microorganisms or their products (13). Furthermore, they have to do in part at least with the development of immunity to the agents mentioned (13), whereas the natural tissue antibody has no discernible relation to the phenomena of immunity to disease.

The natural tissue antibody is distinguishable by its strict affinity for a sedimentable substance extractable from many normal tissue cells. It is not absorbed by sheep erythrocytes, as are the natural Forssman antibodies, and it does not react with alcoholic tissue extracts, as do the natural Wassermann antibodies of rabbit's blood. Furthermore it differs from other so called physiological antibodies in that both it and the substance with which it

reacts are usually present together in the same individual, while the antibodies that react with the blood-group substances, for example, and those that react with the Forssman substances are present only in individuals with tissues presumably devoid of those substances (14).

At first thought it seems strange that an individual can carry in its blood an antibody capable of reacting with a constituent of its own tissues. Indeed, one might suppose that under such circumstances the antibody would necessarily be absorbed and become fixed upon the tissue components for which its affinities fitted it, as actually happens in the paroxysmal hemoglobinuria of syphilitic human beings, in which induced autoantibodies cause lysis of erythrocytes. But this reaction takes place only at temperatures somewhat lower than those normal for the human body (15), and so too when the natural and induced autohemagglutinins of rabbit's blood cause clumping of red cells (16). By contrast the antibody now under consideration reacts at 40°C., and quite as well as at lower temperatures, as recent experiments have shown. Hence other reasons must be sought to explain why it fails to react *in vivo* with the normal tissue constituent.

The natural antibody, once it gets into the blood, may never again come into effective contact with the sedimentable constituents of living cells. This would be true, for example, if the latter were situated within the cell protoplasm or were associated with other cell constituents in such wise as to be shielded. In this relation the fact may be recalled that viruses are protected from the action of specific antiviral antibodies so long as they remain associated with living cells,—a phenomenon that is especially striking in the case of the rabbit papilloma virus, which continues to increase in amount in association with proliferating cells even when these are constantly nourished by blood having great antiviral power (17). The circumstances may be similar also with respect to the substance (or substances) that reacts with Wassermann reagin. The tissues of syphilitic individuals will yield the Wassermann substance as readily as those of normal ones; yet the reagin that circulates in their blood is not demonstrably absorbed *in vivo*. In the light of these findings it scarcely seems surprising that the natural tissue antibody apparently fails to react *in vivo* with the normal tissue substance; and it need not seem strange that the antibodies induced in rabbits against sedimentable kidney substances from alien species fail to exhibit nephrotoxic properties though capable of reacting *in vitro* with sedimentable constituents of rabbit kidney cells (18).

Natural Antibodies and Antigens in Relation to "Non-Specific" Complement Fixation and to Other Serological Complexities

It is obvious that the natural tissue antibody may complicate the results of serological experiments in which rabbit sera are used in mixture with tissue extracts. Since natural antibodies of the same or similar sort may be present in other species (as the sedimentable tissue substances unquestionably are (5)) it seems possible that reactions between them may be responsible for some at least of the so called "non-specific" complement fixation reactions encountered in the past.

The literature on "non-specific" complement fixation reactions has been dealt with by Noguchi (19) and Gussenbauer (20), in various text books (21), and recently by Takenomata (22), Mackie and Finkelstein (23), and Gibson (24). In addition to anticomplementary effects due to chemical inactivation or destruction or adsorption of complement, there are other, so called non-specific, complement fixation reactions in which substances present in the serum of normal animals and man will fix complement in mixture with a variety of "non-antigenic" substances, amongst them peptone, glycogen, various extracts of tissues and bacteria, amino acids, cholesterol, and alcohol.

Many workers have observed that certain "non-specific" complement fixation effects can be more or less readily avoided by empirical means. This has proved true also in the case of the natural tissue antibody. As already mentioned (experiment of Table XIII), certain tissue antigens can be prepared, or diluted, in such a way that reactions with the natural antibody are avoided. The same purpose can often be achieved by diluting the serum. Only occasionally have sera containing the natural tissue antibody been effective in dilutions beyond 1:16 or 1:32, in our experience; whereas the antibodies appearing in rabbit serum in response to foreign antigens often titer much higher, as is generally known.

Still another method long used empirically to avoid "non-specific" complement fixation is to heat the sera to such an extent that the non-specific effects are abolished while the specific antibodies are left unaffected. Noguchi (19), Kolmer (25), and Gibson (24) made much use of this procedure, and Casals and Palacios have recently applied it with noteworthy success in complement fixation tests with various neurotropic viruses and their antibodies (26). The findings of the present work provide a rational basis for the use of heat for this purpose. For they show that heating at 65°C. for 30 minutes destroys the natural antibodies in normal rabbit serum without destroying specific, induced ones. In the experiments of Tables XIII and XIV recourse was had to this procedure to bring into sharp relief the specific reaction between the distinctive substance of the Brown-Pearce tumor and its antibody and other specific antigen-antibody reactions as well.

Duran-Reynals has reported upon a flocculation of tissue extracts by normal and immune sera of fowls and other animals (27). The phenomenon differs notably, however, from that here dealt with. For the flocculations took place only at low temperatures; they occurred when alcoholic as well as saline tissue extracts were employed; the titer of the serum factor responsible for them could be markedly raised by immunization with specific antigens; and the latter proved capable of absorbing concurrently both the non-specific flocculating factor and the specific antibodies.

Many studies attest to the complexity of serological reactions with the fowl tumor agents, and to the close association of these agents with normal tissue constituents (28). In particular it has been shown by numerous workers (28) that the sera of normal adult fowls will not infrequently neutralize the causative agent of Chicken Tumor I, and Andrewes and Amies have observed cross-reactions when the sera of fowls carrying various tumors were mixed with tissue suspensions containing the filtrable agents responsible for them. It is conceivable that natural antibodies of the sort disclosed in the present study or induced ones with similar affinities might be responsible for some of the puzzling reactions, by acting upon normal tissue constituents

with which the disease-producing agents may be associated. The possibility would seem to be brought nearer by the findings of various workers (28) who have noted that the sera of rabbits and goats injected repeatedly with normal fowl tissue will neutralize the filtrable agent responsible for Chicken Tumor I, and also by observations of Chambers and Henle, who have recently shown that antisera prepared by injecting mouse lung particles into rabbits possess the capacity to agglutinate particles of the sort injected, as also to carry down influenza virus Type A when this is present, presumably in association with the normal tissue constituents (29).

Reference has already been made to the fact that the natural antibody can be disregarded in complement fixation tests in which the rabbit papilloma virus and its antibody are concerned (see the experiments of Tables XIII and XIV), for the reason that the "normal" tissue constituent is not present in detectable amounts in papilloma extracts employed as antigen in such tests. It seems likely, however, especially in view of the work of Casals and Palacios already cited (26), that *in vitro* reactions of the natural antibody and the normal tissue constituent may complicate serological tests with other viruses. This actually happened in the experiment of Table XIV, in which normal rabbit sera, containing the natural tissue antibody, reacted with an antigen made from tissues infected with fibroma virus, even though the antigen had been purified by differential ultracentrifugation. It follows that the natural antibody may prove useful as an indicator of the presence of "normal" tissue constituents in virus preparations purified in this way; for, as will be discussed further on, the substance with which it reacts is thrown down in the high-speed centrifuge just as viruses are. In the experiment of Table XIV, the suspension of vaccinial elementary bodies purified by Dr. Smadel was apparently devoid of the normal tissue constituent, and so too was the preparation containing purified papilloma virus, for these did not react with the sera known to contain the natural antibody. It is noteworthy that both viruses were procured from epidermal cells, which have been found to contain comparatively little of the normal tissue constituent with which the natural antibody reacts.

The Constituent of Normal Tissue Cells with Which the Natural Antibody Reacts

An outstanding property of the tissue constituent is that it can be thrown down readily in the high-speed centrifuge, little or none remaining in the supernatant liquid of potent suspensions spun for an hour at speeds of 20,000 to 30,000 R.P.M. (about 29,000 to 65,000 times gravity), as Table VII shows.

Substances readily sedimentable by means of high-speed centrifugation have been isolated from most if not all of the normal and neoplastic plant and animal tissues studied hitherto (5, 32) and recently from a yeast (30) and a bacterium (31). Claude has found (5, 30) that sedimentable materials derived from many normal tissues are complex chemical entities containing large proportions of alcohol-soluble materials (phospholipids) as well as nucleoprotein of the ribose type. When the chemical find-

ings are considered in relation to what is known about cellular antigens (33, 37), it does not seem surprising that the alcohol-soluble fractions should contain substances giving the Wassermann and Forssman reactions, and that the protein components should bear the stamp of the species from which they are derived, as Furth and Kabat have demonstrated (5).

Henle, Chambers, and Groupé (5) have recently cited reasons for supposing that the sedimentable cell constituents may possess distinctive organ-specific characters and have sought evidence for these by injecting the sedimentable substances of various tissues and species into rabbits. The findings of Tables X, XI, and XII of the present paper do not necessarily imply, by contrast, that the sedimentable constituents of various tissues lack organ-specific properties; they indicate, however, that the natural antibody does not distinguish any such. In this relation the fact deserves mention that, with a few noteworthy exceptions, it has proved difficult if not impossible to define organ-specific substances by ordinary serological methods (33, 36); special techniques,—such as those employed in the work with nephrotoxins (34) and in the demonstration of passive anaphylaxis (35),—have usually been required to achieve this end.

In the experiments of the present paper, sera and tissues of the same species (sometimes of the same individual) were used, and immunization procedures were excluded, in order to avoid species- and presumably organ-specific effects. Hence it seems significant that the material with which the natural antibody reacts appears to be the same or closely similar whether derived from one or another of widely various tissues (Tables X, XI, and XII). Several kinds of "heterogenetic" substances are widespread in nature (33), this being true especially of the substance (or substances) that reacts with Wassermann reagin (11); yet the substance with which the natural tissue antibody reacts differs notably from the "heterogenetic" substances heretofore described. For it does not come away into alcohol as many of these do; it is unstable upon standing in saline solution and is destroyed by an amount of heating that has no deleterious effect upon the other substances mentioned (see Table IX); furthermore, though present in many normal tissues, it has not been detected in non-nucleated erythrocytes or in skin. Its properties, viewed as a whole, suggest that it may be a protein. Whether it will prove identical with one or another of the enzymes common to many tissue cells is a problem of immediate interest.

The Sedimentable Constituents of Normal Tissues and the Distinctive Substance of the Brown-Pearce Carcinoma

The sedimentable constituents of normal tissues had not been recognized when the observation was made that the Brown-Pearce tumor contains such a material and that this is distinctive in character and reacts specifically with an antibody which is present only in the blood of animals implanted with the tumor (1). But other sedimentable substances—the viruses—were known then

to be antigenic; and the antigen procured from the Brown-Pearce tumor was found to have several properties strikingly similar to those of certain viruses,—notably its comparatively large particle size and weight as determined by ultrafiltration and ultracentrifugation, and its reaction to changes in pH and to heat. When studied serologically its resemblance to the rabbit papilloma virus (Shope) seemed especially noteworthy, though the two proved wholly distinct from one another, and the Brown-Pearce substance has proved non-pathogenic upon inoculation into normal animals under a variety of conditions (1, 38). Whether the distinctive substance of the Brown-Pearce tumor resembles more the sedimentable constituents of normal tissues than it does the viruses is a question that may be left to the future. In any case its specificity is manifest (see Tables XIII and XIV). More will be said about it in forthcoming papers, in relation both to the normal tissue constituent herein described and to another distinctive sedimentable substance which has recently been found in the V2 carcinoma, a transplantable tumor derived from a virus-induced rabbit papilloma (39).

SUMMARY

Continued serological investigations of the sedimentable constituents of normal and neoplastic tissues have shown that the blood serum of normal rabbits will fix complement in mixture with saline extracts of normal rabbit tissues. The phenomenon has proved referable, not to anticomplementary effects of serum or antigen nor to so called non-specific complement fixation, but to a naturally occurring serum principle, hitherto unrecognized, which reacts specifically *in vitro* with a sedimentable constituent of normal tissue cells.

The principle exists in the blood of nearly all adult rabbits but is absent from that of rabbits less than 1 month old. It can be salted out from serum with ammonium sulfate and is destroyed when heated at 65°C. for 20 to 30 minutes. Its titer was found to run parallel in general with that of two natural antibodies also present in normal rabbit's blood (natural Wassermann reagin, natural anti-sheep hemolysin); but absorption tests showed it to be distinct from these. Because of its properties, the serum principle has been termed the natural tissue antibody.

The substance with which the natural tissue antibody reacts is regularly present in saline extracts of many normal tissues,—those of rabbits and of other species as well. Kidney and liver tissues always yield it in abundance, while spleen, brain, and testicle provide somewhat less; heart and voluntary muscle extracts contain relatively little, and non-nucleated erythrocytes and skin are practically devoid of it. The results of affinity and absorption tests indicate that it is nearly or quite the same from whatever tissue or species derived. It is readily sedimentable in the high-speed centrifuge, little or none

remaining in the supernatant liquid of potent suspensions spun at 25,000 R.P.M. (45,400 *g*) for 1 hour. It either does not come away into alcohol or is inactivated thereby, is readily destroyed by heat (56–70°C. for 30 minutes), and diminishes notably in antigenic potency upon standing overnight in saline suspension or when the tissues containing it are kept in glycerol. Its properties suggest that it may be a protein.

The implications of the findings are discussed in relation to the formation of the natural antibody and its place amongst serological phenomena, to so called "non-specific" fixation of complement and other serological complexities, and with particular reference to the character of the sedimentable constituents of normal and neoplastic tissue cells.

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