TWO HEAT-LABILE FACTORS IN NORMAL SERA WHICH NEUTRALIZE VARIOLA VIRUS.

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THE means by which the immune or the non-immune host attempts to dispose of an infecting virus are obscure. Studies of the fate of inoculated virus within the body of the host throw little light on the actual disposal mechanisms, and the problem is usually approached by making virus neutralization tests in which a mixture of serum from the test animal and virus is inoculated into a suitable indicator animal. In such tests it is customary to heat-inactivate the serum before making the mixtures so that only the heat-stable specific antibody is subjected to test. Most of our knowledge of humoral immunity to virus infection has been derived from such tests.

In contrast to the vast amount of study which has been devoted to heat-stable antibodies, few workers have investigated the rôle of heat-labile serum factors in the immunology of virus infections.

Factors with the capacity to enhance specific neutralization have been described. Thus Gordon (1925) was able in one experiment to demonstrate a marked reduction in virus neutralizing power when a fresh antivaccinial rabbit serum was heated, and further that the loss could be restored, the addition of fresh guinea-pig serum increasing the neutralization 100-fold. However, in two other samples of rabbit serum which Gordon tested, heating did not lessen the neutralizing activity ; Gins (1916) also failed to demonstrate enhanced neutralization with unheated serum. Mueller (1931) showed that the neutralizing capacity of fowl antibody to Rous sarcoma could be intensified and the loss of titre of the heated antiserum restored by the addition of fresh guinea-pig serum. Meyer (1941, cited by Morgan, 1945) showed that the phagocytosis of psittacosis virus was greater with fresh than with old immune serum, and that the effect of heated antiserum was augmented by adding " complement."

Morgan (1945) and Whitman (1947), both working with Western equine encephalomyelitis virus, showed that fresh immune human serum stored at — 70° possesses far greater neutralizing activity than heated serum or serum kept at 4°. By adding fresh guinea-pig serum Morgan could restore the lost activity of sera kept at 4° but not of heated sera. Whitman, however, could restore the lost activity to old and to heated sera by adding fresh normal serum from guinea-pig, monkey or man. Working with dengue virus, Sabin (1950) found that normal guinea-pig, rabbit or human sera could restore the loss incurred by heating an immune serum.

Dozois, Wagner, Chemerda and Andrew (1949) working with encephalomyelitis rabbit antiserum claim to have shown that the potentiating factor in normal rabbit

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serum involved the 2nd, 3rd and 4th component of haemolytic complement. Leymaster and Ward (1949) using mumps virus showed that the chick embryo-protective titre of a mumps antiserum (human or monkey) was markedly reduced by heat inactivation or by absorption with an antigen-antibody precipitate, and that this lost activity could be restored by adding unheated normal human serum but not by heated.

The following workers, however, have failed to demonstrate any potentiating effect with " complement ": Howitt (1934) using horse antiserum, guinea-pig complement, and WEE virus, and Florman and Enders (1942) using rabbit antiserum, fresh normal rabbit serum as complement and vaccinia virus in roller tube cultures of mononuclear cells. Fox (1949) working with *Rickettsia orientalis* likewise found no detectable effect on the degree of neutralization when immune guinea-pig serum was heated, nor when fresh guinea-pig serum was added to the system containing heated immune serum.

In addition to the power of fresh normal sera to potentiate specific virus neutralization by heated immune sera, it is known that normal serum itself may be capable of suppressing virus growth. In the case of poliomyelitis the factor responsible is heat-stable (Jungeblut, 1934). Jungeblut and Engle (1934) showed that the presence of such a heat-stable factor might play some part in determining the resistance of monkeys to poliomyelitis. For the most part, however, the virus inhibitors observed in normal sera have been heat-labile. Thus the viruses of Newcastle disease and of influenza may be inhibited in chick embryos by a heat-labile factor in normal sera (Ginsberg and Horsfall, 1949 ; Howitt, 1950 ; Bang, Foard and Karzon, 1950, 1951 ; Francis, Salk and Brace, 1946 ; Burnet and McCrea, 1946 ; McCrea, 1946 ; Smith and Westwood, 1949). Similar findings have been reported with vaccinia virus (Douglas and Smith, 1930 ; Briody, Ledinko and Stannard, 1951).

Psittacosis virus (Utz, 1948) and the viruses of St. Louis, Japanese B and Russian Far East encephalitis in mice are apparently inhibited by a lipoid fraction of normal sera from several species (Casals and Olitsky, 1947, 1948).

In the recent paper of Briody *et al.*, cited above, it is reported that fresh guinea-pig serum contains a heat-labile factor which inhibits the growth of vaccinia virus on the chorio-allantoic membrane. This factor is absent from mouse sera. It was apparently also present, though in less degree, in the fresh normal rabbit sera which these authors tested.

The variable results recorded by different workers suggest differences in the susceptibility of viruses to heat-labile factors in normal serum and to differences in the sera from different animals, but in addition the nature of the tests and of the animal species used in them probably influence results.

EXPERIMENTAL.

The studies reported here with smallpox virus were undertaken to discover whether fresh normal human sera were all alike in their action on smallpox virus, and whether complement or some similar factor might play a part in the specific neutralization of smallpox virus by immune sera. The ease with which smallpox virus can be grown on the chorio-allantoic membrane, the quantitative estimation of infective virus by pock counts, the lack of haemolytic complement in chick embryos (Polk, Buddingh and Goodpasture, 1938) and their inability to form antibody seemed to offer a suitable method for studying these problems.

Materials and methods.

The variola virus used was a purified elementary body suspension from a chick-embryo-propagated strain isolated in this laboratory from a smallpox case. Virus from the fifth egg passage was used, and a concentration chosen for the tests such as would give about 150 lesions per egg in control mixtures. The vaccinia virus was a rabbit-adapted strain. The cowpox virus was a rabbit-adapted strain which had been transferred to eggs and passed 10 times on the chorio-allantoic membrane.

The strain of herpes simplex virus was isolated from labial herpes and had had 14 egg passages. The normal human sera were obtained by venepuncture from unvaccinated young adults who had not had smallpox. The immune human sera came from adults who had had several successful vaccinations, the most recent being within a year. Sera from normal animals which had not been exposed to infection with viruses of the pock group were also used. They were obtained by venepuncture, cutting the carotid artery of stunned animals, or cardiac puncture under ether anaesthesia.

Blood samples were allowed to clot for 1 hr. at 37°, then transferred to a refrigerator for 1–2 hr., the serum separated and transferred in small quantities to glass ampoules which were sealed and stored in dry ice-ethyl alcohol mixture. A few were stored at about — 50° in dry ice alone. When necessary sera were heat inactivated at 58° for 30 min. The diluting fluid used throughout was 10 per cent broth in saline containing 100 units/ml. penicillin.

Neutralization tests in eggs were made by adding 0·4 ml. virus, suitably diluted, to 0·1 ml. of the serum to be tested and 0·1 ml. broth saline. Where two sera were used in the same mixture the second one replaced the broth saline. After the mixtures had stood at room temperature for 40–60 min. each was distributed on the chorio-allantoic membrane of five to eight 11–13-day–old chick embryos. Lesions were counted after 3 days' incubation at 36°. After discarding dead embryos and occasional badly scarred membranes the average number of lesions per membrane was determined for each mixture, and from this the percentage survival was calculated, assuming 100 per cent survival in the control mixtures of virus and heated normal serum. The survival rate in such control mixtures did not differ from the survival rate when virus was mixed with broth saline alone. Variations from these techniques are recorded in the text.

TABLE I.—*The Inhibition of Smallpox Virus by Unheated Normal Guinea-pig Sera.*

Serum.	Pock counts on individual eggs.					Mean count per egg.	Per cent survival.
1 Unheated	16,	16,	31,	D,	D	21	16
1 Heated	99,	134,	165,	176,	187	152	116
2 Unheated	8,	11,	29,	29,	51	26	20
2 Heated	69,	100,	125,	147,	D	110	84
3 Unheated	32,	40,	48,	55,	80	51	39
4 ,,	12,	23,	45,	68,	D	37	28
5 ,,	14,	27,	33,	80,	(Scarred)	38	29
6 ,,	26,	32,	44,	90,	44	47	36
Normal human (M). Heated	54,	96,	137,	200,	207	131	100
,, ,, (S). ,,	109,	116,	(Scarred),	D,	D		

Control: Average count in mixtures containing heated normal human serum = 131 (i.e., 100 per cent).

(Scarred) = Scarred membrane which was discarded.

D = Dead embryo. Membrane discarded.

RESULTS.

The Occurrence of Inhibiting Factors in Normal Human and Animal Sera.

Table I shows the result of a typical experiment in which six normal unheated guinea-pig sera were tested for neutralizing power against variola virus. It will be seen that all effect significant neutralization, experience having indicated that the neutralization of 50 per cent or more of the virus is significant when 5 or 6 eggs are used for each mixture. The inhibiting power of the sera (Nos. 1 and 2) was destroyed by heating at 58°.

Table II shows the pooled results of tests on sera from different host species.

TABLE II.—*The Occurrence of Inhibiting Factor for Smallpox Virus in Unheated Normal Sera.*

Fresh normal serum from—	Number of animals tested.	Number showing significant neutralization of smallpox virus (i.e., > 50 per cent).
Guinea-pig	19	14
Rabbit	15	12
Man	12	5

In addition to these experiments with variola virus, a few preliminary tests were made with herpes simplex and cowpox viruses using the same technique. The results have been similar to those with variola virus.

A further experiment using vaccinia virus was made by inoculating virus-serum mixtures intradermally into a rabbit and also into a guinea-pig. The lesions in both cases were smaller when the virus was mixed with unheated than when mixed with heated normal human serum.

A Heat-labile Factor in Normal Human and Animal Sera which Potentiates Neutralization of Variola Virus by Specific Heat-stable Antibody.

It was found that if a fresh unheated normal human serum, chosen because it lacked any significant inhibiting power when tested by itself, was added to a mixture of virus and heated immune serum, the extent to which the virus was neutralized was greatly increased. If, however, the normal serum was first inactivated by heat, this potentiating power was lost. Table III shows a typical

TABLE III.—*Smallpox Virus Neutralization by a Heated Immune Serum, Potentiated by Fresh Normal Serum.*

Human sera used.	Pock counts on individual eggs.	Mean.	Per cent survival.
Heated normal + saline	107, 108, 120, 179, 236, 267	169	100
Unheated normal + saline	82, 100, 110, 192, 205, D	138	
,, immune + saline	1, 1, 5, 9, D, D	4	3
Heated immune + saline	15, 37, 40, 46, 72, 97	51	33
,, ,, + heated normal	16, 29, 50, 58, 86, D	48	31
,, ,, + unheated normal	7, 10, 12, 15, D, D	11	7

example of such an experiment. The small difference between the mean counts of heated and unheated normal sera is probably the result of chance, since in another experiment with this same serum the difference was in the reverse direction. However, it appears from our limited observations that in guinea-pig, rabbit and human sera, the natural antibody-like factor mentioned in the preceding section varies in activity, some sera possessing much and others being for practical purposes without such activity.

It will be seen from Table III that an immune serum which has been stored at low temperature loses much of its neutralizing power when heated, and that this power can be partially restored by adding unheated normal but not by heated normal human serum. The failure to achieve complete restoration has characterized all our experiments of this type. There seem to be three possible causes: the immune serum itself may have possessed some heat-labile natural antibody-like factor; it may have possessed heat-labile potentiating factor, more efficient than that provided by adding unheated normal serum from another individual; or some part of the specific acquired antibody may be heat-labile.

It will be seen from Table II that 5 guinea-pig, 3 rabbit and 7 human sera had no significant inhibitory effect on variola virus. Of these, none of the guinea-pig sera, one of the rabbit sera—the only one tested—and all the human sera potentiated neutralization by heated specific human immune serum. The properties of the potentiating factor and of its relation to the naturally occurring inhibiting factor and to haemolytic complement are further considered in the following section.

The next experiment was designed to show that potentiating factor occurs also in sera possessed of natural inhibiting factor. This effect cannot be demonstrated in the direct manner as in Table III, since the potentiating effect is masked by the natural antibody-like effect. Table IV shows the result of an experiment using two

TABLE IV.—*Demonstration of Potentiating Factor in a Normal Human Serum (B) Possessed of the Natural Antibody-like Factor.*

Sera in mixtures.	Pock counts.	Mean.	Per cent survival.
Heated normal B + heated normal B	51, 106, 135, 163, 190	129	100
„ „ B + unheated normal B	30, 32, 36, 41, 83	44	34
Unheated normal B + unheated normal B	(0), 11, 13, 23, 30, 39	23	18
Immune C (heated) + heated normal B	9, 12, 26, 42, 83, 99	45	35
„ „ „ + unheated normal B	0, 1, 1, 2, 3, 4	2	2
„ M „ + heated normal B	13, 14, 68, 69, 77, 87	55	43
„ „ „ + unheated normal B	0, 1, 2, 3, 8, 17	5	4
„ „ „ + immune C heated	9, 21, 43, 44, D, D	29	22

(0) = Badly developed membrane (discarded).

heated immune sera of about equal strength, combined in various ways with a single normal human serum which possesses considerable natural antibody-like effect.

It will be seen that approximately the same degree of virus neutralization is effected when heated immune serum M, heated immune serum C or unheated

normal serum B are mixed with an equal volume of heated normal serum B. When the two heated immune sera were combined, the proportion of virus surviving was almost halved, and similarly when two volumes of the unheated normal serum B were combined, the survival rate was almost halved. But when one volume of either of the immune sera was mixed with one volume of the unheated normal serum, the survival rate was reduced to less than 5 per cent. It appears from this result that the normal serum possesses, in addition to its natural antibody-like effect, considerable power of potentiation. It therefore seems probable that all normal human sera possess potentiating factor.

Some Properties of the Two Heat-labile Components of Normal Human Sera.

The possibility was considered that the natural antibody-like effect shown by some unheated normal human sera might merely represent an additional property of the potentiating factor which is present in all, only becoming evident as an inhibiting factor when present in excess.

The next experiment (Table V) shows that this is not so : normal serum E was known to be completely lacking in natural antibody-like factor, whereas normal

TABLE V.—*The Potentiation of Specific Neutralization of Smallpox Virus by Dilutions of Two Unheated Normal Sera.*

Human sera and dilutions used.	Pock counts.	Mean.	Per cent survival.
Heated normal E + saline	Scar, 54, 57, 87, 115,	78	100
,, immune + ,,	D, 5, 11, 12, 23,	13	17
Unheated normal E + saline	34, 64, 74, 96, 100	74	100
,, ,, A + ,,	D, 11, 12, 15, 31	17	22
Heated immune + unheated normal E 1/1	0, 0, 1, 2	1	1
,, ,, + ,, ,, E 1/5	0, 1, 4, 8	3	4
,, ,, + ,, ,, E 1/25	0, 3, 5, 5	3	4
,, ,, + ,, ,, A 1/1	1, 3, 3, 4	3	4
,, ,, + ,, ,, A 1/5	1, 6, 8, 10	6	8
,, ,, + ,, ,, A 1/25	2, 14, 15, 18	12	16

serum A had this inhibiting factor to the extent that it allowed less than 25 per cent of virus to survive when tested by itself. In contrast, however, the potentiating power of serum E is very good even at a dilution of 1/25, whereas that of serum A, although good when tested undiluted, was waning at 1/5 and was not demonstrable at a dilution of 1/25. It appears therefore that these two serum factors are independent of each other.

Relation to haemolytic complement.—Table VI shows the lack of correlation between haemolytic complement titre and the presence of natural antibody in 7 normal human sera. The presence of potentiating factor is likewise not correlated with the haemolytic complement titre, since it has been shown to be absent from a

TABLE VI.—*The Lack of Correlation between Haemolytic Complement Titre and the Presence of Natural Antibody in Seven Normal Human Sera.*

	Sera tested.						
Serum No.	1	2	3	4	5	6	7
Per cent virus neutralized	84	0	54	30	15	59	73
Complement titre	1/20	1/20	1/20	1/10	1/20	1/10	1/20

guinea-pig serum with a complement titre of 1/30, and yet to be very active in a human serum in which complement could not be detected at a dilution of 1/10.

The two factors seem to be distinct from intact haemolytic complement. Fractionation of complement was not undertaken.

Stability of the two factors.—Both factors have been shown repeatedly to be destroyed by heating for 30 min. at 58°. Ten minutes at 59° seemed equally effective. In one experiment the potentiating factor was only slightly affected by overnight storage at 4° or even at room temperature, and the natural antibody-like factor in another normal human serum seemed to be quite unaffected by the same treatment.

Rapidity of action of the two factors.—Table VII shows that the natural antibody-like factor acts very quickly, a few seconds sufficing for almost maximal effect. There was only slight improvement in neutralization after 2 hr. contact with virus.

TABLE VII.—*Rate of Neutralization of Variola Virus by "Natural Antibody."*

Sera (human).	Time of contact before inoculating mixtures into eggs.	Pock counts.	Mean.	Per cent survival.
Heated normal	30 min.	(4), 51, 66, 98	72	100
Unheated normal	10 sec.	D, 15, 18, 45	26	36
,, ,,	30 min.	4, 11, 22, 43	20	28
,, ,,	120 ,,	11, 12, 15, 31	17	24

In another experiment it was found that adding the potentiating factor immediately before inoculating the eggs to an immune serum/virus mixture which had stood at room temperature for the usual 40–60 min. produced as good neutralization as when the normal serum potentiator was incorporated in the virus-immune serum mixture from the start. Both factors appear therefore to achieve their maximal effect very quickly.

The effect of dilution on the two factors.—Only a few observations have been made, but it appeared that the natural antibody was weak or absent at a serum dilution of 1/5. The potentiating factor on the other hand may still be quite active at a dilution of 1/25 (Table V).

Normal human and rabbit sera, both of which possessed potentiating factor, did not in a dilution of 1/10 enhance the vaccinia haemagglutinin inhibitory action of a human immune serum.

DISCUSSION.

The significance of these two factors in the defence mechanism of the host is unknown. Recently Briody *et al.* have brought forward evidence to suggest that natural antibody to vaccinia virus present in some species may play a part in determining species susceptibility to infection. It may well be that the variable level of the natural antibody-like factor in normal human sera plays some part in determining the severity of smallpox in the unvaccinated. It is probably unimportant in those who have acquired antibody.

The potentiating factor on the other hand is probably important in disposing of invading virus, since it greatly enhances the activity of immune sera. We have not attempted to compare the levels of potentiating factor in normal sera, nor do we know whether these levels are constant in the same individual. We have not

among the small number of human sera examined found any which lacked this factor. It was even present in a child dying of generalized vaccinia through apparent congenital inability to form antibody to the virus.

The failure to restore completely the lost activity of a heated immune serum by the addition of fresh normal serum raises the possibility that some part of the specific acquired antibody may be heat-labile.

In comparing our findings with variola virus with those of Briody *et al.*, who found vaccinia-inhibiting power in all guinea-pig sera examined, a quarter of the guinea-pigs we tested lacked variola-inhibiting factor. Also the levels of inhibiting power which we encountered were apparently lower, although this difference may have arisen from the use of different test virus. The inhibitory activity observed in guinea-pig sera by these workers seemed to be more labile than that found by us in normal human sera against variola virus. They make no mention of a potentiating factor for vaccinia virus. We were unable to demonstrate such in the guinea-pig sera which we tested against variola virus.

SUMMARY.

Normal human sera either fresh or stored at — 50 to — 75° contain a factor which potentiates the specific neutralization of variola virus by heated human immune serum.

In addition some normal human sera also contain a factor which acts like a natural antibody in that it can by itself neutralize variola virus.

Both factors are heat-labile.

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