ON THE ALTERATIONS IN HAEMOLYTIC IMMUNE-BODY WHICH OCCUR DURING THE PROCESS OF IMMUNISATION[1].

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During the process of immunisation with formed elements, *e.g.* red blood corpuscles, the amount of immune-body in the serum of the immune animal may undergo an enormous increase. Thus on injecting a rabbit with ox blood, its serum from possessing practically no immune-body may become so active that 0·00025 c.c. suffices to cause complete lysis of 1 c.c. of 5 per cent. blood suspension in the presence of sufficient complement. The estimation of the amount of immune-body necessary to effect lysis of a given quantity of blood suspension at different periods obviously gives no information as to whether or not the molecules of immune-body undergo in addition a qualitative change in the course of immunisation. As Muir[2] says, "the mere occurrence of haemolysis gives no indication of the total combining affinities" and he has always insisted on the importance of studying the combination of the components concerned in haemolysis by the absorption method. Accordingly we have investigated by this method the changes occurring in the molecules of immune-body during the process of immunisation.

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[2] *Studies on Immunity*, Oxford, 1909.

For this purpose we have chosen the immune-body obtained by injecting washed ox-blood into the peritoneal cavity of rabbits, since in this way a very active immune-body is usually developed, which along with guinea-pig's complement has an extremely powerful toxic effect as judged by the small quantities of both components required to cause haemolysis. Further, the combining properties of such immune-bodies, when raised to a high degree of potency, have already been thoroughly investigated by Muir. Thus, in general, the immune sera studied by Muir were obtained by giving three intraperitoneal injections of the corpuscles of 5, 10 and 15 c.c. of ox-blood at intervals of ten days and then bleeding 7 to 10 days after the last injection. The sera were inactivated by heating at 55° C. Such an immune-body was found to have the following properties:

(1) To the suspension of blood corpuscles from six to ten times the minimum haemolytic dose of immune-body requires to be added before one dose remains free, sufficient time being allowed to permit of the maximum degree of combination.

(2) When the immune-body and complement are added to blood corpuscles there occurs in addition to haemolysis an absorption of the complement. The combined complement does not become dissociated again to any appreciable extent.

(3) The amount of complement absorbed may be much greater than the amount necessary to cause just complete lysis, and the degree of complement fixation depends on the amount of immune-body present. Thus, multiple doses of immune-body together with red corpuscles lead to the union of increased amounts of complement, and in some instances the complement taken up is approximately proportional to the amount of immune-body; in other cases considerable divergence is met with, mainly in the direction of diminished proportional absorption in the case of increasing multiples of immune-body.

Our observations have reference to the points mentioned above. The sera of seven animals have been examined, each being repeatedly tested at different stages of immunisation. The sera were allowed to separate from the blood-clot for 24 hours and were sealed in quill tubes and then heated once for 1¼ hours at 55° C. They were then kept in the dark at room temperature. Tests were made with the same specimen of serum on repeated occasions, so that its properties were usually estimated both recently after withdrawal from the body and also after the lapse of from several weeks to several months. By way of correlating the results several specimens of serum from the

same animal and also from different animals were usually examined simultaneously; and an immune serum of high potency was included in the series. In this way additional information was obtained as to the part played by individual properties of different specimens of red blood corpuscles, especially as regards the effect of age on the immune serum. It will be best to refer to the latter points first of all, since they afford an indication as to the interpretation which should be put upon varying results in consecutive experiments.

Methods.

The procedure followed in the experiments was that adopted by Muir. In order to obtain blood suspensions of fairly uniform density the mixture of washed blood corpuscles and salt solution was centrifugalised until there was a sharp line of demarcation between sediment and supernatant fluid; the latter was then pipetted off and 3 c.c. of sediment were made up to 100 c.c. with salt solution. The guinea-pig's complement was freed from natural immune-body by treatment with an equal volume of washed ox-blood for an hour at 0° C. The haemolytic doses of the immune sera were always estimated just before the experiment and with the same complement and corpuscles; when necessary, to ensure accuracy of measurement, the sera were diluted 10 times or 100 times with salt solution immediately before the estimation. The amount of immune-body necessary to produce just complete lysis in 1¼ hours at 37° C. in the presence of 5–10 doses of complement was taken as the dose.

The dosage of immune-body as affected by individual properties of the red blood corpuscles.

Example. Three specimens of ox-blood were obtained—No. (1) on 6. VI. '10, Nos. (2) and (3) on 7. VI. '10. The blood was kept on ice till 8. VI. '10, when suspensions of the washed corpuscles were prepared and the dose of a potent immune serum tested. Each tube containing 1 c.c. of suspension along with immune-body (I.B.) received 0·05 c.c. of guinea-pig's complement (7 doses) and the reading was taken after 2½ hours at 37° C. The results were as follows:—

R.C.'s	No.	(1)	dose of	I.B.	= 0·0007 c.c.
„	„	(2)	„	„	= 0·0005 „
„	„	(3)	„	„	= 0·0003 „

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These results show that in the presence of excess of complement *the "sensitising action" of a given immune-body varies greatly with different specimens of blood.* Such variations in susceptibility must explain in great part the erratic alterations in dosage of the same immune serum met with when tested at different times (a different specimen of ox-blood and complement was of course employed on each occasion).

Examples. (1) Immune serum from rabbit "B" withdrawn 22. v. '10, four days after the second injection. Table I shows the haemolytic dose in a series of estimations of this immune serum with different samples of blood.

TABLE I.

Blood	Date of estimation	Dose for 1 c.c. R.C.'s
a	25. v. '10	0·003 c.c.
b	1. vi. ,,	0·006 ,,
c	23. vi. ,,	0·002 ,,
d	9. vii. ,,	0·0045 ,,
e	13. vii. ,,	0·0035 ,,
f	3. viii. ,,	0·0045 ,,
g	14. xii. ,,	0·006 ,,

(2) The following example is of interest as the immune-body was exceedingly powerful. Immune serum withdrawn vii. '10, ten days after the third injection.

TABLE II.

Blood	Date of estimation	Dose for 1 c.c. R.C.'s
a'	3. viii. '10	0·0005 c.c.
b'	24. ix. ,,	0·0006 ,,
c'	11. xi. ,,	0·0008 ,,
d'	14. xii. ,,	0·00075 ,,
e'	23. xii. ,,	0·0012 ,,
f'	22. ii. '11	0·0014 ,,
g'	1. iii. ,,	0·0012 ,,

The immune serum in this case appeared to be diminishing in potency. The results of tests which were carried out simultaneously to determine the amount of complement absorbed by red corpuscles along with the immune-body (one and five doses) are given in Table III.

The method of such an absorption experiment is as follows:—To a series of test tubes containing 0·5 c.c. of 5 per cent. blood suspension and a given number of doses of immune-body (usually one and five doses)

varying amounts of complement are added. The tubes are incubated for 1½ hours at 37° C. and are shaken at intervals during this time. Then if lysis is incomplete in any tubes, these are centrifugalised and the fluid is pipetted off into other tubes. To each tube of clear fluid 0·5 c.c. of blood suspension, sensitised previously with five doses of a powerful immune serum from the rabbit, is added. The tubes are again incubated for 1¼ hours and kept at room temperature overnight; the result is read next morning.

TABLE III.

Blood	Amount of complement required to cause just complete lysis of the test corpuscles	
	1 dose of I.B.	5 doses of I.B.
a' (3. VIII. '10)	0·035 c.c.	0·18 c.c.
c' (11. XI. ,,)	0·03 ,,	0·2 ,,
d' (14. XII. ,,)	0·02 ,,	0·09 ,,
e' (23. XII. ,,)	0·045 ,,	0·15 ,,
f' (22. II. '11)	0·03 ,,	0·12 ,,
g' (1. III. ,,)	0·045 ,,	0·13 ,,

When allowance is made for the variations due to different specimens of red corpuscles and complement, it will be seen that the immune-body has not undergone any definite alteration so far as its power of causing combination of complement with the red corpuscles is concerned. Accordingly, these results confirm the view that the immune-body is fairly stable in this respect, and it is clear that if it does deteriorate with age the alteration does not markedly affect the relative amounts of complement absorbed with one dose and with multiple doses. That is to say, deterioration with age might simply be due to a disappearance of a proportion of the molecules without any qualitative alteration.

Alterations in the immune-body during the process of immunisation.

The following examples illustrate the general principle which we have found to hold in all cases. The experiments were all carried out in the manner described above; 0·5 c.c. of blood suspension and immune-body (one or five doses) was mixed with complement; after 1½ hours at 37° C. the presence of free complement in the fluid was tested for by adding corpuscles sensitised with five doses of a powerful mature immune serum (in some cases 0·5 c.c. of test corpuscles was employed, in other experiments 1 c.c., as is noted in the tables).

By "mature" immune-body is meant one which was obtained after repeated injections (usually three), and whose dose was low, 0·001 c.c. or less for 1 c.c. of blood suspension.

Date of experiment, 8. III. '10.
Rabbit "BW," I.B. 28. I. '10, 8 days after 1st injection (4 c.c. blood-sediment), D. = 0·05 c.c.
,, 4. II. '10, 7 ,, 2nd ,, D. = 0·03 c.c.
,, 8. II. '10, 11 ,, ,, ,, D. = 0·003 c.c.
,, 1. III. '10, 7 ,, 3rd ,, D. = 0·008 c.c.
D. of "mature" I.B. = 0·0003 c.c.

Amount of complement in c.c.	Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)							
	1 dose of I.B.				5 doses of I.B.			
	0·01	0·015	0·02	0·025	0·01	0·02	0·035	0·055
"BW" 28. I.	just complete	complete	complete	complete	complete	complete	complete	complete
,, 4. II.	just complete	complete	complete	complete	just complete	complete	complete	complete
,, 8. II.	almost complete	just complete	complete	complete	0	trace	almost complete	complete
,, 1. III.	just complete	complete	complete	complete	0	0	trace	almost complete
Mature I.B.	complete	complete	complete	complete	0	trace	almost complete	complete

Initial lysis of corpuscles in series with 1 D. of I.B. after 1 hour:
"BW" 28. I., 4th tube just complete.
,, 4. II., 4th ,, ,,
,, 8. II., 1st ,, ,,
,, 1. III., 3rd ,, ,,
Mature I.B. 4th ,, ,,

The result of this experiment shows that, (1) the immune-body in the early stages of immunisation (28. I. and 4. II.) is deficient in the power of leading to the combination of complement. Thus, with multiple doses of such an immune-body, little or no more complement may be absorbed than with one dose (in some other instances multiple doses did cause a slight increase in the complement absorption). As immunisation proceeds (8. II.) the power of absorbing complement is acquired, and it persists well marked after the potency of the serum, as measured by the haemolytic dose, has begun to fall (1. III.); (2) the amount of complement necessary to produce lysis in the presence of a small amount of immune-body varies; but in this respect the specimens of the dates 28. I., 4. II., 1. III. and the mature immune-body are practically comparable, although they show such great differences in the amounts of complement absorbed by multiple doses. The

mature immune-body was older than any of the specimens of immune-body "BW." The interval elapsing between the experiment and the withdrawal of specimen 1. III. is comparatively short as compared with the age of the other specimens. The possibility of this factor of age introducing a disturbing effect is eliminated in the following example.

A. Date of experiment, 24. I. '10.

Rabbit "S," I.B. 24. I. '10, 4 days after 1st injection (4 c.c. blood-sediment), D. = 0·0025 c.c. Dose of mature I.B. = 0·00025 c.c.

	Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)				
Amount of complement in c.c.			4½ D. of I.B.		
	0·0125	0·0175	0·025	0·035	0·045
"S" 24. I.	trace	almost complete	just complete	complete	complete
Mature I.B.	trace	trace	marked	very marked	almost complete

B. Date of experiment, 8. III. '10.

Rabbit "S," I.B. 28. I. '10, 8 days after 1st injection, D. = 0·0012 c.c. D. of mature I.B. = 0·0003 c.c.

	Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)								
Amount of complement in c.c.	1 D. of I.B.				4 D. of I.B.				
	0·01	0·015	0·02	0·025	0·01	0·02	0·035	0·055	0·075
"S" 28. I.	almost complete	complete	complete	complete	0	0	trace	almost complete	complete
Mature I.B.	complete	complete	complete	complete	0	trace	almost complete	complete	complete

C. Date of experiment, 1. IV. '10.

Rabbit "S," I.B. 24. I. '10, D. = 0·01 c.c.
28. I. '10, D. = 0·001 c.c.

	Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)						
Amount of complement in c.c.	1 D. of I.B.			5 D. of I.B.			
	0·01	0·02	0·03	0·02	0·035	0·05	0·07
"S" 24. I.	very marked	complete	complete	marked	complete	complete	complete
"S" 28. I.	very marked	complete	complete	0	0	very marked	complete

The results show clearly the deficient power of absorbing complement exerted by the immune-body at the early period of immunisation. This is apparent both when the immune serum 24. I. is tested immediately after withdrawal and also after two months.

The possibility that the degree of complement absorption may be influenced by elements of the serum other than the immune-body had to be considered. Accordingly, the following experiments were carried out, (1) an immune serum was tested at various periods of immunisation,

the time of withdrawal of the specimens being so chosen that a specimen taken while the immune-body content was rising after the first injection, had almost the same haemolytic dose as one taken when the immune-body content was falling, and (2) a potent immune serum was diluted with heated normal rabbit's serum until the volume of serum containing a dose was the same as that of a weak rising immune-serum. The complement absorbing powers of the latter and of the diluted and undiluted potent immune serum were then tested simultaneously.

(1) *Experiment illustrating the complement absorption by rising and falling immune-bodies of approximately equal dosage.*

Date of experiment, 3. VIII. '10.

Rabbit "B," I.B. 9. v. '10, rising, 4 days after 1st injection (4 c.c. blood-sediment), D. = 0·025 c.c.

,, 22. v. '10, ,, 4 ,, 2nd ,, D. = 0·00225 c.c.

,, 7. VII. '10, falling, 26 ,, 3rd ,, D. = 0·006 c.c.

,, 2. VIII. '10, ,, 52 ,, 3rd ,, D. = 0·028 c.c.

D. of mature I.B. = 0·00025 c.c.

Amount of guinea-pig's complement in c.c.	Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)								
	1 D. of I.B.				5 D. of I.B.				
	0·015	0·025	0·035	0·05	0·05	0·07	0·09	0·12	0·15
9. v.	0	0·275	0·6	0·8	0·7	0·875	0·9	complete	complete
22. v.	0	0·4	0·825	complete	0	0·25	0·8	complete	complete
7. VII.	0	0·25	0·5	0·825	0	0	0	0·25	0·7
2. VIII.	0	0	0	0	0	0	0	0	0
Mature I.B.	0	0	0·5	0·875	0	0	0	0·15	0·4

Initial lysis in 1 D. series:

"B," 9. v., 4th tube just complete.

22. v., 1st ,, ,,

7. VII., 3rd ,, ,,

2. VIII., 2nd ,, ,,

Mature I.B. 3rd ,, ,,

Thus, the specimens of immune serum 9. v. and 2. VIII. are almost identical so far as the haemolytic dose is concerned; but the latter leads to the absorption of much more complement. Also (1) the rate of lysis is greater with the falling immune-body and (2) the amount of complement necessary to produce complete lysis in the presence of a limited amount of immune-body (one dose) is less with the falling than with the rising immune-body. As will be seen later, however, there is no definite relationship between the amount of complement necessary to cause complete lysis and the amount of complement absorbed through the agency of a given immune-body.

(2) *Experiment illustrating the effect of diluting the immune-body with inactive serum.*

Date of experiment, 1. III. '11.

Rabbit "GW," I.B. 28. II. '11, 12 days after 3rd injection, D. = 0·0022 c.c.

,, "BH," I.B. 28. II. '11, 15 ,, 1st ,, D. = 0·006 c.c.

With the I.B. of rabbit "GW" two series of tests were made:

(*a*) Without any addition:

(*b*) With the addition of normal rabbit's serum (55° C.), so that 1 D. was contained in 0·006 c.c. of serum.

	Lysis of test corpuscles (0·5 c.c. + 5 D. of I.B.)									
	1 D. of I.B.					5 D. of I.B.				
Amount of guinea-pig's complement in c.c.	0·01	0·02	0·03	0·04	0·05	0·04	0·055	0·075	0·11	0·15
"GW" (*a*) without serum	0·2	0·25	0·3	0·35	0·55	0	0·1	0·25	0·3	0·6
"GW" (*b*) with serum ...	0·2	0·25	0·3	0·35	0·55	0	0·1	0·25	0·3	0·65
"BH"	0·25	0·5	0·75	0·9	complete	0	0·25	0·5	complete	complete

Initial lysis in 1 D. series:

"GW" (*a*) without serum, 2nd tube just complete.

,, (*b*) with ,, 2nd ,, ,,

"BH" 2nd ,, ,,

Thus, the addition of serum to the potent immune-body ("GW") has caused practically no alteration in its power of absorbing complement along with the red corpuscles. Further evidence that the amount of complement absorption is due to the character of the immune-body molecules and is independent of other serum constituents is furnished by the fact that after a single injection of blood corpuscles into a suitable animal the haemolytic power of the serum may increase very rapidly and yet multiple doses of the immune-body may be deficient in the power of causing increased absorption of complement. On the other hand, in some animals both the haemolytic and the complement absorbing power rise rapidly.

Example. Date of experiment, 8. III. '10.

Rabbit "FW," I.B. 1. III. '10, 5 days after 1st injection (4 c.c. blood-sediment), D. = 0·001 c.c.

,, "S," I.B. 28. I. '10, 7 ,, ,, ,, ,, ,, D. = 0·0012 c.c.

	Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)						
	1 D. of I.B.			4 D. of I.B.			
Amount of guinea-pig's complement in c.c.	0·01	0·015	0·02	0·01	0·02	0·035	0·055
"FW"	just complete	complete	complete	0	very marked	complete	complete
"S"	almost complete	just complete	complete	0	0	trace	almost complete

The immune-body in the serum of rabbit "S" at an earlier date after the first injection was deficient in complement-absorbing power, *v.* p. 214.

The independence of the amount of complement necessary to cause lysis with an immune-body and the amount brought into combination through its agency.

The independence of these two functions is well illustrated by the following experiment:—

Date of experiment, 19. v. '10.
Rabbit "B" 9. v. '10, 4 days after first injection (4 c.c. of blood-sediment), D. = 0·035 c.c.
" 17. v. '10, 12 " " " " " D. = 0·003 c.c.
Dose of mature I.B. = 0·0002 c.c.

Amounts of complement in c.c.	Lysis of test corpuscles (1 c.c. + 5 D. of I.B.)						
	1 D. of I.B.			4 D. of I.B.			
	0·01	0·015	0·025	0·02	0·03	0·045	0·06
"B" 9. v.	almost complete	complete	complete	just complete	complete	complete	complete
"B" 17. v.	just complete	complete	complete	just complete	complete	complete	complete
Mature I.B.	trace	almost complete	complete	0	0	distinct	just complete

Initial lysis: most rapid with mature I.B., slowest with "B" 9. v.
"B" 9. v., 1 D. series + 0·025 c.c. complement = not complete.
"B" 17. v., 1 D. " + 0·01 c.c. " = just complete.
Mature I.B., 1 D. " + 0·01 c.c. " = complete.

Thus, while the complement is much more toxic with the specimen 17. v. than with 9. v. the amount of complement absorbed with both is practically equal and much less than what is absorbed by the mature immune-body.

The combining power of immune-body with the red corpuscles.

In several instances the combining power of immune-bodies at early stages of immunisation, for the red corpuscles was tested. We did not attempt to determine differences in the rate of combination, but allowed practically sufficient time for maximal combination.

The following is a representative experiment:—

Date of experiment, 22. II. '11.
Rabbit "BH" I.B. 21. II. '11, 5 days after 1st injection (2 c.c. of blood-sediment), D. = 0·01 c.c.
Rabbit "GW" I.B. 21. II. '11, 8 days after 3rd injection, D. = 0·0015 c.c.
Mature I.B. VII. '10, D. = 0·0007 c.c.

To a series of tubes each containing 0·5 c.c. blood suspension 3, 5, 7, 10 D. of I.B. were added, the tubes were then put at 37° C. for $\frac{3}{4}$ hour and thereafter left for $\frac{3}{4}$ hour at room temperature, being frequently shaken during this time. They were then centrifugalised and the fluid added to 0·5 c.c. blood suspension along with six doses of complement. The amount of lysis in the test corpuscles was read at the end of $1\frac{1}{4}$ hours at 37° C.

Lysis of Test Corpuscles.

Doses of I.B.	3	5	7	10
"BH"	very marked	almost complete	just complete	complete
"GW"	trace	very marked	almost complete	complete
Mature I.B.	very marked	complete	complete	complete

This result shows that provided at least sufficient time is given for combination, the rising immune-body shows no deficiency in its power of combining with the red corpuscles. The complement combining experiment carried out at the same time gave the usual result (Table IV).

TABLE IV.

	Lysis of test corpuscles (0·5 c.c.+5 D. of I.B.)							
	1 D. of I.B.			5 D. of I.B.				
Amount of complement in c.c.	0·015	0·025	0·035	0·035	0·05	0·07	0·1	0·13
"BH"	0·675	complete	complete	0·85	complete	complete	complete	complete
"GW"	0·5	0·9	complete	0·35	0·5	0·6	0·8	complete
Mature I.B.	0·5	0·95	complete	0·3	0·3	0·4	0·85	complete

This shows that the immune-body ("BH") at the early stage has relatively weak powers of leading to absorption of complement when multiple haemolytic doses are used.

In the complement absorption experiments described above the immune-body was added to the corpuscles first and then the complement about 10 minutes later. Muir and Browning[1] had found in a certain case (ox corpuscles, immune-body from the rabbit and ox complement) that the resulting lysis was much more marked when the immune-body was added to the corpuscles a considerable time before the complement. Accordingly, we performed the following experiment to determine whether the length of time elapsing between the addition of the immune-body and the complement to the corpuscles affected the amount of complement absorbed in the case of a weak rising immune-body.

[1] *Vide* Muir, *Studies on Immunity.*

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5 D. of I.B., Rabbit "BH" 21. II. '11, were added to series (*a*) and the mixture of immune-body and corpuscles was allowed to stand two hours at room temperature, then the same amount of I.B. was added to series (*b*) and the complement was added to both series immediately: lysis proceeded slightly more rapidly in series (*a*) than in series (*b*), but the end point was practically the same in both. After incubation for 1½ hours at 37° C. the uncombined complement was tested for in the usual way.

The result showed that the amount of lysis in corresponding tubes in both series was practically identical, that is to say, the amount of complement absorbed was not increased by allowing a long time for combination of the immune-body before adding the complement.

SUMMARY AND CONCLUSIONS.

(1) When rabbits are injected intraperitoneally with ox's red blood corpuscles the haemolytic immune-body which is developed shows qualitative differences at different stages of immunisation.

(2) The immune-body molecules which appear in the serum in the early stage of immunisation (*e.g.* four to eight days after a single injection of 2 to 4 c.c. of red blood corpuscles) are deficient in the power of causing absorption of complement when added to the corresponding blood corpuscles. This is most clearly brought out by the very slight increase in complement absorbed under the influence of multiple doses of immune-body as compared with the amount absorbed by one dose. The deficient complement absorption does not depend to any marked degree on deficient combination of immune-body with the receptors of the red corpuscles.

(3) When, after repeated injections of blood corpuscles, immunisation has been carried to such a stage that an immune-body is produced which is very active in causing absorption of complement, then it is found that on ceasing to give further injections the relative complement-combining power remains high after the immune-body content of the serum as measured by the haemolytic dose has fallen to a very considerable extent.

So far as we are aware the occurrence of such alterations in the properties of immune-body during the process of immunisation has not hitherto been noted.