ON THE HAEMOLYTIC IMMUNE ISOLYSINS OF THE OX AND THEIR RELATION TO THE QUESTION OF INDIVIDUALITY AND BLOOD-RELATIONSHIP.

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The term "Isolysin" was first employed by Ehrlich and Morgenroth in the third of their now classical series of papers entitled "Studies on Haemolysis[1]." It had been shown by Bordet that if the red blood corpuscles of an animal A are injected into another animal B, of a different species, the serum of B develops a haemolysin for the corpuscles of A. This haemolysin being produced in a different species of animal from that yielding the corpuscles, is termed, according to Ehrlich's nomenclature, a heterolysin.

Ehrlich and Morgenroth now set themselves to investigate the results of injecting animals with the red blood corpuscles of other animals of the same species and found that here again a haemolysin was produced which they termed an *isolysin*, as it was formed in animals of the same species as those providing the corpuscles.

Ehrlich and Morgenroth were led to these researches by the following considerations, which are perhaps best given in their own words:

"In pathology, the changes foremost to be considered are those resulting from the absorption, by an organism, of its own cell material. Such occasions are presented by many different diseases. Keeping to the blood, for example, if an individual suffers a considerable subcutaneous haemorrhage or one into a body-cavity, or if part of his blood-corpuscles are destroyed and dissolved by certain blood-poisons, the

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essential conditions, just as in experiment, are given for the reactive formation of substances possessing specific injurious affinities for these blood-cells......

"It is therefore of the highest pathological importance to determine whether the absorption of its own body material can excite reactive changes in the organism, and what the nature of these changes is. The simplest conditions and those most accessible to experimental study are those which arise on the absorption of blood-cells. But here we face a curious dilemma. If an animal organism when injected with blood-cells of foreign species always produces a specific haemolysin for each of these species it must surely be following a natural law; and it is improbable that this law which applies in any particular number of cases should be suspended in the case of blood-cells of the same individual. On the other hand it is not to be denied that the formation of such haemolytic substances would appear dysteological in the highest degree. For example, if, in an individual who has had an extensive haemorrhage into a body-cavity, the absorption of this blood caused the formation of a blood-poison which destroyed the rest of the blood-cells, this would be a phenomenon whose actual occurrence lacks any clinical evidence whatever and one which no one is willing to accept.

"It cannot be doubted that the organism seeks a way out of this difficulty by means of certain regulating contrivances, whose determination will be of the highest interest."

In order to investigate this question Ehrlich and Morgenroth immunised a series of goats with goats' blood. This was done by giving one intraperitoneal injection of a somewhat large volume of blood which had been previously laked by the addition of water. Laked blood was employed as it was thought that the uninjured blood corpuscles of the same species as the animal injected would be destroyed very slowly in the peritoneal cavity of this animal, and that consequently the absorption would be so gradual as to prevent the occurrence of what may be termed an "ictus immunatorius."

It was found in this way that the injection of goats' blood into other goats resulted as a rule in the formation of isolysins, but that in no instance was an autolysin formed, *i.e.* the serum of the injected animal never acquired the property of dissolving its own corpuscles. Altogether thirteen isolytic sera were prepared and their characters studied by means of anti-isolytic serum with the somewhat astonishing result that it was found that they all differed from one another, *i.e.* that they represented different isolysins.

The great interest presented by these isolysins rendered a more extensive examination of their nature and properties most desirable; but the immunisation of a large number of animals in this way is most tedious. Fortunately, however, the writers happened to have at their disposal, in the Institute for the preparation of cattle plague serum at Cairo, about 100 animals which in the course of their immunisation with cattle plague had received large quantities of cattle blood under almost ideal conditions for the formation of isolysins.

These cattle, which were used for the preparation of cattle plague serum, were immunised by Kolle and Turner's method, consisting in a preliminary simultaneous inoculation of cattle plague immune serum and a small quantity of virulent cattle plague blood, followed after a short interval by an intramuscular injection of four litres of virulent blood. These massive injections of four litres were repeated regularly every two months as long as the animal was used for the production of serum.

The cattle were bled for serum 14 days after the large injection of blood and again three times at intervals of a fortnight before the next injection. The animals used for the production of serum were Egyptian cattle and the blood used for their immunisation was that of freshly imported Cyprus cattle. On testing the fresh serum of the cattle which had been immunised in this way on the red blood corpuscles of Cyprus and Egyptian cattle it was found that it showed practically no haemolytic action; but that on the addition of a little fresh guinea-pig serum, it became powerfully haemolytic, the lack of haemolytic action of the fresh serum being due only to the want of a suitable complement. This point appears to have been overlooked by Frei [2], who in referring to cattle plague serum says:

"Only the specific rinderpest anti-bodies were observed, even after the hyperimmunisation of oxen with great quantities of virulent rinderpest blood in order to obtain a strong immune serum, recognised *in vivo* by the successful treatment of animals; in no instance were clinical or anatomical phenomena due to haemolysis or precipitation recorded. We are therefore confronted by the remarkable fact that cattle do not produce isolysins in their blood, *i.e.* substances with the property of dissolving cattle blood *in vivo* or *vitro*, even after the injection of enormous quantities of blood."

In order to get an approximate idea of the strength of the sera of the various animals, a rough test was made of the sera of all the serum producing animals in the Institute, the haemolytic dose being taken as the amount of the serum required to haemolyse in 1 hour at 37° C. 1 c.c. of a 5 % suspension of the corpuscles of one normal Cyprus bull in the presence of 1/10 c.c. of fresh guinea-pig serum.

The results were as follows:

4	animals showed	no haemolysis at 1/10 c.c.
26	„ „	complete haemolysis at 1/10 c.c.
76	„ „	complete haemolysis at 1/100 c.c.
106		

A certain number of sera were also tested at 1/1000 c.c., but in no case was complete haemolysis obtained at this dilution.

The minimum haemolytic dose may therefore in the majority of cases be said to lie between 1/100 and 1/1000 c.c.

If the serum of one immune animal is tested on the red blood corpuscles of a series of different individual cattle marked differences are noted in the susceptibility of the corpuscles of the different individuals, some being highly susceptible, others less so, and others again very resistant.

If now the serum of a second immune animal is tested on the same series of corpuscles similar differences are noted but these are generally not parallel to those obtained with the first serum. Corpuscles which are highly susceptible to one serum may be resistant to the other and vice versa.

This is shown in the following table:

TABLE I.

Showing action of sera of immune cattle on corpuscles of the same individuals.

Immune serum ... ... 0·03 c.c.
Fresh guinea-pig serum ... 0·10 c.c.
5 % suspension of ox corpuscles 1·0 c.c.

		Red blood corpuscles of ox					
		No. 80	No. 86	No. 90	No. 92	No. 99	No. 102
Immune serum of ox	No. 80	0	+ + +	+	tr.	tr.	+
	No. 86	+ +	0	+ + +	+	+ + +	+
	No. 90	+ + +	0	0	+	+ + +	+
	No. 92	+ + +	+ + +	+ + +	0	+ + +	+ + +
	No. 99	+ + +	+ + +	+ + +	+	0	+ + +
	No. 102	0	0	0	0	0	0

tr. = trace of haemolysis.
\+ = definite „
\+ + = marked „
\+ + + = complete „

These results are quite in accordance with what was found by Ehrlich and Morgenroth in their isolytic goat sera.

It is interesting to note that the corpuscles of the immune animals did not, on the whole, appear to show any greater resistance than those of fresh animals, and also that the question of race seemed to have no marked effect. The animals were immunised entirely with the blood of Cyprus cattle, but in spite of this their sera appeared to act equally strongly on the corpuscles of Cyprus, Egyptian and Soudan cattle.

The haemolytic action of the serum of the immune cattle was not limited to the corpuscles of the ox but also acted very powerfully on those of the sheep and goat. The corpuscles of the Egyptian buffalo, camel, rabbit, guinea-pig and man were not affected, at any rate in the presence of guinea-pig complement.

The haemolysin in the serum of the immune cattle is an isolysin, but appears to be practically never an autolysin, *i.e.* it has no action on its own corpuscles. Of all the sera examined by us only in one case was haemolysis observed in the tube containing the immune serum with its own corpuscles, and in this case the amount of haemolysis was slight and may have been due to some accidental circumstance. As a general rule the tube containing the immune serum with its own corpuscles was sharply picked out by the complete absence of even the faintest trace of haemolysis.

This result is in complete accord with what was found by Ehrlich and Morgenroth in their isolytic goat sera.

In the course of our investigations we were much puzzled by the fact that the isolysin present in the serum of the immune cattle does not act with the complement free in the blood of these cattle but appears to require a foreign complement.

We naturally assume that the formation of the isolysin is protective and has for its object the solution and removal of the strange corpuscles; hence in the case of the goat the injection of the animal with the corpuscles of another individual gives rise to an isolysin which, acting in conjunction with the complement normally present in the serum of the goat, causes the solution of the corpuscles which are then easily dealt with.

In the case of the ox, however, we have the formation of an isolysin which has practically no action on ox corpuscles in the presence of fresh ox serum.

It may be added that, by means of a different haemolytic system, it was easy to demonstrate the presence of complement in this serum.

As it was difficult to imagine that the organism would, so to speak, go to the trouble of elaborating a complex isolysin which would not act with the complement available, we were led to enquire whether a suitable complement did not exist elsewhere in the body and with this idea the following experiment was made:

A normal Cyprus bull was injected intravenously with one litre of the mixed serum of ten immune cattle. This serum, although only 24 hours old, showed no action on ox corpuscles *in vitro* if no foreign complement were added, but in the presence of fresh guinea-pig serum was very powerfully haemolytic (0·01 c.c. being sufficient to haemolyse 1 c.c. of 5 $^0/_0$ suspension of ox corpuscles). A few hours after the injection the urine was very darkly haemoglobin stained, showing that a suitable complement had been forthcoming.

On testing the serum of the animal a few days later it was found that although it had now no haemolytic action on its own corpuscles it was distinctly haemolytic for the corpuscles of many other individuals, *i.e.* it was isolytic but not autolytic. It was thus possible, by passive immunisation with the serum, to produce a condition apparently similar to that of the animals actively immunised with corpuscles.

A series of experiments[1] were now made by replacing this "exhaustion" of the serum *in vivo* by exhaustion *in vitro*, the technique being as follows:

The immune serum was mixed with an equal volume of the washed corpuscles with which it was desired to exhaust the serum; the mixture kept at 37° C. for an hour, centrifuged, and the serum again treated in the same way with more washed corpuscles and the process repeated a third time. It was then found that the serum had lost all traces of haemolytic power for the corpuscles in question.

By means of this method the sera of different immune cattle were now exhausted with the corpuscles of various individuals of the same species and the haemolytic power of these sera, after such treatment, was studied on the corpuscles of different individuals.

It was found that if an immune serum is exhausted with corpuscles of an individual (A) it remains haemolytic for the corpuscles of many other individuals, but loses its haemolytic power for the corpuscles of some other individuals as well as for those of (A).

If now a second immune serum is exhausted with the same corpuscles (A) its action on the various corpuscles is not exactly

[1] A preliminary note of these experiments was published in the *Proceedings of the Royal Society*, June, 1910.

parallel to that of the first serum and often shows very marked differences.

This result is to be expected, as it was shown by Ehrlich and Morgenroth that two goats each injected with similar doses of the same goat's blood at the same times gave quite different isolysins. In fact the isolysins formed depend upon two distinct factors:

(*a*) the individuality of the injected corpuscles,
(*b*) the individuality of the animal into which they are injected.

When we consider the enormous number of variations possible in each of these factors we see the almost unlimited possibilities in the resulting sera.

In view of the above it should be possible by taking a mixture of a sufficiently large number of immune sera and exhausting this with the corpuscles of one individual, to obtain a serum which is specific for the corpuscles of this one individual, *i.e.* which has no haemolytic action on these corpuscles, but haemolyses those of all other individuals of the same species. To test this, a mixture was made of the sera of between 60 and 70 immune Egyptian cattle. This mixture was then exhausted with the corpuscles of a normal Cyprus bull and then tested on the washed corpuscles of 20 immune Egyptian cattle, two normal Cyprus cattle and the above mentioned Cyprus bull with whose corpuscles the mixture had been exhausted. For the test equal parts were taken of

(*a*) the exhausted serum,
(*b*) a 5 % suspension of the washed red blood corpuscles,
(*c*) a one-tenth dilution of fresh guinea-pig serum in normal saline.

The tubes were kept at 37° C. for one hour and then left over-night in the ice-safe; after which the results were read off.

It was found then that complete haemolysis had occurred in all the tubes, with the exception of the control tube containing corpuscles of the Cyprus bull with which the serum was exhausted. The exhausted serum was thus able to pick out, quite sharply, the corpuscles of one individual from those of 22 others. Following up these results, a second and more extensive test was made. In this case the same immune serum was used; it was, however, exhausted with the corpuscles of another normal bull.

This exhausted serum was then tested on the corpuscles of 110 different cattle (3 Soudan, 34 Cyprus, 73 Egyptian).

In this test again the tubes showed complete haemolysis with the exception of the one containing the corpuscles for which the serum had been "exhausted." This control tube showed no trace of haemolysis.

A number of other tests has been made by exhausting the serum with the corpuscles of various individuals, and the general rule has so far always held except in the case of close blood-relations, where certain exceptions occur which will be dealt with later.

These results show that the red blood cells of any individual (excluding for the moment the question of close blood-relations) possess characters which differentiate them quite distinctly from the red blood cells of any other individual even of the same species[1].

A consideration of this very striking fact at once leads to speculation as to whether this is not merely one example of a general law and if all the cells of an individual are not, so to speak, stamped with his individuality. We know that this holds for the spermatozoon and the ovum and it can be experimentally demonstrated for the red blood corpuscles, but whether it will prove to be the case for other cells remains to be determined. Unfortunately the other cells present much greater experimental difficulties than the red blood cell whose delicate stroma renders it an ideal object for such observations.

Another point which the above results emphasize is the enormous complexity of the structure of the red blood cells which renders it difficult to imagine that these cells have no other function than the comparatively simple one of acting as oxygen carriers. This question has been pointed out by Ehrlich (9) in his exceedingly suggestive article on "The receptor apparatus of the red blood corpuscles," where he suggests that these cells may be regarded as storage reservoirs in the sense that they temporarily take up the most varied substances derived from the food or from the internal metabolism.

Having found that it was possible to distinguish the red blood corpuscles of non-related individuals of the same species, it was interesting to compare the corpuscles of closely related individuals. The first test was made on the corpuscles of a cow and her calf. The mixed sera of a considerable number of immune cattle were exhausted with the corpuscles of the mother and calf separately and it was found that while

[1] v. Dungern and Hirschfeld (*Zeitschr. f. Immunitätsforschung*, IV., p. 531), using a similar method, have recently investigated the red blood corpuscles of dogs by means of iso-agglutinins. They come to the conclusion that dogs may be divided into several groups according to the agglutination of their corpuscles and conclude that the red blood corpuscles of all the animals belonging to one group have an identical chemical structure.

exhaustion of the serum with the corpuscles of the calf removed the haemolysin for the calf only, exhaustion of the serum with the corpuscles of the cow removed the haemolysin not only for the cow but also for the calf. The continuance of these investigations was much hampered by the difficulty of obtaining, in Egypt, samples of blood from the various members of complete families of cattle or goats; but by the kindness of Mr Littlewood, C.V.I., we had the opportunity of examining an interesting group of sheep. This consisted of three ewes each with one lamb; all the lambs were by the same father, which was fortunately available.

The first test was made on the corpuscles of the father (tup), ewe A, her lamb (lamb *a*) and another sheep not related to the family in question.

A mixture of the sera of about 80 immune animals was twice exhausted with an equal volume of the various corpuscles, and the serum then tested separately on the various corpuscles in the presence of guinea-pig complement as follows:

TABLE II.

Showing action of polyvalent serum, after exhaustion with sheep's corpuscles, on the corpuscles of closely related sheep.

Exhausted immune serum ... 0·3 c.c.
$\frac{1}{10}$ dilution of fresh g.-pig serum ... 0·3 c.c.
5 % suspension of sheep's corpuscles 0·3 c.c.

	Red blood corpuscles of: Tup (father)	Ewe A (mother)	Lamb *a* (child)	Non-related pregnant sheep
Immune serum exhausted with corpuscles of: Tup (father)	0	+ + +	+ + +	+ + +
Ewe A (mother)	+ + +	0	0	0
Lamb *a* (child)	+ +	0	0	0
Non-related pregnant sheep	+ + +	+ + +	+ +	0

It will be noticed that there is a marked parallelism between the lamb and ewe and none between the lamb and father.

Next day the test was continued on the rest of the group as follows (Table III).

On examining the results of the whole test it is seen that while the corpuscles of lamb (*a*) resemble exactly those of the mother, the corpuscles of lambs (*b*) and (*c*) show no resemblance to their mother's but almost exactly resemble those of their father.

TABLE III. *Continuation of Table II.*

Immune serum exhausted with corpuscles of	Red blood corpuscles of									
								Non-related sheep		
	Tup	Ewe A	Lamb *a*	Ewe B	Lamb *b*	Ewe C	Lamb *c*	(1) Tup	(2) Ewe	(3) Ewe
Tup	0	+ + +	+ + +	+	0	+ + +	0	+	0	+ + +
Ewe B	+	+	0	0	0	+ +	+	0	0	0
Lamb *b*	+	+ + +	+ + +	+ +	0	+ + +	+	+ + +	0	0
Ewe C	+ +	0	0	0	0	0	+	0	0	0
Lamb *c*	0	+ + +	+ + +	+ +	0	+ + +	0	+ +	+	+
Non-related sheep:										
(1) Tup	+ +	+	+	+	0	+ + +	+	0	0	0
(2) Ewe	+	+ +	+	+	+	+ +	0	+	0	0

This result is analogous to those obtained by v. Dungern and Hirschfeld[4] who by means of their isoagglutinins found that the offspring of two dogs whose blood belonged to different biochemical groups showed different relations towards their parents. The blood of one pup belonged to the same group as the mother, the blood of another showed the structures of both parents, while two others showed no resemblance to either.

The problem is exceedingly complicated and much work will have to be done before it is safe to generalise, but we believe that the results obtained so far are sufficient to justify the hope that the method may be useful in attacking the question of heredity.

In conclusion we should like to acknowledge our indebtedness to Mr Allen, Veterinary Surgeon to the Serum Institute, who has rendered us invaluable assistance.

Conclusions.

1. The immunisation of the ox with the red blood corpuscles of other oxen gives rise to the formation of a haemolytic amboceptor in the blood of the immunised animals.

2. The amboceptor so formed is an *isolysin* but not an *autolysin*.

3. The amboceptor is fixed by susceptible corpuscles; it is not activated by the complement present in the fresh serum of the ox, but requires the addition of a foreign complement.

4. The race of the animal furnishing the corpuscles appears to have very little influence on the resulting haemolysis.

5. The serum of an animal so immunised acts very differently on the red blood corpuscles of different individual oxen.

6. The sera of different individuals similarly immunised differ from one another in their action on the corpuscles of different individuals.

7. If the serum of a single immunised animal be "exhausted" with excess of the corpuscles of one other individual, the serum loses its power of haemolysing the corpuscles of this individual, while retaining the power of haemolysing the corpuscles of many, but not all, other individuals.

8. If, however, a polyvalent serum be made by mixing the sera of a large number of immunised animals, and this serum is exhausted with the corpuscles of any one individual, the serum entirely loses its power of haemolysing the corpuscles of this individual, but remains strongly haemolytic for all other individuals not closely related to the individual whose corpuscles were employed for the exhaustion of the serum.

(N.B. It is possible that exceptions may be found, but these have not yet been met with except in the cases of close blood relations.)

9. The red blood corpuscles of any individual are thus characterised by a definite individuality of their own and can be distinguished from those of any other individual of the same species.

10. When studied by the above method the red blood corpuscles of closely related animals show interesting relations with one another, which would suggest the application of the method to the study of the question of heredity.

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