NOTES ON THE LEUCOCYTE-REACTION DURING THE IMMUNISATION OF THE HORSE AND GOAT WITH DIPHTHERIA TOXIN.

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In the foregoing paper details have been given of the immunisation with diphtheria toxin of three animals (two horses and one goat). In the case of the horse "Plug" and goat "Mephistopheles" the opportunity was taken of investigating the question of the leucocyte reaction with a view to determine whether any notable changes in the leucocytic formula took place as the result of the frequent injections of toxin and the increased elaboration of antitoxin. The few investigations which have been recorded on the blood-changes during diphtheria immunisation have unfortunately led to somewhat contradictory conclusions.

Nicolas and Courmont (1897) were the first to study this question in any detail. These authors recorded the variations in the total leucocytes during the immunisation of several horses. Immunisation was begun with iodised toxin and continued every second or third day with gradually increasing doses of pure toxin. No fixed rule was followed however. The final dose after about fifty injections was only 60 c.c. toxin. Consequently the intermediate doses of 5 c.c., 10 c.c., 15 c.c., 20 c.c., 30 c.c., 40 c.c., and 50 c.c., were many times repeated. Counts made at rather irregular intervals during the course of immunisation did not show any reaction in the direction of hyperleucocytosis. The average leucocyte count during immunisation varied only very slightly from that previous to the commencement of the inoculations. On two or three occasions, counts were made every two or three hours, after the injection, but here again no notable reaction was evident. Nicolas and Courmont concluded that no conspicuous leucocyte-reaction was to be

detected either at the beginning or at an advanced stage of the injection period and that therefore immunity could be developed without any essential leucocyte change. If hyperleucocytosis results at all it is merely a sign of grave intoxication and indicates that the toxin dose has been too strong.

The work of these authors was severely criticised by Besredka (1898) who laid stress on the necessity of paying attention principally to the behaviour of the polynuclear cells and not of the absolute leucocyte count. Further, while pointing out that Nicolas and Courmont had failed to give any account of the antitoxin yield of their horses, Besredka affirmed that he would not have been surprised at the absence of leucocyte-reaction in the absence of any great development of immunity. It seems doubtful how far this latter criticism of Besredka is justifiable.

This author immunised a goat with diphtheria toxin and showed that each dose of toxin was followed by a conspicuous leucocyte reaction in which the polynuclear cells were mainly implicated. During the later stages, however, there was some evidence that the polynuclear cells did not react so promptly and that the mononuclear cells participated more largely in the reaction.

It may be noted that immunisation was begun with heated toxin and followed up with the unheated fluid. The final dose, after about three months' immunisation, was only 1·5 c.c. of a toxin whose lethal dose for a guinea-pig of 500 gm. was $\frac{1}{50}$ c.c. At the end of this period he was able to demonstrate that ·5 c.c. of the goat serum when mixed with 20 fatal doses of toxin (= ·20 c.c.) protected a guinea-pig of 500 gm. weight. No mention of his method of taking blood-samples from the goat is made by Besredka.

In reply to the latter's criticism Nicolas and Courmont (1898) maintained that their horses had undoubtedly developed antitoxin as a result of the injections. Further, they refer to the temporary paraplegia of Besredka's goat as being due to incautious inoculation. Under such conditions, they assert, a certain degree of hyperleucocytosis would not be an unexpected phenomenon.

A second contribution to the subject was made by Nicolas, Courmont and Prat (1900). Three animals were immunised, a horse, a goat, and a donkey. The polynuclear cells were estimated in addition to the absolute counts. Each animal received increasing doses of toxin up to 17 c.c., which was the final dose. In the goat the initial dose was $\frac{1}{500}$ c.c. of iodised toxin. The counts were made every two or three days, but no hyperleucocytosis was ever demonstrable. Indeed, it

appeared that a hyperleucocytosis associated with a hypopolynucleosis was most in evidence during the immunisation. In summing up their results these authors adhere to their previous statements that immunisation is possible without any essential leucocyte reaction. Butjagin (1902) without giving numerical details, agrees with Nicolas and Courmont, that the elaboration of antitoxin need not be associated with any notable leucocyte reaction.

The leucocyte reaction in the goat.

In the case of "Mephistopheles" (as also of "Plug") the samples were always taken from the jugular vein by cannula, as it was found that samples taken from the ear gave fallacious results. The skin of the goat's ear is exceedingly tough and elastic, so that after puncture of the vein the blood did not flow freely but tended to gather in the subcutaneous tissue, forming a lump which was slowly absorbed. In Table I. are recorded, in relation to the toxin doses the total leucocyte counts and the percentages of the various leucocyte forms during the immunisation of the goat.

From Dec. 1 to Feb. 7 the counts were made every four days, corresponding with the toxin-injections. After the large doses inoculated on Feb. 7 and 12, counts were made every day, in order to determine more accurately the course of the leucocytes in response to massive doses. Also, on several occasions prior to the commencement of immunisation, the leucocytic formula was determined, the percentage of the non-granular cells being about 10% higher than that of the polynuclear cells. On the first day of the injections the relation of the non-granular cells to the polynuclear cells was as 55·2 : 43·6.

Let us consider the period from Dec. 1 to Jan. 16. It will be seen from the table that the effect of one injection had generally disappeared before the next came on, with notable exceptions on Dec. 9, Dec. 30 and Jan. 16. On these latter occasions a marked relative polynuclear leucocytosis still remained after the preceding injection. Also on Dec. 30 and Jan. 16 the absolute leucocyte counts were comparatively high.

In the period from Jan. 19 to Feb. 7 the polynuclear cells predominated markedly on the fourth day after each injection. The daily counts after the injection of 50 c.c. toxin on Feb. 7 and of 100 c.c. toxin on Feb. 12, show clearly that the slight resultant hyperleucocytosis is mainly due to an increase in the polynuclear cells while the mononuclears show only slight fluctuations.

TABLE I. Goat (Mephistopheles).

Date	Toxin	Total leucocytes	Small lymphs.	Large lymphs.	L. monos. and trans.	Non-gran. percentage	Polymorphos.	Eosinophils	Mast-cells
Dec. 1	·001	9,350	51·6	2	1·6	55·2	43·6	·8	·4
5	·002	9,650	48	6·1	3·5	57·6	40	1·7	·03
9	·004	9,200	40·6	2·8	1·8	45·2	53·2	1	·6
13	·01	8,300	38	9·5	6·2	53·7	43·7	1·7	·7
18	·02	6,650	51·8	4·4	1	57·2	41·2	1·2	·4
23	·04	8,350	71·1	1·1	·9	73·1	24·3	2·4	0
27	·1	7,900	48·8	2·9	·1	51·8	45·6	1·8	·6
30	·2	11,000	25·3	3·4	·5	29·2	65	4·8	·9
Jan. 3	·5	7,700	49·8	4·4	1·7	55·9	42·3	·8	·9
8	1	6,850	55·3	8·6	·1	64	33·3	2·6	·1
12	2	6,400	41·9	7·9	2·6	52·4	44·2	2·6	·7
16	3	10,500	23·6	4·6	2·8	31	67·9	·8	·2
19	6	9,500	41·5	5·2	·2	46·9	52	·9	0
23	12	8,300	38·8	1·2	0	40	56·6	2·6	·7
29	25	5,800	39·9	7	1·9	48·8	47·5	3·3	·2
Feb. 3	50	8,000	38·2	1·1	·4	39·7	58·4	1·2	·5
7	50	7,350	45·1	1·8	·8	47·7	50·8	1·1	·4
8	—	7,750	32·3	5·3	1	38·6	60·7	·2	·4
9	—	11,400	36·3	1·5	·9	38·7	60	·6	·6
10	—	7,500	29·3	1·1	1·4	31·8	67·4	·3	·3
12	100	6,400	39	3·4	·9	43·3	55	1·2	·2
13	—	10,600	30·4	·9	·6	31·9	67·6	·3	0
14	—	12,000	30·8	1·4	1·9	34·1	65	·7	0
15	—	8,400	38·4	2·1	·9	41·4	57·3	·9	·3
16	—	9,000	51·7	2·6	0	54·3	43·8	·9	1
19	150	11,400	30·5	·3	0	30·8	68·6	·5	0
20	—	16,000	14·9	·4	0	15·3	84·6	0	0

The absolute values of the non-granular and polynuclear cells after Feb. 7 and 12 are recorded below.

Date	Non-granular	Polynuclear	
Feb. 7	3431	3650	Inoculation of 50 c.c.
8	2926	4620	
9	4332	6840	
10	2325	5025	
11	—	—	
12	2752	3520	Inoculation of 100 c.c.
13	3286	4982	
14	4080	7800	
15	3444	4788	
16	4860	3870	
17	—	—	
18	—	—	
19	3420	7752	Inoculation of 150 c.c.
20	2400	13440	

On the day following the inoculation of 150 c.c. a very marked hyperleucocytosis was present and the polynuclear percentage had mounted as high as 84·6.

Regarding the behaviour of the eosinophile cells and mast-cells no very definite conclusions can be drawn owing to the small numbers of these cells present in the blood of the goat. It would seem, however, that after the injections on Feb. 7, 12, and 19, the eosinophile percentage was considerably reduced.

In Table II. are recorded the leucocyte estimations made during the immunisation of "Plug." Before the commencement of the injections the normal leucocytic formula was ascertained. In marked contrast to the goat, the polynuclear percentage slightly exceeds the non-granular percentage. The leucocytic formula of the horse is also remarkable on account of the high percentage of eosinophile cells.

Let us consider the period from Nov. 17 to Jan. 8.

Although a slight rise in the total leucocyte count was evident on the third day after each of the earlier injections, the percentages of the non-granular and polymorphonuclear cells had invariably returned to their normal values.

On Dec. 4, 15, and 19, however, a marked polynucleosis remained. Also on the 19th the total count remained at the high value of 18,000. It is interesting that the further injection of 8 c.c. toxin on this date did not produce any cumulative effect on the leucocyte reaction as, on the following day, the total count had fallen and the relative polynucleosis had diminished.

TABLE II. Horse (Plug).

Date	Toxin	Total leucocytes	Small lymphs.	Large lymphs.	L. monos. and trans.	Non-gran. percentage	Polymorphos.	Eosinophils	Mast-cells
Nov. 17	·01	8,200	40	2	2·7	44·7	49·4	4·7	1
20	·02	11,650	43·8	1	1·5	46·3	47·1	4·8	1·6
23	·04	12,550	38·8	3·6	3·6	46	48·4	4·4	·9
27	·1	10,166	39·2	3·6	4·2	46	46·3	5·8	·8
30	·2	11,800	42	3·2	3·2	48·4	46·4	4	1
Dec. 4	·5	9,300	36·2	3·3	2	41·5	52·3	4·4	1·7
7	1	10,100	38·5	3·7	1·8	44	49	5·3	1·4
11	2	7,850	45·6	2·1	1·0	48·7	47·6	2·6	·8
15	4	10,050	32·1	5·2	4·5	41·8	53·1	3·8	1
19	8	18,000	15·6	1·6	4·9	22·1	75·5	1·2	1
20	—	12,700	29·6	4·1	3·0	36·7	57·8	4·2	·9
22	15	8,650	46·5	1·8	·8	49·1	47·1	3·1	·5
26	30	10,100	52·6	2·1	·3	55	43	1·1	·6
29	60	10,000	35·8	3·6	3·2	42·6	55	1·8	·7
Jan. 1	120	8,600	33·9	2·0	1·1	37	60	2	·9
4	250	10,000	34·9	3·4	2·4	40·7	55·4	2·2	1·5
8	500	8,300	35·9	1·9	·3	38·1	58·1	2·7	1
9	—	13,750	19·2	·4	0	19·6	79·2	1·1	0
10	—	19,250	17·6	1·9	2·1	21·6	74·8	1·9	1·6
11	—	14,250	26·6	4·2	·7	31·5	65·3	2·6	·4
12	500	8,150	39	2·2	·1	41·3	56·5	1·9	·1
16	800	11,650	36	4	·5	40·5	55·1	3·7	·5
17	—	11,100	15	1·3	·8	17·1	79·4	2·9	·4
18	—	15,800	21·8	2·8	1·7	26·3	70·1	3	·4
19	—	12,800	18·2	2·8	1·6	22·6	74·5	2·4	·3
23	1000	8,400	31·6	3·7	1·8	37·1	59·2	2·7	·9
24	—	19,200	9·9	1·8	·3	12	87·3	·1	·6
25	—	17,100	13·5	·7	3·4	17·6	80·2	1·9	·1
26	—	13,600	28·6	·9	·3	29·8	66·4	2·8	·8
27	—	8,400	42·8	2·5	2·1	47·4	48·1	3·9	·4
29	—	9,250	33·8	2	1·2	37	57·5	5·3	·1
30	—	12,750	30·7	1·2	2	33·9	61	4·3	·7
31	—	8,600	26·9	3	3·3	33·2	62·2	3·9	·5
Feb. 1	—	10,500	33	2·3	1·6	36·9	58·5	3·8	·5
2	—	14,000	31·5	3·8	2·7	38	55·1	5·6	1·1
5	1000	7,150	42·5	3·4	2·1	48	47·6	3·8	·5
6	—	15,500	18·3	·6	·6	19·5	78·6	1·1	·7
7	—	20,300	23·7	1·2	·9	25·8	69·3	3·6	1·1
8	—	12,250	32·5	1·2	1·2	34·9	59·5	3·7	1·8
9	—	8,700	42·4	·4	·9	43·7	47·6	5·7	2·7
10	—	8,500	43	1·8	1·3	46·1	48·9	3·8	1·1

Polynucleosis was also marked on Dec. 29, Jan. 1, 4, and 8. After the injections of 500 c.c., 800 c.c., and 1000 c.c. (*bis*), the blood was examined every day as was done in the case of massive injections in the goat. It will be seen that each of these injections was followed by an enormous rise in the polynuclear percentage although the hyperleucocytosis never exceeded 20,000.

Below are appended the absolute values of the non-granular and polynuclear cells on the days following these massive injections.

After the intravenous injection of 1000 c.c. toxin on March 12, the blood was also examined and it was found that on the day following, the polynuclear percentage had risen to the enormous value of 91·1 while the mononuclear percentage fell to 8·9. On the 14th of March, the polynuclear percentage fell to 83·9 while the mononuclear rose to 15·1.

Date	Non-granulars	Polynuclears	Toxin
Jan. 8	3162	4822	500 c.c.
9	2603	10823	
10	4032	14208	
11	4402	9230	
12	3321	4536	
16	4640	6380	800 c.c.
17	1887	8769	
18	4108	11060	
19	2816	9472	
23	3108	4956	1000 c.c.
24	2304	16704	
25	2907	13680	
26	3944	8976	
27	3948	4032	
29	3404	5244	
30	4191	7747	
31	2838	5332	
Feb. 1	3780	6190	
2	5320	7700	
5	3408	3337	
6	2945	12190	
7	5075	14007	
8	4270	7198	
9	3741	4089	
10	3910	4165	
	Intravenous injection.		
March 12	4300	5200	1000 c.c.
13	1665	16835	
14	2940	16464	

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When we consider the absolute values of the non-granular and polynuclear cells detailed above, it will be evident that the leucocyte response is mainly a polynuclear one. The total mononuclears fall suddenly, but rapidly regain their normal level, while the polynuclears rise slowly to a maximum and then slowly decline. From the fluctuations in the eosinophile and mast-cells it is impossible to draw any reliable inferences.

Summary and Conclusions.

It must be clear then from the above data on the horse and goat that the inoculation of diphtheria toxin produces a definite leucocytic reaction of polynuclear type. In fact the detailed evidence speaks more clearly in favour of a leucocytic reaction than Besredka's own results on the goat which he immunised. The question then arises: How are we to interpret the contradictory conclusions reached by Nicolas and Courmont? Metchnikoff (1905), while referring to the work of these authors in his *Immunity in Infectious Diseases*, maintains that a slight leucocytic reaction is apparent from their own figures and especially from the figures obtained in the early hours succeeding an injection. Of course, Nicolas and Courmont considered these slight changes to be negligible.

It seems to me, that the reason must be sought in the method of immunisation employed.

Nicolas and Courmont immunised their animals with very small doses many times repeated, and by such a method it was doubtless possible to reduce local swelling to a minimum. With no local reaction it would seem quite reasonable to expect an absence of general leucocytic reaction, but, as Metchnikoff maintains, such local tumefaction is never absent in horses which are subjected to increasing doses of toxin and which ultimately develop high grade antitoxin.

Throughout the immunisation of the animals detailed in the foregoing paper local swelling and oedema invariably followed the injections except in the earlier stages of immunisation when small doses were being given.

There is no doubt that, in order to produce a high degree of immunity in an animal, the stimulation must be sufficient. A certain small degree of immunity may always be reached without much leucocytic change, as was apparent in the early stages of "Plug's" immunisation.

It must be admitted, however, that, though the leucocytic reaction may be extremely marked after large doses of toxin, it does not necessarily follow that the antitoxin development will be correspondingly influenced.

Such a phenomenon was apparent in the case of "Plug," a relatively refractory horse.

In the more responsive horses, a parallelism between leucocytic reaction after large doses and increased antitoxin development would certainly be expected, without, however, inferring a causal relationship between these two phenomena. We cannot therefore admit the general applicability of Nicolas and Courmont's views, which merely indicate that cells subjected to small oft-repeated doses lose their power of reaction.

On the other hand there is probably no justification for the criticism brought forward by Besredka with regard to the results of Nicolas and Courmont, viz. that in the absence of antitoxin development, an absence of leucocytic reaction was to be expected.

We may therefore conclude that the leucocytic reaction of polynuclear type which follows the injection of large and increasing doses of diphtheria toxin is merely an evidence of efficient cell-stimulation and may not necessarily be accompanied by increased antitoxin development.

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