DIPHTHERIA ANTI-*GRAVIS* SERUM: ITS ACTION ON EXPERIMENTAL INFECTION AND IN THE TREATMENT OF PATIENTS

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The view, based on clinical experience, has long been held that antitoxin may fail to exert its therapeutic action on diphtheritic infections in man. A number of possible reasons for this failure have been advanced, such as the quality or avidity of the antitoxin, the existence of an additional toxic factor, differences in the amount of toxin produced or the rate of its production by the infecting strains and in the invasive character of many of them. The anomalous relation that is sometimes found to exist between the degree of immunity of a person who has been exposed to the infection and the level of circulating antitoxin in his blood has led to similar speculations. The problem, considered as a whole, concerns active immunization of children as well as the treatment of the disease; the seemingly conflicting data have been reviewed and admirably presented by Hartley and his associates in their report (1950). We therefore think it desirable to give an account of work carried out in 1935–6 jointly with the Monsall Fever Hospital, Manchester, on antibacterial immunity as an agent in the treatment of diphtheria. An anti-*gravis* serum prepared in the horse was tested for its effect in controlling experimental infection in the guinea-pig and in treating patients. The results obtained by us point to the need for a comprehensive study of this aspect of the subject.

A survey of the literature shows that, during the past fifty years, an 'antibacterial' serum has not often been employed in the treatment of diphtheria and that, in the older papers, experimental proof of its efficacy is lacking. Nevertheless, the question whether an antibacterial component is a necessary element in the serum therapy of the disease has frequently been discussed and indeed is referred to by some of the early writers. The evidence brought forward for or against the use of an 'antibacterial' serum cannot easily be appraised, especially in articles published before the description of the three types of *Corynebacterium diphtheriae* by Anderson, Happold, McLeod & Thomson (1931), and the subsequent serological typing of the *gravis* strains (Robinson & Peeney, 1936). Petrie (1934) reviewed the problem of the serum treatment of the severe form of diphtheria and, commenting on the employment of an 'antibacterial' serum, stated his belief that the grounds for the use of such a serum should be re-examined. A few workers had attempted to prepare an 'antibacterial' serum in the horse (Martin, 1903; Dean, 1908; Ramon, Bessemans & de Potter, 1931; Bessemans, Ramon & de Potter, 1931; Ramon & Debré, 1932) but the cultures they used were not strictly

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defined in the immunological sense. The only research known to us which takes into account the three types is that of Sordelli, Manzullo, Navarro Viola & Ferrari (1944). Their conclusions, of which we became aware after this paper had been completed, agree with our own so far as a comparison can be drawn between them. They immunized horses with *gravis*, *intermedius* and *mitis* strains, and carried out protection experiments in guinea-pigs with univalent sera against test-doses of the individual strains used for immunization. Two *gravis* strains gave rise to neutralizing antibodies in the horse which protected guinea-pigs against infection with these strains and with a third *gravis* strain. They found that their antimicrobial sera enhanced the action of antitoxin in experimental infections.

THE PREPARATION OF UNIVALENT 'ANTIBACTERIAL' SERA IN THE HORSE

For the purpose of this investigation three immunological types of serum were prepared in horses at the Lister Institute against strains isolated in Manchester about the same time. These strains were *gravis*, serological type I (Robinson & Peeney, 1936), *intermedius* and *mitis*; their morphological and cultural characters were typical.

Three horses, 'Gravis' (F. 17 years), 'Inter' (F. 14 years) and 'Mitis' (F. 16 years) were immunized with the corresponding bacterial suspensions. These were prepared as follows:

Loeffler's serum in Roux bottles was sown with saline suspensions of the appropriate type, the condensation water having previously been removed. After 18 hr. incubation at 37° C. the growth was transferred to 0·4 % phenol-saline and washed by centrifuging it 3 times; after storage in the refrigerator for a week it was found to be sterile; the final density was 2000×10^6 bacilli per ml. The doses of each suspension were without exception given to the horses intravenously.

A 2 l. bleeding was taken from the horses on 15 October 1934 in order to obtain samples of normal serum for controlling the experimental results, and immediately thereafter each received a routine dose of tetanus toxoid.

The serum from 'Gravis' was alone employed for the clinical and experimental work described in this paper, but details of the immunization of 'Inter' and 'Mitis' are included for comparison (Table 1). Attention is specially directed to the information given in the table for the immunizing courses I, II and III of 'Gravis', because the serum used in the investigation was obtained during and immediately after them.

The horse 'Gravis' received a total of 2190 ml. of the suspension in ninety-two doses during the period 22 October 1934–4 August 1936; this animal remained in good health. The initial dose was 0·1 ml. and the amount was increased to a maximum of 50 ml. during the 1st two immunizing courses; to a maximum of 40 ml. in the 3rd and 4th courses; 35 ml. in the 5th course; 25 ml. in the 6th course; 20 ml. in the 7th course, and 25 ml. in the 8th course. The same variations in the maximum dose as the immunization progressed were recorded for 'Inter' and 'Mitis'. The diminishing scale of dosage indicates that the horses became sensitive to the injections as time went on. The allergic reactions were,

Table 1. *Details of the production of antibacterial horse sera prepared against the* gravis, intermedius *and* mitis *types of* Corynebacterium diphtheriae

Immunizing course		Dosage		Febrile reaction: Evening		Febrile reaction: Morning		Bleedings	
Serial no.	Inclusive dates	Total volume (ml.)	No. of doses	Mean temperature	Mean pulse rate	Mean temperature	Mean pulse rate	Inclusive dates	No. during the period
				Immunization of the horse 'Gravis'					
I	22. x. 34 to 31. i. 35	680	27	101·4 (21)*	45 (21)	100·0 (27)	40 (27)	7. ii. 35	1
II, III	11. ii. 35 to 3. vi. 35	731	22	101·9 (21)	48 (21)	100·2 (21)	40 (21)	25. iii. 35 to 14. vi. 35	4
IV–VIII	7. xi. 35 to 4. viii. 36	779	43	101·2 (33)	45 (33)	100·0 (37)	38 (37)	23. xii. 35 to 12. viii. 36	9
	Totals	2190	92						
				Immunization of the horse 'Inter'					
I	12. xi. 34 to 31. i. 35	729	23	100·9 (19)	39 (19)	99·9 (23)	36 (23)	7. ii. 35	1
II, III	11. ii. 35 to 3. vi. 35	626	22	104·0 (21)	53 (21)	100·7 (21)	43 (21)	25. iii. 35 to 14. vi. 35	4
IV–VI	4. xi. 35 to 15. iv. 36	526	25	103·2 (16)	51 (16)	100·5 (17)	41 (17)	9. xii. 35 to 1. iii. 36	4
	Totals	1881	70						
				Immunization of the horse 'Mitis'					
I	5. xi. 34 to 31. v. 35	700	25	101·7 (20)	45 (20)	100·4 (25)	38 (25)	7. ii. 35	1
II, III	11. ii. 35 to 3. vi. 35	566	21	104·0 (19)	55 (19)	101·9 (19)	44 (19)	25. iii. 35 to 14. vi. 35	4
IV–VIII	4. xi. 35 to 4. viii. 36	699	41	103·1 (32)	51 (31)	100·7 (33)	41 (32)	9. xii. 35 to 25. vi. 36	8
	Totals	1965	87						

* The figures in brackets represent the number of observations from which the mean was derived.

however, never severe and the appetite remained good except when fever was present, but the three horses lost weight in the later stages in spite of careful dieting. The table shows that the temperature and pulse-frequency on the evening of the dose were not much raised at any time in 'Gravis', nor during the 1st immunizing course undergone by 'Inter' and 'Mitis', but that these two horses reacted with definite fever in the later courses. The febrile reaction observed in 'Mitis' during the 2nd and later courses often lasted for 48 hr. The normal pulse-frequency and temperature of the three horses may be taken to be 40 and 100·5° F.

The propriety of continuing the immunization with suspensions of living bacilli was considered, and it was deemed essential, if this procedure were adopted, to protect the horses from a fatal diphtheritic toxaemia by giving them a preliminary series of doses of toxoid so as to produce in them some degree of antitoxic immunity. A disadvantage of the plan was that it would render the serum unsuitable for experimental work on an antibacterial component, although we hoped it might enhance the potency of the serum for clinical use. A series of doses of toxoid was, in fact, interposed between the 3rd and the 4th intravenous course for each horse, and was continued until the serum contained about 100 units of antitoxin per ml. It was, however, decided to avoid, at least temporarily, the injection of living bacilli, since experience at the Lister Institute of immunizing horses with intravenous doses of various kinds of living bacteria, for example, virulent pneumococcus, the smooth 'Vi'-containing *Salmonella typhi* and *Corynebacterium ovis* had shown that despite a maximal concentration of specific antibodies in the blood there is a high degree of risk of a fatal result, if a succession of large doses is given to them.

'Inter' was found dead on the morning of 16 April 1936 after a dose of 25 ml. of the suspension given on the previous day, and 'Mitis' was found dead on the morning of 5 August after a dose of 25 ml. on the previous day. In both, death was unexpected and was probably sudden.

The samples of serum used in the experimental tests were: (1) a sample of unconcentrated serum taken from 'Gravis' on 7 February 1935, and (2) part of a pool of 16 l. of unconcentrated serum obtained from this horse on 25 March 1935, 1 April 1935, 11 June 1935 and 14 June 1935. The remainder of the pool was concentrated and was used for the clinical tests.

The concentration was effected by adding 28 % of solid ammonium sulphate to the serum so as to remove the albumin fraction. The resulting globulin precipitate was dialysed against tap water at room temperature for 3 days; 1 % sodium chloride, 0·35 % tricresol, and 0·35 % ether were added to the dialysis-residue, which was then filtered through a Berkefeld candle. The degree of concentration in terms of the volume of the dialysis-residue was about 4·5 times. The final product in the ampoules showed a good deal of turbidity, especially after storage in the cold room. The turbidity was unavoidable and was due to the presence of the least soluble portion of the euglobulin fraction for, in default of knowledge of the site of the antibody, it was thought better to retain the part that tends to precipitate on standing. Accordingly, care was taken to include the precipitate in the doses given to the patients; even when it was administered intravenously it produced no untoward effects.

EXPERIMENTAL DEMONSTRATION IN THE GUINEA-PIG OF A PROTECTIVE EFFECT FROM INJECTING ANTI-*GRAVIS* SERUM

The two samples of natural anti-*gravis* serum (serological type I), mentioned in the preceding section, were used. These had agglutinin titres of 1:3200 and 1:6400 respectively, and both contained 0·2 i.u. of antitoxin per ml.

For each experiment guinea-pigs of approximately 250 g. were divided into three equal groups. The animals in the 1st group received intracardially, intravenously or intraperitoneally 1% of their body weight of anti-*gravis* serum. Those in the 2nd group—the normal serum control—were each given by the corresponding route 1% of its body weight of normal horse serum; the sample was taken from the same horse before immunization and antitoxin was added to it so that it contained 0·2 i.u./ml. The 3rd group received the test dose of culture alone with no serum.

One hour after the injection of the serum all the animals received subcutaneously into the shaved anterior abdominal wall ten lethal doses of once-washed type I *gravis* bacilli prepared as follows. An 18–24 hr. growth on Loeffler's medium, inoculated after the condensation water had been removed, was suspended in normal saline, the suspension was centrifuged and the deposit re-suspended in saline. The density of the washed suspension was adjusted so that ten lethal doses were contained in 1 ml. The time taken to carry out these procedures was uniform throughout so that variations in viability of the bacteria, due to differences in the time during which they remained in saline before being inoculated, were avoided. A record was kept of the interval that elapsed before death occurred, and cultures from the local lesion, the liver and the heart-blood were made on McLeod's chocolate-tellurite medium.

A summary of ten experiments is given in Table 2, and the results afford grounds for the conclusion that the serum contained a protective antibody. This was borne out by comparing the death rates in the three groups, the survival times, and the frequency with which the organisms were isolated from the liver and the heart-blood.

The low antitoxin content of the anti-*gravis* serum and of the normal control serum was not without effect. This was shown by the much shorter survival time of the controls that received no serum as compared with those in the serum-control group, and also by the fact that a few of the serum-control animals survived. The small amount of antitoxin introduced was, however, apparently not sufficient to modify appreciably the invasive power of the bacteria, since the percentage of positive blood cultures in the two groups of control animals shows little difference. The results obtained from both control groups corresponded well with previous observations on the invasive power of the *gravis*, *mitis* and *intermedius* types when tested for virulence in guinea-pigs (Robinson & Marshall, 1934).

We may reasonably assume that the check on the invasiveness of the organisms and the significantly lower death rate in the experimental animals are attributable to an antibacterial factor in the serum. In all our experiments the serum was given 1 hr. before the test dose, and thus it was in a position to forestall the subsequent

bacterial attack. How far the antibacterial factor would have operated in an already well-established infection was not ascertained. Some tentative observations indicated that the antibacterial action of the serum is to a large extent specific for

Table 2. *The protective effect of* gravis *antibacterial serum in guinea-pigs infected with ten lethal doses of washed bacilli of the* gravis *type injected subcutaneously 1 hr. after the serum*

Exp.	Route of injection of serum	Agglutinin titre	No. of guinea-pigs injected	No. died	Av. time to death (hr.)	No. of times organisms were recovered post-mortem from: Local lesion	Liver	Heart blood
		Gravis antibacterial serum containing 0·2 antitoxin unit per ml.						
1	I.C.	1/3200	8	1	140	1	0	0
2	I.C.	1/3200	10	2	96	2	0	0
3	I.C.	1/3200	10	3	120	2	1	0
4	I.V.	1/3200	10	1	120	1	0	0
5	I.V.	1/3200	10	0	—	0	0	0
6	I.V.	1/3200	6	3	108	3	1	0
7	I.P.	1/6400	10	4	120	3	0	0
8	I.P.	1/6400	10	4	100	4	1	0
9	I.P.	1/6400	10	2	96	2	0	0
10	I.P.	1/6400	10	3	112	2	0	0
			94	23		20	3	0
		Controls. Normal serum plus 0·2 antitoxin unit per ml.						
1	I.C.	0	8	7	72	7	7	2
2	I.C.	0	10	7	63	7	7	3
3	I.C.	0	10	10	68	10	9	4
4	I.V.	0	10	9	60	9	9	3
5	I.V.	0	10	10	58	10	10	5
6	I.V.	0	6	6	62	6	6	2
7	I.P.	0	10	9	65	9	8	3
8	I.P.	0	10	9	70	9	8	4
9	I.P.	0	10	6	70	6	6	2
10	I.P.	0	10	9	64	9	9	3
			94	82		82	79	31
		Controls. No serum						
1	I.C.	0	8	8	44	8	8	3
2	I.C.	0	10	10	42	10	9	3
3	I.C.	0	10	10	48	10	10	3
4	I.V.	0	10	10	40	10	9	4
5	I.V.	0	10	10	38	10	10	6
6	I.V.	0	6	6	40	6	6	3
7	I.P.	0	10	10	44	10	10	4
8	I.P.	0	10	10	46	10	9	3
9	I.P.	0	10	10	48	10	9	3
10	I.P.	0	10	10	42	10	10	4
			94	94		94	90	36

I.C. = intracardially; I.V. = intravenously; I.P. = intraperitoneally.

the different serological types of the *gravis* strains, but this point requires further investigation. Attention was mainly confined to type I which was the predominant *gravis* strain in Great Britain at the time (Robinson & Peeney, 1936).

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CLINICAL TRIAL OF THE ANTI-*GRAVIS* SERUM

Evidence of the efficacy of the serum in treating diphtheria was sought by limiting the trial to cases of a degree of severity which conformed to the definition adopted by the Departmental Committee of the London County Council as 'cases in which the membrane extends beyond the tonsils and anterior pillars to either one or more of the following: palate, uvula, pharynx or post-nasal mucosa and its extensions'; they were referred to as groups I and II by Robinson & Marshall (1934, 1935). Only patients whose symptoms came into this category when admitted to Monsall Hospital were selected for the inquiry provided that they were of suitable age and were infected by the *gravis* strain. In order to avoid possible differences due to age, a comparison was made of treated and untreated cases in each of the following age groups: 0–3 years, 4–5 years, 6–7 years, 8–9 years, 10–11 years and 12–14 years. The sex of the patients was not taken into account.

The scheme followed was to administer to alternate cases in the same age group a dose of anti-*gravis* serum in addition to the antitoxin they would normally have received. The general treatment of the two series of cases was identical. When the first clinical examination of the patient was made he was designated as belonging to series A, that is, the control group, or to B, the specially treated group (Table 3). When two patients of the same age group had to be allotted at the same time, the choice of the control patient was made by spinning a coin.

Table 3. *Summary of cases treated with* gravis *antiserum plus antitoxin and control cases treated with antitoxin only*

Age group (years)	A. Control series			B. Treated series		
	Total	Recovered	Died	Total	Recovered	Died
0–3	4	2	2	2	1	1
4–5	14	8	6	13	9	4
6–7	11	8	3	6	4	2
8–9	5	5	—	5	5	—
10–11	3	3	—	5	4	1
12–14	3	3	—	4	2	2
Total	40	29 (72·5%)	11 (27·5%)	35	25 (71·4%)	10 (28·6%)

An investigation of prevalent bacterial types during the previous three and a half years had provided evidence (Robinson & Marshall, 1934, 1935) that at the time the clinical trial was made the majority of severe cases occurring in the district were likely to be caused by *gravis* strains. Since it was essential to administer the anti-*gravis* serum as early as possible, this was done before the type of the infecting organism was determined; cases afterwards found to be due to a type other than *gravis* were excluded from the analysis.

Injection of the serum did not cause undesirable effects, and, as the trial proceeded, it became manifest that comparatively large doses could be employed with confidence. The usual practice was to give 20–25 ml. either by the intramuscular or intraperitoneal route when the patient was seen for the first time. If, in addition, intravenous medication was decided upon, 1 ml. was injected

together with the antitoxic serum and, if no reaction occurred, the patient received 10 ml. of anti-*gravis* serum after an interval of 0·5–1 hr. This procedure, together with the usual precautions taken when serum is given intravenously, was not attended by any reaction worth recording. The total amount of both kinds of serum was administered as a rule when the patient was examined for the first time. The routine dose of antitoxin was 40,000 units intramuscularly and 60,000 units intravenously; in a few of the cases the dosage was varied, the range being from 30,000 to 80,000 units intramuscularly and from 40,000 to 100,000 units intravenously. A 2nd and occasionally a 3rd injection of the anti-*gravis* serum, nearly always in a dose of 10 ml., was administered to some of the patients. Serum reactions that occurred 9 or 10 days after an injection were neither more marked nor more frequent in the specially treated than in the control series.

The severity of the disease in the two groups of selected patients and the lack of precise knowledge of the kind of serum under test made it unlikely that dramatic results would be obtained. Nevertheless, careful clinical observation led to the conclusion that the anti-*gravis* serum produced an ameliorative action on the local lesion. Thus the fauces became clean more rapidly; on the average, the membrane on the tonsils disappeared 1·5 days earlier than in the control patients. This was particularly noticeable in several cases in which a profuse sanious and purulent nasal discharge ceased after 24–36 hr. The change for the better in the local lesion was accompanied in a few of the cases by a slight temporary improvement in the general condition which encouraged the hope that some of the children who were desperately ill might recover. Again, life was on the average prolonged; of the children who died, those who received the combined treatment lived 0·7 of a day longer than the controls (7 and 6·3 days respectively), notwithstanding that the former cases were admitted 0·4 of a day later in the course of the disease (4·3 and 3·9 days respectively).

Despite its temporary beneficial effects the anti-*gravis* serum did not reduce the fatality rate, for there was no significant difference between the death rates of the two groups (28·6 %; 27·5 %, Table 3). We may add that in both of the groups the patients who recovered were admitted to hospital at an earlier stage of the disease than the patients who died; there was little difference in time between those who were treated with antitoxin and those who received, in addition, the anti-*gravis* serum (3·1 and 3·0 days). Of thirty patients in the control series who survived the acute stage fourteen (46·6 %) developed varying degrees of paresis and one died of diaphragmatic and intercostal paralysis. In the specially treated series twenty-six patients survived the acute stage, twelve of them (46·1 %) developed paresis and one died of diaphragmatic paralysis.

These results show that before the combined treatment began, the toxaemia had progressed so far, and the effect of the toxin had been so great, that the damage could not be overtaken by massive doses of antitoxin together with doses of the anti-*gravis* serum. It should be stated, however, that no method was available for estimating the potency of this serum; the agglutinin titre of similar kinds of serum has no definite relation to its bacteriostatic action, and therefore the dosage was an arbitrary one. The dose of serum given to the patients was much smaller, in

terms of body weight, than that given to the guinea-pigs, even when the concentration effected by removal of the albumin is taken into account. Further, the disease was well established in the patients at the time when the serum was given, whereas in the guinea-pigs the serum was injected an hour before the test-dose of bacteria. These differences may partly explain the relative ineffectiveness of the anti-*gravis* serum in the clinical trial. The question arises whether antitoxin alone, if it could have been given at an earlier stage, would have saved those patients who died. But there is no certainty that recovery would have taken place, since treatment by antitoxin sometimes fails for no apparent reason. Thus some of the children who were admitted on the 2nd day of the illness died, although they had received large doses of antitoxin as well as anti-*gravis* serum. This experience may seem to be a point against the efficacy of the anti-*gravis* serum, but we do not think that such an inference is warranted, because in the preceding account there are indications that support its use. The occasional lack of response to thorough antitoxin treatment makes it advisable, in our view, to carry out further tests of the action of an anti-*gravis* serum.

SUMMARY AND CONCLUSIONS

Antibacterial sera against *C. diphtheriae gravis* (serological type I), *intermedius* and *mitis* were prepared in horses. The details of the immunization and the preparation of the sera are described. The sera contained a minimal amount of antitoxin.

The anti-*gravis* serum in the dose used, when it was given to guinea-pigs 1 hr. before the subcutaneous injection of ten lethal doses of the homologous organism, saved the life of 75% of the animals. Those which died lived longer than the controls, and *C. diphtheriae* was recovered less often from the local lesion and the liver than at the same sites in the controls; it was not recovered from the heart-blood of the animals which received the experimental serum, whereas cultures from the heart-blood were positive in about 40% of the controls.

In these experiments the degree of invasiveness of the strain, as judged by the frequency of its recovery in cultures from the heart-blood and the liver of the control animals, corresponded with earlier observations (Robinson & Marshall, 1934), which showed that in routine virulence tests carried out by the subcutaneous inoculation of guinea-pigs *C. diphtheriae* could often be cultivated from the local lesion, the liver, the pleural fluid and the heart-blood, and that *gravis* and *intermedius* strains were more invasive than *mitis* strains. This was one of the main facts which suggested that an antibacterial factor might prove to be a useful adjunct in the treatment of diphtheria.

The anti-*gravis* serum, when given as a supplement to the routine doses of antitoxin to patients with severe diphtheria, had a definitely beneficial influence on the local lesion. Moreover, the serum improved for a time the general condition of some of the patients, including those who died later. The death rate of the group to which anti-*gravis* serum was administered was similar to that of the control group but, on the other hand, the patients who received the serum lived, on the average, longer than the control patients notwithstanding that treatment of the

former group was begun later in the disease. The clinical evidence in favour of the anti-*gravis* serum cannot be assessed quantitatively, but, since it is based on critical observations, it should not be disregarded.

Consideration of the results as a whole leads us to conclude that the anti-*gravis* serum used in the tests contained a specific antibody which, within limits, had a beneficial action. We suggest, therefore, that the role of the antibacterial factor in the pathogenesis and control of diphtheria merits investigation.

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