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COMBINED *PERTUSSIS*-DIPHTHERIA PROPHYLACTIC ANTIGENS

AN EXPERIMENTAL STUDY TO DETERMINE THE SPECIFIC IMMUNIZING VALUE OF THESE ANTIGENS USED IN COMBINATION

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The use of heterologous additions to antigenic substances has long been recognized as an aid to immunity production. Steabben (1925) stated that the simultaneous injection of colloidal substances, together with an antigen, increased the amount of antibody produced and the rate at which this production rose to a maximum: the colloids caused a greater development of agglutinin at a greater rate.

Ramon (1926) found that the injection of a mixture of specific antigen, such as diphtheria anatoxine (toxoid) with a non-specific substance like tapioca or T.A.B. vaccine increased immunity production. Glenny (1930) showed that an alum precipitated toxoid immunized guinea-pig more readily than the original toxoid. The increased antigenic response was due to delayed absorption and elimination of the inoculum; in certain experiments this increase reached a thousand-fold.

Debré (1932) stated that the addition of a heterologous substance to a diphtheria antigen increased its power of producing immunity. Ramon (1939) referred to this non-specific enhancement of antibody production as 'antigenic synergy' which was associated with a larger effective antigenic surface by absorption of toxoids and toxin by bacterial suspensions.

Parish (1943) stated that there is a better response to tetanus toxoid when it is mixed with T.A.B. vaccine.

The use of alum precipitated diphtheria toxoid (A.P.T.) has reduced the number and size of injections required in immunizing against diphtheria. It is quite probable that any suitable not easily absorbable substance, combined with a diphtheria or other toxoid, would serve the same purpose of enhancing antibody production. It is not surprising, therefore, that workers have found an increased antibody production when diphtheria toxoid is combined with *Haemophilus pertussis* vaccine. A.P.T., which is itself relatively slowly absorbed, might produce an increased antigenic response when combined with *pertussis* vaccine, on account of the further slowing down of the rate of absorption of the diphtheria antigen by the additional suspended material present.

Bordet (1936) was the first to describe the use of a mixture of diphtheria anatoxine and *pertussis* vaccine, but in his brief report supplied neither clinical evidence of protection nor data on immunity tests. Ledingham (1939), commenting on immunization against whooping cough, stated that he saw no reason why diphtheria toxoid should not be administered in combination with pertussis vaccine 'if Ramon's claim holds that the potency of diphtheria toxoid in a mixture with T.A.B. vaccine is in no way interfered with and appears to be enhanced'.

Schutze (1940) immunized guinea-pigs against diphtheria toxin with and without an admixture of *pertussis* vaccine, and both rabbits and guinea-pigs against *H. pertussis* with and without an admixture of diphtheria toxoid. He concluded that not only had the addition of *pertussis* vaccine not interfered with the antigenic efficiency of the A.P.T. used but had greatly increased it. On the other hand, he found that the addition of A.P.T. to *pertussis* vaccine did not increase the antibody titre to *pertussis* over that obtained with the vaccine alone. In short, the antigenic efficiency of the combination was greatly increased for diphtheria whilst it remained unchanged for whooping cough. There is indeed no obvious reason why diphtheria toxoid should increase the antigenic value of *pertussis* vaccine, or why A.P.T. should enhance the immunizing value of a *pertussis* vaccine above that of a simple alum precipitated *pertussis* vaccine alone.

Simon & Craster (1941) reported on the use of an alum precipitated mixture of diphtheria toxoid and *pertussis* vaccine. They found the resulting Schick tests very satisfactory, but did not carry out investigations for *pertussis* antibody production. Lapin (1942) immunized infants simultaneously against diphtheria, whooping cough and tetanus. He obtained an improved immunization against diphtheria and tetanus, and found that the immunity produced against whooping cough, as indicated by serological and mouse protection tests, was definitely greater than that produced by the usual individual immunization. Sauer & Tucker (1942) described the simultaneous administration of diphtheria toxoid and *pertussis* vaccine in separate syringes or in the

same syringe in young children. The results, as determined by the Schick test for diphtheria and the complement fixation test for whooping cough, compared favourably with those obtained after separate injections.

Kendrick (1942) reported upon the use of an alum precipitated mixture of diphtheria toxoid and *pertussis* vaccine in children. She found as good a diphtheria immunity response with the combined vaccine as with the toxoid alone. Complement fixation, agglutination and opsonocytophagic tests showed that *pertussis* antibodies had also been stimulated. Daughtry-Denmark (1942) described the use of a mixture of A.P.T. (20 Lf units) and *pertussis* vaccine (20,000 millions organisms per ml.) given to children in three doses of 1, 2, and 3 ml. at weekly intervals. This worker found the response to diphtheria immunization satisfactory and recommended that in such a combination the *pertussis* vaccine should be present in a strength of 40,000 million organisms per ml.

Mathieson (1942) described his laboratory experiments on guinea-pigs and rabbits with combined *pertussis* and diphtheria antigens carried out to confirm and extend the observation of Schutze. His results showed that the antigenicity of the diphtheria toxoid was increased when the latter was mixed with a suspension of *H. pertussis* and that the combined antigen was as agglutinogenic as the *pertussis* vaccine alone.

Kendrick (1943), continuing her studies of active immunization in children with alum precipitated combined *pertussis* vaccine and diphtheria toxoid, found that a good response was obtained to each antigen. Sauer, Tucker & Markley (1944) studied the immunity response to a mixture of diphtheria toxoid and *pertussis* vaccine over a number of years and found it satisfactory for both antigens, particularly when given in the alum precipitated form.

Miller, Humber & Dowrie (1944) immunized children with combined diphtheria and tetanus toxoids (aluminium hydroxide absorbed) and *pertussis* vaccine. Their studies led them to conclude that the simultaneous administration of these antigens had no disadvantages as compared with their administration in sequence. Foley (1946) carried out a 3-year experiment with combined diphtheria toxoid and *pertussis* vaccine, and concluded that the combined vaccine produced a very good immunity against diphtheria and quite a good protection against whooping cough.

The study of combined whooping cough-diphtheria prophylactics has been carried out in our laboratories for some years, because of the obvious advantages accruing from the use of such combined vaccines in the simultaneous protection against both diseases. Preliminary experiments with laboratory animals having shown that the combination of *pertussis* and diphtheria antigens did not interfere with the immunizing value of either, a combined vaccine was shortly afterwards issued. Further investigations on various combinations of the two antigens have since been made. The findings in our experimental studies are shown below.

PREPARATION OF STOCK ANTIGENS

(1) Haemophilus pertussis *vaccine*

A freshly isolated culture of *H. pertussis* (14802) in phase 1 was grown on Bordet-Gengou medium and a stock vaccine was prepared therefrom (T. 132. 14802) in a strength of 40,000 million organisms per ml. using 'Merthiolate' 1:10,000 as a preservative. For mixture with the diphtheria antigens, this stock suspension was centrifuged and the deposited bacilli were resuspended in the diphtheria antigen solution.

(2) *Diphtheria antigens*

Diphtheria toxin (366) was prepared from *Corynebacterium diphtheriae* (Strain P.W. 8 W.L.P.R.) grown in a peptic digest broth medium containing 0·2 % glucose, 0·6 % maltose, 1·0 % sodium acetate and 0·0024 g. (1 ml.) of ferrous sulphate per 20 l. of broth. The medium was filtered 12 days after inoculation. Flocculation tests showed Lf 56.

(*a*) *Diphtheria anatoxine* (*toxoid*)

The diphtheria toxin was at once transformed into anatoxine by the addition of 5·2 parts of formalin per thousand and incubated for 30 days at 37° C. Flocculation tests showed Lf 50.

(*b*) *Alum precipitated diphtheria toxoid* (A.P.T.) (61).

A portion of the anatoxine was precipitated with 10 % of a 10 % solution of potassium alum. The precipitate was washed four times with normal saline containing 'Merthiolate' 1:10,000.

(3) *Combined* pertussis-*diphtheria prophylactics*

Combined *pertussis*-diphtheria prophylactics were prepared from the above parent stocks as follows:

(*a*) *Diphtheria anatoxine* (Lf 50) + Haemophilus pertussis *vaccine* (20,000 million *per ml.*)

A portion of the anatoxine was used to resuspend a portion of the *H. pertussis* vaccine so that a concentration of 20,000 million organisms per ml. was obtained.

(*b*) *Alum precipitated diphtheria toxoid* + pertussis *vaccine*

The A.P.T. prepared was used to resuspend *H. pertussis* vaccine so that the combined prophylactic contained:

(i) 30,000 million *H. pertussis* per ml.
(ii) 50,000 million *H. pertussis* per ml.

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EFFECT OF MIXED ANTIGENS ON DIPHTHERIA IMMUNITY

Guinea-pigs were used for the immunization experiments with diphtheria and combined diphtheria-*pertussis* antigens. The guinea-pigs were derived from a homogeneous breeding stock all of a pure white strain and weighing about 250 g. at the time of the first inoculation.

The guinea-pigs were immunized as follows:

(1) *With diphtheria anatoxine alone.*

10 guinea-pigs received 1 injection of 2·5 ml.
10 guinea-pigs received 2 injections of 0·2 ml.
10 guinea-pigs received 2 injections of 0·1 ml.
10 guinea-pigs received 2 injections of 0·05 ml.
10 guinea-pigs received 2 injections of 0·01 ml.

(2) *With diphtheria anatoxine and* Haemophilus pertussis *vaccine* 20,000 *million per ml.*

10 guinea-pigs received 2 injections of 0·1 ml*.
10 guinea-pigs received 2 injections of 0·05 ml.
10 guinea-pigs received 2 injections of 0·02 ml.
10 guinea-pigs received 2 injections of 0·01 ml.

(3) *With* A.P.T. *and* Haemophilus pertussis *vaccine* 30,000 *million per ml.*

Forty guinea-pigs were treated in the same way as those receiving anatoxine with *pertussis* vaccine.

(4) *With* A.P.T. *and* Haemophilus pertussis *vaccine* 50,000 *million per ml.*

Forty guinea-pigs were treated in the same way as the preceding two groups.

All injections, with the exception of the single injection of 2·5 ml. of diphtheria anatoxine alone, were given subcutaneously in the abdominal wall in a volume of 1 ml. made up where necessary with normal saline. In each case the second immunizing dose was given 28 days after the first. All the experimental guinea-pigs were submitted to the Schick test 14 days after the administration of the second immunizing dose or after the single dose in the case of the guinea-pigs injected with diphtheria anatoxine alone. Schick negative guinea-pigs were bled 48 hr. after this test and antitoxin determinations were performed on the day of bleeding by Romer's intracutaneous method. The guinea-pigs inoculated with a single dose of diphtheria anatoxine alone, and injected with two doses of 0·01 ml. of diphtheria anatoxine with 20,000 million pertussis per ml. were not bled for titration of antitoxin.

The sera of five Schick negative guinea-pigs in each series were titrated for antitoxin individually, while the sera of the remaining Schick negative guinea-pigs in the series were pooled for that purpose. The sera from Schick negative guinea-pigs, which had received diphtheria anatoxine alone, were titrated individually except the series which had received two injections of 0·2 ml. In this case five sera were titrated individually and the remaining sera pooled for testing.

Tables 1 and 2 show the result of the guinea-pig immunization experiments.

The results shown in Tables 1 and 2 may be summarized thus:

(1) Diphtheria anatoxine, combined with *pertussis* vaccine containing 20,000 million organisms per ml., has a greater antigenic value than diphtheria anatoxine alone; has about the same value as A.P.T. alone and is of less value than A.P.T. combined with *pertussis* vaccine containing 30,000 million organisms per ml.

(2) Alum precipitated toxoid, combined with *pertussis* vaccine containing 30,000 million organisms per ml., has a greater antigenic value than A.P.T. alone or than anatoxine combined with *pertussis* vaccine, and is of about the same value as A.P.T. combined with *pertussis* vaccine containing 50,000 million organisms per ml.

EFFECT OF MIXED ANTIGENS ON IMMUNITY TO *HAEMOPHILUS PERTUSSIS*

Experiments were performed on the immunization of rabbits with various forms of *pertussis* vaccine alone, and with mixtures of *pertussis* vaccine and diphtheria anatoxine and *pertussis* vaccine and A.P.T., in order to discover whether the addition of a diphtheria antigen to the *pertussis* vaccine would improve its antigenic value in the same way that addition of the vaccine to diphtheria antigens improved theirs.

Some of this work was done in 1943, using various batches of reagents. All of it was repeated later using the same reagents as were used in the experiments on diphtheria antitoxin production described above.

Rabbits were immunized by intravenous or subcutaneous injection, and the antibody response was judged on the agglutination titres which their sera developed. Some rabbits failed to produce demonstrable agglutinins. Others gave titres varying between 1:50 and 1:3200. Agglutinin titres commonly fell in the course of a few weeks from their highest titres to low values, but they could be raised again by injection of a further dose of antigen.

A.P.T. mixed with the stronger *pertussis* vaccines generally killed rabbits when given intravenously, as also on occasion did the anatoxine-*pertussis* vaccine mixtures, possibly on account of their formalin content.

* It was found by trial that the addition of *pertussis* vaccine to diphtheria anatoxine or to A.P.T. diminished the amount of diphtheria antigen in a given volume by about 10%. To compensate for such dilution, the actual doses of combined antigen used for injection were 10% greater than the figures shown.

Table 1. *Schick tests on guinea-pigs immunized with*

A. Diphtheria anatoxine alone
B. Diphtheria anatoxine combined with *pertussis* vaccine containing 20,000 million organisms per ml.
C. Alum precipitated toxoid alone.
D. Alum precipitated toxoid combined with *pertussis* vaccine containing 30,000 million organisms per ml.
E. Alum precipitated toxoid combined with *pertussis* vaccine containing 50,000 million organisms per ml.

Doses of antigen in ml.	2·5	0·2+0·2	0·1+0·1	0·05+0·05	0·02+0·02	0·01+0·01
Antigen: A	8	10	5	5	—	2
B	—	—	10	10	9	2
C	—	—	10	10	9	8
D	—	—	10	10	10	10
E	—	—	10	10	10	10

Each figure indicates the number of guinea-pigs which were rendered Schick negative out of a group of ten tested.

Table 2. *Antitoxin titration (units of antitoxin per ml. of serum) on guinea-pigs immunized with*

A. Diphtheria anatoxine alone.
B. Diphtheria anatoxine combined with *pertussis* vaccine containing 20,000 million organisms per ml.
C. Alum precipitated toxoid alone.
D. Alum precipitated toxoid combined with *pertussis* vaccine containing 30,000 million organisms per ml.
E. Alum precipitated toxoid combined with *pertussis* vaccine containing 50,000 million organisms per ml.

Doses of antigen in ml.	0·2+0·2		0·1+0·1		0·05+0·05		0·02+0·02		0·01+0·01	
Antigen: A	1/50	1/25	1/250	1/60	1/250	1/100	—	—	1/500	1/250
	1/10	1/5	1/25	1/10	1/25	1/10	—	—	1/10	1/5
	1/500	1/250	1/25	1/10	1/25	1/10	—	—	—	—
	1/2	1	1/25	1/10	1/25	1/10	—	—	—	—
	1/2	1	1/250	1/100	1/250	1/100	—	—	—	—
	(1/5	1/2)	—	—	—	—	—	—	—	—
	—	—	1/5	1/2	1/2	1	1/5	1/2	—	—
	—	—	2	4	1/25	1/10	1/10	1/5	—	—
B	—	—	1	2	1/10	1/5	1/10	1/5	—	—
	—	—	1/5	1/2	1/2	1	1/5	1/2	—	—
	—	—	1	2	1/5	1/2	1/5	1/2	—	—
	—	—	(1	2)	(1	2)	(1/10	1/5)	—	—
	—	—	1	2	1/5	1/2	1/5	1/2	1/50	1/25
	—	—	1	2	1/5	1/2	1/5	1/2	1/50	1/25
C	—	—	1	2	1/2	1	1/5	1/2	1/2	1/1
	—	—	2	4	1/5	1/2	1/2	1/1	1/50	1/25
	—	—	1/2	1	1/5	1/2	1/25	1/10	1/5	1/2
	—	—	(1	2)	(1/10	1/5)	(1/5	1/2)	(1/10	1/5)
	—	—	4	8	4	8	4	8	2	4
	—	—	4	8	4	8	2	4	1	2
D	—	—	4	8	2	4	2	4	1/2	1
	—	—	1	2	2	4	2	4	2	4
	—	—	4	8	2	4	2	4	1	2
	—	—	(4	8)	(4	8)	(2	4)	(2	4)
	—	—	4	8	4	8	2	4	1	2
	—	—	4	8	4	8	1	2	1/2	1
	—	—	4	8	1	2	2	4	1/5	1/2
E	—	—	4	8	4	8	1/2	1	2	4
	—	—	4	8	4	8	2	4	1	2
	—	—	(4	8)	(4	8)	(2	4)	(1	2)

The first and second figures for each titration indicate the limits between which the actual antitoxin values lay. Values given for each of five individual guinea-pigs in each group. The sixth value, enclosed in parentheses, is the antitoxin value of the pooled sera of the remaining Schick negative guinea-pigs in the group of ten immunized.

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In none of these experiments was any evidence obtained that addition of a diphtheria antigen to the *pertussis* vaccine increased its power to provoke agglutinin production.

RABBITS INOCULATED WITH *PERTUSSIS* VACCINES ALONE

In the earlier work, vaccines were prepared from phase I *H. pertussis* grown on Bordet-Gengou medium. These vaccines, in strengths of 4000 million and 10,000 million organisms per ml., were preserved either with 'Merthiolate' 1:10,000 or with phenol 0·5 % or with formalin 0·2 %.

Suspensions of living *H. pertussis*, 4000 million organisms per ml., were used for other rabbits, in five injections of 0·5 or 1·0 ml. at weekly or longer intervals.

In the later experiments, rabbits were inoculated with the *pertussis* vaccine described on p. 119, which was prepared for use in the guinea-pig experiments on diphtheria antitoxin production. Doses of 0·5 and 1·0 ml. of the vaccine containing 10,000 million organisms per ml. were injected at monthly intervals.

No essential differences in agglutinin production were observed throughout the whole series of rabbits inoculated with *pertussis* vaccine alone.

ALUM PRECIPITATED *PERTUSSIS* VACCINE

Pertussis vaccines preserved with 'Merthiolate' 1:7500, containing 10,000 million and also 20,000 million organisms per ml. were precipitated with alum. 0·5 ml. doses were injected intravenously into rabbits at weekly or monthly intervals. Similar vaccines were given to other rabbits subcutaneously. Some of the rabbits inoculated intravenously died. No significant effect of alum precipitation of the antigen was observed.

PERTUSSIS VACCINE MIXED WITH DIPHTHERIA ANATOXINE

Rabbits were given three weekly injections of *pertussis* vaccine containing 10,000 million and 30,000 million organisms per ml. mixed with diphtheria anatoxine. Some rabbits received the vaccine-anatoxine mixture used in the experiments on diphtheria antitoxin production. Some were inoculated subcutaneously. Titres higher than those obtained with *pertussis* vaccine alone were not obtained.

PERTUSSIS VACCINE MIXED WITH ALUM PRECIPITATED TOXOID

Mixtures of A.P.T. with *pertussis* vaccine containing 10,000 million and 20,000 million organisms per ml. were used in the earlier work. Later, the mixtures containing 30,000 and 50,000 million organisms per ml. used in the diphtheria antitoxin experiments were used, but these regularly proved fatal on intravenous injection.

The conclusion from all of the experiments on the immunization of rabbits against *H. pertussis* is, that there is no evidence that the addition of diphtheria prophylactics to *pertussis* vaccine increases its antigenic potency.

SUMMARY AND CONCLUSIONS

1. Experiments in laboratory animals are described, which were made to determine the specific antibody production following injections of diphtheria and *pertussis* antigens separately or in combination.

2. Agglutinins to *H. pertussis* vaccine were not increased or otherwise changed by combining *H. pertussis* vaccines with either diphtheria anatoxine or A.P.T.

3. A striking enhancement of diphtheria antitoxin production occurred when diphtheria prophylactics were used combined with *pertussis* vaccine. Such improvement in antitoxin production was most marked in the case of diphtheria anatoxine, but was also shown following the use of A.P.T. in combination with *pertussis* vaccine.

4. Diphtheria anatoxine combined with *H. pertussis* vaccine gave no better antitoxin response than did A.P.T. alone.

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REFERENCES

BORDET, J. (1936). A propos du vaccin anticoquelucheux. *Brux. méd.* **16**, 503.

DAUGHTRY-DENMARK, L. (1942). Whooping cough vaccine. *Amer. J. Dis. Child.* **63**, 453.

DEBRÉ, R. (1932). *La vaccination contre la Diphtérie.* Paris: Masson et cie.

FOLEY, A. R. (1946). A three-year experiment with combined diphtheria toxoid-*pertussis* vaccine. *Canad. publ. Hlth. J.* **37**, 259.

GLENNY, A. T. (1930). Insoluble precipitates in diphtheria and tetanus immunization. *Brit. med. J.* **2**, 244.

KENDRICK, P. L. (1942). Use of alum-treated *pertussis* vaccine, and of alum precipitated combined *pertussis* vaccine and diphtheria toxoid for active immunization. *Amer. J. publ. Hlth.* **32**, 615.

KENDRICK, P. L. (1943). A field study of alum-precipitated combined *pertussis* vaccine and diphtheria toxoid for active immunization. *Amer. J. Hyg.* **38**, 193.

LAPIN, J. H. (1942). Combined immunization of infants against diphtheria, tetanus and whooping cough. *Amer. J. Dis. Child.* **63**, 225.

LEDINGHAM, J. C. G. (1939). Prophylactic immunization against measles, scarlet fever, diphtheria, whooping cough and influenza. *Brit. med. J.* **2**, 841.

MATHIESON, D. R. (1942). A laboratory evaluation of a combined antigen for diphtheria and whooping cough prophylaxis. *J. Bact.* **43**, 81.

MILLER, J. J., Jr. & SAITO, T. M. (1942). Concurrent immunization against tetanus, diphtheria and *pertussis*; comparison of fluid and alum precipitated toxoids. *J. Pediat.* **21**, 31.

MILLER, J. J., HUMBER, J. B. & DOWRIE, J. O. (1944). Immunization with combined diphtheria and tetanus toxoids (aluminium hydroxide absorbed) containing *Haemophilus pertussis* vaccine. *J. Pediat.* **24**, 281.

PARISH, H. J. (1943). Discussion on active immunity. II. Bacterial vaccines and toxoids. *Proc. R. Soc. Med.* **36**, 151.

RAMON, G. (1926). Procédés pour accroître la production des antitoxines. *Ann. Inst. Pasteur*, **40**, 1.

RAMON, G. (1939). Mixed vaccinations. *Int. Clin.* **1**, 241.

SAUER, L. W. & TUCKER, W. H. (1942). Simultaneous administration of diphtheria toxoid and *pertussis* vaccine in young children. *Amer. J. publ. Hlth.* **32**, 385.

SAUER, L. W., TUCKER, W. H. & MARKLEY, E. (1944). Immunity response to mixtures of diphtheria toxoid and *pertussis* vaccine. *J. Amer. med. Ass.* **125**, 949.

SCHUTZE, H. (1940). Simultaneous immunization against whooping cough and diphtheria. *Lancet*, **2**, 192.

SIMON, H. & CRASTER, C. V. (1941). Simultaneous immunization with a combined diphtheria-whooping cough vaccine: preliminary report of an eighteen-month study. *J. med. Soc. N.J.*, **38**, 461.

STEABBEN, D. B. (1925). Studies on the physiological action of colloids. I. The action of colloidal substances on blood-elements and antibody content. *Brit. J. exp. Path.* **6**, 1.

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