J. Hyg., Camb. (1976), **76**, 257
Printed in Great Britain

The importance of agglutinin production in mice in the determination of the definitive serotype of *Bordetella pertussis*

BY CAROLINE J. BRONNE-SHANBURY*
The Lister Institute of Preventive Medicine, Elstree, Hertfordshire WD6 3AX

(*Received* 7 *July* 1975)

SUMMARY

A schedule for the routine serotyping of strains of *Bordetella pertussis* based on agglutinin production in mice to the K-antigens has been worked out. Mice have been found as satisfactory as rabbits but far more economical for the production of the very small volumes of serum which are required. Agglutinin production, used in conjunction with direct agglutination, provides definitive information about serotype.

INTRODUCTION

Some isolates of *Bordetella pertussis* which were obtained in the Public Health Laboratory Service Survey (PHLS) (1969) and which gave consistent serotyping results when tested *in vitro* with monospecific typing sera gave different results when tested *in vivo*, that is when used as antigens to elicit agglutinins in rabbits. This phenomenon has also been observed by Holt (1968) who showed that some agglutinogens may become apparent only when a strain has been analysed by agglutinin production. Hitherto rabbits have been used for this purpose but the cost, whilst justifiable for producing monospecific typing sera, makes routine testing of the serotype of isolates very uneconomical.

The observations of Evans & Perkins (1953) that intraperitoneal injection of *B. pertussis* into mice elicited agglutinins suggested that mice would be as sensitive as the rabbits used by Andersen (1952, 1953) and Eldering, Hornbeck & Baker (1957) for producing monospecific agglutinating sera. Evans and Perkins obtained measurable agglutinin titres when two doses of 5×10^9 organisms were injected at a 14-day interval. The mice were bled from the heart 10 to 20 days after the last injection. Since in their experiments no attention was paid to the individual agglutinins, my experiments were carried out to obtain a schedule by which these could be analysed routinely. Some serotyping results of routine analyses of isolates both by direct agglutination and agglutinin production are given.

* Reprint requests to: 12, Flag Walk, Eastcote-Pinner, Middlesex HA5 2EP.

METHODS

Mice

Male TFI mice were used routinely in groups of five of similar weights. Male and female Theilers Original (TO) as well as female Carworth Farms, Lane Petter (CFLP) mice have also been used and gave similar results; female National Institute of Health (NIH) mice were found to be less sensitive than other strains to some agglutinogens.

Bacterial strains

Lyophilized cultures collected and kept at the Lister Institute were used. Some of these are now available from the National Collection of Type Cultures (NCTC) and obtainable from the Curator at the Central Public Health Laboratory, Colindale Avenue, London NW9 5HT.

For the analysis of sera the following strains of known serotype were used for absorption and control: B16, serotype 1,0,3,0,0,0 from Dr Haire (NCTC 10907); 3747 variant, serotype 1,0,0,0,0,0, a spheroplast derived strain described previously (Dolby & Bronne-Shanbury 1975, NCTC 10905); 3747, serotype 1,2,0,0,5,6 from Dr E. K. Andersen (NCTC 10908); 3865, serotype 1,2,0,4,0,0 from Dr E. K. Andersen (NCTC 10906); 5373, serotype 1,2,3,0,0,6 from Dr G. Eldering and D3096 from the PHLS survey (1969).

Detailed investigations of agglutinin production in mice were carried out using two typical isolates of *B. pertussis* collected during the recent PHLS survey (1969). These were D/AD3913, 1,0,3,0,0,0 from Glasgow and D14105, 1,2,0,4,0,0 from Manchester. As examples of the method, 39 further isolates from the same survey were examined; also single colony cultures of laboratory strains.

Dried *B. pertussis* cultures were reconstituted in 1% Bacto-Casamino Acids, Difco (CA), seeded on Bordet-Gengou (BG) plates and incubated at 36° C. They were subcultured three times before being harvested for mouse vaccines, absorption suspensions, or suspensions for agglutination tests.

Production of antisera

Vaccines were prepared from the growth from two BG plates harvested into 10 ml. of saline containing 0·25% formalin. After 24 hr. at room temperature the killed organisms were centrifuged and resuspended in merthiolate saline (0·85% NaCl in 0·006 M phosphate buffer pH 7·2 containing 0·001% merthiolate) to a concentration of 20×10^9 organisms/ml. by opacity. The vaccine was stored at 4° C.

Mice were immunized by intraperitoneal injection of 2×10^9 organisms in 0·2 ml., according to the schedule being investigated (described in results). They were bled from the heart and the pooled serum heated at 56° C. for ½ hr. and stored at 4° C.

Identification of agglutinins in mouse antisera

Antisera were analysed for agglutinins 2–6 by selective absorption with killed suspensions of the strains of known serotype shown in Fig. 1. The suspensions

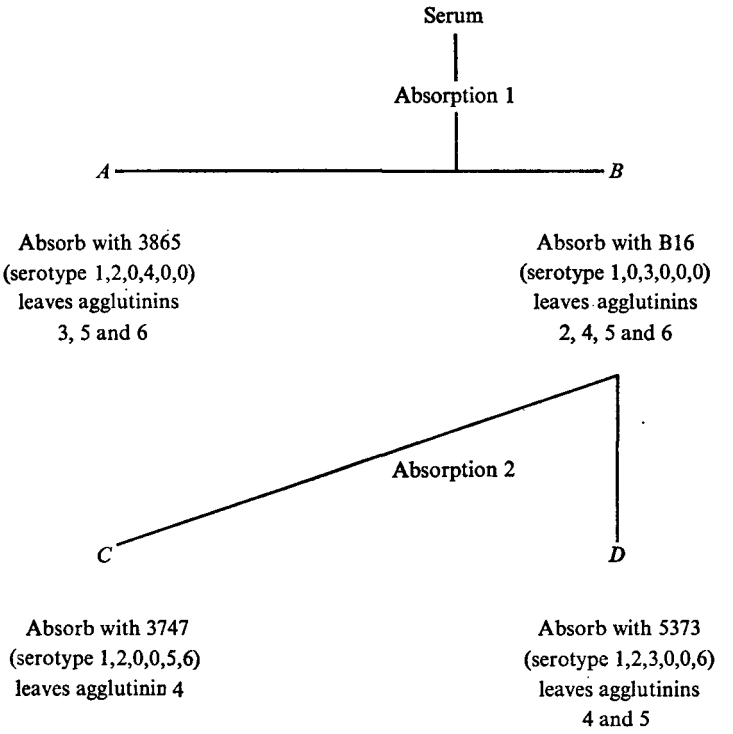

Fig. 1. Selective absorption of agglutinins from antisera.

were distributed so that the centrifuged deposits contained 10×10^9 organisms, suitable for absorbing 0·5–1·0 ml. volumes of sera (as described previously by Dolby & Stephens, 1973 for absorption of child sera). Formalin-killed suspensions were used to absorb agglutinins to the heat-labile antigens whilst agglutinins to the heat-stable antigen were absorbed either using any phase I strain harvested in phosphate buffered saline (PBS) and autoclaved at 10 lb. for 10 min. or during Absorption 1 of the scheme in Figure 1. The absorbed antisera were tested against living suspensions of the strains harvested in PBS and diluted to 10×10^9 organisms/ml. by opacity, in plastic microtitre trays (Disposo, Linbro Chemical Co. Inc., 681 Dixewell Avenue, New Haven, Conn. 06511, USA). These suspensions were checked daily against non-specific typing sera (Bronne-Shanbury, Miller & Standfast, 1976) to ensure that there was no autoagglutination or change in serotype. Possible results are shown in Table 1.

It was assumed that all the isolates investigated contained agglutinogen 1 (Eldering *et al.* 1957). This agglutinin could be tested for directly after absorption of sera with autoclaved suspensions and testing with the 3747 variant containing only agglutinogen 1 (Dolby & Bronne-Shanbury, 1975).

The scheme for selective absorption of agglutinins 2–6 is shown in Fig. 1. Absorptions were carried out in two stages. The first stage, producing absorbed sera A and B, distinguished agglutinin 3 if 5 and 6 were absent, and agglutinin 4 if it were present without 2, but this is a rare occurrence. For identification of

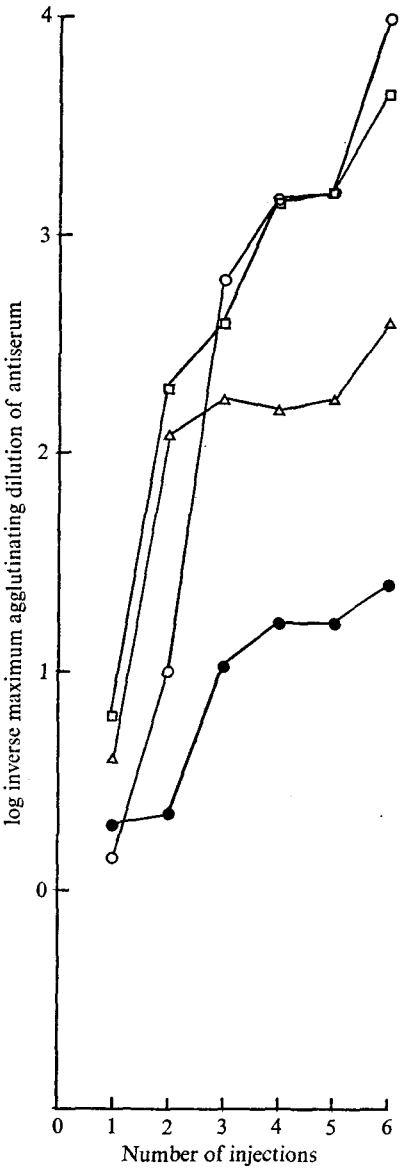

Fig. 2. The effect of the number of injections of vaccine on agglutinin titre in mice. ●, Agglutinin 1; □, agglutinin 2; ○, agglutinin 3; △, agglutinin 4.

agglutinin 4 in the presence of agglutinin 2 and agglutinins 5 and 6, the second-stage absorption producing sera C and D were done.

Although strain 5373 possesses agglutinogen 6, it does so in very small quantities which vary from subculture to subculture. It is therefore difficult to absorb agglutinin 6 when its titre is high and some care must be taken to ensure that a positive agglutination of 3747 with serum D is indeed due to agglutinin 5. Agglutinogen 6 is generally found in the presence of agglutinogen 3 with which it appears

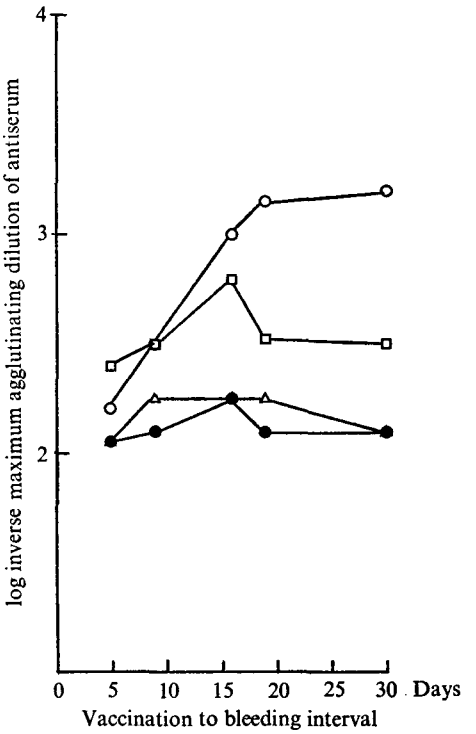

Fig. 3. The effect of the time between the last injection of vaccine and bleeding on the agglutinin titre in mice. ●, Agglutinin 1; □, agglutinin 2; ○, agglutinin 3; △, agglutinin 4.

Table 1. *Cross-absorption of agglutinins*

Serum absorption*	Possible agglutinins	Agglutination of 5373	B16	3747	3865
A	3	+	+	−	−
	5	−	−	+	−
	6	+	−	+	−
B	2	+	−	+	+
	4	−	−	−	+
	5	−	−	+	−
	6	+	−	+	−
C	4	−	−	−	+
D	4	−	−	−	+
	5	−	−	+	−

* Letter refers to absorptions shown in Fig 1.

to be associated in some way (Eldering, Holwerda & Baker, 1967) for when agglutinin 3 is absorbed 6 is also generally absorbed from the serum. Care, therefore, must be taken when all six agglutinins are present. The presence of agglutinogen 6 without agglutinogen 3 is very rare; only one isolate was found at the Lister Institute during the whole PHLS survey (1969).

https://doi.org/10.1017/S0022172400055157 Published online by Cambridge University Press

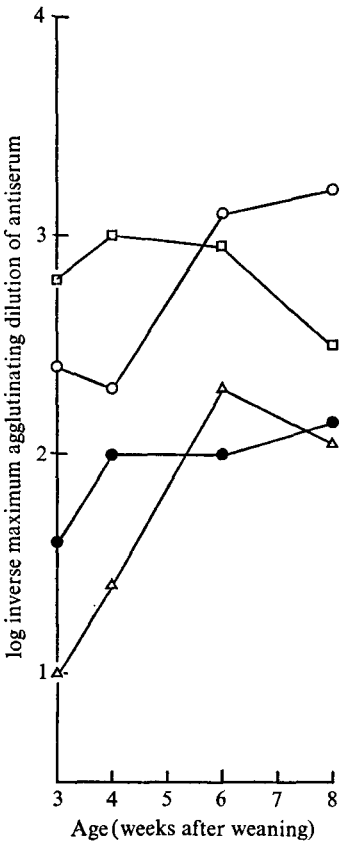

Fig. 4. The effect of the age of mice on the agglutinin titre after vaccination. ●, Agglutinin 1; □, agglutinin 2; ○, agglutinin 3; △, agglutinin 4.

Since agglutinogen 5 is rarely found without 2 (Eldering *et al.* 1967) and agglutinogen 4 is rarely found without 2, it was generally assumed that where agglutinins 4 and 5 were present in the serum, agglutinin 2 was also present; to confirm this strain D3096 was used to absorb agglutinin 4. Absorption with this strain was slow because the agglutinogen was weak. Unfortunately, it was the only 1,0,3,4,0,0 strain available.

RESULTS

Experiments developing a scheme of immunization of mice

Three factors were investigated: (1) the optimum number of injections necessary to raise high titre antisera, (2) the time interval between the last injection and bleeding and (3) the optimum age for the mice. In all cases injections were given at 3- to 4-day intervals. The two vaccines D/AD3913, a 1,0,3,0,0,0 strain, and D14105, a 1,2,0,4,0,0 strain, were used. Tests were performed to identify agglutinins 1, 2, 3 and 4.

A measurable agglutinin titre was found after only one intra-peritoneal injection of *B. pertussis*. The degree of response to the four agglutinogens as estimated by

https://doi.org/10.1017/S0022172400055157 Published online by Cambridge University Press

agglutinin titre is shown in Fig. 2; the titre of all four agglutinins rose sharply at first but began to level off between three and five injections. Fig. 3 shows that by 5 days after the last of four injections the titre of all four agglutinins was already above 1/130 with levelling or a slight increase in titre between 10 and 16 days after the last injection. Thereafter, except in the case of agglutinin 3, there were slight decreases in titre of the agglutinins. From Fig. 4 it can be seen that the age of the mice used affected all the agglutinins, having most influence upon agglutinins 3 and 4. Sensitivity to agglutinins 2 and 4 was reduced from 6 weeks after weaning onwards, whilst sensitivity to agglutinins 1 and 3 continued to increase slowly.

The use of agglutinin production in serotyping

Of 39 isolates tested by direct agglutination, the serotyping results were confirmed by agglutinin production in 21 cases. The other 18 all evoked agglutinins to agglutinogens undetected by direct agglutination. Of the isolates serotyping as 1,0,3,4,0,0 by direct agglutination only one was found which did not produce other agglutinins in mice. Twelve isolates serotyped as 1,0,0,0,0,0 by direct agglutination, but agglutinin production in mice proved only five to be true '1 only' strains. It was of interest that all five had been artificially produced by spheroplast formation (Dolby & Bronne-Shanbury, 1975).

A comparison of the results obtained by agglutinin production with the less informative results provided by direct serotyping is to be found elsewhere (Bronne-Shanbury *et al.* 1976). Use is made of the method to classify the heterogeneous strains belonging to the 1,2,3 serotype groups (Bronne-Shanbury & Dolby, 1976).

This work was presented in part for the degree of Doctor of Philosophy in the University of London and financed by a grant from the Medical Research Council. The author wishes sincerely to thank Dr A. F. B. Standfast and Dr J. M. Dolby for their advice.

REFERENCES

Andersen, E. K. (1952). Serological studies on *Haemophilus pertussis*, *Haemophilus parapertussis* and *Haemophilus bronchiseptica*. Preliminary report. *Acta pathologica et microbiologica scandinavica* **30**, 54–8.

Andersen, E. K. (1953). Serological studies on *Haemophilus pertussis*, *Haemophilus parapertussis* and *Haemophilus bronchiseptica*. *Acta pathologica et microbiologica scandinavica* **33**, 202–24.

Bronne-Shanbury, C. J., Miller, D. & Standfast, A. F. B. (1976). The serotypes of *Bordetella pertussis* isolated in Great Britain between 1941 and 1968 and a comparison with the serotypes observed in other countries over this period. *Journal of Hygiene* **76**, 265–75.

Bronne-Shanbury, C. J. & Dolby, J. M. (1976). The stability of the serotypes of *Bordetella pertussis* with particular reference to serotype 1,2,3,4. *Journal of Hygiene* **76**, 277–86.

Dolby, J. M. & Bronne-Shanbury, C. J. (1975). The use of spheroplast-derived strains to differentiate between *Bordetella pertussis* heat-labile agglutinogens and protective antigens in mice. *Journal of Biological Standardization* **3**, 89–100.

Dolby, J. M. & Stephens, S. (1973). Pertussis antibodies in the sera of children exposed to *Bordetella pertussis* by vaccination or infection. *Journal of Hygiene* **71**, 193–207.

ELDERING, G., HOLWERDA, J. & BAKER, J. (1967). Mouse-protective properties of *Bordetella pertussis* serotypes in passive tests. *Journal of Bacteriology* **93**, 1758–61.

ELDERING, G., HORNBECK, C. & BAKER, J. (1957). A serological study of *Bordetella pertussis* and related species. *Journal of Bacteriology* **74**, 133–6.

EVANS, D. G. & PERKINS, F. T. (1953). An agglutinin-production test in the study of pertussis vaccines. *Journal of Pathology and Bacteriology* **66**, 479–88.

HOLT, L. B. (1968). Pitfalls in the preparation of monotypic agglutinating antisera for *Bordetella pertussis*. *Journal of Medical Microbiology* **1**, 169–80.

PUBLIC HEALTH LABORATORY SERVICE (1969). Efficacy of whooping-cough vaccines used in the United Kingdom before 1968. Preliminary report. *British Medical Journal* iv, 329–33.