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Population studies of diphtheria immunity using antitoxin radioimmunoassay

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SUMMARY

A quantitative method for testing serum diphtheria antitoxin levels was set up using a diphtheria antitoxin radioimmunoassay (RIA). The results of this RIA correlated well with the Schick test in 554 subjects and with intradermal neutralization tests in guinea-pigs in a small group of subjects. The RIA was suitable for use on blood collected by fingerprick on to a disc of standard chromatography paper. These discs could be stored at room temperature for at least 1 month. If storage for more than 6 months was required −20 °C was found to be better. Experience with this RIA in a total of 2349 subjects indicated that it is more accurate, rapid and less costly than Schick testing. The RIA should prove to be the preferred method for testing diphtheria immunity in population surveys.

INTRODUCTION

Immunity to diphtheria may be measured by skin (Schick) testing or by a variety of serological assays of antitoxin (Van Ramshorst, 1971). For population surveys, the Schick test is time-consuming and costly. It requires three visits by the health worker: the first to administer the test and two others to read the test at 3 and 5 days. The subjects who react positively (those who are diphtheria-susceptible) often have a painful red reaction which may persist for days or weeks at the site of the toxin injection. In this study we used a diphtheria antitoxin radioimmunoassay (RIA) to assess diphtheria immunity first in a group of 12-year-old children who were also Schick-tested and later in two groups of children who were not Schick-tested. The test system was then adapted for use on serum eluted from blood collected by fingerprick on to a disc of standard chromatography paper. This simplified procedure is ideal for population surveys of diphtheria immunity.

SUBJECTS AND METHODS

Four groups of subjects were examined.

Group 1 consisted of 554 12-year-old primary-school children from Sydney (Menser *et al.* 1980) who were taking part in an immunization survey during which they had venous blood collected and a standard Schick test performed by intradermal injection of 0·2 ml of Schick Test Toxin (Commonwealth Serum

Laboratories, Parkville, Australia) into the left forearm and 0·2 ml of Schick Control Fluid into the right forearm (Trinca, 1979). The tests were read on the third and fifth days. Twenty of the subjects who were Schick-positive were immunized with three doses of 2 Lf units of diphtheria toxoid. These subjects had venous blood collected for RIA 4 months after the Schick test and at least 6 weeks after each dose of toxoid.

Group 2 consisted of 430 18-year-old high school students from whom venous blood was collected but who were not Schick-tested.

Group 3 comprised 1252 patients aged from 1 to 19 years who were attending the Royal Alexandra Hospital for Children and having blood collected for other reasons.

Group 4 were 113 adult volunteers from whom both venous and fingerprick blood samples were collected.

Diphtheria antitoxin radioimmunoassay. Diphtheria toxin (950 Lf/ml, Commonwealth Serum Laboratories) was purified by Sephadex G-100 chromatography and lyophilized; electrophoresis of purified toxin on polyacrylamide gel containing sodium dodecyl sulphate produced a single homogeneous band. Toxin was labelled with ^{125}I using an adaptation (Bazaral, Goscienski & Hamburger, 1973) of the chloramine-T method of Hunter & Greenwood (1962). Using 20% trichloroacetic acid it could be shown that more than 90% of the ^{125}I used in the assay was bound to the toxin. The capacity of serum to bind diphtheria toxin was determined using the ammonium sulphate method. ^{125}I-labelled diphtheria toxin (10 ng/ml) was added to sera diluted 1 in 20, in duplicate, and incubated overnight at 4 °C. Antigen-antibody complexes were precipitated by the addition of an equal volume of 90% saturated ammonium sulphate. Precipitates were washed with 45% saturated ammonium sulphate and counted in a gamma-counter. Normal rabbit serum was used as the control precipitate; only 5% of the label precipitated in this control. The percentage radioactivity precipitated (% pptn) was calculated for each diluted serum sample (Nelson *et al.* 1978). The assay was standardized against an international standard antitoxin (IU, international units) provided by the Commonwealth Serum Laboratories. After reading against the standard, each serum antitoxin value was corrected for the dilution factor ($< 7\%$ pptn $\equiv$ < 7 mIU/ml; $> 80\%$ pptn $\equiv >$ 100 mIU/ml). An antitoxin level of 10 mIU/ml serum (0·01 IU/ml) is usually accepted as being protective (Ad-hoc Working Group, 1978; Sheffield, Ironside & Abbott, 1978) and it is at about this level, or lower, that the Schick test becomes negative (Ipsen, 1954; Bainton *et al.* 1979). In this study antitoxin values falling between 7 and 12 mIU/ml were considered borderline protective.

Diphtheria antitoxin bioassay. Forty-four serum samples were collected from 11 of 20 diphtheria susceptible subjects, who were initially RIA-negative, before and after each of three doses of 2 Lf units of toxoid. These sera were subjected to intradermal toxin–antitoxin neutralization testing in guinea-pigs in the range 2·5–5120 mIU/ml (Feery *et al.* 1981; Glenny & Llewellyn-Jones, 1931).

Blood collection by fingerprick. Blood was collected by fingerprick on to a standard 22·5 mm diameter circular disc of Whatman No. 3 chromatography paper. Two discs were collected from each subject so one could be retested later. To obtain the correct volume of blood (0·09 ml) it was important to saturate the

Table 1. *Serum levels of diphtheria antitoxin by radioimmunoassay (RIA) correlated with Schick-test results in 554 12-year-old children*

	mIU diphtheria antitoxin per ml serum			
	0	< 7	7–12	> 12
Schick-positive, $n = 59$	27	23	4	5
Schick-negative, $n = 495$	2	11	12	470

disc on both sides. The paper was air-dried and stored at 4 °C. The serum was retrieved from the paper by elution with 1·0 ml phosphate-buffered saline (0·05 M sodium phosphate, 0·1 M sodium chloride, pH 7·5) overnight at 4 °C. Serum (final dilution 1 in 20) was then assayed in duplicate for antitoxin using the radioimmunoassay.

RESULTS

Group 1. The antitoxin levels of these 554 primary-school children are set out in Table 1:63 (11%) had antitoxin levels < 7 mIU/ml; 16 (3%) had borderline levels between 7 and 12 mIU/ml; 169 (31%) had levels between 12–100 mIU/ml and 306 (55%) had levels > 100 mIU/ml. Five of the 59 Schick-positive subjects had antitoxin levels higher than 12 mIU/ml (24, 72 > 100, > 100, > 100 mIU/ml) and were considered to have false positive Schick tests. Among the 495 Schick-negative subjects, 13 had antitoxin levels lower than 7 mIU/ml and were thus below the minimal protective level.

Twenty children whose initial antitoxin level was below the minimal protective value (< 7 mIU/ml) were immunized with toxoid after they had been Schick-tested. Eight of these children had already responded to the Schick-test antigen (0·000 07 μg of diphtheria toxin) by increasing their antitoxin levels to borderline or higher values; the levels in this group of eight, and four others, rose to > 100 mIU/ml after one dose of 2 Lf units of toxoid. The remaining eight children, whose initial antitoxin level was 0 mIU/ml, required two or three doses of 2 Lf units of toxoid to achieve antitoxin levels > 100 mIU/ml. Intradermal neuralization tests in guinea-pigs on 44 sera from 11 of these children correlated extremely well with the antitoxin radioimmunoassay (correlation coefficient = 0·926, $P < 0·001$); the RIA was positive in sera collected from five subjects 1 month after the first dose of toxoid when the bioassay was still negative (Table 2).

Group 2. Of the 430 18-year-old subjects, 31 (7%) had an antitoxin level < 7 mIU/ml; 10 (2%) had borderline levels between 7 and 12 mIU/ml; 127(30%) had levels between 12 and 100 mIU/ml and 262 (61%) had levels > 100 mIU/ml.

Group 3. Of these 1252 patients who were aged between 1 and 19 years, 75 (6%) had antitoxin levels < 7 mIU/ml; 38 (3%) had borderline levels between 7 and 12 mIU/ml; 306 (24%) had levels between 12 and 100 mIU/ml and 833 (67%) had levels > 100 mIU/ml. The percentage of subjects who were non-immune increased with age and 10% of patients aged 12 years or older had levels below the minimal protective level.

Group 4. The antitoxin levels in these 113 adult subjects were spread evenly

Table 2. *Comparison between serum diphtheria antitoxin levels using radioimmunoassay (RIA) and guinea pig intradermal neutralization assay in eleven subjects before and after immunization with toxoid**

Subject no.	Serum sample†	Diphtheria antitoxin in mIU/ml by RIA‡	Diphtheria antitoxin in mIU/ml by intradermal neutralization§
1	a	0	< 2·5
	b	0	< 2·5
	e	> 100	> 100
2	a	0	< 2·5
	b	0	< 2·5
	c‖	> 100	40
	d	7·6	⩽ 2·5
	e	> 100	> 100
3	a	0	< 2·5
	b	0	< 2·5
	c‖	17·6	< 5
	d	> 100	80
	e	> 100	> 100
4	a	0	< 2·5
	b	0	< 2·5
	c	0	< 2·5
	d	48	20
	e	> 100	> 100
5	b	0	< 2·5
	c	80	80
6	a	0	< 2·5
	b	10	10
	c	> 100	> 100
	d	> 100	> 100
	e	> 100	> 100
7	a	0	< 2·5
	b	30	40
	c	> 100	> 100
8	a	0	< 2·5
	b	0	< 2·5
	c‖	22	< 5
	d	> 100	> 100
	e	> 100	> 100
9	a	0	< 2·5
	b	0	< 2·5
	c‖	22	< 5
10	a	0	< 2·5
	b	0	< 2·5
	c‖	25	< 5
	d	> 100	> 100
	e	> 100	> 100
11	a	4	< 2·5
	b	49	40
	c	> 100	> 100

* Correlation coefficient for the two methods 0·926, $P < 0·001$.

† Legend for code letters: a, pre-Schick; b, post Schick; c, post first diphtheria toxoid (2 Lf units); d, post second diphtheria toxoid (2 Lf units); e, post third diphtheria toxoid (2 Lf units).

‡ Assay range 0–⩾ 100 mIU/ml. § Assay range < 2·5–5120 mIU/ml. For this comparison high values given only as > 100 mIU/ml. ‖ Sample positive by RIA while neutralizing assay still negative

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through the range of values 0 to > 100 mIU/ml. There was a very high degree of correlation of results using the two collection methods: venous sample *v.* fingerprick, correlation coefficient = 0·98 ($P < 0·001$). Sera collected on to paper and dried could be stored with no significant loss of antitoxin for at least 1 month at room temperature. Antitoxin levels remained stable for at least 6 months if papers were protected from moisture and stored at −20 °C.

DISCUSSION

It is important to monitor serological levels of immunity to serious infectious disease (Evans, 1980). Schick testing is often used to assess diphtheria immunity but this test has deficiencies when used in large surveys. In 1952, Edsall reported high antitoxin levels in 11 (22 %) of his 50 Schick-positive subjects (Edsall, 1952). In our study, five (10 %) of the 59 Schick-positive children had protective antitoxin levels by RIA. As the RIA correlated very well with the bioassay for sera with low levels of antitoxin, we concluded that the RIA was a more accurate test of diphtheria immunity than the Schick test. Other workers have used the haemagglutination assay for serum antitoxin levels; this assay is also less costly to perform than the Schick test but is subject to variability, particularly in sera with low antitoxin levels (Schubert & Cornell, 1958). Assays based on antitoxin titration on cultivated cells or intradermal neutralization in living animals are accurate tests but are costly for population studies.

A test which can be applied to blood which has been collected by fingerprick on to filter paper and stored for several weeks is advantageous for community surveys. The diphtheria antitoxin radioimmunoassay is very easy to adapt to such conditions and levels remain stable even when the paper discs have been stored at room temperature; storage at room temperature is not satisfactory for the antitoxin assay on cultivated cells (Kriz, Burianova-Vysoka & Roth, 1974). Sera for the RIA may be collected rapidly by fingerprick from large groups of subjects, and untrained personnel can easily learn the technique. Provided they are protected from moisture, samples can be transported safely to central laboratories experienced in radioimmunoassay. In such a laboratory antitoxin assay is relatively simple to set up and the reagents required are less costly than those for Schick testing.

The level of immunity to diphtheria in Australian children was questioned because of a suspected decline in immunization compliance and because many young children have immigrated to the country from regions where routine infant immunization is not readily available. The present surveys have identified groups of school-children with low levels of diphtheria immunity. In one inner-city school 24 % of 12-year-old children were not protected from diphtheria. The fact that 10 % of children over the age of 12 years attending a children's hospital were susceptible was also important. In 1947, Fanning reported an outbreak of diphtheria in a school where 94 % of the children had been immunized and 80 % were Schick-negative, so it may be necessary to maintain high levels of herd immunity to prevent outbreaks in schools.

In this study the radioimmunoassay provided a more accurate, rapid, and less costly method of determining diphtheria immunity than Schick testing. The

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adaptability of this test for the assay of blood collected by fingerprick makes it especially suitable for survey work. It is particularly useful for studies in remote areas or in groups of children in whom venepuncture or Schick testing may be difficult or cause trauma. The RIA correlated very well with the more expensive intradermal neutralization test in the group of 44 sera tested. Detection of antitoxin by the RIA method and not by the neutralization method in five previously negative subjects after their first dose of toxoid may merely indicate the appearance of the non-avid antibodies described by Raynaud (1967). The relationship between these two tests following such an initial dose requires further study, but a close correlation exists after subsequent toxoid doses and also when the subject tested is not in the course of being immunized.

The children who participated in these surveys did so with written parental consent and were volunteers. The studies were carried out with the permission and co-operation of the Director-General of Education and the Chairman of the Health Commission of New South Wales. We thank Dr D. W. Minty of the Commonwealth Serum Laboratories for performing the guinea-pig intradermal neutralization tests. We also thank Miss Mary O'Halloran and the staff of the Biochemistry Department of the Royal Alexandra Hospital for Children for their help with the collection of sera from patients in the Hospital.

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