Detection of antibodies to tetanus toxoid: comparison of a direct haemagglutination method with a radioimmunoassay

AI SHA WANG*, GF BURNS, IJ KRONBORG, IR MACKAY

From the Clinical Research Unit, The Walter and Eliza Hall Institute of Medical Research and the Royal Melbourne Hospital, Melbourne, Victoria, 3050, Australia

SUMMARY Two methods of detecting antibodies to tetanus toxoid were compared, a radioimmunoassay (RIA) employing radiolabelled staphylococcal protein A and a direct haemagglutination (HA) method employing sheep erythrocytes coupled to tetanus toxoid with chromic chloride. These were shown to have a similarly high specificity with the HA method showing slightly higher sensitivity. Haemagglutination offers several additional advantages in terms of simplicity, low cost and less requirement for specialised equipment. The assays were also used to demonstrate a transient IgM response after repeated booster injections with absorbed toxin given to seropositive individuals, and these antibodies were found to be protective in biological tests.

Antibodies to tetanus toxin are a reliable indicator of host protection and the measurement of antitetanus immunoglobulin concentrations is of value in assessing the immune status of individuals at risk, in screening potential donors of immune globulin for passive prophylaxis and in monitoring the efficiency of vaccination programs. In areas where prior immunisation with tetanus toxoid is uncertain, testing for antitetanus antibodies remains of vital importance.

Recently, several groups¹⁻³ have introduced radioimmunoassays (RIA) for the detection of antitetanus antibodies and, while these appear to be very sensitive and specific, they have generally required purified and radiolabelled antibodies to human immunoglobulins. Moreover, the sensitivity of the RIA test has not been directly correlated with the more common assay of passive haemagglutination (HA) with antigen-coupled tanned red blood cells.⁴

We have adopted an RIA employing radiolabelled staphylococcal protein A in a microtitre system for the detection of antitetanus antibodies and have compared this with a HA method which employs tetanus toxoid coupled to sheep erythrocytes with chromic chloride.

Material and methods

SERA

Sera were obtained by venesection from 45 normal

*On sabbatical leave from Capital Hospital, Beijing, Peoples' Republic of China.

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volunteers within the Institute. All the sera were decanted at 56°C and were stored at -20°C until testing. In the immunisation experiments, the volunteers were bled before boosting with tetanus toxoid and then bled again after the time intervals shown in "Results".

TETANUS TOXOID

For immunisation of the volunteers in the boosting experiments absorbed tetanus toxoid (Commonwealth Serum Laboratories (CSL), Melbourne) obtained in 0.5 ml ampoules was employed. The antigen used for coupling to erythrocytes for the HA test and for addition to the plastic plates for the RIA test was non-absorbed tetanus toxoid (CSL, Melbourne) obtained as 1800 limiting flocculation units (LFU)/ml (2700 LFU/mg of protein nitrogen).

HA TEST

Sheep erythrocytes (CSL, Melbourne) obtained in Alsever's solution were washed three times in normal saline, with removal of leucocytes after each wash. The cells were then resuspended to 50% (vol/vol) in normal saline and coupled to tetanus toxoid with chromic chloride as described previously.^{5,6} Briefly, to 0.2 ml of the 50% erythrocytes was added 0.1 ml of 250 LFU/ml toxoid diluted in normal saline and this mixture was mixed on a vortex mixer. Whilst still on the vortex mixer, 0.5 ml of "aged" chromic chloride⁵ at 0.1 mg/ml was added dropwise. The coupled cells were then held at room temperature

for 5 min and washed twice in normal saline and once in phosphate-buffered saline (PBS) before use. Preliminary experiments employing toxoid at 125 LFU/ml, 250 LFU/ml and 500 LFU/ml demonstrated that a slightly higher titre was obtained with 250 LFU/ml than with 125 LFU/ml, but that no increase in titre was obtained when toxoid was used at 500 LFU/ml. Therefore, toxoid at 250 LFU/ml was routinely employed for coupling to the erythrocytes. The erythrocytes were resuspended to 5% (vol/vol) for use in the HA test.

Repeated testing of freshly prepared indicator erythrocytes and coupled erythrocytes which had been held at 4°C as a 5% suspension for up to seven days indicated that the test was very reproducible with different batches of coupled erythrocytes and that these indicator cells could be stored for this time without deterioration.

RIA TEST

The RIA employed used a method with radiolabelled staphylococcal protein A as the second layer.⁷ Various preliminary experiments were performed to standardise the quantity of toxoid added to the plastic plate. These assessed the time and temperature employed for binding of antigen to the plates (for example, Fig. 1), how long the antigen-coated plates could be stored at 4°C, and the optimal time for antibody binding to the coated plates. The results of these experiments were generally clear cut (Fig. 1) and the various conditions were optimised as follows. Each round-bottomed well of the standard microtitre plastic plate (Linbro/Titertek, 76-111-05) received 100 µl of toxoid at 2.5 LFU/ml in distilled water and

Fig. 1 Tetanus toxoid coated to the plastic plate was optimal at 37°C after 2 h in the RIA test.

the antigen-coated plates could be stored for more than seven days without loss of activity. Coating of antigen to the plastic was optimal after allowing the interaction to take place at 37°C (Fig. 1) for 2 h. A plateau of antibody binding to the antigen-coated plate was obtained after 2 h at room temperature. In every determination a control plate coupled with an unrelated antigen (1% bovine serum albumin) was included as a non-specific binding control for each serum tested and the final antibody titre was given as the lowest dilution which gave duplicate counts greater than the maximum mean count in the control.

BIOLOGICAL TEST FOR TETANUS ANTITOXIN (BIOASSAY)

The ability of sera to protect mice from the paralytic effects of a fixed dose of tetanus toxin were compared with a standard preparation of tetanus antitoxin as described in the *British Pharmacopoeia*.⁸

Table 1 Comparison of HA and RIA titres to tetanus toxoid in 45 normal subjects

Subjects	HA	RIA
1 BT	1/2560	1/2560
2 FI	1/655360	1/655360
3 TA	1/40960	1/20480
4 AJ	1/20480	1/10240
5 BE	1/655360	1/81920
6 HD	1/40960	1/10240
7 GR	1/655360	1/163840
8 SB	1/81920	1/40960
9 JM	1/40960	1/10240
10 HH	1/640	1/640
11 AJ	1/20480	1/10240
12 DU	1/40960	1/20480
13 CJ	1/81920	1/20480
14 GR	1/163840	1/20480
15 NR	1/40960	1/10240
16 SL	1/5120	1/1280
17 HS	1/20480	1/20480
18 CA	1/20	1/20
19 AS	1/10240	1/10240
20 AW	1/640	1/320
21 KP	1/40960	1/5120
22 CL	1/2560	1/5120
23 IM	1/20	1/640
24 PA	1/40960	1/10240
25 SA	1/20	1/320
26 HR	1/20480	1/10240
27 CW	1/81920	1/10240
28 GN	1/20480	1/2560
29 NT	1/20480	1/2560
30 DG	1/40960	1/20480
31 PE	1/160	1/640
32 NG	1/320	1/1280
33 WH	1/10240	1/2560
34 WM	1/640	1/640
35 CB	1/640	1/1280
36 WL	1/20480	1/5120
37 TB	1/40960	1/5120
38 SH	1/80	1/80
39 VK	1/2560	1/2560
40 JH	1/40	1/160
41 HR	1/81920	1/20480
42 KM	1/40960	1/10240
43 SB	1/81920	1/10240
44 YA	1/20480	1/10240
45 SZ	1/640	1/320

Fig. 2 Comparison of the antitetanus titre by HA and RIA in a subject boosted with tetanus toxoid.

Results

Serum samples were obtained from 45 normal individuals and the heat-inactivated sera were tested for antitetanus antibody by HA and RIA. The results are presented in Table 1, and it is clear that there was reasonable correlation between the two tests, with the titres from 21 of the samples being identical or within a twofold or less difference in titre. Of the remainder, 19 gave a higher titre when tested by HA, and in only 5 tests was the HA titre lower than the RIA titre. The final endpoint of the HA plates was generally very clear cut and reproducible, and in both tests repeated testing of the sera gave consistent results.

In an experiment three volunteers were boosted with tetanus toxoid and then repeated samples of serum were tested by both HA and RIA. The results from one of these subjects are illustrated in Fig. 2. These tests effectively demonstrated the substantial rise in titre after the booster injections of toxoid, but it was also observed that there was a large discrepancy between the HA and RIA titres in samples immediately after boosting. It was thought that the generally higher titres observed in the HA test might

Table 2 Reduction of HA and RIA titres in sera treated with 2-ME

Subject	Date of Sample	Reciprocal titre			
		HA		RIA	
		Untreated	+ 2-ME	Untreated	+ 2-ME
WA	11/11/80*	320	320	320	160
	20/11/80	327 680	40 960	40 960	10 240
	4/12/80	327 680	81 920	40 960	10 240
	13/1/81*	163 840	163 840	163 840	nd
	22/1/81	>655 360	40 960	20 480	10 240
	20/2/81	>655 360	40 960	20 480	10 240
DU	30/10/80*	320	320	320	160
	11/11/80	163 840	20 480	20 480	10 240
ZL	29/11/81*	640	320	320	320
	8/12/81	40 960	5120	5120	1280

*Booster injection given on this date after serum sample was taken.

Table 3 Correlation of HA titre and the units of mouse protection obtained by bioassay

Subject	Date of sample	Reciprocal HA titre*		Bioassay (units/ml)
		Untreated	+ 2-ME	
IW	11/11/80	320	320	0.32
	20/11/80	327 680	40 960	40.96
	4/12/80	327 680	81 920	40.96
	13/1/81	163 840	163 840	20.48
DU	30/10/80	320	320	0.16
	11/11/80	163 840	20 480	20.48

*Untreated HA titre measures all antitetanus antibodies including IgM, following 2-ME treatment the titre measures principally IgG antibodies. The bioassay results correlate more closely to the untreated HA titre indicating that IgM antibodies are protective.

be due to IgM antibody which would not be detected in the RIA using radiolabelled staphylococcal protein A. This was tested for by 2-mercaptoethanol (2-ME) reduction of the IgM followed by a repeated HA test, and the results (Table 2) clearly validate this premise. Thus 2-ME treatment of the samples reduced the very high HA titres to within one well of the RIA titre whereas the same treatment had no effect on the preimmunisation samples or on the samples taken some eight weeks after boosting. The slight reduction of one to two wells seen in the RIA results after treatment with 2-ME may demonstrate that some IgM was binding the radiolabelled protein A used in this assay, but a one well reduction was also seen in two of the preimmunisation bleeds suggesting a slight non-specific effect of 2-ME. We investigated whether IgM antibodies were likely to be protective *in vivo* by means of mouse protection tests and the results (Table 3) were that the HA titre correlated with the units of protection in the bioassay. This was true whether the major component of the antibody was IgM or IgG.

Discussion

Both of the methods described in this paper, HA and RIA, are demonstrated to be very sensitive for the detection of antitetanus toxoid IgG antibodies. The level of sensitivity was such that at the highest dilutions we were detecting the specific antibody contained in about 0.0023 µg of IgG. The methods were also highly specific. In the RIA, our routine control was an unrelated antigen (bovine serum albumin) coated to the plate, but we also found that normal rabbit serum, which strongly binds protein A, added to the plates coated with tetanus toxoid gave very low background counts. In the HA test, sheep erythrocytes which had not been coupled to tetanus toxoid revealed low levels of natural antibodies, but since this activity was abolished at 1/80 dilutions of serum, it was not necessary to absorb the sera with erythrocytes prior to testing.

A direct comparison of the RIA and HA showed that of the two methods the HA test appeared to be the more sensitive. The HA test offers further advantages. While the RIA employed in this work was simpler than methods described hitherto with radiolabelled antihuman immunoglobulin,¹⁻³ the HA method is simpler to perform, rapid, less expensive in reagents and requires no special equipment such as a gamma counter.

In addition, with the use of 2ME to reduce IgM antibodies, our experiments with volunteers boosted with tetanus toxoid demonstrated that the HA method was effective in revealing IgM antibodies

which are indicative of recent immunisation. These studies also confirm the findings of Hernandez and Burgiss-Wolff⁹ that both primary and secondary vaccination can elicit the formation of transient IgM antibody. Moreover, correlation with mouse protection tests, presumably a more accurate indication of *in vivo* protection, showed that the high HA values after booster injections which were largely the result of IgM antibodies were reflected by greatly increased mouse protection values.

The assays described in this paper were originally developed in order to screen clones of Epstein-Barr virus transformed B lymphocytes from immunised donors for the production of antitetanus toxoid antibodies.³ Such human antibodies produced *in vitro* may eventually prove of great value for passive protection against tetanus. The rapidity and simplicity of the HA method described here make this an ideal procedure for the detection of clones secreting antibody to tetanus toxoid *in vitro*.

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Requests for reprints to: Dr IR Mackay, Clinical Research Unit of The Walter and Eliza Hall Institute of Medical Research and the Royal Melbourne Hospital, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia.