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A possible explanation for the discrepancy between ELISA and neutralising antibodies to tetanus toxin

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Abstract

The structure and protective activity of tetanus antibodies elicited in rabbits after whole-cell pertussis diphtheria–tetanus vaccine (DTPw) vaccination was studied. ELISA antibody levels and toxin neutralisation activity (TNT) were measured in individual serum samples. The ratio of symmetric and asymmetric (functionally monovalent) IgG molecules was determined by concanavalin A (Con A) chromatography. This test is based on the fact that the carbohydrate group responsible for the molecular asymmetry has high affinity for the lectin Con A. Asymmetric molecule ratio was observed to increase with immunisation time, as well as differences between TNT and ELISA levels. All serum samples were overestimated by ELISA as compared to TNT assay, in line with the markedly higher proportion of asymmetric molecules which have lower toxin neutralising activity. Protective levels could not be predicted reasonably from ELISA results below 0.222 IU/ml, because this methodology fails to discriminate between both types of antibodies and only an in vivo serum neutralisation procedure (TNT) reflects the true neutralising serum activity.

Keywords: Antitetanus antibodies; Asymmetric IgG molecules; TNT/ELISA ratio; Whole-cell DTP vaccine

1. Introduction

Originally described as early as 1935 by Heidelberger and Kendall [1] and later by Margni et al. in the 1970s [2–7], the properties of asymmetric, non-precipitating antibodies have been widely reviewed [8,9]. Such antibodies have been demonstrated in all mammalian species studied, following immunisation with a variety of antigens or after parasite or microbial infection. Asymmetric antibodies present a single mannose-rich oligosaccharide group in the Fab region, thus proving univalent and displaying two paratopes whose affinities differ 100-fold, as shown in equilibrium experiments and radiobinding studies with monovalent haptenes [9–11]. In the course of immune response, both symmetric and asymmetric IgG molecules, exerting radically dissimilar neutralising activity, are synthesised in a variable proportion by the same cellular clones. IgG molecules (10 to 15%) in non-immune sera of either human or animal origin are asymmetrically glycosylated [12,13].

Comparative studies on animals repeatedly immunised with soluble or particulate antigen (ovoalbumin insolubilised by treatment with glutaraldehyde or covalently bound to *Salmonella* and *Brucella*) show that asymmetric antibody ratio is higher when the antigen is associated to bacteria and indicate that the physical state of the antigen modulates the relative synthesis of symmetric and asymmetric antibodies [8,14,15].

Studies on rabbits immunised with tetanus toxoid as a component of an acellular vs. a DTPw vaccine

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demonstrated that neutralising activity level in the former was two-fold higher, in agreement with the greater proportion of symmetric antibody with higher neutralising activity [16]. Data from the literature indicate that during pregnancy there is preferential synthesis of asymmetric IgG molecules with antipaternal specificity believed to exert a protective effect upon the foetus and IL-6 has been identified as one of the factors that participate in IgG glycosylation [17]. In this connection, we have reported that in mothers at the time of delivery there is a modulation of tetanus and diphtheria antibody response inducing a higher proportion of asymmetric IgG molecules of lower protective capacity compared to basal levels in non-pregnant women [18].

It had already been observed that at low antibody levels, TNT values diverged more markedly from ELISA titres [19,20], perhaps because the latter test quantifies total antibody content (symmetric plus asymmetric molecules), whereas the former only evaluates the molecules with full neutralising capacity (symmetric antibodies). Although this discrepancy is a well-known phenomenon, its cause still awaits elucidation.

Accordingly, it was of interest to determine, in our rabbit model, whether the lack of correlation between TNT and ELISA values at low antitetanus antibody titres was related to the ratio of asymmetric molecules present in sera from immunised animals.

2. Materials and methods

2.1. Vaccine

Whole-cell pertussis, diphtheria–tetanus vaccine (DTPw) was prepared with 4.6 IU of the second standard for pertussis vaccine (Statens Serum Institute, Copenhagen, Denmark), 7.5 Lf of diphtheria toxoid and 5.0 Lf of tetanus toxoid/0.5 ml dose. Final vaccine contained less than 0.85 mg aluminium as aluminium hydroxide or 0.05 mg thimerosal/dose.

2.2. Animal immunisation

Groups of three New Zealand white rabbits weighing 2.2–2.5 kg were pre-bled (control serum) and inoculated (intramuscularly) at the starting point and 2, 4 and 23 months later with 0.5 ml of DTPw vaccine. Animals were bled weekly and individual serum samples were stored at −20°C until tested.

2.3. Measurement of antibody level by ELISA

Anti-T IgG antibody levels were measured by ELISA in individual serum samples from each animal group. Briefly, non-adsorbed tetanus (Statens Serum Institute, Copenhagen, Denmark) was used as sensitising antigen at 5 μg/ml in phosphate-buffered saline. Six two-fold dilutions of each sample and the reference preparations were assayed. A hyperimmune rabbit serum obtained by intramuscular immunisation with DTPw, titrated in vivo for tetanus antibodies and calibrated in IU/ml against the international equine standard [21] was used as a reference. Adsorbed antibodies were detected with horseradish peroxidase conjugated to goat IgG anti-rabbit IgG (Cappel Lab., Organon Tecknika, Belgium). All determinations were done in triplicate. Absorbance values were measured at 492 nm.

2.4. Measurement of toxin neutralising activity

TNT was performed in mice as described by Craig [21] with minor modifications. Briefly, four two-fold dilutions of each sample were incubated with L+/1000 tetanus toxin for 1 h in darkness at room temperature. Each mixture was injected subcutaneously into four mice. Animals were observed during five days and tetanus symptoms and deaths recorded. Control series of mice injected with toxin mixed with defined amounts of the international standard for tetanus antitoxin were included in each assay and results used to confirm the test dose of the toxin and to correct the antitoxin values obtained. Neutralising international units of serum samples were calculated by the Spearman–Karber method [22] and expressed in IU/ml.

2.5. IgG fraction purification

The IgG fraction from individual serum samples of each animal was isolated using Protein–A-Sepharose (Sigma Chemical Co., St Louis, MO, USA) chromatography, as described by Harlow and Lane [23]. Purified IgG was quantified by spectrophotometric reading at 280 nm. Purity was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting.

2.6. $F(ab')_2$ fragments from IgG fraction

$F(ab')_2$ fragments were obtained by pepsin digestion according to Natvig and Turner [24]. Undigested IgG was removed by Protein A–Sepharose chromatography. Fragment purity was checked by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and immunoblotting. The total $F(ab')_2$ fraction purified from each serum sample were separately processed.

2.7. Measurement of symmetric/asymmetric molecules ratio

This was performed by chromatography in Con A–Sepharose (Sigma Chemical Co., St Louis, MO, USA),

taking advantage of the fact that only asymmetric molecules bind to the lectin through their mannose-rich carbohydrate group [10]. $F(ab')_2$ fragments were used to avoid any possible non-specific interaction among carbohydrates present in the Fc fragment of the whole molecule and Con A. The procedure was carried out as originally described by Leoni et al. [11].

The percentage of total $F(ab')_2$ fragments with asymmetric structure in serum samples was calculated as the difference between protein level of purified $F(ab')_2$ fractions prior to incubation with Con A–Sepharose (symmetric and asymmetric) and pass-through containing symmetric fragments alone (data not shown).

2.8. Statistical analysis

Linear regression analysis was performed on absorbance values recorded for the reference and experimental sample dilutions to calculate ELISA titres. A Mann–Whitney test was applied to analyse antibody titres measured by TNT and ELISA. Kendall's τ correlation coefficient was calculated for the symmetric/asymmetric ratio in ELISA vs. TNT.

3. Results

Individual ELISA and protective activities as well as

Table 1
Relationship among symmetric/asymmetric IgG molecule ratio, antitetanus antibody protective capacity, ELISA antibody level and TNT/ELISA ratio[a]

Weeks after first vaccine dose	Rabbit number	ELISA antibody level (IU/ml)[f]	Antibody protective capacity (IU/ml)	ELISA/TNT ratio	Symmetric/asymmetric IgG ratio
	1	5.68	5.00	1.14	4.65
3[b]	2	5.27	4.20	1.25	4.57
	3	5.22	4.20	1.24	4.13
	1	20.21	16.82	1.20	2.08
11[c]	2	19.68	14.14	1.39	1.96
	3	19.23	11.89	1.62	1.84
	1	25.94	17.80	1.46	1.65
19[d]	2	25.88	17.67	1.46	1.49
	3	25.88	17.67	1.46	1.34
	1	2.05	1.35	1.52	[g]
32	2	2.31	1.48	1.56	[g]
	3	2.36	1.52	1.55	[g]
	1	1.00	0.60	1.67	1.49
40	2	1.20	0.72	1.67	1.59
	3	1.60	1.00	1.60	1.59
	1	0.24	0.140	1.71	1.58
48	2	0.26	0.146	1.78	1.51
	3	0.28	0.150	1.87	1.47
	1	0.200	0.086	2.33	[g]
60	2	0.205	0.090	2.28	[g]
	3	0.231	0.110	2.10	[g]
	1	0.11	0.045	2.44	[g]
84	3	0.14	0.058	2.41	[g]
	1	0.10	0.036	2.78	[g]
88	3	0.10	0.040	2.50	[g]
	1	0.033	0.011	3.00	1.49
92	2	0.043	0.015	2.87	1.47
	1	0.01	0.0026	3.85	1.46
100	2	0.01	0.0027	3.70	1.46
	3	0.01	0.0029	3.45	1.47
	1	39.43	25.00	1.58	1.47
103[e]	2	39.89	25.00	1.60	1.46
	3	41.21	25.46	1.62	1.34

[a] Rabbits were immunised intramuscularly at the starting point, 8, 16 and 100 weeks with 0.5 ml of DTPw vaccine. Animals were bled weekly and all determinations were done in individual serum samples.

[b,-] [c,d,e] Weeks corresponding to peak antibody levels after first, second, third and fourth vaccine inocula, respectively.

[f] Antibody protective capacity values determined by TNT in mice.

[g] Data not available due to limited sample amount.

symmetric/asymmetric and ELISA/TNT ratios are given in Table 1.

For analysing TNT and ELISA values, a variable was constructed which consisted of measuring the log distance from each experimental value to a theoretical curve between TNT and ELISA for which titres by both assays are the same (Fig. 1). The difference between both variables was analysed by the Mann–Whitney test. Differences between TNT and ELISA values proved statistically significant ($p < 0.0001$) and ELISA/TNT ratios were 1.14–3.85 (Table 1). On the other hand, the relationship between antibody titres as measured by TNT and ELISA showed that data fitted the linear regression curve:

$$\log\ \mathrm{TNT} = -0.274 + 1.11\ \log\ \mathrm{ELISA};\ r^2 = 0.9972.$$

On the basis of this curve, 0.222 IU/ml ELISA value was interpolated for 0.1 IU/ml TNT titre.

On determining Kendall's τ correlation coefficient for the symmetric/asymmetric antibody ratio in ELISA vs. TNT, a positive correlation was observed between the ratio vs. both ELISA and TNT ($\tau = 0.44$ and $\tau = 0.443$, respectively) and a negative correlation for the ratio vs. ELISA/TNT ($\tau = -0.685$; $p < 0.01$).

Percentages of total asymmetric $F(ab')^2$ fragments found in vaccinated rabbits increased with immunisation time and were higher than the $12\% \pm 0.93$ value observed in the non-vaccinated group and previously reported ($p < 0.025$; Table 1) [10]. All serum samples were overestimated by ELISA as compared to TNT assay. On occasion, limited sample amount precluded performing Con A chromatography, so that symmetric/asymmetric ratios are not available for a few samples. However, the steadily decreasing trend with immunisation time from 4.65 to 1.34 was readily discerned (Table 1). Concomitantly, as the symmetric/asymmetric ratio decreased, the ELISA/TNT ratio tended to increase up to week 100, just before the fourth dose, after which the ratio dropped to less than half its peak value (Table 1).

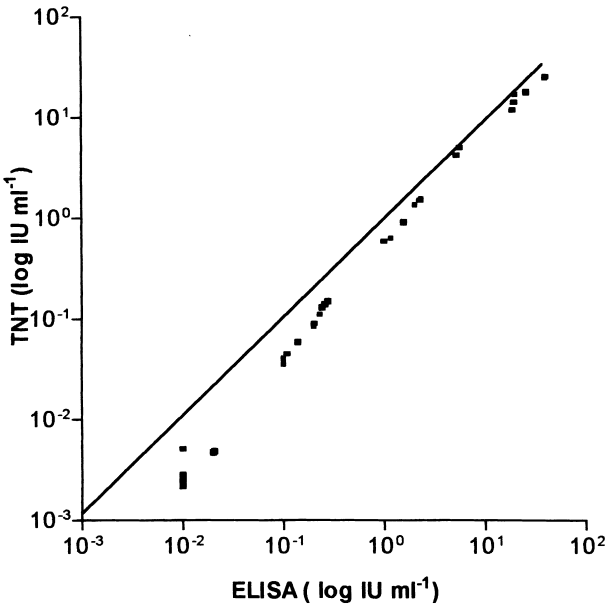

Fig. 1. Relationship between antitetanus antibody levels for DTPw vaccine as measured by TNT and ELISA.

4. Discussion

Protection against tetanus requires a minimum antibody titre of 0.01 IU/ml, which is readily achieved by the application of diverse commercially available vaccines following immunisation schedules [25]. However, one must bear in mind that to evaluate protection against tetanus and diphtheria, not only must the level of produced or transferred specific antibodies be considered but also their capacity to neutralise the toxins [16,18].

The purpose of this research was to determine the potential correlation between serum symmetric/asymmetric antibody ratio and toxin neutralising activity. Special stress was laid on analysing the relation between ELISA antibody levels and TNT values with immunisation time.

Results disclosed a significant difference between both assays. From the analysis of the regression curve it was observed that TNT levels of 0.1 IU/ml corresponded to ELISA values 2.22 times greater, indicating that in order to evaluate the threshold protection status, only titres exceeding 0.222 IU/ml should be considered.

Findings are in agreement with the content of asymmetric antibodies, which have low toxin neutralising capacity, as observed in serum samples. Thus, serum samples at week 100 from animals immunised with either DTPw or acellular DTP vaccine presenting an equal ELISA tetanus antibody level (0.01 IU/ml) but dissimilar symmetric/asymmetric antibody ratio (1.467 vs. 4.273), display widely divergent levels of mean neutralizing activity (0.021 vs. 0.046 IU/ml) [16].

Taking into account that asymmetric molecules may bind firmly by a paratope to the antigen, present results suggest a competitive behaviour between both molecules in binding to the toxin, so that asymmetric antibodies would act as blockers interfering with the neutralising activity exerted by their symmetric counterparts. In support, we have previously demonstrated that the neutralising activity of preparations containing symmetric and asymmetric fragments in the original serum proportion is greater than the value corresponding to purified asymmetric fragments and lower than that of symmetric ones [18].

Simonsen et al. [19,20] found that samples of human

serum with neutralising activity below 0.1 IU/ml were also seriously overestimated by simple ELISA and in order to overcome the problem they developed a competition ELISA with toxin in solution. A very good correlation with neutralising titre was achieved by these authors when sera were assayed twice: once with and once without an excess of purified toxin in the diluent. The difference between the two estimates from each serum sample was considered to be an evaluation of "specific" antibody. In all likelihood, the final result obtained in the above studies corresponds to symmetric antitetanus antibodies alone. However, since a considerable increase in asymmetric IgG molecules has been observed with several particulate immunogens [14,15], it seems reasonable to assume that tetanus toxoid as a component of DTPw vaccine can somehow react with *Bordetella pertussis* and act as a particulate antigen.

Interestingly, in our working conditions, the low neutralising capacity of asymmetric antibodies was evidenced more markedly in serum samples with low antibody titres by ELISA (week 100, just before the fourth dose), in which the ELISA/TNT ratio was 3.45–3.85. Even though a similar symmetric/asymmetric molecule ratio was found in sera at week 103, with an ELISA/TNT ratio of 1.58–1.62, total antibody levels as measured by ELISA were high enough to render negligible the competitive behaviour between symmetric vs. asymmetric molecule binding to the toxin and thus failed to affect serum neutralising capacity.

On the basis of our observations, protective levels could not be predicted reliably from ELISA values below 0.222 IU/ml, since this methodology fails to discriminate between both types of antibodies. Only an in vivo serum neutralisation procedure as TNT reflects the true neutralising serum activity.

Although direct extrapolation of experimental animal data to humans is questionable, to determine symmetric/asymmetric antibody ratios in the relatively few individuals who present tetanus symptoms, despite having neutralising antibody levels approaching 0.1 IU/ml [26,27], would have provided a valuable insight. At any rate, it may be speculated that *B. pertussis* LPS induces IL-6 response, known to mediate IgG glycosylation by modulating glycosyltransferase activity, leading in turn to changes in one of the carbohydrate Fab regions and thus generating an asymmetric molecule. This mechanism is strongly supported by the lack of LPS in the acellular DTP vaccine.

To sum up, a variant of the Argentine Child Vaccination Schedule against diphtheria, tetanus and pertussis was employed. It recommends three doses of DTP vaccine at 2, 4 and 6 weeks of age, plus two booster doses at 18–24 months and 6 yr. In our case the booster dose was applied at 25 months, when the antibody level had dropped to a threshold protective value (0.01 IU/ml). In other words, we sought serum samples with a low ELISA antibody level, obtained in the course of a long-term immunisation schedule to guarantee a high content of asymmetric or blocking antibodies, thus enabling the analysis of the incidence of such antibodies on protective serum activity. Our goal was therefore to achieve in the rabbit model a close analogy with the child awaiting the 18–24 month booster dose.

Recalling that DTPw vaccine will continue to be used for mass immunisation worldwide for some considerable time, since novel acellular DTP vaccines are not yet generally available, we hope that our present observations on the modulation of immune response should help to explain the lack of protection in isolated cases presenting threshold ELISA tetanus antibody levels.

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