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Higher protective activity of Fab′ fragment compared to F(ab′)$_2$ and IgG on experimental tetanus

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Introduction

Purified tetanus antitoxin serum which contains antitetanus bivalent F(ab′)$_2$ fragments is ineffective in interrupting the development of the disease if it is injected during the period when the symptoms appear, that is when the toxin has already been fixed on the neural structures. Circulating F(ab′)$_2$ has some difficulty in passing through the blood–brain barrier; after intravenous or intracarotid injection of fragments from heterologous antitetanus antibodies, F(ab′)$_2$ fragments appear in the spinal fluid only after 15–30 min while Fab′ fragments can be detected after 5 min. Moreover, the titre of Fab′ is 10 times lower than that of F(ab′)$_2$ at 15 min after the injection of the antitoxin (Fedinec 1966; Aleksevich *et al.*, 1976; Aleksevich 1979). Therefore, the transport of the Fab′ fragments through the natural barriers must be somehow facilitated. On the other hand, tetanus toxin bound to its neural receptor keeps both its ability to combine with the antitoxin molecule and to be neutralized without unbinding from its neural receptor (Kryzhanovsky 1970). Using *in vitro* experiments, Kryzhanovsky *et al.* (1972) showed that nine International Units (IU) of Fab′ can neutralize one minimum lethal dose (MLD) of the protagon–toxin complex while 24 IU F(ab′)$_2$ and 60 IU IgG whole molecule are needed to produce the same effect. Therefore, monovalent fragments are more effective in neutralizing the toxin bound to its receptor than the bivalent fragment or the whole molecule. In the present work we report a comparative study of the protective activity *in vivo* of antitoxin sera containing monovalent Fab′ fragments, bivalent F(ab′)$_2$ fragments or whole IgG molecules. We did not isolate the tetanus antitoxin molecules because our main interest was to compare the protective activity of Fab′ fragments with F(ab′)$_2$ fragments present in sera usually used in tetanus therapy.

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Materials and Methods

Antitoxin preparations

Plasma from horses, hyperimmunized against tetanus toxin, was concentrated by precipitation with $(NH_4)_2SO_4$ at 50% (w/v) saturation and contained whole IgG antitetanus molecules. Serum containing bivalent $F(ab')_2$ fragments was obtained by pepsin digestion and simultaneous concentration from the same sample of plasma. Serum containing alkylated monovalent Fab' fragments was obtained by reduction and alkylation of serum containing $F(ab')_2$ fragments diluted with 0.15 M NaCl to 6 g/100 ml. After adjusting the pH to 8.0, 2-mercaptoethanol was added at 0.1 M in N_2. The solution was kept at 5°C for about 24 h and then iodoacetamide was added at 0.1 M. After 2 h at 5°C, the solution was dialysed against 0.15 M NaCl. Serum containing non-alkylated monovalent Fab' fragments was obtained in the same way excluding the addition of iodoacetamide. Sera containing IgG and $F(ab')_2$ antitetanus molecules were produced by Syntex do Brasil S/A. Gel immunodiffusion technique (Ouchterlony 1949) was used to verify the efficiency of the process for obtention of monovalent fragments.

Inoculum

The tetanus toxin when diluted 1:600 000 presented a minimum lethal dose (MLD) as determined in mice. Serial dilutions of the toxin were made in 0.15 M NaCl with 1% peptone. A suspension of spores of *Clostridium tetani* B–89 was also used to induce tetanus in mice and was prepared according to Smith (1964). Minimum lethal dose was determined injecting dilutions from 10^{-1} to 10^{-5} of the spores suspension in freshly prepared and autoclaved 2.5% (w/v) $CaCl_2$ solution.

Biological and chemical controls

The different samples of the antitetanus preparations were titrated by the method of Rosenau and Anderson (1908). One International Unit of tetanus antitoxin is defined as 10 times the smallest volume of the antitoxin solution able to protect a guinea pig from the lethal effects of one L+ dose of tetanus toxin for 96 h. The protein content was determined by the biuret reaction.

Gel filtration of the antitoxin preparations

Estimation of the molecular size (M_r) of the fragments of antibodies was performed by gel filtration through Sephadex G-75 equilibrated with 0.05 M Tris/HCl buffer pH 8.0 containing 0.2 M NaCl and NaN_3. Bovine serum albumin (66 kDa), ovalbumin (45 kDa) and lyzozyme (14 kDa) (Sigma Chemical Co. St. Louis, MO, USA) were used as markers.

In vivo test

Protective activity of the antitetanus preparations was determined from the percentage of survival of mice. Tetanus was induced in groups of 10 to 20 mice (18 to 20 g) by injection into the right hind leg of either 4 MLD of tetanus toxin or 10 MLD of spore suspension of *Cl. tetani*. After 24 and 32 h, depending on the experiment, mice were injected into the left hind leg with antitetanus sera, at doses of 1–26 IU. This dose of toxin was chosen because it reproduces in mice the course of the disease induced by 10 MLD of spores (Smith 1975). The first sign of tetanus (paralysis of the

right hind leg) appeared 20 to 24 h after the injection of toxin or spores. In the unprotected controls and in the inadequately protected mice the disease took from 30 h after the first appearance of signs to progress to fatal tetanus.

Statistical analysis

Statistical analysis of the results from *in vivo* tests, as expressed in percentage of survival of mice, was performed by the probit method (Finny 1978). In situations where this method could not be employed, as when the dose–response curves were not parallel, the LD_{50} was determined using different doses of antitoxin in the range 1 to 26 IU. This was in the case of the experiments employing sera containing bivalent and non-alkylated monovalent fragments injected 32 h after the inoculation of toxin and sera containing the whole molecule and non-alkylated monovalent fragments injected 24 h after the inoculation of toxin.

Results

Characterization of the antitoxin preparations

All the antitoxin preparations were prepared in 0.002% (w/v) phenol and were considered sterile, innocuous and pyrogen-free. The antitoxin titres, the content of proteins and the specific activity of the samples are shown in Table 1.

Table 1 Tetanus antitoxin titres, protein content and specific activity of the serum samples

Antitoxin	Antitoxin titre (IU/ml)	Protein content (mg/ml)	Specific activity (IU/mg)
IgG	300	43	7
$F(ab')_2$	3900	186	21
Non-alkylated Fab'	600	36	17
Alkylated Fab'	480	36	13

To confirm the presence of $F(ab')_2$ and Fab' fragments, the antitoxin preparations were analysed as follows:

Immunodiffusion. All preparations of the monovalent fragments were assayed at the end of the process and also throughout a period of four years to check the stability of the sample. These samples were kept at −20°C and were analysed at irregular intervals, i.e. 2 to 48 months. The samples presented the same immunodiffusion pattern at all times tested. Sera containing monovalent fragments did not present a precipitation line against tetanus toxoid but they maintained their neutralizing activity. All the samples showed a precipitation line after reaction with anti-horse $F(ab')_2$ (Fig. 1).

Gel-filtration in Sephadex G-75. Gel filtration of sera containing bivalent fragments yielded two peaks while sera containing monovalent fragments (alkylated or non-alkylated) yielded three. Peak I of all the samples eluted with the void volume indicating the molecular size (M_r) to be higher than 100 kDa. Peak II common to all samples corresponded to a $M_r = 86$ kDa consistent with the molecular size of $F(ab')_2$

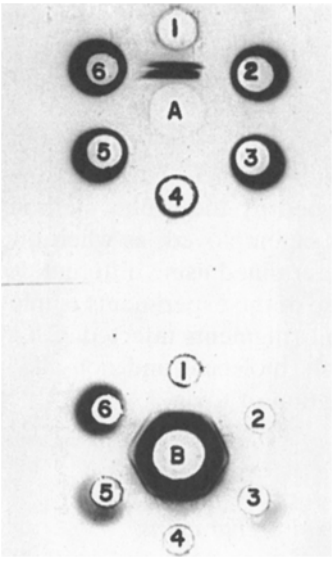

Fig. 1 Agar gel immunodiffusion analysis of tetanus antitoxin sera against tetanus toxoid. A. Tetanus toxoid; B. Anti-horse $F(ab')_2$. 1. $F(ab')_2$ (diluted 1:3); 2. Fab′ (non-alkylated, 48 months at −20°C); 3. Fab′ (non-alkylated, 44 months at −20°C); 4. Fab′ (alkylated, 12 months at −20°C); 5. Fab′ (non-alkylated, 10 months at −20°C); 6. Antirabies Fab′ (unspecific Fab′).

fragments. Peak III obtained in the gel filtrations of sera containing non alkylated or alkylated monovalent fragments corresponded to a $M_r = 56$ and 58 kDa, respectively.

Antitetanus activity. The eluates obtained from gel filtrations were submitted to titration of antitetanus activity and gel immunodiffusion; both results were analysed together in order to confirm the presence of Fab′ tetanus antitoxin. As peak II (86 kDa) from gel filtration of $F(ab')_2$ containing serum precipitated against tetanus toxoid and presented antitetanus activity, it certainly contains the bivalent fragments of antitetanus antibodies. Peak III from gel filtration of sera containing non-alkylated or alkylated monovalent fragments (respectively 56 and 58 kDa) presented 20 IU antitoxin/ml but did not precipitate against tetanus toxoid, and so it can be assumed that it contains the monovalent fragments. Peak II (86 kDa) from these gel filtrations showed 20 IU antitoxin/ml but failed to precipitate in the gel immunodiffusion. In this case two hypotheses were considered and they are discussed below.

Protective activity and relative potency of the antitoxin preparations

The protective activity of non-alkylated Fab′ was compared to $F(ab')_2$, IgG, alkylated Fab′ and also to non-alkylated Fab′ produced from another sample of horse plasma. At 24 and 32 h after the inoculum, whatever the dose of antitoxin employed, the percentage of survival was higher when monovalent fragments were used. A 20 to 30% higher survival rate was obtained when Fab′ was used instead of $F(ab')_2$, employing spores suspension as inoculum. When the inoculum was tetanus toxin, a 20 to 50% higher survival rate was observed in the group receiving Fab′. The same dose of serum containing the whole molecule of tetanus antibodies yielded the lowest percentage of survival. Figure 2 shows the dose–response curves using data obtained

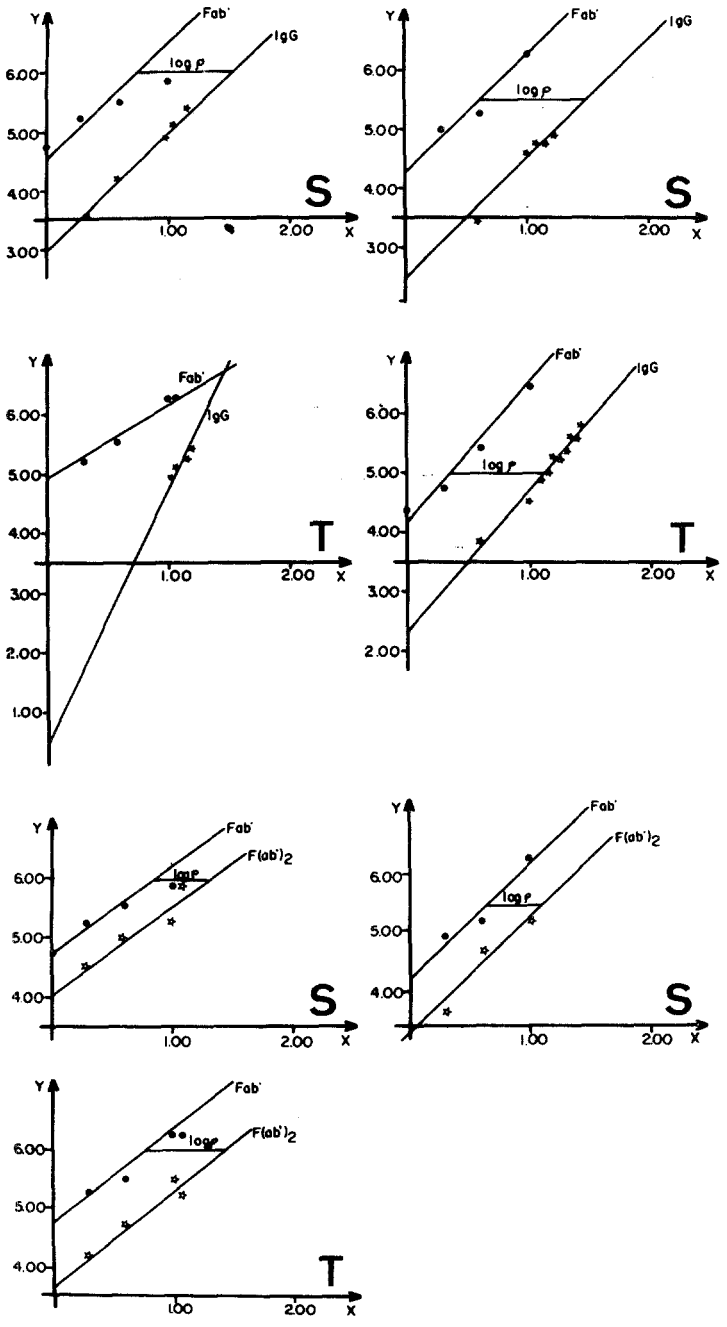

Fig. 2 Dose–responses curves of Fab', $F(ab')_2$ and IgG when they were injected 24 h (left side) or 32 h (right side) after the inoculation of suspension of *Cl. tetani* spores (S) or tetanus toxin (T). Y axis = probit unit, X axis = log (dose). The distance between the straight lines (log ρ) defines the relative potency of the samples

from calculations of the percentage of survival of mice treated with different doses of Fab', $F(ab')_2$ and IgG. Distances between parallel dose–response lines (log ρ) defines the relative potency of the samples and allows their comparison. Using this statistical method, the equivalence between 1 IU of Fab' and the other antitoxins was established (Table 2). In situations where this method could not be applied, the comparison between two samples was made using the values of LD_{50}. The probability level of $P<0.05$ was considered significant. The LD_{50} of Fab' was significantly lower than the LD_{50} of $F(ab')_2$ or IgG indicating that even in these cases the protective activity of the monovalent fragments is higher than that of the bivalent fragment or whole antitoxin molecule. Table 3 shows the results of *in vivo* tests, as expressed as percentage of survival of mice, using different antitoxin doses. Alkylated monovalent fragments (Fab'–SCH_2CONH_2) were less protective than non-alkylated monovalent fragments (Fab'–SH). When two different samples of serum containing Fab'–SH were compared the percentage of survival obtained was analogous (Fig. 3).

Table 2 Equivalence 1 IU of Fab' and $F(ab')_2$ or IgG, established using the statistical method of probit units (Finny 1978)

Experimental conditions		1 IU of Fab' is equivalent to:	
Time	Spores/toxin	$F(ab')_2$	IgG
At 24 h after inoculation	10 MLD spores	2.9 IU	6.1 IU
	4 MLD toxin	4.8 IU	*
At 32 h after inoculation	10 MLD spores	2.9 IU	7.2 IU
	4 MLD toxin	*	5.8 IU

*As the dose–response curves were not parallel under these experimental conditions, the comparison was made using the LD_{50} values.

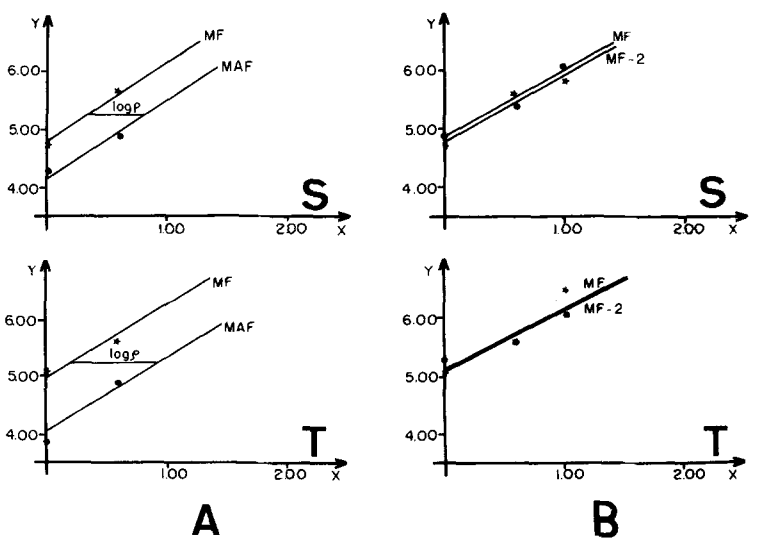

Fig. 3 Dose–responses curves of (A) alkylated (MAF) and non-alkylated (MF) monovalent fragments and (B) two different samples of sera containing non-alkylated monovalent fragments. All samples were injected 24 h after the inoculation of spores (S) or toxin (T). Y axis = probit unit, X axis = log (dose). The distance between the straight lines (log ρ) defines the relative potency of the samples.

Table 3 Percentage survival of mice treated with Fab', $F(ab')_2$ or IgG after the inoculation of tetanus toxin or spores of *Cl. tetani*

	Percentage survival after inocula of:											
	Spores (10 MLD)			Toxin (4 MLD)			Spores (10 MLD)			Toxin (4 MLD)		
Antitoxin (IU)	IgG	$F(ab')_2$	Fab'	IgG	$F(ab')_2$	Fab'	IgG	$F(ab')_2$	Fab'	IgG	$F(ab')_2$	Fab'
1		20	40		0	50			20		0	25
2	0	30	60		20	60		10	50		0	37
4	20	50	70		40	70	6.7	40	60	13.3	0	67
10	46.7	60	80	46.7	70	90	33.3	60	90	33.3	0	90
12	53.3	80	100	53.3	50	90	40			46.7	0	100
14	66.7			60			40			53.3	0	
16				66.7			46.7			60	10	
18										60	15	
20										66.7	25	
22										73.3		
24										73.3		
26										80		

Discussion

Commercial horse antitoxin sera used in tetanus prophylaxis and therapy is usually obtained by simultaneous pepsin digestion and concentration with $(NH_4)_2SO_4$. This process yields a fraction of the serum that migrates from the alpha to the slow gamma region in immunoelectrophoresis (Vaz, 1983). This fraction represents antibodies of several specificities from the common repertoire of the horse that also include the tetanus antibodies. Large amounts of these antibodies are present in horse sera as a consequence of the hyperimmunization against the toxin. Therefore, these sera are not homogeneous. We decided not to isolate the tetanus antitoxin molecules because our interest was to compare *in vivo* the protective activity of sera containing antitetanus monovalent fragments with that of sera usually used in tetanus therapy, that is, sera containing the bivalent fragments.

The process used to obtain monovalent fragments of antibodies from proteolysed hyperimmune sera by treating small volumes with 2-mercaptoethanol and without alkylation has already been developed by Ferri (1967). In our preliminary experiments, the addition of iodoacetamide decreased the pH of the solution which became at first opalescent and then gelatinous. As it is known, alkylation can also be made by dialysis against an iodoacetamide solution when these alterations of the sera are not observed; nevertheless, considering industrial production, where large volumes of sera are used, this step of the process would be very expensive and impractical. Therefore, it was interesting to investigate the protective activity of serum preparations of non-alkylated monovalent fragments, based on previous findings showing that these fragments maintain their antitoxin titre, without any alteration, even after one year. During this period we did not detect any spontaneous recombination by the gel immunodiffusion technique.

Peak II (86 kDa) from gel filtrations of sera, containing non-alkylated or alkylated monovalent fragments, showed antitoxin activity (20 IU/ml); but failed to precipitate against tetanus toxoid. Two hypotheses were considered:

1. This peak contained hybrid bivalent fragments which presented a single combining site derived from tetanus antibodies together with another derived from antibodies with an unknown binding specificity. This could arise as these samples were obtained from horse plasma which contained a pool of antibodies of different specificities and would include tetanus antibodies.
2. Peak II could represent a contamination of Fab′ as the tailing edge of Peak II overlaps the leading edge of peak III.

The comparison between $F(ab')_2$ and Fab′ was made in terms of the amount of antitoxin (expressed in International Units) that produced the same percentage of survival. The results showed that 1 IU of Fab′ was equivalent to 3 IU and to 5 IU of $F(ab')_2$ when mice were inoculated respectively with spores or toxin. When comparing Fab′ with IgG it was observed that 1 IU of Fab′ was equivalent to 6 to 7 IU IgG whatever the inoculum used. These data are in accordance with the results of Kryzhanovsky *et al.* (1972) who were able to neutralize *in vitro* the protagon–toxin complex using the whole molecule of tetanus antibodies and its bivalent and monovalent fragments. These authors observed that the lower the molecular weight of the antitoxins, the smaller the amount needed for a complete neutralization of tetanus toxin already fixed to this unpurified neural receptor (protagon). Their results showed

that 9 IU of Fab' but as much as 24 IU of $F(ab')_2$ and 60 IU of IgG were needed to neutralize 1 MLD of the protagon–toxin complex. Calculating these results in terms of the equivalence of 1 IU of Fab' to the other antitoxins, it can be seen that 1 IU of Fab' is equivalent to 2.7 IU of $F(ab')_2$ and to 6.7 IU of IgG. These values are similar to the ones observed in our *in vivo* experiments.

It was expected that alkylated Fab' would be as efficient as non-alkylated Fab' in protecting mice against tetanus; nevertheless, the percentage of survival of mice treated with the alkylated Fab' fragments was much lower than when non-alkylated fragments were employed. Statistical analysis showed that 1 IU of Fab'–SCH_2CONH_2 is equivalent to 0.2 IU of Fab'–SH. Khavkin *et al.* (1978) studied the important role of the electrochemical properties, resulting from the sum of acidic and basic residues, on the specific function of the whole molecule of immunoglobulins and their bivalent fragments. They showed that amide groups probably make a large contribution to the amidation and deamidation of the immunoglobulins and suggested that they may have effects on both the electrochemical heterogeneity and the affinity of the antibodies. This could explain the lower survival observed when alkylated Fab' fragments were used.

It must be considered that during the production of monovalent fragments there is usually a loss of about 20 to 60% of the antitoxin titre. However, we believe that, in practical terms, the loss of antitoxin titre is overcome by the enhanced protective activity of the monovalent fragments. Also, since the preparation of Fab'–SH includes only one more step in the usual industrial process for production of antitoxic sera, this procedure would be advisable.

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Summary

Horse sera containing anti-tetanus whole IgG molecules, bivalent $F(ab')_2$ fragments and monovalent Fab′ fragments were injected in 24 groups of 10–20 mice to compare their protective activity. When tetanus was induced in the mice, either with toxin or with spore suspension of *Clostridium tetani* 24 or 32 h prior to the injection of the antitoxins, monovalent Fab′ was significantly more efficient in conferring protection against tetanus than $F(ab')_2$ or IgG.

Résumé

Action protectrice accrue du fragment Fab′ par rapport au fragment $F(ab')_2$ et à l'IgG sur le tétanos expérimental.

Des sera de cheval contenant soit des molécules d'IgG entier, des fragments bivalents $F(ab')_2$ ou des fragments monovalents Fab′ ont été injectés dans 24 groupes de 10 à 20 souris afin de comparer leur activité protectrice. Lorsque le tétanos a été induit chez les souris, que ce fût avec la toxine ou avec une suspension de spores de *Clostridium tetani*, 24 ou 32 heures avant l'injection des antitoxines, le fragment monovalent Fab′ s'est révelé plus efficace de manière significative que le fragment $F(ab')_2$ ou l'IgG dans l'octroi de l'activité protectrice contre le tétanos.

Resumen

Mayor actividad protectora frente al tétanos experimental del fragmento Fab′ comparado con $F(ab')_2$ y IgG.

Afín de comparar la actividad protectora frente al tétanos de moléculas de IgG enteras, fragmentos $F(ab')_2$ bivalentes y fragmentos Fab′ monovalentes, se inyectaron, con suero de caballo que contenía las distintas moleculas, 24 grupos de 10 a 20 ratones. Se indujo el tétanos en los ratones 24 o 32 horas antes de la inoculación con antitoxinas, mediante la toxina o bien con una suspensión de esporas de *Clostridium tetani.* El fragmento monovalente Fab′ confirió una protección frente al tétanos significativamente mayor que $F(ab')_2$ y IgG.