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ANTIBODY ACTIVITIES OF 19S AND 7S GLOBULIN FRACTIONS FROM RABBIT ANTISERA TO TETANUS TOXOID

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Summary.—The immune response in rabbits to tetanus toxoid was followed from 10 to 182 days. It was found that 7S became 500 times more efficient in toxin neutralization (TN) and 11 times greater in haemagglutination (HA) than 19S during this time period. No evidence that 19S inhibited 7S and *vice versa* by TN or HA was apparent at the concentrations tested. Precipitation in gel of tetanus toxoid was demonstrated only with 7S.

ANTIBODY activities of the different classes of immunoglobulins indicate that the various antibody classes do not always react equally in the same reaction system. In agglutination, bactericidal reactions, lysis and opsonization, IgM is thought to be more efficient than IgG (Franklin, 1964; Robbins, Kenny and Suter, 1965; Pike, 1967). In complement fixation, neutralization and precipitation, IgG is thought to be more efficient than IgM (Franklin, 1964; Pike, 1967; Dolby and Dolby, 1969).

This study was undertaken to investigate the activities of 19S and 7S rabbit anti-tetanus toxoid globulin fractions by indirect haemagglutination (HA), precipitation in agar gel and toxin neutralization (TN) in mice. This was accomplished by testing at various time intervals rabbit antisera and their globulin fractions following injections of alum precipitated tetanus toxoid. This was to gain insight into the relative activities of the 2 globulin fractions in HA and precipitation of toxoid and protection (neutralization) against tetanus toxin.

The HA and TN tests for antibodies against tetanus toxoid do not always correlate, especially in early sera (Lindqvist, 1968; Edsall and Levine, unpublished data), and probably reflect different activities of antibody classes during the immune response. This may be due to inhibition of, or competition between, antibody classes for combining sites on toxoid-treated red cells and toxin. The HA and TN tests were therefore used to investigate whether 19S inhibits 7S and if 7S inhibits 19S, as indicated with other antigen–antibody systems (Robbins, 1965; Uhr and Finkelstein, 1967; Turner and Krishnapillai, 1968).

IgM is generally considered to be produced early in the immune response, followed by a greater and more persistent IgG response (Bauer and Stavitsky, 1961; Pike, 1967; Uhr and Finkelstein, 1967; Webster, 1968; Gallily and Garvey, 1969). Also, early antibody with agglutinating activity may not have the avidity presumably needed for neutralization of toxin (Taliaferro and Taliaferro, 1961;

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Robbins, 1965; Uhr and Finkelstein, 1967; Siskind and Benacerraf, 1969). In immunization for tetanus, prevention depends on the ability to neutralize bacterial exotoxin with antibody. Hence, this investigation was also directed to determining the major class or classes of antibody capable of protecting against tetanus toxin.

MATERIALS AND METHODS

Tetanus toxoid.—Tetanus toxoid (AL6D), provided by Mr W. C. Latham of the Massachusetts State Laboratory Institute, containing 50 Lf/ml and 0·25 mg protein/ml, was adsorbed to 7·7 mg aluminium phosphate. The purity of the toxoid was 1356 Lf/mg N. This same batch of toxoid (prepared as 25 Lf/ml adsorbed to 3·8 mg aluminium phosphate per dose) has been used in human studies (Edsall *et al.*, 1970).

Immunization of rabbits.—Four rabbits were initially injected subcutaneously (s.c.) at different sites on the back with 2 ml of alum precipitated tetanus toxoid, on Day 42 with 1 ml and on Day 168 with 1 ml of the same preparation. Each rabbit therefore received a total of 200 Lf or 1 mg protein in 3 injections. The 4 rabbits were bled on Days 10, 21, 56, 112 and 182, and their antisera were pooled in equal volumes after each bleeding.

Unrelated control antiserum was obtained by immunizing each of 3 rabbits s.c. with 2 ml of an emulsion containing 1 ml complete Freund's adjuvant (Difco Laboratory, Detroit, Mich.) and 1 ml of 5·0 mg/ml lipopolysaccharide (LPS) *S. typhosa* (Difco Laboratory, Detroit, Mich.). The rabbits were bled after 14 days, and the serum was pooled in equal volumes.

Treatment of antiserum.—Thirty ml of pooled antiserum were precipitated with an equal volume of saturated ammonium sulphate and the precipitate dissolved in distilled water. This was dialysed against several changes of distilled water for 72 hours at 4°. After dialysis and centrifugation, a euglobulin precipitate and pseudoglobulin supernatant were obtained.

Gel filtration.—A Sephadex G-200 column (11·4 × 134·6 cm) was equilibrated with 0·15 mol/l phosphate-buffered saline, pH 7·2 (PBS) at room temperature (RT). The euglobulin precipitate was dissolved in 14 ml of PBS and applied to the column. The void volume was 1200 ml, with an upward flow rate of 60 ml/hour collected in fractions of 14 ml/tube. The protein profile of the eluate was determined at optical density 280 mμ. Only the ascending portion of each 19S fraction was used.

DEAE-cellulose chromatography.—Diethylaminoethyl cellulose (Nutritional Biochemicals Corporation, Cleveland, Ohio) was washed with 1 N NaOH, distilled water, 1 N HCl, distilled water, 0·5 N NaOH, distilled water and equilibrated with 0·0175 mol/l phosphate buffer, pH 7·0. The euglobulin 7S peak from gel filtration on Sephadex G-200 and the pseudoglobulin supernatant were combined, concentrated, dialysed against 0·0175 mol/l phosphate buffer, pH 7·0 and applied to a DEAE column at a ratio of 15 mg protein/ml DEAE. Protein concentrations were estimated by optical density readings at 280 mμ using $E^{1\%}_{280m\mu} = 15$ (Mandy, Rivers and Nisonoff, 1961).

Concentration of globulin fractions.—Eluates from gel filtration and DEAE-cellulose chromatography were concentrated by ultrafiltration using Diaflo PM-10 membranes (Amicon Corporation, Cambridge, Mass.).

Nitrogen determination.—Protein concentrations of the 19S and 7S globulin fractions were determined by the microKjeldahl technique as nitrogen content (Hiller, Plazin and Van Slyke, 1948) multiplied by 6·25.

Preparation of 19S and 7S mixtures for inhibition studies.—To test for inhibition, the 19S and 7S fractions were diluted to predetermined concentrations with PBS for HA and TN testing. Protein composition (mg/ml) of the mixtures is found in Table I.

Immunoelectrophoresis.—Electrophoresis for purity of the 19S and 7S fractions (Table II) and electrophoresis of tetanus toxoid (100 Lf/ml and higher dilutions) was performed for 1½ hours on slides containing 1% purified agar (Difoc Laboratory, Detroit, Mich.) in veronal buffer, pH 8·6. The 19S and 7S fractions (Table II) were precipitated with goat anti-rabbit serum (Hyland Laboratories, Los Angeles, Calif.), goat anti-rabbit IgM and goat anti-rabbit IgG (Cappel Laboratories, Downingtown, Pa.). Toxoid was precipitated with the 19S and 7S fractions (Table II). The gel-coated slides were washed in PBS and then distilled water, stained with 0·1% Ponceau S, destained with 5% acetic acid and dried at RT.

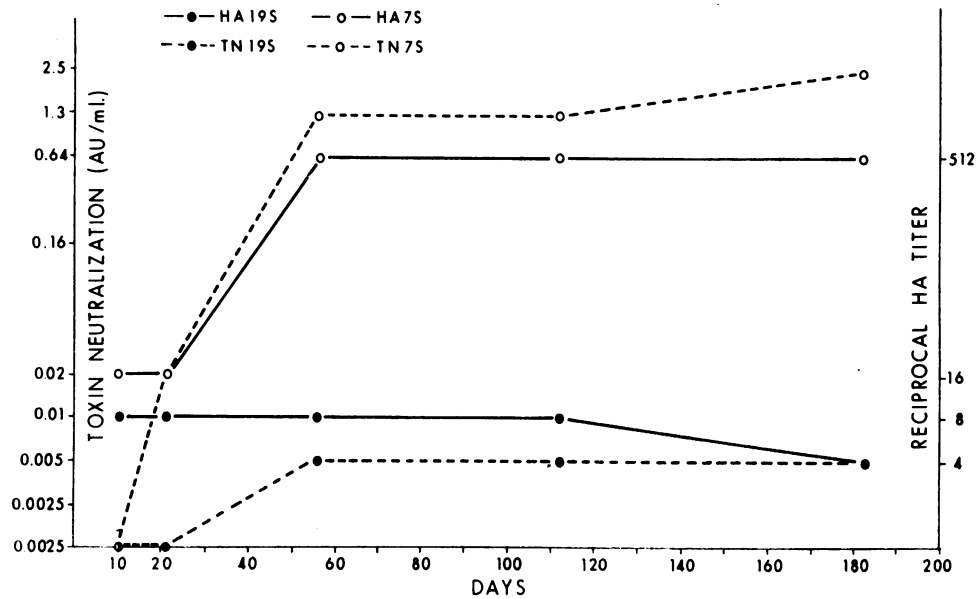

FIG. 1.—19S and 7S (0·5 mg/ml) to tetanus toxoid. HA haemagglutination. TN toxin neutralization.

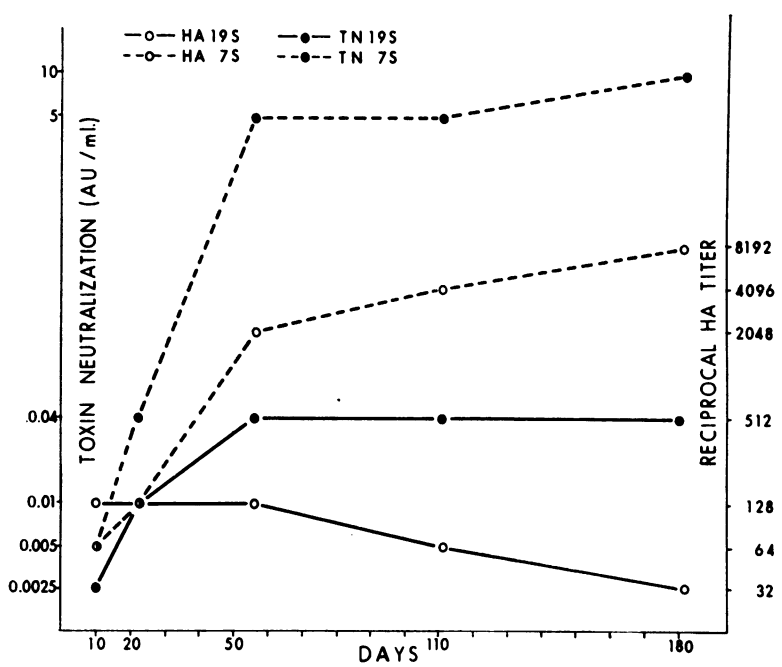

FIG. 2.—19S and 7S (undiluted) to tetanus toxoid. HA haemagglutination. TN toxin neutralization.

Indirect haemagglutination.—The method of Galazka and Abgarowicz (1967) was employed using formalinized and tannin-treated horse red blood cells sensitized with tetanus toxoid (Lot 265). Controls were a standard horse antitoxin from NIH and human tetanus immune globulin.

Agglutination index.—This term refers to the relative agglutinating activity of the 19S and 7S preparations. This was calculated by dividing the reciprocal of the titre by the number of μmol of antibody per ml (Daguillard and Edsall, 1968), assuming a molecular weight of 900,000 for IgM and 155,000 for IgG (Table II).

Tetanus antitoxin determination.—Antitoxin unitage (AU/ml) was determined by TN in mice (Ipsen, 1942) by comparison with standard horse antitoxin from NIH. Two-fold dilutions were titrated beginning at the level of 0·0025 AU/ml, the lowest level readily detectable. The toxin levels used in titration were $L_+/1000$ and $L_+/100$.

Reduction with 2-mercaptoethanol.—Serum samples were reduced with 0·1 mol/l 2-mercaptoethanol (2ME) for 2 hours at RT, then alkylated with 0·1 mol/l iodoacetamide for 2 hours at RT. Control samples contained PBS instead of 2ME. The samples were dialyzed for 3 days against PBS at 4°.

RESULTS

TN titres of unfractionated antisera and the 19S and 7S fractions indicated that 7S was the more important neutralizing fraction (Table II, Fig. 1, 2, 3). TN titres for 7S (0·5 mg/ml) increased from 0·02 AU/ml at 21 days to 2·5 AU/ml at 182 days (Table II, Fig. 1). For Days 10 and 21, 19S (0·5 mg/ml) was agglutinating but not neutralizing (Table II, Fig. 1). TN titres for 19S (0·5 mg/ml) showed a level of 0·005 AU/ml at 56, 112 and 182 days (Table II, Fig. 1). There

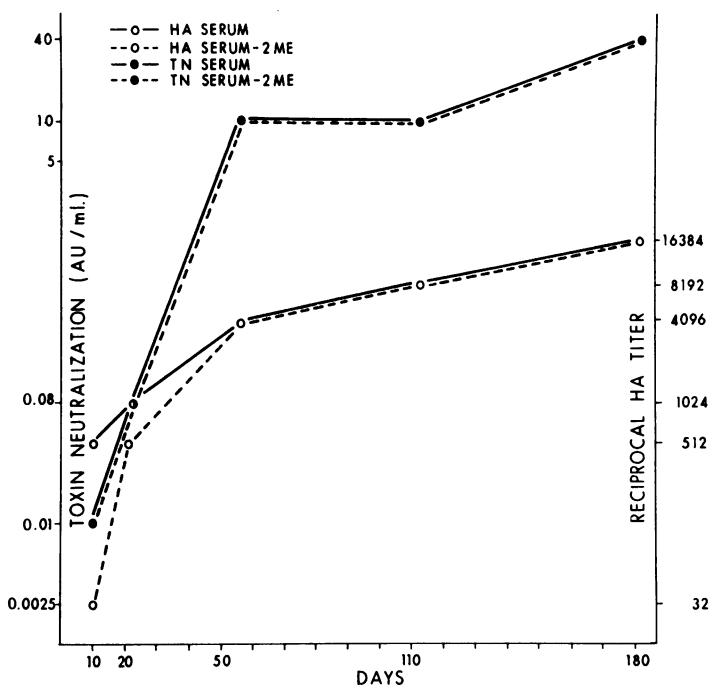

FIG. 3.—Antiserum and 2ME-treated antiserum to tetanus toxoid. HA haemagglutination. TN toxin neutralization.

is a possibility that 19S antitoxin activity could be due to 7S contamination, even though not readily detectable by immunoelectrophoresis (IEP). Less than 1% contamination of the 19S fractions with 7S might account for the 19S activity shown in Tables I and II.

It is generally accepted that IgM is subject to reduction by 2ME whereas IgG is little affected (Deutsch and Morton, 1958). Rabbit antisera from the 5 bleedings were subjected to reduction by 2ME. HA and TN titres, both before and after reduction, are shown in Table II and Fig. 3. These results suggested that by 21 days HA was almost entirely due to 7S. TN titres were the same after reduction as before (Table II, Fig. 3). Apparently 2ME reduction did not affect TN, again indicating that 7S was the important neutralizing fraction.

TABLE I.—*Composition of 19S and 7S Mixtures for Inhibition Studies*

Preparation	Day	Composition of mixtures (mg/ml) 19S	7S	Reciprocal HA titre	Neutralization units (AU/ml)
I	10	0·5	0·05	8	<0·0025
		0·5	0·10	16	<0·0025
		0·5	0·20	16	<0·0025
		0·5	0·40	8	<0·0025
II	10	0·05	0·5	8	<0·0025
		0·10	0·5	8	<0·0025
		0·20	0·5	8	<0·0025
		0·40	0·5	16	<0·0025
III	21	0·5	0·05	8	0·0025
		0·5	0·10	8	0·0025
		0·5	0·20	16	0·01
		0·5	0·40	16	0·02
IV	21	0·05	0·5	8	0·02
		0·10	0·5	16	0·02
		0·20	0·5	16	0·02
		0·40	0·5	16	0·02
V	56	0·5	0·05	64	0·08
		0·5	0·10	64	0·16
		0·5	0·20	128	0·32
		0·5	0·40	256	0·64
VI	56	0·05	0·5	256	1·3
		0·10	0·5	256	1·3
		0·20	0·5	512	1·3
		0·40	0·5	512	1·3
VII	112	0·5	0·05	64	0·16
		0·5	0·10	128	0·16
		0·5	0·20	256	0·32
		0·5	0·40	512	0·64
VIII	112	0·05	0·5	512	1·3
		0·10	0·5	512	1·3
		0·20	0·5	512	1·3
		0·40	0·5	512	1·3
IX	182	0·5	0·05	64	0·32
		0·5	0·10	256	0·64
		0·5	0·20	512	1·3
		0·5	0·40	512	2·5
X	182	0·05	0·5	512	2·5
		0·10	0·5	512	2·5
		0·20	0·5	512	2·5
		0·40	0·5	512	2·5

TABLE II.—*Antitoxin Activity by Day of Response*

Globulin	mg/ml	Day	Reciprocal HA titre	Agglutination index	Neutralization units (AU/ml)
19S	0·5	10	8	14400	<0·0025
	0·5	21	8	14400	<0·0025
	0·5	56	8	14400	0·005
	0·5	112	8	14400	0·005
	0·5	182	4	7200	0·005
7S	0·5	10	16	4960	<0·0025
	0·5	21	16	4960	0·02
	0·5	56	512	158510	1·3
	0·5	112	512	158510	1·3
	0·5	182	512	158510	2·5
19S	2·6	10	128		0·0025
	1·1	21	128		0·01
	1·8	56	128		0·04
	2·8	112	64		0·04
	1·4	182	32		0·04
7S	5·3	10	64		0·005
	1·2	21	128		0·04
	3·5	56	2048		5·0
	2·1	112	4096		5·0
	4·5	182	8192		10·0
Serum		10	512		0·01
		21	1024		0·08
		56	4096		10·0
		112	8192		10·0
		182	16384		40·0
Serum-2ME		10	32		0·01
		21	512		0·08
		56	4096		10·0
		112	8192		10·0
		182	16384		40·0

It was not possible to detect a 19S precipitin arc by IEP. However, a 7S precipitin arc was detected by IEP for Days 56, 112 and 182 (Fig. 4). The 7S precipitin arc was detectable with 100 Lf/ml and 50 Lf/ml toxoid but not with higher dilutions.

Data on preparations I through X for inhibition studies are shown in Table I. In preparation I, two-fold increases of 10-day 7S, in 2 instances, increased by two-fold the HA titres (Table I) when compared with unmixed (0·5 mg/ml) 10-day 19S (Table II). In preparation II, two-fold increases of 10-day 19S, in 3 instances, decreased by two-fold the HA titres when compared with unmixed (0·5 mg/ml) 10-day 7S (Table II). However, two-fold dilution differences may not be significant. In preparations III through X, a pattern seemed to develop in which HA was due to 7S regardless of the presence of 19S (Table I). With preparations III, V, VII and IX, two-fold increases in 7S, in general, gave a two-fold increase in HA titre, suggesting that after 21 days 7S was the important HA fraction.

Control dilutions, for Table I, of unrelated 19S or 7S (LPS) were mixed with the opposite fraction of 19S or 7S to tetanus toxoid. No inhibitory effect of 19S on 7S and *vice versa* was seen by HA or TN. HA and TN remained essentially the same when compared with unmixed 19S and 7S (0·5 mg/ml) to tetanus toxoid (Table II). Unrelated 19S and 7S were negative for tetanus antibodies when tested separately, as expected.

DISCUSSION

High avidity is considered essential in antitoxic and precipitating sera (Taliaferro and Taliaferro, 1961). Robbins *et al.* (1965), working with anti-*Salmonella typhimurium* antibody, consider IgG to be a " more avid " antibody than IgM. Avid antibodies have a low rate of dissociation from protein antigens and are more efficient in neutralization of bacterial toxins than IgM (Robbins *et al.*, 1965).

Bauer and Stavitsky (1961) found by intradermal TN that rabbit 19S diphtheria antitoxin neutralized diphtheria toxin after incubation at 5° but not at 23°. However, Robbins (1965) found that rabbit IgM diphtheria antitoxin did not neutralize diphtheria toxin, although it combined with toxin in the HA test. IgG was capable of both HA and TN of diphtheria toxin. He suggested that when IgM combines with toxin, this combination may prevent further neutralization by IgG. Newcomb and Ishizaka (1967) were unable to demonstrate human IgM diphtheria antitoxin but were able to demonstrate IgG and IgA antitoxin by HA, intradermal TN, and radioactive toxoid-binding methods. Turner and Krishnapillai (1968) found that IgG is more efficient in T4r bacteriophage neutralization than IgM, and that IgM inhibits neutralization of T4r bacteriophage by IgG.

Wolberg, Liu and Adler (1969) found that early IgM and IgG to BSA are less efficient than hyperimmune serum in HA, in that early antibodies require a higher antigen concentration on tannin-treated cells than late antibodies. They speculated that early antibody may be less efficient in binding antigen than late antibody. Grey (1964) measured the binding efficiency of late antibody to BSA and found that there was a maximal binding strength of antibody in relation to time (76–85 days) after primary immunization, regardless of the dosage, route and number of subsequent immunizations.

Heremans, Vaerman and Vaerman (1963) found that human IgG and IgM gave approximately the same agglutinating activity for *Brucella*. They also found that a higher concentration of IgM than IgG was needed in HA for diphtheria antitoxin. Robbins *et al.* (1965) found that the bacterial agglutinating activity of IgM was 22 times that of IgG. Daguillard and Edsall (1968), working with *Salmonella typhi*, found that IgM was more efficient in both agglutination and bactericidal action than IgG. Osler, Mulligan and Rodriguez (1966) found that rabbit anti-HSA IgG obtained from early bleedings possessed little if any HA activity, in contrast to IgG from hyperimmune serum which showed higher agglutinating activity than IgM from the same serum. Lindqvist and Bauer (1966), using primary response antiserum to diazobenzidine-BSA, found that IgM was 10 times more active in HA than IgG. They suggested that the HA efficiency of IgM or IgG may shift throughout the immune response, as the data of Osler *et al.* (1966) implied.

In this investigation, HA titres of 19S (0·5 mg/ml) remained nearly constant throughout the immune response and were never greater than 7S (0·5 mg/ml) even at 10 days (Table II, Fig. 1). However, the agglutination index (Table II) indicated that early 19S HA activity (10-day and 21-day) was 3 times that of early 7S, but late 7S became 11 times more active than 19S in HA during the 10 day–182 day period. 19S HA activity remained virtually constant, but 7S HA activity increased 32 times during the 182-day response (Table II, Fig. 1).

TN by 19S (0·5 mg/ml) remained at 0·005 AU/ml while TN by 7S (0·5 mg/ml) increased from 0·02 AU/ml to 2·5 AU/ml (Table II, Fig. 1). This confirmed the poor neutralizing ability of 19S regardless of the presence or absence of 7S.

Precipitation in gel of 7S and toxoid correlated with the appearance of high neutralizing titres (5 AU/ml) at Day 56 (Table II, Fig. 2, 4). No precipitation in gel was demonstrated with 19S and toxoid. Newcomb and Ishizaka (1967) could not detect IgM diphtheria antitoxin, even by radioimmunodiffusion.

These results indicated a difference in the relationship between early 19S and 7S activity and late 19S and 7S activity. The avidity of late antitoxin was greater

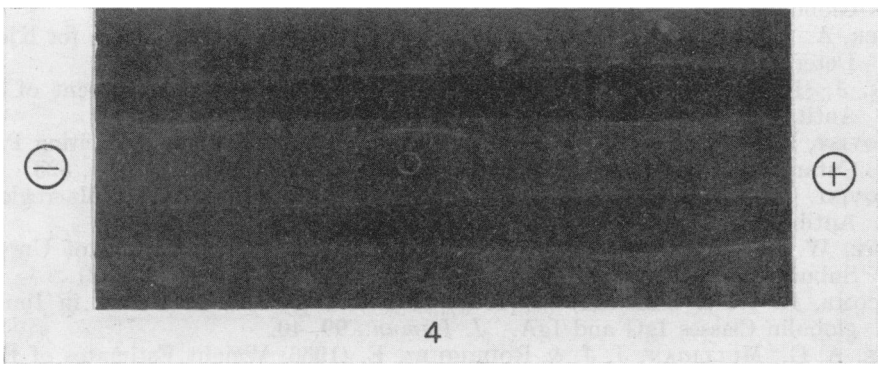

FIG. 4.—Immunoelectrophoresis. 112 day 7S precipitated tetanus toxoid but 112 day 19S did not. The well contained tetanus toxoid (50 Lf/ml); the upper trough contained 7S, and the lower trough contained 19S.

in that late 7S was considerably higher in TN when compared with early antitoxins (Table II, Fig. 1, 2). 7S became 500 times more efficient in TN and 11 times greater in HA than 19S during the 182 day immune response (Table II, Fig. 1). No conclusive evidence that 19S inhibited 7S and *vice versa* by HA or TN was apparent at the concentrations tested (Table I).

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