ANALYSES FOR NEUTRALIZING ACTIVITIES OF IMMUNO- GLOBULIN FRACTIONS IN ANTI-LEPTOSPIRAL HORSE ANTISERUM

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SUMMARY: The 7S and 19S fractions isolated from hyperimmunized antileptospiral horse sera by Sephadex G-200 gel filtration and γ - and T-globulin fractions from the antisera by starch block electrophoresis were studied for neutralizing (leptospiricidal) activities against infection with *Leptospira*. The activity was determined by the intracutaneous method with virulent Shibaura Strain of *Leptospira interrogans* serovar *copenhageni*. No significant difference in regression coefficient was observed between the neutralization line with the 19S globulin fraction and that with the 7S globulin fraction, whereas a significant difference in the regression coefficient was observed when the γ - and T-globulin fractions were compared; the regression coefficient with T-globulin was consistently larger than that with γ -globulin.

INTRODUCTION

Serotherapeutic horse antiserum products have widely been used for treating patients of Weil's disease in Japan. However, until recently no quantitative method for the potency test of the serum product was established. The intracutaneous (ic) method in guinea pigs proposed by Akama et al. (1975) seemed to be useful for quantitative determination of the leptospiricidal activity of the antiserum. The neutralization curves obtained by the ic method were shown to be linear over a wide range of dilution of the leptospiral suspension, but a parallelism of the neutralization lines constructed with various antiserum preparations were not always proved (Akama et al., 1975). This suggests multiple mechanisms involved in the neutralization and the presence of different leptospiricidal antibodies in the antiserum.

This paper reports that regression coefficient of the neutralization lines may be different depending on immunoglobulin classes and suggests that the discrepancy in the regression coefficient among antiserum preparations may result from the different distribution of the immunoglobulins in the preparations.

MATERIALS AND METHODS

Strain: Virulent Shibaura strain belonging to *Leptospira interrogans* serovar *copenhageni* (Arimitsu *et al.*, 1980) was used.

Animals: Healthy female guinea pigs of Hartley/F colony weighing 450–500 g were used for determining the leptospiricidal activity by the ic method, and guinea pigs of about 300 g served for preparing the leptospiral suspension for challenge.

Antiserum preparations: Three antiserum preparations of horse origin, Lots 1223, 31 and 33, were used as starting materials for fractionation (Akama *et al.*, 1975).

Leptospiral suspension: The leptospiral suspension for challenge, liver emulsion of guinea pigs infected with virulent Shibaura strain, was prepared by the procedure described by Mori *et al.* (1974).

Fractionation of antiserum: The antiserum preparations were fractionated by two different procedures, gel filtration on Sephadex G-200 and electrophoresis with starch block as a supporting medium. The fractions obtained from Lots 1223 and 31 were identical with the corresponding ones described in the previous paper (Otani *et al.*, 1980); the others were prepared from Lot 33 by the identical procedures. The two fractions obtained by gel filtration were designated as the 19S globulin and 7S globulin fractions, and the two or three fractions by electrophoresis as the γ - (for Lots 31 and 33) or the γ_2 - and γ_1 - (for Lot 1223) and T-globulin fractions. Each of the fractions obtained by starch block electrophoresis showed a single band when checked by electrophoresis on cellulose acetate membrane.

Determination of the neutralization activity: The antiserum specimen was serially diluted at intervals of 0.125 or 0.25 in logarithm to base 10. Each of the dilutions was mixed well with an equal volume of each of three dilutions of the leptospiral suspension at intervals of logarithmic 0.75. After incubation at 32 C for 60 min, 0.1-ml portions of each mixture were inoculated intracutaneously into the back skin of healthy guinea pigs. Three or more animals were used for each mixture; each animal received 16–20 inoculations. The animals were observed for 7 days for development of hemorrhage at the inoculated sites, and the highest dilution of the serum specimen preventing the development of hemorrhage up to the last day of the observation period was taken as the end point for the leptospiricidal activity. The geometric mean of the end point values was calculated for each of the dilutions of the challenging leptospiral suspension. The results were analyzed statistically.

Cellulose acetate membrane electrophoresis: An amount of 0.01 ml of the serum fraction was placed on cellulose acetate membrane and allowed to migrate for 60 min by a current of 0.6 mA/cm with veronal buffer (pH 8.6, $\mu=0.05$). The membrane was stained with Ponceau 3R, immersed into a Decalin solution, and examined densitometrically.

Determination of protein concentration: Protein concentration of the serum

TABLE I

Neutralization lines with Sephadex G-200 fractions from two antiserum preparations

Specimen	Protein (mg/ml)	Equation ¹⁾
Lot 1223		
Parent preparation	82.86	$y=0.50x-1.51$
19S globulin	2.48	$y=0.44x+0.06$
7S globulin	6.17	$y=0.47x-0.45$
Lot 31		
Parent preparation	94.46	$y=0.55x-1.73$
19S globulin	1.93	$y=0.47x+0.22$
7S globulin	13.09	$y=0.50x-0.99$

1) The equation of the neutralization lines was calculated by the method of least squares.

specimen was estimated by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

RESULTS

Neutralization Lines with Sephadex Fractions

Two parent antiserum preparations, Lots 1223 and 31, and four fractions prepared from them by gel filtration were examined for the neutralization curves. The results are summarized in Table I. Lineality of the lines with all the specimens was not refused; no significant difference was shown among the slopes of the neutralization lines made with the specimens ($p=0.05$) with the common variance (0.146) estimated from the ranges of the values obtained by 47 similar titrations, in which 141 dilutions of the serum specimens were examined.

Neutralization Lines with Electrophoretic Fractions

Three parent antiserum preparations were fractionated by starch block electrophoresis. For Lot 1223, three fractions corresponding to γ_2 , γ_1 and T-globulins were selected and examined for the neutralization lines. Lineality of the lines was confirmed. The results are illustrated in Fig. 1. The slope of the neutralization line constructed with T-globulin differed from those with either parent antiserum or with γ -globulin fractions. Similar results were obtained with Lots 31 and 33 as shown in Table II.

Parallelism of the Neutralization Lines with the Fractions from Three Parent Antiserum Preparations

Differences in regression coefficient of the neutralization lines with the frac-

Fig. 1. Neutralization lines with the fractions from Lot 1223 obtained by electrophoresis.

tions from the three antiserum preparations were statistically analyzed. No significant difference ($p=0.05$) was observed among the coefficients with the parent antisera and their 19S and 7S globulin fractions. As shown in Table III, no significant difference in regression coefficient was demonstrated among the four γ -globulin fractions or among the three T-globulin fractions, whereas a significant difference was observed between regression coefficients with γ -globulin and T-globulin.

Comparison of Neutralizing Activities of Different Immunoglobulins in Parent Antiserum Preparation

The neutralizing activities of the 7S and 19S globulin fractions obtained from the same parent antiserum preparation were calculated with the equations for the neutralization lines (Table I) and common slopes for each parent antiserum series (b for Lot 1223=0.472, b for Lot 31=0.506). The activities of the

TABLE II

Neutralization lines with electrophoretic fractions from three antiserum preparations

Specimen	Protein (mg/ml)	Equation
Lot 1223		
Parent preparation	82.86	$y=0.44x-1.85$
γ_2 -globulin	3.05	$y=0.57x-0.28$
γ_1 -globulin	1.53	$y=0.56x-0.08$
T-globulin	1.45	$y=0.89x+1.25$
Lot 31		
Parent preparation	94.46	$y=0.53x-1.49$
γ -globulin	5.39	$y=0.61x-0.63$
T-globulin	3.87	$y=0.89x+1.81$
Lot 33		
Parent preparation	105.88	$y=0.61x-1.88$
γ -globulin	4.31	$y=0.44x-1.17$
T-globulin	3.15	$y=0.94x+1.06$

TABLE III

Parallelism among the neutralization lines with γ - and T-globulin fractions derived from three antiserum preparations

Parent antiserum	Fraction	Parent antiserum and fraction			
		Lot 31		Lot 33	
		γ	T	γ	T
Lot 1223	γ_1	+	**	+	**
	γ_2	+	**	+	**
	T	*	+	**	+
Lot 31	γ			+	**
	T			**	+

+, No significant deviations from parallelism at $p=0.05$

*, Significant deviations from parallelism at $p=0.05$

**, Significant deviations from parallelism at $p=0.01$

7S and 19S fractions were compared to each other. The leptospiricidal (neutralizing) activity of the 7S fraction was 16.7 times higher than that of the 19S fraction for Lot 1223, and 364 times for Lot 31, on the bases of protein contents of the fractions presented in Table I.

On the other hand, quantitative comparison of the neutralizing activity between T- and γ -globulins is theoretically impossible because of the difference in regression coefficient of the neutralization lines.

DISCUSSION

The antibody activities of immunoglobulins separated from sera of various animals immunized or infected with leptospire were reported by many workers (Hashimoto, 1955; Kikuchi, 1956; Varfolomeyeva et al., 1964; Graves and Faine, 1970; Crawford, 1972; Chiba, 1973; Morris and Hussaini, 1974; Shishkina et al., 1976; Morris et al., 1977; Adler and Faine, 1978; Cinco et al., 1978). However, instructive information is too scanty in the articles to serve for improving the quality of the therapeutic horse antiserum product. Since it was shown that the neutralization lines with various horse antiserum products were not always parallel from one to another (Akama et al., 1975), attempts were made to scrutinize the cause of the disagreement with special reference to immunoglobulin classes.

A significant difference was demonstrated between the slopes of the neutralization lines with the γ - and T-globulin fractions, but not between those with the 19S and 7S globulin fractions; no significant difference was shown among the slopes with the three T-globulin fractions of different origins, showing that the T-globulin fractions have larger regression coefficient of the neutralization lines than the γ -globulin fractions. The difference in regression coefficient of the neutralization lines observed with some antiserum products may be explained by the difference in the active T-globulin contents. Removal of the T-globulin fraction from the therapeutic antiserum product may be helpful in reducing the disagreement in the regression coefficient of the neutralization line. However, since T-globulin might have so-called leptospiral antitoxin, understanding of the roles of the immunoglobulin classes in the therapeutic horse antiserum product should be circumspective.

It remains to be studied whether there is any difference in the mechanisms of inactivating leptospire among the antibodies of different immunoglobulin classes.

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