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Rubella Virus: Neutralizing Antibody in Commercial Gamma Globulin

Abstract. *In 19 lots of standard commercial gamma globulin from humans, the titers of antibody neutralizing rubella virus varied from 256 to 2048. The titers could not be correlated with geographic location of the donors, method of extraction, or tissue source. Samples of gamma globulin from rubella convalescents had titers of 4096. These titers were approximately 20 times higher than those in serum specimens from patients ill with natural or experimental infection. Serum antibody appears to persist for a number of years at nearly the original titers.*

Rubella virus causes a variety of congenital defects that arise during a significant proportion of pregnancies where the infection occurred during the first trimester (*1*). To prevent the development of these defects, a number of investigators have recommended the administration of human γ-globulin to exposed, pregnant women (*2*). However, Korns (*3*) and Grayston and Watten (*4*) have reported variation in the protective value of different lots of γ-globulin for individuals exposed to rubella. We now have information on the amounts of rubella-neutralizing antibody in various lots of commercial γ-globulin and relate this information to serum antibody titers in natural and experimental infection.

In the past, the assay of rubella antibody in γ-globulin and human serum was not possible. Recently, the isolation of the virus and the detection of neutralizing antibody in patients with rubella have been described (*5*). Virus grown in tissue culture has been reported to produce clinical rubella in volunteers who did not have neutralizing antibody (*6*). It was also reported that the virus could be re-isolated from these volunteers; that antibody developed following natural and experimental infection; and that clinical disease did not occur after the administration of the virus to individuals who already had antibody. The neutralization test was over 90 percent reproducible. Virus has been isolated from college students and antibody has been detected in their sera (*7*). The experimental disease is reported to have been produced in African green monkeys (*8*).

Seventeen lots of γ-globulin were obtained from manufacturers in the United States and two lots from a producer in Sweden. Fifteen of these lots were received in the lyophilized state and were reconstituted to a concentration of 16.5 percent with Hanks balanced salt solution and stored at 4°C. The other lots (D and F), containing merthiolate (1:10,000) as a preservative, were prepared for clinical use in the liquid form by the manufacturer. Of these, two were produced in this country and contained 16.5 percent γ-globulin. The remaining two lots were prepared in Sweden at a concentration of 12 percent γ-globulin. One of these latter lots was identified as Swedish convalescent rubella γ-globulin and was derived from plasma collected from military recruits approximately 4 months after clinical illness (*9*). An additional special lot of γ-globulin from rubella convalescents was prepared for reference purposes with plasma obtained from four volunteers 2 months after they developed clinical rubella as a result of the intranasal administration of 100 $TCID_{50}$ (tissue culture infective dose—50 percent effective) of safety-tested RV strain rubella virus (*10*). These volunteers were among those participating in studies similar to those reported previously (*6*). Initially, the toxicity of the merthiolate contained in the lots prepared for clinical use was considered a potential source of difficulty in the tissue-culture systems needed for the titrations. However, in practice, it became apparent that the high titers of antibody encountered in the tests with γ-globulin permitted sufficient dilution of the preservative so that this material did not interfere with the titrations. In addition to the γ-globulin specimens, 30 serums were collected from patients with natural or experimental rubella and stored at −20°C.

The RV strain of rubella after five passages in tissue culture was used for these investigations (*6*). Standard titrations were made with rubella virus diluted 1 to 3.2 (0.5 log). For box titrations, serial twofold dilutions of γ-globulin or serum were tested with 0.5 log dilutions of rubella virus in the range of 0.5 to 3.0 logs.

In performing the neutralization tests, heat inactivated, diluted γ-globulin or serum was combined with an equal volume of the appropriate dilution of rubella virus and incubated at room temperature for 1 hour. Then, 0.2 ml por-

Table 1. Rubella-neutralizing antibody titers of gamma globulin prepared by seven manufacturers (A, B, C, D, E, F, G). The values in parenthesis represent the concentration of the gamma globulin preparation used in the test. P, plasma; Pc, placenta; C, plasma from rubella convalescent patients; CE, experimental rubella convalescent patients' plasma.

Lot	Source	Population		Neutralizing antibody titer*
		Number	Location	
	(A) Ethanol extraction, 16.5 percent			
1	P	8,000	Japan	256
2	P	8,000	Japan	427
3	P	8,000	Japan	512
4	P	8,000	Japan	2048
5	P	8,000	Japan	2048
6	P	8,000	Japan	2048
	(B) Methanol extraction, 16.5 percent			
1	Pc	13,000	Urban east U.S.	256
2	Pc	13,000	Urban east U.S.	256
3	Pc	13,000	Urban east U.S.	341
4	Pc	13,000	Urban east U.S.	2048
	(C) Methanol extraction, 16.5 percent			
1	Pc	15,000	Midwest and west U.S.	256
2	Pc	38,000	Midwest and west U.S.	256
	(D) Salt fractionation, 16.5 percent			
1	P	2,800	East U.S.	512
2	P	2,800	East U.S.	1024
	(E) Ethanol extraction, 16.5 percent			
1	P	60	Los Angeles	256
2	P		Los Angeles, Oklahoma, Chicago	12
	(F) Ethanol extraction, 12 percent			
1	C		Sweden	512
2	C		Sweden	4096
	(G) DEAE Sephadex column			
†	CE	4	East U.S.	4096

*Reciprocal mean of three determinations by serial twofold dilutions of gamma globulin. Titers reproducible within twofold variation.
† Reference gamma globulin.

tions of this mixture were inoculated into tubes containing primary kidney tissue cultures from African green monkeys (*11*). Each tube contained 1.5 ml of maintenance medium, consisting of Eagle's basal medium to which was added per milliliter, penicillin, 100 units; streptomycin, 10 mg; amphotericin B, 0.05 U; polymyxin B, 1 mg; and also glutamine, 0.2 mmoles; and 0.2 percent hyperimmune SV-5 rabbit antiserum (*11*). These cultures were incubated in a stationary position for 8 days at 37°C after which time the maintenance media was changed. No γ-globulin or serum was added at this time for testing. Twenty-four hours later, the cultures were inoculated with 100 $TCID_{50}$ of Coxsackie A-9 virus and the tubes were again incubated at 37°C. After 3 days, the cultures were observed and the presence of cytopathogenic effect due to the Coxsackie A-9 virus was interpreted as indicating neutralization of the interfering effect of the rubella virus.

Several factors were of considerable importance in the performance of the neutralization tests. One of the foremost was the quantity of rubella virus used in the titrations. Box titrations of the γ-globulin and serum demonstrated that a change of dilution of 0.5 log in the amount of rubella virus used in the test resulted in approximately an eightfold change in antibody titer (Fig. 1). The most satisfactory titers were obtained with 0.5 to 1 log of the RV strain of rubella virus. With this amount of virus, γ-globulin and serum could be titrated comparatively. When greater amounts of rubella virus were used, small amounts of antibody could not be detected. When smaller amounts of virus were used, the titers appeared extremely high and variable. The box titrations reported in Fig. 1 were conducted with γ-globulin and serum samples of high titer. Thus antibody could be detected with concentrations of rubella virus in excess of the amounts in 1.0 log dilutions. Samples which contain less antibody, however, required the use of lower concentrations of rubella virus. Neutralization-index titrations did not afford sufficient comparative data since small differences in antibody titer could not be readily detected. Several parallel titrations of each sample of γ-globulin and serum with 0.5 log dilutions of virus were necessary to insure inclusion of titrations utilizing 0.5 to 1 log of rubella virus in the test. This was due to the variability of the

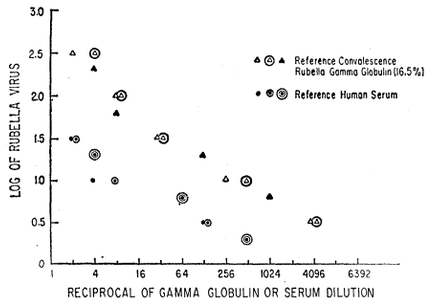

Fig. 1. Titers of a lot of γ-globulin and a sample of human serum with various concentrations of RV strain rubella virus determined in three different tests (Test 1, triangle, dot; test 2, circled triangle and circled dot; test 3, solid triangle and double circle dot).

titer of the virus in successive titrations even when portions of the same virus pool were used. Reference convalescent γ-globulin (titer of 4096 with 0.5 log rubella virus) and reference human serum (titer of 128 with 0.5 log rubella virus) were included as controls. The inclusion of these reference materials permitted the determination of relative titers based on these standards when the quantity of rubella virus in a titration was not exactly 0.5 log but was in the optimal range of 0.5 to 1 log. Another factor of importance was the variation in the number of cells in the tissue cultures. Strict control of cell concentrations in the tissue culture was essential for reliable results. Also, control serum tests were performed in each case to rule out neutralization of the indicator virus due to Coxsackie A-9 antibody in the serum. The omission of serum after the change of maintenance medium, however, resulted in tests in which Coxsackie A-9 antibody rarely interfered with the determination of rubella antibody. With careful attention to the aforementioned factors, titrations of γ-globulin and serum were reproducible within twofold variation (plus or minus one dilution).

Table 2. Rubella neutralizing antibody in young adult males with natural and experimental infection.

No. of cases	Neutralizing antibody titer*
Natural infection†	
3	16
2	32
1	64
4	128
Experimental infection†	
1	16
3	32
1	64
5	128
Natural infection (10 to 15 years earlier‡)	
3	16
4	32
2	64
1	128

* Determined with primary kidney tissue cultures from the African green monkeys, 0.5 to 1.0 log RV strain rubella virus; serial dilutions of serum; and 100 $TCID_{50}$ Coxsackie A-9 virus as indicator system. † Specimens obtained 3 to 4 weeks after onset of rash. ‡ Date of rubella reported in case history.

Antibody content of the standard lots of commercial γ-globulin (Table 1) showed an eightfold range in titer (256 to 2048). There was no evidence that the antibody titers could be correlated with the geographic location of the donors (Japan, regions of the United States, Sweden), with the method of preparation (ethanol and methanol extraction, salt fractionation), with the number of donors (60 to 38,000), or the tissue source of the material (placenta, plasma). Outdated plasma as a source of γ-globulin (Manufacturer D, lots 1 and 2) demonstrated titers in the same general range as those of other lots prepared from comparatively fresh plasma or placental sources. The antibody content of the Swedish preparation of rubella γ-globulin from convalescents was higher (twofold) than that of any other commercial lot tested. Since this sample was prepared to a concentration of only 12 percent γ-globulin, it is quite possible that the titer was significantly higher than any of the other titers reported. The reference convalescent γ-globulin prepared by the DEAE Sephadex Column method also demonstrated a high titer (4096). However, because of the special technique used in the preparation of this material, it is not possible to compare it directly with the commercial lots.

Serum specimens were obtained from ten military recruits with natural rubella disease within 24 hours from the onset of rash and 3 weeks afterwards. Similar paired serums from acute and convalescent phases of the disease were collected from ten young adult male volunteers who participated in studies of experimental rubella similar to those previously reported (*6*). All ten men developed clinical rubella. In addition, serum specimens were obtained from ten individuals who gave a history of rubella 10 to 15 years earlier. On the basis of the results of these studies (Table 2), it would appear that the neutralizing antibody titers 3 weeks after natural or experimental infection are of the same magnitude and are quite similar to those detected in patients

10 to 15 years after recuperation from natural rubella. These findings suggest that neutralizing antibody persists at a relatively unchanged concentration for an extended period of time after natural infection. The amounts of antibody in human serum specimens (16 to 128) and those in standard commercial γ-globulin (256 to 2048) are consistent with the increase in concentration of γ-globulin (20 times) which occurs during the manufacturing process.

The variation in the protective value of the different lots of γ-globulin for rubella previously reported by several investigators (*2, 3, 4*) may be a reflection of the eight-fold difference in neutralizing antibody which was found in the lots of γ-globulin assayed in this investigation. Other factors, however, such as dosage and time of administration of γ-globulin, must be considered in this connection. Future studies of the protective value of lots of γ-globulin are necessary to establish the validity of the selection of specific lots for clinical use on the basis of antibody titrations.

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Molecular Heterogeneity of Enzymes: Malate Dehydrogenase of Euglena

Abstract. *Experiments with analogs of the coenzyme nicotinamide adenine dinucleotide demonstrate that the molecular forms of malate dehydrogenase in* Euglena *vary with the nutritional environment. Electrophoretic separations on starch gel show that* Euglena *grown on autotrophic medium has a malate dehydrogenase which is lacking in* Euglena *grown on heterotrophic medium.*

Analogs of the coenzyme, nicotinamide adenine dinucleotide (NAD), can be used to differentiate the molecular forms of dehydrogenases linked to pyridine nucleotides (*1–3*). The activities of a dehydrogenase are determined in the presence of the coenzyme and in the presence of individual analogs of the coenzyme. Differences in the ratios of activities of NAD and analogs with the same dehydrogenase from different sources indicate a difference in the molecular structure of the enzyme from the different sources. Kaplan and coworkers have used the technique to distinguish molecular forms of malate dehydrogenase (*1, 2*) and lactate dehydrogenase (*2, 3*). They found molecular heterogeneity of the same dehydrogenase from similar tissues of different species and from different tissues of the same species. The former phenomenon indicates that molecular heterogeneity of enzymes may be of value to the study of systematics. We studied malate dehydrogenase in *Euglena* to learn the effect of environment upon the molecular forms of dehydrogenase. This organism was chosen because of its controversial taxonomic position and because its nutritional environment can be varied by growing it on either an autotrophic or a heterotrophic medium.

Euglena gracilis variety *bacillaris* (*4*) was grown without aeration in erlenmeyer flasks at *p*H 3.5 on the heterotrophic medium described by Greenblatt and Schiff (*5*). Malate and glutamate serve as the sources of carbon for this medium. The cultures were exposed to continuous white fluorescent light and were maintained at 21°C to 24°C. The autotrophic cultures were grown in Fernbach flasks on a similar medium except that it lacked malate and glutamate and that 5-percent CO_2 in air was bubbled through it. Heterotrophic cultures were harvested at the end of 5 days and the slower-growing autotrophic cultures at the end of 10 days. After the cells were washed twice with phosphate buffer, *p*H 7.5, they were suspended in 0.2*M* phosphate buffer, *p*H 7.5, containing 0.001*M* disodium ethylenediaminetetraacetate (EDTA); the cells were then disrupted to form an extract, in a French pressure cell at 3 to 4 tons pressure (*6*). The extract was clarified by centrifugation at 35,000*g* for 30 minutes. To remove interfering endogenous activity, the protein was precipitated by addition of ammonium sulfate to 90-percent saturation and this precipitate, suspended in 0.1*M* phosphate buffer, *p*H 7.5, containing 0.001*M* EDTA, was used as the source of malate dehydrogenase.

Kinetic measurements of malate dehydrogenase activity were made in silica cuvettes with a Beckman DB spectrophotometer linked to a recorder. The reaction mixtures consisted of 0.09*M* bicarbonate-carbonate buffer (*p*H 10.2), 0.006*M* or 0.05*M* or 0.10*M* sodium malate, 0.00025*M* NAD or coenzyme analog—3-pyridinealdehyde adenine dinucleotide (PAAD), 3-acetylpyridine adenine dinucleotide (APAD), 3-pyridinealdehyde hypoxanthine dinucleotide (PAHxD), or nicotinamide hypoxanthine dinucleotide (NHxD)—and malate dehydrogenase suspension to make a total volume of 3.0 ml. The enzyme was added at zero time and the activity was related to the initial rate of increase in optical density at the absorption peak for the reduced coenzyme or analog (*1, 7*).

Ratios of reaction rates with NAD and its analogs were determined with a fixed amount of malate dehydrogenase. Data showing large differences in ratios for the enzyme from the two different types of *Euglena* cultures are reproduced in Table 1.

The data in Table 1 indicate that the malate dehydrogenase of *Euglena* grown on autotrophic medium is different from the malate dehydrogenase of *Euglena* grown on heterotrophic medium. For example, with the analog 3-acetylpyridine adenine dinucleotide, substrate inhibition was observed with the heterotrophic *Euglena* enzyme but not with the autotrophic *Euglena* enzyme. At low malate concentration the heterotrophic *Euglena* enzyme reacted only slightly with 3-pyridinealdehyde hypoxanthine dinucleotide in contrast to a low but measurable rate in the case of the autotrophic *Euglena* enzyme. The other data also show large differences