both rabbits (designated as AbH4 and AbH5, respectively). The L-chains were prepared from I^{125}-labeled antibodies from the A3A5 rabbit (designated as $AbL5I^{125}$) and from I^{131}-labeled antibodies from the A3A4 animal (designated as $AbL4I^{131}$). An equimolar mixture was prepared from AbH4, $AbL5I^{125}$, and $AbL4I^{131}$, and a similar mixture from AbH5, $AbL5I^{125}$, and $AbL4I^{131}$. The recombination procedure was carried out on samples of each mixture in the presence and absence of the hapten N-2,4-dinitrophenyl ϵ-aminocaproic acid (DNP-aminocaproate) (*5*). Samples of the recombined material were analyzed on sucrose density gradients, and from the amount of I^{125} and I^{131} in the 7*S* peak and from the specific activities of each of the labeled L-chains the molar ratios, AbL5/AbL4, were calculated. The reformed 7*S* molecules contained nearly the same ratio of AbL5/AbL4 that was present in the original mixture (Table 2). Thus recombination occurred at random and was not influenced by hapten.

Subsequently, the active recombined antibodies were isolated from the mixtures as follows. Precipitates were produced at equivalence between the recombined antibodies and the dinitrophenyl groups conjugated to bovine γ_2-globulin. Unrecombined L-chains do not precipitate or coprecipitate under these conditions (*5*). The precipitates were washed and then redissolved in a sufficient amount of the aforementioned DNP-aminocaproate to provide a 200-fold excess of the dinitrophenyl group. About 40 percent of the calculated amount of 7*S* molecules in the mixture were eluted from the precipitate when recombination was performed in the absence of hapten, and 55 percent when the recombination was performed in the presence of hapten. From the amount of I^{125} and I^{131} in the isolated precipitating antibodies, molar ratios of AbL5 to AbL4 were again calculated. The specifically eluted reconstituted antibodies contained nearly the same ratio of AbL5 to AbL4 that was present in the original mixture (Table 2). Hence the active antibody molecules contain the same molar ratio of L5- to L4-chains as that of the total population of recombined molecules.

Antiserums to the allotypic markers were used to precipitate hybrid molecules exactly as already described for the γ_2-globulin obtained prior to immunization. Antiserum to A5 was added to the active antibodies recovered from a mixture of AbH4, $AbL5I^{125}$, and $AbL4I^{131}$, and similarly antiserum to A4 was added to the active antibodies isolated from a mixture of AbH5, $AbL5I^{125}$, $AbL4I^{131}$. The data (Table 2) indicate that hybrid antibody molecules were produced under the conditions of recombination, and the values observed approximate those calculated for random recombination of the AbL4- and AbL5-chains with the added H-chains. The presence of the hapten had no effect. Thus by this assay at least the antibody molecules with two different L-chains were as active as antibodies with identical L-chains.

In additional experiments the γL4-chains of the γ_2-globulin of unimmunized animals were used in the appropriate mixtures in place of the AbL4-chains. In the presence of the hapten, AbH-chains preferentially recombine with AbL-chains from an equimolar mixture of AbL- and nonspecific γL-chains (*5*). Precipitating antibodies were isolated from a mixture of AbH4, $AbL5I^{125}$, and $\gamma L4I^{131}$ and from a mixture of AbH5, $AbL5I^{125}$, and $\gamma L4I^{131}$. Nonspecific γL-chains were present in the eluted antibody molecules (*5*). Thus the AbL5- and the γL4-chains had formed hybrid molecules with both kinds of AbH chains even though the hapten was present during recombination.

Single cells from germinal centers of lymphoid follicles as well as single mature plasma cells produce γ_2-globulin molecules with both A4 and A5 markers in rabbits heterozygous for these allotypes (*8*). On the other hand, the individual γ_2-globulin molecules from such animals contain only one type of L-chain. Structural restrictions due to allotypic differences do not prevent the formation in vitro of hybrid molecules. Recombination studies with "half-molecules" (one H-chain bound to one L-chain) of rabbit γ_2-globulin results in the formation of hybrid molecules containing different H-chain and L-chain allotypic markers (*9*). Similarly, in our study hybrid-functioning antibody molecules and hybrid γ_2-globulin molecules were produced when separated H- and L-chains were recombined.

Human type I and type II L-chains can form hybrid γ_2-globulin molecules (*10*), although the peptide sequence of type I and type II L-chains are vastly different (*11*). On the other hand the molecules formed in vivo do not have two different L-chains (*12*).

These observations suggest that structural restrictions are not responsible for formation in vivo of functional antibody molecules with two allotypically identical L-chains and imply that the cause of this is related to selective polypeptide synthesis.

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References and Notes

1. J. B. Fleischman, R. H. Pain, R. R. Porter, *Arch. Biochem.* Suppl. 1, 174 (1962).
2. A. Feinstein, P. G. Gell, A. S. Kelus, *Nature* **200**, 653 (1963); G. W. Stemke, *Science* **145**, 403 (1964); R. A. Reisfeld, S. Dray, A. Nisonoff, *Immunochemistry*, in press.
3. S. Dray and A. Nisonoff, *Proc. Soc. Exp. Biol. Med.* **113**, 20 (1963); A. M. Gilman, A. Nisonoff, S. Dray, *Immunochemistry* **1**, 109 (1964).
4. D. E. Olins and G. M. Edelman, *J. Exp. Med.* **119**, 789 (1964); J. A. Gally and G. M. Edelman, *ibid.*, p. 817; O. Roholt, K. Onoue, D. Pressman, *Proc. Nat. Acad. Sci. U.S.* **51**, 173 (1964).
5. H. Metzger and M. Mannik, *J. Exp. Med.* **120**, 765 (1964).
6. We thank Dr. S. Dray for these rabbits and for providing antiserums to the A4 and A5 allotypic markers.
7. F. S. Farah, M. Kern, H. N. Eisen, *J. Exp. Med.* **112**, 1195 (1960).
8. J. E. Colberg and S. Dray, *Immunology* **7**, 273 (1964).
9. S. K. Seth, A. Nisonoff, S. Dray, *Immunochemistry*, in press.
10. M. Mannik, in preparation.
11. N. Hilschmann, personal communication; F. W. Putnam and C. W. Easley, *Federation Proc.* **22**, 657 (1963).
12. M. Mannik and H. G. Kunkel, *J. Exp. Med.* **117**, 213 (1963); J. L. Fahey, *J. Immunology* **91**, 438 (1963).

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Rubella Complement Fixation Test

Abstract. *Complement-fixing antigens specific for rubella were made in tissue cultures of RK-13 rabbit kidney cells and primary cultures of kidney from the African green monkey. The antigens were prepared with the infected cell monolayer diluted to 30 percent with supernatant fluid and frozen and thawed three times. Complement-fixing antibody appeared in patients with rubella soon after the termination of rash and persisted for at least 8 months. In 8 out of 12 individuals who had the disease 10 to 20 years before they were tested, no complement-fixing antibody was found, but neutralizing antibody was present.*

Serologic investigations with rubella have been restricted because of the lack of rapid and inexpensive tests such as complement fixation or hemagglutination. Although several neutralization tests are available, they are technically difficult to perform and require at least 1 week for completion (*1*, *2*). An in-

Table 1. Titers of packed-cell antigens.

Time of harvest (days)	Antigen titers (units)	
	RK-13 tissue cultures	Primary cultures of African green-monkey kidney
1½	1†	No activity
2	8	No activity
3	2	No activity
4	1–2	1
7	Trace–1	4
10	*	4
14		1

* Cytopathogenic effect at this time. † Reciprocal of last dilution giving 3+ reaction.

direct fluorescent-antibody test was reported recently which is quite rapid, but requires considerable proficiency and technical effort for reliable results (*3*). We now describe a complement-fixation test for rubella in which pooled infected cells are used. Such cells were used by Pereira *et al.* (*4*) for preparing potent adenovirus infectious particles and antigens and, more recently, by the Laboratory of Infectious Diseases for demonstrating "cell-associated" complement-fixing antigens in "packed cell" preparations of tissue cultures infected with adenovirus types 12 and 18, SV 40 virus, and with avian sarcoma viruses (*5*).

Rubella antigens were prepared in continuously cultured rabbit kidney cells (strain RK-13) (*6*) and in primary tissue cultures of kidney from the African green monkey (*7*). In each instance, the cultures were grown and maintained in 32-ounce (907 ml) prescription bottles containing 35 to 40 ml of media (*8*). The rubella virus used in these studies was the RV strain (*2, 9–11*). The cultures were harvested at intervals and antigens associated with cellular material and "fluid" antigens were prepared.

To prepare the packed-cell antigen, all the maintenance medium was carefully removed and the cell monolayer (about 0.7 ml per bottle) was scraped from the glass with a rubber spatula. This material was centrifuged at 600*g* for 10 minutes and the sediment (approximately 0.5 ml) was suspended in the supernatant fluid, the final concentration of the cells being 30 percent. The suspension was frozen and thawed 3 times; this was the cell-associated antigen.

The maintenance medium removed during the preparation of the cell-associated antigen was then used for preparing the fluid material for antigen determinations. Some of the medium removed was centrifuged at 105,000*g* for 3 hours; the supernatant was then removed and the sediment resuspended in supernatant fluid so that the volume was one-tenth of the original volume. The medium before centrifugation is referred to as the 1× fluid antigen, and, after centrifugation, as the 10× fluid antigen. Both preparations (1× and 10×) were frozen and thawed 3 times and tested for antigenicity.

As an alternative but satisfactory method for preparing packed-cell antigens, we utilized infected cultures which were frozen and thawed 3 times. The repeated freezing freed the cells from the bottles. The fluid and cell debris were then centrifuged at 600*g* for 10 minutes, and the sediment was resuspended to a 30-percent concentration in the supernatant fluid, this constituting the antigen.

Serum specimens were obtained during studies of rubella in military personnel (*2*); during epidemics in Alaska (*12*); from patients in the Collaborative Perinatal Research Study (*10*); from local patients with rubella; and from children with various other illnesses (*13*).

Complement-fixation tests were performed according to the microtechnique described previously (*14*), spiral loops and disposable plastic plates being used. For antigen titration, 0.025-ml serial dilutions of the antigen were made with the use of the loops. To each antigen dilution, 0.025 ml of serum (appropriately diluted) and 2 exact units of complement (0.025 ml) were added, and the mixtures were incubated overnight at 4°C. The hemolytic system (0.05 ml) was then added to each mixture which was then incubated at 37°C for 1 hour and afterward at 4°C for 4 hours. For serum titrations, serial dilutions of inactivated serums were made with the loops, and these dilutions were titrated against 4 units of antigen.

Neutralization tests were conducted according to the enterovirus interference technique in primary tissue cultures of kidney from the African green monkey (*9*). For these tests, roller-tube cultures were each inoculated with 0.2 ml of a mixture containing 0.5 $\log_{10}$ of the RV strain rubella virus (*9*) and a dilution of inactivated serum. After 8 days the maintenance medium was changed, and on the 9th day the cultures were inoculated with 100 $TCID_{50}$ (tissue culture infectious dose, 50-percent effective) of Coxsackie A-9

Table 2. Titers of complement-fixing (CF) antibody in serums obtained from individuals with natural rubella.

Age of individual(s) (years)	Time serum obtained in relation to onset of rash	CF antibody titer	Neutralizing antibody titer
Individuals from Maryland, Virginia, and Illinois			
6	Day of rash	<4	<4
	1 month later	16*	8
18	Day of rash	<4	<4
	1 month later	32	16
26	Day of rash	<4	<4
	3 weeks later	32	8
	8 months later	32	8
28	Day of rash	<4	<4
	1 month later	64	16
	8 months later	64	16
Individuals from Pribilof Islands, Alaska			
7	10 days before	<4	<4
	Day of rash	<4	<4
	4 months later	32	≥16
12	7 days before	<4	<4
	3rd day of rash	4	<4
	4 months later	32	8
14	10 days before	<4	<4
	2nd day of rash	8	<4
	4 months later	64	16
Pregnant woman with rubella during first trimester			
26	1 month before	<4	<4
	2nd day of rash	4	Trace
	1 month later	16	8
	2 months later	32	16
	4 months later	32	16
	8 months later	32	16
Individuals with rubella 10 to 20 years previously			
23, 24	(Two adult males)	<4	16, 16
21	(One adult male)	4	16
20–36	(Three pregnant females)	4, 4, 4	16, 16, 32
18–32	(Six pregnant females)	<4	16–32
26	(One pregnant female)	<4	<4

* Reciprocal of last dilution giving 3+ reaction.

Table 3. Titers of complement-fixing (CF) antibody in serums from individuals with myxovirus infections other than rubella.

Age of individuals(s) (years)	Infection	CF antibody titer	Neutralizing antibody titer*
22	Influenza A[2]	<4†	16§
		<4‡	16
8	Parainfluenza type 1	4†	32
		4‡	32
18	Parainfluenza type 2	<4†	16
		<4‡	16
19	Parainfluenza type 3	<4†	<4
		<4‡	<4
6	Mumps	8†	16
		8‡	16
5, 6	Measles (rubeola) (two patients)	<4†	<4
		<4‡	<4
2	Respiratory syncytial condition)	<4†	<4
		<4‡	<4

* Confirmed by serological tests. † Serum obtained on day of disease or rash. ‡Serum obtained 3 weeks after disease or day of rash. § Reciprocal of last dilution giving 3+ reaction.

virus. The cultures were examined 3 days later. The appearance of a cytopathogenic effect due to Coxsackie A-9 virus was interpreted as indicating neutralization of the interfering effect of the rubella virus.

Only the packed-cell antigens had demonstrable antigenicity in the complement-fixation tests. The highest titers of antigen were prepared from cells harvested 2 days after inoculation of RK-13 cultures and 7 to 10 days after inoculation of primary cultures of kidney from African green monkeys (Table 1). The serum used for these tests was the first specimen with a high titer obtained from a 28-year-old patient from Maryland 1 month after the rash appeared (Table 2).

When tested against serum specimens from individuals with rubella, the titers of complement-fixing antibody paralleled, but were generally lower than, the neutralizing antibody titers (Table 2). The time of development of complement-fixing antibody was slightly later than that of neutralizing antibody since in none of four cases was there definite evidence of the development of complement-fixing antibody during the time of rash. Complement-fixing antibody persisted for at least 8 months, but was detectable in only four of 12 specimens obtained from normal individuals and pregnant women who had the disease 10 to 20 years previously; however, all 12 specimens contained neutralizing antibdy. There was no evidence of heterologous reaction with the seven other myxoviruses tested (Table 3). Paired serum specimens from monkeys which were experimentally infected with rubella in a previous study showed the development of CF antibody in the serum specimens taken one month after the intranasal inoculation of the virus (*2*).

Several facts concerning the packed-cell complement-fixing antigens emerged from these investigations. First, the titer of the virus inoculated in the cultures for antigen production should be no less than the titers we used; with smaller quantities of virus, only low titers of antigens could be produced. Second, the time of maximum production of complement-fixing antigen differed for the cell lines used. Similar cell-associated antigens were prepared with other tissues which support the growth of the virus, including $LLCMK_2$ cells and the chronically infected RCC-$LLCMK_2$ cells (*3*). In each case, the day on which the maximum amount of complement-fixing antigen was produced differed but was within the range of 2 to 10 days after inoculation. Third, complement-fixing antibody persisted for a least 8 months but diminished after 10 to 20 years. This differs from neutralizing antibody which persisted for many years (*10*). The presence of complement-fixing antibody in 4 of 12 patients who had neutralizing antibody but no known recent rubella is probably due to the continuation of complement-fixing antibody in these individuals for a long period of time; however, it may indicate relatively recent infections which were not recognized by the patients, or infection with an antigenically related virus.

The complement-fixation test has direct clinical application since it can provide rapid serological confirmation of the diagnosis of rubella. The specificity of the test should make it particularly useful for epidemiological investigations including large-scale studies of the efficacy of gamma globulin and new vaccines in the prevention of disease and the prevention of possible damage to the foetus. Antibody tests such as virus neutralization may still be necessary to identify patients who experienced rubella a number of years before being studied.

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References and Notes

1. P. D. Parkman, E. L. Buescher, M. D. Artenstein, *Proc. Soc. Exptl. Biol. Med.* **111**, 225 (1962); T. H. Weller and F. A. Neva, *ibid.*, p. 215; K. McCarthy, C. H. Taylor-Robinson, I. G. Furminger, *Lancet* **1963-II**, 21 (1963).
2. J. L. Sever, G. M. Schiff, R. G. Traub, *J. Am. Med. Assoc.* **182**, 663 (1962).
3. G. C. Brown and H. F. Maassab, *Science* **145**, 943 (1964).
4. H. G. Pereira and R. C. Valentine, *Gen. Microbiol.* **19**, 178 (1958); H. G. Pereira, A. C. Allison, C. P. Farthing, *Nature* **103**, 895 (1959).
5. M. D. Hoggan, W. P. Rowe, P. H. Black, R. J. Huebner, *Proc. Natl. Acad. Sci.*, in press; R. J. Huebner, in *Perspectives in Virology IV* (Wiley, New York, in press); P. S. Sarma, H. C. Turner, R. J. Huebner, *Virology* **23**, 313 (1964).
6. The rabbit kidney cultures (strain RK-13) were provided originally through the courtesy of Dr. J. A. Dudgeon, Hospital for Sick Children, London, England.
7. The monkey kidney was obtained commercially from Microbiological Associates, Inc., Bethesda, Md., and Flow Laboratories, Rockville, Md.
8. For the RK-13 rabbit kidney cells, the growth medium consisted of Medium 199 and calf serum, 95 and 5 percent, respectively. For the maintenance medium, the calf serum was reduced to 2 percent. The cells were incubated at 34°C, and were inoculated with 4 to 6 ml containing 10^4 to 10^6 virus per 0.2 ml. The virus had been grown in RK-13 cells or in kidney tissue from the African green monkey. The growth medium for the kidney tissue from the African green monkey consisted of Eagle's medium, 89.8 percent; calf serum, 10 percent; SV-5 antiserum, 0.2 percent. The maintenance medium consisted of Eagle's basal medium, 99.8 percent; and SV-5 antiserum, 0.2 percent. The cells were incubated at 36°C, and were inoculated with 4 to 8 ml containing 10^3 to $10^{4.5}$ virus per 0.2 ml, the virus having been grown previously in kidney tissue from the same species of monkey.
9. G. M. Schiff, J. L. Sever, R. J. Huebner, *Science* **142**, 58 (1963).
10. J. L. Sever, G. M. Schiff, R. J. Huebner, *Obstet. Gynecol.* **23**, 153 (1964).
11. C. L. Cusumano, G. M. Schiff, J. L. Sever, R. J. Huebner, *J. Pediat.* **65**, 138 (1964).
12. J. A. Brody, J. L. Sever, R. McAlister, G. M. Schiff, R. Cutting, *J. Am. Med. Assoc.*, **191**, 619 (1965); J. L. Sever, J. A. Brody, G. M. Schiff, R. McAlister, R. Cutting, *ibid.*, p. 624; J. A. Brody, J. L. Sever, G. M. Schiff, *New Engl. J. Med.* **272**, 127 (1965).
13. Obtained through the courtesy of Dr. Albert B. Kapikian, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.
14. J. L. Sever, A. C. Ley, F. Wolman, B. M. Caplan, P. W. Crockett, H. C. Turner, *Am. J. Clin. Pathol.* **41**, 167 (1964).

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Density-Gradient Centrifugation: Non-Ideal Sedimentation and the Interaction of Major and Minor Components

Abstract. *A small amount of southern bean mosaic virus was contained in a narrow zone after density-gradient centrifugation, but in a much wider zone after centrifugation with a large amount of a second virus. Zone-spreading of a major component by non-ideal sedimentation in density-gradient centrifugation can cause zone spreading of a minor component that the major component overlaps.*

The width of a zone of virus or protein after sucrose density-gradient centrifugation increases as the amount in the zone increases (*1*). The amount of virus or protein in a zone of a given width is only a few percent of that which would be present if the negative density-gradient which is due to the virus or protein concentration gradient were equal to the positive density gradient due to the sucrose concentration gradient. Brakke (*1*) postulated that the increase in zone width as the amount of virus or protein in-