occur *in vivo;* further, it suggests that in studies of viremia leucocytes should not be excluded from specimens to be tested.

The observations with ECHO 9 and measles viruses do not justify the assumption that leucocytes are universally susceptible to viruses which are known to produce viremia. In the case of infection with type 1 poliovirus, viremia has been well documented(8). However, experiments similar to those now reported and conducted recently in this laboratory have not provided evidence that type 1 poliovirus multiplies in leucocytes.

Whereas leucopenia is associated with measles, in human infection due to ECHO 9 virus the peripheral white count is usually within normal limits or slightly diminished and may rarely be elevated early in the illness (6,7); consequently, although it is suggested that both viruses may infect leucocytes *in vivo*, it is not clear that such infection influences leucocyte survival.

Summary. Propagation of ECHO 9 virus in suspensions of human blood leucocytes has been described.

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Demonstration of Serological Specificity in the Reaction of Individual Gamma Globulins with Rheumatoid Factors by Hemagglutination.* (27054)

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Two general serological methods utilizing human gamma globulin for detection of rheumatoid factors in human serum are now being used. Human gamma globulin isolated by the Cohn alcohol fractionation method (Fraction II) from pooled plasma may be coated on latex particles(1) or may be attached to red blood cells by means of a tannic acid bond(2). Although the sensitivity of the several techniques utilizing pooled gamma globulin vary, they are all characterized by an absence of specificity for the several types of rheumatoid factor as defined by the second method in general use. The Gm system defines the rheumatoid factors by means of human Rh_0 positive red cells coated with selected incomplete anti-D sera(3). The rheumatoid factors are defined as anti-Gm factors of differing types depending on the Gm characteristics of the anti-D coat. Rheumatoid sera of the Gm (a+) type may be negative by this test or may show a marked prozone reaction. Harboe (4) has reported that such sera become positive and lose their prozone reaction when the euglobulin fraction is studied free of the coexisting specific inhibitor contained in the serum pseudoglobulin fraction.

Past efforts to utilize 7S gamma globulin isolated by physical and chemical means from individuals as a cell coating material to detect the several types of rheumatoid factors as defined by the Gm system have been unsuccessful (5,6,7). Isolated gamma globulin fractions from individuals have been either nonreactive or when partially denatured by heating or

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other means have shown a reactivity similar to that of pooled commercial gamma globulin in its non-specificity.

The present study was undertaken in an effort to utilize tannic acid-treated sheep erythrocytes coated with 7S gamma globulin obtained from normal and rheumatoid individuals to detect serologically distinguishable rheumatoid factors.

Material and methods. Rheumatoid sera: Its euglobulin and pseudoglobulin fractions. Sera were obtained from patients with classical peripheral rheumatoid arthritis (R-sera). Rheumatoid factor activity was measured by the tanned cell procedure† of Heller *et al.*(2) after heat inactivation of serum complement and absorption with washed sheep erythocytes. The euglobulin and pseudoglobulin‡ fractions of serum were obtained by the technique described by Ziff *et al.*(8) using dialysis against a M/150 phosphate citrate buffer pH 6.3. The precipitated euglobulin fraction after being washed in the same buffer was dissolved in a volume of pH 8.0 phosphate saline buffer equal to that of the original amount of serum used. Denatured material which would not dissolve was discarded.

Titers are expressed as the $\log_2$ of the highest dilution causing agglutination using a 1:28 initial dilution in a pH 8.0 phosphate saline buffer containing 1:2,000 bovine serum albumin.

Isolation of gamma globulin. One serum from a patient with peripheral rheumatoid arthritis (R4360) and 2 normal sera were used as the sources of gamma globulin. The 2 normal sera, kindly provided by Dr. Hugh Fudenberg, were N4226, 7S Gm type Gm 1(a+b+x−) Gm 2(a−b+) and N4227, 7S Gm type Gm 1(a−b+x−) Gm 2(a−b+). The 7S gamma globulin from individual sera was obtained in 2 chromatographic steps.

a) Gel filtration utilizing Sephadex G-25 (9,10) was used to separate proteins soluble in 0.004 M pH 8.0 phosphate saline (approximately 95% of all serum protein—designated peak I) from those gamma globulins not soluble at this low buffer electrolyte concentration.

b) The 7S gamma globulin was isolated from peak I by means of anionic exchange chromatography on diethylaminoethylecellulose (DEAE)§ as described previously(9).

Isolated protein was preserved by quick freezing in a dry ice-acetone bath. The 7S gamma globulin character of the isolated protein was established by analytic ultracentrifugation and immunoelectrophoresis against a horse antiserum to normal human serum(10).

Coating of cells. Washed sheep red cells were coated with the individual gamma globulins using the method described by Heller(2). A rabbit antiserum to pooled human 7S gamma globulin was used to establish that the cells were coated. The tannic acid-treated cells were exposed to a concentration of 1.0 mg of protein/ml.

Inhibition procedure. A 0.5 ml volume of dilutions of whole serum or its pseudoglobulin fraction was used to inhibit agglutination by 0.5 ml of a stated concentration of rheumatoid serum. The dilution of the agglutinating serum was added to the serial dilutions of the inhibiting material. The tubes were incubated at 37°C in a water bath for 1 hour and 0.5 ml of a 0.25% concentration of the coated cells then added. The rack was then maintained at 4°C overnight. Complete agglutination is designated as 2, indicating a flat blanket of cells, while total inhibition refers to a tight button of cells and is designated as zero.

Results. Cells coated with the 7S gamma globulin from R4360 as obtained from the chromatographic procedures and after different degrees of heating of the isolated globulin were tested against 3 R sera which showed strongly positive tests using cells coated with commercial pooled gamma globulin. The results shown in Table I indicate that all cells were about equally coated as indicated by the rabbit antiserum but that the strength of agglutination by R sera was much weaker with the unheated gamma globulin than with that heated to 48°C for 10 minutes. Heating gamma globulin to 63°C for 5 minutes causes loss of the specificity of the reactions as indicated by serum R4499 giving a pattern and end point identical with cells coated with pooled commercial gamma globulin. For these reasons all

† Commercial pooled human globulin obtained through the courtesy of American National Red Cross, Washington, D. C.

‡ This fraction also contains the serum albumin.

§ Coarse mesh, 0.9 mEq/g, obtained from Brown & Co., New Hampshire.

TABLE I. Agglutination Pattern of R Sera Tested with Cells Coated with Unheated and Heated Gamma Globulin from R4360.

Serum R	γ-R4360* cell coating	Log_2 dilution of serum (Initial dilution—1:28)							
		1	2	3	4	5	6	7	8
4501	Unheated	0	0	1	1	1	1	0	0
	48°C-10 min	2	2	2	2	2	2	1	0
	63°C- 5 "	2	2	2	2	2	2	2	0
2285	Unheated	0	0	0	1	1	1	0	0
	48°C-10 min	1	1	2	2	2	2	0	0
	63°C- 5 "	2	2	2	2	2	2	2	2
4499	Unheated	0	0	0	0	0	0	0	0
	48°C-10 min	0	0	0	0	0	0	0	0
	63°C- 5 "	2	2	2	2	2	2	1	0

* Log_2 titer using rabbit antiserum to human gamma globulin.
Cells coated with γ unheated - 5.
" " " γ heated to 48°C-10 min—6.
" " " γ heated to 63°C- 5 min—6.

cells used in the following studies are coated with protein solutions after heating the protein in a concentration of 1 mg of protein/ml to 48°C for 10 minutes. No grossly detectable clouding of the solution is produced by this degree of heating.

TABLE II. Agglutination of Cells Coated with Individual Gamma Globulins Tested Against 5 R Sera and their Euglobulin Fractions.

Serum R		Log_2 titer of cells coated with:			
		Pooled γ	γ-R4360	γ-N4226	γ-N4227
4501	Whole serum	7	7	6	0
	Euglobulin	7	9	8	0
4405	Whole serum	8	0	0	0
	Euglobulin	12	0	0	0
2285	Whole serum	8	6	7	0
	Euglobulin	8	7	8	9
4373	Whole serum	9	0	0	0
	Euglobulin	12	6	12	0
4647	Whole serum	8	6	0	0
	Euglobulin	10	8	7	0
Rabbit antiserum		5	6	8	6

Table II lists the titer of 5 whole R sera and their euglobulin fractions expressed as the log_2 of the dilution at the end point. All positive tubes showed a maximum agglutination pattern. The 5 sera used all showed about the same end point to cells coated with pooled gamma globulin and showed the same or higher titers when their euglobulin fraction was tested with these cells. The results using the cells coated with individual gamma globulins show that no individual coat duplicates the coating of pooled gamma globulin. Serum R2285 is the only one reacting as the euglobulin to all coats while the other 4 each have one or more instances of a totally negative test when using euglobin. Serum R4405 lacks rheumatoid factor for all of the individual gamma globulins. The presence of a specificity of the 3 individual coats is shown both in the results using whole serum as well as the euglobulin fractions. Serum R2285 is the only one in this panel having rheumatoid factor reactive with the determinants of γ-N4227 and this does not appear related to the euglobulin of highest reactivity with pooled gamma globulin coated cells.

Three R sera having strongly positive tests to pooled gamma coated cells but no titer to cells coated with γ-N4226 were selected to test the inhibiting capacity of R sera and its pseudoglobulin fraction against serum R4501 which agglutinates γ-N4226 coated cells. Table IIIa shows that 2 of these sera contain rheumatoid factor elements in their euglobulin capable of reacting with γ-N4226 coated cells. The pseudoglobulin fractions show no reactivity.

TABLE III. Inhibition of Rheumatoid Serum.

a) Characteristics of inhibiting sera

Serum R		Log_2 titer cells coated with γ-N4226
4647	Whole serum	0
	Euglobulin	7
	Pseudoglobulin	0
4405	Whole serum	0
	Euglobulin	0
	Pseudoglobulin	0
4373	Whole serum	0
	Euglobulin	12
	Pseudoglobulin	0

b)

Inhibiting material	Log_2 dilution of inhibiting material*†‡ (Initial dilution 1:100)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
R4647 Whole serum	1	2	2	2	2	2	2	2	2	2	2	2	2	2
Pseudoglobulin	1	2	2	2	2	2	2	2	2	2	2	2	2	2
R4405 Whole serum	0	0	0	0	0	0	0	0	0	0	1	1	2	2
Pseudoglobulin	0	0	0	0	0	0	0	0	0	0	1	2	2	2
R4373 Whole serum	0	0	0	0	0	0	0	0	0	1	1	1	2	2
Pseudoglobulin	0	0	0	0	0	0	0	0	0	1	2	2	2	2

* Cells coated with γ-N4226.
† Agglutinating serum R4501—0.5 ml of 1:56 dilution in each tube.
‡ Control: .5 ml of 1:56 dilution of R4501;
.5 ml of diluent;
.5 ml of γ-N4226 coated cells.
Result: 2+ agglutination.

The dilution of R4501 to be inhibited (1:56) provides a control tube showing maximum agglutination using 0.5 ml of buffer in place of the dilution of inhibiting serum. Serial dilution of the inhibiting materials were all started at a 1:100 dilution relative to the whole serum. Table IIIb shows that a striking difference in inhibitory capacity exists for serum R4647 which shows little or no inhibition ability compared to sera R4405 and R4373. The inhibitory capacity of the pseudoglobulin fractions parallels those of the whole sera. All 3 of the sera examined for inhibitory capacity showed moderately elevated serum gamma globulin on paper electrophoresis.

Discussion. Although the 7S gamma globulin isolated chromatographically from individual normal and rheumatoid sera shows the ability to coat tannic sheep erythrocytes as indicated by a rabbit antiserum, the pattern of agglutination of such coated cells tested against rheumatoid serum was extremely difficult to read. Prior studies employing chemical and heating methods for the aggregation of gamma globulin have shown increased reactivity after such treatment as judged by the magnitude of the precipitation reaction between gamma globulin and rheumatoid sera(5,11,12). No direct investigation of the aggregate content of the chromatographically isolated gamma globulin was made in the present study. Our objective was to obtain preparations of gamma globulin from individual sera which would exhibit a spectrum of reactivity to different rheumatoid sera similar to that observed in the Gm system utilizing select anti-D sera to coat human Rh(+) cells. Serum R4499 proved a valuable reagent since it agglutinated cells coated with pooled gamma globulin yet failed to agglutinate cells coated with γ-R4360. Heating γ-R4360 to 48°C for 10 minutes provided a coating material which gave a maximum agglutination pattern with R sera No. 4501 and R2285 yet showed no reaction with R4499. This differential reactivity was lost by heating γ-R4360 to 63°C for 5 minutes prior to coating cells (Table I). If pooled gamma globulin contains all possible determinant groupings and this accounts for its reactivity with all R sera, it is difficult to conceive how heat can impart such heterogeneity to the gamma globulin of a single individual which demonstrates a high order of selectivity after lesser degrees of heating. A possible explanation may lie in the exposure of a portion of the gamma globulin molecule which reacts with different rheumatoid factors and itself lacks determinant specificity. Aliquots of these same samples of individual gamma globulins which were frozen and thawed several times or permitted to remain at room temperature or at 37°C for several hours showed the same augmentation of reactivity as produced by heating to 48°C for 10 minutes. This latter procedure would appear to provide a more reproducible reagent and is used throughout the present investigation.

The results shown in Table II indicate that the totality of reactivity found in the pooled gamma globulin coated cells is not found in any of the 3 individual gamma globulins. Three types of reaction patterns are noted in this study. In those sera which show strong reactivity to an individual gamma globulin coat in both the whole serum and its euglobulin fraction, the pseudoglobulin of that serum is presumed to be deficient in the inhibitor specific for the rheumatoid factor of the serum which is reacting with an individual gamma globulin coated cell. The frequent observation of higher titers for the euglobulin fraction compared to the whole serum would suggest this is a quantitative rather than qualitative lack. This does not imply that the serum is deficient in inhibitors complementary to other rheumatoid factor specificities. This is demonstrated in the reaction patterns of sera R2285 and 4647. The presence of strong agglutination ability by the euglobulin with none demonstrable by the whole serum is taken to indicate an amount of inhibitor in the pseudoglobulin at least sufficient to react with all of that rheumatoid factor specificity present. Finally, the absence of agglutinating ability in both the whole serum and its euglobulin fraction for a cell coated with an individual gamma globulin indicates the absence of rheumatoid factor capable of reacting with that particular cell coating. From the results shown in Table II, γ-R4360 would appear more like γ-N4226 than γ-N4227. Rheumatoid sera having rheumatoid factor specificity complementary to the determinant groups of γ-N4227 would appear to be uncommon compared to those reactive with γ-R4360 and γ-N4226.

Based on the reasoning presented for the direct agglutination results, 2 of the 3 sera selected to study inhibitory capacity against R4501 (Table IIIa,b) show inhibitory capacity. The 3rd serum R4405 lacking reactive rheumatoid factor for γ-N4226 used as a coating material cannot be shown to have inhibitory activity for the specificity of this test system by direct agglutination.

The results shown in Table IIIb indicate that serum R4405, although lacking rheumatoid factor activity for cells coated for γ-N4226 does contain significant amounts of an inhibitor for the γ-N4226 versus serum R4501 test system. Although sera R4647 and R4373 show a qualitatively similar reaction pattern in Table IIIa they show a completely opposite inhibitory capacity in Table IIIb. The inhibitory capacity of the pseudoglobulin fractions of these sera were examined, to rule out the possibility that the lack of inhibitory capacity in serum R4647 is due to an exact balance of inhibitory material and rheumatoid factor in the whole serum. A possible explanation for the behavior of these 3 sera studied for inhibitory capacity based on multiple specificities for both inhibitory activity and rheumatoid factor activity is shown in Table IV.

TABLE IV. Possible Mechanism of Inhibition and Non-Inhibition.

	System to be inhibited	
	Cell coat γ-N4226	Agglutinating serum R4501
	1, 2	Anti 1
	Specificity of inhibiting sera	
	Inhibition specificity	Rheumatoid factor specificity for cell coating γ-N4226
Inhibiting serum R4405	1	Lacks anti 1 & anti 2
" " R4373	1	Anti 1
Non-inhibiting serum R4647	2	" 2

The ability of serum R4647 to show slight inhibitory ability is reminiscent of the findings of Harboe(13) that anti-Gm(a) and anti-Gm(x) inhibiting substances can be found in small amounts in Gm(a − x −) sera.

Whether or not the specificity of gamma globulin obtained from individuals when coated on tannic acid-treated red cells parallels that reported utilizing the sensitized human Rh(+) cell system cannot be stated. Some type of specificity is apparent both by direct agglutination and by inhibition. This technique should permit classification of 7S gamma globulin types in both normal and rheumatoid individuals as well as classification of different types of rheumatoid factors without recourse to rare anti-D cell typing sera. The presence of several types of rheumatoid factor in the same serum has been noted previously by chemical fractionation(14,15) as well as by Gm typing (16,17,18,19). The phenomenon of coexisting inhibitory material with rheumatoid factor in the same serum as noted in the Gm system especially in Gm(a +) individuals(4) seems also to apply to the present system.

Summary. Gamma globulin (7S) isolated by chromatographic techniques from 2 normal individuals and from one patient with rheumatoid arthritis coats tannic acid-treated sheep erythrocytes and detects rheumatoid factors with a specificity not found using pooled human gamma globulin. The serological specificity of individual gamma globulin preparations is destroyed by heating the protein to 63°C for 5 minutes. The specificity is also observed in an inhibition system using whole rheumatoid serum or its pseudoglobulin fraction. The results may be explainable by the presence of multiple determinants in both the 7S gamma globulin isolated from individuals as well as in the rheumatoid factor complex found in individual rheumatoid sera.

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Interactions of the Oral Microbiota
I. A System for the Defined Study of Mixed Cultures.* (27055)

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The net effect of a mixed microbial population is often greater or smaller than the simple sum of activities of the individual species growing in pure culture. It is therefore not possible to predict with certainty the influence of a single species, whose biochemical and growth characteristics have been determined solely in pure culture, on one or more different species. To the best of our knowledge there are no satisfactory techniques which allow measurement of interactions between 2 or more species of microorganisms growing in a dynamic state similar to that of nature and which at the same time permit control over basic factors of growth and population.

Until recently, attempts to assess bacterial interactions *in vitro* have fallen into 2 groups: a) growth in closed systems with selective isolation of components at varying intervals as reported by Annear(1) among others, and b) effect of metabolites produced by one species on another, the organisms being separated by a permeable membrane or similar barrier as described by Wilson and Goaz(8). A quantitative aspect was approached by Rosebury(7) with his relatively simple "drop pair" technique in which each of 2 different species diluted over a one-thousand-fold gradient are cultured as overlapping pairs on agar plates. While this technique can be evaluated mathematically, it is restricted to 2 species at a time and yields little information on products formed.

A limiting aspect of these methods (closed systems) is the lack of control over growth dynamics. Continuous culture permits controlling this factor and was suggested for early mixed populations by Rogers and Whittier(6). More recently, Zybrycki and Spaulding(9) constructed a simple continuous system for mixed cultures but cited no data on the interactions observed. A more sophisticated concept of continuous culture stems from the contribution of the chemostat by Novick and Szilard (5). The basic premise of this development assumes that multiplication of organisms in the growth cell is directly proportional to the rate at which some critical limiting nutrient is admitted to this cell. Novick(4) has also discussed the growth of pure cultures in a 2-stage arrangement in which the output of a chemostat is fed to a second growth cell. If nutrients in the second system are not limited, it becomes possible to define mathematically the steady state growth which results so that:†

$$X_2^\circ k^\circ m - F_1 + F_2/V_2 + \frac{F_1}{V_2} X_1^\circ = 0$$

In which: X_1° and X_2° respectively are the bacterial populations entering and leaving the

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† Mathematical symbols employed are those of the American Standards Assn. as cited by Gerhardt and Bartlett(3).