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IMMUNOCHEMICAL STUDIES OF HUMAN SERUM GAMMA GLOBULINS *

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The establishment of the quantitative precipitin reaction as an analytical-chemical procedure by Heidelberger and Kendall (1) was followed in 1937 by Kendall's original use of the method in the estimation of human serum proteins in health and disease (2, 3). A major drawback to the use of this immunochemical method for obtaining meaningful human serum protein values has concerned the purity or homogeneity of the specific protein used as the antigen (4). In particular, the heterogeneity of the human gamma globulin fraction, as defined electrophoretically, has been pointed out (5). Cohn, Deutsch and Wetter have demonstrated distinctive immunochemical behavior in various subfractions of the human serum gamma globulins (6). In these studies, however, the principal differences in immunochemical behavior of the subfractions were noted in zones of antigen excess; this zone is not employed in the studies reported herein.

Although numerous physical, chemical and immunological criteria have been set forth for the establishment of protein purity, the lack of information regarding protein structure does not permit a clear definition of a single human protein species. In general, the proteins comprising the human serum gamma globulins separated by ethanol fractionation have an isoelectric distribution of pH 5.7 to 7.5; their electrophoretic mobilities range from 0.97 to 2.64. The marked antigenic similarity, indeed almost antigenic identity, of the members of this family of proteins in

the zone of antibody excess, has been well documented (7, 8). For the purposes of this study it was felt that a physically or chemically homogeneous gamma globulin preparation, possessing immunologic homogeneity in the zone of antibody excess, might be employed for preparation of antibody to be used in a quantitative precipitin test for estimation of gamma-reactive globulin in human fluids. Because of the inadequacy of other methods, it was considered likely that this technique might be especially useful when the concentration of the gamma globulin to be measured is very low.

The physical, chemical and immunological similarities of the human gamma globulins and most specific antibodies are well documented (9, 10). On the biological level, the clinical entity agammaglobulinemia provides strong evidence in support of the concept that gamma globulin and most antibodies arise from a common source (11, 12). Accordingly, immunochemical estimations, patterned directly after the methods of Heidelberger and Kendall (1), have been applied to the study of agammaglobulinemic and normal human sera, and reference comparisons to electrophoretic observations have been accomplished. The purposes of the present study were several-fold and included the following: 1. To establish the efficacy of the quantitative precipitin reaction for the accurate estimation of human serum gamma globulins. 2. To measure the serum concentrations of gamma-reactive globulin in normal humans. 3. To describe the quantitative gamma globulin deficit in patients with both congenital and acquired agammaglobulinemia. 4. To establish the biological survival time of parenterally administered gamma globulin in humans by immunochemical means. 5. To study the appearance of gamma-reactive globulins in the newborn baby of a mother with acquired agammaglobulinemia, and to relate the appearance of gamma globulin

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to the time of onset of specific immune globulin synthesis and release; to study the disposition of maternally transmitted gamma globulin by the agammaglobulinemic offspring of an immunologically normal female who had previously borne a son afflicted with the congenital form of the disease. 6. To compare maternal and cord serum concentrations of gamma-reactive globulin.

MATERIALS AND METHODS

Sixty immunologically normal children ranging in age from one day to 15 years: These children were patients at the University of Minnesota Hospitals, Minneapolis General Hospital and the Ancker Hospital of St. Paul, who had been admitted for study or treatment of congenital anomalies or of trauma. None had been troubled with recurrent infections, and in no case was there reason to suspect an abnormality of protein synthesis or an immunological deficit. Twenty-four immunologically normal, healthy adults: These subjects were laboratory and hospital personnel, ranging in age from 19 to 34 years. Thirty-one maternal cord serum pairs: In all cases, the maternal blood was drawn within two hours of parturition. Sera from 16 patients with agammaglobulinemia: Nine of these were patients with the congenital form of the disease, and seven with the acquired form. The subjects chosen for study of gamma globulin half-life determinations included four congenitally agammaglobulinemic patients and a normal newborn who was bled frequently during the neonatal period. All subjects were free of clinical infections at the time these studies were performed.

After preinjection bleedings, each subject in the half-life study was given gamma globulin in various dosages intramuscularly. Venous blood was drawn thereafter at varying intervals. The bloods were allowed to clot at room temperatures for one hour, placed at 2° C. overnight, the sera separated by centrifugation in the cold, quick-frozen at -70° C., and stored at -26° C. until ready for use.

The decline of maternally transmitted gamma globulin was assessed by studying the male offspring of an apparently normal mother who had previously borne a congenitally agammaglobulinemic male. In this study, blood samples were drawn from the jugular vein of the baby in the neonatal period and at intervals throughout the first year.

The appearance of gamma globulin in the newborn and its quantitative characteristics during the first year of life were determined in the unique situation of a normal female offspring of a patient with acquired agammaglobulinemia, reported upon in detail elsewhere (13, 14). Frequent venous blood samples were obtained from the baby's jugular vein throughout the first year of life.

Total serum proteins were determined by the biuret method of Weichselbaum (15). Quantitative precipitin determinations for human gamma-reactive globulin were

performed as outlined by Kabat and Mayer (16). A highly purified gamma globulin preparation obtained by ethanol fractionation was kindly supplied by Dr. J. T. Edsall, and served as antigen in these studies. The method of Kendall (2) for alum precipitation of the antigen and that of Jager, Smith, Nickerson and Brown (17) for the immunization schedule were adopted. Anti-human gamma globulin was produced in albino mixed hybrid rabbits weighing 4 to 5 Kg. The alum precipitated antigen was administered intravenously only and in a concentration of 1 mg. per ml. over a three week period. Each animal received a total of 12 mg. of the alum precipitated material. On the eighth day following the last injection of antigen, each animal was exsanguinated by cardiac puncture under sterile conditions. The bloods obtained were allowed to clot at room temperatures for one hour, placed at 2° C. overnight, the clots then rimmed and the tubes centrifuged at 1,500 rpm for 20 minutes in an International PR-1 centrifuge; the sera were decanted and pooled after preliminary testing for antibody content. The pool was then recentrifuged at 2,000 rpm for 20 minutes, decanted, and merthiolate was added to a final concentration of 1 part in 10,000. The pool was then heated at 56° C. for 30 minutes, allowed to come to room temperature, dispensed into sterile glass tubes, quick-frozen in an alcohol-acetone dry ice bath, and stored at -26° C. until ready for study. Prior to use in these and all subsequent determinations, sera and antisera were clarified by centrifugation for one hour in the cold at 3,000 rpm.

Absorption-precipitin study. A solution of the gamma globulin was prepared in nitrogen-free diluent, consisting of 0.01 molar phosphate at pH 7.4, and brought to ionic strength 0.145 with sodium chloride. The concentration of gamma globulin in this solution was established by repeated micro-Kjeldahl analyses; volumetric dilutions of this standard were then prepared. Quantitative precipitin determinations were carried out by adding 1 ml. of each antigen dilution to 1 ml. of antiserum in standard heavy-walled precipitin tubes. After thorough mixing by agitation, the tubes were capped with parafilm, and allowed to incubate for one hour at 37° C., then placed at 0 to 4° C. for eight days. The tubes were agitated daily. They were then centrifuged for one hour in the cold at 3,000 rpm, and the supernates poured off and tested for the presence of antigen and antibody. All procedures were carried out in the cold. The tubes were allowed to drain over filter paper for 15 minutes, and the precipitates were washed by resuspension in 0.145 N nitrogen-free NaCl. This washing procedure was repeated twice. The washed specific precipitates were digested with 0.5 ml. digestion solution and analyzed for nitrogen by the micro-Kjeldahl method. Repeated analyses, using the same as well as different lots of antiserum, yielded results agreeing within less than 5 per cent in all instances, and in most instances, the agreement was within less than 2 per cent. Reference was thenceforth made to the zone of antibody excess in the precipitin curves thus constructed. One hundred-fifty ml. of rab-

TABLE I
Serum gamma globulin concentrations in normal humans:
Immunochemical estimations

Group	Number	Gamma globulin mg. %	Range
Normal infants 1 day-7 wks.	13	663.19	417.90-1140.62
Normal infants 3-6 mo.	9	398.3	214.84- 621.09
Normal infants 6-15 mo.	14	731.2	279.69-1125.00
Normal children 19-54 mo.	18	882.37	440.62-1541.60
Normal children 7-15 yrs.	6	1151.9	833.40-1312.50
Normal adults 19-34 yrs.	24	1124.3	734.00-1687.50

bit anti-human gamma globulin serum was treated with 75 mg. of lyophilized agammaglobulinemic serum which had been found to contain 2.5 mg. per cent of gamma globulin, incubated for one hour at 37° C. and then stored at 0 to 4° C. for eight days, with daily agitation. Sterile procedure was used throughout. The treated antiserum was then centrifuged at 3,000 rpm for one hour in the cold and decanted. Seventy-five ml. of this once absorbed antiserum was again absorbed by the same technique. Finally, aliquots of the unabsorbed, once and twice absorbed antisera were decomplexed with crystalline BSA-rabbit-anti-BSA.¹ The latter reagents were added in precise proportions corresponding to their equivalence point as determined by previous quantitative precipitin studies. Separate quantitative precipitin determinations were then performed using each of the antisera with dilutions of the standard gamma globulin solution.

Figure 1 demonstrates the close agreement among the amounts of gamma globulin precipitated in the zone of antibody excess with the three antisera, and serves to demonstrate that even the unabsorbed antiserum possessed sufficient immunological homogeneity for use in determinations of gamma globulin concentrations. As may be seen from the figure, no decrease in the amount of precipitate occurring in the zone of antibody excess took place when absorbed serum was used. These findings also suggest that the gamma globulin preparation used as antigen did not contain other protein constituents in significant amounts.

Free electrophoresis was carried out in a Klett Model E Tiselius apparatus at 4° C. against veronal buffer, pH 8.6, ionic strength 0.1; the gamma globulin used as antigen was found to be symmetrically monophoretic. Paper electrophoresis was carried out in a Spinco Model R series B paper electrophoresis apparatus, and the patterns obtained in the Spinco Analytrol. This study, too, revealed that the gamma globulin fraction employed as antigen was homogeneous.

¹ Armour and Co., bovine plasma albumin, crystallized.

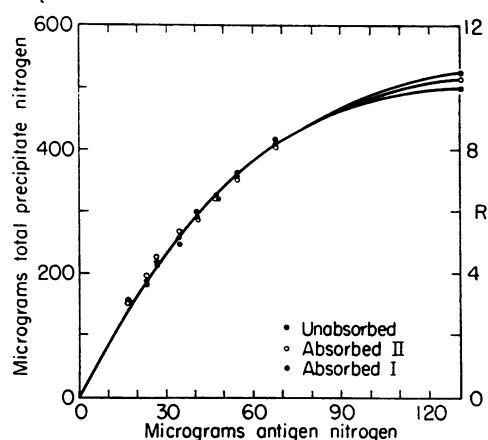


FIG. 1. ABSORPTION-PRECIPTIN STUDY OF RABBIT ANTI-HUMAN GAMMA GLOBULIN SYSTEM

Studies performed with Oudin (18), double diffusion, and Ouchterlony (18) methods revealed a single band of precipitate at equivalence when the antiserum was tested against the standard human gamma globulin antigen, normal human sera and agammaglobulinemic sera.

RESULTS AND DISCUSSION

Summarized in Table I are the gamma globulin concentrations as determined immunochemically on normal infants, children and adults. The distribution of gamma globulin levels is also shown as the stippled area on Figure 3.

Table II describes the quantitative immunological deficit in 16 agammaglobulinemic patients. All agammaglobulinemic patients have some gamma globulin as measured in this way; the serum gamma globulin concentrations in patients with congenital agammaglobulinemia ranged from 2.5 to 16.0 mg. per cent while those of patients with acquired agammaglobulinemia ranged from 12.0 to 97.0 mg. per cent. Although some overlap between the two groups was observed, it will be seen that the adult agammaglobulinemics generally possess higher concentrations of gamma globulin in their serum than do the children with the congenital form of the disease.

Figure 2 summarizes the data obtained in the immunochemical estimation of the biologic half-life of parenterally administered gamma globulin in patients with agammaglobulinemia. In this study, serum gamma globulin concentrations were plotted as a semilogarithmic function of time following the injection of various doses of gamma globulin. These patients ranged in age from one

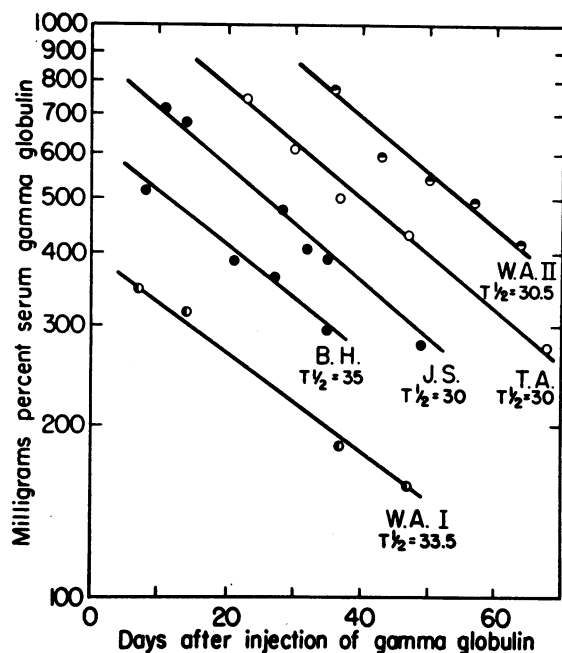


FIG. 2. BIOLOGIC HALF-LIFE OF HUMAN GAMMA GLOBULIN IN PATIENTS WITH AGAMMAGLOBULINEMIA

to seven years. The biologic half-life of gamma globulin in these patients ranged from 30 to 35 days and appeared to be independent of age, concentration of the protein, or time after injection.

Figure 3 describes the appearance of gamma globulin in the normal female offspring of a patient with acquired agammaglobulinemia, and its decline in the agammaglobulinemic male offspring of an apparently normal female. In the first instance, the gamma globulin concentration of the maternal blood did not differ significantly from that of the cord blood, nor were the isohemagglutinins or antibodies to any of the antigens with which the mother had been stimulated observed in the baby's serum. Antigenic stimulation was provided the baby by weekly injections of typhoid-paratyphoid vaccine, and diphtheria, pertussis and tetanus vaccine during the first two months and monthly thereafter. The baby was born agammaglobulinemic and remained so throughout the first 40 days of life. On approximately the fortieth day of life, the serum gamma globulin concentration was found to be 115 mg. per cent and it rose rapidly thereafter. Eighteen days following the first increase in gamma globulin concentration and at a time when the concentration of this

TABLE II
Immunochemical estimations of serum gamma globulin concentrations in patients with agammaglobulinemia

Form of disease, Subject	Sex	Age yrs.	Gamma globulin concentration mg. %
Congenital agammaglobulinemia			
T. A.	M	1	14.0
J. S.	M	2	2.5
W. A.	M	6	4.4
B. H.	M	6	10.0
E. S.	M	7	11.5
R. J.	M	3	16.5
R. D.	M	3	13.4
S. E.	M	11½	8.8
J. Si.	M	1	10.0
			Mean: 10.1
			Range: 2.5-16.0
Acquired agammaglobulinemia			
L. L.	F	30	12.0
F. H.	M	58	40.0
K. N.	M	35	97.0
A. L.	M	20	65.5
L. Z.	F	56	30.0
M. O'B.	F	63	19.0
B. A.	F	67	74.0

Mean: 48.2
Range: 12.0-97.0

protein was nearly 200 mg. per cent, antibodies to H antigen were first demonstrated in significant titer. Thereafter, the baby formed agglutinins to O and B antigens, formed diphtheria antitoxin, and became Schick negative at four months of age. It is of interest that appreciable quantities of gamma globulin were present for a considerable period preceding the appearance of demonstrable levels of circulating antibodies.²

The decline of maternally transmitted gamma globulin in the agammaglobulinemic son of a normal female emphasizes several points of interest. First, this child who cannot form gamma globulins begins life with at least a normal concentration of this protein in the serum. The logarithmic decline of gamma globulin in this baby was uniformly linear throughout the first 10 months of

² Since this study has been completed, another female child was born to the same agammaglobulinemic mother. Similar studies of gamma globulin concentration revealed a curve almost identical to that of the baby described here except that the initial increment in circulating gamma globulin concentration occurred between 20 and 28 days after birth rather than between 35 and 42 days as was the case with this baby.

life, at which time the serum concentrations fell within the range observed among other agammaglobulinemic children. The half-life of gamma globulin in this child throughout the first 10 months of life is comparable to that seen in agammaglobulinemic patients given gamma globulin parenterally.

In Figure 4 are shown semilogarithmic plots of the decline of gamma globulin concentration of a normal offspring of a patient who had previously borne a son found to be agammaglobulinemic. In this graph, the decline of gamma globulin concentration is compared to the decline observed following injection of gamma globulin in a six year old agammaglobulinemic boy. During the first 80 days of life, the curve in this patient was similar in slope to that defining the half-life of injected gamma globulin in the agammaglobulinemic patients and virtually identical to that defining the decline in gamma globulin concentration in an agammaglobulinemic baby born of an apparently normal mother (Figure 3).

Table III summarizes the serum values on 31 maternal blood-cord blood pairs taken at parturition. The differences between the two groups of sera with respect to total protein concentration and gamma globulin concentration estimated both electrophoretically and immunologically were small. Although the gamma globulin concentrations determined by the immunochemical method were slightly higher in the cord serum than in the maternal serum, the difference between the two groups was not statistically signifi-

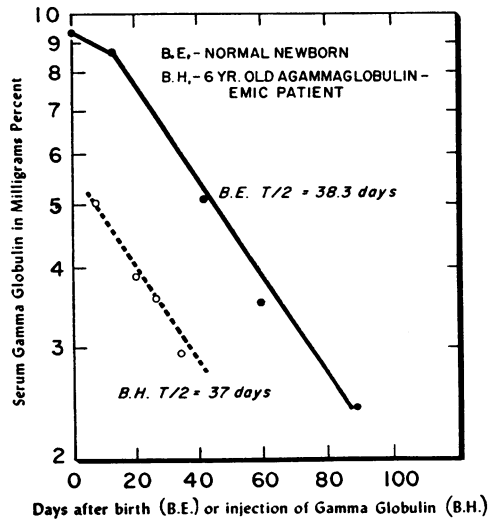


FIG. 4. BIOLOGIC SURVIVAL TIMES OF GAMMA GLOBULIN IN A CONGENITAL AGAMMAGLOBULINEMIC PATIENT COMPARED WITH DECLINE IN A NORMAL NEONATE

cant. These observations, although consistent with their interpretations, provide little support for the conclusions of Moore, Martin du Pan and Buxton, and others who suggest that active transport and/or local placental production of gamma globulins are responsible for elevated cord levels noted with electrophoretic methods (19).

DISCUSSION

The immunoelectrophoretic observations of Grabar and Williams (20, 21) indicate that human gamma globulins may be immunologically homogeneous and physico-chemically heterogeneous. The immunological studies in the agar precipitin systems performed here support this contention. Furthermore, the absence of significant amounts of serum proteins other than gamma globulins from the material used as the antigen in these studies, as well as the specificity of the antibodies produced in the rabbit against this human gamma globulin, is suggested by the absorption-precipitin study.

Previous studies comparing results of the gamma globulin levels obtained with the immunochemical method and gamma globulin levels obtained by electrophoretic analyses have revealed that higher concentrations are obtained with the former technique. This finding has been variously attributed to the presence of gamma-reactive globulin under

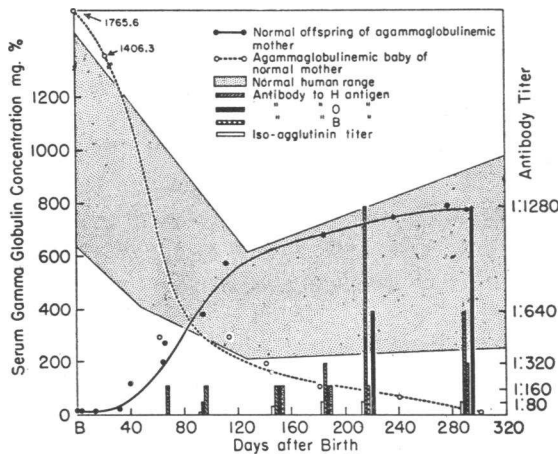


FIG. 3. APPEARANCE OF GAMMA GLOBULIN AND ANTIBODY SYNTHESIS IN THE NEWBORN PERIOD

TABLE III
Serum values on 31 maternal-cord samples taken at delivery

	Total serum protein		Electrophoretic gamma globulin		Immunochemical gamma globulin	
	Maternal	Cord	Maternal	Cord	Maternal	Cord
	<i>Gm. %</i>		<i>mg. %</i>		<i>mg. %</i>	
Mean	6.965	6.529	940.4	1139.7	978.7	1081.8
S. D.	0.81	1.28	264	320.8	296.4	285.9
Range	5.6–	4.85–	580–	510–	509.4–	645.3–
	9.3	10.2	1826	2070	1762.5	2187.5
p Value	0.1096		0.0076		0.1645	

the alpha and beta globulin peaks of the electrophoretic spectrum. When paper electrophoretic methods are used, albumin tailing, higher affinity of the gamma globulins for the dyes used, and the application artifact introduce errors which prohibit strict quantitation. The data presented here, however, in agreement with those of Goodman and co-workers (22), suggest that immunochemical and electrophoretic values are of the same order of magnitude within the usual ranges of concentrations observed in human sera. The immunochemical method, being more sensitive and more precise, affords clearer insight into subtle differences in protein concentrations, and is particularly valuable in assessing very low concentrations of protein.

Among the sera of patients suffering from the severe handicap in protein synthesis, agammaglobulinemia, we have observed no instance in which gamma globulin was not detectable by precipitin methods in liquid-liquid or in semisolid media. The finding of extreme hypogammaglobulinemia rather than complete absence of the protein in the sera of these patients by immunochemical means was reported upon in the original description of the disease. Our observations support this conclusion and indicate that neither impurities in antigens used nor cross reactivity among the serum proteins account for the small amounts of gamma globulin measured in each case.

The mean biologic half-life of parenterally administered gamma globulin, established by the immunochemical method, reveals a value which is comparable to that reported by Bruton, Apt, Gitlin and Janeway (11) in agammaglobulinemia by a similar technique, and by others in normal humans using immunologic, electrophoretic

and salt precipitation techniques, respectively (23–25).

The appearance in the newborn of apparently nonimmune, gamma-reactive globulin in appreciable quantities well before the appearance of specific serum antibody might suggest that only some gamma globulin is antibody. However, the existence of this apparently nonimmune globulin may well represent a response to antigens which are ubiquitous and for which testing was not accomplished. The failure of this child to synthesize gamma-reactive globulin during the first 40 days of life is also apparent from these data. Further data collected in this laboratory on a similar case revealed initial appearance of serum gamma globulin between 21 and 28 days of life.

The immunochemical studies reported herein are in essential agreement with other studies performed using different methods to reveal the variation in serum gamma globulin concentrations throughout infancy and childhood. Normal infants display a variable interval, lasting between two and six months, prior to the assumption of full immunological responsibility. During this interval, the gamma globulin concentrations may decline normally to values slightly above 200 mg. per cent. Certainly most children show no apparent ill effects of the immunological inadequacy or the low gamma globulin levels. Failure to assume active gamma globulin synthesis, or delay in development of this function, is followed by further decline in gamma globulin concentration. This state has been referred to as the transient hypogammaglobulinemia of infancy and is characterized clinically, just as is clinical agammaglobulinemia, by extreme susceptibility to bacterial infection. Similarly, a child subsequently proved to be agammaglobulinemic showed con-

tinued decline in gamma globulin concentration at an almost identical rate to that observed early in the neonatal period in children subsequently shown to be immunologically normal. In this instance, however, the decline continued until levels under 10 mg. per cent were reached. This child developed increased susceptibility to infection when the gamma globulin level descended below 100 mg. per cent (Figure 3). From these and other observations, it would appear that the critical level of serum gamma globulin concentration is of the order of 100 mg. per cent.

SUMMARY

Procedures for the quantitative estimation of gamma globulins in human sera used in this laboratory are presented. Data suggesting the adequate homogeneity of a gamma globulin preparation for use in immunochemical estimations are presented. Normal values for the gamma globulin concentration in newborns, infants, children and adults, as determined by immunochemical methods, are presented and compared with electrophoretic values. Gamma globulin concentrations in 16 patients with agammaglobulinemia are shown. The half-life of injected gamma globulin in four congenitally agammaglobulinemic patients, as determined by the immunochemical method, is documented. The assumption of gamma globulin synthesis and immunological responsibility in the unique instance of a normal offspring of an agammaglobulinemic patient is described and compared with a "reciprocal" instance of an agammaglobulinemic offspring of an immunologically normal female. The rate of decline of gamma globulin concentration during the first two and one-half months of life observed in an immunologically normal child was found to be similar to that of parenterally injected gamma globulin in agammaglobulinemic patients and similar to the decline of gamma globulin concentration observed during the neonatal period in an agammaglobulinemic child.

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