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Immunoglobulin Levels in Serum of Normal Infants and Pre-School Children as Determined by Immunochemical Analysis

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Standards for determining serum immunoglobulin (Ig) levels in infants and pre-school children are now possible as a result of data compiled and presented in this paper. These can be compared with adult standards used in this hospital and presented in earlier issues of the MEDICAL JOURNAL. The Hospital's laboratory technique is compared with other methods for measuring IgM, IgA and IgG serum levels.

Introduction

With the development of immunochemical methods of analysis, several different technics¹⁻¹¹ have been employed to study the concentrations of serum immunoglobulins (IgM, IgA and IgG) in normal individuals and in various disease states. Previous reports from this laboratory⁸ have presented a practical technic to determine serum immunoglobulin levels in adults. The present paper records serum immunoglobulin levels in normal infants from birth to five years of age. Adult values previously determined were expressed in mg/100 ml. of serum relative to a standard serum to which arbitrary immunoglobulin values were given. Pediatric serum values which have been determined by using the same method are readily compared with adult serum.

Various methods for determining immunoglobulins in serum are summarized in Table I. They fall into three distinct groups. Group-1 consists of methods based on the *simple diffusion* in agar method of Oudin.¹² Group-2 consists of methods based on the *double-diffusion* in agar method of Ouchterlony.¹³ Group-3 is a miscellaneous group of methods unique to individual laboratories.

In the *simple diffusion methods*, the antiserum is incorporated into the gel matrix. Gel containing antiserum is poured into tubes or onto flat plates and the test serum is layered over it or placed in holes cut in the gel. The concentration of individual immunoglobulins is measured as a function of the distance traveled by a precipitin

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LoGrippto, Manson and Sharpless

band within a standard time. Employing this method, Heremans¹ reported serum values for IgG, IgA and IgM (Table I). Huntley² studied fifty normal children, ages 5-18 years, for IgG by this technic but did not include IgA and IgM values. The simple diffusion principle has also been employed in the form of radial plates by Fahey⁴ and by Stiehm and Fudenberg.⁵ The latter workers studied immunoglobulin levels in 260 children under five years of age and an additional 36 individuals from 6-16 years of age comparing their immunoglobulin values with those of 30 normal adults (Table I). The work of Stiehm and Fudenberg⁵ gives the range of variation found in normal infants and children. Data for the first year of life are arranged in age groups of 1-3 months, 4-6 months and 7-12 months.

In the *double diffusion methods*, both the antiserum and the test serum diffuse through the agar. The position at which precipitation band forms is an index of the concentration of the individual immunoglobulin in the test serum. Gell⁶ and Soothill⁷ employed this method. Six different dilutions of antiserum were placed in agar holes

A SUMMARY OF METHODS FOR DETERMINING
SERUM IMMUNOGLOBULIN (Ig) VALUES IN MAN

Table I

CONDITIONS			mg/100 milliliters of Serum									AUTHORS	
METHOD	TYPE OF STANDARD	AGE AND (NUMBER)	IgG			IgA			IgM				
			Mean	S. D.	Coef/Var.	Mean	S. D.	Coef/Var.	Mean	S. D.	Coef/Var.		
SIMPLE DIFFUSION													
1. Tubes	Purified Ig	25-45 yrs (25-normal)	1000	200	20%	112	41	37%	75	20	27%	Heremans 1960 (1)	
	Commercial Ig & Biuret protein	5-18 yrs (50-normal)	1410	260	18%	NOT GIVEN						Huntley 1963 (2)	
	Pooled serum standardized against purified Ig	Adults(?) (54-normal)	1194	Not Calculated		395	Not Calculated		191	Not Calculated		Claman & Merrill 1964 (3)	
	2. Radial Plates	Norm. serum standardized against purified Ig	Adults(?) (30-normal)	1240	220	17%	280	70	25%	120	35	28%	Fahey & McKelvey 1965 (4)
		Purified Ig Kjeldahl Protein	Adults(?) (30-normal) newborn to 16 yrs (296)	1158	305	26%	200	61	31%	99	27	27%	Stiehm & Fudenberg 1966 (5)
Normal Range of Variations with respective age Groups													
DOUBLE DIFFUSION													
3. Holes in Agar	Purified gamma-globulin	Not Studied	NOT STUDIED									Gell 1957 (6)	
	Purified Glob. & a Cryoglobulin	Adults (20-normal)	1232	250	20%	NO VALUES GIVEN			145% (of Std. Serum)			Soothill 1962 (7)	
4. Plastic Template on Agar	Pooled norm. serum with arbitrary values attached	Adults(?) (48-normal)	1094	237	24%	82	26	32%	69	20	29%	Sharpless & LoGrippto 1965 (8)	
MISCELLANEOUS													
5. Immune-inhibition and immuno-electrophoresis		Infants(98) Children(25) Adults(29)	EXPRESSED IN UNITS									West, et al. 1961(9), 1962(10)	
6. Isotopic I ¹²⁵ labeled Ig immune-inhibition		Adults(?) (19-normal)	1263	270	13%	394	91	23%	116	55	47%	Fahey & Lawrence 1963 (11)	

Serum of Infants and Children

cut around a central hole containing appropriately diluted test serum. The hexagonal-shaped line of precipitation which was formed is characteristic of the concentration of the antigen in the central hole. An estimate of the concentration of the test antigen was obtained by comparing its pattern with diluted control sera. Soothill studied the levels of IgM and IgG in 20 adults. No attempt was made to study IgA values.

Indirect immune inhibition procedures have been reported by West^{9,10} and by Fahey.¹¹ West's method is a combination of immune inhibition and immunoelectrophoresis. Test serum is mixed with a standardized antiserum and the degree of loss of specific precipitin-antibody measured by immunoelectrophoresis.

Fahey's method employs radioactive-labeled globulins in an immune-inhibition method. Test serum and antiserum are incubated and the precipitate removed. The degree of loss of specific antibody is then measured by reacting treated antiserum with radio isotope labeled globulins. The amount of radioactivity in the supernatant after centrifugation is a measure of this loss. Fahey¹¹ expressed values for immunoglobulins in 19 normal adults and his data are expressed in milligram percent, making them comparable to other reported data (Table I).

The technic for the data in this report is a micro-double diffusion in agar method which has been reported in detail.⁸ A general review is given under "Materials and Methods".

Materials and Methods

In a cross-section study, the technic developed in this laboratory was used to investigate serum immunoglobulin levels in normal children from birth to age five years. Seventy full-term infants (60 newborn and 10 one-week-old), whose mothers had had a normal ante-partum delivery and post-partum course were studied. Blood samples were also obtained from 108 normal infants and children, who showed normal growth and development, normal physical findings and who had had no recent infection. Blood samples were obtained on different children at different ages during the first year of life and from ages one to five years, in order to get a quick cross-section of age groups. The sterile venous blood was assayed immediately or soon after one freezing and thawing. A total of 178 infants and children was studied.

Materials and equipment used in this procedure are readily available from commercial sources.⁸ The micro-double diffusion in agar technic utilizes a five-well plastic template* arranged with a central well and four peripheral wells, which are equidistant from each other and 4 mm. from the central well and have a capacity of 0.025 ml. Molten agar is placed on a glass microscope slide, allowed to gel slightly and overlaid with the plastic template. Mono-immune antiserum for each immunoglobulin is placed in the central well and four different dilutions of the patient's serum are placed in the peripheral wells (Figure 1). Dilutions used depend on the particular immunoglobulin being determined, the titer of the antiserum and the concentration of immunoglobulin in the test serum.⁸ Proper dilutions are ascertained by setting up preliminary slides with diluted reference serum. Appropriately greater or lesser dilutions are used for

* Available from: Mann Research Labs, 136 Liberty St., New York, N.Y. 10006

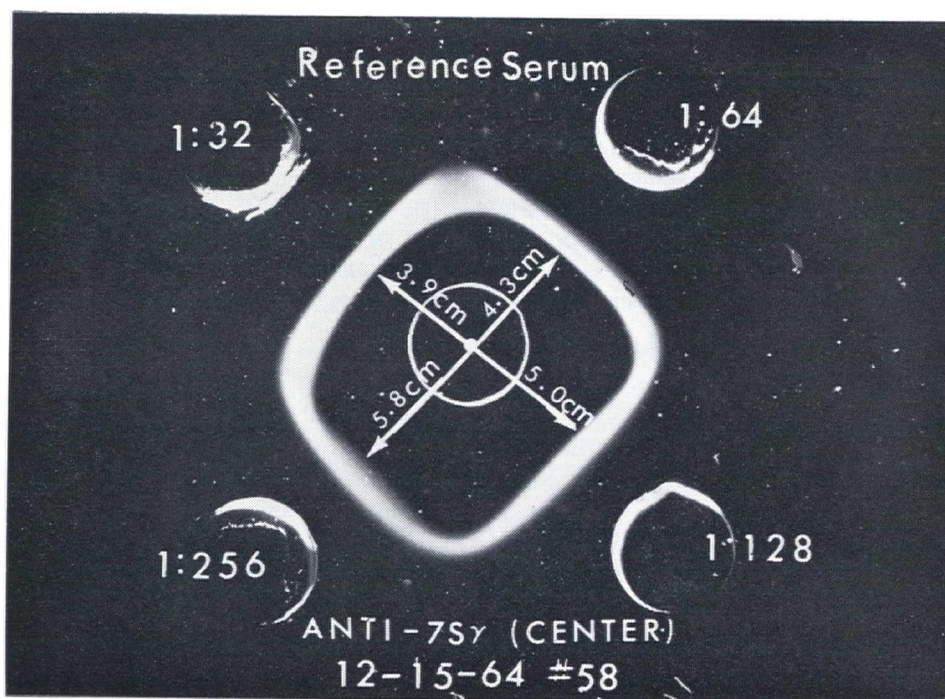


Figure 1

Precipitation pattern obtained with anti-IgG ($7S\gamma$) globulin in the central well and diluted reference serum in the peripheral wells. Pattern obtained when the serum dilution series has been selected so that $7S\gamma$ globulin and its antiserum are present in approximately equivalent concentrations. Actual distance measurements are indicated by arrows.

abnormal sera. After 40-48 hours incubation in a moist chamber, the slides are removed and the templates lifted. With magnification, the precipitation bands and the imprint of the five template wells on the agar surface are clearly seen. The bands are measured from the center of the central well to the inner edge of the precipitation band. With proper dilutions, the precipitation pattern appears as a single band forming the perimeter of an eccentric four-sided figure about the central well. The distance of the band from the central well is inversely proportional to the immunoglobulin concentration in the diluted serum in the peripheral wells. This dimension is utilized for quantitation. When the immunoglobulin concentration is high, the band is located close to the central well. When concentration is reduced, the band is located proportionally further from the center. Mono-immune antisera for the specific immunoglobulins are purchased from commercial sources. The advantages of this technic are its simplicity and speed for volume work in a hospital clinical laboratory, the reproducible results and the availability of reliable materials from commercial sources.

Values are expressed in milligrams per 100 ml. of serum relative to a standard serum to which arbitrary values have been given. To make clinically significant alterations readily apparent, normal values must be determined for each laboratory.

Serum of Infants and Children

Table II
SERUM IMMUNOGLOBULIN LEVELS IN INFANTS AND PRE-SCHOOL CHILDREN DETERMINED BY MICRO-DOUBLE-DIFFUSION IN AGAR

AGE RANGE	NO. OF SAMPLES	Immunoglobulin (Ig) Values*					
		IgM		IgA		IgG	
		MEAN	S. D. **	MEAN	S. D.	MEAN	S. D.
Cord Serum	IgM-60 IgA-60 IgG-59	0-25	----	0-8	----	1013 (omitted 2037)**	+305
1 week	IgM-10 IgA-10 IgG-9	12	<u>+7</u>	0	----	816 (omitted 1654)	+210
1 month	IgM-9 IgA-9 IgG-9	33	<u>+8</u>	Trace to 5	----	473	+148
2 months	IgM-10 IgA-10 IgG-10	32	<u>+10</u>	4	<u>+2</u>	344	+74
4 months	IgM-10 IgA-10 IgG-10	45	<u>+17</u>	5	<u>+3</u>	315	+98
6 months	IgM-10 IgA-9 IgG-10	51	<u>+31</u>	9 (omitted 26)	<u>+3</u>	389	+182
8 months	IgM-10 IgA-10 IgG-10	53	<u>+13</u>	7	<u>+5</u>	366	+105
10 months	IgM-10 IgA-10 IgG-10	54	<u>+14</u>	8	<u>+5</u>	409	+113
1 year	IgM-10 IgA-10 IgG-10	58	<u>+19</u>	13	<u>+5</u>	524	+129
2 years	IgM-9 IgA-9 IgG-9	61	<u>+14</u>	17	<u>+13</u>	656	+121
3 years	IgM-9 IgA-9 IgG-10	75 (omitted 43)	<u>+8</u>	27 (omitted 72)	<u>+6</u>	726	+285
4 years	IgM-10 IgA-10 IgG-10	68	<u>+19</u>	37	<u>+18</u>	792	+315
5 years	IgM-9 IgA-9 IgG-10	85 (omitted 128)	+11	43 (omitted 90)	<u>+13</u>	820	+219

*Expressed as milligrams per 100 ml of serum relative to a standardized serum.
** Values which deviated more than 4 times the average deviation from the mean were omitted.

Results

Serum immunoglobulin levels (IgM, IgA and IgG) of full-term infants and pre-school children are summarized in Table II. The determinations in serum were made from (a) 60 cord serums; (b) 10 infants one week of age; (c) 10 infants in each of the age groups one, two, four, six, eight, ten and twelve months; (d) 10 children each in the groups from two to five years. The number of samples for each immunoglobulin determination in the respective age groups is given together with the number of samples assayed for each component. The mean values and the standard deviation calculated

LoGrippe, Manson and Sharpless

for each immunoglobulin vary with the age of the child and the respective immunoglobulin. The results shown in parentheses and indicated as "omitted" are markedly deviate values.

Figures 2, 3 and 4 are scattergrams for IgM, IgA and IgG respectively, showing individual serum values and the actual range for each age group. The figures are plotted to compare the serum immunoglobulin levels in the pre-school age group with the normal adult range.

Immunoglobulin-M: In the newborn infants, the concentration of immunoglobulin-M in the cord serum is low in comparison with adult values. A mean value cannot be calculated. Of 60 serums tested, six had no detectable IgM in undiluted serum,

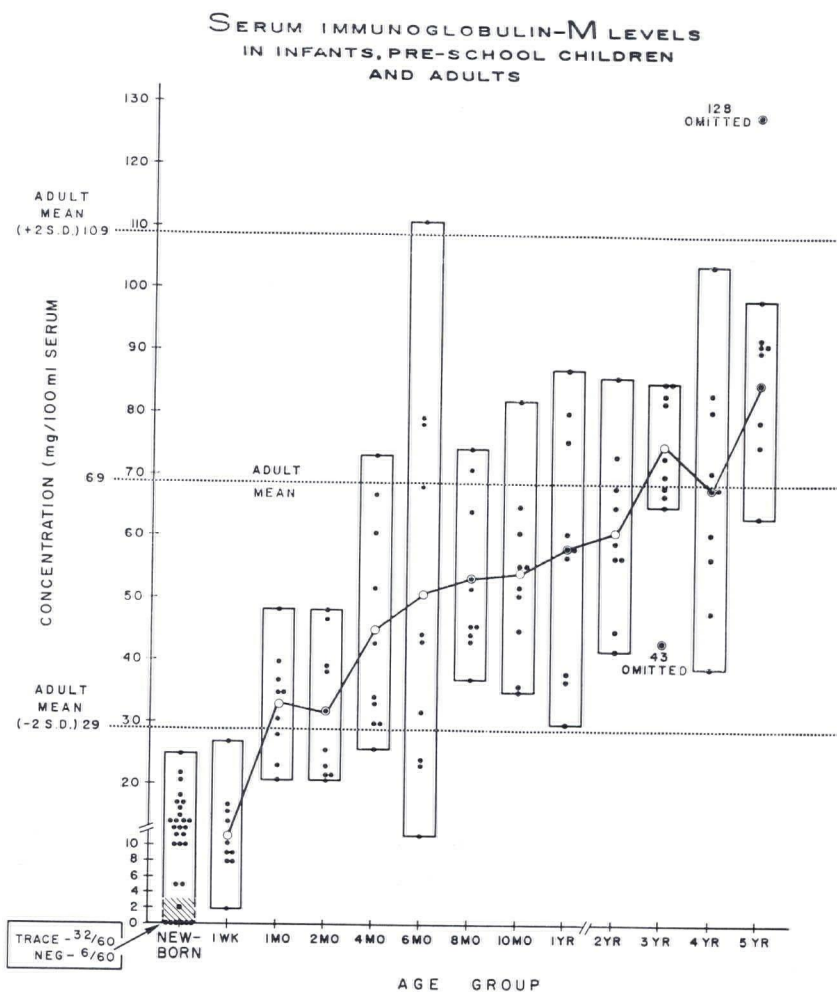


Figure 2

Serum of Infants and Children

32 had only trace quantities and the remaining 22 had values ranging from 5 to 25 mg/100 ml. Negative values indicate no detectable precipitation band with undiluted serum. Trace quantities indicate a precipitation band with undiluted serum samples but less than 2.5 mg/100 ml. of IgM, the minimal quantity measurable by this technic. In the first week of life (4-7 days) IgM values are measurable, having a calculated mean value of 12 mg/100 ml. (± 7 mg/100 ml. as 1 SD). This component may reach the lower range of adult values within one to two months but does not attain the adult mean value until the third year of life. There is a gradual increase in mean values from two months to three years of life with the widest scatter seen at six months.

Immunoglobulin-A: This component was not detectable in 15 of the 60 cord serums tested and appeared in trace quantities in 43 cord serums. The remaining two cord serums had values of 3 mg/100 ml. and 8 mg/100 ml. respectively. Similar values were found at one week of age; nine out of 10 serums were negative for IgA and one serum showed trace quantities. IgA makes its appearance by the first month of life.

SERUM IMMUNOGLOBULIN-A LEVELS IN INFANTS, PRE-SCHOOL CHILDREN AND ADULTS

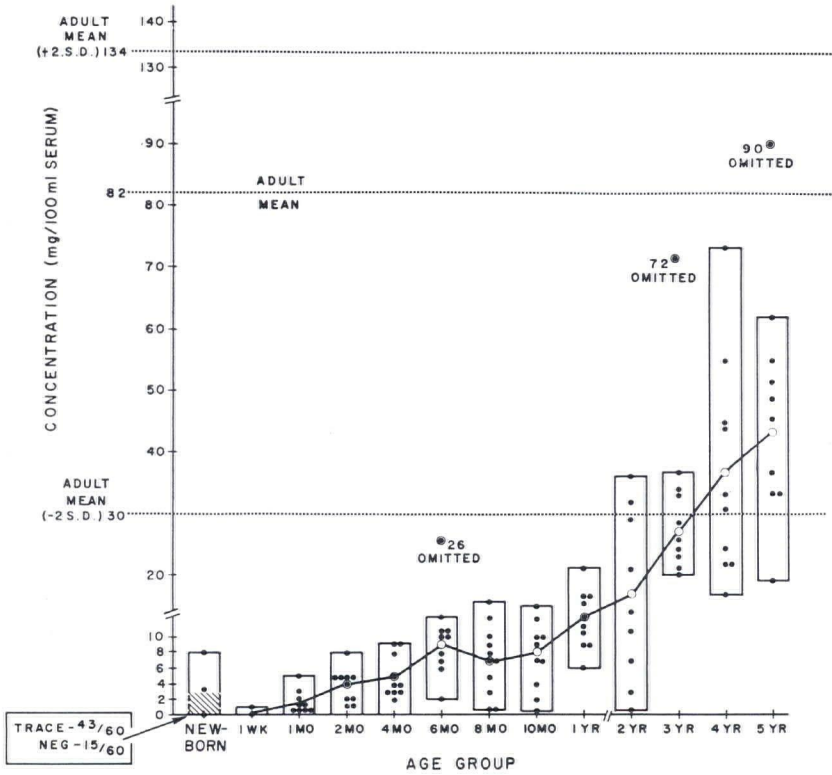


Figure 3

LoGrippo, Manson and Sharpless

A mean value of 4 mg% IgA could be calculated at age two months. IgA does not increase much above this level until six months of age and does not attain a value within the adult normal range until three years of age. Even then the IgA values are still low.

Immunoglobulin-G values were found to be within the adult range of serum levels in sixty cord serums, the mean being 1013 mg/100 ml. The mean in 10 sera during the first week of life was 816 mg/100 ml. Cord serum values were comparable to the adult mean values (1004 mg/100 ml.) but dropped by approximately 200 mg. % during the first week of life. There is a progressive decline in the passively transferred maternal immunoglobulin-G component so that by the first month of life, the

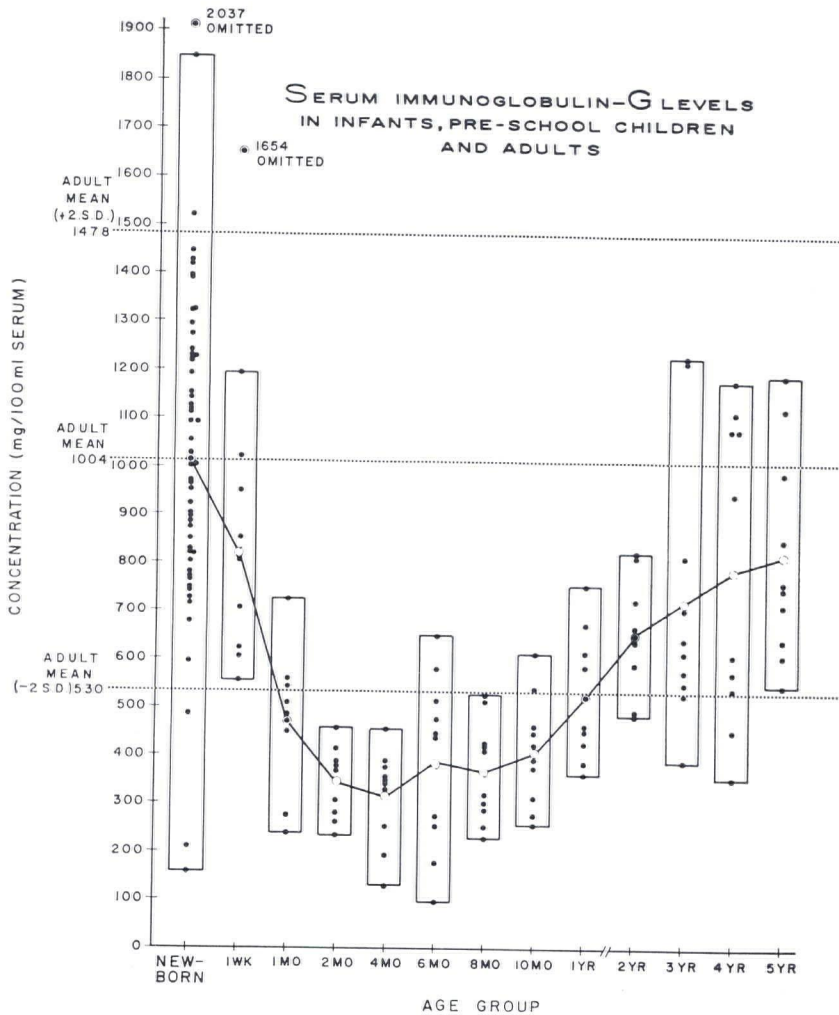


Figure 4

Serum of Infants and Children

mean value (473 mg.%) is approximately 50% of birth values. IgG mean levels decreased to 344 mg. and 315 mg.% during the second and fourth months, respectively. From 6 to 12 months, the level gradually increased but did not reach the lower range of adult values until the second, third, and fourth years of life. The scattergram shows a fairly wide range of individual variation with the widest ranges at one week, at six months and after three years.

Discussion

Table I shows several methods available for measuring serum immunoglobulins. Some are complex while others, being simple and rapid, are suitable to routine clinical laboratory service. It is apparent that marked variations occur in both *mean values* and *standard deviations* of two major components (IgA and IgM). The IgG values are in relatively close agreement with all the methods. The variations in IgA and IgM values require an explanation.

It would be presumptuous to say that one method is better than the other or simpler to perform. Any technic is suitable for a particular laboratory which has mastered the procedure and obtained reproducible results. It is important that each procedure be

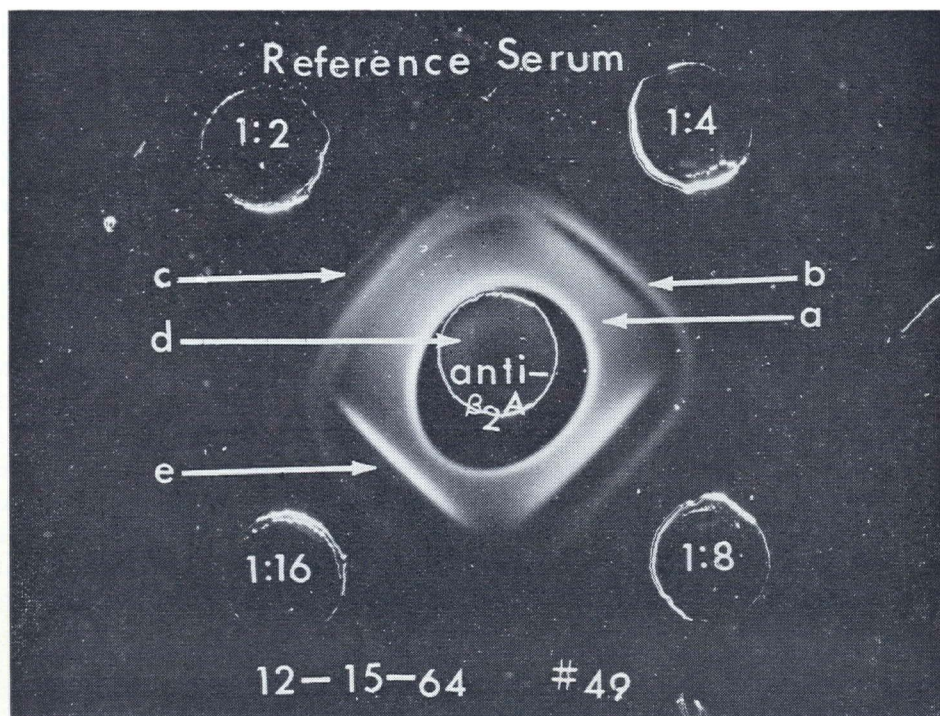


Figure 5a

Precipitation pattern obtained with anti-IgA (β_2A) globulin in the central well and diluted reference serum in the peripheral wells. The specific band (a) and four non-specific bands (b, c, d and e) obtained with this antiserum are indicated by arrows. Pattern obtained when the serum dilution is too low. The specific band is wide and ill-defined and the non-specific bands are prominent.

capable of detecting marked variations from normal to facilitate the diagnosis of various clinical entities with immunoglobulin disturbances. Nevertheless, we found it necessary to modify the technics available in order to establish a consistent standard for comparison. The lack of conformity results from the variation in titer and specificity of mono-immune antisera for their respective immunoglobulins regardless of their source. These variables must be under laboratory control if reproducible results are to be maintained.

Figure 5a illustrates a major source of error with mono-immune antisera and may explain the occurrence of false high values for the IgA component, particularly in cord serum when values are so low that undiluted serum must be tested. In figure 5a the micro-double diffusion technic demonstrates five precipitation bands using commercial antiserum (designated as specific for IgA) in the central well and a standard reference serum in the peripheral wells. The two-fold dilutions in the peripheral wells

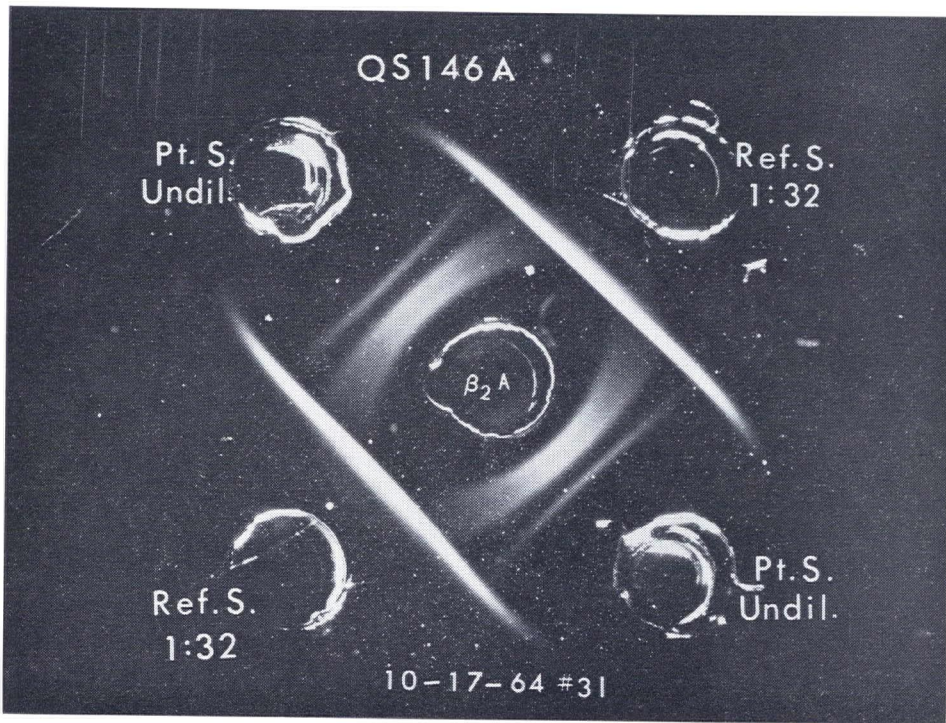


Figure 5b

Photograph illustrating the presence of non-specific precipitating antibodies in the antiserum to IgA (β_2A globulin). Two peripheral wells contained reference serum diluted 1:32. The other two wells contained undiluted patient serum. Failure of the prominent specific band obtained with reference serum to fuse with any of the bands obtained with patient serum clearly shows that these bands are non-specific. Very slight turning of the tips of the specific band toward the wells containing patient serum may indicate that IgA (β_2A globulin) is present in very minute quantity, too low to produce visible precipitation. Failure to include reference serum on the slide could lead to erroneous interpretation of a normal IgA concentration instead of the decrease clearly illustrated.

Serum of Infants and Children

allow varying concentrations of the other serum components to diffuse more adequately and to precipitate in the zone of equivalence for their respective antigen-antibody complex. Although four of the five precipitation bands are minor and the IgA component is the major band, such antisera can produce erroneous measurements, indicating higher values for the IgA component. Thus, any technic which incorporates antiserum in the gel matrix and measures single precipitation bands as the index to the IgA value, may prove unreliable. Micro-double diffusion technic for IgA values in cord serum are negative or demonstrate only trace quantities (less than 2-5 mg%). When low values for IgA are being determined, undiluted patient serum must be used. Figure 5b clearly shows how nonspecific precipitation bands could be measured when antiserum is incorporated into the gel matrix.

Many sources of error are inherent in immuno-chemical technics. Unless the method of analysis takes these non-specific bands into consideration with each determination made, serum immunoglobulin standards will vary in *mean values, standard deviations and coefficient of variation*. With the micro-double diffusion technic reported here, it is easy and inexpensive to make duplicate determinations. Moreover, each determination gives an average value of six to eight separate measurements (Figure 1). Reproducibility is thereby increased without added time and materials. In addition, interference from non-immunoglobulin serum components is readily eliminated as a potential source of error, especially with pathological serum. It is possible to run a control on the same slide in such a manner that the precipitation band of the unknown serum fuses with that of the control serum, thus establishing unequivocally the identity of the unknown band. (Figure 5b).

Summary

Utilizing a micro-double diffusion immunochemical technic developed in this laboratory, the concentrations of serum immunoglobulins (IgM, IgA and IgG), have been measured in cord sera from newborns and sera from 118 normal infants at various ages from birth to five years. The values in normal infants and children are reported according to age, together with statistical analysis of these data. The age-related changes in the concentrations of the several immunoglobulins studied are discussed without reference to their clinical significance. The study seeks instead to establish the normal limits of immunoglobulin concentration in children as measured by our method. These data thus provide a standard with which immunoglobulin values in disease states may be compared.

Pitfalls in the immunochemical methods of measuring serum immunoglobulins are discussed together with methods of avoiding them. The necessity for maintaining an adequate control for these technics is emphasized if consistent results are to be obtained.

LoGrippto, Manson and Sharpless

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