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An international reference preparation for human serum immunoglobulins G, A and M: content of immunoglobulins by weight

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An International Reference Preparation for Human Serum Immunoglobulins G, A, and M has been established by the World Health Organization and international units have been assigned to it. This paper describes international collaborative assays carried out by 10 specialized laboratories, which attempted to define the immunoglobulin contents of the International Reference Preparation by weight. For all immunoglobulins the estimates of contents by weight were imprecise, largely owing to heterogeneity of estimates between laboratories. Mean estimates of immunoglobulin contents by weight are given, but it is considered that the results of assays of immunoglobulin against the International Reference Preparation or related preparations are more precisely expressed in terms of international units.

A research standard for human serum immunoglobulins G, A, and M, preparation 67/86, has been described previously (Rowe, Anderson & Grab, 1970); it consists of freeze-dried diluted pooled human serum. This material was considered to be suitable for a standard for quantitative determinations of IgG, IgA, and IgM using the single radial diffusion technique or similar immunochemical procedures. It was established by the Division of Biological Standards, National Institute for Medical Research, London, as the British Research Standard for Human Serum Immunoglobulins G, A, and M. Subsequently, the WHO Expert Committee on Biological Standardization (1971) established part of this material as the International Reference Preparation of Human Immunoglobulins IgG, IgA, and IgM. Units of potency of the three immunoglobulins were assigned to the British Research Standard, and the WHO Expert Committee on Biological Standardization subsequently defined international units of the same size for the International Reference Preparation. These international units were each defined as the activity contained in 0.8147 mg of the International Reference Preparation. This paper describes collaborative assays that have been carried out to estimate the content of IgG, IgA, and IgM in the International Reference Preparation by weight, and thus to estimate that weight of active material of each immunoglobulin in it that corresponds to 1 unit of potency, i.e., to 0.8147 mg of the International Reference Preparation.

The most direct approach to the assessment of the content (by weight) of immunoglobulins per ampoule of preparation would be to isolate each of the immunoglobulins and to determine the weight of each isolated protein. This approach is not possible at present, since current techniques do not permit the isolation of immunoglobulins from serum with high recovery and in high purity. Accordingly the immunoglobulin content was estimated by comparing solutions of the International Reference Preparation with solutions of purified immunoglobulins at stated concentrations, using immunochemical techniques. These estimations were made independently by each of a number of laboratories, which were invited to participate in a collaborative assay. The results were analysed centrally by the World Health Organization.

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THE COLLABORATIVE STUDY

This was carried out by the 10 laboratories listed in the Annex, together with the names of the responsible workers. In this report the laboratories are indicated by a code number, which is not related to their order in the Annex. Each laboratory received ampoules of the International Reference Preparation packed in insulated containers containing solid carbon dioxide. In the laboratories, the preparation was stored at −20°C or colder. Prior to analysis, the ampoule contents were reconstituted by the addition of 1 ml of distilled water per ampoule. Each laboratory compared the reconstituted preparation with isolated purified immunoglobulins at stated concentrations prepared in the same laboratory,[1] using the immunochemical techniques commonly employed in that laboratory.

Details of the isolated proteins used for calibration are given in Table 1. Myeloma proteins or M-macroglobulins of Waldenström (monoclonal proteins) were sometimes used as calibrating proteins, but most preparations of IgG came from normal or pathological sera from which myeloma proteins were absent (polyclonal proteins). No laboratory reported impurities in the calibrating proteins on analysis by various physicochemical and immunochemical methods except for Laboratory 7, which found trace contaminants in its IgA and IgM preparations. The concentration of protein in the isolated preparation was usually estimated from its content of nitrogen, or from optical density measurements at 280 nm. The conversion factors used for the calculation of the concentration of an individual immunoglobulin by a particular method were identical in all laboratories, except in the case of IgA. In calculating the concentration of IgA from optical densities at 280 nm, Laboratories 1, 3, and 4 used an extinction coefficient (E 280 nm) of 13.4, whereas Laboratory 9 used a coefficient of 15.0 (Table 1). Usually the reconstituted International Reference Preparation was compared directly with the isolated purified immunoglobulins, but sometimes the comparison was indirect, using an intermediate preparation that had been calibrated previously against isolated purified immunoglobulins.

Details of antisera and immunochemical techniques are given in Table 2. All antisera were judged to be specific by gel-diffusion tests, but Laboratory 6 reported that their antisera to IgA and IgM contained antibodies to immunoglobulin light chains detectable by a haemagglutination test. The immunochemical technique employed was usually that of single radial diffusion and in most cases the diffusion of antigen was allowed to proceed until the zones of precipitate reached their maximum size.

Statistical analysis

The results reported by eight laboratories have been submitted to the full conventional statistical treatment of biological assays. The analysis was carried out on the WHO IBM 360/40 computer.

Two laboratories (8 and 9) used assay methods not comparable with the conventional parallel-line or slope-ratio assay methods and their data could not be included in the standard computer processing of the assay results. The concentration estimates reported by these two laboratories are shown in Table 4, but were not included in the general analysis.

Statistical information on the type and the design of the assays performed in the eight collaborating laboratories retained for the analysis is summarized in Table 3. Results of 78 assays [2] were reported by these laboratories. The number of replicates in each assay varied between 1 and 3, but was 2 in the majority of cases.[3]

The average dose–response lines for each preparation were graphically produced by the computer plotter. The process was repeated with various combinations of different transformations of the dose and response metameters. On the basis of the visual evidence provided by the graphs, a decision was made for each laboratory on the appropriate transformation system to be adopted and the corresponding type of statistical technique to be applied, whether parallel-line or slope-ratio assay (see Table 3). The graphs showed also that in several parallel-line assays (mainly when IgM was tested) the lowest concentrations were responsible for the statistical invalidity of the assay. Such concentrations were excluded from the final analysis.

For each individual assay, the concentration (in mg/ml) of immunoglobulin in the reconstituted International Reference Preparation was calculated

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[1] Except for IgM prepared by Laboratory 10, which was compared with the International Reference Preparation by Laboratory 7.

[2] A set of experiments carried out on one plate was considered to be one assay. In one laboratory the International Reference Preparation was tested simultaneously on the same plate against two different calibrating proteins.

[3] When in the analysis of variance the residual error could not be calculated from the between-replicate variation, it was taken as the mean square for deviations from linearity.

as well as the precision of the concentration estimate (statistical weight). The statistical significance of departure from linearity and parallelism[1] of dose–response regression lines was tested by applying the F-ratio to the relevant sums of squares of the analysis of variance.

Formal statistical invalidity was observed in many assays. However, a close examination of the situation revealed that the statistical significance of departure from parallelism[1] or from linearity was presumably due to the error variance being smaller than in the other assays which showed no significant departure.

In addition, immunoglobulin concentrations estimated from assays formally invalid were generally not responsible for within-laboratory statistical heterogeneity. It was therefore considered reasonable to retain the results of all assays in the calculation by laboratory of the final mean immunoglobulin concentrations of the reconstituted International Reference Preparation.

The homogeneity of the concentration estimates obtained within each laboratory was studied separately by the χ^2 test (Humphrey et al., 1953) for each immunoglobulin and each calibrating protein.

For the results that did not show heterogeneity, the average logarithmic concentration was computed by weighting each logarithmic concentration value with the reciprocal of its variance derived from the internal evidence of the assay. The variance of the average concentration was then simply the reciprocal of the total of the individual weights.

For the laboratories whose results were found to be heterogeneous, the between-assay variance was computed and the weight of each logarithmic concentration value was redetermined by taking the reciprocal of the total variance, i.e., including both within- and between-assay variances according to the method described by Bliss (1952). The new weights were applied to the individual logarithmic concentration, and its variance was taken as the reciprocal of the sum of the new individual weights.

RESULTS

The test results, consolidated by calibrating proteins used within each laboratory, are presented in Table 4. The mean concentrations were generally based on combinations of 2–5 individual estimates. The χ^2 test showed that, within any one calibrating protein in a given laboratory, homogeneity was good in the majority of cases. Thus of 10 mean concentration estimates for IgG, 2 were heterogeneous; of 6 mean concentration estimates for IgA, 1 was heterogeneous; and of 9 mean concentration estimates for IgM, 4 were heterogeneous. (Heterogeneity was assessed at the 5% probability level of statistical significance.)

It should be noted that statistically significant between-estimate heterogeneity could be observed, even when the individual estimates were reasonably close, if the assays were particularly precise—that is, if the statistical weight per estimate was high. It is seen in Table 4 that the statistical weights varied considerably from laboratory to laboratory and in particular were relatively large for Laboratory 4, whose estimates were heterogeneous for IgG and IgA. As far as Laboratories 2 and 4 are concerned, further analysis of variance indicated that variation between different antisera used by these laboratories was also a possible cause of heterogeneity between individual estimates.

The magnitudes of the discrepancies among concentration estimates from laboratories where individual estimates were heterogeneous between themselves are indicated in Table 5, which shows the ranges of values obtained. The small ranges from Laboratory 4 confirm that it was the statistical precision of the individual estimates rather than a wide range of estimated values that was responsible for the highly significant heterogeneity of individual concentration estimates from this laboratory.

As already mentioned, immunoglobulin concentrations in the International Reference Preparation reported by Laboratories 8 and 9 are also given in Table 4. However, these estimates were not included in the computation of the final combined estimate of mean concentration, because the assay methods used by these two laboratories were not comparable with those used by the other laboratories. As shown in Table 2, Laboratory 9 used a different extinction coefficient for the calibration of the IgA concentration of the calibrating protein from that used by other laboratories. On the basis of an extinction coefficient of 13.4 the IgA content of the reconstituted International Reference Preparation estimated by Laboratory 9 would become 1.41 mg/ml.

The overall mean immunoglobulin concentrations in the reconstituted International Reference Preparation were calculated by combining the weighted mean

[1] Tested on the component for intersection in the slope-ratio assays.

Table 1. Isolation and characteristics of

	Laboratory			
	1	2	3	4
IgG				
calibrating protein code no.	—	1	1	1
source	—	serum	pooled serum	pooled serum
monoclonal (MC) or polyclonal (PC)	—	PC	PC	PC
techniques of isolation	—	pseudoglobulin $(NH_4)_2SO_4$, DEAE, aggregates removed by UC	DEAE	DEAE
physicochemical tests on isolated proteins	—	none	UC, free-solution EL, gel-filtration	UC, no aggregates
immunochemical tests on isolated proteins	—	IE and DD against anti-G,A,M and ATHS, 1 line only	IE and DD against ATHS, 1 line only	IE and DD against 3 ATHS, 1 line only: antigen 7.20 mg/ml
methods for estimation of total protein, and conversion factor	—	nitrogen, 16 % N	OD 280 nm, E = 13.8	OD 280 nm, E = 13.8
time and temperature of storage from isolation to comparison	—	14 days including preparation, 4°C	1 day, 4–6°C	14 days, 4°C
direct or indirect [a]	—	direct	direct	direct
IgA				
calibrating protein code no.	1, 2, 3, 4	—	1	1, 2, 3
source	serum	—	pooled serum	serum
monoclonal (MC) or polyclonal (PC)	PC	—	PC	PC
techniques of isolation	Zn, DEAE–Sephadex, G200	—	Zn, $(NH_4)_2SO_4$, EL, G150	1—pseudoglobulin, $(NH_4)_2SO_4$ ppt., CM, TEAE 2,3—pseudoglobulin, Zn, EL, $(NH_4)_2SO_4$
physicochemical tests on isolated proteins	none	—	UC, free-solution EL, gel-filtration	UC, 10 % polymers
immunochemical tests on isolated proteins	IE against ATHS IgA only	—	IE and DD against ATHS IgA only	IE and DD against 3 ATHS IgA line only conc. >10 mg/ml

* Abbreviations: ATHS = anti-total human serum; EL = electrophoresis; IE = immunoelectrophoresis; DD = double diffusion analysis in agar; Zn = precipitation by Zn++; 0 = information not available; DEAE (TEAE) = chromatography on DEAE (TEAE) cellulose; G 50, G100, G200 = chromatography on Sephadex G50, G100, G200; CM Sephadex = chromatography on carboxymethyl Sephadex; UC = ultracentrifugation; SRD = quantitative analysis by single radial diffusion.

immunoglobulins used for calibration *

code no.

5	6	7	8	9	10
IgG					
1	1, 2, 3, 4, 5	1	1	—	—
normal serum	1—normal serum 2, 3, 4, 5—pathological sera	plasma	serum	—	—
PC	1, 4, 5—PC; 2, 3—MC	PC	PC	—	—
Cohn FII	Cohn FII, DEAE and CM Sephadex	DEAE, EL	commercial preparation	—	—
UC, 1 peak	UC, cellulose acetate, starch gel EL	none	none	—	—
IE, haemagglutination inhibition	IE and DD	DD, SRD with various antisera. No impurity	IE and DD. No impurity	—	—
nitrogen	dry weight and refractive increment	nitrogen, 16 % N	nitrogen 16 % N	—	—
14–28 days, frozen	1–4 years, −70°C	1–6 months, −20°C	4 years, −70°C	—	—
direct	1, 2, 3—direct 4,5—indirect	direct	indirect	—	—
IgA					
1	1, 2	1, 2	1	1	—
Serum	0	plasma	serum	ascitic fluid	—
MC	1—MC; 2—PC	MC	PC	PC	—
0	0	DEAE, EL, G200	Pseudoglobulin, Zn, EL, $(NH_4)_2SO_4$	EL, Zn, DEAE, G200	—
0	UC, cellular acetate starch gel EL	none	none	UC, homogeneous	—
IE and haemagglutination inhibition	IE and DD	1—IE, pure, SRD 0.6 % IgG 2—IE, pure, SRD 0.6 % IgG	IE and DD, pure	IE and DD, homogeneous	—

[a] Method of comparison with isolated purified preparations: " direct " indicates that the isolated proteins were compared directly with the International Reference Preparation; " indirect " indicates that the isolated proteins were used to calibrate an intermediate material, usually human serum, which was subsequently compared with the International Reference Preparation. Time and temperature of storage refer in this case to the intermediate material.

Table

	Laboratory			
	1	2	3	4
	IgA (*continued*)			
methods of estimation of total protein and conversion factor	Biuret using albumin standard, OD 280 nm, E = 13.4	—	OD 280 nm, E = 13.4	OD 280 nm, E = 13.4, nitrogen, 16 % N
time and temperature of storage from isolation to comparison	1, 2—6–8 days at 4°C 3—several months at −20°C 4—3 years at −20°C	—	1 day, 4°C	lab. standard, −30°C
direct or indirect [a]	1,2,3—direct 4—indirect	—	direct	indirect
	IgM			
calibrating protein code no.	—	—	1	—
source	—	—	pooled serum	—
monoclonal (MC) or polyclonal (PC)	—	—	PC	—
techniques of isolation	—	—	$(NH_4)_2SO_4$, Euglobulin, kaolin	—
physicochemical tests on isolated protein	—	—	UC, EL	—
immunochemical tests on isolated protein	—	—	IE and DD: no impurities	—
method for estimation of total protein and conversion factor	—	—	OD 280 nm, E = 13.3	—
time and temperature of storage from isolation to comparison	—	—	1 day, 4°C	—
direct or indirect [a]	—	—	direct	—

concentrations of Laboratories 1–7 and 10. The results of Laboratory 6 for immunoglobulins A and M were not included in the computation of the overall mean because of their surprisingly high value for IgA and low value for IgM (Table 4), which seems to indicate a lack of comparability with the other laboratories in the determination of the immunoglobulin content in the calibrating materials. Weighted and unweighted overall means were in good agreement (Table 6). The heterogeneity of between-laboratory estimates was significant for all immunoglobulins at the 1% probability level.

The extremely large between-laboratory differences in the precision of the original concentration estimates (see the average statistical weights per estimate shown in Table 4) prevented the correct determination of the statistical precision of the overall weighted mean concentration. The 95% confidence interval

1 (*continued*)

code no.					
5	6	7	8	9	10
		IgA (*continued*)			
OD 280 nm, E = 13.4	refractive increment	nitrogen 16 % N	nitrogen, 16 % N	OD 280 nm, E = 15.0	—
0	1–4 years, −70°C	1— 7 days; 2—1 month; −20°C	4 years, −70°C	a few days, repeated after a few weeks, unfrozen and frozen	—
indirect	indirect	direct	indirect	direct	—
		IgM			
1	1, 2, 3, 4	1, 2	1	—	1
normal serum	serum	plasma	serum	—	serum
PC	1, 2—MC 3, 4—PC	MC	MC	—	PC
Na_2SO_4, EL, G200	G200, DEAE, CM Sephadex	1—G200, EL 2—Euglobulin, DEAE	0	—	EL, DEAE, G200 EL G200
0	UC, cellulose acetate, starch-gel EL	None	None	—	UC, single peak, S = 18.85
IE and haemagglutination inhibition	IE and DD, pure	1—IE and DD, pure 2—trace non-Igs on IE	IE and DD Pure	—	DD Pure
nitrogen	dry weight and refractive increment	nitrogen, 16 % N	nitrogen, 16 % N	—	interferometry
0	1—4 years, −70°C	1—4 months 2—1 month, −20°C	4 years, −70°C	—	0: not frozen
direct	1,2—direct 3,4—indirect	direct	indirect	—	direct

was therefore estimated for the overall unweighted mean concentration only (Table 6).

The immunoglobulin activity of the International Reference Preparation has been defined in terms of units. Each ampoule of the International Reference Preparation contains on average 100 units of IgG and of IgA and of IgM, and when the contents of one ampoule of the International Reference Preparation are dissolved by adding 1 ml of distilled water the resulting solution (of calculated volume 1.06 ml) contains 94.4 units per ml of each of these immunoglobulins (Rowe, Anderson & Grab, 1970). The weight of each immunoglobulin corresponding to 1 unit of activity of the International Reference Preparation has been calculated from the overall unweighted mean concentrations in the reconstituted International Reference Preparation shown in Table 6.

Table 2. The antisera and the quantitative immunochemical methods used in the assays *

Laboratory code no.	Immunoglobulin	Antisera				Method of quantitative test			
		Species	Source of immunogen	Absorbent	Test for specificity [a]	Single radial diffusion or other test	If SRD		
							Diffusion time (hours)	Finality [b]	Highest concentration tested [c]
1	IgA	sheep	Colostral, PC	cord serum	IE, DD	SRD	72	yes	1 in 2
2	IgG	goat [d]	0	0	IE	other [e]	—	—	—
3	IgG IgA IgM	rabbit rabbit rabbit	serum, PC	appropriate immunoglobulins	IE, DD, SRD	SRD	> 50	yes	1 in 40 1 in 8 1 in 2
4	IgG IgA	goat and rabbit goat and rabbit	serum, PC; milk, PC; and serum, MC	L-chains and IgA L-chains and IgG —all polyclonal	IE, DD	SRD	96	yes	1 in 25 1 in 5
5	IgG IgA IgM	rabbit rabbit rabbit	serum, PC serum, MC pooled serum, MC	0 0 0	IE	SRD	0 0 0	yes yes yes	1 in 4 1 in 1 1 in 1
6	IgG IgA IgM	goat goat goat	serum, PC serum, MC serum, MC	L-chains IgA def. serum and L-chains L-chains	IE, DD, SRD	SRD	4 6 24	no	1 in 1
7	IgG IgA IgM	sheep goat rabbit	serum, PC serum, MC sera, MC [g]	0 0 0	IE, DD	SRD	17 17 72	no	1 in 1
8	IgG IgA IgM	goat [d] goat [d] goat [d]	0	0	IE, DD	SRD	4 16–20 16–20	no yes yes	1 in 1
9	IgA	rabbit	ascitic fluid	IgG	IE, DD, CF	other [f]	—	—	0
10	IgM	rabbit	sera, MC [g]	0	IE, DD	SRD	72	no	1 in 1

* CF = complement fixation, other abbreviations as in Table 1.
[a] All antisera were specific in the tests indicated; the anti-IgA and anti-IgM from Lab. 6 reacted with light chains as judged by haemagglutination tests.
[b] Whether or not diffusion proceeded to maximum ring diameter.
[c] Highest concentration of the reference preparation 67/86.
[d] Commercial antisera.
[e] A modified Oudin test.
[f] Quantitative complement fixation.
[g] The immunogen consisted of 2 monoclonal proteins isolated from different human sera.

Table 3. Basic statistical information on the assays of the reconstituted International Reference Preparation performed in different laboratories

Laboratory code number	Statistical type of assay	Transformation used		Number of assays analysed per antigen				Number of dilutions [a] per preparation	Number of replicates per dilution and per preparation
		Dose	Response	Total	IgG	IgA	IgM		
1	slope ratio	—	square	4	—	4	—	4	2
2	parallel line	log	—	3	3	—	—	3–6	2–3
3	slope ratio	—	square	11	4	3	4	2–4	1 or 2
4	slope ratio	—	—	8	4	4	—	5–6	2 or 3
5	slope ratio	—	square	6	2	2	2	3–5	1
6	parallel line	log	square	35	16	5	14	3–6	2
7	parallel line	log	square	8	2	4	2	3–6	2
10	parallel line	log	square root	3	—	—	3	4	2

[a] No. retained for the analysis.

On this basis 1 unit of activity of IgG corresponded to 80.4 μg of isolated IgG with 95% confidence interval 69.2–93.3 μg; 1 unit of IgA corresponded to 14.2 μg of isolated IgA with 95% confidence interval 12.1–16.6 μg; and 1 unit of IgM corresponded to 8.47 μg of isolated IgM with 95% confidence interval 6.99–10.1 μg (Table 7).

DISCUSSION

Estimates of immunoglobulin concentrations, in terms of mg/ml, of the reconstituted International Reference Preparation carried out by the 10 collaborating laboratories showed a wide range of values (Table 4). IgG ranged from 5.3 to 11.5 mg/ml, IgA from 0.98 to 3.19 mg/ml, and IgM from 0.19 to 0.95 mg/ml. Thus, even laboratories that specialize in immunoglobulin preparation and in quantitative immunochemical measurement failed to achieve close agreement between themselves. In general, estimates made in one laboratory using a single batch of calibrating protein and a single antiserum were homogeneous, whereas highly significant between-laboratory heterogeneity was the rule. The overall heterogeneity of estimates appeared likely to be due to (1) the use of different calibrating proteins, (2) the use of different antisera, and (3) other aspects of the assay systems.

Studies in some laboratories showed that their calibrating proteins were themselves heterogeneous. In addition, comparison with data from the previous study of 67/86 (Rowe, Anderson & Grab, 1970) also indicated that important heterogeneity of this type did in fact occur. In the present study, when the International Reference Preparation, which is part of the batch of material 67/86, was assayed against the various purified calibrating proteins in 8 laboratories the 95% confidence intervals of the estimated concentrations, expressed as percentages of the mean, were 30.0% for IgG, 31.7% for IgA, and 36.7% for IgM.[1] When sera from 6 healthy donors were assayed against the International Reference Preparation (68/160–68/166, Table 8, Rowe, Anderson & Grab, 1970), the means of the values for the 95% confidence intervals of the estimated potencies, calculated separately as the percentage of the mean potency of each serum,[1] were 7.8% for IgG, 5.8% for IgA, and 9.8% for IgM. Eleven laboratories took part in the latter study. In both studies the individual laboratories used their own antisera and their own modifications of various quantitative techniques. This approximately four-fold increase in the relative 95% confidence intervals observed in the assay of the calibrating proteins against the International Reference Preparation, compared with the assay of the normal sera against the International Reference Preparation, strongly suggests that the various calibrating proteins were more heterogeneous between themselves than were the immunoglobulins of the

[1] Calculated as: (Range of 95% confidence limits/mean) × 100.

Table 4. Mean immunoglobulin concentration in the reconstituted International Reference Preparation with respect to the calibrating proteins used in each laboratory

Laboratory code number	Calibrating protein code no.	Number of estimates	Within laboratory χ^2 heterogeneity test [a]	Weighted mean concentration (mg/ml)	Average statistical weight per concentration estimate
			Immunoglobulin G		
2	1	3	1.76	8.8	1 040.9
3	1	4	0.01	8.2	45.0
4	1	4	46.63 **	8.4 [b]	4 381.3 [c]
5	1	2	0.05	11.5	15.5
6	1	2	1.63	7.6	380.4
6	2	2	0.84	5.3	244.1
6	3	2	2.33	7.3	230.1
6	4	5	12.31 *	6.2 [b]	129.6 [c]
6	5	5	8.32	6.8	324.6
7	1	2	1.23	7.3	10 700.9
8	—	—	—	9.2 [d]	—
			Immunoglobulin A		
1	1	1	—	1.36	7 122.8
1	2	1	—	1.22	9 732.6
1	3	1	—	1.40	8 903.8
1	4	1	—	1.83	22 479.9
3	1	3	0.47	1.72	289.0
4	1	4	18.38 **	1.54 [b]	6 821.8 [c]
5	1	2	0.08	0.98	5 293.7
6	1	5	0.54	3.19	602.2
7	1	2	0.00	1.08	3 802.0
7	2	2	0.98	1.16	2 570.3
8	—	—	—	1.97 [d]	—
9	—	—	—	1.58 [d]	—
			Immunoglobulin M		
3	1	4	6.24	0.95	3 870.8
5	1	2	1.12	0.87	2 230.2
6	1	2	0.18	0.28	118.0
6	2	2	10.60 **	0.22 [b]	53.2 [c]
6	3	5	1.54	0.50	758.0
6	4	5	11.06 *	0.19 [b]	83.4 [c]
7	1	2	6.37 *	0.82 [b]	296.7 [c]
7	2	2	2.12	0.68	1 955.9
10	1	3	7.90 *	0.69 [b]	654.3 [c]
8	—	—	—	0.64 [d]	—

[a] * = statistically significant at 5 % probability level
** = statistically significant at 1 % probability level.
[b] Weighted with statistical weights adjusted for within-laboratory heterogeneity.
[c] Average of adjusted statistical weights.
[d] Concentration reported by the laboratory, not directly comparable with the weighted mean concentrations calculated for the other laboratories.

Table 5. Range of estimates for immunoglobulin concentration of the reconstituted International Reference Preparation in laboratories with significantly heterogeneous mean concentration *

Laboratory code number	Calibrating protein code number	Number of estimates	Concentration estimate (mg/ml)	
			Mean [a]	Range
		Immunoglobulin G		
4	1	4	8.4	8.1–8.7
6	4	5	6.2	5.2–8.8
		Immunoglobulin A		
4	1	4	1.54	1.48–1.57
		Immunoglobulin M		
6	2	2	0.22	0.17–0.27
6	4	5	0.19	0.14–0.28
7	1	2	0.82	0.75–0.91
10	1	3	0.69	0.63–0.76

* At 5 % probability level of statistical significance.
[a] Weighted with statistical weights adjusted for within-laboratory heterogeneity.

normal sera, and that this heterogeneity was likely to be an important cause of the variability of estimates of immunoglobulin concentrations of the International Reference Preparation when assayed against these materials. The heterogeneity of the calibrating proteins could arise from variability of methods used to estimate the protein content of their solutions, as well as from variability of their immunochemical reactivity.

Significant heterogeneity was sometimes observed when different antisera were used in one laboratory for comparison of the International Reference Preparation with the same calibrating protein. When Laboratory 4 used two different antisera to IgG, the mean concentrations, estimated against the same calibrating protein, were 8.62 and 8.12 mg/ml and a χ^2 test showed significant between-antiserum heterogeneity for these figures. However, when Laboratory 4 used two different antisera to IgA to assess the International Reference Preparation against a single calibrating protein, the mean concentration estimates of 1.53 and 1.55 mg/ml did not differ significantly. These findings suggest that variability between antisera may sometimes occur, and can be a significant cause of heterogeneity of estimates of concentration. A similar conclusion was reached previously, concerning the use of different antisera to assay immunoglobulins in various normal human sera against the International Reference Preparation (Rowe, Anderson & Grab, 1970).

The heterogeneity of the concentration estimates obtained in this study precludes the precise determination of the concentrations of IgG, IgA, and IgM by weight in the International Reference Preparation. Hence, while it is recommended that concentrations of immunoglobulins in other preparations should be estimated by assaying them against the International Reference Preparation, the results of this comparison will be expressed most precisely in units per ml, not in mg per ml. The unit for each immunoglobulin was defined previously (Rowe, Anderson & Grab, 1970).

Furthermore, since a large part of the heterogeneity appears to arise from differences between different

Table 6. Overall weighted and unweighted estimated mean immunoglobulin concentrations in the reconstituted International Reference Preparation and the corresponding 95 % confidence intervals of the unweighted means

Immuno-globulin	Number of concentration estimates	Overall weighted mean concentration (mg/ml)	Overall unweighted mean concentration (mg/ml)	95 % confidence interval for the unweighted mean concentration (mg/ml)	Heterogeneity between laboratory estimates [a]
IgG	10	7.60	7.59	6.53–8.81	**
IgA	9 [b]	1.33	1.34	1.14–1.57	**
IgM	5 [b]	0.84	0.80	0.66–0.95	**

[a] ** Statistically significant at 1% probability level.
[b] Excluding Laboratory 6.

Table 7. Immunoglobulin contents of the International Reference Preparation and of working standards 67/95, 67/97, and 67/99

Preparation	IU/ ampoule	IU/ml [a]	Weight (μg) [b] per unit
	Immunoglobulin G		
67/86	100	94.4	80.4 (69.2–93.3)
67/95	101	95.3	—
67/97	102	96.2	—
67/99	102	96.2	—
	Immunoglobulin A		
67/86	100	94.4	14.2 (12.1–16.6)
67/95	101	95.3	—
67/97	101	95.3	—
67/99	102	96.2	—
	Immunoglobulin M		
67/86	100	94.4	8.47 (6.99–10.1)
67/95	102	96.2	—
67/97	102	96.2	—
67/99	102	96.2	—

[a] Refers to a solution prepared by dissolving the contents of 1 ampoule in 1 ml of distilled water.

[b] Unweighted mean estimates and 95 % confidence intervals, derived from Table 6.

preparations of calibrating proteins used in different laboratories, it seems that, with methods in their present state, the estimation of immunoglobulins in terms of weight is inherently imprecise. With this reservation clearly in mind, we have calculated the mean estimates of the immunoglobulin contents of a solution prepared by adding 1 ml of distilled water per ampoule of the International Reference Preparation. These calculated figures have wide 95% confidence intervals (Table 6). It should be realized that future advances in methods may allow a review of these figures, but that the potency in terms of international units would not be subject to such revision.

In addition to the International Reference Preparation, part of the same batch 67/86 has been established as the British Research Standard for Immunoglobulins G, A, and M. From the same bulk of material further preparations 67/95, 67/96, and 67/99 have been made, and are issued as working standards. Their potencies, in international units relative to the International Reference Preparation, have been estimated (Rowe, Anderson & Grab, 1970) and these values are listed for convenience in Table 7. (Values for the potencies of immunoglobulins in preparations 67/95 and 67/97 have been derived by direct comparison of activities of immunoglobulins in them with the International Reference Preparation; values for the potencies of immunoglobulins in preparation 67/99 have been calculated from the relative mean weights of ampoule contents of 67/99 and 67/86, assuming the same activity of immunoglobulin per unit weight of contents.) As previously emphasized, immunoglobulin concentrations can be expressed most precisely as international units per ml rather than as mg/ml. If, however, it is considered essential to express concentration by weight, values for the working standards may best be derived from their unit contents and the weight of immunoglobulin estimated to correspond to 1 unit in the International Reference Preparation.

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RÉSUMÉ

PRÉPARATION INTERNATIONALE DE RÉFÉRENCE POUR LES IMMUNOGLOBULINES SÉRIQUES HUMAINES IgG, IgA et IgM: TENEUR EN IMMUNOGLOBULINES EXPRIMÉE EN POIDS

La concentration de chacune des immunoglobulines IgG, IgA et IgM contenues dans la préparation internationale de référence a été estimée en milligrammes par millilitre au cours d'un essai collectif auquel ont participé 10 laboratoires. On a eu recours à des méthodes immunochimiques pour comparer des solutions de la préparation

internationale avec des préparations d'immunoglobulines purifiées de concentrations déterminées.

Pour toutes les immunoglobulines, on a obtenu une gamme étendue de valeurs estimées de sorte que l'évaluation n'a pu être faite avec précision. Les teneurs moyennes en immunoglobulines d'une solution de la préparation internationale de référence reconstituée par addition d'1 ml d'eau distillée par ampoule s'établissaient à 7,59 mg/ml avec un intervalle de confiance 95% de 6,53–8,81 mg/ml pour l'IgG, à 1,34 mg/ml avec un intervalle de confiance 95% de 1,14–1,57 mg/ml pour l'IgA, et à 0,80 mg/ml avec un intervalle de confiance 95% de 0,66–0,95 mg/ml pour l'IgM. Il s'ensuit que 1 unité internationale d'IgG correspond à 80,4 μg d'IgG purifiée, avec un intervalle de confiance 95% de 69,2–93,3 μg, 1 unité internationale d'IgA correspond à 14,2 μg d'IgA purifiée, avec un intervalle de confiance 95% de 12,1–16,6 μg, et 1 unité internationale d'IgM correspond à 8,47 μg d'IgM purifiée, avec un intervalle de confiance 95% de 6,99–10,1 μg. L'ampleur de l'intervalle de confiance 95% reflète la très forte hétérogénéité des estimations obtenues dans les divers laboratoires. Il faut chercher la raison de ces divergences dans le fait qu'on a utilisé, en guise d'étalon, des préparations d'immunoglobulines purifiées différentes, et aussi des antisérums différents.

Etant donné l'imprécision des estimations pondérales de la teneur en immunoglobulines, il est recommandé d'exprimer les concentrations d'immunoglobulines dans les préparations, par comparaison avec la préparation internationale de référence, en unités internationales et non en milligrammes par millilitre.

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Annex

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