Collaborative study* to recalibrate the International Reference Preparation of Anti-D Immunoglobulin

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SUMMARY A collaborative study involving nine independent assays by eight laboratories has recalibrated the anti-D concentration of the International Reference Preparation of Anti-D Immunoglobulin (68/419) in terms of the International Standard for Anti-Rho (anti-D) Incomplete Blood Typing Serum (64/16). This study was carried out when it was found that 68/419 had been calibrated not against 64/16, as originally intended, but inadvertently against another preparation. Based on the results, a revised rounded off value of 300 IU anti-D per ampoule of 68/419 was assigned by the Expert Committee on Biological Standardisation of WHO at its 30th meeting.

Many of the known variables in anti-D quantitation using the AutoAnalyzer were considered in the preparation of the protocol for this study. The remarkably close agreement of the results indicated that the format can be used as an acceptable model for interlaboratory studies in the future.

The international unit (IU) of anti-D is defined by the anti-D content of the International Standard for Anti-Rho (anti-D) Incomplete Blood Typing Serum (Code No. 64/16), which contains 32 IU per ampoule. The above standard has been used to assay:

1. The International Reference Preparation for Anti-D Immunoglobulin (68/419) previously estimated to contain 150 IU anti-D per ampoule. In addition, this preparation was estimated to contain 60 µg anti-D per ampoule by the isotope labelling method; thus 1 µg anti-D was estimated to be equivalent to 2.5 IU;

2. The British Working Standard for Anti-D Antibodies (72/229), which was estimated to contain 11.5 IU anti-D per ampoule (Brozovic, Greaves, Mussett, Wybrow, Goldsmith, and Gunson, unpublished).

Comparative assays of the anti-D content of 68/419 and 72/229, initiated by two of the authors (HHG and PJB), revealed discrepancies in the assigned anti-D concentrations, suggesting that the value of 150 IU anti-D for 68/419 had been underestimated. These findings were confirmed by colleagues at another Regional Transfusion Centre (Jenkins and Blakeman, personal communication). Since 68/419 had been established as the International Reference Preparation of Anti-Rh(D) Immunoglobulin by the WHO Expert Committee on Biological Standardisation with an assigned potency of 150 IU per ampoule, it was considered important to carry out a further collaborative study to check the calibration of 68/419 in terms of international units. The results of that study are reported in this paper.

*Participants in the study

BJ DAWES: Blood Group Reference Laboratory, London

WJ JENKINS: Regional Transfusion Centre, Brentwood

D LEE: Transfusion Centre, Lancaster

R MITCHELL: Regional Transfusion Centre, Glasgow

LAD TOVEY: Regional Transfusion Centre, Leeds

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W WAGSTAFF: Regional Transfusion Centre, Sheffield

THE AUTHORS: Regional Transfusion Centre, Oxford and the National Institute for Biological Standards and Control

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Description of materials

Each participant was informed of the nature of each freeze-dried test preparation, but the ampoules carried a letter code only:


Code $A_1/A_2$ Anti-Rho (Anti-D) Incomplete Blood Typing Serum (64/16), containing 32 IU per ampoule

$B_1/B_2$ British Working Standard for Anti-D Antibodies (72/229)

$C_1/C_2$ International Reference Preparation of Anti-D Immunoglobulin (68/419)

**Design of collaborative study**

Eight laboratories, identified by code number, took part in the study. One laboratory carried out two independent sets of assays using different concentrations of test red cells (5% and 20%). For the purpose of presentation and analysis, these two sets of assays were treated as though they came from separate laboratories (4 and 5). Each laboratory was asked to follow a protocol for the study so that the methods could be standardised.

**Assays of Anti-D**

These were performed using the AutoAnalyzer incorporating a manifold and based on the method devised by Marsh et al.\(^3\) but maintaining the incubation coils at 37°C and the settling coils at a constant temperature below 20°C.\(^4\) Pump tubes were changed before starting the assays. The upper and lower limits of the recorder pen were set while red cell diluent solution was flowing through the colorimeter cell. There was no expansion of any part of the range. Pre-bromelised test red cells were obtained from a pool of at least four D-positive samples less than 5 days old prepared for use on each day of the exercise. The pool comprised cells from one of the following probable genotypes: $R_1R_1$, $R_2R_2$, $R_1R_2$. Other reagents were those used routinely but were required to be kept constant (i.e., from the same batch) throughout the exercise. The sampling rate did not exceed 33 per hour. When 5% test red cell suspensions were used, an 8 mm flow-through cell and 410 nm filter were substituted.

**Starting material for preparation of dilutions for assay**

This was obtained by placing the entire contents of one ampoule in a volumetric flask (50 ml for ampoules coded A or B and 1000 ml for C) containing the diluent 0·5% bovine serum albumin (BSA) in 0·15 M NaCl, which had been shown to be suitable for anti-D assay using the AutoAnalyzer.\(^5\) The freeze-dried material was dissolved at room temperature with gentle shaking to avoid frothing, after which diluent was added to the exact volume.

For each assay, four independent dilutions were prepared as accurately as possible from the starting material.

**Experimental design**

The experimental design covered five days. On day 1, ampoules $A_1$, $B_1$, and $C_1$ were reconstituted as above, and three aliquots of each were separated, two of which were immediately frozen to −30°C or below for use on days 2 and 3. The remaining aliquots were used to select four dilutions of each of the three preparations which satisfied the criteria for optimal assay of anti-D, viz, each dilution should give a linear OD response, and the degree of agglutination should be within the range 15-50%. On each of days 2 and 3, one aliquot of each preparation was thawed, and two independent sets of the four chosen dilutions were prepared from it. These were then assayed in balanced order ($A_1B_1C_1B_1A_1$ on day 2, and $C_1B_1A_1B_1C_1$ on day 3). On day 4, ampoules $A_2$, $B_2$, and $C_2$ were reconstituted, and two aliquots of each were separated, one being immediately frozen for use on day 5. Assays were then performed on days 4 and 5 in exactly the same manner as on days 2 and 3. Thus, each laboratory performed a total of four assays.

Results were recorded on standard forms, which were returned together with the trace from the AutoAnalyzer recorder.

**Methods of statistical analysis**

All assays were analysed as multiple parallel line assays relating optical density response to log dilution. Tests of statistical validity were performed by analysis of variance and for each valid assay estimates of potency in international units per ampoule were obtained for each preparation by comparison with the standard 64/16. Within each laboratory the individual log potency estimates for each preparation were combined after testing statistical homogeneity. If the homogeneity test indicated that the variation between log potency estimates could be accounted for entirely by the variation in $\Delta$OD within individual assays, the estimates were combined by taking a weighted mean, the weights being the reciprocals of the variances of the individual estimates. Otherwise the log potency estimates were combined by taking their unweighted arithmetic mean. In either case appropriate confidence limits were calculated.

**Results**

**Statistical validity of assays**

A small number of significant ($P < 0.05$) departures from parallelism or linearity of the relationships
between ΔOD and log dilution were found. In most instances the variation was attributable to random error, and the assays were accepted as valid. In laboratory 6, however, two assay responses were excluded as being wildly aberrant, and the data were re-analysed for the relevant assays. One further assay from this laboratory was rejected as statistically invalid because of significant non-parallelism (P < 0.01).

Data from laboratories 4 and 5 (in reality the same laboratory, see above) frequently showed evidence of significant departures from parallelism and linearity but with no discernible pattern or trend. Most probably, this is explained by slight, systematic underestimation of the residual error, and on this basis the potency estimates were accepted as valid.

**CALIBRATION OF PREPARATION 68/419 IN TERMS OF 64/16**

The combined potency estimates from each laboratory together with their 95% confidence limits are shown in Table 1 and their distribution is illustrated in Figure 1. The potency estimates ranged from 283 to 357 IU per ampoule with an overall mean of 311 IU per ampoule. There was significant heterogeneity between the combined log potency estimates for the different laboratories (P < 0.001).

The overall combined potency estimate for 68/419 is significantly different from the previously assigned value of 150 IU per ampoule.

**Table 1 Combined potency estimates for 68/419**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Combined potency (IU/ampoule)</th>
<th>95% Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>320</td>
<td>314-327</td>
</tr>
<tr>
<td>2</td>
<td>307</td>
<td>274-345</td>
</tr>
<tr>
<td>3</td>
<td>286</td>
<td>278-293</td>
</tr>
<tr>
<td>4</td>
<td>283</td>
<td>271-296</td>
</tr>
<tr>
<td>5</td>
<td>314</td>
<td>297-331</td>
</tr>
<tr>
<td>6</td>
<td>339</td>
<td>322-357</td>
</tr>
<tr>
<td>7</td>
<td>304</td>
<td>290-318</td>
</tr>
<tr>
<td>8</td>
<td>299</td>
<td>291-307</td>
</tr>
<tr>
<td>9</td>
<td>357</td>
<td>345-369</td>
</tr>
<tr>
<td>Overall</td>
<td>311</td>
<td>294-330</td>
</tr>
</tbody>
</table>

**CALIBRATION OF PREPARATION 72/229 IN TERMS OF 64/16**

Table 2 shows the combined estimates for each laboratory, and their distribution is illustrated in Figure 2. The potency estimates ranged from 8-8 to 12-4 IU per ampoule with an overall mean of 10-8 IU per ampoule. The variation between laboratories was more pronounced for the comparison of 72/229 with 64/16 than for that of 68/419, and there was a suggestion of bimodality; not surprisingly, there was significant heterogeneity between the combined log potency estimates for the different laboratories (P < 0.001).

**Table 2 Combined potency estimates for 72/229**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Combined potency (IU/ampoule)</th>
<th>95% Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11-3</td>
<td>10-7-12-0</td>
</tr>
<tr>
<td>2</td>
<td>12-1</td>
<td>11-1-13-3</td>
</tr>
<tr>
<td>3</td>
<td>9-9</td>
<td>9-7-10-2</td>
</tr>
<tr>
<td>4</td>
<td>8-8</td>
<td>8-7-9-0</td>
</tr>
<tr>
<td>5</td>
<td>9-9</td>
<td>9-4-10-4</td>
</tr>
<tr>
<td>6</td>
<td>12-1</td>
<td>11-5-12-8</td>
</tr>
<tr>
<td>7</td>
<td>9-9</td>
<td>9-3-10-5</td>
</tr>
<tr>
<td>8</td>
<td>11-4</td>
<td>11-1-11-7</td>
</tr>
<tr>
<td>9</td>
<td>12-4</td>
<td>12-0-12-8</td>
</tr>
<tr>
<td>Overall</td>
<td>10-8</td>
<td>9-9-11-9</td>
</tr>
</tbody>
</table>

**Fig. 2 Distribution of combined potency estimates 72/229.**

The overall combined potency estimate for 72/229 was consistent with the previously assigned figure of 11-5 IU per ampoule in that the 95% confidence limits (9-9 to 11-9) enclosed this value.

**EFFECT OF TEST RED CELL CONCENTRATION ON ANTI-D ASSAY**

This was assessed by comparison of the results obtained in laboratories 4 and 5 where all aspects of the assay remained constant except for the use of 5% and 20% red cell suspension respectively. There were significant differences between the combined potency estimates for both 68/419 and 72/229 (P < 0.01). It is noteworthy, however, that with respect to 68/419 the difference between the combined potency estimates expressed as a percentage of the
mean is 10.4% and the comparable value for 72/229 is 11.8%.

Discussion

The principal aim of the study was to check the calibration of the anti-D potency of 68/419 against the standard 64/16. It is clear from the results obtained that this had not been assessed correctly in the previous study. Investigations into the cause of the error have revealed that, inadvertently, 68/419 had been calibrated against the wrong material. The value of 311 IU anti-D per ampoule found in the present study has been rounded down to 300 IU anti-D per ampoule, and the latter potency has been accepted by the Expert Committee for Biological Standardisation of the WHO to define the anti-D content of the International Reference Preparation 68/419. Since the value of 60 μg anti-D per ampoule determined previously was carried out independently by the isotope labelling method, this value remains correct. Thus it is estimated that 1 μg anti-D is equivalent to 5.0 IU and not 2.5 IU as previously reported, or 2.8 IU. The latter conversion factor has been used widely in the past to express the anti-D content of 64/16 in terms of micrograms. It is recommended that standards based on 64/16 should be calibrated in terms of international units.

It was possible also in the present study to demonstrate that the results of anti-D assay of 72/229 in terms of 64/16 did not differ significantly from the previously assigned potency of 11.5 IU anti-D per ampoule.

Various workers have commented on the variables involved in the anti-D assay using the AutoAnalyzer. The results of inter-laboratory anti-D assays can vary up to ±100% of the mean (Gunson, unpublished observation) if care is not taken to standardise the method. Participants in the present study were asked to follow a detailed protocol, and the maximum percentage deviation of any individual laboratory’s combined potency estimate from the overall mean was 15% for 68/419 and 19% for 72/229.

While the aim of the collaborative study to redefine the anti-D content of 68/419 was achieved, the above results also indicate that the protocol devised could form an acceptable model for future calibration studies of anti-D standards.

Of the variables, error in preparation of dilutions is probably the most important factor and, in particular, the reconstitution of freeze-dried material. The reconstitution procedures used in the present study are recommended for routine use since aliquots of the reconstituted material can be frozen at −40°C for up to three months without loss of potency.

This results in economy in the use of the standards and, with respect to the British Working Standard 72/229, enough material is available for its use as a true working standard and not purely as reference material. Use of a uniform standard for anti-D assays of plasma and serum will also help to improve reproducibility. The International Reference Preparation 68/419 is suitable only for anti-D assay of immunoglobulin and should not be used as a standard for assays carried out using plasma or serum.

An opportunity was taken in the study to compare the use of 5% with 20% test cell suspensions in the assay. Although there was a significant difference between the two combined potency estimates for both 68/419 and 72/229, in practice the differences were relatively small in absolute terms. We have concluded that either red cell suspension can be used satisfactorily, and the 5% suspension has certain advantages both in economy of test cells and in increasing the sensitivity of the method when assaying low concentrations of anti-D in individual test antisera. This can be particularly valuable in the anti-D assay of certain antenatal samples.

We acknowledge the contribution made by the participants in this study and thank Drs DR Bangham and DP Thomas for helpful advice, and Mr MJ Tydeman for assistance in the statistical analyses.

References

8. Gunson HH, Phillips PK, Stratton F. The adverse
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