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The British reference preparation for influenza virus haemagglutinin

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SUMMARY

The two methods for measuring the haemagglutinin content of an influenza virus suspension are the haemagglutinating (HA) and chick cell agglutinating (CCA) techniques and both measure the same biological activity. With the establishment of an international reference preparation for influenza virus haemagglutinin (type A), however, it seems logical to express the haemagglutinin content of influenza vaccines in international units. Accordingly a collaborative study was arranged in order to obtain agreement on the number of units to be assigned to a British reference preparation for influenza haemagglutinin. It was agreed that the preparation contains 190 i.u. per ampoule and 1 i.u. is contained in 0·0622 mg. of the dried material.

INTRODUCTION

It is agreed that there is a correlation between the haemagglutinin content of whole virus influenza vaccines and the ability of the vaccine to protect against the disease, but for many years there has been a controversy over the most accurate method of measuring the potency of influenza vaccine. The haemagglutinin content, which has been taken as an index of potency, has been expressed either as haemagglutinating activity (HA) or chick cell agglutinating activity (CCA). Both measure the ability of the virus to agglutinate red blood cells of the chicken but the absolute values of the activity differ by the two methods.

The World Health Organization (Report, 1968) established an International Unit of influenza haemagglutinin activity and it seemed logical therefore to start expressing the potency of influenza vaccine in International Units rather than in HA or CCA. The World Health Organization International Laboratory for Biological Standardization in Copenhagen has now arranged two series of collaborative assays on a large number of influenza virus vaccines and the results of these studies are soon to be published (Krag & Weis Bentzon, 1971). In order to calibrate a proposed British Reference Preparation in International Units, therefore, a collaborative study was arranged and the purpose of this report is to present the findings and establish the Reference Preparation.

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DESIGN OF THE STUDY

The laboratories and work programme

Four laboratories took part in the study, three of them determined the activity of the vaccines by the HA and three of them by the CCA method.

Each laboratory was requested to determine the activity of five coded preparations A to E in International Units by comparison with the International Reference Preparation and to obtain a ratio of activity for each preparation with the proposed British Reference Preparation. The laboratories were also asked to include in each titration their own working reference preparation.

On each of 3 working days each coded preparation and the proposed British Reference Preparation were titrated in parallel using dilution steps differing by about 30%. On a fourth working day the titrations were repeated but on this occasion the International Reference Preparation was included which was known to contain 200 International Units per ampoule. The International Reference Preparation was to be used in accordance with the recommendations of the World Health Organization collaborative assay of 1968.

The vaccines

The following vaccines were distributed:

Vaccine A. Allantoic fluid containing A_2/ENG/76/66, centrifuged and distributed in 1 ml. volumes in ampoules. Snap frozen and stored at $-70°$ C.

Vaccine B. Allantoic fluid containing B/ENG/5/66, centrifuged and distributed in 1 ml. volumes in ampoules. Snap frozen and stored at $-70°$ C.

Vaccine C. The proposed British Reference Preparation reconstituted with distilled water and distributed in 1 ml. volumes in ampoules. Snap frozen and stored at $-70°$ C.

Vaccine D. Vaccine C diluted 1/4 in distilled water was distributed in 1 ml. volumes in ampoules. Snap frozen and stored at $-70°$ C.

Vaccine E. Normal allantoic fluid from 10-day-old embryonated hen's eggs centrifuged and distributed in 1 ml. volumes in ampoules. Snap frozen and stored at $-70°$ C.

Proposed British Reference Preparation. Distributed as a freeze-dried preparation.

International Reference Preparation. Distributed as a freeze-dried preparation and containing 200 i.u./ampoule.

Before despatch the vaccines were transferred from the $-70°$ C. refrigerator and packed into expanded polystyrene boxes containing solid carbon dioxide. Laboratories 1 and 2 received the vaccines still frozen and they were stored at $-60°$ C. Laboratories 3 and 4 received the vaccines thawed but cold. Laboratory 3 refroze the vaccines at $-70°$ C. whereas Laboratory 4 stored the vaccines at $+4$ °C. before starting the assays.

ASSAY SYSTEMS

Haemagglutination titrations

The haemagglutinin content was titrated by the method recommended by the World Health Organization (1953).

Diluted preparations were titrated in WHO pattern perspex plates using two-fold dilutions in 0·25 ml. of M/100 phosphate buffered saline, pH 7·2. To each cup 0·25 ml. of an 0·5 % chick erythrocyte suspension were added, and the end point read as that dilution giving 50 % haemagglutination according to the pattern method. The end point of activity was read after standing for 1 hr. at room temperature (20–22° C.) and where the end point fell between dilutions, it was calculated by interpolation.

Erythrocyte suspensions were standardized by colorimetric or densitometric methods, according to the routine practice in each laboratory. A pool of erythrocytes was made from several fowls and used for from 1 to 3 days.

All preparations were titrated in parallel on the same day.

Chick cell agglutination estimations (*densitometric readings*)

The CCA estimations were based on the original principles of Hirst and Pickles (1942) and modified by Miller (1965) which eliminates the subjective reading of the pattern test and substitutes an objective reading of optical densities (O.D.).

For these estimations, different types of colorimeter or densitometer were used in the different laboratories. The 50 % end-point was determined graphically or by calculation as that dilution lying half way between the O.D. reading of a standard suspension of chick red cells and the O.D. reading of a maximally agglutinated standard suspension of chick red cells.

RESULTS

Haemagglutination titrations

The statistical analysis of the results obtained by measuring the haemagglutinin activity of the virus preparations is shown in Tables 1 to 3 and Fig. 1.

Preparations A, B, C and D were compared with the proposed British Reference Preparation and since it was known that preparation E was a control preparation containing normal allantoic fluid, it has been excluded from any statistical analysis.

In Table 1 the details of the assays comparing preparations A, B, C, D, the proposed British Reference Preparation and the International Reference Preparation are shown. In addition Laboratory 1 included two of their own preparations and these have also been included in the results.

Potency ratios of preparations A, B, C and D were estimated in terms of the proposed British Reference Preparation using the log titres for each preparation measured on the same day or, in the case of Laboratory 1, log titres for the preparations in the assays in which the same chick erythrocytes were used. The variance of each potency was calculated from the pooled variance between log titres of each preparation assayed at the same time and the weights (reciprocal of the variance) were estimated.

When tests for homogeneity of the log potencies of each preparation from each laboratory were carried out it was found that the only estimates of log potency that were homogeneous within a laboratory were preparation D from Laboratories 2 and 3 and preparation A from Laboratory 1. Thus unweighted geometric mean potencies and confidence limits based on the direct estimate of variance of the

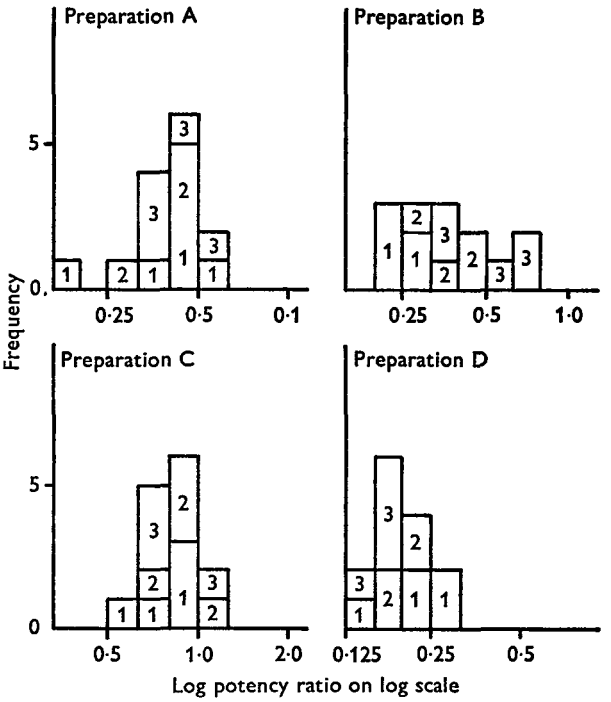

Fig. 1. Frequency distribution of log potency ratios of preparations A, B, C and D in terms of the proposed British Reference Preparation (HA method)

Table 1. *Details of the thirteen assays by the HA test method included in the analysis*

Laboratory number	Preparations compared	No. of assays
1	A, B, C, D, BRP 2 lab. preps.	3
	A, B, C, D, BRP IRP, 2 lab. preps.	2
2	A, B, C, D, BRP	3
	A, B, C, D, BRP IRP	1
3	A, B, C, D, BRP	3
	A, B, C, D, BRP IRP	1

BRP = Proposed British Reference Preparation.
IRP = International Reference Preparation.
Lab. preps. = various laboratory preparations.

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log potencies have been computed for each of the four preparations from the three laboratories. These results are shown in Table 2 and frequency distributions of the log potencies, one for each preparation, are given in Fig. 1.

Table 2. *Potency ratios of preparations in terms of the proposed British Reference Preparation by the HA test method*

Preparation	Laboratory 1	Laboratory 2	Laboratory 3	Combined results
A	0·375 0·219–0·640	0·390 0·280–0·542	0·409 0·328–0·510	0·391 0·333–0·460
B	0·245 0·221–0·271	0·364 0·249–0·534	0·503 0·351–0·720	0·354 0·285–0·440
C	0·751 0·554–1·018	0·846 0·624–1·146	1·017 0·563–1·835	0·804 0·716–0·903
D	0·227 0·158–0·328	0·197 0·166–0·240	0·168 0·149–0·190	0·196 0·172–0·223
X*	0·207 0·175–0·245	—	—	—
Y*	8·199 7·216–9·315	—	—	—

Values given as geometric mean potency ratios and 95% confidence limits based on a direct estimate of variance of the log potency ratios.

* Preparations from Lab. 1.

Table 3. *Potency of the proposed British Reference Preparation in terms of the International Reference Preparation by the HA test method*

Laboratory number	Potency ratio	Overall unweighted mean potency ratio and 95% confidence limits	Potency i.u. per ampoule
1	0·950 0·680	—	190 136
2	1·197	0·946 0·645–1·387	239
3	1·037	—	207

Preparation C, which was the reconstituted and refrozen proposed British Reference Preparation, should have had a ratio of 1 with the proposed British Reference and the results agree quite well with this. It is also interesting to note that preparation D, which was a 1/4 dilution of C, has indeed been shown to have a ratio of about 1:4 to the proposed British Reference Preparation.

The only preparation for which the variability between the mean log potencies from the three laboratories is significantly greater ($0{\cdot}01 > p > 0{\cdot}001$) than the variability within the laboratories is preparation B. The overall unweighted geometric mean potency ratios and confidence limits are also given in Table 2 and the results of the two additional preparations assayed by Laboratory 1 have been included.

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There were only four occasions when the proposed British Reference Preparation and the International Reference Preparation were compared at the same time and so within laboratory variability of the log potencies could not be examined. Weights for the log potencies were estimated in the same manner as described above, but the test for homogeneity showed the log potencies to be heterogeneous and so an overall unweighted mean potency ratio has been computed together with confidence limits based on the direct estimate of variance. The individual potencies and the overall results are given in Table 3.

Chick cell agglutination assays (densitometric method)

The results of the statistical analysis of the CCA assays for preparations A, B, C and D in terms of the proposed British Reference Preparation are shown in Tables 4–6 and Fig. 2.

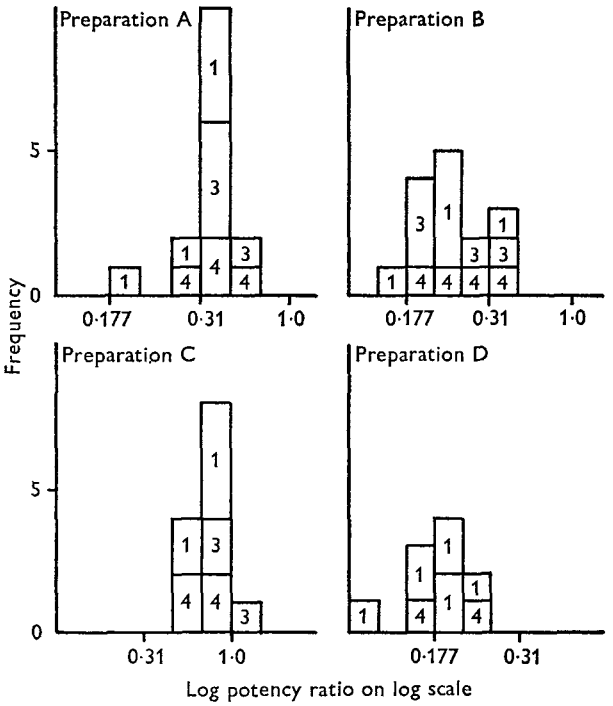

Fig. 2. Frequency distribution of log potency ratios of preparations A, B, C and D in terms of the proposed British Reference Preparation (CCA method)

All CCA assays have been analysed as parallel line assays (Finney, 1952). Each log dose response line was examined by eye and statistical analysis carried out on the data taken from the linear portion of it, omitting the data from the doses that gave no response or a maximum response. Thus the reader may not necessarily obtain exactly the same results by comparing end points of titrations.

In Table 4 the details of the assays comparing preparations A, B, C, D, the proposed British Reference Preparation and the International Reference Prepara-

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tion are shown. In addition each laboratory used its own laboratory preparation as a house standard.

Potency ratios of preparations A, B, C and D were estimated in terms of the proposed British Reference Preparation using the log titres measured on the same day or, in the case of laboratory 1, log titres for the preparations in the assays in which the same chick erythrocytes were used. For the same reasons as given for the results from the HA assays, confidence limits based on the direct estimates of variance of the log potencies have been computed and all results have been

Table 4. *Details of the fifteen assays by the CCA test method included in the analysis*

Laboratory number	Preparations compared	No. of assays
1	A, B, C, D, BRP, 2 lab. preps.	4
	A, B, C, D, BRP, IRP, 2 lab. preps.	2
3	A, B, C, BRP, lab. prep.	3
	A, B, BRP, lab. prep.	1
	A, B, BRP, IRP, lab. prep.	1
4	A, B, C, D, BRP, lab. prep.	3
	A, B, C, D, BRP, lab. prep. IRP	1

BRP = Proposed British Reference Preparation.
IRP = International Reference Preparation.
Lab. prep. = various laboratory preparations.

Table 5. *Potency ratios of preparations in terms of proposed British Reference Preparation by the CCA test method*

Preparation	Laboratory 1	Laboratory 3†	Laboratory 4†	Combined potency ratio
A	0·301 0·226–0·400	0·384 0·327–0·450	0·371 0·273–0·505	0·345 0·304–0·399
B	0·244 0·191–0·311	0·241 0·173–0·337	0·251 0·182–0·345	0·245 0·214–0·279
C	0·769 0·638–0·927	0·866 0·622–1·210	0·704 0·572–0·866	0·769 0·694–0·858
D	0·177 0·135–0·232	—	0·199 0·158–0·251	0·186 0·159–0·221
X* Lab. standards	0·237 0·177–0·317	2·171 1·519–3·102	1·821 1·598–2·075	—
Y*	7·617 6·058–9·557	—	—	—

Values given are geometric mean potency ratios and 95% confidence limits based on a direct estimate of variance of the log potency ratios.

* Various laboratory preparations.

† Mean and range.

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presented in the same manner. The results are shown in Table 5 and the frequency distribution of the log potencies for each preparation are shown in Fig. 2. Again the results show that the ratio of preparation C to the proposed British Reference Preparation approximates to unity and that the ratio of D is about 1:4.

As with the HA assay method, there were only four occasions when the proposed British Reference Preparation and the International Reference Preparation were compared at the same time and so within laboratory variability of the log potencies could not be examined. The overall unweighted mean potency ratios and ranges however, are shown in Table 6.

Table 6. *Potency of the proposed British Reference Preparation in terms of the International Reference Preparation by the CCA test method*

Laboratory	Potency ratio	Overall unweighted mean potency ratio and range	Potency i.u. per ampoule
1	0·948	0·905	189
3	1·041	0·718–1·040	208
4	0·718		143

CONCLUSIONS

The collaborative assay of five unknown virus preparations by four laboratories using two methods of assay (the HA and CCA) has shown that the absolute values of haemagglutinating activity obtained in any laboratory at any time are dependent upon many factors such as the method of test, and the quality and quantity of the red blood cells used in the test. The results are much more uniform, however, when a reference preparation to which a unit has been assigned is included in each test.

From the results of the HA tests it was shown that the British Reference Preparation had an estimated potency of 189·2 International Units per ampoule with confidence limits of 129·0–277·4 i.u. and the CCA results were in agreement with this.

The British Reference Preparation 64/13 for haemagglutination, which is a stable dried preparation of influenza virus with ampoule to ampoule uniformity, therefore, has been assigned a potency of 190 i.u. per ampoule. The dried weight in each ampoule is 11·82 mg. (range 11·59 to 12·08 mg) and therefore, 1 i.u. is contained in 0·0622 mg.

It would be meaningless to assign an HA or CCA unit in terms of International Units since these ratios differ depending upon the test system and the laboratory doing the test.

We are grateful to Beecham Research Laboratories, Evans Medical Ltd. and Philips Duphar for taking part in the collaborative assay.

Evans Medical Ltd. very kindly supplied the virus suspension for the British Reference Preparation, which was dried by the Biological Standards Division of the National Institute for Medical Research.

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