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International Reference Preparation of Rheumatoid Arthritis Serum

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The National Institute for Medical Research, London, England, was requested by the WHO Expert Committee on Biological Standardization to arrange a collaborative study of the serum pool they had obtained, to determine its suitability to serve as an international reference preparation of rheumatoid arthritis serum. A batch of this serum was assayed by 11 laboratories in 7 countries against 30 test preparations. On the basis of the results obtained, the serum has been established as the International Reference Preparation of Rheumatoid Arthritis Serum and the International Unit of Rheumatoid Arthritis Serum has been defined as the activity contained in 0.171 mg of the international reference preparation.

A description is also given of the British reference preparation of rabbit antibody to sheep red blood cells (amboceptor) and this material was also tested in the collaborative study.

After the meeting of the 10th International Congress of Rheumatology in Rome, an international study of 1 positive and 1 negative pool of human sera taken from 50 rheumatoid arthritis patients and 50 normal control patients, respectively, was undertaken by Bozsoky (1963). The titre, reported by 19 laboratories, for the positive serum varied from 32 to about 30 000 (940-fold) by the sheep cell agglutination test, and from 64 to 20 000 (310-fold) by the latex fixation test in 14 laboratories. The technique of test varied considerably between laboratories. Also, Nasou et al. (1963) found considerable variation between estimates of titre of rheumatoid factor by the F.II bentonite flocculation test, as performed in 6 laboratories.

Collection of material designed to form a British standard serum was commenced in April 1963 and the WHO Expert Committee on Biological Standardization (1964) requested the National Institute for Medical Research in collaboration with the World Health Organization to study the suitability of this material to serve as an international reference preparation.

It was thought that the use of a reference preparation of rheumatoid factor activity might be useful in correlating results between laboratories and within laboratories, and that relative potencies estimated against a reference preparation would be more uniform than the titres hitherto estimated.

International Reference Preparation of Rheumatoid Arthritis Serum

During 1963, sera from patients with rheumatoid arthritis were requested from a number of centres in the United Kingdom of Great Britain and Northern Ireland. Samples of serum were sent by post, without refrigeration, but after receipt were stored at −20°C. The sera contained 0.01% of sodium azide and were judged to be of medium titre on the evidence of titrations of rheumatoid factor carried out in the donating laboratories. Later, 197 sera were thawed, pooled and filtered through Millipore membranes of average pore diameter 0.45 μ and the pool was divided into 3 batches, A, B and C. In January and February 1964, batches A and B were each filled in 0.2-ml volumes into approximately 3500 ampoules and batch C into a smaller number of ampoules. The material was freeze-dried in the ampoules and subjected to 2–3 weeks secondary desiccation over P_2O_5 *in vacuo*; the

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ampoules were filled with dry pure nitrogen and sealed hermetically. The mean wet weight of the contents of 45 ampoules of batch A determined during filling was 201.9 mg and the range was 199.4 mg–204.1 mg. The dry weight of contents was determined on 6 ampoules of batch A, giving a mean of 17.1 mg and a range of 16.3 mg–17.45 mg. Batch A, coded 64/1, was of 3500 ampoules and was set aside as the proposed international reference preparation of rheumatoid arthritis serum. Batch B was coded 64/2 and reserved as the First British Standard for Rheumatoid Arthritis Serum. The mean wet weight of contents of 64/2 was 201.6 mg and the range was 199.5 mg–204.7 mg.

Rabbit antibody to sheep red blood cells (amboceptor)

A total of 25 sandy-lop rabbits was immunized at the National Institute for Medical Research by a series of 5 or 6 injections of sheep red blood cells. Each injection was of 5 ml of 5% washed sheep red blood cells and all but a few of the injections were given by the intravenous route. Some trouble with anaphylactic reactions was experienced during the immunization. Five days after the last injection the rabbits were bled and their sera were pooled. In 1965, part of the pool of sera was diluted in physiological saline by adding 3180 ml of saline, pH 6.7, to 1060 ml of serum. The diluted serum was distributed into ampoules by volume (1.0 ml) and freeze-dried. The mean wet weight of the contents of 55 ampoules determined during filling was 1.016 g with a total range of $\pm$ 0.71%. The whole freeze-drying batch, comprising 3000 ampoules, was set aside as the first British reference preparation of rabbit antibody to sheep red blood cells (amboceptor). It was coded 66/236. This material was designed to serve as antigen for coating red cells in the sheep red cell agglutination test for rheumatoid factor. It was assigned a unitage of 1000 British units per ampoule.

Suggested technique for the sheep cell agglutination test

Information submitted by a number of laboratories in the United Kingdom was collated and a suggested technique of test for use in Part I of the collaborative assay was compiled at the National Institute for Medical Research. After the completion of Part I of the collaborative study, a conference of participants from the United Kingdom made further suggestions, and a modified technique of test was described. This modification was recommended for use in Part II of the collaborative study alongside each local technique. Details of the recommended technique are given in Annex 1 at the end of this paper.

THE COLLABORATIVE STUDY

A collaborative study of batch A of rheumatoid arthritis serum (64/1) was undertaken by 11 laboratories in 7 countries. The study was conducted in 2 parts.

Part I was a preliminary enquiry regarding (*a*) reproducibility of results between and within laboratories by the use of the sheep cell agglutination test when a reference preparation of rheumatoid arthritis serum was used, (*b*) the effect of using either a local technique or a common suggested technique for the sheep cell agglutination test and (*c*) the profiles of pool II amboceptor and of each local amboceptor, that is, the rate at which the apparent titre of the proposed international reference preparation for rheumatoid arthritis serum decreased when the concentration of amboceptor, used to coat red cells, was progressively reduced. The higher this rate, the steeper the profile of the amboceptor.

Part II of the collaborative assay was a more formal examination of several questions, primarily (*a*) whether any standard was useful for the estimation of rheumatoid arthritis serum activity and (*b*) if a standard was useful, whether 64/1 would serve as such a standard.

Participants were asked to assay, in terms of the proposed international reference preparation, the same material which had been degraded by heat and also 30 coded human sera, thought to contain low-, medium- and high-titre rheumatoid factor. These sera had been collected from patients with rheumatoid arthritis, by laboratory 8. They were freeze-dried at the National Institute for Medical Research.

In both parts of the collaborative study, participants were asked to use the sheep cell agglutination test according to the recommended techniques. It should be noted that the recommended technique was different for Part I and Part II, one difference being that in Part II the concentration of red cells used was half the concentration recommended for Part I: this might have been expected to increase by 2-fold the apparent titre of rheumatoid factor in Part II. Participants were also asked to use the sheep cell agglutination test done by the local technique as used by each laboratory and also other tests, e.g., the latex fixation test, as performed in each local laboratory. In the event, the 11 participants who

submitted results for the sheep cell agglutination test used the suggested techniques and 10 also used their own local technique.

In using the suggested technique in Part II, 4 participants did not inactivate the sera (laboratories no. 1, 2, 4 and 10) and 1 participant (laboratory 4) did not absorb sera with red cells. Laboratory 1 absorbed sera only whenever it was deemed necessary because of non-specific agglutination.

The results from participants who used these modifications of the suggested technique were close to the means of the results of all other laboratories.

RESULTS AND STATISTICAL ANALYSIS

Results were received from 10 laboratories in Part I and from 11 in Part II. The names of participants are listed in Annex 2. Each laboratory is designated by a code number that bears no relation to the order of the listing of participants.

Rabbit amboceptor

In Part I the titre of the preparation of rabbit amboceptor (66/236) varied between 200 and 800 between laboratories, and in Part II from 200 to 1600 under comparable conditions. This was considered a satisfactory agreement when one designated method was used.

The profile of amboceptor gives some index of its quality and other things being equal an amboceptor with a less steep slope is more useful in the sheep cell agglutination test. The slope of 66/236, as determined by the participants, was approximately the same as that of the amboceptors in general use in the various laboratories. In both Part I and Part II, amboceptor 66/236 detected in good titre all the rheumatoid factor sera which had been tested and found positive by other methods. The titration end-points of rheumatoid factor and of 66/236 were sharp and 66/236 was found to be easy to reconstitute to a clear solution. For these reasons it was considered to be a suitable material to serve as a reference preparation for rabbit antibody to sheep red blood cells (amboceptor) and in 1965 it was established as the British reference preparation for this activity.

The proposed international reference preparation of rheumatoid arthritis serum

In the original requests to participants, particular emphasis was placed on the use of the sheep cell agglutination test and most of the results submitted were obtained by the use of some modification of this test.

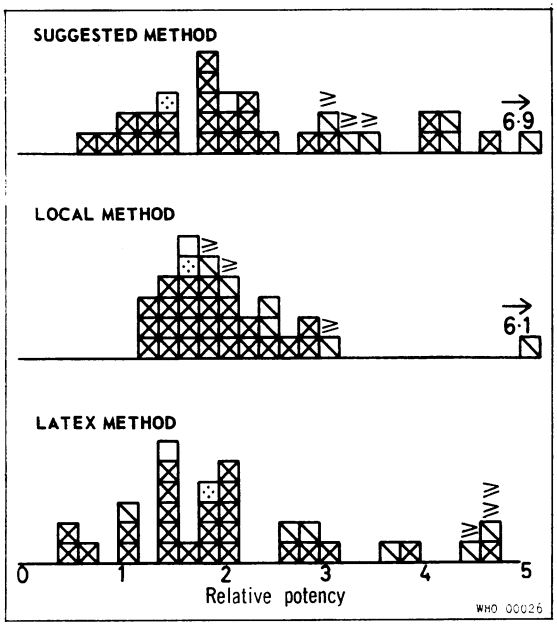
In Part I the inter-laboratory variation in estimated titres of the proposed international reference preparation of rheumatoid arthritis serum was from 320 to 1600. Each laboratory made 5 estimates of the titre of 1 sample and obtained an intra-laboratory variation of up to 4-fold. Furthermore, the relative potency of coded dilutions of the proposed international reference preparation were estimated by each laboratory within 2-fold above or below the real dilutions. It was of interest that the local methods of the sheep cell agglutination test produced as reliable and consistent results as did the method suggested by the National Institute for Medical Research.

In Part II of the collaborative study, results were received from 11 laboratories and a preliminary assessment of these results was presented in 1965 to the WHO Expert Committee on Biological Standardization (1966). Results were later submitted to further statistical analysis in Copenhagen and this was reported to the Expert Committee in 1968. The following is a summary of the statistical analysis.

The 30 test sera were considered in 2 groups—24 medium or strongly reactive sera and 6 weak sera—and the basic analysis concerned the results of the 24 strong sera examined by 8 of the 11 laboratories. Log potencies (to the base 2) of the sera in relation to the average of all 24 sera were calculated (see accompanying figure). The within-laboratory variance of the log potencies was: for the suggested method 0.60, for the local method 0.45 and for the latex method 0.77. These were about 3 times greater than the average variances (0.18) due to experimental error assessed from duplicate readings. The range of log potencies was found to be normally distributed within 95% limits for the local method. For the other methods the distribution was less normal with a somewhat wider scatter. It was considered that a standard was useful in this test, and that where a standard was used in relation to the local methods the proposed reference preparation of rheumatoid arthritis serum (64/1) was a suitable material for such a standard.

It is often suggested that an attempt by all laboratories to use a single common method, here exemplified by the " suggested method ", would increase apparent agreement between laboratories. In the present instance this was the case, the variation of

DISTRIBUTION OF POTENCY RANGE FOR 32 HUMAN SERA FROM RHEUMATOID ARTHRITIS PATIENTS [a]



Weak sera
Medium strength sera
Serum A heated to 26°C
Serum A heated to 37°C

[a] The suggested method was used in laboratories no. 1-7 and 9; local methods were used in laboratories no. 1-7 and 9; the latex method was used in laboratories no. 2, 4, 5, 7 and 9.

The sign ⩾ denotes those cases in which, for one or more laboratories, the titrations were incomplete and for which only a lower limit can be given.

titres between laboratories being reduced to some extent. However, the suggested method yielded less consistent estimates of potency relative to 64/1 than did the local methods, with all sera (see the table and the accompanying figure).

Results obtained by a few laboratories which used the latex method varied more widely than those obtained by other methods, the variance of the average differences between titres of sera being 0.901: this was significantly higher than the expected value of 0.244. This is assumed to mean that the latex method reflected other properties of the sera than did the suggested and local methods for the sheep cell agglutination test. It is noted that in the survey reported by Bozsoky (1963) results with the latex test varied less than with the sheep cell agglutination test.

It may be relevant that in the sheep cell agglutination test rabbit globulin is the reactant and in the latex test, human globulin. Furthermore the colloidal stability of latex particles is a much more critical factor in determining the end-point of agglutination than is the stability of red blood cells.

For each of the 30 patients who provided test sera, a list of clinical signs and symptoms was made. These included the duration of the clinical signs and symptoms, the age at onset, the sex, the functional capacity estimated clinically, the haemoglobin concentration of the blood, the erythrocyte sedimentation rate, the treatment given to the patient, whether chloroquine or prednisolone or phenylbutazone was administered, and whether the clinical condition was

DISTRIBUTION OF RANGES OF TITRE AND RELATIVE POTENCY FOR 24 SERA FOR SUGGESTED METHOD AND FOR LOCAL METHOD

Method of estimation	Ranges				Average range
	≤2.0	2.01–4.0	4.01–6.0	>6.0	
			$\log_2$-titres [a]		
Suggested method (8 laboratories)	7 (29)	14 (58)	3 (13)	0	2.92
Local method (8 laboratories)	0	0	13 (54)	11 (46)	6.26
			$\log_2$-potencies relative to the average of 24 sera [a]		
Suggested method (8 laboratories)	14 (58)	7 (29)	3 (13)	0	2.13
Local method (8 laboratories)	15 (63)	9 (37)	0	0	1.91

[a] Figures in parentheses show the percentage distribution.

stationary or variable, as estimated by the erythrocyte sedimentation rate or the sheep cell agglutination test (as done in the local laboratories) or from the clinical condition. No correlation was observed between these features and the strength of the serum as measured by the sheep cell agglutination test in the collaborative study, except that the 3 weakest sera were connected with short duration and low index for loss of functional capacity.

The proposed international reference preparation of rheumatoid arthritis serum, 64/1, was, of course, a pool of 197 sera. The average potency of 24 strong or medium sera in relation to this proposed reference preparation varied according to the geographical location of the testing laboratory, when the suggested method for the sheep cell agglutination test was used. The estimates were consistently higher in 4 continental European laboratories, numbered 2, 4, 6 and 7, than in the 5 laboratories in the United Kingdom and the 1 laboratory in the USA. One continental European laboratory, no. 11, did not fit into this pattern. No explanation of this geographical effect is immediately apparent, but the question will be considered in a paper now in preparation by S. G. Anderson and S. Cooper.

The proposed reference preparation, which had been stored at +26°C was not significantly less potent than the material stored at —10°C. However, the same preparation stored at 37°C for 6 months was found to have decreased in potency to between 24% and 38% of its original potency. The value of K_{-10} has been estimated according to the method described by Jerne & Perry (1956). Using the results obtained by the local method (average titres for laboratories numbered 1–7 and 9) the estimated value is 0.01 $\log_2$ units per month. This means that a reduction to 50% is expected after 100 months or about 8 years of storage at −10°C. The 95% limits of error are, however, 1 year to 250 years. The values found by Jerne & Perry for freeze-dried antitoxins ranged between 2000 and 20 000 years.

Three laboratories provided information about the titres which they considered just positive in the sheep cell agglutination test, that is, the lowest serum potencies taken to indicate a diagnosis of rheumatoid arthritis in adults. The critical potency calculated for laboratory 7 was 6 units per ml, for laboratory 8 it was 15 units per ml and for laboratory 5 it was 32 units per ml. One of the present authors (V. H.) accepts a value of 12.5 units per ml as the lowest potency of diagnostic value.

DISCUSSION AND CONCLUSIONS

The sera from 197 rheumatoid arthritis patients were pooled, distributed into ampoules and freeze-dried, and the dried material was used in the United Kingdom as the British national standard. In 1964 the World Health Organization accepted the material as the International Reference Preparation of Rheumatoid Arthritis Serum. In 1968, in the light of the further statistical analyses of the data obtained from the collaborative study, and in accordance with the authorization given in 1968 by the WHO Expert Committee on Biological Standardization (1969), the International Unit of Rheumatoid Arthritis Serum was defined and made equal to the British Unit so that 1 International Unit was contained in 0.171 mg of dry powder. For practical purposes it can be taken that each ampoule of the international reference preparation contains 100 International Units.

The contents of each ampoule may be reconstituted by the addition of 0.2 ml of either distilled water or buffered saline, and the whole contents of the ampoule should be washed out with buffered saline repeatedly to make a total volume of say 4 ml. This solution will then contain 25 IU per ml.

In a collaborative assay of the proposed international reference preparation of rheumatoid arthritis serum the British reference preparation of rabbit antibody to sheep red blood cells (amboceptor) was included. Acceptable results were obtained with both substances by 11 laboratories in 5 countries. The end-points of the sheep cell agglutination test were satisfactorily clear.

A statistical analysis of the results of the collaborative assay concluded that the proposed international reference preparation was useful as a reference in reducing the effect of inter-laboratory differences in sheep cell agglutination titres determined by local methods. There was some evidence that the latex test used by 4 of the laboratories provided less concordant results and measured activity different from that measured by the sheep cell agglutination test. The variations in relative potencies encountered with the local methods with medium and strong sera lay within a 14-fold range. For weak sera in some laboratories larger variations occurred. The stability of the reference preparation was judged to be satisfactory but it was thought that the preparation should be kept under observation from this point of view.

ACKNOWLEDGEMENTS

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RÉSUMÉ

PRÉPARATION INTERNATIONALE DE RÉFÉRENCE DE SÉRUM ANTI-ARTHRITE RHUMATOÏDE

En 1963, le Comité OMS d'experts de la Standardisation biologique avait demandé au National Institute for Medical Research de Londres d'organiser, en collaboration avec l'OMS, l'étude collective d'un mélange lyophilisé de 197 sérums prélevés chez des malades atteints d'arthrite rhumatoïde. Cette préparation a fait l'objet, dans 11 laboratoires de 7 pays, d'un titrage comparatif vis-à-vis de 30 préparations d'épreuve. Les résultats en ont été analysés au Statens Seruminstitut de Copenhague. Sur la base de cette analyse, le Comité OMS d'experts de la Standardisation biologique a décidé de constituer le matériel considéré en préparation internationale de référence de sérum anti-arthrite rhumatoïde. L'unité internationale de sérum anti-arthrite rhumatoïde a été définie comme l'activité de 0,171 mg de la préparation internationale de référence.

Au cours de cette étude, les laboratoires participants ont utilisé la préparation d'ambocepteur adoptée en 1965 comme préparation britannique de référence d'anticorps de lapin anti-érythrocytes de mouton.

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Annex 1

A SUGGESTED TECHNIQUE FOR THE SHEEP CELL AGGLUTINATION TEST FOR RHEUMATOID FACTORS FOR USE IN THE COLLABORATIVE ASSAY OF THE PROPOSED REFERENCE PREPARATION

REAGENTS

Alsever's solution

Dextrose	20.5 g	
Sodium citrate	8.0 g	(Analar $Na_3C_6H_5O_7 \cdot 2H_2O$ MW 294.11)
Citric acid	0.552 g	(Analar $H_3C_6H_5O_7 \cdot H_2O$ MW 210.15)
Sodium chloride	4.2 g	
Water to	1000 ml	
Seitz filter	pH 6.1	

Phosphate-buffered saline

Prepare as 3 separate solutions:

(a) Sodium dihydrogen phosphate 24.6 gm per litre (Analar $NaH_2PO_4 \cdot 2H_2O$: MW 156.01)

(b) Disodium hydrogen phosphate 22.4 gm per litre (Analar Na_2HPO_4: MW 141.97)

(c) Sodium chloride 8.5 gm per litre

Mix *(a)* and *(b)* to give pH 7.4 and then mix 1 part of *(a)* + *(b)* with 9 parts of *(c)*.

Sheep cells

Take sheep blood into Alsever's solution and store at 4°C. Wash 3 times, each time making up to 5% in phosphate-buffered saline, and centrifuging on the bench at 500 *g* for 15 min. After 3 washings the supernatant should be clear and free from colour. If this condition is not fulfilled the cells should be discarded. Make up as 1% cells and as 25% cells in phosphate-buffered saline.

N.B.: 1% = 2×10^8 cells per ml

The International Reference Preparation of Rheumatoid Arthritis Serum

The reconstitution of this reference preparation is discussed in the body of the paper.

The British reference preparation of rabbit antibody to sheep red blood cells (amboceptor)

Dilute original serum 1: 100 in physiological saline. Store in aliquots at −20°C. Thaw for use but do not re-freeze.

Test rheumatoid serum

Clot blood at 37°C for 1 hour, and spin at 500 *g* for 20 minutes. Store serum at −20°C. Thaw in water-bath at room temperature. Heat at 56°C for 30 min before use.

PROCEDURE

Absorption of serum

Spin 1.0 ml of 25% sheep cells at 500 *g* for 15 min. Remove supernatant. Add 0.75 ml of a 1: 5 dilution of serum in phosphate-buffered saline. Shake and leave at room temperature for 60 min, then at 4°C for 60 min. Spin at 500 *g* for 15 min at 4°C. Supernatant is considered as a 1: 5 dilution of serum.

Titration of amboceptor (see note below)

Dilute amboceptor in doubling dilutions in phosphate-buffered saline. Add equal volumes of 1% cells. Leave at room temperature for 15 min. Shake. Leave at 4°C for 2 hours. Read patterns of agglutination. Use amboceptor at one-quarter of the concentration of the " + " end-point. At this dilution, amboceptor should show no agglutination of red cells.

Sensitization of red cells

Mix equal volumes of 1% cells and amboceptor at the determined dilution, both in phosphate-buffered saline. Incubate at room temperature for 15 min. If it is desired to store these cells for a short while (e.g., half an hour) this should be at 4°C.

Titration of test serum

Set up doubling dilutions of sera to be tested in phosphate-buffered saline. Include a positive control and a negative control serum. Add an equal volume of 0.5% unsensitized cells to the first tube of each serum dilution. Add an equal volume of 0.5% sensitized cells to all but the first tube of each serum dilution. Leave at 4°C for 18 hours. Read the end-point as the initial dilution of serum in the tube showing a " + " pattern of agglutination. By the initial dilution is meant the dilution of serum before the addition of red cells. The unsensitized cells should show no agglutination.

COMMENTS

The above technique has been compiled from a number of suggestions submitted by laboratories in the United Kingdom. However, the technique assumes the availability of certain laboratory conditions. For more routine use, such as for the testing of large numbers of unknown sera, several simplifications of the method may be desired.

For example rheumatoid sera may be obtained under less rigid conditions of clotting and separation from clot. Amboceptor may be stored satisfactorily for a reasonable time at 4°C, especially in the presence of glycerol or

phenol saline. Similarly, rheumatoid sera may be stored at 4°C for a short while. Titration of amboceptor may not be necessary each time the routine testing of sera has to be performed. Once determined, the appropriate dilution of amboceptor may be used repeatedly, and checked only occasionally.

For purposes of standardization it is recommended that all sera be absorbed with sheep cells, and that phosphate-buffered saline be used as diluent, in order to avoid introducing any other biological agent. However, for routine use it is found that most sera do not require to be absorbed with red cells, and that the proportion requiring absorption is much reduced if 2.5% sheep serum in saline is used as a diluent.

Annex 2

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