Rev. Inst. Med. trop. São Paulo
10(4):209-213, julho-agôsto, 1968

UDC 616.31-07
616-073.56

QUANTITATIVE STUDIES OF COMPLEMENT FIXATION

II — Relative potencies in Complement Fixation as Established in Statistically Controlled Assays

M. SIQUEIRA(1), M. B. ESTEVES(2), P. M. FREIRE(3) and O. G. BIER(4)

SUMMARY

Comparative titrations of five rabbit anti-bovine serum albumin antisera were performed, one of the antisera having been adopted as a reference standard. Data obtained in replicate tests performed on different days and with different batches of complement and red cells provided a sound statistical basis for assessment of titers and relative potencies from a *parallel line assay*.

INTRODUCTION

In a preceding paper [1] the quantitative method described by MAYER et al. [6] was applied to the study of complement (C') — fixation in syphilis with cardiolipin antigen and led to establishment of a linear relationship between the logarithm of the amount of serum and the number of 50 per cent units of complement ($C'H_{50}$) fixed by a constant, optimal amount of antigen. Parallel lines obtained in replicate experiments with 8 different syphilitic sera provided the basis for a statistically controlled assay in which the potencies of the sera could be rigorously compared in relation to one of them taken as a reference standard.

A simplified method with the same characteristics of immunological precision inherent in the method used in [1] was described in the first paper of this series [2]. In the present paper this simplified method was used for estimating relative potencies in C'-fixation experiments with a soluble antigen, crystalline bovine serum-albumin (BSA), and its homologous antibody, rabbit anti-BSA.

MATERIALS AND METHODS

Antigen — Crystallized bovine serum-albumin obtained from Pentex Inc., Kankakee, Ill., was used without further purification. A stock solution containing 0.01 mg of BSA N per ml was preserved frozen at -18°C and diluted as required for the experiments.

Antisera — Five samples of rabbit anti-BSA (21, 22, 23, 24, and 25), containing respectively 0.707, 0.510, 0.479, 1.080, and 0.169 mg AbN/ml, were used. Sera were diluted to contain 40 μg AbN/ml and kept in the freezer at -18°C. Adequate dilutions of these stock solutions were prepared according to results of preliminary tests.

C'-fixation method — The method used for the quantitative study of C' fixation is fully

(1) Associate Professor of Microbiology and Immunology, Escola Paulista de Medicina, São Paulo, Brasil
(2) Assistant, Dept. of Immunology, Instituto Biológico, São Paulo, Brasil
(3) Assistant, Dept. of Virology, Instituto Biológico, São Paulo, Brasil
(4) Professor of Microbiology and Immunology, Escola Paulista de Medicina, São Paulo, Brasil

described in [2]. In preliminary tests the final volume was reduced to 1 ml and reading of supernates was performed with the aid of visual color standards. Data for statistical analysis were obtained from replicate experiments with 2 $C'H_{50}$ and a final volume of 5 ml.

Spectrophotometric readings of supernates were converted into percentages of hemolysis. When these percentages, after adequate transformation (probit, logit, or inverse sine), were plotted against the logarithm of the corresponding amounts of serum, they showed a linear relationship. In the range of the experimental data (20 to 80%), the above mentioned transformations were interchangeable. Angular transformation was adopted for the data subject to statistical analysis since weighting coefficients do not have to be used in calculation of dose-response lines, and the amount of information in each angle is independent of its own value. The methods used for statistical treatment of the data are described in [3].

RESULTS

Experiments with constant C' and red cells — A first series of experiments was carried out with the same batches of complement and red cells. Representative of this experimental material, are the results of a series of assays in which 4 test sera were compared with a reference standard serum. For each assay, the reference standard and 2 test sera were chosen to provide two independent comparisons for a given test serum. Sera were tested at three different dilutions in the range of partial fixation corresponding to 20-80 per cent hemolysis; and in the whole series of assays, a constant ratio (1.1) was maintained between successive dilutions. For each dose of serum, four replicates were tested with independent dilutions of reagents and separate pipettes.

The homogeneity of variances of the angle values observed at each dose level was tested by chi-square (Bartlett's test) and found satisfactory; therefore, a pooled error variance was used in subsequent analysis of the data.

The average "empirical angle" for each dose was then plotted against the logarithm of the corresponding amount of serum. For the data of the assays, a total of 12 provisional regression lines were fitted, by inspection, with a common slope. The "expected angles" read from these lines for each dose level were then replaced by "working angles", and these were used in the calculation of regression lines. The scatter of the "working angles" about the lines when reduced to the same units of the error variance and compared with the latter was found significant ($F = 2072$, $P = 0.025$). Consequently, the variance resulting from scatter about the lines was used to assess the departure from parallelism. A non-significant F-value (0.923) indicated that a common slope ($b = -329.337$) could be applied to all lines.

Relative potencies were estimated from the values of M, the horizontal distance between the lines. The antilogs of M, when multiplied by 100, represent the potencies as percentages of the "standard" (Table I). The precision of the M values was determined according to BLISS [4], using as an estimate of the assay error the pooled variance resulting from scatter about individual lines and departure from parallelism ($s^2 = 3.2995$ with 23 degrees of freedom). Table I gives the confidence limits of relative potencies for the individual assays, as well as the combined values for each test serum. Overlapping confidence limits indicated agreement between the two independent determinations of potency for sera 21, 23, and 24. In the case of serum 25, a significant difference was found between the potency values of the two assays, although the absolute difference amounts to only 2.1%.

Experiments with variable C' and red cells — To check the reproducibility of the results in tests carried out with different preparations of complement and red cells, 9 assays were performed using the combinations of 3 different batches of C' (I, II, and III) and the cells of 3 different sheep (A, B, and C).

Sera 21, 23, and 25 were taken as "Unknowns" and compared in quadruplicated tests at 3 or more dose levels with serum 22, adopted as a "Standard".

The logits of average percentages of hemolysis were plotted against the logarithm of the corresponding serum dilution, and

TABLE I

Relative potencies of five antisera in statistically controlled assays

Standard: Serum 22 (100 per cent)
Unknowns: Sera 21, 23, 24 and 25

Assay no.	Sera	Potencies btw. confidence limits for P = 0.05		
I	21	70.6	*72.1*	73.5
II		71.3	*72.8*	74.2
Combined		71.4	*72.5*	73.5
I	23	139	*142*	145
II		141	*144*	148
Combined		141	*143*	146
I	24	124	*126*	128
II		125	*127*	129
Combined		125	*126*	128
I	25	87.7	*88.7*	89.7
II		89.9	*90.8*	91.7
Combined		87.7	*89.8*	91.9

from the resulting straight lines, titers and slopes were graphically estimated. Relative potencies were calculated as a percentage of the titer of serum 22, used as a reference standard (Table II).

As shown in Table II, there was good agreement between the results obtained in the various assays, the limits of variation of titers and relative potencies being encompassed within the limits of normal variation (Table III).

With the exception of assay no. 5, variations of slope were always within ± 10%.

DISCUSSION

The results presented in this paper confirm earlier findings [1] by pointing out the possibility of estimating C'-fixing potencies on the basis of parallel, straight log-dose response lines fitted to the data for a reference preparation and for the "Unknown" according to well established statistical procedures.

The small value found for $\lambda = s/b$ (0.0055 ± 0.0008) indicates a high degree of precision in this assay technique, as compared with other assays [5], e.g., insulin in the rabbit (0.140 ± 0.016), digitalis in the cat (0.065 ± 0.004), or histamine in the guinea pig gut (0.034 ± 0.004). It is pertinent to recall that previous results with syphilitic sera and cardiolipin antigen [1] obtained by using the quantitative method of MAYER et al. [6] yielded a value of $\lambda = 0.094 \pm 0.0012$. This value indicates a lower degree of precision than that obtained in the present series of experiments with the simplified method described in [2].

The regression lines that provided the basis for establishment of relative potencies were obtained by plotting the logarithm of the amount of serum against the inverse sine of the percentage of lysis. Maltaner's supposed linear relationship between the amount of antibody and the amount of C' required for 50 per cent hemolysis is only an approximation, since this relationship is in fact represented by a sigmoid line.

SIQUEIRA, M.; ESTEVES, M. B.; FREIRE, P. M. & BIER, O. G. — Quantitative studies of complement fixation. II — Relative potencies in complement fixation as established in statistically controlled assays. *Rev. Inst. Med. trop. São Paulo* **10**:209-213, 1968.

TABLE II

Data for the experiments with variable C' and red cells

Assay Nº	Cells	C'	Serum	Titer	Relative Potency %	Slope
1	A	I	22	127	100	.110
			21	96	76	.110
			23	182	143	.100
			25	116	91	.110
2	A	II	22	140	100	.090
			21	100	71	.100
			23	190	136	.085
			25	120	86	.100
3	A	III	22	147	100	.090
			21	110	75	.110
			23	210	143	.100
			25	140	95	.110
4	B	I	22	130	100	.100
			21	98	75	.090
			23	185	142	.110
			25	122	94	.115
5	B	II	22	135	100	.090
			21	100	74	.120
			23	185	137	.110
			25	127	94	.100
6	B	III	22	145	100	.090
			21	110	76	.110
			23	200	138	.100
			25	140	96	.100
7	C	I	22	140	100	.135
			21	103	74	.115
			23	192	137	.135
			25	125	89	.155
8	C	II	22	130	100	.090
			21	98	75	.090
			23	190	146	.095
			25	120	92	.100
9	C	III	22	132	100	.100
			21	100	76	.085
			23	187	142	.085
			25	125	95	.100

TABLE III

Means ($\bar{x}$) and standard deviations ($s_{\bar{x}}$) for the data of Table II

Serum no.	Titers		Confidence limits for P = 0.05	Relative Potencies		Confidence limits for P = 0.05
	$\bar{x}$	$s_{\bar{x}}$		$\bar{x}$	$s_{\bar{x}}$	
21	101.7	4.8	90.7 — 112.7	74.6	1.5	71.2 — 78
23	191.2	8.2	172.3 — 210.1	140.4	3.3	132.8 — 148
25	126.1	8.0	107.7 — 144.5	92.4	3.1	85.3 — 99.5

Deviations from linearity as well as from parallelism in the previously mentioned regression lines may affect the precision or even invalidate any comparison of C'-fixing potencies based upon a *parallel line assay*, as reported by THOMPSON [7] for experimental date obtained by MALTANER and his collaborators. This was not the case, however, for the data obtained in the present set of experiments with a purified, soluble antigen and its corresponding rabbit antiserum.

No significant variation was observed in regard to titers and relative potencies in replicated tests with different batches of complement and red cells.

RESUMO

Estudos quantitativos sôbre fixação de complemento. II — Determinação de potências fixadoras relativas em ensaios estatìsticamente controlados

Utilizando-se o método descrito na contribuição I desta série, quatro soros de coelho anti-soralbumina bovina foram comparados com um quinto sôro adotado como padrão de referência.

Os dados obtidos em provas replicadas, realizadas em dias diferentes ou com lotes diversos de complemento e de glóbulos vermelhos, permitiram dar sólido apoio estatístico à conclusão de que a avaliação quantitativa da reação de fixação do complemento pode ser feita através de um ensaio de linhas paralelas, situando-se entre os bio-ensaios do maior grau de precisão.

REFERENCES

1. BIER, O. G.; SIQUEIRA, M. & FURLANETTO, R. S. — Quantitative complement fixation in syphilis as a statistically controlled assay. *J. Immun.* **74**:51-56, 1955.

2. BIER, O. G.; SIQUEIRA, M. & ESTEVES, M. B. — Quantitative studies of complement fixation. I — A simplified and accurate procedure based on 50 per cent hemolytic end point. *Rev. Inst. Med. trop. São Paulo* **10**: 199-208, 1968.

3. BLISS, C. I. — *The Statistic of Bioassay.* New York, Academic Press Inc., 1952.

4. BLISS, C. I. — Confidence limits for measuring the precision of bioassays. *Biometrics* **12**:491-526, 1956.

5. BLISS, C. I. & CATTEL, M. — Biological assay. *Ann. Rev. Physiol.* **5**:479-539, 1943.

6. MAYER, M. M.; OSLER, A. G.; BIER, O. G. & HEIDELBERGER, M. — Quantitative studies of complement fixation. I — A method. *J. Immun.* **59**:195-206, 1947.

7. THOMPSON, W. R. — On the use of parallel or non-parallel systems of transformed curves of bioassay: illustration in the quantitative complement fixation test. *Biometrics* **4**:197-210, 1948.

Recebido para publicação em 1/3/1968.