J. Hyg., Camb. (1962), **60**, 333
Printed in Great Britain

The titration of toxoplasma-antibody*

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(*Received* 19 *February* 1962)

INTRODUCTION

When infection with *Toxoplasma gondii* (hereafter called the parasite) has taken place, antibodies can be demonstrated in the serum of the patient for many years (van Soestbergen, 1957). The antibody content of the serum can be measured by a serological method (the dye test) which is based upon the observation that the parasites on coming into contact with antibody under appropriate conditions, lose their affinity to methylene blue (Sabin & Feldman, 1948). The parasites which react with the antibody will be referred to as the unstained parasites in contradistinction to the stained parasites.

The significance of the dye test as a diagnostic tool and as a method for studying the reaction between a parasite and its antibody can hardly be underestimated. The test has, by making possible the laboratory diagnosis of toxoplasmosis, greatly stimulated interest in the infection but is however still subject to certain suspicions with respect to its reliability and specificity.

The parasites give a quantal response to the stimulus of the antibody (van Soestbergen, 1956). In analysing quantal response data it is necessary to consider the distribution of tolerances over the population studied.

The purpose of this paper is to communicate results of a recent statistical study on the nature of this tolerance distribution. The fraction of unstained parasites is a function of the time during which the parasites are incubated with the antibody. The dependence of the values derived for the parameters of the tolerance distribution on the length of the incubation period has been investigated.

METHODS

A strain of *T. gondii*, isolated from a patient and cultivated intracerebrally in a pure strain of albino mice, was used; the same strain of mouse was employed throughout. The suspension of the organisms used for the titration of the sera was obtained from the peritoneal exudate of mice that had been inoculated intraperitoneally 3 days previously with toxoplasma-infected cerebral material. The exudate was collected by abdominal tapping; it contained large numbers of free

* This paper is based on the Ph.D. dissertation of Dr A. A. van Soestbergen. The original dissertation is available from the Health Research Organization TNO, The Hague, and from the library of the State University, Leyden. The work was aided by a grant from the Health Research Organization TNO, The Hague, The Netherlands.

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parasites and small numbers which remained within the phagocytes present in the exudate and were thus protected from the action of antibody.

To obtain sufficient serum for the duration of the investigation blood was taken from a patient whose serum on preliminary investigation exhibited a high titre of toxoplasma-antibody. The serum, after centrifuging off the corpuscles, was divided into small lots and preserved at $-20°$ C. The antibody is not destroyed by heating to 56° C. for 30 min.

The parasites respond to the stimulus of the antibody only in the presence of an activator. This activator is present in fresh human serum and is destroyed by heating to 56° C. for 30 min. Activator serum which must be entirely or almost entirely devoid of toxoplasma-antibody does not lose much of its activator component in the course of 6 months if preserved at $-30°$ C.

The serum, preheated to 56° C. for 30 min., was titrated for antibody content by pipetting 0·1 ml. of each of a number of dilutions in saline into agglutinating tubes and adding 0·3 ml. of a 1:10 mixture of a suspension of parasites and activator. The tubes were incubated at 38° C. in a water-bath. A shaking mechanism ensured thorough mixing of the contents of the tubes during the incubation. The reaction between the parasites and the antibody was stopped after a certain time by addition of 0·2 ml. of a dye mixture consisting of a 3:10 dilution of a saturated alcoholic methylene blue solution in a soda-borax buffer, pH 11 (973 ml. of a 0·53 % (w/v) solution of Na_2CO_3 and 27 ml. of a 1·91 % (w/v) solution of $H_2B_4O_7$). The titrations were read the following day by counting a sample of n parasites from each tube and noting the number r_z of unstained parasites present. The serum dilution is a linear function of the antibody content of the serum and is hereafter indicated by z. For each serum dilution incubated during a time t a value $(p_z)_t = (r_z)_t/n$ was calculated. The whole series of $(p_z)_t$ values obtained for different serum dilutions z incubated during the same length of time t constituted the results of a titration. The serum was titrated at 11 different values of t. In the present investigation t varied between 10 and 360 min.

RESULTS AND DISCUSSION

Data obtained with 11 titrations of the same toxoplasma-antibody containing serum are collected in Table 1. The time during which the tubes were held at 38° C. varied between 10 and 360 min. as indicated. For each time t the fractions p_z were calculated. The ratio of p_z to $(1-p_z)$ can be expressed as a function of t. This relationship is described by

$$\frac{(p_z)_t}{(1-p_z)_t} = t^{b_z}, \tag{1}$$

where b_z represents the slope of the straight line drawn through the points if

$$\log [(p_z)_t/(1-p_z)_t]$$

is plotted against $\log t$. The relationship is a reasonable approximation of the data as is illustrated in Fig. 1. It appears from Fig. 1 that b_z has a maximum value for the serum dilutions 1/48, 1/64 and 1/96. This maximum value is $b_M = 0·685$.

https://doi.org/10.1017/S0022172400020441 Published online by Cambridge University Press

A minimum value of $b_N = 0{\cdot}320$ is obtained with the titration where the antibody containing serum has been replaced by saline (the control tubes). The parasites in these tubes have lost their affinity to the dye because of antibody present in the peritoneal exudate developed by the mouse on which the parasites had been cultivated and because of antibody present in the activator serum. It may be

Table 1. *Titration of toxoplasma-antibody. The number of unstained parasites* (r) *observed in random samples of* n *parasites in serum dilutions incubated with the parasites during different lengths of time*

Serum dilution		10 min.		20 min.		30 min.		40 min.		50 min.		60 min.	
z	$x(=\log z)$	n	r	n	r	n	r	n	r	n	r	n	r
1/48	−1·681	600	364	600	463	700	556	700	579	600	521	600	518
1/64	−1·806	600	370	700	509	600	474	600	511	600	479	600	520
1/96	−1·982	600	351	600	425	600	465	600	500	600	493	600	510
1/256	−2·408	400	236	—	—	400	294	—	—	400	298	400	319
1/384	−2·584	—	—	400	239	—	—	400	275	—	—	400	303
1/512	−2·709	400	140	—	—	400	229	—	—	400	266	500	314
1/768	−2·885	—	—	400	166	—	—	400	194	—	—	400	184
1/1024	−3·009	400	45	—	—	500	150	—	—	500	140	500	156
1/1536	−3·186	—	—	400	46	—	—	400	54	—	—	400	44
1/2048	−3·311	400	34	—	—	400	60	—	—	500	51	400	59
1/3072	−3·487	—	—	400	28	—	—	400	39	—	—	400	43
1/4096	−3·612	400	42	—	—	400	40	—	—	400	44	400	42
Control		600	49	600	37	600	53	600	53	600	73	600	72

		120 min.		180 min.		240 min.		300 min.		360 min.	
		n	r	n	r	n	r	n	r	n	r
1/48	−1·681	600	537	600	527	600	550	600	567	600	566
1/64	−1·806	600	536	600	539	600	558	600	569	600	571
1/96	−1·982	600	514	600	531	600	553	600	560	600	558
1/256	−2·408	400	326	400	335	—	—	400	368	—	—
1/384	−2·584	400	317	400	335	500	391	—	—	400	332
1/512	−2·709	400	259	400	306	—	—	400	319	—	—
1/768	−2·885	400	220	400	206	400	225	—	—	400	255
1/1024	−3·009	700	257	500	163	—	—	400	185	—	—
1/1536	−3·186	500	103	400	81	500	95	—	—	400	115
1/2048	−3·311	400	44	400	65	—	—	500	94	—	—
1/3072	−3·487	400	46	400	54	400	49	—	—	400	83
1/4096	−3·612	400	52	400	56	—	—	400	61	—	—
Control		600	61	600	80	700	96	600	75	600	104

assumed that these parasites would also have remained unstained if antibody containing serum had been present in the tube. Expected maximum and minimum values of $(p_z)_t$ can be derived from the values of b_M and b_N. These values are denoted by $(P_M)_t$ and $(P_N)_t$. The maximum number of parasites expected to have reacted with the antibody in the serum after an incubation period t is $n(P_M)_t - n(P_N)_t$. The actual number of unstained parasites due to the action of the antibody present in the serum at a certain dilution z is $n(p_z)_t - n(P_N)_t$. The fraction of parasites

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remaining unstained because of the action of the antibody in the serum, calculated as a fraction of the maximum number expected after an incubation period t, is

$$(p'_z)_t = \frac{(p_z)_t - (P_N)_t}{(P_M)_t - (P_N)_t}. \tag{2}$$

The fractions $(p'_z)_t$ plotted against the logarithm of z appear to follow (approximately) a cumulative normal curve described by

$$(p'_z)_t = \frac{1}{\sigma\sqrt{(2\pi)}} \int_{-\infty}^{x} e^{-(x-\mu)^2/2\sigma^2} dx, \tag{3}$$

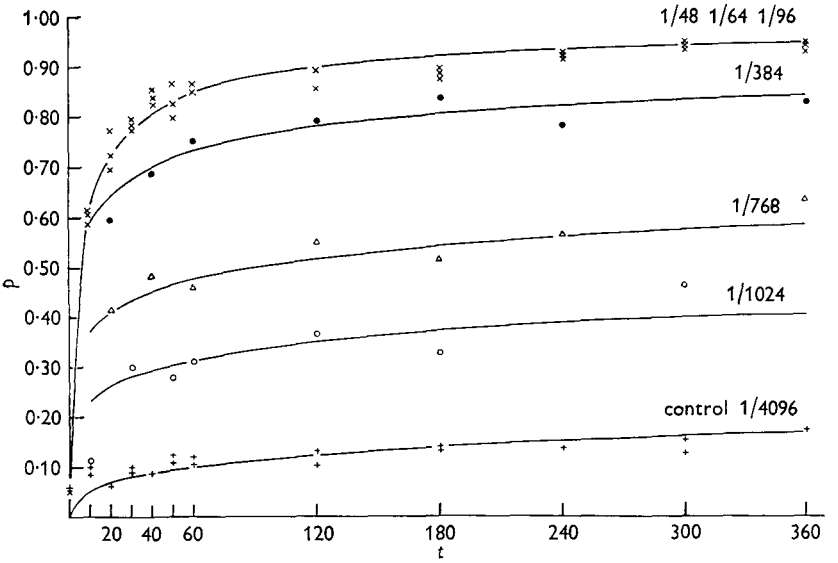

Fig. 1. Toxoplasma-antibody titrations. The relationship between the fraction unstained parasites and the incubation time. p = the fraction unstained parasites; t = the incubation time in minutes.

where x represents the log of the serum dilution z; μ and σ are parameters of the equation; μ is the value of x for $(p'_z)_t = 0{\cdot}5$ and represents a log LD50 or the logarithm of the titre of the serum; σ^2 is an expression for the variance of the distribution of $(p'_z)_t$ on x; estimates of μ and σ are hereafter indicated by u and s.

The agreement between the experimental data and the relationship described by equation (3) was subjected to statistical analysis.

Many classes of data, especially those dependent upon a quantal response to a stimulus, have been found amenable to treatment on the hypothesis that the tolerance, or a simple transformation of the tolerance, of a subject is normally distributed in respect of a given stimulus. The relationship described by equation (3) was verified by plotting the probits of the fractions $(p'_z)_t$ obtained for a specified value of t against the log of the serum dilution in which case a linear relationship is expected. The use of the log serum dilutions to measure the antibody content in these titrations requires no more justification than that it introduces a simplification into the analysis.

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The theory of probit analysis, based upon the calculation of maximum likelihood estimates of the parameters, has been extensively treated by Finney (1952). The application of this method, if part of the population responds even in the absence of the stimulus and another part fails to respond however high the stimulus, has been given by Finney (1949) as a general theorem. The method finds application in the treatment of data obtained with toxoplasma-antibody titrations. By analysing the data in accordance with this method the following are obtained: a χ^2-test on the assumption of the normal distribution of tolerances; estimates of the parameters μ and σ; variances of the sampling distributions of these estimates; corrections on the estimates of the 'nuisance' parameters $(P_M)_t$ and $(P_N)_t$.

On account of the rather extensive calculations involved if corrections on the estimates $(P_M)_t$ and $(P_N)_t$ have to be derived from the data it was decided to consider the values obtained from b_M and b_N as sufficiently exact to warrant a probit analysis of the data only estimating μ and σ. Allowance for the fact that the probits of the fractions do not all carry the same weight was made by introducing a weighting factor:

$$w = Z^2[(P_M)_t - (P_N)_t]^2/(P_z Q_z)_t,$$

where $(P_z Q_z)_t$ is the expected fraction of parasites remaining unstained multiplied by the fraction of parasites stained, at a serum dilution z and an incubation time t. Z, the ordinate corresponding to a probability P of a normal distribution, has been tabulated (Fisher & Yates, 1957). A representative example of the probits of $(p'_z)_t$ plotted against x, taken from the data collected in Table 1, is given in Fig. 2. The incubation time t for this titration was 60 min. The results of probit analysis of all the data are collected in Table 2. The calculations by which these results were obtained have been published in full detail elsewhere (van Soestbergen, 1956). All calculations were carried out in duplicate. The difference in magnitude of the confidence intervals of the estimates presented in Table 2 is due to the fact that the number, magnitude and weight of the samples were not all identical.

The χ^2-values collected in Table 2 are strongly indicative of heterogeneity. It has been demonstrated that the probit regression line is representative for the relation between the tolerance-probit and the logarithm of the serum dilution for samples not exceeding 150 parasites (van Soestbergen, 1956). Larger samples giving very small confidence intervals around the estimates of the fractions of unstained parasites are relatively too exact, and small errors in the dilution technique and in the counting process appear as disturbing factors. In these cases a significant χ^2 results. In the present investigation all of the samples investigated were very large, numbering 400–700 parasites per sample (values of n in Table 1). The large number of significant χ^2-values is mainly attributed to the fact that too many subjects were included in each sample.

The usual time during which parasites and antibody-containing serum are incubated in the course of routine titrations is 1–2 hr. It is evident from Table 2 that b_p, the slope of the probit line, does not change significantly for incubation periods varying between 10 and 360 min. The values of u (= log LD 50) are almost equal for the titrations carried out with incubation periods varying between

20 and 240 min. The titres obtained with the titrations carried out at incubation periods of 300 and 360 min. are a little too low in comparison, and the titre of the 10 min. titration is too high. It may be concluded that a large variation in incubation time has no influence on the value of μ nor on the value of σ.

The method of analysis of data obtained with toxoplasma-antibody titrations

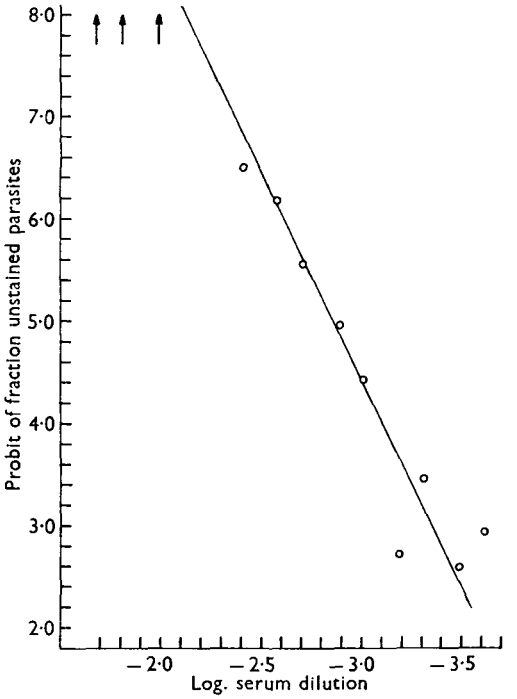

Fig. 2. Toxoplasma-antibody titrations. The relationship between the fraction of parasites unstained because of antibody in the serum and the logarithm of the serum dilution.

Table 2. *Titration of toxoplasma-antibody. Estimates of the parameters of the tolerance distribution of the parasites on the logarithm of the serum dilution obtained with titrations incubated during different lengths of time.*

(χ^2 has been calculated on the assumption of a normal distribution of tolerances.)

t (min.)	log LD 50			Slope of the probit line b_p			χ^2	P
10	−2·81	< −2·68	< −2·55	1·84	< 3·12	< 4·41	17·044	0·0006
20	−2·94	< −2·86	< −2·78	2·46	< 4·00	< 5·55	7·00	0·03
30	−2·94	< −2·87	< −2·80	2·05	< 3·10	< 4·16	2·168	0·54
40	−2·94	< −2·87	< −2·80	2·66	< 4·06	< 5·47	8·414	0·014
50	−2·90	< −2·85	< −2·81	3·22	< 4·17	< 5·13	13·881	0·003
60	−2·88	< −2·85	< −2·83	3·60	< 4·10	< 4·62	13·793	0·055
120	−2·89	< −2·86	< −2·84	3·16	< 3·63	< 4·10	27·817	0·0003
180	−2·89	< −2·86	< −2·84	3·73	< 4·20	< 4·68	22·783	0·002
240	−2·91	< −2·84	< −2·78	2·70	< 3·82	< 4·95	17·337	0·00015
300	−2·97	< −2·93	< −2·88	3·29	< 4·05	< 4·80	2·26	0·13
360	−3·00	< −2·92	< −2·85	2·18	< 3·29	< 4·39	5·98	0·05

https://doi.org/10.1017/S0022172400020441 Published online by Cambridge University Press

presented in this paper has been used to follow the antibody development in a patient through a number of years commencing a few days after the infection with *T. gondii* had occurred (van Soestbergen, 1957).

SUMMARY

In toxoplasma-antibody titrations with the dye-test the tolerances of the parasite *Toxoplasma gondii* are approximately normally distributed on the logarithm of the serum dilution. Log LD 50 (the logarithm of the titre), the variance of the tolerance distribution and the variances of the sample distributions were estimated by probit analysis of data obtained with titrations of a serum which contained the antibody. An empirical expression was derived relating the fraction of parasites attacked by the antibody to the time of incubation with the antibody. Approximately the same values for the titre and the variance of the distribution were obtained for every incubation period between 20 min. and 4 hr.

The author is indebted to Mr J. C. A. Zaat and to Dr E. F. Drion of the Statistical Division of the Central Organization TNO for helpful discussions and assistance with the statistical calculations. The technical assistance of Miss A. K. Fast is gratefully acknowledged.

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