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Antibody Production Studied by Means of the Localized Haemolysis in Gel (LHG) Assay

II. ASSAY PROCEDURE

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Summary. The localized haemolysis in gel (LHG or plaque) assay has been investigated and it has been shown that the best condition for the assay is a plate made up from a standard tissue culture medium (199) in an agarose gel. Several convenient but sub-optimal alternatives are described. Other variables that were investigated were the concentration of indicator erythrocytes in the assay plate, the amount of complement added, the incubation time and the neutralization of xenogeneic developing serum by free mouse serum γ-globulins. The reasons for using $\log(x+1)$ transformed data for statistical analysis are also presented.

INTRODUCTION

The LHG assay is one method of estimating the number of antibody-producing cells in an animal. It is based on the observation that when lymphoid cells and sheep red blood cells (sheep RBC) are placed together in a thin gel, areas of lysis (plaques) can be detected around those lymphoid cells producing antibody to the sheep RBC (Jerne, Nordin and Henry, 1963). Some plaque-forming cells (PFC) are detected by the simple addition of complement; others are not seen unless an anti-immunoglobulin serum is added as well (Dresser and Wortis, 1965; Šterzl and Říha, 1965; Weiler, Melletz and Breuninger-Peck, 1965).

The degree to which the number of PFC approximates the actual number of antibody-forming cells is called the absolute efficiency of the assay. This will depend in part on the similarity of *in vitro* to *in vivo* conditions affecting the number of antibody molecules produced by each cell and the sensitivity of the technique. Hege and Cole (1966), among others, have attempted to make an estimate of absolute plaquing efficiency. It is also true that the number of PFC will depend on the *in vitro* conditions for a particular experiment. For instance, the degree of anti-complementarity of the gel is a critical variable (Jerne *et al.*, 1963). The degree to which the number of PFC found under one set of conditions approximates to the number of PFC found under a standard set of conditions is called the relative plaquing efficiency. It is possible that in an alteration of assay conditions, ten times more plaques might be detected than under the standard conditions, although this may only mean an increase of from 1 to 10 per cent in absolute efficiency.

In this paper we describe experiments using spleen cells from mice immunized with sheep RBC in which a variety of changes in assay conditions affect the relative plaquing efficiency. It is hoped that the conclusions drawn from the use of this antigen will apply

to the use of other erythrocytes or erythrocytes coated with lipopolysaccharide (Halliday and Webb, 1965; Landy, Sanderson and Jackson, 1965; Möller, 1965), coupled with hapten (Merchant and Hraba, 1966) or with protein (Dresser and Wortis, 1967).

MATERIALS AND METHODS

Male CBA mice 13–16 weeks of age were immunized by an intraperitoneal injection of 4×10^7 sheep RBC. A pool of lymphoid cells prepared from three to five spleens was used for each experiment. Details of the standard procedure have been given elsewhere (Dresser and Wortis, 1967), but can be summarized as follows: a layer of 1·2 per cent agarose (L'Industrie Biologique Française) dissolved in Dulbecco's phosphate buffered saline was made in a polystyrene Petri dish. In some experiments Difco Bacto agar plus 0·5 mg/ml DEAE dextran (Pharmacia) was substituted for agarose. A second layer of 0·6 per cent agarose in Gey's solution with both sheep RBC and spleen cells was then added. The sheep RBC were usually 0·1 ml of a 20 per cent suspension. The RBC suspension was made up from the packed cell volume after centrifugation at 1750 ***g*** for 5 minutes. This was mistakenly quoted as 450 ***g*** in an earlier paper (Wortis, Taylor and Dresser, 1966). When the tubes containing the top layer are kept at 46° for an appreciable time they become slightly alkaline. The effect of this was investigated by gassing the individual tube with carbon dioxide and then capping, thereby maintaining a slightly acid pH. There was no detectable effect ($P > 0{\cdot}4$). The plates were incubated for 2 hours at 37° in a humid atmosphere of 4 per cent carbon dioxide and 96 per cent air. Then complement was added and the plates incubated for another 45–60 minutes. Plaques were counted using a conductivity colony counter and dark ground illumination. In experiments in which a developing serum was used, the serum was added directly to the molten top layer prior to preparing the plate for incubation. The serum in these experiments was a rabbit, anti-mouse immunoglobulin described previously (Wortis *et al.*, 1966). Details of changes from the standard procedure are given where appropriate in the next section.

EXPERIMENTS AND RESULTS

A plot of log variance against log mean shows a closely linear relationship over a wide range, for both direct PFC/plate and developed PFC/plate. Fig. 1 illustrates this relationship for direct PFC, and it can be seen that the slope of the line is nearly 1·5, suggesting a transformation to $x^{0{\cdot}25}$ prior to analysis (Tukey, 1957). The logarithmic transformation actually used is a reasonable approximation to this and much simpler to interpret. A $\log(x+1)$ transformation has been made (Quenouille, 1950), and we have used data in this form for the calculation of all means and for all *t*-tests. The same considerations apply when calculating the number of PFC per spleen (also per other organs or per animal).

The relative number of PFC obtained from a single spleen cell suspension depends on the buffers used in the assay plates, the composition of the gel and the presence or absence of an enriched medium (Table 1). It can be seen that agarose gives higher numbers of PFC than agar plus DEAE dextran; that Gey's solution is better than either veronal saline buffer (VSB, Kabat and Meyer, 1961), or Dulbecco's phosphate buffered saline (PBS); and that Parker's 199 tissue culture medium is somewhat better than Gey's. Furthermore, in practice agarose is easier to use than agar-DEAE because it does not

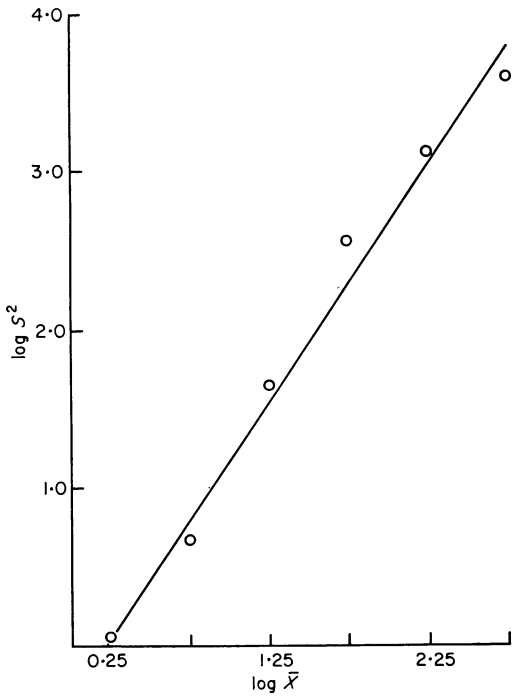

FIG. 1. A plot of log mean direct PFC per plate ($\bar{X}$) against log variance (S^2).

froth so readily on being brought to the boil, it dissolves and remains as a sol at lower temperatures, it spreads more easily, and, in our experience, the plaques are clearer. Although 199 medium is superior to Gey's, for the sake of simplicity we have decided to use Gey's solution and agarose in as many of our future experiments as possible.

It is clear that wide differences in the concentrations of the indicator erythrocytes (sheep RBC) do not affect the number of PFC in the assay plates (Table 2). For this reason we believe that it is sufficient to prepare suspensions of indicator erythrocytes on the basis of packed cell volume.

The effect of adding different concentrations of fresh guinea-pig serum as a source of complement is shown in Fig. 2. It is clear that 1 ml of 10 per cent 'complement' (in Gey's solution) is optimal and leaves a sufficient margin to allow for minor differences between different batches of guinea-pig serum. Complement can be added at any time in the first 5 hours of incubation, providing that the total time of incubation at 37° is not less than 2 hours. Complement added after more than 5 hours incubation results in fewer direct PFC being detected (Fig. 3). The number of developed PFC/plate does not change over a 5-hour period of incubation (Fig. 4).

We have made a standard PFC suspension and preserved this at −79°. The cells were from spleens of CBA mice immunized 5 days previously with 4×10^6 sheep RBC i.p. The spleen cells were prepared in the usual way, suspended in 10 per cent foetal calf serum, 10 per cent dimethyl sulphoxide and Gey's solution, and frozen to −79° in a Lovelock cooler (Ashwood-Smith, 1964; Smith, 1965); the cooling rate in a Lovelock

TABLE 1

EFFECT OF COMPOSITION OF GEL OR BUFFER ON PFC COUNT: SUMMARY OF FOUR SEPARATE EXPERIMENTS

Experiment No.	Top layer		Bottom layer		No. of plates	PFC/plate derived mean*	PFC/plate mean†	Standard deviation	P‡ <
	Gel	Buffer	Gel	Buffer					
1.1	Agarose	Gey's	Agarose	PBS	6	155	2·1935	0·0573	—
1.2	Agarose	Gey's	Agarose	Gey's	6	134	2·1312	0·0643	0·05
1.4	Agarose	Gey's	Agarose	VSB	6	98	1·9954	0·1207	0·005
1.8	Agarose	Gey's	Agar/DEAE	PBS	6	47	1·6810	0·0974	0·001
1.3	Agarose	PBS	Agarose	PBS	6	82	1·9205	0·0756	0·001
1.7	Agarose	199	Agarose	PBS	6	146	1·1662	0·0463	0·4
1.11	Agarose	199	Agarose	199	6	151	1·1815	0·0670	0·8
1.5	Agarose	199	Agarose	Gey's	6	125	2·1006	0·0607	0·05
3.6	Agarose	Gey's	Agarose	PBS	9	78	1·8958	0·0239	—
3.8	Agar/DEAE	199	Agar/DEAE	199	8	71	1·8583	0·0527	0·1
48.24	Agarose	Gey's	Agarose	PBS	7	110	2·0459	0·0469	—
48.25	Agarose	Gey's	Agar	PBS	7	1	0·3586	0·2492	0·001
48.28	Agarose	Gey's	Agarose	199	6	147	2·1619	0·1086	0·02
48.22	Agar/DEAE	Gey's	Agarose	PBS	7	111	2·0506	0·0545	0·99
48.23	Agar/DEAE	Gey's	Agar	PBS	7	49	1·7012	0·1068	0·001
48.26	Agar/DEAE	199	Agar	199	7	37	1·5829	0·0904	0·001
48.27	Agarose	199	Agar	199	7	1	0·2726	0·0743	0·001
50.3	Agarose	Gey's	Agarose	PBS	6	502	2·7015	0·0252	—
50.4	Agarose	Gey's	Agar/DEAE	PBS	6	367	2·5659	0·0648	0·005
50.9	Agarose	Gey's	Agarose	199	6	606	2·7831	0·0536	0·01
50.10	Agarose	199	Agarose	PBS	6	374	2·7598	0·0804	0·001
50.7	Agarose	199	Agarose	199	6	637	2·8049	0·0396	0·001
50.8	Agarose	199	Agar/DEAE	199	6	516	2·7137	0·0721	0·1
50.5	Agar/DEAE	199	Agarose	199	6	645	2·8102	0·0374	0·001
50.6	Agar/DEAE	199	Agar/DEAE	199	6	485	2·6863	0·0262	0·4
50.1	Agar/DEAE	Gey's	Agarose	PBS	5	487	2·6885	0·0378	0·95
50.2	Agar/DEAE	Gey's	Agar/DEAE	PBS	5	360	2·5581	0·0378	0·001

* Derived mean = antilog mean -1.

† Mean $= \frac{\Sigma \log (x+1)}{n}$.

‡ Upper limit for P in a t-test in which the null hypothesis was that both the experimental group and the standard group were samples taken from the same population.

TABLE 2

EFFECT OF SHEEP RBC CONCENTRATION IN TOP LAYER ON PFC COUNTS: TWO EXPERIMENTS

Experiment No.	Per cent sheep RBC	PFC/plate derived mean	No. of plates	P*
48.1	2·00	104	6	0·2–0·1
48.2	1·75	126	6	0·3–0·2
48.3	1·50	122	6	0·7–0·6
48.4	1·25	139	6	0·4–0·3
48.5	1·00	125	6	—
48.6	0·75	144	6	0·4–0·3
48.7	0·50	117	6	0·7–0·6
12.1	1·50	168	5	0·8–0·7
12.2	1·25	147	6	0·4–0·3
12.3	1·00	165	9	—
12.4	0·75	126	5	0·025–0·02

* For a two tailed t-test *vs.* 1·00 per cent.

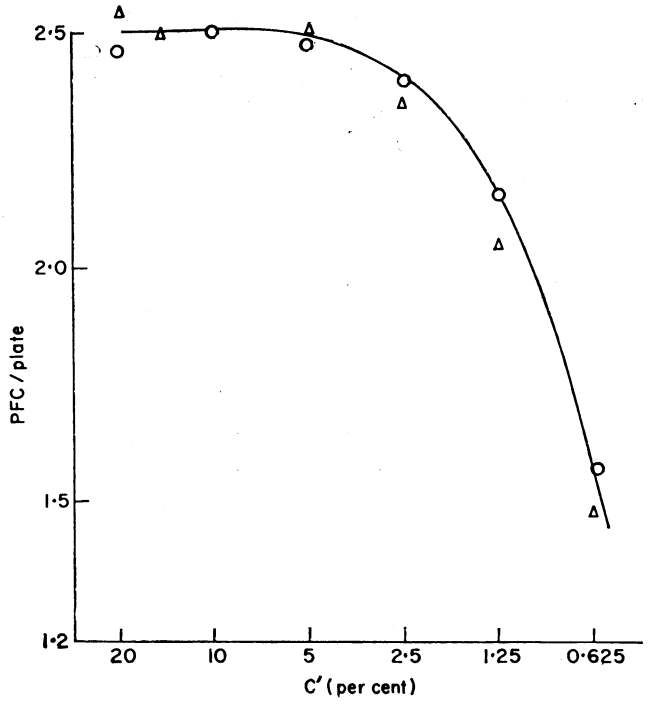

FIG. 2. The effect of complement concentration on the log mean direct PFC per plate. Standard plates (see Table 1, Experiment No. 1.1). One millilitre of complement diluted in Gey's solution added to each plate. △, Fresh guinea-pig serum; ○, preserved (freeze-dried) guinea-pig serum (Burroughs Wellcome). Four plates per point, 9-cm Petri dishes.

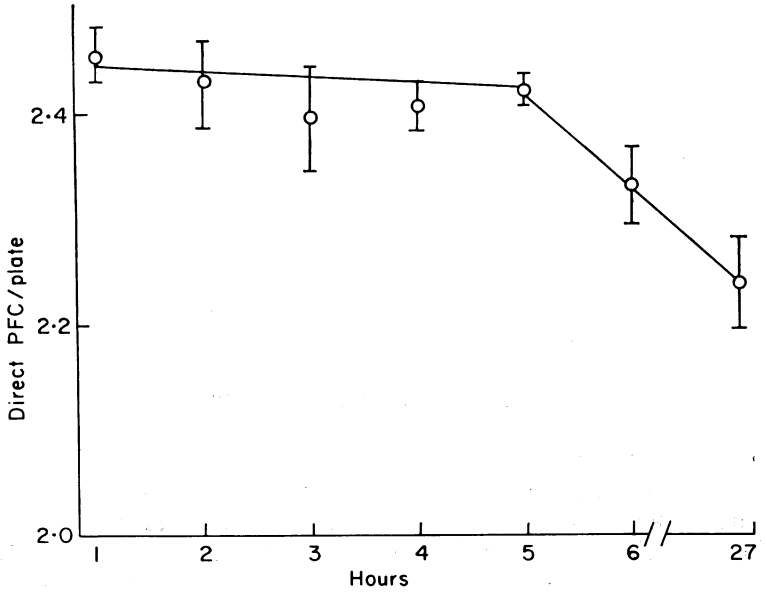

FIG. 3. Effect of time of incubation at 37° on the log mean direct PFC per plate. Time is hours before complement added; a further 45 minutes incubation is given after addition of complement. Five plates per point. Mean and one standard deviation.

cooler is 1° per minute to −15°, and at 4° per minute from −20° to 79°. After only 2 weeks of preservation the number of PFC per 10^6 spleen cells did not differ significantly from the number detected a fortnight earlier, before preservation. After 5 months of preservation in a dry ice (solid CO_2)cabinet, the 'viable' cell count had dropped three-fold, and the number of PFC per 10^6 'viable' cells was significantly less than 4½ months earlier. We have investigated the use of ampoules of frozen spleen cells as 'standards', to check day to day variation in the assay. The results are summarized in Table 3.

TABLE 3
DATA BASED ON PFC/10^6 PRESERVED SPLEEN CELLS, AFTER 5 MONTHS OF PRESERVATION (FIVE POINTS PER GROUP, EACH POINT THE GEOMETRIC MEAN OF SIX ASSAY PLATES)

	S^2	D.F.	F	P
Variance within 1 day	5873·5	4	1·917	Not significant
Variance from day to day	11258·5	4		

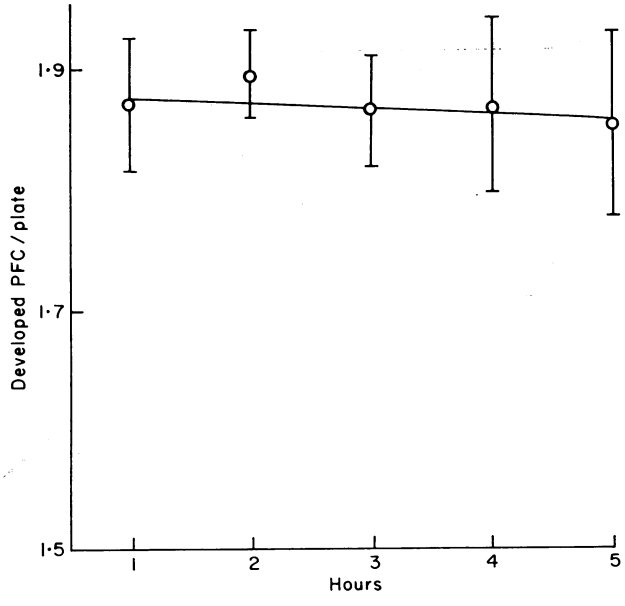

FIG. 4. As Fig. 3, but with developed PFC.

Nussenzweig, Merryman and Benacerraf (1964) have suggested that some classes of mouse antibody will not fix guinea-pig complement unless a small amount of fresh mouse serum is present. Table 4 shows that the addition of fresh mouse serum to guinea-pig complement does not affect the number of PFC seen on any assay plate. Care was taken to look both at 4 and 18 days after immunization, and at both developed and direct PFC. Furthermore, 10 per cent fresh mouse serum (CBA) is not a source of sufficient complement to cause haemolysis in this system.

TABLE 4

TEST OF THE ABILITY OF FRESH CBA SERUM EITHER ALONE OR IN ASSOCIATION WITH GUINEA-PIG COMPLEMENT TO PROMOTE PLAQUE FORMATION

Experiment No.	Type of PFC	ml of serum added		PFC/plate derived mean	*P*
		Mouse	Guinea-pig		
25.00	Direct	0·2	—	0	Decrease
25.1	Direct	0.02	0·18	132	Decrease
25.2	Direct	0·002	0·2	112	Decrease
25.3	Direct	0·0002	0·2	133	Decrease
25.4	Direct	—	0·2	138	—
25.5	Developed	0·2	—	0	Decrease
25.6	Developed	0·02	0·18	117	0·2–0·15
25.7	Developed	0·002	0·2	101	Decrease
25.8	Developed	0·0002	0·2	101	Decrease
25.9	Developed	—	0·2	107	Decrease
25.10	Direct	0·2	—	0	Decrease
25.11	Direct	0·02	0·18	7·8	0·4–0·35
25.12	Direct	0·002	0·2	5·8	Decrease
25·13	Direct	0·0002	0·2	4·9	Decrease
25.14	Direct	—	0·2	6·5	—
25.16	Developed	0·2	—	0	Decrease
25.17	Developed	0·02	0·18	72	No change
25.18	Developed	0·002	0·2	69	Decrease
25.19	Developed	0·0002	0·2	62	Decrease
25.20	Developed	—	0·2	72	—

For experiments 25.00–25.9 cells were taken from mice immunized 4 days previously.

For experiments 25.10–25.20 cells were taken from mice immunized 18 days previously. *P* values are for a one tailed *t*-test *vs.* the standard method based on the hypothesis that the addition of mouse complement would increase the number of plaques.

It is a possibility that the presence of serum in a lymphoid cell suspension affects plaque counts by providing either free antibody directed against the indicator erythrocytes (sheep RBC) or by providing a more favourable environment for the PFCs. Table 5 shows that neither normal mouse serum nor an anti-sheep RBC serum from CBA mice, when incorporated in the top layer solution at concentrations likely to be encountered when using spleen cell suspensions, affected the numbers of direct PFC detected. Direct PFC means those PFC which form visible plaques without the necessity of using an anti-immunoglobulin developing serum. When such a developing serum is used a further hazard lies in the possibility that free mouse immunoglobulins in the medium might absorb out some or all of the developing (or inhibiting) antibodies. Interaction of the 'developing' antibody with free mouse immunoglobulins to form soluble complexes, would lower the amount of γ-globulin complex formed on the indicator erythrocytes and thus greatly decrease complement fixation and subsequent plaque formation. This has been investigated experimentally, and the result is presented in Fig. 5. Aliquots of washed spleen cells from CBA mice immunized with 4×10^7 sheep RBC i.p. 11 days previously, were plated in top layers containing different ($\log_2$ dilutions) of normal CBA mouse serum. A standard amount of developing serum, prepared in rabbits against mouse '7S' γ-globulins, was added to each top layer. It can be seen that 0·06 per cent (6) NMS greatly inhibits the amount of development seen, when compared with 0·001 per cent (12) NMS

TABLE 5

EFFECT OF NORMAL CBA SERUM OR CBA ANTI-SHEEP RBC IN THE TOP LAYER ON THE NUMBER OF DIRECT PFC DETECTED

Experiment No.	Per cent serum		PFC/plate derived mean	No. of plates	*P**
13.23	4·0	Normal	79	4	0·2–0·1
13.24	1·0	Normal	73	4	0·1–0·05
13.25	0.25	Normal	91	5	0·98–0·95
13.26	0·06	Normal	76	4	0·3–0·2
13.27	0·015	Normal	71	4	0·3–0·2
13.29	—		92	4	—
13.30	4·0	Anti-sheep RBC	102†	3	0·4–0·3
13.31	1·0	Anti-sheep RBC	76	4	0·3–0·2
13.32	0·25	Anti-sheep RBC	83	4	0·4–0·3
13.33	0·06	Anti-sheep RBC	89	4	0·8–0·7
13.34	0·015	Anti-sheep RBC	86	4	0·7–0·6
13.29	—		92	4	—

* For a two tailed *t*-test *vs.* standard method (13.29).
† Some generalized lysis of the whole plate.

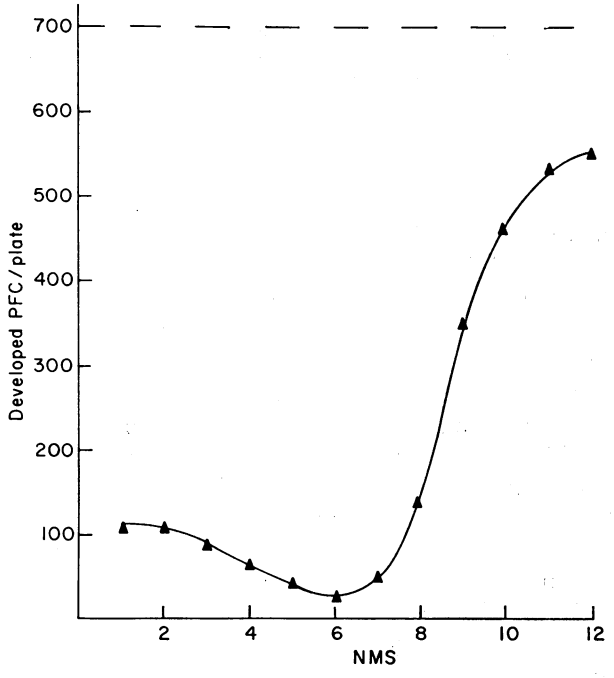

FIG. 5. The effect of fresh normal mouse serum (NMS) from CBA mice added to the top layer on the log mean developed PFC per plate. Dashed line indicates the number of developed PFC per plate from a suspension of washed spleen cells (CBA, 11 days post-immunization 4×10^7 sheep RBC i.p.). Six plates per point. NMS-2 is 1 per cent, 3 is 0·5 per cent, and 4 is 0·25 per cent, etc.

or no NMS at all (dashed line). A 'developing' effect of concentrations of NMS greater than 0·06 per cent can also be seen, and this will be discussed below.

'Washing' of suspensions of lymphoid cells, to remove mouse serum proteins, is, therefore, considered to be essential. This is carried out by suspending the cells in 12–15 ml of Gey's solution immediately after the cell suspension has been prepared. The suspension is centrifuged at 600 ***g*** for 5 minutes and the cells resuspended in an appropriate volume of Gey's solution before being stored in an ice water bath. There appears to be no detectable effect on the plaque count when the time interval between killing the animal and placing the washed suspension in an ice water bath is not longer than 15 minutes (at a room temperature of 20°). We found that plunging the spleen directly into Gey's solution at 0° significantly reduced the plaque count.

Fig. 5 shows that 1 per cent (2) fresh CBA serum either 'develops' or allows the development of more plaques than 0·06 per cent (6) of the same serum. It is unlikely that the rabbit developing serum should be more effective in the presence of 1 per cent NMS than 0·06 per cent, especially as further reduction in the concentration of NMS greatly reduces the inhibitory effect on development. This conclusion was clearly supported in a parallel experiment where 1 per cent of the same CBA serum caused a similar degree of development in the absence of rabbit developing serum. This was seen when the fresh CBA serum was added both at the start of incubation, or after 2 hours, immediately before the addition of guinea-pig complement. An aliquot of the same batch of CBA serum, which was heat inactivated at 56° for ½ hour, did not show any 'development' at a concentration of 1 per cent when added either before or after incubation. The 'development' (two- to three-fold) by 1 per cent fresh CBA serum is clearly not due to nutritional factors improving the synthesis of antibody by cells *in vitro* (late addition effect) but could be due to a synergic effect of CBA complement with guinea-pig complement, perhaps for a small minority of PFC producing one particular class or sub-class of antibody. We failed to detect such an effect in the experiments summarized in Table 4.

The use of developing sera to give developed PFC or to inhibit direct PFC is a complex problem depending on the specificity of the sera, their concentration and time of addition to the plates. These phenomena will be described in a subsequent paper in this series.

DISCUSSION

Several good alternative methods exist for carrying out the LHG assay. When it is necessary to obtain very high relative plaquing efficiencies, tissue culture media with agarose are much the best, and this should be used if any attempt is to be made to estimate the absolute plaquing efficiency and hence the total number of cells making antibody of a given specificity. Relative plaquing efficiencies higher than those for gel-media can be obtained when liquid-media are employed (Ingraham and Bussard, 1964; Cunningham, 1965). This may be especially true if in the future an estimate of the amount of antibody synthesized by an individual cell is to be estimated, being calculated on the basis of plaque size and taking into account other variables as antibody class (size), binding energy and the number of available antigenic sites for antibody attachment to the indicator erythrocyte.

If comparisons are to be made within the LHG system, for instance the number of PFC found in a lymphoid organ at different times after immunization, then the less expensive and somewhat easier alternative of using agarose in Gey's solution is acceptable.

It is important to note that using the standard method described here there are reasonable margins of safety with respect to the concentration of both sheep-RBC and guinea-pig complement which are used. This means that it is reasonably safe to compare results obtained on different days, a conclusion which has been confirmed when we used aliquots of a frozen standard PFC preparation.

The sources of variation which lead to a variance which is greater than the mean has not been explored experimentally; a variance equal to the mean would be expected in a Poisson distribution. The observed discrepancy could be due to at least two factors, artefacts in the gel and clumps of lymphoid cells. Armitage (1957) in a statistical account of viral pock-counts has pointed out the sources of variation in a situation analogous to our own. The discrepancy just mentioned may be due to artefacts in the gel which have a proportionally greater effect when the true count is low. That they are counted as true plaques is due to a systematic error of discrimination by the person counting, and this failure of discrimination raises an interesting point concerning the threshold size of a plaque, which would result in it being recognized and counted. We have scanned plates under a binocular microscope and failed to detect any plaques not already counted by the standard method.

A cell releasing only a few antibody molecules, for instance, would probably not be detectable in a semi-solid medium, although in the liquid medium technique of Cunningham (1965) very very small plaques, due to the lysis of as few as fifty erythrocytes, can be detected microscopically. On *a priori* grounds it seems likely that for a given thickness of top layer and concentration of sheep RBC, and for any one class of antibody, plaque size is related to the amount of antibody synthesized by the lymphoid cell. Systematic changes in the size of visible plaques for different classes of antibody, at different times after immunization or treatment of CBA mice, have been noted. It is clear that plaque size will be an important variable to investigate in the future.

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