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

III. MOUSE CELLS PRODUCING FIVE DIFFERENT CLASSES OF ANTIBODY

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Summary. By the use of antiglobulin sera of known specificity mouse cells releasing γM, γG_1, γG_{2a}, γG_{2b} or γA antibody to sheep erythrocytes can be identified in the LHG assay. The properties of these sera are described. Cells releasing γG_1 or γG_{2b} antibody appear as soon after immunization as cells releasing γM antibody. High doses of sheep RBC (1×10^{10}) were needed to stimulate the appearance of γA releasing cells in the spleen after intraperitoneal immunization.

INTRODUCTION

Cells producing haemolytic antibody (plaque-forming cells or PFC) are detected in the LHG assay. *Direct* PFC are those cells producing antibody which cause complement-dependent lysis of the target red blood cells without there being any need for the addition of a specific anti-(immuno)globulin serum (Jerne, Nordin and Henry, 1963; Ingraham and Bussard, 1964). In contrast, *indirect* PFC are those cells producing an antibody which requires such an antiglobulin serum to render it haemolytic, thereby giving rise to a visible plaque on the addition of complement (Dresser and Wortis, 1965; Sterzl and Říha, 1965; Weiler, Melletz and Breuninger-Peck, 1965).

Five classes of immunoglobulin have been identified in mice. These are γM, γG_1, γG_{2a}, γG_{2b} and γA (Fahey, Wunderlich and Mishell, 1964a, b). These classes of immunoglobulin carry mixtures of common and class-specific antigenic determinants on their heavy (H) polypeptide chains, called μ, γ_1, γ_{2a}, γ_{2b} and α, respectively.

In this paper we show that antisera specific for γ_1-, γ_{2a}-, γ_{2b}- and α-chains can be used as developing sera to make indirect plaques visible but that an anti-μ serum only reduces the number of direct plaques detected. Sera which react with all classes by virtue of their specificity for common antigenic determinants on the Fab fragment of the antibody molecules, both inhibit plaques around direct PFC and develop plaques around indirect PFC. The timing and magnitude of the splenic response of CBA mice to sheep red blood cells (sheep RBC), in terms of the numbers of PFC, are presented here for each class of antibody. Populations of cells were tested to see if individual cells were making more than one class of γG antibody.

MATERIALS AND METHODS

CBA male mice, aged 3½–4½ months, were used in these experiments. The antigen used for both immunization and as target cells in the LHG assay, or as antigen for *in vitro* tests,

was sheep RBC. Unless otherwise stated in the text, immunization was by intraperitoneal (ip) injection of 4×10^7 sheep RBC. The LHG assay and the statistical treatment of the data [log $(x+1)$ transformation] was carried out as described previously (Dresser and Wortis, 1967; Wortis, Taylor and Dresser, 1968).

Table 1 gives details of the various antisera used for development or inhibition of plaques. Crude antisera raised in rabbits were absorbed with appropriate myeloma γ-globulins co-valently coupled to polyamino-polystyrene (Webb and La Presle, 1961; Warner, Herzenberg and Goldstein, 1966). After absorption these sera and an anti-allotype serum were tested for their ability to precipitate ^{125}I-labelled myeloma γ-globulins (Weiler, Hofstra, Szentivanyi, Blaisdell and Talmage, 1960; Herzenberg, Warner and Herzenberg, 1965). The antisera used in our experiments had no detectable precipitating activity when tested with myeloma globulin of classes other than of the same class as the myeloma used for immunization. In the case of the anti-allotype serum (anti-Ig-1a) where this particular

TABLE 1

PREPARATION OF ANTISERA

Specificity	Antigen	Source of antiserum	Absorbed with:
M	4-day bleed of anti-*Pseudomonas* NCMB 406*	Rabbit	MPC-1 (A) MPC-11 (G_{2b}) RPC-5 (G_{2a})
G_1	MOPC-21	Rabbit	MPC-1 MPC-11 RPC-5
G_{2a}	Myeloma 5563	Rabbit	MPC-25 (G_1)
G_{2a} (Ig-1a)	Hyperimmune CBA anti-*B. pertussis**	C57BL/6 mice	–
G_{2b}	MPC-11	Rabbit	5563 MPC-25
A	MPC-1	Rabbit	MPC-11 RPC-5
Fab	Papain digest of normal serum 7S	Rabbit	–
Polyspecific	5563	Rabbit	–

* The antibody was mixed with the bacteria, incubated for ½ hour, washed and the coated bacteria injected i.p.

allotype determinant is restricted to the γG_{2a} class of γ-globulin (Fahey *et al.*, 1964a, b) only myeloma globulins of this class were precipitated. Care was taken to ensure that the antisera were not directed against idiotypic (myeloma specific) determinants, by using wherever possible more than one myeloma of any one class in the tests for specificity. We are very grateful to Drs Leonore A. and Leonard A. Herzenberg who tested all our specific sera and supplied and absorbed the anti-γ_1, -γ_{2b} and -α sera. In our experience a great deal of care is needed in preparing specific antisera for developing PFC. If sera are absorbed prior to use, insoluble absorbents should be used to avoid the presence of soluble complexes which may disassociate in the gel producing either inhibition or development of PFC. Also, all specific sera should be independently assayed by a system capable of detecting 1 μg or less of antibody.

Antisera to sheep RBC were raised in CBA mice. Sera obtained 4 and 10 days after immunization were pooled separately and fractionated according to molecular size on Sephadex G-200, in 0·6 M buffer, pH 8·0. The '19S' antibody-rich serum protein from the 4-day pool and the '7S' antibody from the 10-day pool, were concentrated to the original serum volume and used in *in vitro* experiments.

All haemagglutination and haemolysin titrations were carried out in veronal saline buffer (VSB) (Kabat and Meyer, 1961). Details of the experimental protocol are in Fig. 1, where is can be seen that there are two titration procedures: *early* addition of developing (or inhibiting) antiglobulin serum is prior to mixing the sheep RBC and the anti-sheep RBC antiserum, whereas *late* addition means that the sheep RBC and mouse anti-sheep RBC mixture was incubated for some time before the addition of the antiglobulin.

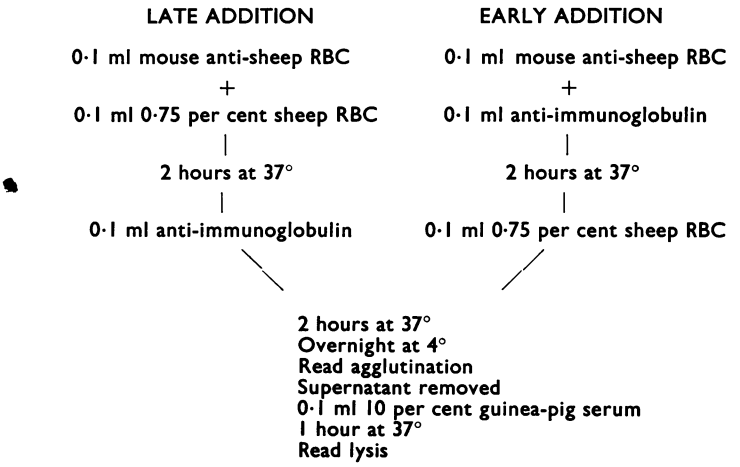

FIG. 1. A scheme for haemagglutination and haemolysin titrations showing the difference in procedure between late and early addition of developing (anti-immunoglobulin) serum.

RESULTS

THE CHANGE IN HAEMAGGLUTINATION AND HAEMOLYSIN TITRES RESULTING FROM THE ADDITION OF ANTI-(IMMUNO)GLOBULIN SERUM

When '7S' CBA anti-sheep RBC was titrated *in vitro* with and without developing antiserum, the polyspecific, the anti-γ_1-chain, the anti-Ig-1a and the anti-Fab sera increased both the haemagglutination and the haemolytic titres. The effect on lysis was greater when the antiserum was added late rather than early. These results are given in detail in Table 2.

In contrast, when '19S' CBA anti-sheep RBC was used, the polyspecific, the anti-Fab and the anti-μ-chain antisera all decreased the haemolytic titres; the decrease being the most clearly seen when an early addition of the antiglobulin serum was made. Even with late addition of developing serum and with concentrations which gave a marked increase in haemolytic titre of '7S' antibody, no increase in the lytic titre of '19S' antibody was seen. The details of these titrations are given in Table 3, where it can also be seen that the anti-μ-chain serum increased the agglutination titre but not the lytic titre of the '19S' anti-sheep RBC antiserum.

DEVELOPMENT OF VISIBLE PLAQUES BY SPECIFIC ANTISERA

For titrations carried out in LHG assay plates, the developing or inhibiting sera were first serially diluted in Dulbecco's (with Ca^{++} and Mg^{++}) phosphate buffered saline

TABLE 2

EFFECT OF ANTI-IMMUNOGLOBULIN ON HAEMAGGLUTINATION AND HAEMOLYSIS BY 7S ANTIBODY

Addition†		Agglutination*		Lysis*	
		Early	Late	Early	Late
None		8		8	
Polyspecific	1	8	13	7	12
	2	9	13	9	12
	3	8	8	8	8
	4	8	8	8	8
	5	Not done	8	Not done	8
Anti-γG_{2a}	1	10	12	10	12
	2	11	10	12	12
	3	8	10	9	10
	4	8	9	8	8
	5	Not done	8	Not done	8
None		7		8	
Anti-γG_1	1	7	11	9	12
	2	7	7	11	12
	3	7	7	8	8
	4	7	7	8	8
	5	Not done	7	Not done	8
Anti-Fab	1	7	10	7	12
	2	8	10	9	13
	3	10	9	10	12
	4	7	8	8	8
	5	Not done	7	Not done	8

* Titre expressed as $-\log_2$ of last positive tube.

† Dilution of sera expressed as tube number where tube 1 was 1 : 50 and five-fold dilutions were made.

TABLE 3

EFFECT OF ANTI-IMMUNOGLOBULIN ON HAEMAGGLUTINATION AND HAEMOLYSIS BY 19S ANTIBODY

Addition†		Agglutination*		Lysis*	
		Early	Late	Early	Late
None		4		7	
Polyspecific	1	4	4	4	6
	2	4	4	5	6
	3	4	4	6	7
	4	4	4	6	7
	5	Not done	4	Not done	7
Anti-Fab	1	4	5	4	6
	2	4	4	5	7
	3	4	4	6	7
	4	4	4	7	7
	5	Not done	4	Not done	7
Anti-μ	1	4	7	4	6
	2	4	7	5	6
	3	4	5	6	7
	4	4	4	6	7
	5	Not done	4	Not done	7

* Titre expressed as $-\log_2$ of last positive tube.

† Dilution of sera expressed as tube number where tube 1 was 1 : 50 and five-fold dilutions were made.

(PBS) and 0·1 ml of the diluted antiserum was added to each 2-ml volume of molten (44°) LHG top layer. When the LHG plates were made up with spleen cells from mice immunized with 4×10^7 sheep RBC (i.p.) 9 days previously, antisera specific for γ_1-, γ_{2a}- and γ_{2b}-chains developed plaques (Fig. 2a, b, c and d, respectively). The anti-Fab and the polyspecific serum also developed plaques but the titration curves differed qualitatively from those in Fig. 2 (a–d), in having a maximum in the middle dilution ranges (Fig. 3 a and b). The anti-μ-chain serum did not develop plaques and actually inhibited the formation of direct plaques at this time (Fig. 4a).

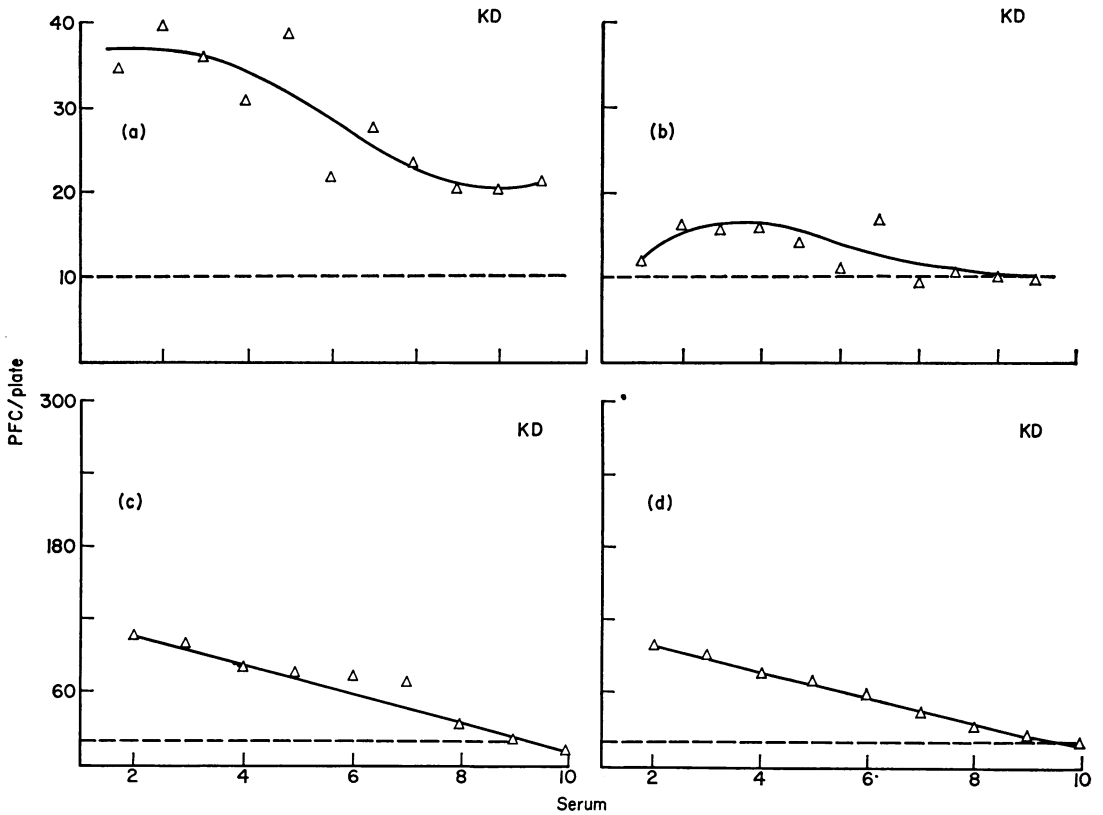

FIG. 2. Titration of the developing activity of four anti-immunoglobulin sera added to spleen cells from mice immunized with 4×10^7 sheep RBC (i.p.) 9 days previously. In each graph the ordinate is number of PFC/assay plate, the abscissa is a $\log_2$ dilution of serum where 1 represents a final concentration of the serum in the top layer of 1 per cent, and 2 will be 0·5 per cent, and so on. The broken line represents the number of PFC on untreated plates (direct PFC) and each separate point is the mean [log $(x+1)$] of four assay plates. (a) Anti-γG_1; (b) anti-γA; (c) anti-γG_{2a}; (d) anti-γG_{2b}. KI is the inhibition constant: that is, the proportion of direct PFC inhibited at a given concentration of antiserum. KD is the development constant: that is, the correction factor needed when a developing serum is used at sub-optimal concentration (Wortis, Taylor and Dresser, 1966).

THE INHIBITION OF DIRECT PLAQUE FORMATION BY SPECIFIC ANTISERA

The development of '7S' plaques by antisera of appropriate specificity was studied using cells from mice immunized 9–11 days previously. The reason for this was partly the data of Adler (1965) who showed that '7S' antibodies in the circulation were high at

this time and partly our own data (Wortis, Taylor and Dresser, 1966) which showed that at this time the ratio of developed to direct PFC was greatest. An earlier paper (Dresser and Wortis, 1965) indicated that spleen cells taken from CBA mice 2 days after immunization formed only direct plaques and that these plaques could be inhibited by relatively high concentrations of a polyspecific 'developing' serum. Furthermore, little or no '7S' antibody can be detected in the circulation up to 4 days after immunization (Adler, 1965). It, therefore, seemed appropriate to investigate the phenomenon of the inhibition of direct plaques using spleen cells of mice immunized 2 days previously.

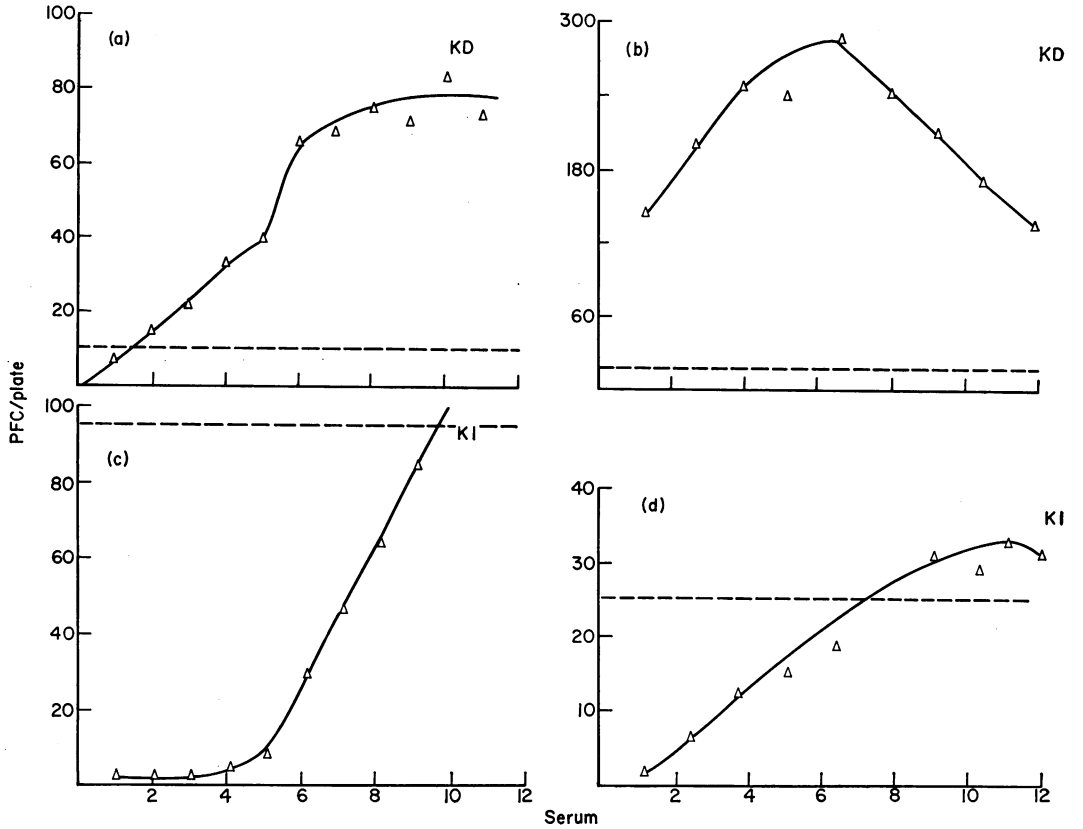

FIG. 3. Similar to Fig. 2 for (a) and (b). (c) and (d) used cells from mice immunized with 4×10^7 sheep RBC (i.p.) 2 days previously. (a) and (c) anti-Fab, (b) and (d) polyspecific anti-mouse immunoglobulin serum.

Anti-γ_1-, -α-, -γ_{2a}- and -γ_{2b}-chain sera do not decrease the number of 2-day PFC detectable as visible plaques (Fig. 5 a–d, respectively), but anti-Fab and the polyspecific antiserum were inhibitory at the higher concentrations (Fig. 3c and d). An anti-μ-chain serum inhibited virtually all 2-day direct plaques, but only half of the direct plaques formed by day-9 cells; this discrepancy will be discussed (Fig. 4).

The inhibitory effect on plaque formation of 2-day spleen cells was investigated further by absorbing the polyspecific antiserum with mouse Fab fragment (Fig. 6). It can be seen that the inhibitory property is removed by such absorption but that the capacity to develop plaques in day-9 cells is not affected.

Since virtually all direct plaques formed 2 days after immunization were inhibited by the anti-μ-chain serum (Fig. 4b), it is concluded that these PFC were producing γM antibody (IgM). In contrast, this same anti-μ-chain serum only inhibited about half the direct plaques 9 days after immunization (Fig. 4a). This difference might be due to one or more of the following alternatives: the 9-day cells produce more antibody; there are some (indirect) γM-producing cells 9 days after immunization; or finally, by day 9 there are

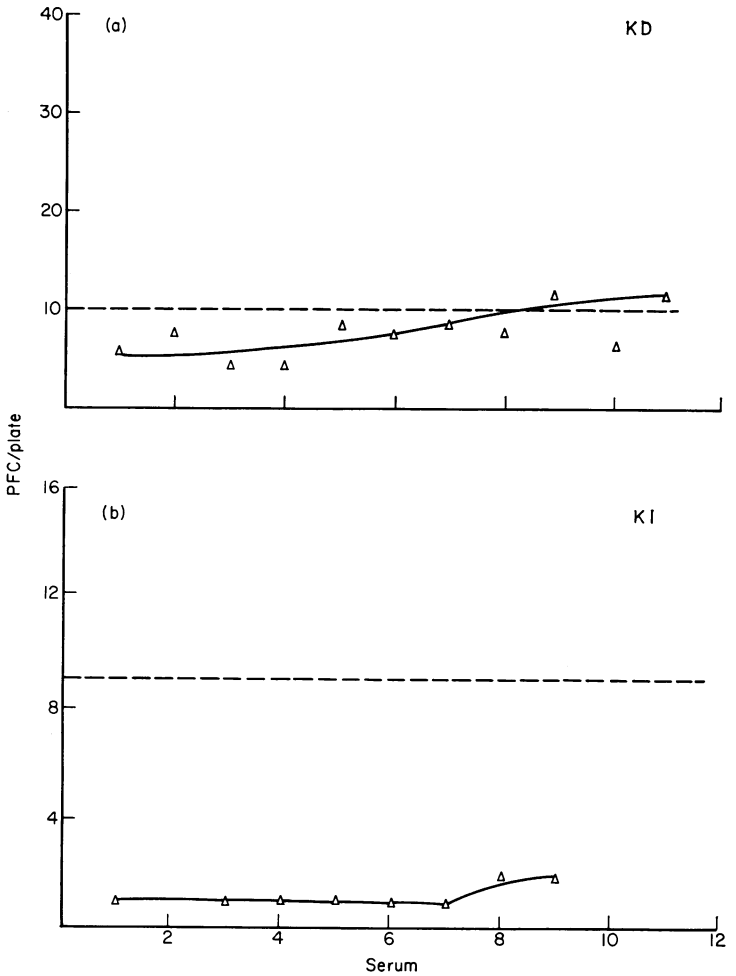

FIG. 4. Titration of the developing and inhibiting activity of a specific anti-γM serum against: (a) cells from mice immunized 9 days previously, and (b) 2 days previously.

some direct PFC produced by non-γM antibody. The first possibility seems unlikely because a concentration of anti-μ serum 100 times greater than was required to inhibit day-2 plaques, still failed to suppress more than half the day-9 direct plaques. The second alternative was tested in the following experiment: spleen cells from mice immunized 12 days previously were made up into LHG plates, incubated for 1 hour, then 1 ml of anti-μ-chain serum (dilutions from 1 to 0·002 per cent) was added and spread over the surface of the

plate, followed 1 hour later by an addition of complement. By comparison with control plates not receiving anti-μ-chain serum, there was neither a decrease nor an increase in the numbers of PFC. We conclude that our anti-μ-chain serum does not develop indirect γM PFC. In another experiment, spleen cells taken 4 days after immunization were plated with complement added at the start of incubation, so that direct plaques could form as soon as the haemolytic antibody was released by the PFC. This is a standard feature of the version of the LHG technique employing a carboxymethyl cellulose gum to suspend PFC and target cells (Ingraham and Bussard, 1964). After 2 hours of incubation we added a

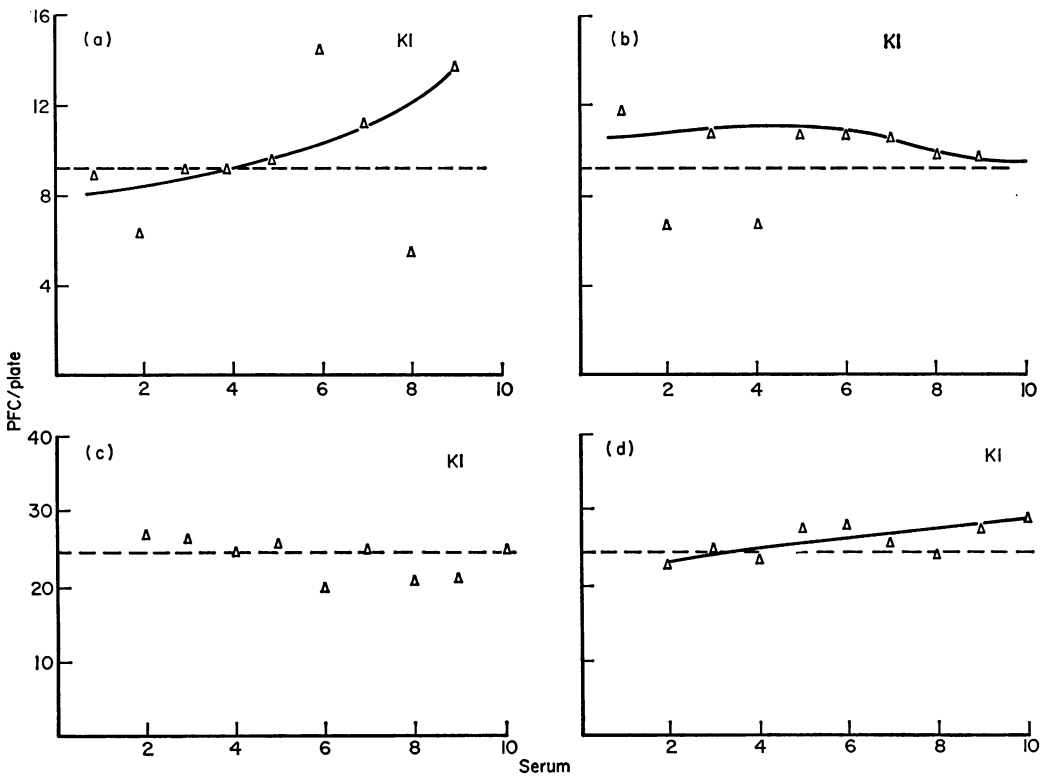

FIG. 5. Same sera as in Fig. 2 titrated with cells from mice immunized with 4×10^7 sheep RBC (i.p.) 2 days previously. (a) Anti-γG_1; (b) anti-γA; (c) anti-γG_{2a}; (d) anti-γG_{2b}.

highly inhibitory dilution of anti-μ-chain serum to half the plates and a further 2 hours later made a second addition of complement to all the plates. There was no difference in the number of plaques seen on the plates of the two groups.

THE ANTIGEN DOSE RESPONSE FOR DIFFERENT CLASSES OF ANTIBODY

Large groups of mice were injected (i.p.) with different doses of sheep RBC. At intervals after the injection, four mice from each group were killed and their spleens individually assayed for PFC. For each spleen cell suspension duplicate assay plates were set up for each

antiglobulin serum being used, and a pair of plates without serum were used to estimate the number of direct PFC for each spleen cell suspension. The resulting time–response curves for each class of PFC and dose of antigen were summarized by integrating each curve and plotting the result of this integration ('PFC/days') for each class of PFC, against the dose of antigen. The integration was carried out by plotting the mean values on an arithmetic scale and assuming that a straight line joined each successive point, and calculating the area beneath the curve as the sum of the areas of the contiguous trapezia from day-2 to day-18. (Wortis *et al.*, 1966). The dose–response curves from four experiments are shown in Fig. 7, where it can be seen that even on these limited criteria, each class is distinctive. In this figure the values for the individual experiments are plotted separately and a line drawn through each experimental value. It can be seen that the response of

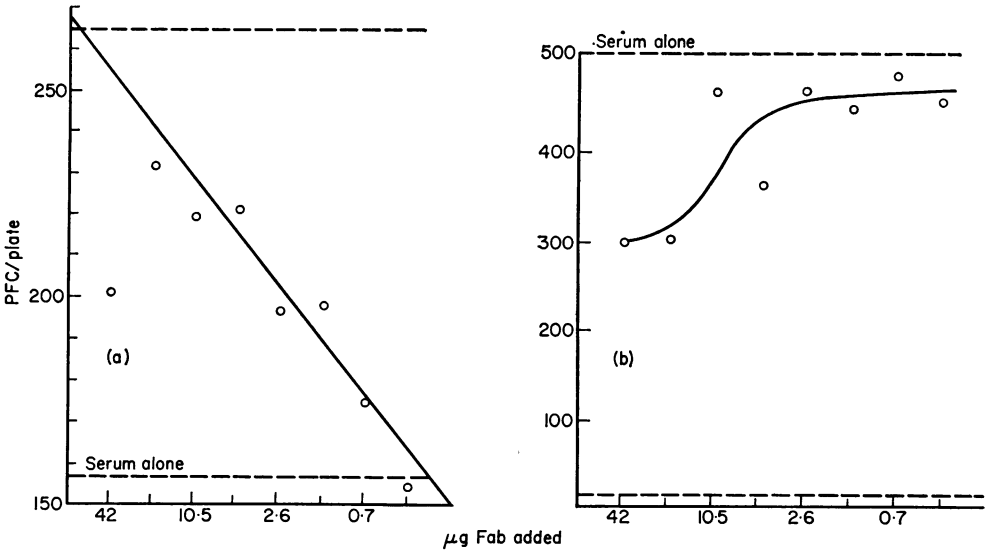

FIG. 6. The suppressive effect of Fab fragment on the inhibitory and developing activity of a polyspecific anti-immunoglobulin serum. (a) Using spleen cells from mice immunized 2 days previously. The upper broken line shows the number of direct PFC, and the lower broken line the number of direct PFC remaining when enough antiserum was added to the top layer to make the final concentration 0·1 per cent. The individual points represent the mean (from four plates) PFC per plate for plates containing the indicated amount of Fab in addition to antiserum. (b) Similar to (a) but using cells from mice immunized 9 days previously. However, in contrast to (a) the lower broken line is the number of direct PFC (on an untreated plate) and the upper broken line is the total development seen when the polyspecific antiserum concentration is 0·1 per cent. It must be noted that 42 μg of the Fab preparation used here had a significant suppressive effect on the formation of PFC in the absence of any developing serum. It is clear that with this polyspecific serum most of the inhibitory activity is due to anti-Fab antibody, and most of the developing activity is due to anti-Fc activity.

all three γG classes are displaced to the right of the response in terms of direct PFC; more antigen is required to produce a given level of γG PFC than is needed to produce the same level of direct PFC. The responses of the three γG classes are very similar over the antigen dose range 4×10^5 to 4×10^7 sheep RBC injected (i.p.). At higher doses there is a plateau, or in some experiments a fall, in the number of γG_{2a} PFC. Similarly, at these high doses, the direct PFC show no further increase in response. In contrast, the number of γG_1 and γG_{2b} PFC are still increasing, although the rise in the latter is not so marked as in the γG_1 response.

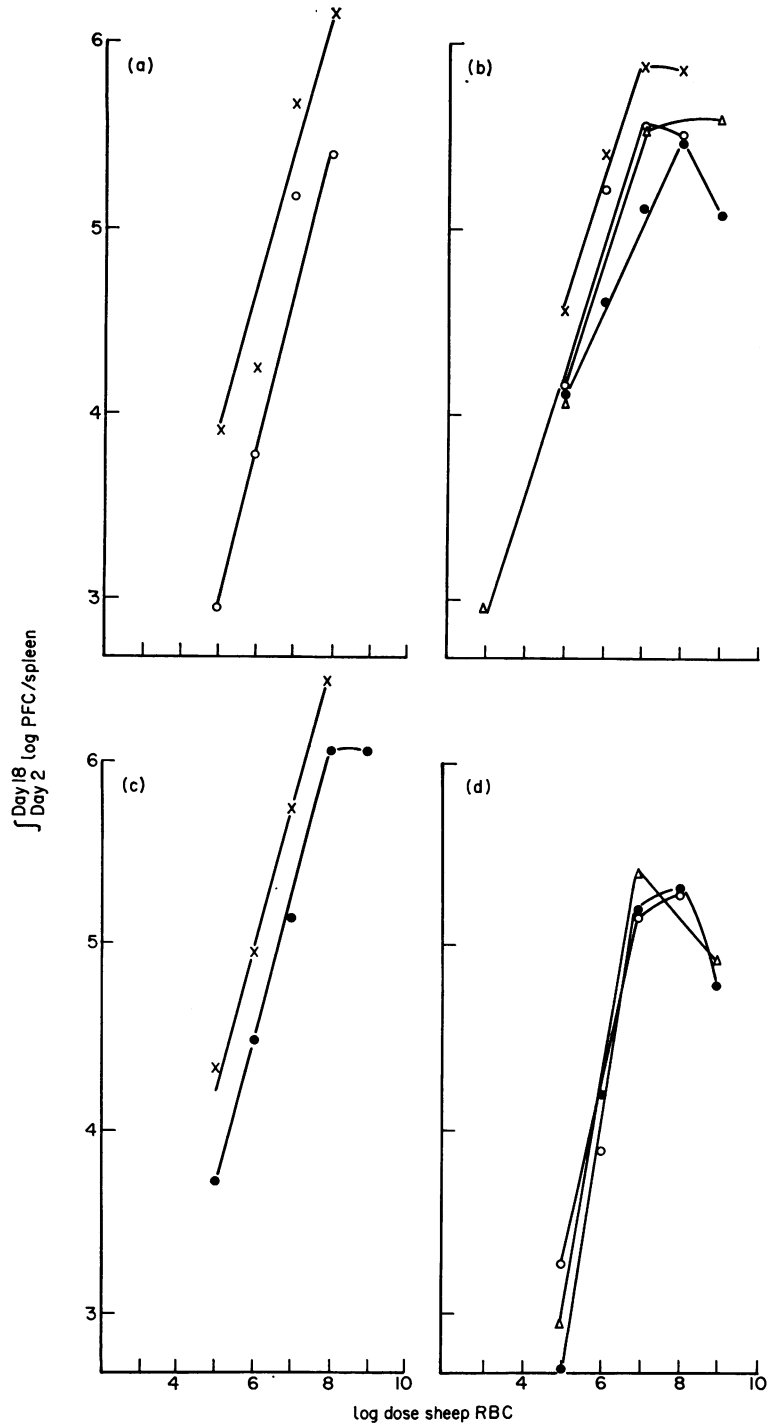

FIG. 7. In which the total PFC response ($\int_2^{18}$ PFC/spleen) to different doses of sheep RBC is shown. (a) γG_1-PFC, two separate experiments; (b) direct (γM)PFC, four separate experiments; (c) γG_{2b}-PFC, two separate experiments. (d) γG_{2a}-PFC, three separate experiments. The same experiment is represented by the same symbol in each portion of the figure. Each point is derived from the integration of an entire time–response curve, such as that shown in Fig. 8. Both the γM and the γG_{2a} responses fail to show increased levels of response when the antigen dose is increased beyond 4×10^7. In the abscissa the numbers indicated are the index n, where the actual dose of sheep RBC injected is 4×10^n.

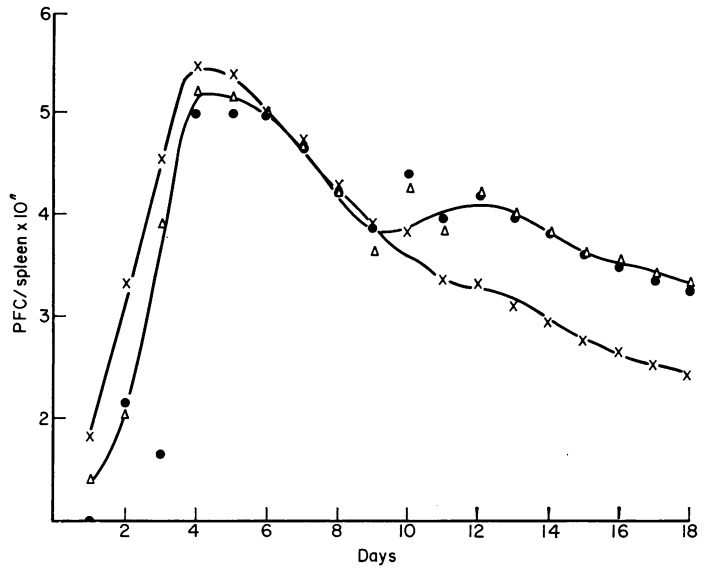

FIG. 8. The numbers of PFC found in CBA mouse spleen at intervals after the injection (i.p.) of 4×10^7 sheep RBC. Each point is the geometric mean derived from four mice. The numbers of γG_1-(△) and γG_{2b}-PFC (●) are so similar that only one curve has been drawn through these points. γG_1- and γG_{2b}-PFC appear as early as γM PFC (×).

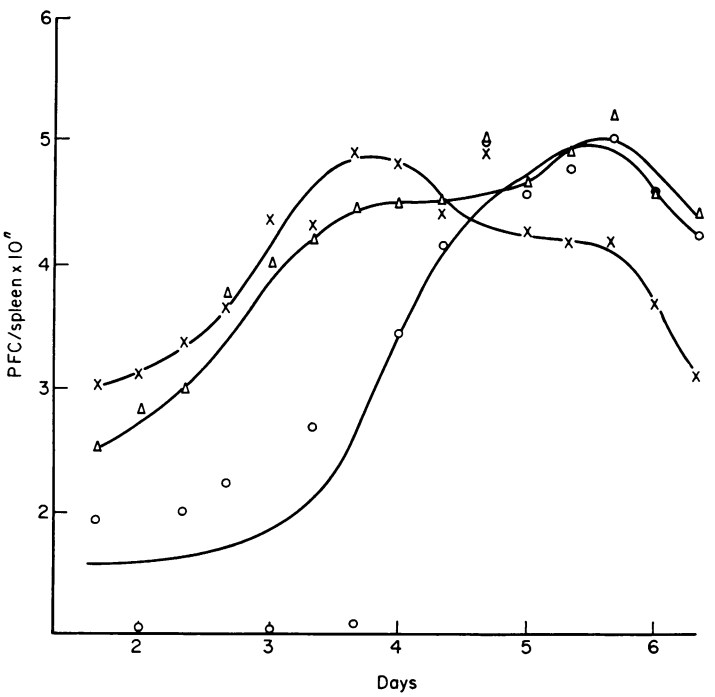

FIG. 9. The numbers of PFC found in CBA mouse spleen at 8-hour intervals following the injection (i.p.) of 4×10^7 sheep RBC. γG_1-PFC (△) appear as early as γM PFC (×), but γG_{2a} (○) appear at least 24 hours later.

THE TIME OF APPEARANCE OF PFC OF DIFFERENT CLASSES

After the intraperitoneal injection of 4×10^7 sheep RBC, γG_1 PFC appeared before other indirect PFC and reached a peak simultaneously with the γM (direct) PFC. Two experiments showing this, together with an experiment which shows that γG_{2b} appears earlier than γG_{2a}, are illustrated in Figs. 8 and 9.

Our experiments with a developing serum specific for γA were limited by the small amount of serum remaining after it had been found that γA PFC only appeared in significant numbers after high doses of sheep RBC had been injected (4×10^9, i.p.). Fig. 10 shows that after a high dose of sheep RBC the peak of γA PFC cannot have been reached before the 9th day after immunization.

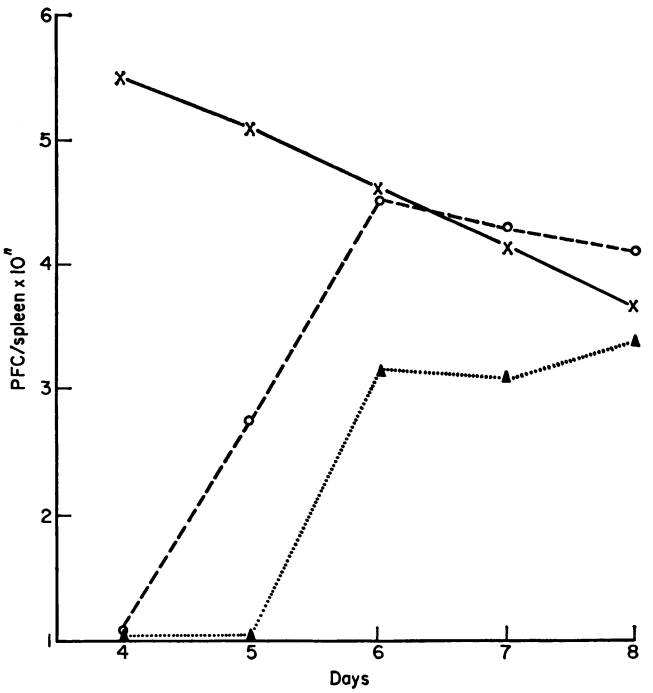

FIG. 10. The numbers of γA (▲), γG_{2a} (○) and γM PFC (×) found in mouse spleen at intervals after the injection (i.p.) of 1×10^{10} sheep RBC. The experiment ended, due to lack of specific anti-γA serum, before it could be established whether or not the numbers of PFC seen on day 8 represented a peak or plateau.

EVIDENCE THAT ONE PFC PRODUCES ONLY ONE CLASS OF γG ANTIBODY AT ANY ONE TIME

LHG assay plates were prepared with spleen cells from mice immunized 9 or 10 days earlier with 4×10^7 sheep RBC. For each individual spleen cell suspension plates were set up without any developing serum (to detect direct γM plaque-forming cells), with a specific rabbit anti-γ_{2a} and another group of plates with an anti-γ_{2b} serum, and a fourth group with a mixture of these two antisera. It seemed likely that if different cells made the two classes of antibody then the developing effect of the two sera would be additive, whereas a non-additive effect could be interpreted as indication that an individual cell

made both sub-classes of γG_2 antibody. There were thirty PFC developed with the anti-γG_{2b} serum, fifteen developed with the anti-γG_{2b} serum and sixty-two with the sera combined. This result is compatible with the sera having an additive effect. The result of a similar experiment using antisera directed against γ_{2a}-, γ_{2b}- and γ_1-chains, led to the same conclusion (Table 4). It is concluded from these experiments that the vast majority of plaque-forming cells (PFC) make antibody of only one class although it is realized that a few PFC could be making more than one class of antibody.

TABLE 4

TEST FOR ADDITIVITY OF ANTI-γG_1 -γG_{2a} AND -γG_{2b} SERA

	Expected	Observed	P (t test)*
$\gamma G_1 + \gamma G_{2a}$	78	67	$>0{\cdot}4$
$\gamma G_1 + \gamma G_{2b}$	34	37	$>0{\cdot}5$
$\gamma G_{2a} + \gamma G_{2b}$	72	81	$>0{\cdot}5$
$\gamma G_1 + \gamma G_{2a} + \gamma G_{2b}$	92	103	0·02–0·01

* Two-tailed test.

The conclusion drawn from the summation experiment was confirmed in a small preliminary replica LHG experiment. Spleen cells from mice immunized by an intraperitoneal injection of 4×10^7 sheep RBC, 9 days previously, were made up into a concentrated suspension in Gey's solution (30×10^6/ml) and 0·02 ml of this suspension was sandwiched between two thin layers of 0·7 per cent Agarose containing sheep RBC. After 2 hours incubation at 37° the two layers were separated and briefly washed in Gey's solution. The two layers were then treated with different developing sera or in controls with the same or no developing serum. The numbers of coincident plaques seen in the pairs of layers were entirely consistent with these being due to direct PFC. Several methods of scoring replica plaques were tried: the most satisfactory method was photography under dark-ground illumination, making a print of one replica and a mirror image print of the other. Coinciding plaques can easily be checked off by laying one print on top of another and picking through with a pin. An example of a control pair of replica plates is shown in Fig. 11; we scored thirty-six coinciding plaques, three doubtful and three occurring on one-half and not the other in such a control experiment.*

DISCUSSION

Řiha (1964) showed that an antiglobulin serum of xenogeneic origin could augment the haemolytic titre of an anti-sheep RBC serum. His finding led to the use of such antiglobulin sera for the development of visible plaques round cells producing non-haemolytic antibody in the LHG assay procedure. We have confirmed Řiha's original finding by showing that the haemolytic titre of mouse '7S' anti-sheep RBC antibody can be increased by a 'developing serum' directed against determinants on either or both the Fc and Fab portions of the antibody molecules. On the other hand, we have shown that '19S' anti-sheep RBC titres are not augmented in this way and may in fact be decreased. The demonstration of the inhibition and augmentation of the lytic titres as a result of the specific

* Recently, Dr J. Ivanyi, working in this laboratory, has greatly improved the technique increasing the number of coinciding plaques as control replicas to > 98 per cent. Direct PFC were suppressed by the method of Plotz, Talal and Asofsky (1968b), and when this was done and one-half developed for γG_1 and the other for γG_{2a}, only six out of 448 plaques appeared on both plates.

action of the antiglobulin serum added to the test serum and the classes of anti-sheep RBC antibody present, provides an explanation for the observed effects of antiglobulin sera on the development of visible plaques round PFC.

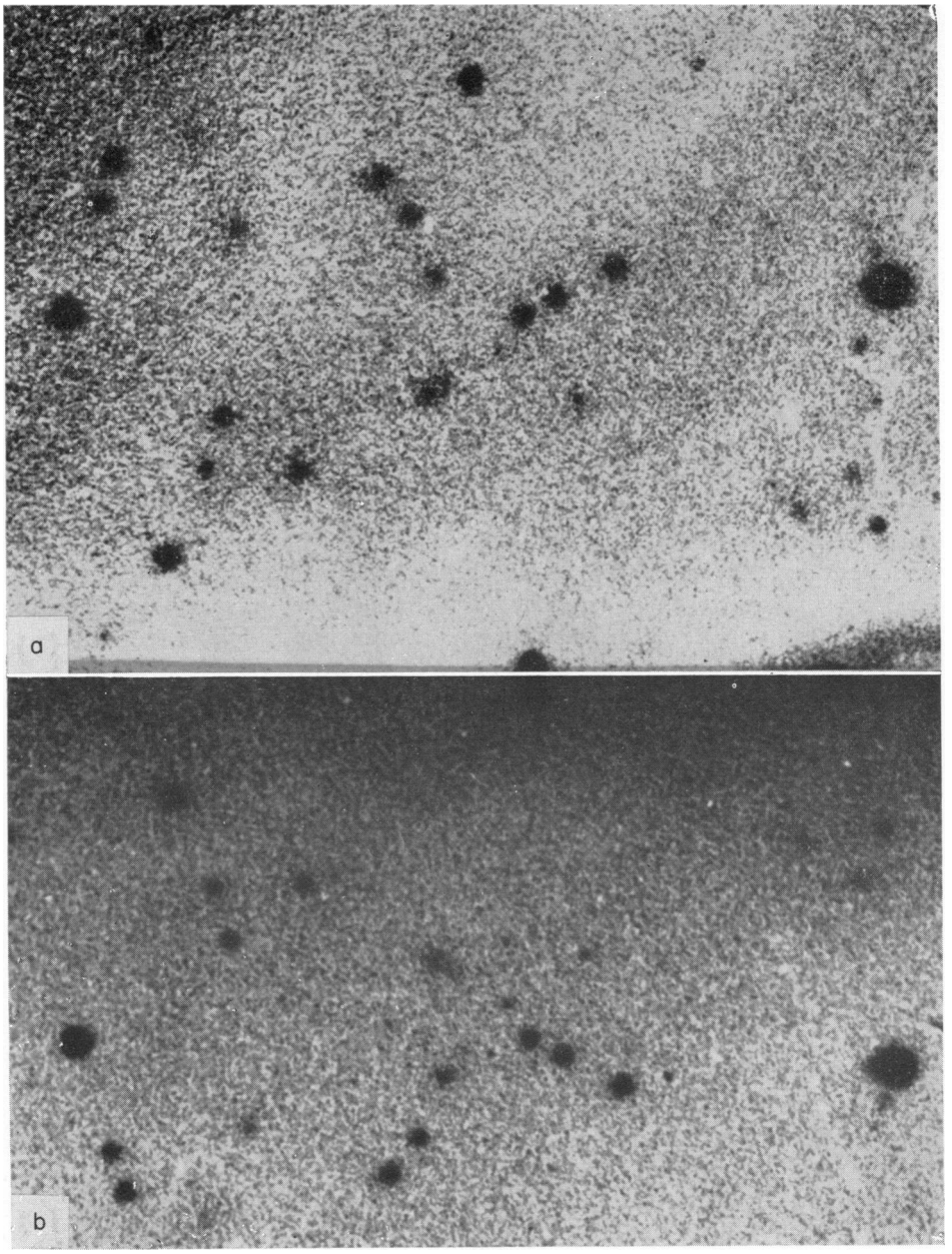

FIG. 11. The top and bottom layers (sheep RBC in agarose) of a sandwich of spleen cells from a mouse immunized 4 days previously. The sandwich in this preliminary control experiment was incubated for 2 hours, separated, washed, had complement added to each half which was then incubated at 37° for a further 40 minutes. Each direct (γM) PFC formed a plaque on both layers.

Antiglobulin sera specific for determinants on the Fc portion of H-chains of γG or γA molecules, developed plaques round PFC, which were presumably producing antibody of these classes. Such antisera did not inhibit direct plaques, whereas anti-μ-chain sera inhibited (direct) plaques but did not develop plaques. Sera reacting with Fab determinants, which are found on molecules of all classes, both inhibited direct plaques and developed indirect plaques. Similarly, a polyspecific antiserum which developed indirect PFC inhibited direct PFC mainly by virtue of its anti-Fab activity (see Fig. 6).

The original observations of Dresser and Wortis (1965), that plaques formed a few days after immunization were highly susceptible to inhibition by a polyspecific serum which would develop (indirect) plaques later on, has been extended to show that it is γM (direct) plaques which are inhibited by an antiserum ('developing serum') specific for μ-chain or Fab fragment. It is clear from the experiments with humoral antibodies *in vitro*, involving the augmentation of haemolytic titres, that there is no need to postulate any effect of the antiglobulin serum on the cells themselves, to explain either development or inhibition of plaques. These conclusions are in complete agreement with Ingraham, Siegel, Watanabe and Todd (1967) and provide an alternative to the explanation offered by Chou, Cinader and Dubiski (1967). It seems likely that high concentrations of anti-γM antisera inhibit lytic titres of γM antibody and the formation of direct plaques by decreasing the access of antigen to antibody or of complement to γM complement binding sites. A further effect in LHG plates might be the prevention of diffusion of γM antibody away from the PFC, thus accounting for the almost complete suppression of day-2 direct PFC when the inhibitory serum is added at the start of incubation.

We have not detected cells releasing γM antibody with low haemolytic efficiency. Two sub-classes of γM have been found in rabbits (Svehag, 1964; Hoyer, Borsos, Rapp and Vannier, 1968) and guinea-pigs (Hyslop and Roeder, 1966). Plotz *et al.* (1968b) have detected indirect γM PFC in the mouse and demonstrated two classes of γM (Plotz, Colten and Talal, 1968a). Their developing sera were raised against either 'normal' γM or against the γM from myeloma MOPC 104E, whereas our sera were raised against 19S antibody produced 4 days after immunization with bacteria (Pseudomonas NCMB 406—a marine bacterium which does not share antigen specificities with any known mammalian pseudomonad) (Hobbs, Cann, Gowland and Byers, 1964). Thus the possibility remains that the antiserum we describe here does not react with γM of low haemolytic efficiency.

Evidence has been presented that within the γG sub-classes single antibody-producing cells are limited to producing antibody of one (sub-) class at a time. This observation is in conformity with the generally accepted current belief, based on the experiments of Mellors and Korngold (1963), Bernier and Cebra (1965), Pernis, Chiappino, Kelus and Gell (1965) and Nussenzweig, Green, Vassalli and Benacerraf (1968) that there is selective activation of a single immunoglobulin gene on one chromosome.

Plotz *et al.* (1968b) have reported that γG_1 and γG_2 PFC appear on the 6th day following immunization (i.p.) with 4×10^8 sheep RBC. Our data show that γG_1 and γG_{2b} PFC are present as early as day 3 and reach a peak by day 5. We believe that the differences in these experiments may be due to differences in the preparation of the specific antisera. Unlike the sera used by Plotz *et al.* (1968a, b) ours had been absorbed with insoluble absorbent and checked against day-2 cells for residual inhibitory activity.

We were not able to detect γA PFC in the spleen of mice immunized with less than 4×10^9 sheep RBC. This confirms the report of Walters and Jackson (1968). However, they found

γA PFC only on days 5 and 6, while we found no γA PFC until day 7. The source of this difference is open to question but in addition to the points made with reference to the experiments of Plotz *et al.* (1968b) the possibility remains that there are different subclasses of γA-globulin.

Previous workers have shown that the relative contribution of each class to the entire pool of circulating antibody can differ with the interval between immunization and sampling the resulting antiserum (Bauer and Stavitsky, 1961); with the state of the antigen (Nossal, Ada and Austin, 1964; Torrigiani and Roitt, 1965); with the presence or absence of an adjuvant (White, Jenkins and Wilkinson, 1963); with the strain (Barth, McLaughlin and Fahey, 1965) or age of mouse (Makinodan and Peterson, 1966). It is now clear, from the experiments reported here, that these differences are largely a function of the numbers of antibody-forming cells, rather than a function of the differential catabolism of antibody of different classes or of their distribution after synthesis. A further conclusion that can be

TABLE 5

SUMMARY OF THE CLASS SPECIFIC RESPONSES OF CBA MICE TO SRBC, IN TERMS OF THE NUMBERS OF PFC IN THE SPLEEN

Class	Day peak reached after 4×10^7 SRBC (i.p.)	Optimum dose SRBC	Thymus dependence*	Effect of 2×10^9 pertussis†
γM	4	4×10^7	×10	×5
γG_1	4–5	4×10^9 (?)	×100	×15
γG_{2a}	6	4×10^7	×20	×15
γG_{2b}	4–5	4×10^8	×20 (?)	‡
γA	8 or later §	1×10^{10} (?)	‡	‡

* Taylor and Wortis (1968).

† Dresser, Anderson and Wortis (unpublished observations).

‡ Not known.

§ After 1×10^{10} SRBC (i.p.).

reached is that there is a relationship between the class of immunoglobulin a cell produces and the life history of the cell (Table 5). There are at least three possible alternative mechanisms:

(1) The antigen-sensitive cell is multipotential with regard to class. Following stimulation by antigen, daughters of this cell make each class in sequence. This situation was suggested by Nossal, Szenberg, Ada and Austin (1964) to explain the production of '19S' and '7S' antibody. The simultaneous production of γM, γG_1 and γG_{2b} makes this an unlikely possibility.

(2) The antigen-sensitive cell is multipotential. Commitment with regard to class could occur at the time of contact with antigen, and the class of antibody eventually produced could then depend on the nature of this contact. It has been suggested by Wilkinson, Fleming and White (1967) that antigen arriving at a lymphoid organ by a lymphatic supply would stimulate a '7S' response, that arriving in the bone marrow (via the blood stream) stimulating a '19S' response. It is not difficult to imagine how such peripheral factors could affect the way in which antigen selected particular H-chain cistrons, which in turn are linked to genes controlling cellular physiology.

(3) The antigen-sensitive cells are pre-differentiated both with regard to the class of antibody to be produced and the physiological nature of the cellular response to antigen.

The available evidence is compatible with this view and the current tendency is to accept such model hypotheses. It would be unfortunate if such uncritical acceptance of a hypothesis like this were to delay investigations of the basic mechanism of differentiation.

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