Immunology, 1969, **16**, 399.

Effect of the Number of Haptens Coupled to Each Erythrocyte on Haemolytic Plaque Formation

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(*Received 4th September* 1968)

Summary. We varied the density of a hapten coupled to sheep erythrocytes (SRC), and used different hapten–erythrocyte conjugates to study single antibody forming cells from rabbits. Both direct and indirect (developed by anti-IgG) plaques were seen. Hapten density influenced both plaque number and size. With aliquots of the same lymphoid cell suspension heavily coupled SRC yielded more direct and more indirect plaques than lightly coupled SRC. This tendency decreased with continuing immunization. Free hapten inhibited the formation of plaques more easily in lightly than in heavily coupled erythrocytes. Direct plaques were more dependent on a dense epitope coat than indirect ones. Our data indicated, that cells producing IgG antibody could produce direct plaques.

To explain our data we suggest that the product of some anti-hapten producing cells needs the collaboration of several combining sites to bind efficiently onto the hapten–SRC. They are dependent on a dense coat. The product of other cells has a higher affinity per site and binds efficiently onto the lightly coupled SRC.

INTRODUCTION

Localized haemolysis in gel (LHG) is a sensitive means of detecting single antibody-producing cells. It has been used to measure the immune response to erythrocytes (Jerne, Nordin and Henry, 1963; Ingraham and Bussard, 1964), and to lipopolysaccharides (Landy, Sanderson and Jackson, 1965), a polypeptide or proteins (Walsh, Mauer and Egan, 1967; Golub, Mishell, Weigle and Dutton, 1968) and haptens (Merchant and Hraba, 1966; Harrel and Merchant, 1967) using modified sheep red blood cells (SRC).

It was originally thought that IgM-producing cells could be measured by the number of direct plaques, and IgG-producing cells by the increase in plaque numbers after addition of anti-IgG (Šterzl and Riha, 1965; Dresser and Wortis, 1965). Some recent evidence suggests that this theory should be modified in two ways. First Hege and Cole thought that a few IgG-producing cells could cause direct plaques (Hege and Cole, 1966). More recently Plotz and co-workers found that anti-IgM increased the number of plaques (Plotz, Talal and Asofsky, 1968). This suggested that not all IgM-producing cells can form direct plaques. There are reasons to suppose (Mäkelä, Cross and Ruoslahti, 1969) that the epitope density on SRC might affect the reactions of IgG and IgM differently. The effects of epitope (Jerne, 1960) density have not been investigated in the haemolytic plaque system since all the earlier workers used a single procedure to coat their erythrocytes with antigens. Consequently we decided to study the effect of hapten density on the

LHG assay. SRC were treated with several concentrations of dinitrophenyl acetic acid (NNP)–azide (Brownstone, Mitchison and Pitt-Rivers, 1966) and used in the LHG assay and for testing IgM and IgG anti-NNP antibodies.

EXPERIMENTAL METHODS

SRC were coupled to NNP–azide in 0·12 M carbonate–bicarbonate buffer, pH 9·25. A 10 per cent suspension of three times washed SRC was mixed with NNP–azide concentrations of 1, 0·1, 0·01 or 0·001 mg/ml. The coupled SRC were called No. 1, No. 2, No. 3 and No. 4, respectively. The coupling reaction was allowed to proceed overnight at 4°. The following day the coupled SRC were washed three times and used as a 25 per cent suspension for the LHG assay or as a 1 per cent suspension for haemolytic titrations. For each experiment a new batch of coupled SRC was prepared.

We used NNP–chicken globulin conjugate (NNP–CG) to immunize rabbits. For a primary stimulation rabbits received 0·5 mg of alum-precipitated antigen, injected dorsally into the foot. The draining popliteal lymph nodes were removed at varying intervals, under ether anaesthesia, and the lymph node cells used for the assay. For the secondary response rabbits, which had received antigen in the foot and from which the popliteal lymph nodes had been removed, received 0·5 mg NNP–CG i.v. Their spleen cells were used for the assay. For the tertiary response the rabbits first received 1 mg NNP–CG i.p. together with Pertussis vaccine, and later were immunized twice as for the secondary response. The interval between successive immunizations varied from 4 to 8 weeks.

Anti-rabbit IgM antibody was prepared as described earlier (Mäkelä, Kostiainen, Kopenen and Ruoslahti, 1967). Volumes of 1 ml were absorbed first with 0·07 and then with 0·025 ml of rabbit colostrum. The antiserum was diluted 1 : 10 for the plaque assay. It was devoid of anti-IgG activity at this dilution as can be seen from Table 1.

TABLE 1

INABILITY OF OUR 'ANTI-IgM' TO REPLACE ANTI-IgG AS A PLAQUE-ENHANCING AGENT

Antiserum	No cells plated	Total No. of plaques	No. of plaques/10^6 lymphoid cells
None	6×10^6	11	1·8
Anti-IgG, 1 : 4	2×10^6	349	174
Anti-IgM, 1 : 10 twice absorbed	6×10^6	7	1·1
Anti-IgM, 1 : 10, three times absorbed	6×10^6	7	1·1

Cells from rabbit No. 683 taken 32 days after the second antigen injection were plated with No. 3 SRC.

Anti-IgG was prepared by immunizing a sheep with γ-chains, prepared by the method of Fleischman, Pain and Porter (1962). Two injections, each of 2 mg of the γ-chains, were given 3 weeks apart, and the sheep was bled 10 days after the second. The serum was absorbed with the IgM fraction prepared by Sephadex G-200 filtration. In various control experiments the inhibition of direct PFC by anti-IgG was found to vary from 0 to 5 per cent. Because the reduction was so small we did not use a correction factor when we calculated the number of indirect PFC.

Single cell studies

The LHG assay used was essentially the same as that described by Landy *et al.* (1965) except that the cells were plated on microslides to increase the sensitivity of the assay. Lymphoid cell and coupled SRC suspensions were prepared in Dulbecco's phosphate-buffered saline. They were first tested to find the dilution that gave up to 200 plaques/slide with all four types of coupled SRC when the suspension volume was varied from 0·01 to 0·03 ml/slide. Lymphoid cells (0·06–0·18 ml), coupled SRC (0·06 ml), 5 per cent agarose (0·84 ml) and anti-IgG (0·06 ml) were mixed in small test tubes at 45°. In some cases anti-IgM, free hapten or Dulbecco's phosphate-buffered saline was used instead of anti-IgG. Aliquots of 0·19 ml of the mixture were spread on the pre-heated microslides. They were then incubated for 2 hours in humidified chambers at 37° and developed with a 1 : 80 dilution of guinea-pig complement for 1 hour. The slides were stored at 4° until counted with 2× magnification.

RESULTS

HAEMOLYSIS OF NNP-COUPLED SRC BY IgM AND IgG SERUM ANTIBODIES

We filtered three rabbit anti-NNP sera through Sephadex G-200 gel (Mäkelä *et al.*, 1967) and determined the haemolytic titres of the first two main protein peaks. The peaks are called IgM and IgG in this paper. IgM fractions were absorbed with packed SRC (15 : 1 v/v) to remove the natural haemolytic antibodies against SRC. SRC with different hapten coats were incubated in doubling dilutions of serum. Complement was added to one set of tubes after an hour's incubation, and the direct haemolytic titres were read after a further hour's incubation at 37°. To determine the indirect haemolytic titres the anti-NNP sensitized SRC in a duplicate set of tubes were washed three times with saline, and then incubated with anti-IgG and complement for 1 hour at 37°. The results of these titrations are given in Table 2. Three conclusions can be drawn from them:

TABLE 2

LYSIS OF NNP-COUPLED SRC BY IgM AND IgG FRACTIONS OF ANTI-NNP SERA

Type of immune response	Red cell type	Direct titres		Titres with anti-IgG enhancement*	
		IgM	IgG	IgM	IgG
Primary response	No. 1	64	512	–	–
	No. 2	32	4	64	2048
	No. 3	4	0	0	64
	No. 4	0	0	0	4
Secondary response	No. 1	256	256	–	–
	No. 2	8	4	4	1024
	No. 3	2	0	0	128
	No. 4	0	0	0	64
Tertiary response	No. 1	–	256	–	–
	No. 2	–	0	–	2048
	No. 3	–	0	–	1024
	No. 4	–	0	–	16

*SRC were incubated in IgM or IgG fraction, washed three times, then anti-IgG and complement were added.

(1) The density of NNP receptors on SRC strongly affected the sensitivity of the cells to immune lysis. The sensitivity of the cells to anti-NNP antibodies declined in the order No. 1> No. 2> No. 3> No. 4. No. 4 cells were not sensitized to direct lysis by the serum

antibody concentrations used, but they gave positive results in the plaque assay to be described later.

(2) IgM and IgG antibodies reacted differently to variations in hapten density. IgM antibody sensitized Nos. 1, 2 and 3 cells to direct complement lysis, but IgG antibody only Nos. 1 and 2. However, IgG antibody must have been absorbed onto Nos. 3 and 4 cells because the addition of anti-IgG caused lysis.

(3) The haemolytic titres of IgG fractions were considerably enhanced by anti-IgG while those of IgM fractions were not. Anti-IgM was not used in these experiments.

STUDIES OF SINGLE CELLS

When we plated anti-NNP lymphoid cells with our NNP-coupled SRC we found haemolytic plaques resembling those seen with antilipopolysaccharide lymphoid cells (Pasanen and Epstein, 1967). We believe that these plaques were caused by a reaction between anti-NNP antibodies and NNP on the erythrocytes for the following reasons:

Plaque counts with lymphoid cells from NNP-immunized animals were up to 1000 times higher than controls (non-immunized or immunized less than 3 days previously).

Free hapten (NNP-aminocaproic acid) almost completely inhibited plaque formation at a concentration of 10^{-5}M. With some lymphoid cell suspensions a concentration of 10^{-7}M inhibited 99 per cent of the activity.

TABLE 3

NUMBER* OF DIRECT AND INDIRECT PLAQUES (USING ANTI-IgG) AT DIFFERENT STAGES OF IMMUNIZATION (VARIOUS COUPLED SRC WERE USED FOR TESTING)

Day of response and identification of rabbit	Type of coupled SRC							
	Direct plaques				Indirect plaques			
	No. 1	No. 2	No. 3	No. 4	No. 1	No. 2	No. 3	No. 4
Day 0, No. 563	4	–	4	3	0	–	0	0
Day 4, No. 316	17	46	7	–	39	26	2	–
Day 5, No. 333	144	75	12	2	697	382	12	–
Day 5, No. 801	292	148	4	–	472	228	2	–
Day 6, No. 310	279	204	90	–	–	797	267	–
Day 11, No. 311	692	117	24	6	2202	2031	1442	2
Day 13, No. 303	2953	830	513	64	3367	4870	4580	1006
Day 24, No. 313	–	188	34	3	–	1064	1105	1
Day 26, No. 308	187	378	41	0	423	322	769	194
Sec. 12, No. 311	1043	967	715	14	747	1271	1548	1651
Sec. 32, No. 683	31	42	2	3	111	168	172	161
Tert. 9, No. 88	183	387	12	8	562	850	955	627

Sec., Secondary response; Tert., tertiary response.

* Per 10^6 nucleated cells.

Usually more plaques were seen with heavily coupled SRC than with lightly coupled ones (Table 3). There were two qualifications to this general rule:

(1) Direct plaques were more dependent than indirect plaques on a high degree of coupling: there were very few direct plaques when No. 4 SRC were used.

(2) Lymphoid cells taken early after immunization only lysed heavily coupled SRC, while cells taken later, or after two injections of antigen, also produced plaques with lightly coupled SRC (Table 3).

The data in Table 3 suggest that with No. 1 and perhaps No. 2 SRC direct plaques may be formed even with IgG antibody. Thus more than half the No. 1 SRC plaques, produced by the lymphoid cells of rabbit No. 311 after the peak of its secondary response were direct. A quarter of the No. 1 SRC plaques produced by the cells of rabbit No. 88 at the peak of its tertiary response were direct. Yet gel-filtration studies (unpublished) showed that the corresponding serum antibodies and the serum antibodies of many other rabbits at these stages of immunization were almost exclusively IgG. Another fact suggesting that IgG was responsible for these direct plaques is that when the hapten density was reduced (No. 3 SRC) they disappeared while the number of indirect plaques (using anti-IgG) simultaneously increased. These single cell data agree with the data in Table 2 that indicate that IgG serum fractions can directly sensitize heavily coupled SRC for complement lysis.

To obtain further information about the nature of the direct anti-NNP plaques we used anti-rabbit IgM, which inhibits some or all IgM plaques (Dresser and Wortis, 1967). Anti-IgM did not inhibit the type of direct plaques which we suspected to be produced by IgG (Table 4). The data thus agree with the supposition drawn from Table 3 that a portion of direct plaques with heavily coupled SRC may be due to IgG antibody.

TABLE 4

PER CENT REDUCTION OF DIRECT PLAQUES BY ANTI-RABBIT IgM

	Day of response	Identification of animal	No. 1 SRC	No. 2 SRC	No. 3 SRC	No. 4 SRC
	5	335	85	95	83	–
	5	801	91	89	92	–
	13	303	−16	6	97	100
	24	313	4	–	–	–
	26	308	− 9	− 6	88	–
Sec.	12	311	− 4	−37	−29	–

Sec., Secondary response.

When the lymphoid cells were taken late after immunization and the erythrocytes were heavily coupled, anti-IgM sometimes increased the plaque number (Table 4). On the other hand anti-IgM strongly inhibited direct plaques produced by lymphocytes shortly after immunization. These cells are known to synthesize mainly IgM antibody (Mäkelä, Kostiainen, Koponen and Ruoslahti, 1967). With one exception (rabbit No. 311) they also inhibited direct plaques in lightly coupled SRC. Although the anti-IgM sometimes increased the plaque number the plaques obtained in the presence of it were turbid and smaller than plaques produced by the same lymphoid cells in the absence of anti-IgM.

PLAQUE SIZE

The degree of coupling affected mean plaque size as well as plaque number. With heavily coupled erythrocytes very small plaques were formed but with lightly coupled erythrocytes the plaques were larger. We believe that this was because heavily coupled erythrocytes absorbed more anti-NNP than lightly coupled ones, and thus restricted antibody diffusion. Some lymphoid cells apparently produced fewer plaques with No. 1 than No. 2 SRC and this may have been because the smaller plaques with No. 1 SRC were more easily missed.

HAPTEN INHIBITION

Inhibition of plaque formation by free hapten was used to control the specificity of the plaques. 10^{-8}–10^{6}M concentrations of NNP-aminocaproic acid,* when incorporated in the agar, reduced the plaque count to 20 per cent of the original or less (Fig. 1). Even the lowest concentration tested, 10^{-8}M, reduced counts to almost nil with some populations of lymphoid cells.

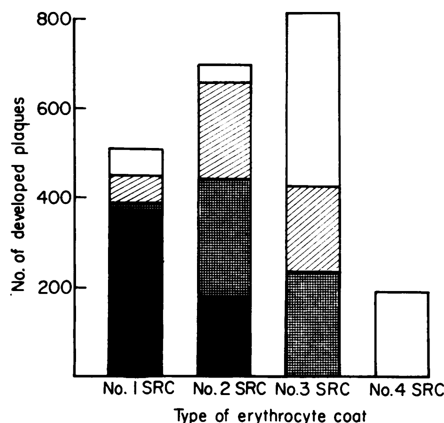

FIG. 1. Plaque formation is more easily inhibited by free hapten in lightly coupled than in heavily coupled erythrocytes. Lymphoid cells were taken from rabbit No. 308 26 days after primary immunization. Complete columns represent the total number of plaques with different hapten–erythrocyte conjugates. Open areas represent plaques that were inhibited by 10^{-8}M (final concentration) NNP hapten, hatched areas those surviving 10^{-8}M but not 10^{-7}M, stripped areas those surviving 10^{-7} but not 10^{-6}M, and solid areas those resistant to 10^{-6}M hapten.

The data in Fig. 1 suggest that free hapten is a more efficient inhibitor of plaque formation when the SRC are lightly coupled than when they are heavily coupled. For instance 10^{-8}M NNP-aminocaproic acid inhibited all the No. 4 SRC plaques and 48 per cent of the No. 3 SRC plaques, but only 6 per cent of No. 2 and 10 per cent of No. 1 SRC plaques.

DISCUSSION

Our data demonstrate that the epitope density on erythrocytes strongly affects the number and nature of plaques in the LHG assay. They emphasize the importance of standardized coupling procedures when hapten-coupled erythrocytes are used to detect cells producing anti-hapten antibody.

We wish to discuss four of our findings: (i) cells not producing IgM antibody can produce direct plaques, (ii) free hapten can inhibit the formation of plaques more easily in lightly than in heavily coupled erythrocytes, (iii) plaques caused by early antibodies are more dependent on a dense epitope coat on the erythrocytes than those caused by late antibodies and (iv) direct plaques are more dependent on a dense epitope coat than indirect ones (using anti-IgG).

* The NNP–azide conjugated to chicken globulin for the immunogen reacted predominantly with the ε-amino groups of lysines. The predominant hapten in the immunogen was thus ε-amino NNP–lysine. As free ε-amino NNP–lysine was difficult to prepare—it was difficult to avoid formation of α-amino NNP–lysine—we prepared the closely related NNP–ε-aminocaproic acid instead.

The data strongly suggest that when the epitope density on the erythrocytes is high enough IgG-producing cells can give direct plaques in the LGH assay, depending on the assay conditions. At lower epitope densities the direct plaques disappeared while the number of indirect plaques (using anti-IgG) increased by a roughly corresponding number. This agrees with the data of Borsos and Rapp (1965) and Humphrey and Dourmashkin (1965) that two IgG molecules in close proximity must interact to produce haemolysis. According to Humphrey's calculations a minimum of 820 molecules of IgG on the surface of an erythrocyte is needed to produce this close proximity (Humphrey, 1967). Our data can be explained by assuming that the hapten density on the No. 1 SRC was high enough to allow the absorption of more than the critical number of IgG anti-NNP molecules on each erythrocyte. However with Nos. 3 and 4 SRC the hapten density was not high enough for this, and anti-IgG was needed for lysis (Table 3).

Our data and the data of Plotz *et al.* (1968) show that both IgM and IgG plaques can be either direct or require antiglobulin-enhancement. The fact that IgM antibodies bind complement more efficiently than IgG antibodies probably explains why IgM plaques *generally* tend to be direct and IgG plaques indirect (Dresser and Wortis, 1967).

As well as determining the absolute upper limit of antibody molecules that can be loaded onto the surface of an erythrocyte the epitope density may be important in determining the highest number of valencies that one antibody molecule can use to bind to the erythrocyte surface (Mäkelä, Ruoslahti and Ehnholm, 1969). When the density is high, an IgG molecule may use two valencies and an IgM molecule even more. With a very low density the minimum number of epitopes that an antibody molecule can reach may be one. This factor may be unimportant when the antibody affinity is very high and one site produces sufficient binding energy. However, our data suggest that it is critical in some circumstances. On this basis we can explain the two following findings which seem difficult to explain otherwise.

One is that free (monovalent) hapten was a poorer inhibitor of plaque-formation with heavily coupled SRC than with lightly coupled SRC (Fig. 1). Free hapten is expected to be a poor competitor whenever the antibodies can attach themselves to an erythrocyte by multiple bonds (Mäkelä *et al.*, 1969).

The second observation is that the reactions of early antibodies depend more on a high hapten-density on SRC than those of late antibodies (Tables 2 and 3). To eliminate the complication caused by class differences we shall only discuss the indirect plaques developed by anti-IgG. For instance 4 or 5 days after primary immunization lymphoid cells gave no indirect plaques (using anti-IgG) with No. 4 SRC, and with No. 3 SRC only 5 per cent of the numbers seen with Nos. 1 or 2 SRC. Later in the primary response, most direct and indirect IgG plaques could be demonstrated with No. 3 SRC but not with No. 4 SRC. Lymphoid cells taken during the secondary and tertiary responses produced almost as many IgG plaques with No. 4 SRC as with No. 1 SRC. This difference could be demonstrated using the early and late cells of a single animal (rabbit No. 311, Table 3). The explanation might be that the hapten density permits efficient double bond formation with No. 1 SRC but not with No. 4 SRC. Some double bond formation may be possible with Nos. 2 and 3 SRC but it is inefficient. Double bonding may be essential for efficient binding of early IgG antibodies while late antibodies may have an affinity high enough for efficient binding using only one site.

We found that a high hapten density was more necessary for direct plaques than for those developed with anti-IgG (Table 3). This may be because a higher proportion of direct

plaques are caused by IgM. This might indicate that multiple bonds between antigen and antibody molecules are more often important to IgM than to IgG molecules. However at this early stage in the work we do not fully understand this and some other points.

Another puzzling finding is shown in Table 4. Anti-IgM sometimes reduced the number of plaques (probably IgM plaques), but sometimes (especially late in the course of immunization) enhanced it. The fact that this increase was accompanied by a decrease in the average plaque size and turbidity of the plaques may suggest that there was always some inhibition. There are also some discrepancies between the data of other workers. For instance Dresser and Wortis (1967) found that anti-IgM always reduced the number of plaques while Plotz *et al.* (1968) found that it always increased the number.

We do not know the absolute NNP-densities on our SRC, nor did other investigators know the densities of the particular epitopes they used. The observations of Humphrey (1967) suggest, however, that the density of the Forssman receptors on sheep erythrocytes is high enough to allow IgG anti-SRC antibodies to produce direct plaques. Their hapten density would thus resemble the density of our Nos. 1 and 2 erythrocytes.

ACKNOWLEDGMENTS

We thank Mrs Anja Saarela for excellent technical assistance. The work was supported by the Finnish Medical Research Council.

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