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The Induction of Haemolysin Producing Cells *in Vitro*: Inhibition by Antiglobulin Antisera

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Summary. The primary immune response (PFC) of mouse spleen cell cultures to sheep red cells was inhibited by anti-IgG, anti-Fab and anti-IgM, but not by anti-Fc or anti-allotype sera. The inhibition of PFC was independent of complement and could be prevented by addition of normal mouse serum.

INTRODUCTION

It has been postulated (Burnet, 1959; Bretscher and Cohn, 1968) that precursor cells—the cells which will differentiate, divide and secrete antibody molecules following contact with immunogen—have receptor molecules incorporated into their cell surface. To allow selective recognition of immunogen the antigen-binding sites of these molecules must have the same specificity as the binding sites of the secreted immunoglobulins. This hypothesis is supported by a variety of experimental findings: antigen dependent stimulation of DNA synthesis in spleen cell suspensions from immunized donors (Dutton and Eady, 1964), inhibition of initiation of the immune response by incubation with hapten *in vitro* (Mitchison, 1967), the demonstration that cells responsible for a specific immune response can be fractionated on antigen-coated columns (Wigzell and Anderssen, 1969), the destruction of antigen-sensitive cells by cell-bound radioactive flagellin (Ada and Byrt, 1969) or the blockage of the receptor molecules by specific affinity labeling reagents (Segal, Globerson, Feldman, Haimovich and Givol, 1969; Plotz, 1969). There is also evidence that these antigen recognition molecules are immunoglobulins or similar molecules and are bound to the cell membrane: thus, lymphoid cells can be stimulated by antiglobulin antibodies (Sell, 1967; Oppenheim, Rogentine and Terry, 1969), by anti-allotype antibodies (Sell and Gell, 1965), by anti-light chain sera (Greaves, Torrigiani and Roitt, 1969) or by anti-H chain sera (Skamene and Ivanyi, 1969). At lower concentrations, anti-light chain sera can block the response of lymphoid cells to antigen (Greaves *et al.*, 1969).

In the present study we have investigated the effect of antisera to various mouse immunoglobulin preparations on the *in vitro* response of spleen cell suspensions obtained from normal mice to foreign erythrocytes. It was found that anti-mouse IgG, anti-mouse Fab(IgG) and anti-mouse IgM inhibited the development of haemolysin producing cells, whereas anti-mouse Fc(IgG) did not have this blocking effect. Similar inhibition of the immune response has been reported recently by Fuji and Jerne (1969) and by Lesley and Dutton (1970).

MATERIALS AND METHODS

Mice

Normally ($C_{57}Bl/6$ Rij × DBA/2 Rij)F_1 mice, 8–12 weeks old, were used; in the experiments assaying the effect of anti-allotype sera $C_{57}Bl/6$ Rij and CBA/CafNimr females were used.

Spleen cell cultures

Spleen suspensions were prepared and washed 2–3 times in Hanks's Balanced Salt Solution. The cells were suspended in complete medium (Eagle's suspension medium, enriched with nonessential amino-acids, glutamine, pyruvate, antibiotics and 5 per cent foetal calf serum) and cultivated in plastic petri dishes (Falcon No. 3001) according to Mishell and Dutton (1967). In most cases the antiglobulin sera were added before the immunogen.

Erythrocytes

Sheep (SRBC) or horse (HRBC) erythrocytes were stored in Alsever's solution no longer than 4 weeks and were washed twice in BSS before use.

Cell harvest and assay for haemolysin producing cells

The cells were harvested after 4 or 5 days culture. The number of haemolysin producing cells (PFC) was determined by a modification of the technique described by Jerne, Nordin and Henry (1963) (Mishell and Dutton, 1967). The number of PFC was calculated per 10^6 recovered cells.

Preparation of the anti-globulin sera

Rabbit anti-mouse globulin serum: Rabbits were immunized with Na_2SO_4-precipitated and Sephadex G-200-fractionated mouse ascites globulin.

Rabbit anti-mouse Fab(γG_{2a}) and anti-mouse Fc(γG_{2a}) sera were prepared by Dr G. Riethmüller, Tübingen, by column fractionation of papain-digested myeloma globulin (MOPC 5).

Rabbit-anti-mouse IgM-serum: Rabbits were immunized with myeloma-globulin (MOPC 104E), prepared by Dr B. A. Askonas, Mill Hill, England. (Immunoelectrophoresis precipitation revealed lines against mouse-IgM and mouse albumin but not against mouse IgG.)

Mouse anti-allotype sera (anti-allotype CBA *a* $C_{57}Bl$, Code G2b, and $C_{57}Bl$ *a* CBA, Code Gla) was prepared by Dr N. A. Mitchison, Mill Hill.

All these sera—and isogenic mouse serum—were heat-inactivated (1 hour at 56°), diluted 1:5 in BSS and filtered through washed Millipore filters, 0·22 μ pore size. In some experiments the sera were absorbed twice with sheep erythrocytes (0·1 ml packed cells per ml serum).

RESULTS

THE ADDITION OF ANTI-IgG SERUM TO SPLEEN CELL CULTURES

The spleen cell suspensions were cultured with SRBC as immunogen. The kinetics of the development of IgM-haemolysin producing cells, and the dependence of the dose of the immunogen have been previously described (Mishell and Dutton, 1967).

When rabbit anti-mouse globulin serum was added to the cultures at the start of the culture period the number of PFC that could be detected 4–5 days later was reduced (Table 1). The degree of reduction of PFC was dependent on the amount of antiserum added. Preincubation of the antiserum and absorption with SRBC did not alter the quality of the antiserum. Equally, varying the dose of the immunogen—that is, adding between 3×10^4 and 3×10^7 SRBC to each culture—did not influence the effect of the antiserum (Table 2). Normal rabbit serum, taken from the rabbits before immunization with mouse globulin, did not inhibit the development of PFC.

The inhibition of the development of PFC did not depend on the activity of added complement. Inactivation of the complement system by heating the serum (1 hour at 56°)

TABLE 1

INHIBITION OF THE DEVELOPMENT OF HAEMOLYSIN PRODUCING CELLS BY ADDITION OF ANTI-MOUSE IgG SERUM

Volume of Anti-IgG added	Volume of mouse serum added	PFC, day 4	PFC, day 5
—	—	500	625
0·002 ml	—	690	710
0·004 ml	—	570	720
0·01 ml	—	200	450
0·02 ml	—	35	210
0·04 ml	—	2	61
0·02 ml	0·002 ml	160	420
0·02 ml	0·01 ml	515	605
—	0·01 ml	740	690

The antiserum (rabbit anti-mouse IgG serum) and the mouse serum was added to the spleen cell suspension ($1{\cdot}5 \times 10^7$ cells/culture) 20 minutes before addition of the SRBC (3×10^6). Assay of PFC 4 and 5 days later.

TABLE 2

EFFECT OF ABSORPTION OF THE ANTISERUM WITH SRBC, OF DIFFERENT DOSES OF SRBC AND OF THE COMPLEMENT SYSTEM OF THE INHIBITION OF THE IMMUNE RESPONSE

Volume of added sera	SRBC Added	PFC, day 4
—	—	61
—	3×10^4	290
—	3×10^6	380
—	3×10^7	360
0·005 ml antiserum	3×10^4	61
0·005 ml antiserum	3×10^6	55
0·005 ml antiserum	3×10^7	70
0·01 ml antiserum	3×10^4	22
0·01 ml antiserum	3×10^6	7
0·01 ml antiserum	3×10^7	25
0·01 ml normal rabbit serum	3×10^4	330
0·01 ml normal rabbit serum	3×10^6	320
0·01 ml normal rabbit serum	3×10^7	355
0·005 ml antiserum, pretreated with ovalbumin-antiovalbumin complex	3×10^6	41
0·01 ml antiserum, pretreated with ovalbumin-antiovalbumin complex	3×10^6	10

Rabbit anti-mouse globulin serum or homologous normal rabbit serum (taken before immunization) was added to the spleen cell cultures ($1{\cdot}4 \times 10^7$ cells/ml). In some experiments the antiserum was incubated with a washed complex of ovalbumin–antiovalbumin (30 minutes at 37°) in addition to the usual heat-inactivation of the complement system.

or by addition of ovalbumin–anti-ovalbumin complex did not influence the blocking effect of the antiserum (Table 2).

Normal, isogenic mouse serum neutralized and prevented the inhibition of the development of PFC caused by anti-globulin serum. This neutralization of the antiserum depends on the dose of added mouse serum. When normal mouse serum was added 1–2 days after the beginning of the culture period with anti-globulin serum there was a partial reversal of the inhibition of the development of PFC (Table 3).

Table 3

Dependence of the inhibition on the time of addition of the antiserum

Volume of anti-IgG serum added	Time of addition	PFC, day 4
—	—	450
0·04 ml	Immediately	19
0·01 ml	Immediately	74
0·04 ml	24 hours later	80
0·01 ml	24 hours later	125
0·04 ml	48 hours later	310
0·01 ml	48 hours later	480
0·04 ml	72 hours later	680
0·01 ml	72 hours later	750
Normal rabbit serum		
0·04 ml	Immediately	495
0·04 ml	24 hours later	410
0·04 ml	48 hours later	460
0·04 ml	72 hours later	390
0·04 ml anti-IgG serum	Immediately	
and 0·02 ml mouse serum	Immediately	490
and 0·02 ml mouse serum	After 24 hours	210
and 0·02 ml mouse serum	After 48 hours	64

The sera were added at time zero to the cultures ($1{\cdot}3 \times 10^7$ spleen cells and 3×10^6 SRBC/culture) or 1–3 days thereafter. Assay for PFC on 4th day.

Antiserum blocked the rise of PFC only if added to the cultures early (Table 3). A quantity of antiserum—sufficient to block the response almost completely if added at the start of the culture—was much less effective if added 24 hours after the beginning of the culture period or later. After 72 hours of culturing the antiserum had no inhibiting effect. On the contrary, antiserum added on the 3rd or 4th day of culture led in many experiments to an increased number of PFC; this increase could not be demonstrated by addition of normal rabbit serum (Table 3).

Addition of anti-Fab-fragment serum, anti-Fc fragment serum or anti-allotype serum to spleen cell cultures

Rabbits were immunized with the Fab fragment or Fc fragment of γG_{2a}-myeloma globulin. The antisera were added to the spleen cell cultures, and their effect was compared with the inhibition of the development of PFC by anti-globulin serum (Table 4). Rabbit anti-mouse Fab fragment serum inhibited the rise of PFC during the culture period whereas rabbit anti-mouse Fc serum had only a slight effect. Anti-allotype serum, which had shown clear precipitation lines in the agar-double-diffusion assay, did not inhibit the immune response (Table 5).

TABLE 4

INHIBITION OF THE DEVELOPMENT OF HAEMOLYSIN PRODUCING CELLS BY ADDITION OF ANTI-FAB AND ANTI-FC SERUM

Volume of Antiserum added	PFC		
	Cultures without mouse serum	Cultures with 0·02 ml mouse serum	Cultures with 0·01 ml mouse serum
—	300	340	236
0·0025 ml anti-IgG*	75	—	—
0·005 ml anti-IgG	38	288	228
0·01 ml anti-IgG	10	—	—
0·02 ml anti-IgG	0	140	129
0·0025 ml anti-Fab†	220	—	—
0·005 ml anti-Fab	54	220	87
0·01 ml anti-Fab	33	149	48
0·02 ml anti-Fab	4	146	12
0·005 ml anti-Fc‡	270	270	—
0·01 ml anti-Fc	260	270	310
0·02 ml anti-Fc	134	310	340

The antiserum and the mouse serum were added to the cultures ($1{\cdot}3 \times 10^7$ spleen cells). The immunogen (3×10^6 SRBC) was added 20 minutes later. PFC were assayed on day 4.

* Rabbit anti-mouse IgG serum, inactivated

† Rabbit anti-mouse Fab (γG_{2a}) serum, inactivated

‡ Rabbit anti-mouse Fc (γG_{2a}) serum, inactivated

TABLE 5

EFFECT OF ANTI-ALLOTYPE SERUM ON THE SPLEEN CELL CULTURE RESPONSE

Spleen cells	Volume of antiserum		PFC, day 4
	CBA *a* $C_{57}Bl$	$C_{57}Bl$ *a* CBA	
BDF_1	—	—	440
	0·01 ml	—	480
	0·02 ml	—	520
	—	0·01 ml	510
	—	0·02 ml	460
$C_{57}Bl/6$	—	—	260
	0·02 ml	—	180
	—	0·02 ml	220
CBA	—	—	520
	0·02 ml	—	480
	—	0·02 ml	580

The anti-allotype serum was dialysed 48 hours against phosphate-buffered saline, run through a Sephadex G-25 column (to remove the merthiolate) and filtered. It was added to cell cultures of $C_{57}Bl/6$ spleen ($1{\cdot}3 \times 10^7$ cells/ml), of CBA spleen ($1{\cdot}5 \times 10^7$ cells/ml) and of BDF_1 spleen ($1{\cdot}1 \times 10^7$ cells/ml). 3×10^6 SRBC had been added; the PFC/10^6 were counted 4 days later.

INHIBITION OF THE DEVELOPMENT OF PFC BY ANTI-IgM SERUM

Antiserum, prepared in rabbits against MOPC 104E myeloma protein (μ and λ chains), inhibited the development of PFC in the spleen cell cultures (Table 6); the inhibition could be reversed by simultaneous addition of isogenic mouse serum.

Table 6

Inhibition by anti-IgM serum

Volume of antiserum* added	Volume of mouse serum added	PFC, day 4
—	—	206
0·002 ml	—	220
0·004 ml	—	54
0·01 ml	—	38
0·02 ml	—	2
0·04 ml	—	0
0·004 ml	0·01 ml	290
0·01 ml	0·01 ml	43
0·02 ml	0·01 ml	3
0·01 ml	0·02 ml	234
0·02 ml	0·02 ml	150
0·04 ml	0·02 ml	8
0·01 ml	0·04 ml	320
0·02 ml	0·04 ml	146
0·04 ml	0·04 ml	69

The antiserum (inactivated) and the mouse serum (inactivated) were added to the spleen cell cultures ($1{\cdot}1 \times 10^7$ cells) 20 min before the SRBC. Assay for PFC after 4 days of culture.

* Rabbit-anti-γM(MOPC 104 E) serum.

DISCUSSION

A number of experiments have been cited (Introduction) which are compatible with the hypothesis that antigen-receptor molecules are present in the cell membrane of lymphoid cells, and that these receptor molecules have many qualities in common with humoral antibody molecules. To learn more about the receptor molecules a variety of antisera against mouse globulins or globulin fractions were added to spleen cell cultures stimulated by erythrocyte antigens. It was expected that attachment of the antibody to the receptor molecules would block the induction of the haemolysin response. Rabbit anti-mouse IgG serum added to the spleen cell cultures did reduce the number of PFC developing 3–4 days later; it was shown that (Table 2) anti-SRBC antibodies (Henry and Jerne, 1967) were not responsible for this effect. Action of the complement system did not appear to be necessary.

Inhibition was most effective if antiserum was added at the beginning of the culture period or if the spleen cells were preincubated with the antiserum. Antiserum added 2–3 days later had little or no blocking effect, perhaps because it was neutralized by soluble globulins already synthesized and secreted. These experiments, therefore, do not answer the question whether antigen receptor molecules are necessary only during the induction process or whether their presence is necessary continuously during cell division and antibody synthesis.

In many cultures incubated with small amounts of antiserum there were few PFC detectable after 3 days, but thereafter the number of PFC increased faster than in control cultures. Similarly, if the antiserum was added 3 days after culturing the number of PFC rose faster than in control cultures. In this phase of the *in vitro* response the stimulatory effect of antiserum might be due to the neutralization of newly synthesized antibody (Henry and Jerne, 1967; Moller and Wigzell, 1965; Uhr and Baumann, 1961).

Of the different antisera which we have tested anti-IgG, anti-Fab fragment and anti-

IgM serum did block the development of PFC. The anti-IgG and anti-Fab had affinity mainly for κ and γ chains; our anti-IgM had affinity for λ and μ chains only. The PFC assayed in the cultures by the direct plaque assay (Jerne *et al.*, 1963) are considered to be IgM secreting cells. Since most of the cells are producing κ light chains it seems possible that the haemolysin response can be blocked by anti-μ chain activity, and by anti-κ chain activity (this has been recently demonstrated with a purified anti-κ chain serum (Lesley *et al.*, 1970)). It cannot be blocked by anti-Fc fragment serum or by anti-allotype serum, either because the Fc fragment of the receptor molecules is not available to the antibody, or because these receptor molecules have H-chains different from the secreted molecules (IgX).

The results show that an immunoglobulin molecule is in some way involved in the initiation and continuation of the response. It is possible that the molecule is not cell-bound but has the properties of a 'carrier' antibody (Henry and Jerne, 1967; Bretscher and Cohn, 1968), for which the argument would be equally applicable. Since anti-Fc did not block the immune response *in vitro*, these antibody molecules with 'helper function' either do not play an essential role during the induction phase, or they carry an H chain not recognized by our anti-Fc serum.

Since anti-allotype sera did not inhibit the haemolysin response they cannot be used to test the hypothesis that two different lymphoid cells have to cooperate during the immune response. Similarly, calculations of the number of receptor molecules on the surface of our antigen-sensitive or antigen-reactive cells are not yet possible since we do not know the affinity of the antisera for the cell-bound receptor molecules, whether there is steric hindrance of the antibody binding of cell-bound receptor molecules, or if the cooperating cells carry different receptor molecules. It is hoped to answer some of the questions by studying the immune response in rabbit spleen cells in which allotypic markers are known to exist on the L-chain and on the Fd fragment of the H-chain.

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