Immunology, 1972, **22**, 259.

Effect of Antibodies Against Immunoglobulins and the Theta Antigen on the Specific and Non-Specific Stimulation of Mouse Spleen Cells *in Vitro*

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(*Received 7th May* 1971)

Summary. Normal mouse spleen cells were stimulated in culture by phytohaemagglutinin (PHA), pokeweed mitogen (PWM), allogeneic cells; keyhole limpet haemocyanin (KLH) sensitized cells by the specific antigen. The stimulation of the cells was measured by [^{3}H]thymidine incorporation into the TCA precipitable fraction of the cultures. In this system, the effect of treating the cells with an antibody against the theta antigen and an antibody against immunoglobulin, with or without complement, was investigated. Treatment of the cells with antisera and complement or without complement gave similar results. The secondary *in vitro* stimulation with soluble antigen KLH could be markedly reduced with both anti-θ and anti-immunoglobulin serum. The response to allogeneic cells in the mixed lymphocyte reaction was only reduced by anti-θ serum and not by anti-immunoglobulin serum. No definite effect could be demonstrated by either antibody on the non-specific stimulation by PHA or PWM.

INTRODUCTION

The immune response of mice to certain antigens requires the co-operation of at least two distinct cell types. In transfer experiments to irradiated recipients, marrow-derived cells generate large numbers of antibody producing cells only in the presence of thymus derived cells and antigen (Miller and Mitchell, 1969). The exact mechanisms of co-operation, however, are not known. Recently, markers have been recognized which are specific for thymus cells and others, probably specific for some of the bone-marrow-dependent cells. The θ antigen is found on the surface of thymus and thymus-derived peripheral lymphoid cells in several strains of mice such as C3H, BALB/c and C57Bl/6 (Reif and Allen, 1966). Specific antibodies against θ-C3H can be produced in AKR mice which have the same H-2-locus (Raff, 1969; Takahashi, Carswell and Thorbecke, 1970). Immunoglobulin-like molecules have been recognized on the outer cell surface of lymphocytes of the peripheral lymphoid organs and the blood but only very rarely on thymus cells (Coombs, Gurner, McConnell and Munro, 1970; Raff, 1970b; Rabellino, Colon, Grey and Unanue, 1971). These immunoglobulin-like molecules can react with antigen (Wigzell and Mäkelä, 1970). Circumstantial evidence makes it likely that the cells displaying such immunoglobulin-like receptor molecules are bone-marrow-derived thymus independent lymphocytes (Rabellino *et al.*, 1971).

Antibodies to immunoglobulins have been used to inhibit DNA production *in vitro* of sensitized lymphoid cells of the rabbit after re-exposure to the antigen (Daguillard and Richter, 1970). Antibodies against the θ antigen are able to block the capacity of sensitized spleen cells to confer immune reactivity to irradiated syngeneic recipients (Raff, 1970a), and to block *in vitro* sensitization to sheep red blood cells (Schimpl and Wecker, 1970).

In the following experiments, the effects of antibodies against immunoglobulin and θ antigen on the *in vitro* reactivity of mouse spleen cells were investigated. Sensitized and normal spleen cells were cultured in the presence of specific antigen, allogeneic cells and non-specific stimulants, and their reactivity assessed by the measurement of [^{3}H]thymidine incorporation. Some of the cells were brought in contact with these antibodies before or during the culture and their reactivity compared to control cells.

MATERIALS AND METHODS

Animals

Inbred mice of the BALB/c and the C3H strain were obtained from the Tierfarm AG Sisseln, and of the C57Bl/6 and the AKR strain from the Institut für biologisch-medizinische Forschung AG, Füllinsdorf, Switzerland. They were maintained on a standard laboratory diet and water *ad libitum*. In all experiments, female mice of initial age 5–8 weeks were used. Rabbits were obtained from a local breeder.

Cell suspensions

Mice were anaesthetized with ether, bled, and killed by cervical dislocation. Spleen or thymus were removed aseptically and minced in Petri dishes. After being gently squeezed, the cells were washed from the tissues with an excess of Eagle's minimum essential medium (MEM) (Baltimore Biological Laboratory), supplemented with L-glutamine (292 μg/ml), penicillin-G (100 U/ml), streptomycin (100 μg/ml) and buffered with -N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid (HEPES, Calbiochem). The cells were then transferred to conical centrifuge tubes, fat and remaining tissue pieces removed and washed once by centrifugation.

Immunization

BALB/c mice were immunized by one intraperitoneal injection of 0.1 mg of keyhole limpet haemocyanin (Calbiochem) (KLH) adsorbed on bentonite by the method of Gallily and Garvey (1968). The sera showed titres of at least 1/620 3 weeks after immunization when tested with the tanned cell haemagglutination method as described previously (Vischer and Stastny, 1967). Mice were used for culture 3–8 weeks after immunization.

Anti-θ C3H antibodies (anti-θ) were produced by injecting 10^8 C3H thymus cells three to four times at monthly intervals; 10^9 pertussis organisms were added to the first injection. Mice were bled 1 week after the last injection. Two pools were used during the experiments reported in this paper; one was used for incubations in the presence of guinea-pig serum as a source of complement and the other was used for adding to cultures. Normal AKR serum was obtained by orbital bleeding of the same mice before immunization. Specificity of the anti-θ was assessed by the indirect fluorescence method with viable lymphnode and thymus cells as described by Raff (1970b). AKR cells were consistently

negative and staining of (BALB/c × C57Bl/6) F1 or C3H cells gave results similar to those reported by Raff (1970b).

For the production of an antiserum against mouse immunoglobulins (anti-Ig), rabbits were immunized with mouse serum fraction II (Mann Research Laboratories). On immunoelectrophoresis, the anti-Ig reacted strongly with immunoglobulins and faintly with transferrin and some minor β-globulin components. One pool was used for all experiments. Normal rabbit serum came from a pool obtained from several rabbits. All sera were incubated at 56° for 30 minutes before use.

Cultures

Triplicate samples of 10^7 spleen cells were cultured in 1 ml of MEM enriched with 5 per cent normal rabbit serum. 12 × 75 mm polystyrene tubes were used for the cultures (Falcon Plastics). In some tubes, the same cells were cultured in the presence of 5 μl of a 1/10 dilution of phytohaemagglutinin-P (PHA) (Difco), 5 μl pokeweed mitogen (PWM) (Grand Island Biological Co.), or 5 μl of KLH (0.5 mg/ml in MEM). In mixed lymphocyte cultures, 5×10^6 BALB/c spleen cells were cultured together with an equal number of C57Bl/6 cells.

In some experiments, cell suspensions were incubated before culture for 45 minutes at 37° with 0·1 ml of antiserum or the corresponding normal serum and 0·2 ml of guinea-pig serum as a source of complement (Difco) per ml at a cell concentration of 25×10^6/ml made up with MEM. The cells were subsequently washed three times in an excess of MEM enriched with 1 per cent rabbit serum, and cultured at a concentration of 15×10^6/ml, calculated from the initial suspension. Cell death after incubation determined by the trypan blue test was about 10 per cent after treatment with complement and normal serum, 30 per cent after treatment with anti-θ and 20 per cent after treatment with anti-Ig.

In other experiments, cell suspensions were incubated for 2 hours at 37° with anti-Ig or normal rabbit serum under the conditions described above omitting guinea-pig serum. Anti-θ (pool 2) was added to other cultures (10 μl/culture); the control tubes received normal AKR serum.

Measurement of [^{3}H]thymidine incorporation

To measure the rate of DNA synthesis of the cultured cells, 0·5 μCi of [^{3}H]thymidine (The Radiochemical Centre, Amersham, specific activity: 5·0 Ci/mM) was added 16 hours before termination of cultures. Zero-time samples, in which the radioactive label is added after termination of the cultures were included in each experiment. At the end of the culture period, 10 per cent of cold trichloracetic acid was added to the cultures and the resulting precipitate washed twice with 5 per cent trichloracetic acid and once with cold absolute ethanol. The residue was then dissolved in 0·5 ml of Soluene (Packard) and mixed with 10 ml counting solution (5 g PPO, 0·4 g dimethyl-POPOP, 100 ml Toluol). Radioactivity was determined in a Nuclear Chicago liquid scintillation counter at 46 per cent efficiency.

The results are expressed as the mean of the net counts per minute of triplicate cultures ± the standard deviation (SD). Sometimes, the ratio of counts per minute of stimulated cultures to counts in the corresponding control cultures is also given. Tables 1–6 represent one experiment each using the same original cell pool of autogeneic or syngeneic cells. All experiments reported have been done at least twice with similar results.

RESULTS

INFLUENCE OF ANTI-θ AND ANTI-IG ON THE STIMULATION OF SPLEEN CELLS OF KLH IMMUNIZED MICE EXPOSED TO SPECIFIC ANTIGEN *in vitro*

Incubation of sensitized spleen cell suspensions with anti-θ or anti-Ig together with complement before culturing resulted in a markedly reduced stimulative response after exposure to the specific antigen KLH (Table 1). Both the absolute incorporation of [^{3}H]thymidine and the ratio of the counts after stimulation by antigen to counts without antigen were reduced. Similarly, addition of neat anti-θ during the culture time clearly reduced the response when compared to cultures where normal AKR serum was added (Table 2). Addition of anti-Ig to the cultures has by itself a stimulatory effect on [^{3}H]-

TABLE 1

INHIBITION OF ANTIGEN INDUCED LYMPHOCYTE STIMULATION BY PREINCUBATION OF SENSITIZED CELLS WITH ANTIBODIES AGAINST IMMUNOGLOBULIN OR THE θ-ANTIGEN AND COMPLEMENT*

Treatment with C and:	s̄ KLH c/min (±SD)†	c̄ KLH c/min (±SD)†	Ratio c̄/s̄
Normal mouse serum	2210 (±162)	22533 (±647)	10·2
Anti-θ	469 (±24)	1355 (±10)	2·9
Normal rabbit serum	3497 (±143)	34033 (±1790)	9·7
Rabbit anti-mouse Ig	2239 (±134)	7956 (±1094)	3·5

s̄ = without; c̄ = with.

* Spleen cells of BALB/c mice immunized with KLH and exposed *in vitro* to the antigen.

† Mean net counts/min. of triplicate cultures ± standard deviation. 0·5 μCi [^{3}H]thymidine was added at 60 hours and radioactivity of TCA precipitable fraction measured at 72 hours.

TABLE 2

EFFECT OF ADDING ANTI-θ TO CULTURES STIMULATED BY SPECIFIC ANTIGEN*

	s̄ KLH c/min (±SD)†	c̄ KLH c/min (±SD)†	Ratio c̄/s̄
Cultures with 10 μl of normal serum added	4241 (±1082)	39079 (±1793)	9·2
Cultures with 10 μl of anti-θ	4426 (±36)	19135 (±934)	4·3

* Spleen cells of BALB/c mice immunized with KLH and exposed *in vitro* to the antigen.

† See Table 1.

thymidine incorporation. However, incubation of the cells with anti-Ig for 2 hours in the absence of active complement results in no cell loss and no spontaneous stimulation and was sufficient to reduce the response to antigen to a considerable degree (Table 3).

INFLUENCE OF ANTI-θ AND ANTI-IG ON THE MIXED LYMPHOCYTE REACTION

In contrast to the stimulation *in vitro* induced by the specific soluble antigen KLH, preincubation with anti-Ig and complement had no effect on the [^{3}H]thymidine incorporation during the mixed lymphocyte reaction using two strains of mice differing at the H-2-locus. Anti-θ and complement however had a marked effect (Table 4). Similarly, preincubation with anti-Ig in the absence of active complement did not alter the reactivity. Addition of anti-θ however reduced the reaction (Table 5).

EFFECT OF ANTI-θ AND ANTI-Ig ON THE NON-SPECIFIC STIMULATION OF SPLEEN CELLS INDUCED BY PHA AND PWM

Incubation of the cell suspension with anti-θ or anti-Ig together with complement did not much decrease the [^{3}H]thymidine incorporation after stimulation with PHA or PWM (Table 6). This experiment has been repeated three other times with similar results, using two different lots of anti-θ. Similar results were obtained after preincubation with anti-Ig in the absence of active complement or when anti-θ was added to the cultures

TABLE 3

EFFECT OF PREINCUBATION WITH ANTI-Ig ON THE SPECIFIC STIMULATION BY SOLUBLE ANTIGEN *in vitro**

	$\bar{s}$ KLH c/min (±SD)†	$\bar{c}$ KLH c/min (±SD)†	Ratio $\bar{c}/\bar{s}$
Cultures incubated with normal rabbit serum	239 (±48)	1212 (±171)	5·0
Cultures incubated with anti-Ig	130 (±10)	266 (±14)	2·0

* Spleen cells of BALB/c mice immunized with KLH and exposed *in vitro* to the antigen. Before culturing, the cells were incubated with inactivated anti-Ig in the absence of complement and subsequently washed.
† See Table 1.

TABLE 4

EFFECT OF PREINCUBATION WITH ANTI-θ OR ANTI-Ig AND COMPLEMENT ON THE MIXED LYMPHOCYTE REACTION

Treatment with C and:	BALB/c c/min (±SD)†	C57Bl c/min (±SD)†	C57Bl × BALB/c c/min (±SD)†	Ratio*
Normal mouse serum	1333 (±169)	962 (±110)	7077 (±1339)	6·2
Anti-θ	1235 (±108)	634 (±144)	2155 (±187)	2·3
Normal rabbit serum	1170 (±270)	805 (±64)	6779 (±394)	6·9
Rabbit anti-mouse Ig	1164 (±59)	700 (±43)	5641 (±566)	6·2

* Ratio of column 3 to half of the sum of columns 1 and 2.
† See Table 1.

TABLE 5

EFFECT OF ADDING ANTI-θ TO THE MIXED LYMPHOCYTE REACTION*

	BALB/c c/min (±SD)†	C57Bl/6 c/min (±SD)†	BALB × C57Bl/6 c/min (±SD)†
Normal AKR serum added	394 (±36)	316 (±25)	1414 (±69)
Anti-θ added	193 (±46)	155 (±6)	297 (±84)

* 10^7 spleen cells per culture.
† See Table 1.

(Table 7). The absolute counts obtained in all cultures with antibodies are lower. The ratio of counts of the stimulated cultures per counts of non-stimulated cultures rather increased than decreased in the experiments with complement.

DISCUSSION

Daguillard and Richter (1970) demonstrated in the rabbit that the treatment of lymphoid cells with anti-Ig and complement or treatment with the same antibody in the

absence of complement reduced the specific proliferation of sensitized cells when exposed to specific antigen *in vitro*. Our results confirm their findings in another species, the mouse. Similar results were obtained in a primary *in vitro* immune response with a specific anti-κ chain serum in the mouse by Lesley and Dutton (1970).

Daguillard and Richter (1970) observed as well that preincubation of cells with anti-Ig during a short time was not sufficient to induce stimulation of the cells by anti-Ig. This stimulant needs to be present during the whole time of culture. Although the fixation of antibody on the cell surface proceeds within minutes as demonstrated by the immunofluorescence method, additional reactions taking more time are required for stimulation of the cells. The explanation of this phenomenon remains speculative at the present time. Lattice formation on the cell surface or reaction of the antibody with freshly divided cells during the course of culture could be required for stimulation.

TABLE 6

EFFECT OF PREINCUBATION WITH ANTI-θ OR ANTI-IG AND COMPLEMENT ON THE UNSPECIFIC LYMPHOCYTE STIMULATION*

Treatment with C′ and:	$\bar{s}$ c/min (±SD)†	$\bar{c}$ PHA c/min (±SD)†	Ratio	$\bar{c}$ PWM c/min (±SD)†	Ratio
Normal mouse serum	401 (±20)	19397 (±1971)	48	17498 (±1816)	44
Anti-θ	190 (±14)	13659 (±1311)	72	9609 (±564)	50
Normal rabbit serum	397 (±31)	14541 (±961)	37	16065 (±1071)	41
Rabbit anti-mouse Ig	193 (±17)	11881 (±691)	67	11058 (±1317)	52

* Balb/c spleen cells. Stimulants present during the whole time of culture.
† See Table 1.

TABLE 7

EFFECT OF ANTI-IG AND ANTI-θ ON NON-SPECIFIC STIMULATION OF MOUSE SPLEEN CELLS

	$\bar{s}$ c/min (±SD)†	PHA c/min (±SD)†	Ratio PHA/$\bar{s}$	PWM c/min (±SD)†	Ratio PWM/$\bar{s}$
Normal rabbit serum*	184 (±25)	416 (±98)	2·3	705 (±108)	3·8
Anti-Ig*	106 (±6)	254 (±10)	2·4	522 (±80)	4·9
Normal mouse serum‡	1074 (±205)	32711 (±5530)	30·5	14537 (±2379)	13·5
Anti-θ‡	1027 (±84)	25327 (±2558)	24·6	14997 (±3164)	14·6

* Culture No. 216. Cells incubated for 2 hours with normal rabbit serum or anti-Ig. Stimulants were only added during the 2 hours of incubation. Cells washed subsequently three times.
† See Table 1.
‡ Culture No. 220. 10 μl normal mouse serum or anti-θ added to cultures. Stimulants present during the whole culture period.

The reduction of the KLH response after pretreatment with anti-Ig and complement cannot solely be due to the decreased number of cells as pretreatment without complement where no significant cell loss in comparison with the control cell population occurs, gives the same effect. Blocking of immunoglobulin-like receptors on the surface of lymphoid cells or elimination of these cells seems the most likely mechanism. These immunoglobulin receptors probably react with antigens (Wigzell and Mäkelä, 1970). Similarly, elimination of θ-bearing, thymus derived cells significantly decreased their proliferative secondary response induced *in vitro* by KLH. Again, addition of anti-θ in the absence of active complement had a similar effect, making the difference in cell numbers in the experiments with complement a secondary factor. Takahashi *et al.* (1970) obtained

similar results *in vivo* by transferring anti-θ incubated lymphoid cells to irradiated recipients; both the primary and the secondary antibody responses were diminished.

From the above experiments it can be concluded that the stimulation of [^{3}H]thymidine incorporation during the secondary response *in vitro* to KLH is based both on cells with Ig receptors and cells carrying the θ antigen. These experiments do not throw light on the question of whether only one or both cell types react primarily with the antigen. Nonspecific mitogens released into the medium after stimulation of a few cells with the antigen might account for the activity of the other cells (Imrie and Mueller, 1968; Larralde, 1970). It is not known whether θ or Ig-receptor-bearing cells produce such mitogens and which cell type responds to them. Experiments elucidating these questions are currently under way.

The mixed lymphocyte reaction, believed to involve the cell-mediated immune system, could easily be suppressed both by adding anti-θ or by treating the cells with the antibody and complement before culturing. Anti-Ig had no effect. This is in contrast with the results of Greaves, Torrigiani and Roitt (1969) who were able to suppress the mixed lymphocyte reaction of human peripheral lymphocytes with certain antisera against human light chains. Only one anti-mouse-Ig was used in our experiments. However, this anti-Ig was highly efficient in suppressing the response to KLH *in vitro*. Our results indicate a basic difference between the stimulation of [^{3}H]thymidine incorporation *in vitro* during a secondary response to KLH and during a response to allogeneic cells. If any Ig receptors are needed for the mixed lymphocyte reaction, they clearly have to be different from those of importance to the secondary response to KLH.

The failure significantly to reduce the reaction to PHA or PWM with anti-Ig is consistent with the report of Daguillard and Richter (1970) who showed in the rabbit that different cell types reacted with PHA and with soluble antigens and that the PHA response could not be suppressed by anti-Ig. Surprisingly, however, treatment of mouse spleen cells with anti-θ and complement did not abolish the response to PHA or PWM. The antisera used were those which were highly reactive with the specific stimulants. The response to PHA is significantly reduced with cells obtained from neonatally thymectomized rats (Rieke, 1966) and mice (Takiguchi, Adler and Smith, 1971). PWM in our system reacts optimally with cells obtained from the thymus when compared with cells from other lymphoid organs (Vischer, unpublished data). From recent reports, however, it seems likely that cells active in the mixed lymphocyte reaction can be separated from the cells responding optimally to PHA (Colley, Shih Wu and Waksman, 1970), and that different cell types as determined by density gradient separation react to this mitogen (Takiguchi *et al.*, 1971). Not all these cells might carry enough θ antigen on their surface to be inactivated by the anti-θ used in the present experiments.

The present experiments demonstrate a basic difference between the secondary response to soluble antigen *in vitro* and the response to allogeneic cells. The system using specific suppression of thymus-derived cells and some of the bone-marrow-derived cells promise a wide application in elucidating the mechanism and regulation of the *in vitro* lymphocyte reaction.

ACKNOWLEDGMENTS

The excellent technical collaboration of Miss Doris Plüss and Miss Margret Haag is highly appreciated.

This work was supported by a grant from the Swiss National Fund for Scientific Research.

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