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Role of membrane structures in B-lymphocyte activation

THE EFFECT OF BINDING ANTI-IMMUNOGLOBULIN, AGGREGATED IgG AND IMMUNE COMPLEXES

R. RAMASAMY *Department of Immunology, A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

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Summary. It is shown that the binding of bivalent anti-immunoglobulin preparations to the surface immunoglobulin of murine B lymphocytes as well as the binding of aggregates of IgG or immune complexes does not lead to detectable increase in cell division in microcultures. Treating lymphocytes with immune complexes and aggregated IgG does not abolish the subsequent mitogenic response induced by LPS. The background mitosis observed in culture is inhibited with anti-immunoglobulin antibodies.

INTRODUCTION

It has been suggested that Fc receptors are identical to or closely associated on the B-lymphocyte membrane with antigens coded for by genes of the I region in the histocompatibility locus (Dickler and Sachs, 1974; Basten, Miller and Abrahams, 1975). The I region is involved in the control of the antibody response to many antigens. Genes mapping in the I region of the histocompatibility locus have been reported to code for antigen-specific, T lymphocyte-derived molecules (antigen-specific T-cell factor) as well as the corresponding acceptor molecules on B lymphocytes (Munro and Taussig, 1975). A hypothesis has also been put forward that the Fc receptor on a B-lymphocyte functions as a 'pro-receptor' for antigen receptor, i.e. an integral membrane component which serves to anchor immunoglobulin receptors to the cell membrane (Ramasamy, Munro & Milstein, 1974). Thus a close relationship is claimed between structures involved in the B-lymphocyte response to antigen and Fc receptors.

Correspondence: Dr R. Ramasamy, Department of Immunology, A.R.C. Institute of Animal Physiology, Babraham, Cambridge CB2 4AT.

The binding of antigen–IgG complexes or aggregated IgG to a B cell through the Fc receptor mimics the action of binding multivalent antigen or aggregated monovalent antigen. The difference is in the nature of the immunoglobulin receptor on the B-cell membrane. Thus the immunoglobulin receptor on B cells is reported to be IgM subunit-like molecules on the basis of cross-reactivity with serum IgM and sizing on polyacrylamide–SDS gels (Marchalonis and Cone, 1973; Vitetta and Uhr, 1973).

In view of these observations and since cell division is a feature of antigen-induced B-lymphocyte activation, experiments were undertaken to determine whether the binding of antigen–antibody complexes and aggregated IgG to Fc receptors results in the activation of B lymphocytes to cell division. The results show that binding of IgG aggregates and immune complexes to murine B cells does not lead to the rapid cell division which is readily induced by the B-cell mitogen, *Salmonella typhosa* lipopolysaccharide (LPS).

Anti-immunoglobulin sera have been shown to activate lymphocytes to division in rabbits (Sell, Rowe and Gell, 1965) and chickens (Weber, 1975), but not in mice (Elson, Singh and Taylor, 1973; Greaves, Janossy, Feldmann and Doenhoff, 1974). The results described in this investigation confirm that anti-immunoglobulin treatment does not activate a detectable proportion of murine B lymphocytes to division. However, an inhibition of the background rate of cell division is observed.

MATERIALS AND METHODS

LPS

Bacto-lipopolysaccharide *S. typhosa* (LPS), prepared by the Westphal method was obtained from Difco Laboratories, Surrey. The LPS was dissolved in phosphate-buffered saline (PBS) and millipore filtered for sterilization.

Rabbit anti-mouse IgG

A rabbit antiserum to mouse IgG was obtained by immunization with purified Adj PC5 myeloma protein (IgG 2a/kappa) in Freund's complete adjuvant. A Fab_2 fraction of the serum obtained by 18 per cent sodium sulphate precipitation followed by DEAE–cellulose chromatography was digested with pepsin in 0·1 M sodium acetate buffer, pH 4·0. One to 2 mg of pepsin was added for every 50 mg of protein. The pepsin digest was then fractionated on a Sephadex G-200 column. The fractions were tested for the presence of an Fc determinant by a two dimensional immunodiffusion test. This involved the detection of a spur due to antigenic determinants on the Fc, on comparing intact IgG with fractions from the pepsin digest using anti-rabbit IgG serum. Fractions which showed no trace of Fc by this test were then pooled, tested for antibody activity, and termed the Fab_2 anti-mouse IgG preparation. The Fab_2 preparation was then absorbed three times with mouse thymocytes, dialysed against PBS and sterilized by millipore filtration. A Fab_2 fraction of a sheep antiserum to mouse Fab prepared in a similar manner was used in some experiments.

When whole anti-IgG serum was used in the experiments, the antiserum was absorbed with mouse thymocytes and heat-inactivated at 56° for 30 min. This was done to eliminate the binding of natural antibodies to mouse cells present in rabbit serum and the lytic effects of complement.

Rabbit IgG aggregates

Freeze-dried rabbit IgG (Schwartz-Mann, Orangeburg, New York) was made up to 5 mg/ml in PBS and absorbed three times with mouse thymocytes. The solution was then sterilized by millipore filtration and heated to 63° to induce clearly visible opalescence. To determine the extent of aggregation, a sample was tested by analytical centrifugation. A comparison with an unaggregated solution of IgG at 5 mg/ml showed that only about 50 per cent of the original IgG was still present as monomeric IgG material while the rest had been converted to large polymers.

Immune complexes

Immune complexes were preformed by adding a heat-inactivated rabbit anti-ferritin serum which had been preabsorbed with mouse thymocytes, to an equivalent amount of ferritin as determined by a prior immune precipitation titration. Aliquots were then added to the cultures.

Addition of reagents to culture

Reagents in PBS were usually added in 10–20 μl aliquots to 200 μl of culture. Radioactive thymidine was added in 20 μl of tissue culture medium. In the experiment to determine the effects of pretreating lymphocytes with anti-immunoglobulin and aggregates of rabbit IgG on a subsequent LPS response, the anti-immunoglobulin and aggregated IgG were added at the beginning of culture followed by LPS after 18–24 h.

Microculture

Spleen cells from nu/nu mice obtained from the MRC Laboratory Animal Centre, Carshalton, Surrey, or the Department of Veterinary Medicine, University of Cambridge, were used as a source of B cells in the experiments. Spleen cells from B mice, i.e. CBA mice which had been thymectomized, irradiated at 850 R and reconstructed with bone marrow cells and then the spleens used as a source of B cells, gave results similar to those described here. The cells were washed in RPMI 1640 medium and resuspended in culture medium at $2–3 \times 10^6$ cells per ml. This cell concentration was found to be optimal in the experimental system. The culture medium was RPMI 1640 containing 5 or 10 per cent heat-inactivated foetal calf serum with added pyruvate, glutamate, mercaptoethanol at a final concentration of 5×10^{-5}M, anti-fungal agent and

antibiotics. The cultures were gassed with a 5 per cent CO_2:95 per cent air mixture and no further nutrients were subsequently added.

The cells were cultured in Nunc tissue culture microtest plates with approximately $4–6 \times 10^5$ cells in a well in 0·2 ml of medium. Each test was done in triplicate cultures.

Determination of rates of DNA synthesis

One microcurie of (methyl) [^{3}H]thymidine from the Radiochemical Centre, Amersham, Bucks, diluted with non-radioactive thymidine to give a specific activity of 50 mCi/millimole was added to each well 6 h prior to harvesting. The cells were harvested mechanically onto Whatman glass fibre paper discs. The discs were allowed to dry, immersed in 2 ml of scintillant containing 4 g of PPO (diphenyloxazole) and 0·1 g POPOP (1,4-di(2-(5-phenyloxazolyl))) benzene dissolved in a litre of toluene and counted in a Packard Liquid Scintillation Counter.

Expression of results

The results are expressed as the mean of three replicate cultures ± the standard error of the mean. The stimulation index refers to the ratio of thymidine incorporation in test cultures to the thymidine incorporation in cultures where no reagent or only PBS was added.

RESULTS

Effect of rabbit IgG aggregates

The effects of adding a heat-aggregated rabbit IgG preparation which contained a significant proportion of aggregates was investigated and the result of an experiment summarized in Table 1. The results show that aggregated IgG induces no significant activating effect comparable to LPS on thymidine incorporation. Pretreating B cells with aggregated IgG for 18–24 h does not abolish the subsequent response of the cells to LPS. This is in contrast to the effects of the anti-mouse IgG serum which produces a marked inhibition of the LPS response and the background rate of thymidine incorporation. The ability of the rabbit IgG used in this experiment to bind to murine spleen cells was checked by immunofluorescence staining of cells treated with aggregates, with a fluorescein-conjugated goat anti-rabbit IgG serum.

Table 1. Effect of rabbit anti-mouse IgG serum and aggregated rabbit IgG on the LPS response

Treatment	c.p.m. Incorporated at 66–72 h (± s.e.)	c.p.m. Incorporated at 90–96 h (± s.e.)
PBS (20 μl)	394 ± 89	221 ± 9
LPS (100 μg/ml)	3356 ± 91 (8·5)	2674 ± 194 (12·0)
Aggregated IgG (500 μg/ml)	400 ± 56 (1·0)	151 ± 12 (0·7)
Aggregated IgG + LPS (100 μg/ml)	2100 ± 24 (5·3)	n.d.
Rabbit anti-IgG 1/20 dilution	55 ± 2 (0·1)	81 ± 24 (0·4)
Rabbit anti-IgG + LPS (100 μg/ml)	54 ± 5 (0·1)	33 ± 2 (0·1)

LPS added 18–24 h after the other reagents. The rabbit anti-mouse IgG used was undiluted antiserum absorbed with thymus cells from BALB/c mice and then heat-inactivated at 56° for 30 min. The final concentration in culture was 1/20 of serum concentration. The rabbit IgG used in this experiment was preabsorbed with thymus cells from BALB/c mice. The nuwber in brackets is the stimulation index.

n.d. = Not determined.

s.e. = Standard error of the mean.

Effect of immune complexes

The effect of adding immune complexes, preformed at equivalence, to the spleen cell cultures was investigated and the result of an experiment summarized in Table 2. The results show that there is an inhibition of the background thymidine incorporation between 42 and 48 h produced by the rabbit antiserum to ferritin. The addition of immune complexes did not result in increased thymidine incorporation compared to the addition of an equivalent amount of anti-ferritin serum. The addition of LPS, however, did result in an increased thymidine incorporation which could not be abolished by the addition of immune complexes.

Effect of anti-immunoglobulin on nu/nu spleen cells

In order to investigate the effect of binding anti-immunoglobulin, a Fab_2 preparation of a rabbit anti-mouse IgG serum was added to spleen cell cultures at three different concentrations. A control series of cultures to which LPS was added was included in order to estimate the potential for thymidine incorporation in the cultures. The results (Table 3) clearly show that no stimulation of mitosis

Table 2. Effect of immune complexes

Treatment	c.p.m. Incorporated at 42–48 h (±s.e.)	c.p.m. Incorporated at 66–72 h (±s.e.)
No addition	1307±36	771±168
Ferritin (10 μg/ml)	1329±32 (1·0)	898±314 (1·2)
Rabbit anti-ferritin (1/10 dilution)	316±4 (0·2)	753±99 (1·0)
Rabbit anti-ferritin (1/40 dilution)	490±9 (0·4)	1155±118 (1·5)
Immune complexes (1/10 dilution)	341±11 (0·3)	768±12 (1·0)
Immune complexes (1/40 dilution)	557±38 (0·4)	1462±143 (1·9)
LPS (100 μg/ml)	5142±175 (3·9)	2275±27 (3·0)
LPS (100 μg/ml) + complexes (1/40 dilution)	4685±151 (3·6)	2421±88 (3·1)

Immune complexes were formed at equivalence by adding ferritin to undiluted anti-ferritin serum. A 1/10 dilution of immune complexes is approximately a 1/10 dilution of anti-ferritin serum with added antigen. The LPS was added at the beginning of the culture. The number in parentheses is the stimulation index.

s.e. = Standard error of the mean.

Table 3. Effect of the Fab_2 fraction of rabbit anti-mouse IgG serum

Treatment	c.p.m. Incorporated at 66–72 h (±s.e.)	c.p.m. Incorporated at 90–96 h (±s.e.)
No treatment	844±23	1100±163
LPS (100 μg/ml)	3639±465 (4·3)	6584±364 (6·0)
Fab_2 anti-mouse IgG		
1·25 mg/ml	122±35 (0·1)	334±94 (0·3)
0·125 mg/ml	641±44 (0·8)	950±38 (0·9)
0·0125 mg/ml	879±90 (1·0)	1236±14 (1·1)

The number in parentheses is the stimulation index.

s.e. = Standard error of the mean.

takes place on anti-IgG treatment. Thus the binding of anti-IgG reagents which might be expected to mimic the effects of antigen apparently does not activate murine B spleen cells into mitosis. There is instead of a stimulation, a dose-dependent inhibition of thymidine incorporation compared to cultures where no anti-IgG was added. This effect is probably not dependent on complement since a heat-inactivated serum and Fab_2 fragments were used in the experiment, although the synthesis of

Table 4. Effect of the Fab_2 fraction of a sheep anti-mouse Fab and rabbit anti-mouse IgG serum

Pretreatment	c.p.m. Incorporated at 42–48 h (±s.e.)	c.p.m. Incorporated at 66–72 h (±s.e.)
No addition	425±5	202±13
LPS (100 μg/ml)	2610±331 (6·1)	791±71 (3·9)
Sheep Fab_2 anti-mouse Fab		
25 μg/ml	111±4 (0·3)	106±19 (0·5)
2·5 μg/ml	347±23 (0·8)	215±11 (1·1)
Rabbit anti-IgG serum		
1/20 dilution	135±28 (0·3)	68±4 (0·3)
Rabbit anti-ovalbumin serum		
1/20 dilution	203±25 (0·5)	197±24 (1·0)

The number in parentheses is the stimulation index.

s.e. = Standard error of the mean.

heat-labile complement components in the cultures and complement fixation to the Fab_2 antibodies through the alternative pathway is impossible to exclude.

A similar dose-dependent inhibition was observed when a Fab_2 fraction of a sheep anti-mouse Fab serum and a whole rabbit anti-IgG serum was used (Table 4). Although a rabbit antiserum to ovalbumin used at the same dilution also produced an inhibition of thymidine incorporation, this was observed only between 42 and 48 h of culture but not between 66 and 72 h. The magnitude of the inhibition was smaller than that observed with anti-IgG serum. A similar inhibition is produced between 42 and 48 h of culture, but not between 66 and 72 h by a rabbit antiserum to ferritin (Table 2). The rabbit anti-IgG serum, however, produced a marked inhibition of thymidine incorporation up to 96 h in culture (Tables 1 and 4). It appears therefore that while there is a non-specific inhibition of background thymidine incorporation resulting from the addition of rabbit serum to the cultures there is an additional, specific inhibition produced by the binding of anti-immunoglobulin antibodies to the spleen cells.

DISCUSSION

The results show that the binding of immune complexes, formed from rabbit antisera, and rabbit IgG

aggregates to the Fc receptors on murine B lymphocytes does not lead to a detectable increase in cell division. The binding of IgG aggregates and immune complexes also does not abolish the mitogenic response to LPS. Although LPS is reported to activate only a proportion of B lymphocytes (Janossy and Greaves, 1975) the mitogenic potential of the cells in culture was measured by the response to LPS. The addition of LPS to the cultures results in up to a 12-fold but usually a 4-6-fold increase in thymidine incorporation over background.

It has been suggested that the Fc receptor on B lymphocytes functions as a proreceptor for immunoglobulin which acts as antigen receptor on B lymphocytes (Ramasamy *et al.*, 1974). It has also been reported that the binding of antigen–antibody complexes in the absence of complement is mitogenic for human peripheral blood leucocytes (Möller, 1969). Thus it was possible that the mitogenic signal delivered to a B lymphocyte by antigen was mediated through an aggregation of surface immunoglobulin which in turn led to an aggregation of membrane-localized Fc receptors (Ramasamy *et al.*, 1974). The results obtained in the experiments described here apparently do not support a simple model of this type for B-cell activation. These results confirm an earlier report that, in mice, the binding of ligands to Fc receptors does not result in the activation of B lymphocytes to mitosis (Möller and Coutinho, 1975).

Although B lymphocytes have been activated into cell division with anti-immunoglobulin sera in rabbits (Sell *et al.*, 1965) and chickens (Weber, 1975), this has not been observed in mice (Greaves *et al.*, 1974; Elson *et al.*, 1973). The results obtained here confirm that at least a large fraction of murine B lymphocytes cannot be activated to cell division by anti-immunoglobulin treatment alone. Under similar conditions the B lymphocytes are able to respond to LPS.

The rabbit lymphocytes which respond to anti-immunoglobulin are apparently unable, under the culture conditions used, to develop rough endoplasmic reticulum (Marcusson & Roitt, 1969) or to synthesize immunoglobulin for secretion (Sell, 1970). It has been reported that anti-immunoglobulin treated rabbit cells synthesize IgG antibodies to a hapten if, in addition, soluble T-cell factors are supplied (Kishimoto & Ishizaka, 1975). It may well be that murine B cells require T cell-derived or other non-specific help in order to respond by cell division to treatment with anti-immunoglobulins.

The inhibition of the background thymidine incorporation by anti-immunoglobulin treatment needs explanation. A similar inhibition of the foetal calf serum and PPD (purified protein derivatives of tuberculin) induced mitosis in spleen cells from nude mice has been reported previously (Andersson, Bullock & Melchers, 1974). It is possible that a part of the background cell division is in response to an antigen-independent mitogenic effect of foetal calf serum and that this is inhibited by the anti-immunoglobulins. This explanation is consistent with the possibility of a tolerogenic signal being mediated through the Ig receptors. An alternative explanation is that this is due to an anti-immunoglobulin-induced blocking of the recognition of antigens present in foetal calf serum.

The inability of anti-immunoglobulins to activate B cells to division is consistent with the suggestions that at least one of the obligatory signals required for activating the majority of murine B cells to cell division may be a non-specific one, i.e. not mediated directly through the immunoglobulin receptors (Coutinho, 1975; Möller, 1975) or that the signal for activation is very critically dependent on the nature (e.g. the time course or the intensity) of the interaction of antigen with the immunoglobulin receptors.

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