

ROLE OF C'3 AND Fc RECEPTORS IN B-LYMPHOCYTE ACTIVATION*

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Although it was previously generally accepted that the immunoglobulin receptors on B lymphocytes were directly responsible for at least the initial steps in lymphocyte activation, the findings that thymus-independent antigens were at the same time polyclonal B-cell activators (PBA),¹ capable of inducing polyclonal antibody synthesis in high concentrations (7), lead to the realization that at least one triggering receptor is not the immunoglobulin receptor (5, 6). Actually, most of the available evidence suggests that Ig receptors do not activate B cells, although they possess a passive focussing function, allowing them to selectively concentrate to their surface the activating signals present on TI molecules or secreted by accessory cells (6, 8, 9). The evidence for this one nonspecific signal hypothesis (9) can be summarized as follows.

Haptens coupled to PBA induce specific TI responses at low concentrations and polyclonal activation at high concentrations, which cause paralysis of the specific antihapten response (5, 6). Free hapten does not prevent induction of antihapten or polyclonal responses induced by hapten-PBA conjugates, indicating that Ig receptors do not by themselves deliver paralytogenic signals, but free hapten prevents induction of specific antihapten responses by low (but not high) conjugate concentrations, indicating that the Ig receptors function to focus the triggering signal to the B cells (5, 6). Subthreshold concentrations of hapten-PBA conjugates added to B cells together with low concentrations of unsubstituted PBA cause a specific antihapten response (footnote 2), which could not be induced by any substance alone, suggesting that triggering was provided both by the carrier and the hapten-PBA conjugate and that these worked additively in the induction of specific antihapten responses. In addition, immune responses induced by PBA could never be suppressed by the addition of free TD-antigen or hapten, showing that the Ig receptors neither induced nor prevented induction by competent stimuli (5, 28), whereas haptenic molecules for PBA, such as low molecular weight dextrans, effectively suppressed induction of specific and polyclonal antibody

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¹Abbreviations used in this paper: BSS, balanced salt solution; LPS, lipopolysaccharide; PBA, polyclonal B-cell activators; PFC, plaque-forming cells; PPD, purified protein of derivative; RFC, rosette-forming cell; S. Dex, stearyl-dextran; TI, thymus independent.

²Coutinho, A., E. Gronowicz, and G. Möller. Mechanism of B-cell activation and paralysis by thymus-independent antigens: additive effects between NNP-LPS and LPS in the specific response to the hapten. *Scand. J. Immunol.* In press.

synthesis (6, 8), because they interfered with the correct (non-Ig) triggering sites at the cell surface.

Several lines of evidence suggest that Ig receptors by themselves are not the triggering structures. Thus, attempts to induce specific antibody synthesis to haptens and hapten (TD) protein conjugates by adding them to lymphocytes in soluble or precipitated form or by fixing the antigens onto nonmitogenic matrixes, such as Sepharose particles, have failed, although the same haptens coupled to PBA carriers always induced specific synthesis (28). Clearly, the carrier and not the hapten (or the hapten density) was primarily responsible for induction of antibody synthesis, and the carriers differed with regard to their PBA properties, the protein carriers used completely lacking PBA properties. This is further supported by findings using protein carriers having PBA properties (KLH) coupled to insoluble matrixes (15) which were immunogenic *in vitro*.

The final evidence comes from studies on genetic nonresponders to a PBA (lipopolysaccharide [LPS]). The strain C3H/HeJ is resistant to the toxic effects of LPS and does not respond with polyclonal antibody synthesis to LPS (34). This defect is a property of the B cells. The C3H/HeJ mice fail to mount a specific immune response to haptens coupled to LPS, but not to the same or related haptens coupled to other PBA molecules or to thymus-dependent antigens (33, 35, footnotes 3 and 4). Thus, the V gene repertoire to the antigens studied is normal in the animals, but they fail to produce antibodies because they cannot respond to carrier delivering the triggering signal. This finding definitely rules out Ig receptor-mediated triggering by itself and cannot be accommodated by any concept of Ig cross-linking or receptor redistribution hypothesis. The genetic evidence also demonstrates that PBA properties are necessary for immunogenicity and focus attention on the nature of the non-Ig structures responsible for triggering of B cells.

As a first attempt to study the structures responsible for immunocyte triggering, attention was focussed on already well characterized surface receptors, which have been implicated in B-cell activation. These include the C'3 and Fc receptors, in addition to the Ig receptors, the latter already being excluded. It has been suggested that C'3 receptors are triggering structures, to a large extent based on the finding that many, but not all PBA have the capacity to bypass the normal sequence of complement fixation and are capable of directly activating C'3 (13, 30). Although different findings have been made regarding the role of C'3 receptors in TI and TD responses (12, 13, 16, 19, 30), it was considered important to investigate carefully whether these receptors by themselves are competent to activate B cells or whether they were capable of interfering with activation induced by competent PBA.

The Fc receptors have also received attention as triggering structures, because they may function as receptors for Ig (1, 10) and they appear to be in close association with the Ia antigens on B cells, the latter structures being considered to be of importance in lymphocyte activation (11, 14). Therefore, studies were executed to elucidate the role of Fc receptors in B-cell activation.

³ Coutinho, A., E. Gronowicz, and B. Sultzer. 1973. Genetical control of B-cell responses: selective unresponsiveness to lipopolysaccharide. *Scand. J. Immunol.* In press.

⁴ Coutinho, A., and E. Gronowicz. Genetical control of B-cell responses. III. Requirement for functional mitogenicity of the antigen in thymus-independent specific responses. Manuscript submitted for publication.

Materials and Methods

Mice. Mice of the inbred strains A, B10.5M, CBA, and F₁ hybrids between these strains were used in different experiments.

Mitogens. Lipopolysaccharide from *Escherichia coli* O55.B5 prepared by the phenol-water extraction method was obtained from Dr. T. Holme, Dept. of Bacteriology, Karolinska Institute, Stockholm. Purified-protein derivative of tuberculin (PPD) was obtained from the State Serum Institute, Copenhagen, Denmark as a preservative-free solution containing 1 mg/ml. Stearoyl-dextran (S. Dex) (7×10^4 mol wt, 1 stearyl group/20 glucose units) was obtained from Dr. W. Richter, Pharmacia, Uppsala. Pneumococcal polysaccharide SIII was purchased from Burroughs Wellcome, England, and obtained as a lyophilized powder. Dextran sulphate from Pharmacia Fine Chemicals, Uppsala (5×10^5 mol wt, sulphur content 17%) was used in some experiments.

Preparation of Lymphocytes. Spleens were removed from mice, cut in pieces, and pressed through a 60 mesh stainless steel screen into ice-cold balanced salt solution (BSS). After brief sedimentation, the cells in the supernates were washed three times in BSS and subsequently suspended in culture medium to the desired cell concentration. Cellular and viability counts were performed in Burker haemocytometers after staining the damaged cells with 0.02% trypan blue.

Medium. The medium used was Eagle's minimum essential medium in Earle's solution, supplemented with glutamin, nonessential amino acids and pyruvate, and containing 100 IU of penicillin and 100 μ g of streptomycin per ml, respectively, as described by Mishell and Dutton (24). The medium was further buffered by 10 mM of Hepes, and the pH adjusted to 7.0-7.2. All these reagents were obtained from Flow Laboratories, Irvine, Scotland. Experiments were carried out in serum-free medium or occasionally in the presence of 10% fetal calf serum (Rehatuin, batch 238).

Assay of DNA Synthesis. Cultures were given a 24-hr pulse of [³H]thymidine (The Radiochemical Centre, Amersham, England) with a spec act of 5 Ci/mmol, diluted in culture medium in order to have a concentration of 2 μ Ci/ml in the cultures. Tube cultures were harvested by pouring them onto Sartorius membranes (0.8 μ pore size) in a Millipore multi-manifold sample collector. The cells were washed in 15 ml of 0.9% saline and the filters placed in scintillation vials to dry overnight. 5 ml of scintillation fluid was added to each vial and the samples counted in a scintillation spectrometer (Tri-Carb Packard, Packard Instrument Co., Downers Grove, Ill.).

Lymphocyte Cultures. Induction of antibody synthesis was always performed in 3 cm diameter plastic petri dishes (Nunc, Denmark) using a cell concentration of 10^7 spleen cells/ml/culture, set up in triplicates. Cultures were incubated at 37°C in plastic boxes filled with a mixture of 10% CO₂, 83% N₂ and 7% O₂, and rocked on a platform at 8 oscillations per minute.

Assay of Antibody Synthesis. A modification (4) of the Jerne plaque assay (19) was used to quantitate cellular antibody production in culture. Cells harvested with a plastic policeman were washed once in cold BSS and adjusted to the desired cell concentration. 0.6 ml of 0.5% agar (Difco Bacto Agar, Difco Laboratories, Detroit, Mich.) in BSS containing 0.05% DEAE-dextran (Pharmacia Fine Chemicals, Uppsala, Sweden) was added to haemolysis tubes, kept at 46°C. Thereafter, 0.05 ml of the indicator red cells diluted 1:8 in BSS, 0.2 ml of the cell suspension and 0.05 ml of guinea pig serum diluted in 1:4 in BSS were added to each tube. The mixture was plated in three 0.2-ml spread sports on plastic petri dishes of 9 cm diameter, and incubated for 3 h at 37°C. Plaque-forming cells were counted using indirect light.

Different red cells were used as indicators in the assay. Sheep red blood cells, always obtained from the same donor, were stored in sterile alsever's solution and washed twice in BSS before use. For haptentation of the red cells (using NNP or fluorescein isothiocyanate-FITC), extensively washed sheep blood was suspended in a carbonate-bicarbonate buffer, pH 9.2 (20% vol/vol) and kept with permanent stirring at room temperature. The reactive forms of the haptens were then added and the reaction allowed to proceed for 40 min. The haptentated red cells were finally extensively washed and used in the plaque assay. A concentration of 0.1 mg/ml was used with NNP-acid and with FITC, the concentration varied between 5 and 0.1 mg/ml depending on whether low or high affinity antibodies should be detected.

Conjugation of FITC to Various Carriers. FITC (BDH Chemicals, Isomer no. 1) was conjugated to proteins according to the method of Bergqvist and Schilling (2). In short, FITC was dissolved in bicarbonate buffer pH 9.23. The FITC concentrations ranged from 10 to 0.1 mg/ml. 3.3 ml of the FITC solutions were enclosed in dialysis bags. The bags were immersed into 10 ml of HGG or HSA solutions

containing 10 mg protein per ml. The proteins used were previously dissolved in the same bicarbonate buffer, pH 9.23. The mixture was stirred overnight for 24 h at 0°C and thereafter the protein solution was added to a Sephadex G-25 column and eluted with phosphate buffer pH 7. The protein solutions were filtered to sterility and made up to protein concentration of 2 mg/ml.

LPS could not be efficiently labeled by the above procedure and therefore the following method was adopted. 10 mg/ml of LPS was dissolved in bicarbonate buffer pH 9.23 and in a separate vessel 2 mg/ml of FITC were dissolved in the same buffer. To 1 ml LPS solution was added 3 ml of FITC solution. The mixture was stirred overnight and LPS separated from nonreacted FITC by passage through Sephadex G-25 column. For stronger labeling 20 mg of LPS was directly dissolved in 2 ml FITC solution (10 mg/ml bicarbonate buffer, pH 9.23). The content of FITC per mole of protein or LPS was kindly determined spectrophotometrically by Dr. R. Bergqvist according to the method of McKinney et al. (22). The following preparations were used (molar ratio of FITC to carrier) FITC₁₀-HGG and FITC₁₀-LPS. In the case of LPS a mol wt of 10⁶ was assumed for the calculations.

Antisera. Antisera for the elaboration of Fc rosette-forming cells (RFC) was obtained from a rabbit after 6 wk intravenous injections of 1 ml of a 25% suspension of sheep red blood cells (SRBC). The serum was separated on a Sephadex G-200 column (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) and the 7S fraction was collected and concentrated to original serum volume by negative pressure dialysis. A 19S fraction from a rabbit anti-SRBC antiserum was obtained from Dr. Knut Lidman, National Bacteriology Laboratory, Stockholm, Sweden.

Antiserum to FITC was prepared by injecting mice with 400 µg alum-precipitated FITC₁₀-HGG intraperitoneally four times at weekly intervals. The mice were bled 6 days after the last injection. The serum was used unseparated or the 7S fraction after Sephadex G-200 was employed.

Determination of Fc-Binding Lymphocytes. In order to study the number of lymphocytes capable of binding to the Fc portion of antibody molecules, the following procedure was followed (27). 1 ml of SRBC (1%) was mixed with the 7S fraction of a rabbit anti-SRBC antibody so that the final antiserum dilution was 1/100 and incubated for 30 min at room temperature. Thereafter, the suspension was cooled in ice and washed three times in the cold. 0.1 ml of a 1% suspension of the antibody-coated SRBC was added to 0.1 ml of a suspension of lymphocytes (25 × 10⁶ cells/ml) and finally 0.5 ml BSS was admixed. The cells were centrifuged at 750 rpm in the cold and carefully resuspended by a pasteur pipette, and the number of Fc rosettes was determined in the microscope. When Fc-binding cells were enumerated using FITC₁₀-HGG or FITC₁₀-LPS various concentrations of FITC-HGG (250 µg/ml to 25 µg/ml) were mixed with different concentrations (undiluted to 1/6) of the antiserum for 30 min at 37°C and thereafter 10⁶ lymphocytes were added to the mixture for another 30 min. After three washings the number of fluorescent cells were counted. An analogous procedure was followed with FITC₁₀-LPS although 2 mg/ml of the antigen was used with various concentrations of the 7S fraction of the anti-FITC-HGG antiserum.

Determination of C'3-Binding Lymphocytes. In short, the following procedure was followed. 1 ml of a 1% SRBC solution was treated with a rabbit anti-SRBC IgM preparation at a final dilution of 1/640 for 30 min at 37°C. The SRBC were then washed in the cold three times and resuspended to the original volume. Subsequently, fresh strain A mouse serum as a source of complement, was added to the suspension at a final dilution of 1/5. The suspension was incubated for another 30 min at 37°C, and then the SRBC were again washed three times and diluted to 1% in BSS. The antiserum employed did not cause RFC formation in the absence of added mouse sera as a source of complement.

Experimental Procedure. The experiments aimed at determining whether C'3 or Fc receptors were directly involved in lymphocyte activation or could suppress activation induced by PBA. The C'3 receptors were studied in two ways: lymphocytes (10⁷/ml) were treated with 100 µg/ml of S. Dex for 30 min at 37°C and thereafter washed extensively. Half of the cells were incubated with fresh mouse serum as a source of complement (15 × 10⁷ cells/ml in an equal volume of serum), or heat-inactivated mouse serum (56°C for 45 min) or with BSS. The cells were again washed and thereafter cultivated in serum-free medium in the presence or absence of PBA. In parallel, the ability of the treated cells to bind Fc and C'3-coated SRBC was determined.

A second method to study the role of C'3 receptors was to incubate different PBA (2 mg/ml) with fresh or heat-inactivated mouse serum (or in some experiments guinea pig serum as a source of complement) or BSS. The PBA were then directly added to cells in Mishell-Dutton cultures and the response determined 2 days later. Finally, mixtures of different concentrations of anti-FITC-HGG and FITC₁₀-HGG (250 or 25 µg/ml) were treated with fresh or inactivated mouse serum or BSS and

added to Mishell-Dutton cultures in the presence or absence of PBA and the response again determined 2 days later.

The role of Fc receptors were analyzed in an analogous way using mouse anti-FITC serum or the 7S fraction of this serum and FITC₁₀-HGG or FITC₁₀-LPS. A mixture of these reagents was added to cultured cells in the presence of PBA to determine whether the treated cells were activated to polyclonal antibody synthesis or whether the response to PBA was altered.

A second method employed SRBC coated with the 7S fraction of a rabbit anti-SRBC diluted 1/100 or 1/1,000 as described above. The SRBC and lymphocytes were mixed in proportions as described for the Fc rosette assay, centrifuged and carefully resuspended. The mixture was cultivated alone or with LPS or PPD in serum-free cultures. Appropriate controls were included as will be described later.

Results

C'3 Receptors do not Activate Lymphocytes or Prevent their Triggering by Different PBA. Stearoyl dextran binds to cell membranes and has the ability to fix complement onto the cell membrane. This was demonstrated by first treating SRBC with various concentrations of S. Dex, washing the cells three times, and thereafter adding guinea pig serum as a source of complement. It was found that red cells treated with S. Dex. (in concentrations from 1 mg/ml to 15 µg/ml) were completely lysed by complement, whereas noncomplement treated red cells were completely unaffected. Most likely the lipid part of the molecule dissolves in the membrane and remains attached after extensive washing and thereafter S. Dex is capable of binding complement, leading to lysis of the cells. Analogous studies were made with lymphocytes. The degree of lysis after addition of guinea pig complement was much smaller (35%). S. Dex itself had no toxic effect on lymphocytes. These results demonstrate that S. Dex binds to lymphocytes and red cells and these subsequently become capable of fixing guinea pig complement.

As a next step it was studied whether mouse complement would be fixed in an analogous way. Since mouse serum is poorly lytic it was investigated whether S. Dex treated mouse lymphocytes, subsequently mixed with mouse serum as a source of complement would interfere with the ability of such cells to bind C'3-coated red cells in a rosette assay. As can be seen in Table I this was the case,

TABLE I
Inhibition of C'3 Binding by Stearoyl Dextran and Fresh Mouse Serum

Treatment of lymphocytes*	RFC binding	
	Fc	C'3
	%	%
S. Dex plus BSS	36	29
S. Dex plus heat-inactivated serum	36	32
S. Dex plus fresh serum	40	6

* 10⁷ lymphocytes were incubated with 100 µg S. Dex for 30 min at room temperature. After two washings the cells were incubated with BSS, heat-inactivated or normal serum, respectively, for 30 min at 37°C. After three washings the cells were rosetted with SRBC treated to reveal Fc or C'3 binding lymphocytes.

whereas the binding of Fc-coated red cells was the same as in untreated cells. Controls treated with heat-inactivated serum or with S. Dex alone did not inhibit C'3 or Fc rosette formation.

Having established that the C'3 receptors indeed react with complement by the procedure used it was investigated whether C'3 receptors could cause activation of polyclonal antibody synthesis after having reacted with complement. As shown in Fig. 1 this was not the case. Thus, there was no increase in the plaque-forming cell (PFC) response against FITC, NNP, or SRBC of lymphocytes treated with S. Dex alone or S. Dex and fresh mouse serum. Neither did binding of C'3 to spleen cells prevent induction of polyclonal activation induced by PBA, such as LPS or PPD (Fig. 1). Thus, there was no evidence for a triggering role of C'3 receptors nor was there any indication that the C'3 receptors constituted part of the triggering receptors, since blocked C'3 receptors did not interfere with PBA activation.

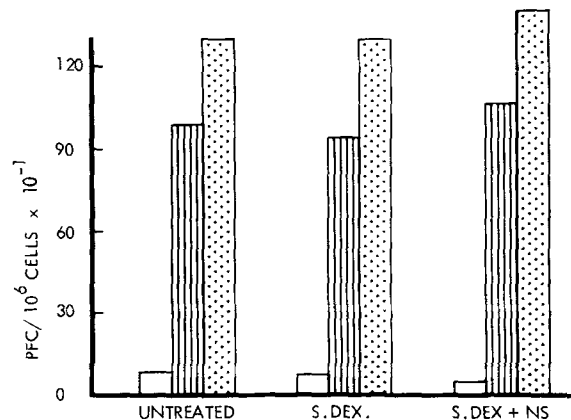


FIG. 1. Failure of C'3 receptors to activate lymphocytes or to prevent their activation by LPS or PPD. Lymphocytes were untreated or treated in vitro with 1 mg of S. Dex or S. Dex plus fresh mouse serum as a source of complement and thereafter cultivated in serum-free medium for 2 days (□). In addition, the cells were given 100 μ g LPS (▨) or PPD (▩) in culture and the polyclonal antibody response against FITC-SRC determined at day 2.

C'3 Receptors have a Passive Focusing Function in Lymphocyte Activation by Competent Ligands. As already mentioned, several PBA have the capacity of bypassing the normal sequence of complement fixation and directly activating and binding C'3. Actually this finding constituted the basis for the postulate that C'3 receptors are responsible for lymphocyte activation. In order to study the role of C'3 in activation of lymphocytes by PBA the following types of experiments were performed. PBA were mixed with fresh mouse serum or guinea-pig serum as sources of complement for 30 min at 37°C and thereafter various concentrations of the mixtures were added to spleen cell cultures in serum-free medium and the polyclonal antibody responses were measured 2 days later or, alternatively, induction of DNA synthesis was determined 3 days later. Controls consisted of PBA mixed with heat-inactivated serum, or with BSS. Several experiments were carried out to study whether fresh and heat-inactivated serum had differential effects on lymphocytes in culture, but it was not possible to show that with regard

to cell survival, response to T mitogen, background antibody synthesis, induction of primary antibody responses to SRBC.

Experiments were performed with LPS, SIII, PPD, and S. Dex and the results were essentially similar with all of them. Thus, PBA incubated with normal serum did not result in an increased number of PFC or greater degree of DNA synthesis, irrespective of whether the serum was heat-inactivated or not. On the contrary, there was quite often a slight reduction of the total number of PFC by serum treatment as compared to the response after treatment with BSS, although to a small extent only. However, fresh mouse serum-treated PBA always caused a shift in the dose response profile as compared to PBA treated with inactivated serum or with BSS. Thus, 10- to 100-fold lower concentrations of the PBA resulted in optimal stimulation in the presence of fresh serum and there was a distinct suppression of the response at concentrations which were optimal in the presence of BSS or heat-inactivated serum (Figs. 2-5). These findings were

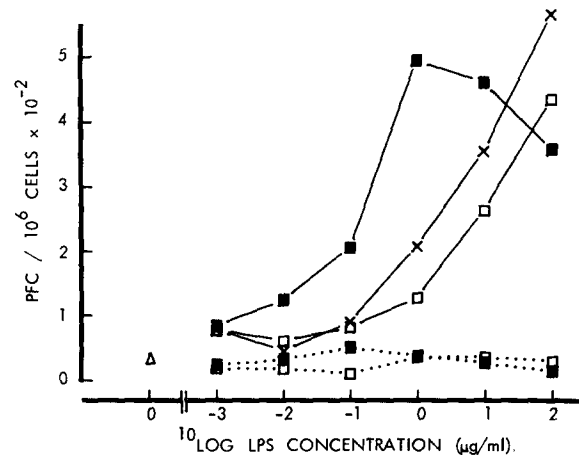


FIG. 2. LPS was mixed with fresh mouse serum (■—■), heat-inactivated serum (□—□) or BSS (x—x) and thereafter different concentrations of the mixtures were added to spleen cells in serum-free culture. In addition, the same concentrations of fresh (■...■) or inactivated (□...□) serum were added alone to the cultures. The anti-FITC response was determined at day 2. The background response in untreated cultures is also shown (Δ).

consistently made when normal mouse serum was the source of complement. However, when guinea pig complement was employed, there was sometimes a very weak induction of polyclonal antibody synthesis, presumably because of complement-mediated toxic effects on the lymphocytes. In principle, the results were analogous to those reported with mouse serum (Fig. 6). Thus, PBA that had bound C'3 caused a shift in the dose response curve, lower concentrations of the PBA being needed for optimal activation.

The effect of PBA complement complexes to decrease the PBA concentration needed for optimal concentration is presumably due to a focussing function of the C'3 receptors, which allows more PBA molecules that have bound C'3 to be fixed to the lymphocytes at any PBA concentration. This interpretation is supported by direct experiments demonstrating binding of FITC₁₀-LPS to lymphocytes in the presence of fresh but not heat-inactivated mouse serum as a source of

LYMPHOCYTE-TRIGGERING RECEPTORS

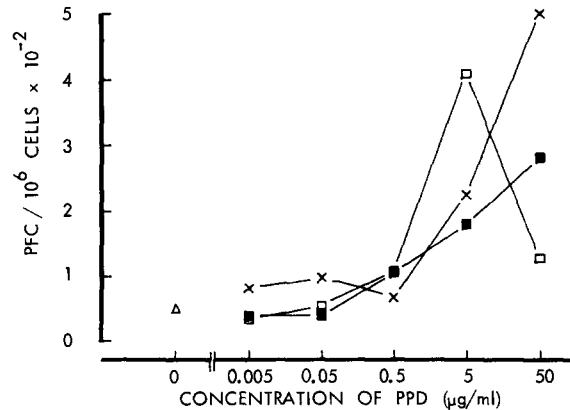


FIG. 3. PPD was mixed with fresh serum (□-□) heat-inactivated serum (■-■) or BSS (x-x) and thereafter different concentrations of the mixtures were added to serum-free cultures of spleen cells. The response against FITC was determined at day 3. The background is also shown (Δ).

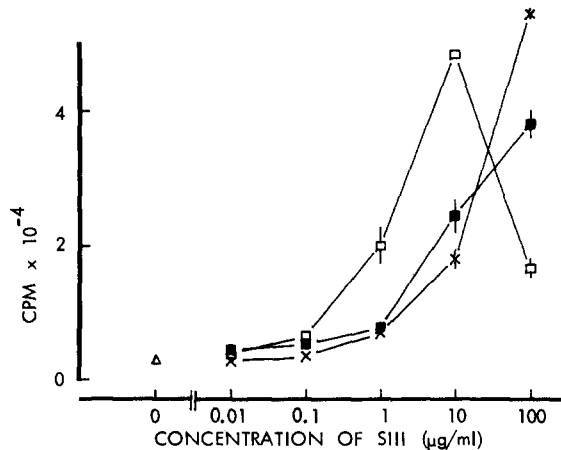


FIG. 4. Same as Fig. 3, except that SIII was used and that DNA synthesis was determined at day 3.

complement. FITC₁₀-LPS was treated with fresh or inactivated mouse serum and thereafter mixed with spleen cells and the number of cells binding to fluorescent LPS was determined in a fluorescent microscope. It was found that FITC₁₀-LPS incubated with fresh mouse serum fixed to about 35% of the lymphocytes, whereas LPS incubated with heat-inactivated serum only bound to 2%. As a second step it was investigated whether lymphocytes which had reacted with fresh normal serum-treated LPS would be capable of binding SRBC prepared to reveal C'3 or Fc binding lymphocytes. The results (Table II) show that fresh serum-treated LPS completely abolished binding of C'3 RFC, whereas LPS itself, LPS treated with heat-inactivated serum or fresh serum itself did not have this effect. Fc-binding lymphocytes were not significantly affected by any treatment.

It seems likely that the C'3 receptors will have a passive focussing role in immunocyte triggering by PBA in the presence of complement in the same way as

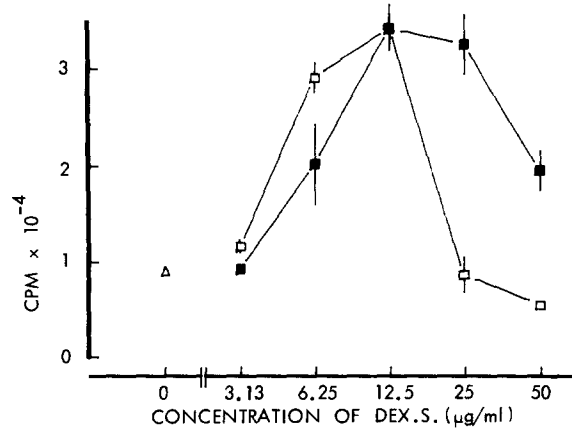


FIG. 5. Same as Fig. 4 using S. Dex.

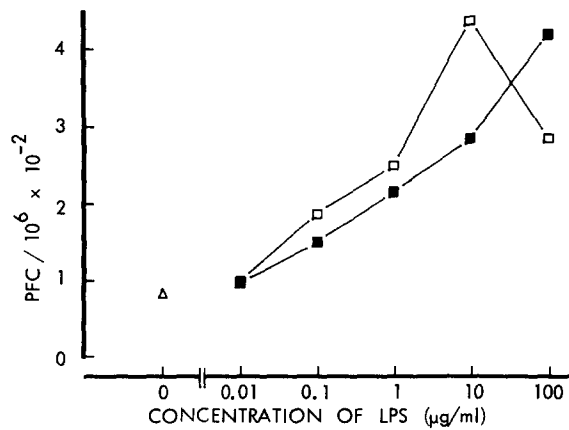


FIG. 6. Same as Fig. 5, but using LPS, measuring polyclonal antibody synthesis and using guinea pig serum as a source of complement.

TABLE II
Inhibition of C'3 Binding by LPS and Fresh Mouse Serum

Treatment of lymphocytes*	RFC binding	
	Fc	C'3
	%	%
Fresh serum plus BSS	23	14
LPS plus BSS	32	25
LPS plus inactivated serum	23	27
LPS plus fresh serum	19	2

* LPS (2 mg/ml) was first mixed with an equal vol of BSS, heat-inactivated or fresh mouse serum, respectively, for 30 min at 37°C. Thereafter, 5×10^6 lymphocytes were added to the mixtures and incubation continued for 30 min. The cells were washed and rosetted with SRBC treated to reveal Fc or C'3 binding lymphocytes.

the Ig receptors. However, the Ig receptors can decrease the optimally stimulating concentration of a PBA by a factor of 10^3 - 10^6 , whereas C'3 receptors only caused a 10- to 100-fold difference in the optimal concentrations. This most likely reflects the relative affinities of the binding to the two types of receptors involved.

Fc Receptors do not Activate Lymphocytes. Two approaches were used to investigate whether binding to Fc receptors would activate lymphocytes to polyclonal antibody synthesis. In the first, SRBC were coated with two concentrations (1/100 and 1/1,000) of the 7S fraction of a rabbit anti-SRBC serum. Subsequently, the washed red cells were mixed with spleen cells as in the procedure for the detection of Fc-binding lymphocytes, the mixture was centrifuged and resuspended, and thereafter cultivated in serum-free medium. Polyclonal antibody responses were determined 2 days later. As can be seen in Fig. 7 there was no evidence for induction of polyclonal antibody synthesis, nor

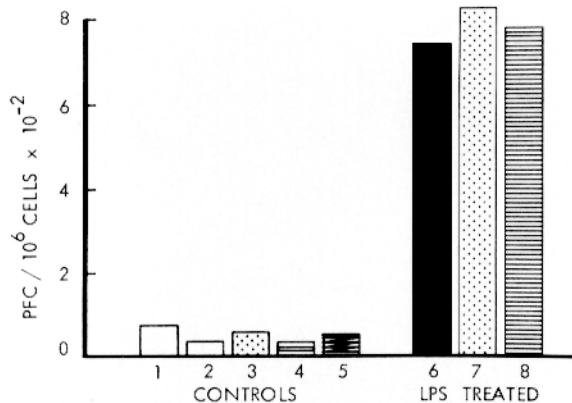


FIG. 7. Failure of Fc receptors to induce polyclonal antibody synthesis or to suppress such responses induced by LPS. Serum-free cultures were set up without addition to medium (controls) or with $100 \mu\text{g}$ LPS (LPS treated). Before culture the lymphocytes were untreated (1), treated with SRBC (2), with SRBC sensitized with a 1/100 (3) or 1/1,000 (4) dilution of 7S anti-SRBC serum or treated with the antiserum diluted 1/100 alone (5). Untreated lymphocytes were given LPS in culture (6) as were cells rosetted with antiserum diluted 1/100 (7) and 1/1,000 (8). The response against FITC was determined at day 2.

was there an increased number of PFC detectable against the antigen (SRBC) added to the cultures (Fig. 8). Thus, the Fc receptors did not appear to activate lymphocytes into polyclonal or specific antibody synthesis.

In order to study whether occupied Fc receptors would interfere with polyclonal activation-induced PBA, the rosetted lymphocytes were treated with LPS or PPD at optimal concentrations in serum-free medium. There was no indication of suppression of polyclonal antibody synthesis (Fig. 7) nor was the LPS-induced response to SRBC in the presence of Fc-coated SRBC significantly changed as compared to non-SRBC-treated controls (Fig. 8).

In the second system, a highly thymus-dependent antigen (FITC₁₀-HGG) was employed in conjugation with a specific anti-FITC-HGG serum or the 7S fraction of this serum. It was first studied whether an antigen antibody complex of this type would adhere to lymphocytes as has been reported by others (1, 10, 11).

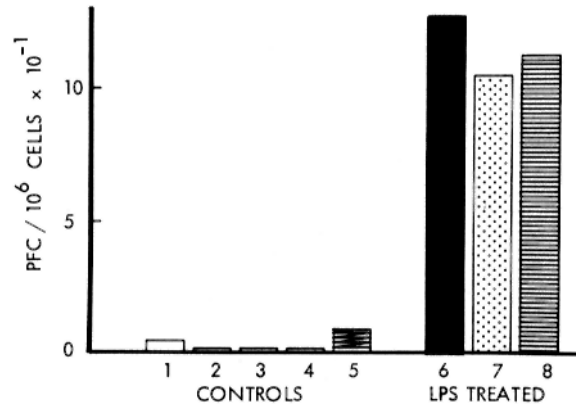


FIG. 8. Same as Fig. 7, but measuring specific anti-SRBC responses.

FITC₁₀-HGG at a concentration of 250 or 25 $\mu\text{g/ml}$ was mixed with various concentrations (1/2 to 1/16) of the antiserum or the 7S fraction of it. After 30 min at 37°C 10⁶ spleen cells were added to the mixture, which was incubated for 30 min. After washings the cells were inspected in a fluorescent microscope. It was found that between 39–50% of the cells had bound fluorescent material in all the mixtures tested, although the intensity of the binding decreased with decreasing antigen concentrations. There was no difference between unfractionated serum and the 7S fraction. When antiserum was replaced with normal serum there was no detectable binding to any cells. Thus, the antigen-antibody complexes used bound to lymphocytes, most likely to the Fc receptors on B cells as described by others (1, 10, 11).

In order to study whether such antigen-antibody complexes would by themselves activate lymphocytes into polyclonal antibody synthesis or prevent activation by competent ligands, several different types of experiments were carried out. FITC₁₀-HGG in concentrations ranging from 25 to 0.0025 $\mu\text{g/ml}$ were added to cultures which also received antiserum or the 7S fraction of the antiserum in concentrations ranging from 1/10 to 1/10,000. In the controls normal mouse serum or the 7S fraction from normal serum replaced the antiserum. In addition, the antigen alone or the antiserum alone was used in various concentrations. Alternatively, immune complexes were first formed *in vivo* by incubation for 30 min at 37°C and thereafter they were added in different concentrations to serum-free spleen cell cultures. The polyclonal response (against SRBC) was measured at day 2 or 4. In addition, the anti-FITC response was measured at the same time periods. As shown in Table III, there was no induction of antibody synthesis. Quite often the background increased, but this was observed to the same extent in cultures given normal serum, or antibody alone and most likely represented a serum effect. High concentrations of antigen was often suppressive as seen before with this antigen (28). The experimental conditions covered 10⁴-fold concentrations of antigen and antibody, respectively, but there was no induction of polyclonal antibody or specific antibody synthesis.

These complexes did not interfere to any significant extent with the ability of LPS to induce polyclonal antibody synthesis as shown in Table III and Fig. 9 and

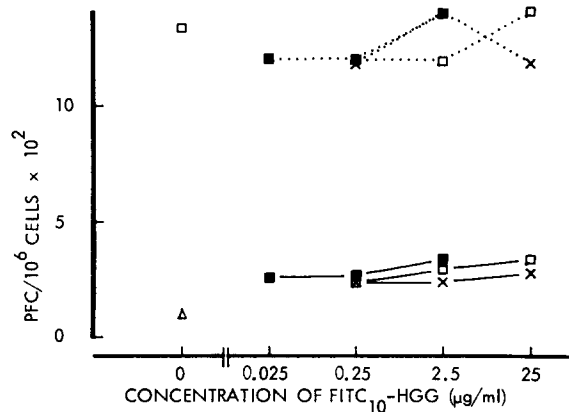


FIG. 9. Failure of antigen-antibody complexes to induce polyclonal antibody synthesis or suppress the response to LPS. Complexes of 250 μg (□) or 25 μg (■) of FITC₁₀-HGG and the 7S fraction of mouse anti-FITC₁₀-HGG were formed *in vitro*. Thereafter different concentrations of the complexes were added to cultures (—). The same complexes were also added to cultures given 100 μg LPS (····). One complex was incubated with fresh mouse serum as a source of complement and thereafter added to cultures (x). The background (Δ) as well as the response to LPS itself is also shown (□).

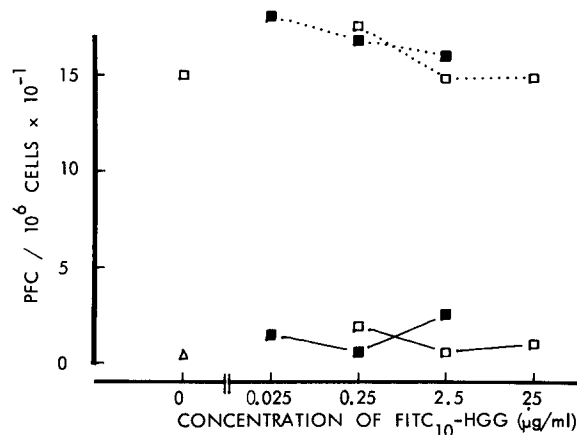


FIG. 10. Same as Fig. 9, using another batch of antigen and a different antiserum.

complexes. Thus, the simultaneous binding to both Fc and C'3 receptors cannot activate lymphocytes.

Discussion

It has been established that polyclonal antibody synthesis can be induced by PBA acting on nonspecific receptor(s). The evidence strongly suggests also that specific antibody synthesis is induced by one nonspecific signal identical to that activating cells polyclonally (5, 7, 8, 9). Thus, specific thymus-independent antibody synthesis is induced by antigenic determinants coupled to PBA molecules, but only under conditions that allow expression of the PBA property. Hapten-LPS conjugates are capable of inducing specific antihapten responses only if the LPS molecule is a PBA, but not if it has been chemically modified so

as to lose PBA properties (alkaline hydrolysis), even though the hapten density was the same in the two preparations (17). Also, hapten-LPS conjugates are not immunogenic in mice which are genetically unresponsive to the PBA property of LPS, whereas a normal response can be obtained in these mice against the same hapten coupled to other PBA carriers or to TD antigens (6, 8, 33, 35, footnotes 3 and 4). These findings exclude any triggering mechanism based only on interaction with the Ig receptors (e.g. cross-linking) as the activating event. In a recent report (15) it has been claimed that insolubilized antigen is capable of achieving triggering of B cells into specific antibody synthesis. The experiments made use of hapten-KLH conjugates coupled to Sepharose beads. Since KLH has been shown to be a PBA (8) it is to be expected that a hapten conjugated to KLH would be immunogenic and the observed difference between soluble and insolubilized conjugates is not unexpected (for discussion see 8), since KLH is a weak PBA which may require both Ig focussing and high local concentrations if the experimental conditions are unfavorable (e.g. carried out in the presence of serum). It is noteworthy that the direct test of the hypothesis put forward (cross-linking of Ig receptor causes B-cell triggering), namely the insolubilization of the hapten directly to Sepharose beads (without the presence of the weak PBA protein carrier), was not carried out. Instead the experiments were performed with a completely different insoluble matrix (polyacrylamid). However, it is known that polyacrylic acid is capable of interacting with the PBA receptors (8).

The two-signal model (3) of B-cell activation is made highly unlikely by several findings (for discussion see 8). Thus, PBA induces antibody response even though there is no complementarity between the PBA and the combining site of the Ig receptors (7). The specific antihapten response to a hapten conjugated to a PBA is abolished by the simultaneous addition of free hapten (5), although the two-signal hypothesis would not predict that. The hapten-blocked cells are not paralyzed, however, since high concentrations of the hapten-PBA conjugate induce antihapten responses (5). Finally, all attempts but one (36) to induce specific antihapten responses by hapten-completely TD antigen complexes (signal one) together with PBA 8 (signal 2) have failed (7, 27). Synergy between antigen and PBA has been observed repeatedly, but the positive reports have been carried out *in vivo* (8, 33, footnote 5) or else *in vitro* with red cell antigens (20, 31, 32). In the latter case it has been shown that the effect depends on helper cells (T cells and adherent cells) and does not represent a direct effect on B cells (20). It is obviously necessary to establish that two substances act on the same B cells in order to substantiate a two-signal concept, but so far this has not been performed. Thus, the synergy experiments performed do not support a two-signal hypothesis. Actually, they argue against this concept when considered together.

Presently, only the one-signal hypothesis (9) can explain the experimental findings in a satisfactory way. This concept postulates the existence of a nonspecific triggering receptor. It is therefore essential to identify the nature of this receptor. This paper represents such an attempt and attention was focussed on two well characterized receptors, namely the Fc and C'3 receptor. Both have

⁵ Persson, U., and E. Möller. The effect of LPS on the primary immune response against the hapten NNP. Manuscript submitted for publication.

been implicated as triggering receptors. It was initially claimed that the C'3 receptor was the actual triggering receptor (13), but recent findings make this unlikely and it is presently suggested that these receptors only have a function in TD responses, and this function is not believed to directly trigger B cells but rather to increase the binding of the antigen to the B cells (12, 13, 16, 18, 30).

The studies presented above exclude C'3 receptors as direct triggering receptors, but support the notion that C'3 receptors may have a focussing function, allowing a more efficient binding to B cells of substances that have bound C'3. This may make the triggering event more efficient when the antigen is TI, since lower concentrations of PBA are sufficient for optimal triggering. It also allows paralysis to be induced at lower concentrations. An analogous role of C'3 may operate in TD responses. However, in comparison with the focussing function of the Ig receptors, the C'3 receptors are inefficient and probably they do not play a major role in immunogenicity.

Fc receptors have also been suggested to play an essential role in lymphocyte activation. This was initially based on the findings that antigen-antibody complexes were competent to induce DNA synthesis in blood lymphocytes (26). Furthermore, the Fc receptors (21, 23, 29) are responsible for induction of antibody-induced, lymphocyte-mediated cytotoxicity (25), even though the nature of the activated cells (non-T) remains obscure. Finally, the findings that Ia antigens appear to be physically linked on the cell membrane to the Fc receptors (11), since anti-Ia antiserum prevents binding of Fc to the B cells (11) and that the same antiserum have the ability to suppress at least some immunological recognition phenomena, such as the MLC reaction (14), have focussed interest on the possible triggering role of the Fc (or Ia) structures. The findings in this paper do support the notion that Fc receptors are competent to trigger B cells directly, not the idea that the Fc receptors constitute part of the triggering sites, since blocked Fc receptors failed to influence polyclonal and specific induction by PBA. Again, the Fc receptors may have a passive focussing function by attracting competent ligands which have bound antibody to the surface in a higher concentration than could otherwise be achieved, but this role is probably of minor importance. The nature of the nonspecific B-cell triggering structure responsible for induction of antibody synthesis is still unknown.

Summary

Attempts were made to identify the non-Ig lymphocyte receptor responsible for B-cell induction by antigen and polyclonal B-cell activators (PBA). As a first step, the role of C'3 and Fc receptors was analyzed. It was shown that complement could be fixed onto B cells to such an extent that the lymphocytes could not bind complement-coated red cells, but this did not result in induction of polyclonal antibody synthesis, nor did it inhibit the lymphocytes response to PBA.

However, the C'3 receptors possessed a passive focussing role in the induction of polyclonal antibody responses. Thus, PBA that had fixed complement activated polyclonal responses at lower concentrations than the same substances that had not fixed complement. Most likely the dual binding of PBA molecules to B cells by the PBA and the C'3 receptors caused more PBA molecules to be

bound to each cell. However, the focussing function of the C'3 receptors was several orders of magnitude smaller than that of the Ig receptors.

Analogous studies were carried out with Fc receptors. Binding of different types of antigen-antibody complexes did not cause activation of polyclonal or specific antibody synthesis, nor did it significantly interfere with induction of antibody synthesis by PBA substances.

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