筑波大学

医学博士学位論文

IgM-SPECIFIC BINDING LYMPHOKINE

—— IgM-BINDING FACTOR ——

(IgMに特異的に結合するリンフォカイン－IgM結合因子－)

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ABSTRACT

Incubation of concanavalin A (Con A)-activated thymocytes from normal mice resulted in the release of soluble factors which selectively inhibited rosette formation of overnight-cultured human peripheral blood lymphocytes (PBL) with ox erythrocytes (Eo) sensitized with rabbit IgM. Rosette formation of PBL with TNP-coupled Eo sensitized with mouse monoclonal IgM antibody against DNP was also inhibited with these factors. The factors were specifically absorbed with mouse IgM-Sepharose column and could be eluted from the column at acid pH. They did not bind to mouse IgG-Sepharose column, nor inhibited rosette formation of PBL with Eo sensitized with rabbit IgG. From these properties of the factors we designated them "IgM-binding factors". The culture supernatants of Con A-activated thymocytes also contained IgG-binding factor which specifically bound to Eo sensitized with rabbit IgG. Addition of mouse IgM to the culture of Con A-activated thymocytes enhanced the formation of IgM-binding factors, and normal thymocytes and spleen cells treated with IgM also formed the factors. Molecular weights of IgM-binding factors were approximately 70,000 and 35,000 daltons estimated by gel filtration with TSK G3000SW column. IgM-binding factor of the each molecular size suppressed the IgM plaque-forming cell (PFC) response of sheep red blood cell (SRBC)-primed spleen cells to SRBC. The IgG PFC response of these cells to SRBC was also suppressed but much less effectively by the factors.

INTRODUCTION

Recent studies in isotype-specific regulation of antibody production by T cells have indicated that T cell factors having affinity for antibodies in a particular isotype regulate the production of antibodies in the corresponding isotype. Fridman and his coworkers have shown that soluble factors having affinity for IgG (Ig binding factors, IgG-BF) were formed by T cells bearing Fc receptors specific for IgG (FcγR) and suppressed IgG and IgM response (1,2). Ishizaka and his coworkers have shown that IgE-binding factors formed by FcεR positive T cells specifically regulated IgE response (3,4,5). Yodoi and his coworkers have shown that IgA-binding factor formed by FcαR positive T cells specifically suppressed IgA response (6,7). These results indicate that an immunoglobulin-binding factor for a particular isotype is formed by a T cell bearing Fc receptors (FcR) specific for the corresponding class of immunoglobulin.

Because the existence of T cells bearing Fc receptors specific for IgM (FcμR) has been reported in several species (8,9,10,11), we wondered if these T cells might form a soluble factor having specific for IgM (IgM-binding factor). In this study we have employed the rosette inhibition assay using overnight-cultured human peripheral blood lymphocytes (PBL) as a source of FcμR positive cells to detect murine IgM-binding factor. We have examined the existence of IgM-binding factor in supernatants of concanavalin A (Con A)-activated thymocytes which had been shown to contain suppressor T cells for IgM response (12). The results show that the stimulation with Con A and/or IgM induces the production by thymocytes of IgM-binding factors and that the factors were formed by splenic T cells treated with IgM. Biological activities of IgM-binding factors were also examined. Results show that the IgM-binding factors suppressed IgM response more intensively than IgG response.

MATERIALS AND METHODS

Immunogulobulins and antibodies

1. Mouse monoclonal IgM antibody specific for DNP

Mouse monoclonal IgM antibody specific for the DNP hapten was obtained from the ascites of Balb/c mice that had been injected with the hybridoma cell line 35-7D. The 35-7D B cell hybridoma was established as described below.

Media and salt solution for hybridization were prepared as follows. Dulbecco's modified Eagles Medium (DMEM) was purchased from Gibco (Cat. No. 320-1965). HAT-medium and HT-medium were prepared by adding 1/100 volume of the respective 100x solutions of aminopterin (4×10^{-5} M solution in H_2O) and hypoxanthine(10^{-2} M solution in H_2O)-thymidine(1.6×10^{-3} M solution in H_2O) to DMEM. GKN-solution contained per 1 liter distilled water: 8 g NaCl, 0.4 g KCl, 1.77 g $NaH_2HPO_4 \cdot 2H_2O$, 0.69 g $NaH_2PO_4 \cdot H_2O$; 2 g glucose, 0.01 g phenol red. Polyethylene glycol (M.W. 4000, Nakarai) was melted and sterilized by autoclaving or boiling for 30 min and was cooled to 50°C, then combined with an equal volume of GKN. The resulting 50 % PEG-solution was used for hybridization. A Balb/c mouse (10 wk of age, purchased from Shizuoka Laboratory Animal (Shizuoka, Japan)) was primed with an i.p. injection of 100 μg DNP-keyhole limpet hemocyanin (KLH) included in 0.1 ml of alum. Eighteen days later, the mouse was received a booster i.p. injection of 10 μg DNP-KLH dissolved in phosphate buffered saline (PBS, pH 7.2). Three days after the booster injection, the mouse was killed by axilla dislocation and spleen was transfered into a plastic petri dish containing GKN. The cells were teased from the capsule with a spatula, washed two times with GKN and were fused with SP2/0-Ag14 cells by the method of Galfre et al(13). 10^8 spleen cells were

obtained and mixed with 3 x 10^7 SP2/0-Ag14 in a 50 ml conical tube (Falcon 2070), and spun for 10 min at 1,000 rpm at room temperature. All the supernatant was carefully withdrawn. A total of 0.5 ml 50 % PEG was added dropwise to the pellet under agitation (to brake up the pellet) over a period of one minute at room temperature. After 2 min with gently rotating by hand, 2 ml of GKN was added during 2 min, then 7 ml of GKN was added over a period of 3 min. After centrifuge for 10 min at 1,100 rpm, the pellet was resuspend in 28 ml of DMEM containing 10 % heat-inactivated fetal calf serum (FCS, M. A. Bioproducts, Walkersville, MD), 3 mM glutamine, 1 mM pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycine. One hundred μl aliquots were distributed into 280 wells of 96 well plates (Costar Tissue Culture Cluster Cat. No.3596, 205 Broadway, Cambridge, Mass., U.S.A.). The cells in the trays were cultured in a humidified air at 37°C in an atmosphare of 5 % CO_2. After 24 hr 0.1 ml HAT-medium was added in each well and cultured. After 3 days and twice a week thereafter, 0.1 ml medium was removed and replaced with HAT-medium. After 7 to 10 days the wells were inspected for hybrids and the HAT medium was replaced by HT-medium. Hybridomas that were secreting mouse Ig were selected by enzyme immunoassay with the use of Cytoscreen G (Seragen, Boston, MA) and 96-well polystylene plates (Sumitomo Bakelite, Tokyo, Japan) coated with DNP-bovine gamma globulin. Subcloning of B cell hybridomas was carried out in soft agar. About 150 hybridoma cells were seeded on a petridish containing 5 ml of 0.24 % agarose (Seakem, LE. agarose) in DMEM-15 % FCS and cultured. After 2 weeks colonies were observed and single clones were picked and expanded in liquid medium. Goat antisera specific for either mouse IgM, IgG1, or IgG2a and IgG2b (heavy chain specific, Nordic Immunological Laboratories, Tilburg, Netherland) were used for Ouchterlony immunodifusion test in 1 % agarose (Behring Institute) in

DPBS to determin the isotype of antibody secreted from hybridomas.

To purify IgM antibody from the ascites fluid, DNP-bovine serum albumin (BSA) conjugated with CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) was used as an immunoadsorbent. The ascites fluid was diluted with the equal volume of 10 mM Tris-HCl buffer, pH 8.0, containing 0.3 M NaCl, and was fractionated by the precipitation with 50 % saturated ammonium sulfate. The precipitated proteins were dialyzed against the Tris buffer, and were applied to the DNP-BSA-Sepharose column. The column was washed with the Tris buffer until the optical density at 280 nm of the effluent became less than 0.04. The bound protein was eluted with 0.1 M dinitrophenol (pH 8.2), and was dialyzed extensively against the Tris buffer and concentrated.

2. Rabbit anti-ox erythrocyte IgM and IgG antibodies

Rabbit IgM and IgG antibodies against ox erythrocytes (Eo) were obtained by immunization of two rabbits with Eo. Five % suspension (v/v) of Eo in saline (5 ml) was injected to each rabbit. One week later the animals were boosted by another injection of the same amount of Eo, and bled at one week after the boost. The IgM and IgG fractions of the antisera were obtained by the precipitation with 42 % saturated ammonium sulfate followed by gel filtration on a TSK G3000SW column with a high pressure liquid chromatograph (HPLC) from Beckman (Model 344). Protein eluted as the first peak from the column with Dulbecco's PBS (DPBS) at a flow rate of 3 ml/min was mostly IgM, and was concentrated. The second protein peak eluted from the column corresponding to the IgG fraction was pooled and dialyzed against 0.0175 M Na-phosphate buffer (pH 6.3), and applied to DEAE-Sephacel (Pharmacia) column chromatography. Protein eluted from the column with the same buffer was pooled and concentrated. No contaminating IgG or IgM could be detected in the purified IgM or IgG

preparation, respectively, by immunodiffusion test with goat anti-rabbit IgG (γ-chain specific) antiserum and anti-rabbit IgM (μ-chain specific) antiserum (Cappel, Malven, PA).

3. Mouse normal IgM and IgG

Mouse peripheral blood IgM and IgG were obtained from normal Balb/c mouse sera. The purification of IgM and IgG were performed by the same manner applied for the purification of rabbit anti-Eo antibodies described above exept that 0.04 M K-phosphate buffer (pH 8.0) containing 0.03 M NaCl was used for elution buffer in IgG purification by DEAE-Sephacel column chromatography. The purified IgM and IgG contained no detectable IgG and IgM, respectively, by immunodiffusion test with goat anti-mouse IgG antisera and anti-mouse IgM serum (Nordic).

4. Rabbit anti-mouse Ig antibody

Rabbit IgG antibodies against mouse Ig used for plaque assay were obtained by immunization of rabbits with mouse Ig fractionated from normal mouse sera by the precipitation with 40 % saturated ammonium sulfate. The immunization protocol was similar to that described by Henry and Good (14). Each rabbit was injected with 4 mg of protein containing mouse IgG included in complete Freund's adjuvant, dividing the emulsion among 4 intramuscular sites on the back of the animal. The injections were repeated one month later. Four weeks after the second immunization, the animals were bled. The antisera were heat-inactivated (56°C, 30 min) and absorbed with 1/10 volume of packed SRBC at 4°C for 30 min, then fractionated with 40 % saturated ammonium sulfate. IgG was purified by gel filtration and DEAE-Sephacel column chromatography.

Formation of IgM-binding factors

Normal thymocytes were obtained from male Balb/c mice (6 to 8 wk of age). Thymus were pooled in petridishes containing medium and broken up with frosted slideglasses. The cells were washed two times with RPMI1640 medium (Nissui, Tokyo, Japan) containing 5 % FCS, and resuspended in RPMI1640 medium supplemented with 5% FCS, 15 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH7.2, 3 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycine. Five ml aliquots of cell suspensions (4 x 10^6 cells/ml) were cultured for 48 hr in the presence or absence of 2.5 µg/ml Con A (Pharmacia Fine Chemicals, Uppsala, Sweden) in 12 well tissue culture clusters (Linbro, Cat. No. 76-053-05, Flow Laboratories, Inc. A Flow General Company McLean, Virginia 22102 U.S.A.) as descrived previously (12). After washing three times with RPMI1640 medium containing 5 % FCS, the cells were resuspended in a fresh RPMI1640 medium (5 x 10^6 cells/ml) supplemented as described above except that the concentration of FCS was decreased to 1 %, and cultured for another 24 hr in the presence or absence of 10 µg/ml of mouse IgM. The culture supernatants were filtered through Diaflo YM100 membranes (Amicon, Tokyo, Japan) to remove molecules larger than 1 x 10^5 daltons. The culture filtrates (CF) were concentrated 1/5 of the original volume of culture supernatants by ultrafiltration using Diaflo YM5 membranes, and were used as sources of IgM-binding factors.

In some experiments IgM-binding factors were obtained from thymocytes and spleen cells stimulated with IgM. Thymocytes and spleen cells from normal Balb/c mice (8-10 wk of age) were suspended in RPMI1640 medium containing 1 % FCS and 10 µg/ml IgM (2 x 10^7 cells/ml). The cells were cultured for 24 hr in 12 well tissue culture plates. The culture supernatants containing IgM-binding factors were treated as the same methods described above.

Fractionation of IgM-binding factors

Con A-activated thymocytes were cultured for 24 hr in the presence of 10 μg/ml murine peripheral blood IgM to obtain culture filtrates. The gel filtration of concentrated CF was carried out on a TSK G3000SW column which had been calibrated with bovine gamma globulin (M.W. 160,000), BSA (M.W. 67,000), ovalbumine (M.W.45,000), chymotrypsinogen (M.W. 25,000), cytochrome C (M.W. 13,000). Nine ml fractions were pooled separately, and each pooled fraction or the unfractionated CF was applied to mouse IgM-Sepharose. IgM-Sepharose was prepared by coupling 10 mg IgM from normal Balb/c mice with 5 ml of CNBr-activated Sepharose 4B. Mouse IgG-Sepharose was prepared in the same manner using IgG from normal mice. A 2 ml of a concentrated and pooled fraction was mixed with 1 ml of IgM-coated Sepharose. The mixture was kept at 4°C for 4 hr with constant rotation, and then transfered to into a small column (1 cm diameter, Biorad, Richmond, CA). The effluent and the washing with DPBS were pooled. The beads were then eluted with 50 mM glycine-HCl buffer, pH 3.0. The eluate was collected into a tube that contained an appropriate volume of 1 M Tris-HCl buffer, pH 8.0 to neutralize the pH of the eluate. The effluent and the eluate were then concentrated to 2 ml and dialyzed against DPBS. In some experiments, they were further dialysed against RPMI1640 medium. In the same fashion, effluents and eluates from IgG-Sepharose of CF were prepared.

Culture of human peripheral blood lymphocytes (PBL)

Heparinized peripheral blood was obtained from healthy volunteers. The blood was centrifuged at 2,000 rpm for 5 min at room temperature. After plasma was depleted, RPMI1640 medium was added to the original blood volume and the suspension was layered on Ficoll-Paque (Pharmacia Fine Chemicals) and centrifuged at 1,350 rpm at room temperature for 30 min. Intermediate layer containing

lymphocytes was carefully collected and the cells were washed two times with RPMI1640 medium. The cells were suspended in TC199 medium (Nissui) containing 20 % FCS, 100 U/ml penicillin and 100 μg/ml streptomycine (1 x 10^6 cells/ml) and cultured for 24 hr. The cells were washed and used for rosette assays.

Formation of rosettes and rosette inhibition assay

Ox erythrocytes sensitized with rabbit IgM (Eo-rIgM) or IgG (Eo-rIgG) were employed as indicator cells to detect FcμR(+) cells or FcγR(+) cells. Subagglutinating dose (1/2 dilution of final hemagglutination titer) of each antibody preparation was used for sensitization of Eo. Usually 10 to 30 μg/ml of antibody was used to sensitize Eo suspended (1 %) in DPBS.

TNP-coupled Eo sensitized with mouse monoclonal IgM (E'-mIgM) were also used for the detection of FcμR(+) cells. TNP-coupled Eo was prepared by the method of Rittenberg and Pratt (15). One half ml of packed Eo was mixed with 3.5 ml of 0.28 M cacodylate buffer, pH 6.9, containing 50 mg of trinitrobenzene-sulfonic acid (Nakarai, Kyoto, Japan). The tube containing the cells was rotated gently for 30 min at room temperature with almi foil wrapping, then centrifuged at 1,600 rpm for 5 min. The supernatant was removed, then the cells were suspended in DPBS and centrifuged. To the cell pellet, 60 mg glycylglycine in 5 ml DPBS was added and kept standing for 10 min at room temperature. Then the cells were washed three times with DPBS and used as TNP-coupled Eo (E'). For sensitization, 1% suspension of E' was incubated with 50 μg/ml monoclonal IgM anti-DNP antibody for 30 min at 37°C.

The three kinds of sensitized ox erythrocytes were washed three times with DPBS before use in rosette assay. FcμR(+) cells or FcγR(+) cells were detected with rosette formation by a modified version of the method described by Greenberg and Lyndyard (16). Cultured and washed PBL were mixed with

sensitized erythrocytes, and centrifuged at 1,000 rpm for 5 min. The mixture was kept on ice for 60 min. A pellet was gently mixed with a drop of 0.2 % toluidine blue, and a suspension was examined in a hemocytometer (Fuchs Rosenthal, Kayagaki Works Co., Ltd., Tokyo, Japan). Usually, 1,000 to 2,000 cells were counted to enumerate the percentage of rosette forming cells, and each sample was set up in triplicate. A positive rosette forming cell was defined as a cell having at least three red cells on the surface. In order to test the ability of CF or fractionated samples to inhibit rosette formation, 30 µl of CF was added to 21 µl of 1 % sensitized-Eo suspension, and the suspension was incubated for 2 hr on ice before 15 µl of PBL (2 x 10^7 cells/ml in TC199 containing 20 % FCS) was mixed.

Enzyme treatment

IgM-binding factors were treated with several enzymes. Concentrated CF (5 ml) was applieded to IgM-Sepharose column and the eluate (5 ml) was dialyzed against DPBS. One ml aliquots of IgM-binding factors were treated with 5 U trypsin (Sigma, Insoluble Trypsin from bovine pancreas), 5 U ribonucrease A (Sigma, Insoluble Ribonucrease from bovine pancreas), 40 U pronase (13 mg pronase E purchased from Kaken Chemical Co., Tokyo, Japan, which coupled with 0.5 ml CNBr-activated Sepharose 4B), or 40 U papain (250 U papain purchased from Sigma, which coupled with 0.5 ml CNBr-activated Sepharose 4B) at 37°C for 60 min. Supernatants were dialyzed against DPBS and assessed for their ability to inhibit IgM-rosette formation.

Spleen cell fractionation

Splenic lymphocytes were obtained by passing the spleen cell suspension (9 ml of 5 x 10^7 cells/ml in RPMI1640 containing 5 % FCS) through a 10 ml volume

of Sephadex G-10 column (Pharmacia) to deplet adherent cells. B cells in the splenic lymphocytes were deleted by using tissue culture dishes coated with the $F(ab')_2$ fragments of goat anti-mouse IgG. The dishes were prepared as follows. Tissue culture dishes (Falcon, 3003 Opilux Tissue Culture Dish, 10 cm diameter), containing 5 ml of 1 mg/ml $F(ab')_2$ fragment goat anti-mouse IgG (Cappel, heavy and light chain specific) in borate buffered saline (61.9 g H_3BO_3, 43.9 g NaCl dissolved in 6 liter distilled water, adjusted to pH 8.0 by NaOH) were incubated at 4°C overnight. After aspiration of the solution, the dishes were washed two times with RPMI1640 medium and washed one time with RPMI1640 medium containing 5 % FCS. After 1-hr incubation of the G-10 passed spleen cells in the anti-IgG-coated dishes, the nonadherent cells were recovered and applied to the another anti-IgG-coated dishes.

To enumerate surface Ig positive cells, fractionated cells were incubated with fluorescein conjugated $F(ab')_2$ fragment of goat anti-mouse IgG (Cappel, heavy and light chain specific) for 30 min on ice, then the cells were washed three times with DPBS and examined under a fluorescent microscope.

Antibody response

The effect of IgM-binding factor or control preparations on antibody response was studied. Normal Balb/c mice (8 to 16 wk of age) were immunized with an i.v. injection of 0.5 ml of SRBC suspension (1.25 % in saline). Eleven days later, spleen cells were obtained from the mice. The cells were suspended in RPMI1640 medium supplemented with 5 % FCS, 15 mM HEPES buffer, pH7.2, 3 mM glutamine, 0.1 mM MEM-nonessential amino acids (Gibco), 1 x MEM vitamins (Gibco), 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycine, and 0.2 ml aliquots cell suspension (4×10^6

cells/ml) were cultured in 96-well tissue culture plates (Costar) in the presence or absence of 3 x 10^6 cells/ml of SRBC at 37°C, 5 % CO_2, and 95 % room air. 0.16 ml of a cell suspension was mixed with 0.04 ml of a preparation of IgM-binding factor or a control preparation at the beginnig of the culture.

Plaque assay

Plaque-forming cells (PFC) secreting IgM or 7S antibodies were assayed by the method of Cunningham (17). Fifty µl washed spleen cell suspension in baranced salt solution (BSS) containing 5 % FCS was mixed with 40 µl GPS solution (0.3 ml guinea pig serum + 0.942 ml BSS containing 5 % FCS) and 9 µl 20 % SRBC in saline, and the mixture was poured into a Cunningham chamber. Paraffin (M.P. 62-64°C, Wako Pure Chemical Industries, Ltd.) and white vaseline (Nakarai) mixture was pre-warmed to melt, and the Cunningham chamber was seeled with the mixture. The chamber was incubated at 37°C in a humidified air for 1.5 hr to be able to detect plaques. IgM PFC were developed as direct plaque formers. In parallel Cunningham chamber slides, dilutions of rabbit IgG antibody against mouse Ig optimal for the development of indirect 7S PFC were included in the assay mixtures. When calculating the number of 7S PFC, it was necessary to make corrections to account for the inhibition of IgM plaques by anti-mouse Ig antibody:

$$\text{Correction factor} = \frac{\text{IgM plaques measured with complement plus anti-Ig}}{\text{IgM plaques measured with complement only}}$$

$$\text{7S plaques} = (\text{total number of plaques obtained by indirect assay}) - (\text{number of direct plaques} \times \text{correction factor})$$

We tentativelly used the number of 7S PFC as the number of IgG PFC, because rabbit anti-mouse γ-chain specific antiserum (Cappel) gave essentially the same numbers of 7S PFC to that obtained by using rabbit IgG antibodies against mouse IgG (whole molecule).

RESULTS

Formation of IgM-binding factor by Con-A stimulated thymocytes

Normal thymocytes were cultured in the presence or absence of 2.5 μg/ml Con A. After 48 hr, the cells were washed, resuspended in a fresh culture medium, and cultured for another 24 hr in the absence of Con A. The culture supernatants were filtered through Diaflo YM100 membranes to remove IgM molecules, and the culture filtrates (CF) were concentrated 5-folds and assessed for their ability to inhibit the formation of IgM rosettes and IgG rosettes. As shown in Table 1, CF of Con A-activated thymocytes inhibited the formation of both IgM rosettes and IgG rosettes, whereas CF of unstimulated thymocytes had little effect on rosette formation on the both types.

The experiment shown in Table 1 also suggested that the formation of the IgM rosette-inhibiting factor was enhanced in the presence of IgM. When 10 μg/ml IgM was present in the second culture period, CF obtained after 24-hr culture had a higher ability to inhibit IgM rosette formation than CF of the same thymocytes cultured in IgM-free medium (The IgM rosette inhibiting activity was observed significantly in CF from culture supernatants of not only Con A-activated thymocytes but also normal thymocytes treated with IgM.). However, CF of Con A-activated thymocytes cultured with IgM contained significantly less IgG rosette-inhibiting factor than CF of the same cells cultured in IgM-free medium. A control filtrate was obtained by filtration of medium which contained 10 μg/ml IgM followed by 5-fold concentration. The control filtrates failed to inhibit the formation of either IgM rosettes nor IgG rosettes.

Experiments were carried out to determine whether the IgM rosette-inhibiting factor might have affinity for mouse IgM. Culture supernatant was obtained by 24-hr incubation of Con A-activated thymocytes with IgM. The culture supernatant was filtered through Diaflo YM100 membranes, concentrated 5-fold by ultra-

filtration using Diaflo YM5 membrane, and dialyzed against DPBS. A 2-ml aliquot of the preparation was added to each of 1 ml Sepharose coupled with 2 mg normal mouse IgM and 1 ml Sepharose coupled with 2 mg normal mouse IgG, and the suspensions were mixed for 4 hr at 4°C. From the each mixture the effluent and the eluate with glycine-HCl buffer, pH 3.0, were pooled, and the ability to inhibit IgM rosette formation or IgG rosette formation was studied.

As shown in Table 2, the ability of CF to inhibit IgM rosette formation was recovered in the eluate from IgM-Sepharose and in the effluent from IgG-Sepharose. On the other hand, the ability of CF to inhibit IgG rosette formation was recovered from in the effluent from IgM-Sepharose and in the eluate from IgG-Sepharose. The latter ability seemed to be the existence of IgG-binding factor described by Fridman et al. (1). Using Sepharose beads coupled with affinity-purified monolconal IgM, the distribution of rosette inhibiting activities between the effluent and the eluate fractions from the beads was essentially the same to that was obtained by using normal mouse IgM-coupled Sepharose as shown in Table 2 (data not shown). It appears that IgM rosette inhibiting factor has affinity specific for IgM and thus inhibits rosette formation with Eo-IgM. Therefore we designated this new factor IgM-binding factor.

To confirm that IgM-binding factor, which was absorbed with IgM-Sepharose and eluted with acidic buffer from the column, does not have affinity for IgG-Sepharose, the eluate of CF from IgM-Sepharose was fractionated on IgG-Sepharose and the effluent and the eluate with acidic buffer were assessed for the ability to inhibit IgM rosette formation. As shown in Table 3, essentially all IgM-binding factor in the sample was recovered in the effluent from IgG-Sepharose. This result indicates that IgM-binding factor specifically binds to IgM.

Sensitivity of IgM-binding factor to proteolytic enzymes

IgM-binding factor recovered from IgM-Sepharose was treated with immobilized enzymes. The protein nature of IgM-binding factor was confirmed by the sensitivity of IgM-rosette inhibiting activity to trypsin, chymotrypsin, and papain, but not to ribonuclease A treatment (Table 4).

Cell source of IgM-binding factor

Experiments were carried out to study whether IgM-binding factor was formed by lymphoid cells other than thymocytes. Thymocytes, spleen cells, and mesenteric lymph node cells were cultured for 24 hr in the presence of 10 μg/ml mouse IgM. Culture supernatants were filtered through YM100 membranes, concentrated 5-fold by using YM5, and assessed for their ability to inhibit IgM rosette inhibition. As shown in Table 5, both CF of thymocytes and CF of spleen cells inhibited the formation of IgM rosettes. CF of mesenteric lymph node cells, however, had little effect on IgM rosette formation. The CF of thymocytes and spleen cells were further fractionated into IgM-Sepharose. The results were obtained that IgM-rosette inhibiting activity in CF of thymocytes and spleen cells was recovered in the eluates from the IgM-Sepharose (data not shown).

Experiments were carried out to study the cell source of the IgM-binding factor. Spleen cells from normal mice were fractionated into nonadherent cells by using Sephadex G-10 column, and nonadherent cells were further fractionated into B cells and T cells by using anti-mouse Ig coated-dishes. Each fraction as well as unfractionated spleen cells was cultured with 10 μg/ml mouse IgM. After 24 hr, the supernatants were obtained, and concentrated CF were assessed for their ability to inhibit IgM rosette formation. As shown in Table 6, the CF preparations from whole spleen cell fraction and nonadherent cell fraction inhibited the IgM rosettes. Also CF preparation from B cell depleted fraction,

which would contain almost T cells, inhibited the IgM rosettes.

Suppression of the IgM response by IgM-binding factor

Con A-activated thymocytes were cultured with IgM for 24 hr, and the culture supernatant was filtered through YM100 membranes. IgM-binding factor and IgG-binding factor in the CF were purified by affinity chromatography using an IgM-Sepharose column and an IgG-Sepharose column, respectively, and their effects on the IgM response and the IgG response of SRBC-primed spleen cells to SRBC were studied. It can be seen in Table 7 that IgM-binding factor recovered in the eluate from IgM-Sepharose markedly suppressed the IgM response, whereas it had little effect on the IgG response. On the other hand, the effect of IgG-binding factor recovered in the eluate from IgG-Sepharose was not remarkable but the factor significantly suppressed the IgG response. The IgM response was not suppressed significantly by the factor.

Molecular sizes of IgM-binding factors

The molecular weight of IgM-binding factor was estimated by gel filtration. Con A-stimulated thymocytes were cultured with 10 μg/ml IgM for 24 hr, and culture supernatants were filtered through YM100 membranes. The filtrates were concentrated 10-fold and 1.5 ml of the preparation was fractionated by TSK G3000SW column using the HPLC system. Fractions shown at the bottom of Fig. 2 were pooled separately, and each pooled fraction was tested for the presence of IgM-binding factor.

The results of the experiments (see Fig. 1) indicate that two separate fractions contained IgM-binding factor; one was eluted from the column just before BSA (M.W. 67,000) and another IgM-binding factor fraction was obtained between ovalbumin (M.W. 45,000) and chymotrypsinogen (M.W. 25,000). These

fractions had the ability to inhibit the formation of IgM rosettes, and also had the ability to suppress the IgM PFC response and the IgG response of SRBC-primed spleen cells to SRBC. Suppressive effects by these fractions on the IgG response were less intensive than that on the IgM response.

DISCUSSION

The data presented in this report indicated that IgM-binding factors are formed by murine thymocytes activated with Con A. Because the IgM-binding factors have affinity specific for IgM but not for IgG, they are clearly different from a factor reported by Molenaar et al. (18), which had affinity for both IgM and IgG.

As it has been shown that the other immunoglobulin-binding factors already found were produced by T cells bearing FcR specific for the corresponding isotype, it is reasonable to study the role of FcμR positive T cells in the production of IgM-binding factors. However, we have not succeeded in isolating murine FcμR positive T cells by rosette formation because IgM rosettes by murine T cells are extremely fragile to be fractionated by gradient centrifugation (19). The extreme fragility was also a reason why I have not employed murine lymphocytes for routine rosette assays to detect murine IgM-binding factors. In contrast, human FcμR positive lymphocytes that I employed form stable IgM-rosettes (8). Addition of mouse IgM to the cultures of Con A-activated thymocytes enhanced the formation of IgM-binding factors (Table 1). Formation of IgM-binding factors was also observed when normal thymocytes and spleen cells were cultured with IgM (Table 5). Similar phenomena were found in the system of the other immunoglobulin-binding factor formation in vitro. The factor formation can be induced by stimulation of lymphocytes with corresponding isotype of immunoglobulin (5,6, 20). Table 5 also shows that the formation of IgM-binding factors by mesenteric lymph node cells was not observed. The reason why the factor formation could not be observed might be do to that the factor was absorbed by IgM in culture. But on the other hand, B cell-depleted spleen cell fraction, in which only T cells would exist, could form IgM-binding factors when the cells were cultured with IgM (Table 6). These findings support the notion that IgM-binding factors

are produced by FcμR positive T cells. Andersson et al. reported that rosette formation between murine lymphocytes and SRBC sensitized with murine IgM was inhibited by soluble murine IgM-antigen complexes or human IgM $(Fc)_{5\mu}$ but not by free murine IgM, human Fab_{μ} fragment, or antigen alone (19). Rosette formation between human lymphocytes and SRBC sensitized with rabbit IgM was, however inhibited by murine myeloma IgM (MOPC 104E), or by sera obtained from mice injected with IgM-producing hybridoma, or even by normal mouse sera. The discrepancy between the two reports may be partly do to the difference in the combination of species from which they prepared lymphocytes and antibodies. A free IgM molecules is stellate with five $(Fab')_{2\mu}$ moieties and a central $(Fc)_{5\mu}$ disc (21), and has segmental flexibility (22). When IgM is antigen-bound, "staple" configrations can be taken (21). These reports suggest that free IgM has less affinity for FcμR on lymphocytes than the exposed $(Fc)_{5\mu}$ or IgM with configurational change. Therefore, IgM molecules present in murine serum at the concentration of 0.1 to 0.8 mg/ml (normal Balb/c; ref. 23,24) may not induce the formation of IgM-binding factors in vivo until they form complexes or change their configuration. As purified IgM tends to be denatured or aggregated easily in the buffer with physiological ionic strength, the formation of IgM-binding factors might be enhanced by those denatured or aggregated IgM which might be spontaneously induced in the purified IgM preparations in vitro. On the other hand, the formation of IgG-rosette inhibiting factor was decreased by the addition of IgM to the culture (Table 1). This phenomenon may be correlated with the factors that one T cell can be switched from the expression of FcγR to the expression of FcμR (25), and that the transition of FcγR positive cells to FcεR positive cells can be induced by addition of IgE to the culture (3).

Table 7 showed the suppressive activity of IgM response by IgM-binding factors. To detect clear IgM suppressive activity, it was necessary to purify

the factors from the crude culture supernatant. Similar phenomena were reported by Fridman et al. (27), and by Yodoi et al. (6) in the study of IgG-binding factors and IgA-binding factor, respectively. The suppressive effect of IgM-binding factors on IgM response was in good agreement with previous report that Con A-activated thymocytes suppressed primary IgM response against SRBC in vitro (12). The weak but significant suppressive effect on IgG response by IgM-binding factors (Table 7 and Fig. 1) might be the result of binding of the factors to IgM expressed on "early memory" cells for IgG synthesis (28).

The molecular weights of IgM-binding factors were determined to be approximately 70,000 and 35,000 daltons by gel filtration (Fig. 1). The result suggests that IgM-binding factors exist in at least two molecular formers. The relationship between the two formers is not unclear. Among the other immunoglobulin-binding factors, IgG-binding factors were shown to have similar molecular weights. Recently Vaquero et al. reported that messenger RNA coding for suppressive IgG-binding factor obtained from a murine T cell hybridoma was translated into two polypeptides having MW 78,000 and 37,000 daltons, and that the larger might correspond to dimers of the smaller (26).

It has been shown that lipomodulin, a phospholipase inhibitory protein, plays an important role in including suppressor cells from thymocytes by Con A (12). It has been also shown that lipomodulin plays a role as glycosylation-inhibiting factor in including IgE-suppressive factor (29,30). Among the immunoglobulin-binding factors reported, IgE-binding factors could be classified into at least two groups, i.e. IgE-suppressive factor (5) and IgE-potentiating factor (4) according to their effects on IgE synthesis, whereas the others were found to be suppressive to the antibody synthesis in the corresponding classes (1,6,20). IgE-potentiating factor was produced in the presence of glycosylation-enhancing factor which was shown to be a kallikrein-like enzyme formed by a subset of T cells (31,32). Vitetta and her coworkers have reported the existence of

a soluble factor ($BCDF_{\mu}$) which selectively induce IgM secretion in B cells (33). There might exist IgM-binding factors having similar function to $BCDF_{\mu}$.

IgD is the last class of immunoglobulin, to which a specific binding factor has not been found. Although the role of IgD expressed on B cells is not clear yet, signals transmitted through IgD and IgM must be involved in the control of the production of IgM antibody and IgG antibody.

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NOTES

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2. Abbreviations used:

Con A	-	concanavalin A
PBL	-	peripheral blood lymphocytes
TNP	-	trinitrophenyl
DNP	-	dinitrophenyl
Eo	-	ox erythrocytes
Eo-rIgM	-	ox erythrocytes sensitized with rabbit IgM
Eo-rIgG	-	ox erythrocytes sensitized with rabbit IgG
E'-mIgM	-	TNP-coupled ox erythrocytes sensitized with mouse monoclonal IgM
PFC	-	plaque-forming cell(s)
SRBC	-	sheep red blood cell(s)
FcR	-	Fc receptors
FcγR	-	Fc receptors specific for IgG
FcεR	-	Fc receptors specific for IgE
FcαR	-	Fc receptors specific for IgA
FcμR	-	Fc receptors specific for IgM
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
CF	-	culture filtrate(s)
KLH	-	keyhole limpet hemocyanin
alum	-	alminum hydroxide gel
BSA	-	bovine serum albumin

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Table 1

Formation of IgM-rosette inhibiting factor and IgG-rosette inhibiting factor by Con A-activated thymocytes[a]

Thymocytes treated with	IgM in culture	Inhibition of rosette formation[b] E'-mIgM RFC[c]	Eo-rIgM RFC[d]	Eo-rIgG RFC[e]
		%	%	%
None[f]	-	4	N.D.	1
	+	8	16	10
Con A	-	12	24	35
	+	37	42	14
control CF[g]	+	N.D.	0	0

a) Normal thymocytes were precultured for 48 hr in the presence or absence of 2.5 μg/ml Con A. The cells were washed, and incubated for 24 hr in the presence or absence of 10 μg/ml IgM to obtain culture filtrates.

b) Either E'-mIgM, Eo-rIgM or Eo-rIgG was used for rosette formation with 24 hr-cultured human PBL in the presence of concentrated culture filtrates.

c, d, e) Percentage of rosette forming cells in the absence of culture filtrates was 13%(c), 23%(d), and 12%(e), respectively.

f) Normal thymocytes were precultured in the absence of Con A.

g) A medium containing 10 μg/ml IgM was filtrated through a Diaflo YM100 membrane, and was concentrated 5 folds.

Table 2

Absorption of IgM-rosette inhibiting factor and IgG-rosette inhibiting factor by IgM-Sepharose and IgG-Sepharose, respectively[a]

Thymocytes cultured with	Fraction added to the mixture[b]	Inhibition of rosette formation	
		Eo-rIgM RFC[c]	Eo-rIgG RFC[d]
		%	%
None	Unfractionated	15	0
Con A	Unfractionated	41	19
IgM-Sepharose	Effluent	0	24
	Eluate	41	0
IgG-Sepharose	Effluent	31	1
	Eluate	0	26

a) Thymocytes were precultured for 48 hr in the presence or absence of 2.5 μg/ml Con A. The cells were cultured with 10 μg/ml IgM for another 24 hr to obtain culture filtrates. The filtrates were then concentrated. An aliquot of the culture filtrate from Con A-activated thymocytes was applied either IgM-Sepharose or IgG-Sepharose. The distribution of rosette inhibiting factors between the effluent and the eluate fractions was examined.

b) Human PBL cultured for 24 hr were mixed with either Eo-rIgM or Eo-rIgG in the presence of each fraction, and the mixture was incubated to form rosettes.

c,d) Seventeen % of human PBL formed rosettes with either Eo-rIgM or Eo-rIgG when they were incubated in a control medium.

Table 3

Failure of absorption of IgM-binding factor by IgG-Sepharose[a]

Fractionated with	Fraction added	RFC[b] with Eo-rIgM	Rosette inhibition
		%	%
IgM-Sepharose	Eluate	11.6	30
IgG-Sepharose	Effluent	11.4	31
	Eluate	17.0	0
Medium control		16.6	

a) CF from ConA-activated thymocytes cultured with 10 μg/ml IgM was absorbed with IgM-Sepharose, and the eluate from the column was obtained. An aliquot of the eluate was further fractionated with IgG-Sepharose. Each fraction was assessed for its ability to inhibit IgM-rosette formation.

b) RFC; rosette forming cells.

Table 4

Sensitivity of IgM-binding factor to proteolytic enzymes[a]

IgM-binding factor treated with	RFC with Eo-rIgM	Rosette inhibition
	%	%
none	12.7	41
trypsin	21.5	0
pronase	18.9	12
papain	17.6	18
ribonuclease A	10.8	50
medium control	21.4	

a) IgM-binding factor purified with IgM-Sepharose was treated with agarose-bound enzymes (see Materials and Methods), and was assessed for the ability to inhibit IgM-rosette formation.

Table 5

Source of IgM-bindng factor[a]

Source of CF	IgM in culture	RFC with Eo-rIgM	Rosette inhibition
		%	%
Thymocytes	-	14.6	3
	+	9.1	40
Spleen cells	-	15.0	0
	+	10.6	30
Mesenteric lymph node cells	-	15.5	0
	+	14.6	3
Medium control		15.1	

a) Thymocytes, spleen cells, and mesenteric lymph node cells were obtained from normal mice. Each cell suspension (2×10^7 cells/ml) was cultured for 24 hr in the presence or absence of 10 µg/ml IgM to obtain culture filtrates (CF). Then CF were assessed for their ability to inhibit IgM rosettes.

Table 6

Cell source of IgM-binding factor

Spleen cell fraction	sIg^+ cells	RFC with Eo-rIgM	Rosette inhibition
	%	%	%
Unfractionated[a]	40	15.2	31
Nonadherent cells[b]	41	13.5	39
B cell depleted Fr.[c]	1	14.9	33
B cell enriched Fr.[c]	59	19.5	12
Medium control		22.1	

a) Normal spleen cells were cultured for 24 hr in the presence of 10 μg/ml IgM to obtain culture filtrates.

b) Spleen cells were passed through Sephadex G-10 column to deplet adherent cells and the column-passed cells were cultured for 24 hr in the presence of 10 μg/ml IgM.

c) Sephadex G-10 passed cells were further fractionated into B cell depleted fraction by using anti-mouse Ig-coated dishes. Cells nonadhered to dishes (B cell depleted Fr.) and recovered cells from the dishes (B cell enriched Fr.) were obtained, and were cultured for 24 hr in the presence of 10 μg/ml IgM.

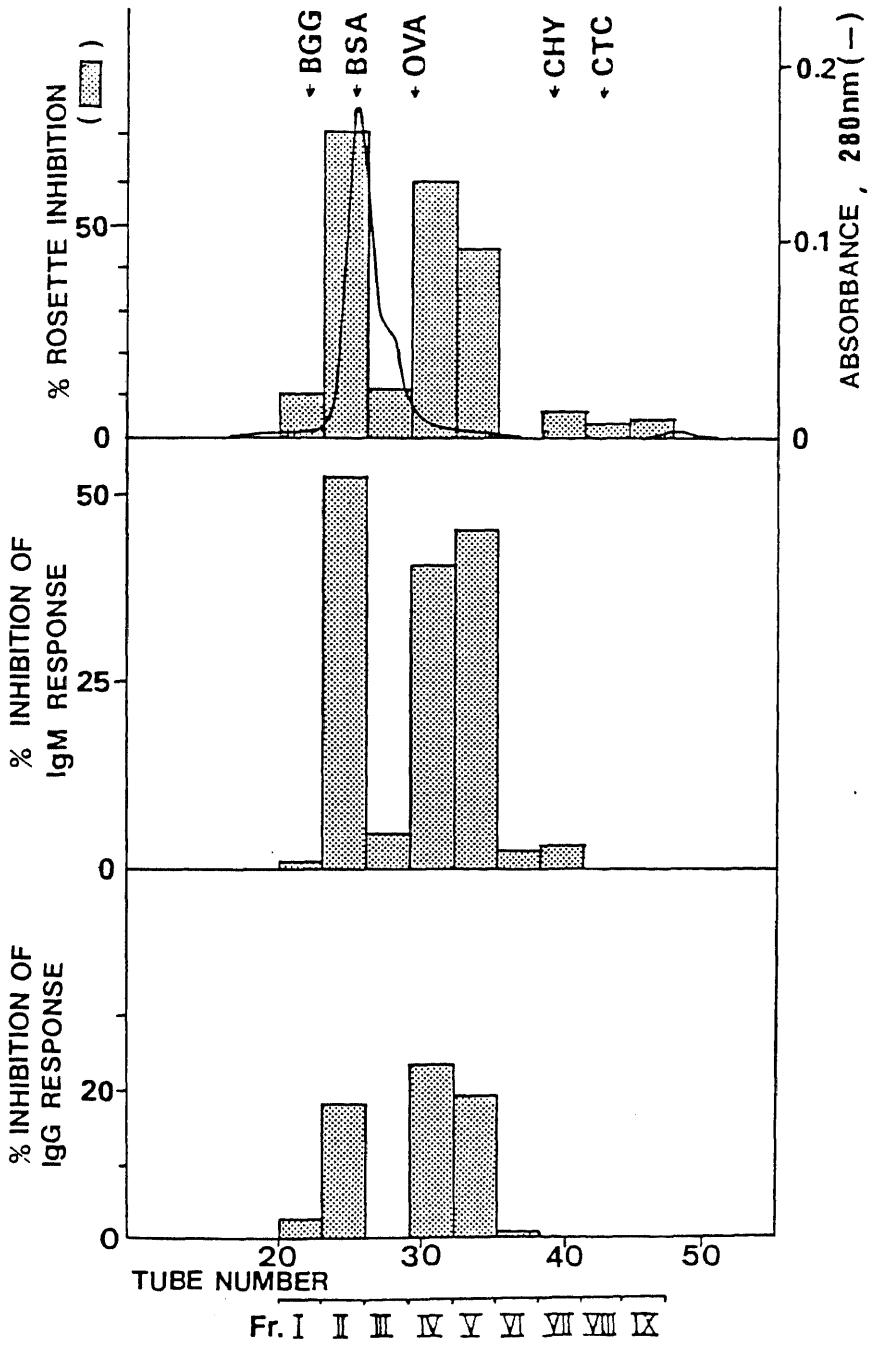
Table 7

Effects of IgM-binding factor and IgG-binding factor on the secondary *in vitro* anti-SRBC IgM response and IgG-response

Sample added	% inhibition of IgM-rosette	% inhibition of IgG-rosette	SRBC in culture	Anti-SRBC PFC IgM response	Anti-SRBC PFC IgG response
Medium			-	83 + 38	108 + 14
Medium			+	1775 + 100	1892 + 52
IgM-BF	38	0	+	850 + 101	1633 + 38
IgG-BF	0	40	+	1667 + 126	917 + 95

a) One hundred µl of IgM-binding factor preparation or IgG-binding factor preparation was added to each of 100 µl culture of 6 x 10^5 SRBC and 6 x 10^5 spleen cells obtained 11 days after SRBC-immunization. Four days later the cells were harvested, and the direct IgM PFC and the indirect IgG PFC per culture were measured. Each determination represents the mean of triplicate cultures (and the standard deviation).

Fig. 1


BGG
BSA
OVA
CHY
CTC
% ROSETTE INHIBITION ( )
ABSORBANCE, 280nm(—)
0.2
0.1
0
50
% INHIBITION OF IgM RESPONSE
25
% INHIBITION OF IgG RESPONSE
20
20
30
40
50
TUBE NUMBER
Fr. I II III IV V VI VII VIII IX

LEGEND

Fig. 1 : Gel filtration of a culture filtrate of Con A-activated thymocytes. Normal thymocytes were precultured with 2.5 μg/ml Con A for 48 hr. The Con A-activated cells were incubated with 10 μg/ml IgM for 24 hr to obtain a culture filtrate. The culture filtrate was concentrated 10-fold, and 1.2 ml of the concentrated culture filtrate was applied to a TSK G3000SW column. Each eluate fraction was then mixed with IgM-Sepharose to obtain acid-eluates, and the acid-eluates were assessed for the presence of IgM-binding factors by IgM-rosette inhibition (panel A). Effect of the acid-eluate obtained from each fraction on IgM response (panel B) and IgG response (panel C) was also determined by the same method described in the Table 7. Each determination represents the mean of triplicate cultures, and is expressed as percent inhibition compared to the cultures receiving only medium (11,047 $\pm$ 151 IgM PFC/culture and 14,080 $\pm$ 538 IgG PFC/culture). IgM-binding factors in the unfractionated culture filtrate were also obtained by using IgM-Sepharose. IgM PFC and IgG PFC in the cultures received the factors were 5,107 $\pm$ 205, 11,693 $\pm$ 854/culture, respectively.