B-LYMPHOCYTE ACTIVATION BY THE Fc REGION OF IgG*

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Lymphocytes of different species have receptors for the Fc portion of immunoglobulin molecules. The binding to these Fc receptors has been shown for antigen-antibody complexes, heat-aggregated IgG, and also for Fc fragments of IgG (1). Autologous and heterologous aggregated IgG, as well as various antigen-antibody complexes, appear to bind and compete for the same receptor. Thus, the effect of aggregated human IgG on mouse spleen cells may be used as a model for the interaction of antigen-antibody complexes with the Fc receptor.

The biological function of the Fc receptor of lymphocytes is not known, although numerous observations are consistent with the concept that this receptor may play a role in the regulation of the humoral immune response. Enhancing (2–4) and inhibiting (5, 6) effects of antibody on specific immune responses (7) depend in part on the Fc portion of the antibody as shown by several authors (8–12). Certain antigen-antibody complexes can also enhance (13) or inhibit (13–16) in vitro lymphocyte transformation. Studies on the direct activation of normal, unsensitized lymphocytes by antigen-antibody complexes have produced conflicting results. Bloch-Shtacher et al. (17) and Möller (18) reported that antigen-antibody complexes could stimulate DNA synthesis in normal human peripheral blood lymphocytes. However, this finding could not be repeated with mouse cells (19, 20), and it was concluded that lymphocytes were not directly activated by the Fc receptor (19).

The aim of the present work was to determine the ability of different forms and fragments of human IgG (HGG)[1] to activate normal mouse spleen cells by developing an in vitro culture system. The findings demonstrate that the Fc region of IgG, when modified by heat aggregation or when separated by enzy-

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[1] *Abbreviations used in this paper:* AHGG, aggregated human gamma globulin; AMGG, aggregated mouse gamma globulin; ATS, anti-thymocyte serum; BSS, balanced salt solution; C, complement; Con A, concanavalin A; cpm, counts per minute; DHGG, deaggregated human gamma globulin; FcR, Fc receptor; FCS, fetal calf serum; GRBC, goat erythrocyte; HGG, human gamma globulin; Ig, immunoglobulin; LPS, lipopolysaccharide; MGG, mouse gamma globulin; PBS, phosphate-buffered saline; PFC, plaque-forming cell; RBC, erythrocyte; SRBC, sheep erythrocyte; TNP, 2,4,6-trinitrophenyl.

matic cleavage from IgG, can induce strong stimulation of blast transformation, DNA synthesis, and polyclonal antibody formation.

Materials and Methods

Mice. A/J male mice, 8–12 wk of age, were obtained from The Jackson Laboratory, Bar Harbor, Maine. BALB/c nu/nu mice were kindly provided by Dr. M. B. A. Oldstone, Scripps Clinic and Research Foundation.

Spleen Cell Cultures and Assays. Spleens were prepared as single cell suspensions by pressing the tissue through a 50-mesh stainless steel screen into cold sterile balanced salt solution (BSS). After two washes in BSS and removal of clumps, the cells were suspended in RPMI-1640 medium (Flow Laboratories, Inc., Rockville, Md.), supplemented with 2 mM L-glutamine, penicillin, and streptomycin (Microbiological Associates, Bethesda, Md.), 5×10^{-5}M 2-mercaptoethanol, and 0.5% fresh autologous mouse serum.

(*a*) DNA SYNTHESIS. Triplicate cultures of 0.2 ml containing 5×10^5 spleen cells were incubated in flat bottom microtiter plates (3040 Micro Test II, Falcon Products, Oxnard, Calif.) at 37°C and 5% CO_2. The cultures were pulsed with 1 μCi tritiated thymidine ([^{3}H]TdR) (New England Nuclear, Boston, Mass., NET-027)/0.05 ml after 4 days of incubation (unless stated otherwise) and were harvested 18 h later with an automatic cell harvester (model M24V, Brandel, Rockville, Md.). The strips were dried, placed in 5 ml Aquasol (New England Nuclear), and counted on a scintillation counter. Results are expressed as mean counts per minute (cpm) of triplicate cultures ± standard error.

(*b*) ANTIBODY-FORMING CELLS. For generation of plaque-forming cells (PFC), spleen cells were incubated as triplicate 1-ml cultures containing 5×10^6 cells in tissue culture plates (3008) MultiWell, Falcon Products). PFC were assayed by a modification (21) of the Jerne and Nordin plaque assay (22). For detection of hemolytic plaques to HGG, goat erythrocytes (GRBC) (Colorado Serum Co., Denver, Colo.) were conjugated to GRBC-absorbed Cohn Fraction II HGG by using water-soluble carbodiimide (Story Chemical Co., Muskegon, Mich.) (21). Heavily conjugated 2,4,6-trinitrophenyl (TNP)-burro erythrocytes (TNP-BRBC) were prepared according to the method of Kettman and Dutton (23). Guinea pig serum was the source of complement and rabbit anti-mouse IgG was used for the detection of indirect PFC. Results of the plaque assay are expressed as mean PFC/10^7 original cells of duplicate pools.

(*c*) ENUMERATION OF BLAST CELLS. Individual lymphocyte cultures were transferred and spread on microscope slides. Air-dried smears were stained with methyl green pyronin Y to facilitate the counting of large pyroninophilic blast cells.

Immunological Reagents

(*a*) IgG. HGG was obtained as Cohn Fraction II through the courtesy of the American Red Cross National Fractionation Center with the partial support of National Institutes of Health grant HE 13881 (HEM) and purified on DEAE-cellulose by elution with 0.01 M phosphate buffer pH 8.0.

Mouse IgG (MGG) (20 mg/ml) was obtained from Cappel Laboratories, Cochranville, Pa.

HGG and MGG were aggregated by heating at 63°C for 30 min. Deaggregated HGG (DHGG) was obtained by ultracentrifugation of DEAE purified HGG for 2½ h at 150,000 *g* as previously described (24).

(*b*) IgG FRAGMENTS. $F(ab')_2$ fragments were prepared by pepsin digestion of HGG as described by Nisonoff et al. (25). To obtain Fab and Fc fragments, HGG was digested with papain for 18 h (26) and purified on a DEAE-cellulose column by stepwise elution with a modification (27, 28) of the method described by Franklin (29). To remove undigested IgG (less than 5%), the Fab fraction was subsequently chromatographed on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.). Purity was confirmed by Ouchterlony analysis with rabbit anti-human Fc or Fab.

Goat Fc and Fab fragments were prepared by the same method as described for fragments of human IgG, except that papain digestion was extended to 22 h.

Rabbit Fc and Fab (from IgG) were isolated after incubation with papain for 18 h by gradient elution on a CM cellulose column according to Porter (26). Fc fragments were further purified by dialysis against cold phosphate-buffered saline (PBS), and the Fc fragments were recovered in the

form of crystals. Both Fab and Fc fractions were immunochemically pure, as determined by Ouchterlony analysis.

IgG and all reagents were filter sterilized before incubation for enzyme digestion. Fab and Fc fragments were refiltered after purification and concentration.

(*c*) PREPARATION OF ROSETTING REAGENT FOR REMOVAL OF Ig-BEARING CELLS. Rosetting reagent was kindly provided by Doctors S. M. Walker and G. Meinke, Scripps Clinic and Research Foundation. $F(ab')_2$ fragments of goat Ig anti-mouse $F(ab')_2$, which precipitated all classes of Ig-bearing kappa light chains (as demonstrated by Ouchterlony analysis), were prepared by pepsin digestion of purified antibodies eluted from an $F(ab')_2$ mouse IgG-Sepharose 4B immunoabsorbent column (Pharmacia Fine Chemicals).

1 mg of sterile filtered $F(ab')_2$ fragments in 0.5 ml PBS was added to 0.1 ml glutaraldehyde fixed (30), packed sheep erythrocytes (SRBC) in 5 ml of 0.1 M sodium-acetate buffer, pH 5.0, and the mixture rotated overnight at room temperature. The cells were then washed six times with PBS and stored in PBS with 0.1% sodium azide at 4°C. The reagent is stable for several months when prepared under sterile conditions.

(*d*) RABBIT IgG ANTI-OX RBC. Rabbits were immunized with increasing amounts (0.5–3 ml) of 10% ox erythrocytes (RBC) in PBS twice a week for 1 mo. The IgG fraction was isolated from the serum by ammonium sulphate precipitation and DEAE-column chromatography.

Sucrose Density Ultracentrifugation. The sample to be separated by ultracentrifugation was applied to the top of a linear sucrose gradient of 5–20% sucrose (wt/vol) in 0.05 M Tris buffer, pH 7.5 in a 12.5-ml tube. The samples were centrifuged at 35,000 rpm in a Beckman SW 41 rotor (Beckman Instruments, Inc., Cedar Grove, N. J.) at 4°C for 20 h. Markers in a control tube included rabbit IgG labeled with ^{131}I, sheep hemoglobin, and human light chains (a gift from Dr. H. Spiegelberg, Scripps Clinic and Research Foundation). The position of the light chain marker was determined by Ouchterlony analysis. The fractions collected from the gradient were pooled according to their optical density (280 nm) and the position of the markers, dialysed overnight against PBS and filter sterilized.

Preparation of Different Lymphoid Cell Populations

(*a*) ANTI-T CELL SERUM (ATS) AND COMPLEMENT (C) TREATMENT. Rabbit anti-mouse thymocyte serum (lot no. 15038, Microbiological Associates, Bethesda, Md.) was absorbed with a myeloma cell line (XS-63) as previously described (31). Spleen cells were incubated at a concentration of 30×10^6/ml in BSS with a 1:30 dilution of antiserum for 30 min at 4°C. The cells were centrifuged and resuspended to 30×10^6/ml in a 1:5 dilution of guinea pig complement (Pel Freeze Bio-Animals, Inc., Rogers, Ark.) and incubated for 30 min at 37°C. The complement was preabsorbed for 30 min at 4°C with 1:5 packed volume of A/J RBC, and then with a mixture of thymocytes and spleen cells. Control cultures consisted of cells treated with normal rabbit serum and complement. After three washes in BSS, the cells were resuspended in culture medium.

(*b*) NYLON WOOL COLUMNS. Nylon wool (FT-242, Fenwal Laboratories, Inc., Morton Grove, Ill.) was boiled in 0.2 N HCl for 30 min and rinsed repeatedly in distilled water over a period of 5 days. Filtration of cells through nylon wool columns (12 ml syringe containing 0.6 g nylon wool) was carried out as described by Julius et al. (32). The columns were loaded with $80–100 \times 10^6$ cells, from which $10–18 \times 10^6$ viable nonadherent cells were recovered. The effluent cells were washed twice in cold BSS before being resuspended in culture medium.

(*c*) ROSETTING OF Ig-BEARING CELLS. To remove RBC and dead cells before rosetting, spleen cells were purified by Ficoll-Isopaque density gradient centrifugation (33). For rosette formation, 5×10^7 purified lymphocytes were mixed with 5×10^9 goat anti-mouse Ig coated SRBC (prepared as described above) in 1 ml BSS containing 5% fetal calf serum (FCS) and centrifuged at 4°C for 30 min. The pellet was gently resuspended and the rosetted cells removed by centrifugation for 8 min on Ficoll-Isopaque (34). The cells recovered at the interface (about 20% of the original population) were washed twice in BSS before resuspension in culture medium.

(*d*) REMOVAL OF Fc RECEPTOR-POSITIVE CELLS. Spleen cells were depleted of Fc receptor-positive cells by using ox RBC treated with a subagglutinating dose of rabbit IgG anti-ox RBC (35). Ficoll-Isopaque purified spleen cells (5×10^6 cells/ml of RPMI-1640 (Flow Laboratories, Inc.) supplemented with 5% FCS) were incubated at a ratio of 1:25 with ox RBC coated with rabbit IgG anti-ox RBC for 5 min at 37°C and subsequently spun at 140 *g*. The pellet was kept at room temperature for 30 min, gently resuspended, and layered on a Ficoll-Isopaque gradient, and then

spun at 400 *g* for 8 min. Fc-negative lymphoid cells were collected at the interface, washed twice in BSS, and resuspended at the desired concentration in culture medium. Fc-positive cells forming the rosettes found in the pellet were carefully resuspended in a small amount of medium and submitted to rapid osmotic lysis in H_2O to lyse the ox RBC, and washed twice in BSS before counting in culture medium.

The percent of Fc-positive lymphoid cells in the spleen determined by this method varied between 45 and 60%. Both Fc-positive and -negative populations were more than 95% pure as found by rerosetting them with rabbit IgG anti-ox RBC coated ox RBC.

(*e*) PLASTIC ADHERENT SPLEEN CELLS. Spleen cells (5×10^7 cells/10 ml) were cultured in plastic Petri dishes (no. 3003, Falcon Products) in RPMI-1640 (Flow Laboratories, Inc.) supplemented with 0.5% fresh mouse serum. After 1 h at 37°C (in CO_2 incubator) the nonadherent cells were removed and recultured twice on a fresh Petri dish for an additional hour. The adherent spleen cells were also recultured in fresh medium and removed with a rubber policeman after 1 h, then washed twice before resuspension in culture medium.

Tolerance Induction. For induction of tolerance mice received 2.5 mg DHGG by intraperitoneal injection (24).

Mitogens. Concanavalin A (Con A) (Pharmacia Fine Chemicals) was added at a final concentration of 0.5 μg/0.2 ml per well. Lipopolysaccharide (LPS) (*Escherichia coli* 055:B5, Difco Laboratories, Detroit, Mich.) was added at a final concentration of 10 μg/0.2 ml per well.

Results

The Proliferative Response to HGG and Fragments of HGG. To study the effect of different preparations and fragments of IgG on lymphocytes, normal mouse spleen cells were cultured for 4 days in the presence of HGG or HGG fragments. Fig. 1 shows that aggregated HGG stimulated the rate of DNA synthesis after 4 days of incubation. DHGG had no apparent effect at low concentrations and suppressed the background proliferation of normal spleen cells at high concentrations. Similarly, soluble HGG which contains some aggregates had no significant effect on DNA synthesis. To determine which portion of the immunoglobulin molecule was responsible for the stimulatory effect of aggregated HGG, fragments of IgG including Fab, $F(ab')_2$, and Fc were tested. As seen in Fig. 1, only the Fc region of IgG was able to trigger increased [^{3}H]thymidine incorporation. Further, the stimulation by isolated Fc fragments was more than 100-fold, or 10 times higher than the stimulation obtained with intact, aggregated IgG at optimal concentration. $F(ab')_2$ and Fab fragments, either soluble or heat-aggregated, were not stimulatory over a wide dose range.

In addition to [^{3}H]thymidine incorporation, the stimulation was also assessed microscopically. Fc stimulated cultures showed an average of 38% blast cells compared to 2% in control cultures at the peak of the response (day 5).

Kinetics of the Proliferative Response. The mitogenic response to Fc is characterized by a peak of DNA synthesis between days 4 and 5 of culture in contrast to the LPS response which shows an earlier peak between days 1 and 3 (Fig. 2). Only the time-courses at optimal Fc and LPS concentrations are shown in Fig. 2, since they were similar at all concentrations tested.

Response to Fragments of Goat and Rabbit IgG and to Aggregated Mouse Gamma Globulin (AMGG). Fig. 3 shows that the strong proliferative effect shown with human Fc can also be observed with Fc fragments from goat IgG. Rabbit Fc fragments were found to crystallize at the pH and protein concentration required for cell cultures. This may explain the complete lack of stimulation by rabbit Fc fragments (Fig. 3). AMGG was also stimulatory for mouse spleen cells and produced an eightfold stimulation with 10 μg of AMGG per culture.

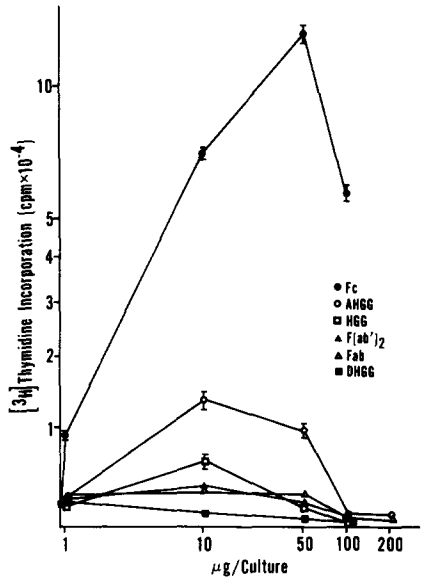

FIG. 1. Stimulation of normal mouse spleen cells with HGG and fragments of HGG. Triplicate cultures were pulsed with 1 μCi of [^{3}H]thymidine after 4 days of incubation and harvested 18 h later. HGG (□), AHGG (○), DHGG (■), Fc (●), F(ab')$_2$ (▲), and Fab (△) were prepared as described in Materials and Methods.

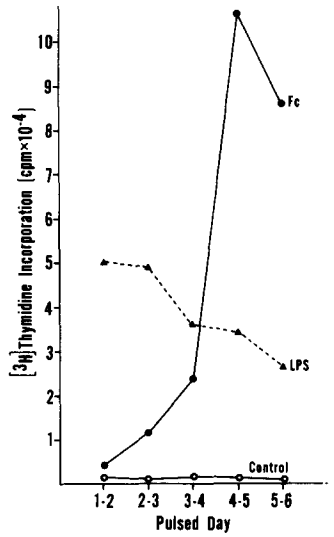

FIG. 2. Kinetics of the proliferative response induced by Fc fragments. Cultures were pulsed with 1 μCi of [^{3}H]thymidine for 18 h after 1, 2, 3, 4, and 5 days of incubation. Spleen cells cultured alone (○ – ○), with 10 μg Fc (from human IgG)/culture (● – ●), or with 10 μg LPS/culture (▲ – ▲).

Sucrose Density Gradient Centrifugation. Evidence for association of the mitogenic effect with Fc, a protein with a sedimentation rate of 3.5S, rather than with contaminating LPS of high sedimentation rate was sought. Fc fragment preparations were submitted to sucrose density centrifugation and differ-

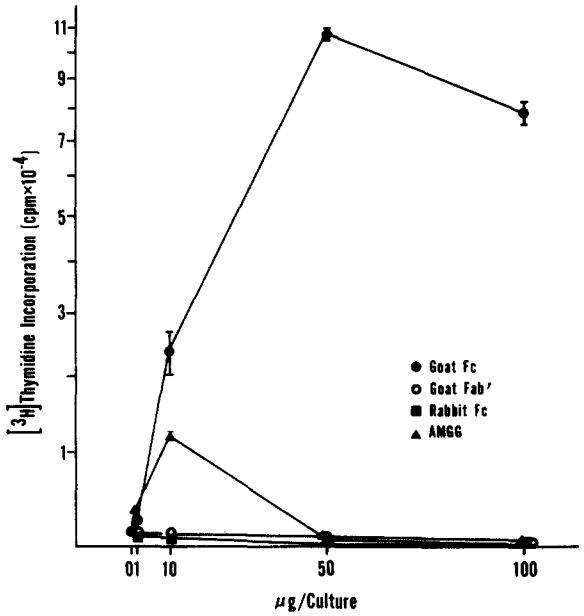

FIG. 3. Stimulation of DNA synthesis in spleen cells with Fc (●—●) and Fab (○—○) fragments of goat IgG, rabbit Fc (■—■), and AMGG (▲—▲).

ent pools were tested for their capacity to induce proliferation in mouse spleen cells. As seen in Fig. 4, the maximal activity was found in pool 5 which had the highest protein concentration, migrating between 3 and 5S. On Ouchterlony analysis it gave a single precipitin line against goat anti-human serum which fused with the precipitin line obtained with goat anti-human Fc.

Role of Serum. Hartmann and Bokisch (36) have demonstrated stimulation of DNA synthesis and blast transformation in mouse lymphocyte cultures by isolated human C3b. The stimulation by Fc fragments described here, follows the same kinetics as reported for C3b (36). Therefore, the possibility that Fc fragments may mediate their stimulatory effect through complement binding was investigated. The findings shown in Fig. 5 do not support a major role for complement in this system, since heat-inactivated mouse serum was as effective as fresh serum in supporting the stimulation. In the absence of serum, the cpm of stimulated as well as control cultures were lower than in the presence of 0.5% mouse serum. Nevertheless, highly significant stimulation (46-fold) was obtained in serum-free cultures (Fig. 5).

Nature of the Spleen Cells Responding to Fc Fragments

(*a*) SPLENIC T AND B LYMPHOCYTES. Several approaches were used to define the population of Fc responsive spleen cells. As shown in Table I, rabbit anti-mouse thymocyte serum and complement pretreatment of spleen cells which inhibited the Con A response by 90%, had no effect on the response to Fc fragments. This, together with the results in Table II showing congenitally athymic mice (nu/nu) which do not possess any detectable Con A responsive T cells, but which respond well to Fc fragments, suggests that non-T cells, most likely B cells, are activated by Fc. While the results indicate that T cells are not

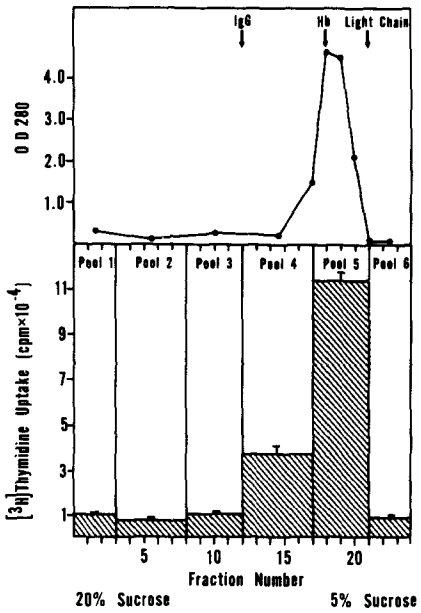

FIG. 4. Sucrose density gradient centrifugation of Fc preparation (from HGG). The protein concentration of pool 5 was adjusted to 10 μg/0.1 ml for addition to cultures. The same dilution as for pool 5 was performed on each pool. Triplicate cultures were pulsed with [^{3}H]thymidine for 18 h after 4 days of incubation (hatched bars). ● – ● Fc protein concentration.

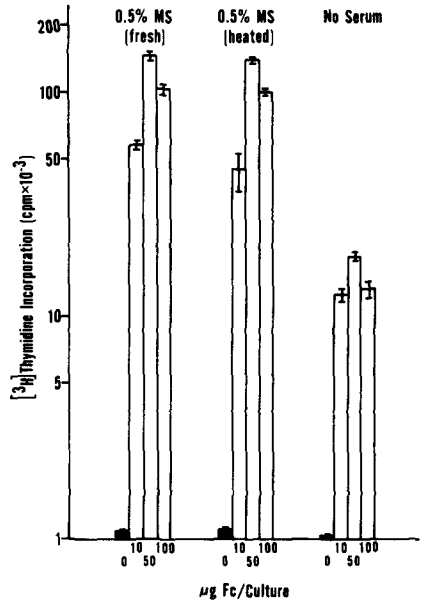

FIG. 5. Effect of serum source on Fc induced proliferation. Control cultures (■) and Fc stimulated spleen cell cultures (□) were pulsed with [^{3}H]thymidine for 18 h after 4 days of incubation. Mouse serum was used fresh (prepared from mouse blood obtained by cardiac puncture), or heat-inactivated at 56°C for 30 min.

TABLE I
Effect of Rabbit ATS and C Pretreatment on the Response to Fc Fragments

	cpm[^{3}H]thymidine uptake (± SE)				
	Day 2-3			Day 4-5	
	–	Con A	LPS	–	Fc, 50 μg
NRS* and C-treated spleen	1,508 (± 251)	107,673 (± 10,528)	56,905 (± 1,670)	2,163 (± 46)	122,974 (± 929)
ATS and C-treated spleen	3,429 (± 232)	13,742 (± 400)	37,777 (± 666)	4,765 (± 532)	124,060 (± 3,334)

* NRS, normal rabbit serum.

TABLE II
Response of Spleen Cells from Nude Mice to Fc Fragments

	cpm[^{3}H]thymidine uptake (± SE)				
	Day 2-3			Day 3-4	
	–	Con A, 0.5 μg	LPS, 10 μg	–	Fc, 10 μg
BALB/c nu/+	957 (± 101)	154,997 (± 4,402)	35,707 (± 804)	1,010 (± 95)	35,254 (± 1,083)
BALB/c nu/nu	2,348 (± 72)	2,815 (± 525)	36,025 (± 505)	1,588 (± 114)	38,610 (± 1,796)

required for the proliferative response to Fc, they do not rule out the presence of Fc responsive cells among splenic T cells. To test the proliferative response of T cells, spleens were depleted of B cells by two different methods: nylon wool column fractionation and rosetting with anti-mouse Ig coated SRBC.

Spleen cells which do not adhere to nylon wool have been shown to be highly enriched for T cells (32). In the experiment shown in Table III, the nylon nonadherent spleen cells were 90% T cells as determined by cytotoxicity after treatment of the cells with rabbit anti-mouse thymocyte serum and complement. This T-cell-enriched population responded well to Con A, but poorly to LPS and to Fc fragments (Table III). Fc responsive spleen cells are therefore nylon wool adherent or dependent on an adherent cell type.

Since nylon wool columns also retain a portion of the T cells which are loaded, they may retain Fc responsive T cells. Therefore, an alternative approach, the selective removal of surface Ig-positive spleen cells, was applied. This was achieved by allowing Ig-bearing spleen cells to form rosettes with SRBC coated with $F(ab')_2$ fragments of goat anti-mouse immunoglobulin. The rosettes could then be removed by Ficoll-Isopaque gradient centrifugation. The cells remaining at the Ficoll interface were found to be more than 95% depleted of Ig-positive cells upon rerosetting with the same reagent. Their capacity to respond to the B-cell mitogen LPS was highly reduced (93% inhibition, Table IV) while their response to the T-cell mitogen Con A was only slightly (11% inhibition in the experiment shown in Table IV) or not significantly affected. The response to Fc

TABLE III
Effect of Nylon Wool Column Separation of Spleen Cells on the Response to Fc Fragments

	cpm[^{3}H]thymidine uptake (± SE)					
	Day 2-3				Day 4-5	
	–	Con A	LPS	Fc, 10 μg	–	Fc, 10 μg
Unseparated spleen cells	1,001 (± 54)	132,072 (± 1,867)	21,394 (± 1,423)	6,018 (± 518)	2,032 (± 87)	46,027 (± 1,634)
Nylon wool nonadherent spleen cells	280 (± 10)	149,589 (± 2,046)	1,104 (± 126)	921 (± 31)	771 (± 17)	1,514 (± 46)

TABLE IV
Effect of Removal of Ig$^+$ Spleen Cells on the Proliferative Response to Fc Fragments

	cpm[^{3}H]thymidine uptake (± SE)					
	Day 2-3				Day 4-5	
	–	Con A	LPS	Fc, 50 μg	–	Fc, 50 μg
Control spleen cells*	1,740 (± 48)	142,538 (± 3,006)	37,423 (± 2,584)	14,343 (± 452)	1,852 (± 73)	118,920 (± 7,093)
Ig$^-$ spleen cells‡	745 (± 83)	125,886 (± 5,218)	3,299 (± 176)	1,438 (± 52)	2,073 (± 77)	5,276 (± 160)

* Ficoll-Isopaque-purified spleen cells.
‡ Ficoll-Isopaque-purified spleen cells after removel of Ig$^+$ spleen cells by rosetting with anti-mouse Ig coated SRBC.

fragments was reduced by more than 95% after removal of Ig$^+$ spleen cells (Table IV). This effect is most evident at the peak of the proliferative response to Fc, on day 5 of culture.

(*b*) MACROPHAGES. The term macrophage is used here for plastic adherent spleen cells. Since macrophages possess Fc receptors (37), it was of interest to examine their role in the response to Fc fragments. With the culture technique for the present study, by using medium supplemented with 2-mercaptoethanol, depletion of plastic adherent cells had no significant effect on the mitogenic response to Fc and LPS (Table V). Although the adherent cell-enriched fraction showed higher [^{3}H]thymidine incorporation than the macrophage-depleted fraction, the ratio of experimental cpm over control cpm did not significantly differ in the two groups. This suggests that plastic adherent cells do not play a major role in the response to Fc. The need for a small number of adherent cells can, however, not be excluded, since the depletion is never complete.

(*c*) Fc RECEPTOR-BEARING SPLEEN CELLS. Spleen cells were segregated into Fc receptor-positive (FcR+) lymphocytes and Fc receptor-negative (FcR−) lymphocytes by using ox erythrocytes coated with rabbit IgG anti-ox RBC as described in the Materials and Methods.

In the FcR− spleen cell fraction the response to Fc was reduced by 84% (Fig. 6). The total activity of unseparated spleen cells was recovered in the FcR+ fraction (Fig. 6).

Antibody Production in Spleen Cell Cultures Stimulated by Fc Fragments. The proliferation which was observed upon stimulation with Fc fragments could represent the clonal expansion of HGG-specific antibody forming cell

TABLE V
Effect of Macrophage Depletion on the Response to Fc Fragments

	cpm[^{3}H]thymidine incorporation (± SE)					
	Day 2-3			Day 4-5		
	Control	LPS, 10 μg	Ratio*	Control	Fc, 10 μg	Ratio
Unseparated spleen cells	1,283 (± 116)	35,182 (± 2,470)	27.4	1,271 (± 94)	41,061 (± 4,525)	32.3
Plastic adherent spleen cells	680 (± 86)	71,354 (± 3,272)	104.9	1,388 (± 46)	53,041 (± 956)	38.2
Nonadherent spleen cells	217 (± 9)	33,045 (± 1,565)	152.3	826 (± 169)	39,266 (± 4,620)	47.5

* Ratio = experimental (+ LPS or Fc) cpm/control (+ medium) cpm.

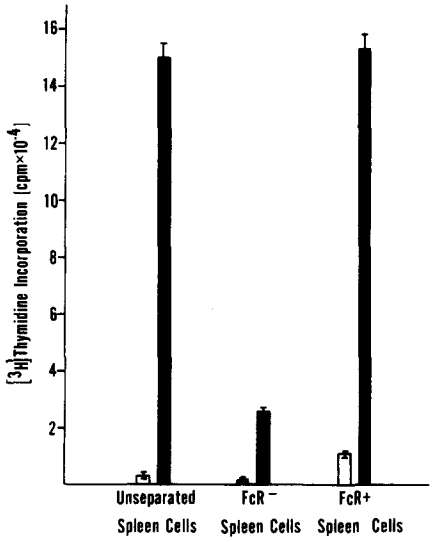

FIG. 6. Proliferative response to human Fc fragments by Fc receptor-bearing spleen cells (FcR+) and Fc receptor negative spleen cells (FcR−). Unseparated spleen cells represent Ficoll-Isopaque purified, but not rosetted spleen cells. Spleen cells cultured alone (□) and in the presence of 10 μg Fc per culture (■) were pulsed with [^{3}H]thymidine on day 4 of culture and harvested 18 h later.
The Con A response of the FcR− spleen cells was intact (304,757 ± 11,467 cpm vs. 287,210 ± 346 cpm of unseparated controls). The response to LPS was reduced from 64,513 ± 1,326 cpm to 14,089 ± 336 cpm.

precursors. Therefore, the primary antibody response to the antigens expressed in the Fc portion of HGG was studied. For this purpose, spleen cells were cultured in the presence or absence of Fc fragments for 5 days and subsequently assayed for PFC against HGG coupled to GRBC. These HGG-GRBC served as effective target cells in the same experiment by using cells from HGG-, Fab-, and Fc-primed animals (not shown). Results of two representative experiments with normal spleen cells are shown in Table VI. No direct or indirect PFC-specific for HGG were detected.

TABLE VI
Antibody Response Induced by Fc Fragments

	Direct PFC/10^7 cells cultured			Indirect PFC/10^7 anti-HGG
	anti-HGG*	anti-GRBC	anti-TNP‡	
Exp 1				
Normal spleen cells control	<1	<1	–	<1
Normal spleen cells + 50 μg Fc/ml	<1	153	–	<1
Exp 2				
Normal spleen cells control	<1	1	<1	<1
Normal spleen cells + 150 μg Fc/ml	<1	260	204	<1

* Plaques against unconjugated GRBC were subtracted from the number of plaques obtained against HGG-conjugated GRBC.
‡ The anti-TNP response was determined against BRBC heavily conjugated with TNP. No plaques to BRBC were detectable.

TABLE VII
Proliferative Response to Fc Fragments by HGG Tolerant Spleen Cells

	cpm[^{3}H]thymidine uptake (± SE) Day 4–5		Ratio*
	Control	Fc, 10 μg	
Normal spleen cells in normal mouse serum	2,056 (± 137)	155,769 (± 3,764)	75.8
Tolerant spleen cells in normal mouse serum	2,022 (± 10)	145,696 (± 2,958)	72.1
Tolerant spleen cells in tolerant mouse serum	1,958 (± 171)	136,580 (± 1,779)	69.8

* Ratio = experimental cpm/control cpm.

Polyclonal Antibody Synthesis. As seen in Table VI, a marked increase in the number of PFC against GRBC and TNP-BRBC was observed in spleen cell cultures stimulated with Fc fragments. This polyclonal activation was not detectable on days 2 and 3 of culture, and was optimal on day 5.

Proliferative Response to Fc Fragments by HGG Tolerant Spleen Cells. Further evidence against an HGG-specific response is provided by the experiment shown in Table VII. It has previously been shown that A/J mice tolerized with 2.5 mg of DHGG remain specifically unresponsive to in vivo challenge with AHGG for several months (24). From adoptive transfer experiments it is known that tolerance to HGG exists at both the T- and B-cell level (38). The mice for the present study were used 10 days after tolerance induction, a time when both B cells and T cells are unresponsive to HGG. The presence of tolerance was established in other mice from the same group by subsequent challenge with aggregated human gamma globulin (AHGG). As shown in Table VII, HGG tolerant spleen cells were able to respond to Fc fragments as well as normal cells. This implies that the proliferative response to Fc is not dependent on HGG-specific PFC precursors.

Discussion

These studies show that AHGG as well as Fc fragments of the same immunoglobulin preparation are able to induce proliferation of murine spleen cells in

vitro as assessed by [^{3}H]thymidine incorporation and enumeration of blast cells in the cultures. The activity of the isolated Fc fragment is much higher (at least 10-fold) than the activity of aggregates containing the parent IgG molecule. This higher activity on a weight basis of Fc fragments as compared to AHGG could well be related to the number of Fc sites available, since it is likely that fewer sites are exposed in aggregated IgG. Thus, the concentration of AHGG required for optimal stimulation by its Fc sites may not be reached before the high amounts of protein added create adverse culture conditions.

The finding that Fab fragments or $F(ab')_2$ fragments, either soluble or heat aggregated, were unable to induce a response, emphasizes the requirement for the Fc region as a trigger of proliferation. In addition, some change, probably structural, has to occur in the Fc part of the molecule since unaggregated IgG is inactive. In this regard, similar requirements exist for binding of IgG to Fc receptors. Many studies suggest that to activate stable Fc binding, some change either by aggregation or complexing of antibody with antigen is necessary (39–42). Such a conformational change in the Fc portion of the IgG molecule seems to occur upon binding of antigen to the Fab portion of IgG (43). Some direct evidence for a physical change has also been presented (44, 45). Perhaps the modification after the interaction of IgG antibody with antigen, as well as the alteration of IgG by heat aggregation and the splitting of Fc from the parent molecule by papain digestion, all expose a site in the Fc portion that is masked in the intact molecule. By analogy to the change required for more avid binding to plasma membrane components, it could be postulated that a similar change is required to induce cell proliferation. However, the two events are not necessarily dependent on the same molecular structure.

Immune complexes and aggregated IgG are also known to bind and activate complement components (46), and Fc fragments have the property to bind at least C1q (47). Since an analogy between the structural requirements for binding of complement and for mitogenic stimulation by Fc may exist, and also because stimulation of DNA synthesis in mouse spleen cells by soluble human C3b has been reported (36), it was important to define the role of complement in Fc-dependent mitogenesis. Our results suggest that complement does not play a major role, since the stimulation by Fc does not require the addition of fresh serum as a source of complement components. It is, however, not possible to completely rule out a role of complement, since some complement components are heat stable or could be synthesized by lymphoid cells in the serum-free cultures.

A further problem, the possible contamination of the Fc preparation with LPS, was considered. Many facts argue against a contamination with LPS being responsible for the mitogenic effect of Fc. The DHGG, HGG, Fab, and $F(ab')_2$ fractions prepared from the same lot of HGG and in the case of Fab and $F(ab')_2$, after filtration, dialysis, and concentration similar to the treatment of Fc fragments, were devoid of stimulatory activity. It is therefore highly unlikely that only Fc preparations contained LPS. Furthermore, when the Fc preparation was run through a sucrose density gradient, the peak of activity was associated with the Fc peak, where LPS would have migrated to the bottom of the gradient, or where binding of LPS to Fc would have changed the sedimentation rate of Fc.

Further, there is a clear difference between the time-course of the in vitro response to Fc and the typical kinetics of a mitogenic response to LPS or Con A. It is not known why the strong mitogenic response to Fc takes 4–5 days to reach peak proliferation, while the peak response to Con A and LPS can be observed after 1–2 days of culture. It could reflect the presence of only a small population of Fc fragment responsive cells in the spleen which takes longer to expand. Alternatively, the stimulation may depend on further processing of the Fc fragment in culture.

All the criteria used in this study for the identification of different lymphoid populations in the mouse spleen suggest that Fc responsive cells are among the surface Ig-positive B-lymphocyte population. Although Fc receptors have also been demonstrated on a T-cell subpopulation of murine spleen (1), all attempts to stimulate normal splenic T cells remaining after removal of B cells showed that they were not activated by Fc fragments. The small stimulation (twofold) observed in the T-cell-enriched fraction of nylon wool filtered and anti-Ig rosetted spleen cells cannot be attributed to T cells, since these fractions are not completely free of B cells as indicated by their weak response to LPS. The affinity of Fc fragments for T cells may be too weak to trigger proliferation. Alternatively, Fc could trigger a different function in T cells which may not be manifested by increased DNA synthesis.

The strong stimulatory property is not restricted to human Fc since goat Fc displays the same effect. Such lack of species specificity is not surprising since it has also been reported for the binding of aggregated IgG to lymphoid cells bearing Fc receptors (1). This effect is further not restricted to heterologous aggregated IgG since AMGG also induced proliferation of autologous spleen cells. The blastogenesis thus seems not related to a clonal expansion of Fc-specific antibody-forming cell precursors. The failure to detect specific PFC against HGG after 4–7 days of stimulation with Fc confirms this point. Moreover, cells from mice tolerant to HGG at both the T- and B-cell level were stimulated to the same extent as normal spleen cells.

In contrast, Fc stimulation results in polyclonal activation as indicated by the increased number of PFC against GRBC and TNP. It therefore appears that the Fc portion of IgG can act like B-cell mitogens and polyclonal activators.

The molecular events in the triggering of proliferation by Fc fragments have to be defined. An obvious hypothesis is that Fc delivers its signal by binding and cross-linking surface Fc receptors on lymphocytes. Alternatively, the mitogenic signal of the modified Fc may not depend on an interaction with the Fc receptor. A direct interaction of Fc fragments with the Fc receptor has not been demonstrated in this study, and, although the data suggest that the response is a function of Fc receptor-bearing lymphocytes, the relationship between Fc binding and cell activation remains to be studied. Our in vitro observation may represent a model for the induction and amplification of the antibody response. Thus, it may mimic a signal that is delivered to the cell through an allosteric change in the Fc portion of the surface immunoglobulin upon binding of specific antigen. Further, the mitogenic potential of Fc may provide the postulated second signal (48) required to trigger antigen-specific B cells, the first signal being provided by antigen binding and the second signal by an interaction of antibody with the antigen and the cell's Fc receptor. This would trigger clonal

expansion of antigen-specific B lymphocytes and thereby enhance the antibody response.

Summary

Strong stimulation of DNA synthesis (up to 150-fold) and blast transformation can be induced in mouse spleen cells by Fc fragments of human IgG. The mitogenic response is optimal on day 5 of culture and is dependent on the concentration of Fc fragments with a sedimentation rate of 3–5S. Intact IgG is also stimulatory, but only when modified by heat aggregation, and produces only a 10-fold increase in [^{3}H]thymidine uptake. The stimulation by aggregated IgG is dependent on the Fc portion, since aggregated (or soluble) Fab or F(ab')$_2$ fragments are inactive.

The results show that the response is T-cell independent and that it is a function of nylon wool adherent, surface Ig-positive, Fc receptor-bearing B lymphocytes.

Fc fragments do not induce plaque-forming cells to human IgG in normal mouse spleen cell cultures, but rather trigger polyclonal antibody synthesis (anti-goat erythrocytes, anti-2,4,6-trinitrophenyl). It is postulated that the Fc region of antibodies plays a role in the regulation of the humoral immune response by triggering clonal expansion of B lymphocytes.

Note Added in Proof: Recently we have been able to prepare Fc fragments from mouse IgG by using a slight modification (2-h papain digestion) of the method described for human Fc fragments. The mouse Fc fragments were as stimulatory as human Fc fragments.

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