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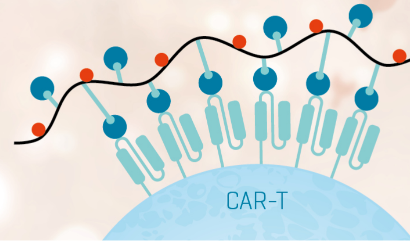

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J Immunol (1980) 125 (1): 226–231.
https://doi.org/10.4049/jimmunol.125.1.226

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0022-1767/80/1251-0226$02.00/0
THE JOURNAL OF IMMUNOLOGY
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Vol. 125, No. 1, July 1980
Printed in U.S.A.

AGGREGATED HUMAN γ-GLOBULIN-INDUCED PROLIFERATION AND POLYCLONAL ACTIVATION OF MURINE B LYMPHOCYTES[1]

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Murine splenic B lymphocytes are stimulated to proliferate and undergo polyclonal activation in the presence of heat-aggregated human γ-globulin (AHGG). Splenic macrophages are required to generate the proliferative and polyclonal antibody responses. The polyclonal response as opposed to the proliferative response is dependent upon the presence of T lymphocytes. The stimulatory molecule responsible for the AHGG-induced proliferative and polyclonal activation is derived from incubation of AHGG with splenic macrophages. The biologically active moiety is derived from the Fc portion of the HGG molecule and is approximately 14,000 m.w. The method of aggregation is critical for the generation of stimulatory HGG preparations. Heat aggregation is the only method that produced a biologically active preparation.

The Fc portion of antibody, whether in the form of Fc fragments (1-6) or an antigen-antibody complex (1, 2, 7-17), has been shown to be important in both *in vivo* and *in vitro* activation and/or regulation of bone marrow-derived (B) lymphocyte responses. The regulatory phenomena of immune complexes do not appear to be activated by intact native immunoglobulin (Ig), isolated Fab fragments, or $F(ab')_2$ fragments.

Furthermore, murine splenic bone marrow-derived (B) cells are induced to proliferate when exposed to Fc fragments derived from papain-digested human Ig (1-6). Further study of this phenomenon has revealed that the activation of B cells by Fc fragments occurs through a 14,000 m.w. cleavage product, termed Fc subfragment (5). This Fc subfragment is produced by incubation of Fc fragments with splenic macrophages. Moreover, the Fc fragment and subfragment are capable, in the presence of a thymus-derived (T) cell signal, of inducing murine B cells to make antibody polyclonally (6).

Immune complexes and aggregated Ig are known to have a wide variety of biologic properties (18) that include the ability to induce mouse B cells to proliferate (1, 2). The purpose of this study was to investigate whether aggregated human γ-globulin (AHGG)[4] was capable of stimulating a polyclonal antibody response, whether the proliferative and polyclonal responses occur via a stimulatory molecule similar to that which is responsible for the Fc fragment-induced response, and to determine the requirement for T cells. Evidence is presented to show that AHGG induces a T cell-dependent polyclonal response, and both the proliferative and polyclonal antibody responses occur through a 14,000 m.w. molecule derived from the Fc portion of the aggregated Ig.

MATERIALS AND METHODS

Animals. Male mice of the inbred C57BL/6St strain were obtained from L. C. Strong Laboratories (Del Mar, Calif.). Inbred C57BL/6 nude mice (N_4F_4) were obtained from the Scripps Clinic and Research Foundation breeding colony. All mice were between 8 and 10 weeks of age. New Zealand White rabbits were purchased from Rancho Conejo (Vista, Calif.).

Preparation of AHGG. Pooled IgG was obtained as Cohn fraction II through the courtesy of the American Red Cross National Foundation Center with the partial support of National Institutes of Health Grant HE-138801 and was purified by DEAE cellulose chromatography with 0.01 M phosphate buffer, pH 8.0, used as the eluent.

Heat. Human γ-globulin (HGG) was heat aggregated as described previously (19). The HGG was adjusted to a concentration of 20 mg/ml in 0.01 M phosphate buffer, pH 8.0, and maintained at a temperature of 63°C for 25 min. The heated material was then left at 4°C for 24 hr before collection of the AHGG. The aggregates were precipitated twice with 0.62 M sodium sulfate and dialyzed extensively against phosphate-buffered saline (PBS), 0.001 M phosphate, pH 7.2, 0.15 M NaCl before use.

Organic Solvents. Aggregation of HGG with organic solvents was done by the method of Waldesbuhl *et al.* (20). DEAE-purified HGG was brought to a concentration of 20 mg/ml in 0.15 M saline and was mixed with an equal volume of ethanol, acetone, or dimethylsulfoxide (DMSO) (J. T. Baker Chemical Co., Phillipsburg, N. J.). The solutions were maintained at 4°C for 1 hr before the collection of the precipitates. Treatment of HGG with the organic solvents resulted in a visible white precipitate. The precipitates were washed and dialyzed extensively against PBS before use.

Preparation of Fc fragments. Fc fragments were obtained by a 5-hr digestion of HGG with papain (Sigma Chemical Co., St.

Received for publication January 18, 1980.
Accepted for publication March 24, 1980.
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[1] This is Publication No. 2033 from the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California. This work was supported in part by the United States Public Health Service Grants AI-07007 and AI/CA-15761, American Cancer Society Grant IM-42I, and Biomedical Research Support Program Grant RRO-5514.

[2] Recipient of Damon Runyon-Walter Winchell Cancer Fund Postdoctoral Fellowship DRG-239-F, and United States Public Health Service Postdoctoral Fellowship AI-05813.

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[4] Abbreviations used in this paper: HGG, human γ-globulin; AHGG, aggregated human γ-globulin; B cell, bone marrow-derived lymphocyte; DMSO, dimethyl sulfoxide; 2-ME, 2-mercaptoethanol; EDTA, ethylenediaminetetraacetic acid.

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Louis, Mo.) in the presence of L-cysteine (Sigma) and ethylenediaminetetraacetic acid (EDTA) (J. T. Baker Chemical Co.) (21). After digestion, the material was chromatographed on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N. J.) to remove any undigested IgG. The Fc and Fab fragments were then separated from each other by DEAE chromatography (22).

Preparation of adherent cell supernatant. Stimulatory adherent cell supernatant was prepared as previously described (5). A single cell suspension was prepared by teasing apart the spleens into cold PBS. The suspension was allowed to stand for 5 min on ice to allow clumps to settle out. The cells were washed twice and resuspended to a concentration of 2×10^7/ml in RPMI 1640 (Flow Laboratories, Rockville, Md.), supplemented with 2 mM L-glutamine, 1% BME vitamins (Grand Island Biological Co., Grand Island, N. Y.), 100 units penicillin, and 100 μg streptomycin (Microbiological Associates, Bethesda, Md.), 5×10^{-5} M 2-mercaptoethanol (2-ME) and 5% fetal calf serum (FCS) (GIBCO). One-milliliter samples were allowed to adhere to tissue culture dishes (3001, Falcon Plastics, Oxnard, Calif.) for 1 hr in an atmosphere of 5% CO_2 at 37°C. The cells were washed extensively with RPMI 1640 to remove nonadherent cells before the addition of AHGG. AHGG (1.5 mg) in 2 ml RPMI 1640 supplemented with 2 mM L-glutamine, 1% vitamin, 100 units penicillin, 100 μg streptomycin, 5×10^{-5} M 2-ME, and 0.5% fresh normal mouse serum was added to the adherent cell layer. The supernatant material was collected after a 5-hr incubation and centrifuged at 1500 × G for 10 min in a refrigerated centrifuge and sterilized by filtration through a 0.22-μ filter before chromatographic separation.

Chromatography of the adherent cell supernatant. The adherent cell supernatant was chromatographed on a Sephadex G-50 superfine (Pharmacia) column (1.2 × 30 cm) with a bed volume of 20 ml. The material was eluted with PBS at a flow rate of 30 ml/hr, and 0.5-ml samples were collected. The column was calibrated with the following standards: blue dextran (>50,000 m.w.) (Pharmacia), deoxyribonuclease (31,000 m.w.) (Sigma), and lysozyme (14,300 m.w.) (Calbiochem-Behring Corp., La Jolla, Calif.). The m.w. of the mitogenic material was calculated from a plot of the elution volumes *vs* the logarithm of the m.w. of the standards. Mitogenic activity was found to have a m.w. of approximately 14,000.

Affinity chromatography of the mitogenic material. Agarose beads (Biogel A50m, Bio Rad Laboratories, Richmond, Calif.) were activated with cyanogen bromide, and affinity column purified anti-Fc was conjugated at a concentration of 1 to 2 mg/ml activated agarose in 0.1 M phosphate buffer, pH 6.6 (23). Anti-Fc was generated in rabbits with Fc fragments derived from the IgG1 myeloma protein and was shown to be specific for Fc by immunodiffusion. Seven to 8 ml of conjugated agarose were then placed into a 10-ml column and equilibrated with PBS, pH 7.2. The material containing the 14,000 m.w. mitogen subfragments derived from the Sephadex chromatographic separation of the adherent cell supernatant was pooled before affinity column filtration. The pooled material was filtered through the anti-Fc column, and the bound material was removed from the affinity column by elution with 0.1 M acetic acid. The eluted material was immediately neutralized to pH 7 with 1 N NaOH and dialyzed against PBS for 24 hr. The material was concentrated to the original volume by vacuum dialysis, sterilized by filtration, and stored at −70°C before use.

Depletion of macrophages by Sephadex G-10 filtration. Spleen cells were filtered through columns of Sephadex G-10 (Pharmacia) by the method of Ly and Mishell (24). Briefly, 5 × 10^7 spleen cells in 2 ml RPMI 1640 supplemented with 5% FCS were filtered through a column containing 9 ml of Sephadex G-10 that was previously equilibrated with RPMI 1640. The number of residual macrophages present was monitored by esterase staining (25). Sephadex G-10 passage reduced the esterase-positive cells from approximately 7 to 10% to <0.1%.

Spleen cell proliferation assay. The method of Berman and Weigle (1) was used for measuring spleen cell proliferation induced by Fc fragments, adherent cell supernatants, endotoxin (LPS) (*Escherichia coli* 055:B5) (Difco Laboratories, Detroit, Mich.), and concanavalin A (Miles Laboratories, Inc., Elkhart, Ind.). Cells were suspended to a concentration of 5×10^6/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 1% BME vitamins, 100 units penicillin, 100 μg streptomycin, 5×10^{-5} M 2-ME, and 0.5% fresh normal mouse serum. Duplicate cultures of 5×10^5 cells/0.2 ml were incubated in flat-bottom microtiter plates (3040 Micro Test II, Falcon) at 37°C in 5% CO_2. The cultures were pulsed with 1 μCi tritiated thymidine ([^{3}H]TdR) 5 Ci/mM (Amersham/Searle, Arlington Heights, Ill.)/0.25 ml after 2 days of incubation, unless otherwise stated, and harvested 18 hr later with an automated cell harvester (M 24 V, Brandel, Rockville, Md.). The filters were dried, placed in 3 ml scintillation fluid (OCS, Amersham/Searle), and counted in a Beckman LS-230 scintillation counter. The results are expressed as mean counts per minute (cpm) minus the background of duplicate cultures ± standard error. Each experiment was performed a minimum of three times, and the experiments are representative of all the data.

Polyclonal antibody response assay. For the generation of the polyclonal plaque-forming cell (PFC), response spleen cells were suspended to a concentration of 6×10^6/ml in RPMI 1640 supplemented as described in the proliferation assay with the addition of 7.5% FCS. Duplicate cultures of 6×10^5 cells/0.3 ml were incubated in microtiter plates (3040 Microtest II, Falcon) at 37°C in 5% CO_2. The duplicate cultures were harvested on day 3 and assayed for a response to 2,4,6-trinitrophenyl (TNP) by the slide modification of the Jerne and Nordin plaque assay (26). Heavily conjugated TNP-sheep red blood cells (TNP-SRBC) were prepared according to the method of Kettman and Dutton (27) and were used as the indicator RBC. Guinea pig serum (Pel-Freez, Rogers, Ark.) was the source of complement (C) to develop the direct or IgM plaques. Results of the plaque-forming assay are expressed as mean PFC/10^6 original cells of duplicate pools ± standard error. Each experiment was performed a minimum of three times, and the experiments shown are representative of all the data.

RESULTS

AHGG-induced proliferation and polyclonal antibody production. Fc fragments derived from mammalian Ig induced murine splenic B lymphocytes to undergo proliferation and differentiation to antibody-producing cells in the presence of T lymphocytes and macrophages (6). In addition, heat-aggregated HGG has also been shown to induce a proliferative response in murine B cell populations (1, 2), but neither the stimulatory molecule nor the nature of the response has been investigated. The results in Table I compare heat-aggregated HGG and Fc fragments derived from the same preparation of HGG for their ability to induce murine spleen cells to proliferate and produce polyclonal antibody. The heat-aggregated preparation was capable of stimulating spleen cell cultures, whereas untreated HGG was nonstimulatory (data not shown).

To determine whether the method used to aggregate the

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TABLE I

The ability of AHGG to induce proliferation and polyclonal antibody production

Stimulator	cpm ± S.E.[a]	Direct Anti-TNP PFC/ 10^6 Cultured Cells ± S.E.[a]
	2,792 ± 385	6 ± 2
AHGG[b]	31,002 ± 1,939	116 ± 8
Fc fragments[c]	45,555 ± 1,401	143 ± 6

[a] Proliferation and polyclonal antibody production were measured on day 3 of culture.

[b] 100 μg/culture heat AHGG.

[c] 100 μg/culture Fc fragments from HGG.

HGG was critical for the production of a stimulatory preparation, various techniques for aggregation were compared. Heating HGG at 63°C for 30 min resulted in an aggregated preparation that was very stimulatory when compared with untreated HGG (Table II). In contrast, aggregation by the organic solvents, ethanol, acetone, and DMSO resulted in nonstimulatory HGG preparations (Table II). Hereafter, all references to AHGG will refer to the heat-aggregated HGG preparations. Moreover, organic solvent-treated HGG failed to block the AHGG-induced response (data not shown).

To ascertain the optimal concentration of AHGG needed for the generation of both the proliferative and polyclonal antibody responses, increasing amounts of AHGG were added to murine spleen cell cultures, and the response was measured on day 3 of culture. The optimal concentration of AHGG for both proliferation and polyclonal antibody production was 100 μg/culture (Fig. 1). When concentrations of AHGG greater than 100 μg/culture were employed, suppression of both the proliferative and polyclonal responses occurred. To determine the optimal day for assaying the response to AHGG, proliferative response was found to peak on day 2 to 3 of culture and then decline rapidly on day 4 (Fig. 2). Similarly, the polyclonal antibody response peaked on day 3 of culture (Fig. 2).

Characterization of the cell requirement for the proliferative and polyclonal antibody response. Macrophages have been shown to be essential for the Fc fragment-induced proliferative and polyclonal antibody response (5, 6). Spleen cell preparations were depleted of macrophages by filtration through columns of Sephadex G-10 and then assayed for their ability to proliferate and produce polyclonal antibody to AHGG. The results indicate that both the proliferative and polyclonal responses have a mandatory requirement for adherent cells[5] (Table III). Depletion of macrophages resulted in a dramatic reduction in the proliferative (2476 cpm *vs* 55,713 cpm) as well as the polyclonal (34 PFC *vs* 104 PFC) responses when compared with untreated control spleen cell cultures.

To determine the need for T lymphocytes in the AHGG-induced proliferative and anti-TNP polyclonal responses, nude (nu/nu) and littermate (nu/+) mice were employed. When spleen cells from nu/nu mice were incubated with AHGG, a significant proliferative response ensued, whereas the anti-TNP polyclonal response was not significantly different from the background control response (Table IV). In contrast, when nu/+ spleen cell preparations were tested with the same concentration of AHGG, both significant proliferative and polyclonal antibody responses were observed (Table IV). These results indicate that the requirements for proliferation do not appear to be as stringent as polyclonal antibody production.

Characterization of the stimulatory molecule derived from AHGG. Recent work from this laboratory has characterized a 14,000 m.w. subfragment derived from Fc fragments as the entity responsible for Fc-induced proliferation and polyclonal activation (5, 6). Macrophage monolayers were incubated with AHGG for various periods of time, and the supernatant material was assessed for the ability to stimulate spleen cell cultures. The optimal incubation time for the generation of a maximal proliferative response (22,904 cpm) and anti-TNP response (125 PFC) was 5 hr (Table V). Spleen cell cultures receiving normal macrophage supernate did not respond above background controls. Because macrophage-depleted cultures were found to respond to Fc subfragments, but not to Fc fragments, Sephadex G-10-filtered spleen cells were used in the assay cultures. The AHGG macrophage supernatant was chromatographed on Sephadex G-50 superfine, and the fractions that induced stimulation were pooled. Both the proliferative and polyclonal activities were found to reside at a m.w. of approximately 14,000

[5] These cells have been previously characterized as splenic macrophages (5).

TABLE II

Comparison of various methods of aggregation for the generation of mitogenic AHGG

Aggregation Method	HGG[a]	cpm ± S.E.[b]	Direct Anti-TNP PFC/ 10^6 Cultured Cells ± S.E.[b]
None		2,544 ± 489	40 ± 12
None	1	2,343 ± 568	ND[c]
None	10	1,888 ± 378	44 ± 3
None	100	2,039 ± 82	53 ± 4
Heat	1	2,932 ± 282	ND
Heat	10	7,959 ± 103	ND
Heat	100	26,599 ± 293	244 ± 12
Ethanol	1	2,565 ± 312	ND
Ethanol	10	2,738 ± 203	37 ± 4
Ethanol	100	3,099 ± 628	40 ± 6
Acetone	1	3,100 ± 894	ND
Acetone	10	2,804 ± 20	36 ± 6
Acetone	100	2,731 ± 416	50 ± 2
DMSO	1	3,422 ± 63	ND
DMSO	10	2,970 ± 800	50 ± 11
DMSO	100	5,121 ± 1,500	34 ± 8

[a] μg/culture.

[b] Proliferation and polyclonal antibody production was measured on day 3 of culture.

[c] Not done.

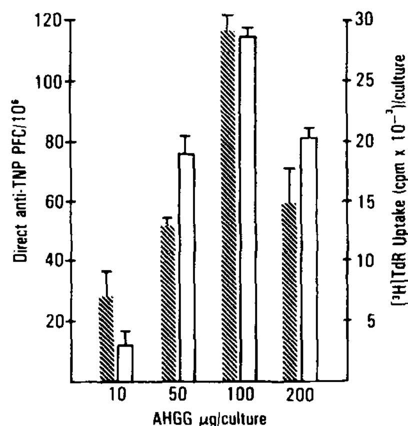

Figure 1. The polyclonal (cross-hatched bars) and proliferative (open bars) responses were measured on day 3 of culture.

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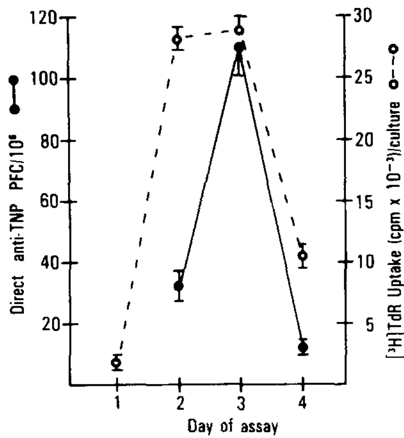

Figure 2. The polyclonal (●——●) and proliferative (O-----O) responses were measured from day 1 to day 4 of culture. AHGG was added at a concentration of 100 μg/culture.

TABLE III

The requirement for macrophages in the AHGG induced proliferative and polyclonal antibody responses

Spleen Cell Treatment	AHGG[a]	cpm ± S.E.[b]	Direct Anti-TNP PFC/10[6] Cultured Cells ± S.E.[b]
None	−	3,314 ± 1,110	3 ± 2
None	+	55,713 ± 2,273	104 ± 4
Sephadex G-10 filtered	−	2,476 ± 1,041	25 ± 5
Sephadex G-10 filtered	+	5,873 ± 752	34 ± 12

[a] 100 μg heat AHGG/culture.

[b] Proliferation and polyclonal antibody production were measured on day 3 of culture.

(data not shown). When these fractions were pooled and assayed on Sephadex G-10-filtered spleen cell populations, both a significant proliferative and polyclonal antibody response was observed (Table VI). To determine whether the 14,000 m.w. material was derived from AHGG, it was filtered through an anti-human Fc affinity column, and the effluent and eluate were assayed for the ability to induce both proliferative and polyclonal antibody responses. The anti-Fc column effluent induced a much lower response than the untreated material (Table VI). The eluate material contained the stimulatory activity indicating that the 14,000 m.w. material was derived from the Fc piece of AHGG.

DISCUSSION

Murine splenic B lymphocytes undergo proliferation and differentiation to polyclonal antibody-producing cells upon stimulation with heat-aggregated HGG. These findings are similar to the result obtained with Fc fragments (1-6).

The polyclonal antibody response as opposed to the proliferative response requires the presence of T lymphocytes. Although the exact nature of the T cell signal in the AHGG-induced polyclonal response is unclear at this time, the T cell signal in the Fc fragment-induced response appears to be responsible for the differentiation of the proliferating cells to antibody-producing cells (6) and can be substituted for by a highly purified concanavalin A-induced T cell-replacing factor.[6]

[6] Thoman, M. L., E. L. Morgan, and W. O. Weigle. Polyclonal activation of murine B lymphocytes by Fc fragments. II. Replacement of T cells by a soluble helper T cell replacing ractor (TRF). Manuscript submitted for publication.

A T cell involvement in polyclonal antibody responses has been demonstrated previously in rabbit (28), mouse (29), and human (30) models. Shinohara and Kern (28) observed that the addition of thymus cells to rabbit spleen cells resulted in an enhancement of the bacterial lipopolysaccharide- (LPS) induced polyclonal antibody response. In addition, Goodman and Weigle (29) reported that the presence of T cells boosted the murine B cell polyclonal response induced by LPS. It should be noted, however, that these systems (24, 29), as opposed to our system, do not have a mandatory requirement for T cells in the generation of the response.

The stimulatory molecule responsible for AHGG-induced proliferative and polyclonal antibody responses is derived from incubation of the AHGG with splenic macrophages. That macrophages are an essential component of the AHGG as well as the Fc fragment-induced proliferative and polyclonal responses (6) is unique, in that the responses induced by other polyclonal B cell activators appear to be macrophage independent (31-35). The mitogenic material does not appear to be a macrophage factor, because it binds to and can be eluted from an anti-Fc

TABLE IV

The differential requirement for T cells in the AHGG-induced proliferative and polyclonal antibody responses

Source of Cells	AHGG[a]	cpm ± S.E.[b]	Direct Anti-TNP PFC/10[6] ± S.E.[b]
nu/nu	−	4,065 ± 598	33 ± 6
nu/+	−	5,538 ± 445	8 ± 2
nu/nu	+	38,331 ± 354	36 ± 3
nu/+	+	37,058 ± 3,007	120 ± 6

[a] 100 μg/culture.

[b] Culture was assayed for proliferation and polyclonal antibody on day 3 of culture. The lack of T cells in nu/nu mice was monitored with Con A.

TABLE V

The capacity of AHGG stimulated macrophage supernatant to stimulate Sephadex G-10 filtered spleen cells

Stimulator[a]	cpm ± S.E.[b]	Direct anti-TNP PFC/10[6] Cultured Cells ± S.E.[b]
	2,175 ± 287	24 ± 11
Mø supernatant without AHGG	3,585 ± 101	38 ± 8
Mø supernatant with AHGG	22,904 ± 198	125 ± 3

[a] Fifty microliters of supernatant material derived from incubation of a macrophage monolayer with or without heat AHGG for 5 hr.

[b] Cultures were assayed for proliferation and polyclonal antibody production on day 3 of culture.

TABLE VI

The ability of the stimulatory material from the macrophage supernatant to bind to an anti-Fc affinity column[a]

Treatment of Stimulatory Material[b]	cpm ± S.E.	Direct Anti-TNP PFC/10[6] Cultured Cells ± S.E.
	1,597 ± 241	5 ± 2
None	26,270 ± 1,913	109 ± 3
Anti-Fc column effluent	6,338 ± 62	27 ± 11
Anti-Fc column eluate	27,477 ± 102	105 ± 4

[a] Sephadex G-10 filtered spleen cells were incubated with 50 μl of the stimulatory material and assayed for proliferation and polyclonal antibody production on day 3 of culture.

[b] The 14,000 mw pool from the Sephadex G-50 chromatographic separation of AHGG macrophage supernatant.

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affinity column. This observation is important, since biologically active factors derived from macrophages have similar m.w. (36–39). The 14,000 m.w. product derived from the incubation of macrophages with AHGG appears to be identical to the Fc subfragment derived from the interaction of Fc fragments and macrophages (5). These results suggest that stimulation of B cells by AHGG and Fc fragments occur through the same pathway. These data lend support to our hypothesis (5) that the regulation of B cell activation by Fc fragments, aggregated Ig, and antigen-antibody complexes occur through the same pathway, with the key being the Fc portion of the antibody molecule. The addition of ovalbumin mouse anti-ovalbumin complexes to murine spleen cell cultures results in a significant proliferative and polyclonal antibody response (Morgan and Weigle, manuscript in preparation). Experiments are currently in progress to ascertain whether the immune complex-induced activation of murine B cells occurs through a 14,000 m.w. subfragment of the antibody molecule.

The method of aggregation is critical for the generation of stimulatory HGG preparations. Of the methods tested, only heating produced an AHGG preparation that induced proliferation and polyclonal activation. The organic solvents used to aggregate the HGG have been shown to produce biologically active aggregates (19). Waldesbuhl *et al.* (20) found that mixing the organic solvents DMSO, ethanol, and acetone with HGG resulted in aggregates with high anti-complementary activity. The reason for the inability of the aggregated preparations to induce proliferative and polyclonal antibody responses is unclear. One possibility is that since both these responses appear to require enzymatic cleavage of AHGG, the organic solvents may aggregate the HGG in such a manner that the enzyme-binding site(s) is not exposed. Heating of the HGG probably exposes the cleavage site that is recognized by the macrophage enzyme(s), which results in the 14,000 m.w. biologically active polypeptide.

It is important to determine whether C is involved in the AHGG-induced responses, since immune complexes and AHGG as well as Fc fragments are capable of binding and activating C components (40, 41), and soluble C3b is capable of stimulating DNA synthesis in mouse spleen cells (42). Although C does not appear to play a role in the Fc fragment-induced proliferative response (1, 2), work is currently in progress to further assess the need for C components in polyclonal activation.

Acknowledgments. The authors wish to thank Nancy Kantor for excellent technical assistance, and Janet Kuhns and Mary Chalmers for secretarial expertise.

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