THE REQUIREMENT FOR ADHERENT CELLS
IN THE Fc FRAGMENT-INDUCED PROLIFERATIVE
RESPONSE OF MURINE SPLEEN CELLS*

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Mouse spleen cells can be stimulated to proliferate, undergo blast transformation,
and synthesize immunoglobulin in response to Fc fragments derived from human,
murine, and other mammalian immunoglobulins (Ig) (1–3). Proliferation is unique to
Fc, as Fab or F(ab')2 fragments are unable to induce proliferation. In studies with Fc
fragments from various classes of human myeloma proteins, IgG and IgA stimulate
best, whereas IgD was intermediate and IgM induces only a weak proliferative
response (2). In addition, it was observed that Fc fragments prepared from human
IgE do not stimulate mouse spleen cells above background. The cells stimulated to
proliferate by Fc fragments are presumed to be B cells, as they bear Ig receptors for
the Fc portion of Ig, are nylon wool nonadherent, and are not susceptible to lysis by
anti-thymus serum treatment (1, 3).

The studies in this report demonstrate that the proliferative response of mouse B
cells induced by Fc fragments occurs only when plastic adherent cells are present. In
addition, spleen cells from C3H/HeJ mice are unresponsive to Fc fragments as a
result of defect in the splenic adherent cell population.

Materials and Methods

Animals. Male mice of the inbred A/J and C3H/HeN strains were obtained from the
Scripps Clinic and Research Foundation breeding colony. C3H/HeJ mice were obtained from
The Jackson Laboratories (Bar Harbor, Maine). C3H/St mice were obtained from L. C. Strong
Laboratory (Del Mar, CA). All mice were between 8–10 weeks of age. New Zealand white
rabbits were purchased from Rancho Conejo (Vista, Calif.).

Preparation of Fc Fragments. A human IgG1 myeloma protein (Fi) was a gift from Dr. Hans
L. Spiegelberg, Scripps Clinic and Research Foundation. Fc fragments were obtained by
papain digestion of the IgG1 (Sigma Chemical Co., St. Louis, Mo.) for 5 h (4). The Fc and Fab
fragments were then separated from each other by DEAE chromatography (5, 6).

Preparation of Antisera. Antisera to IgG1 and bovine serum albumin (BSA)1 (Reheis Chemical
Co., Chicago, Ill.) were prepared by immunizing rabbits with four intramuscular injections

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‡ Recipient of Damon Runyon-Walter Winchell Cancer Fund Postdoctoral Fellowship DRG-239-F.
1 Abbreviations used in this paper: BSA, bovine serum albumin; BSS, balanced salt solution; FCS, fetal calf
serum; [3H]Tdr, tritiated thymidine.
each (5 mg/injection) of either IgG1 or BSA in incomplete Freund's adjuvant over a 2-mo period. The rabbits were exsanguinated 7–10 d after the final injection.

**Spleen Cell Proliferation Assay.** The method of Berman and Weigle (1) was used for measuring spleen cell proliferation induced by Fc fragments and endotoxin (E. coli O55:B5 (Difco Laboratories, Detroit, Mich.). Triplicate cultures of 5 × 10^6 cells/0.2 ml were incubated in flat-bottom microtiter plates (3040 Micro Test II, Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.) at 37°C in 5% CO₂. The cultures were pulsed with 1 μCi tritiated thymidine ([³H]Tdr) 5 Ci/mM (Amersham/Searle, Arlington Heights, Ill.)/0.05 ml after 2 d of incubation, unless otherwise stated, harvested 18 h later, and processed for scintillation counting. The results are expressed as mean counts per minute (cpm) minus the background of triplicate cultures ± standard error. In certain experiments spleen cells in 0.05 ml RPMI-1640 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) (Grand Island Biological Co.) were allowed to adhere to the microtiter wells for 30 min in an atmosphere of 5% CO₂ at 37°C. Before adherence, the dead cells and erythrocytes were removed by Ficoll-Isopaque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N.J.) centrifugation (7). The wells were washed extensively with RPMI-1640 to remove nonadherent cells, after which Sephadex G-10-filtered cells (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) were added to the wells.

**Removal of Adherent Cells.** Adherent cells were removed from the spleen cell population by two different methods. Spleen cells were filtered through columns of Sephadex G-10 by the method of Ly and Mishell (8). The number of residual adherent cells present was monitored by esterase staining (9). In addition, cells were depleted by adherence to plastic. 5 × 10^7 spleen cells in 1 ml of balanced salt solution (BSS) + 10% FCS were allowed to adhere to tissue culture dishes (3003, Falcon Labware, Div. of Becton, Dickinson, & Co.) for 30 min in 5% CO₂ at 37°C. The nonadherent cells were carefully removed at the end of the 30-min incubation.

**Anti-T-Cell Serum and Complement Treatment.** T-cell-depleted spleen cell populations were prepared by the use of rabbit anti-thymocyte serum (Microbiological Associates, Walkersville, Md.) as previously described (1).

**Spleen Cell Irradiation.** Spleen cells, at a concentration of 5 × 10^7/ml in phosphate-buffered saline (PBS) + 10% FCS, were exposed to 2,000 R of irradiation from Gamma Cell 40 small animal irradiator (Atomic Energy of Canada Ltd., Ottawa, Canada) delivered at 107 R/min.

**Measurement of Fc-Fragment Binding.** Fc-fragment binding was measured as described previously.² Spleen cells were suspended in BSS supplemented with 10% FCS. 0.3 ml of the cell suspension, 0.1 ml BSS, and 0.1 ml of ¹²⁵I-labeled Fc fragments, iodinated by the method of McConahey and Dixon (10) were added to a 12 × 75-mm tube (Falcon Labware). This mixture was incubated for 15 min at 37°C with occasional shaking, followed by layering the suspension onto 1 ml of FCS in a 1.5 ml polystyrene conical centrifuge tube (Brinkmann Instruments, Inc., Westbury, N.Y.). The tubes were centrifuged at 3,000 g for 5 min, the FCS was aspirated from the cell pellet, and the tip of the tube, with the cell pellet, cut off with a hot wire. The amount of ¹²⁵I bound was determined by counting the pellet in a model 1195 gamma counter (Searle Diagnostics Inc., subsid. of G. D. Searle & Co., Des Plaines, Ill.). Fab fragments did not bind above background in this assay system.

**Affinity Chromatography.** Agarose beads (Bio-gel A50m, Bio-Rad Laboratories, Richmond, Calif.) were activated with cyanogen bromide and either affinity-purified anti-IgG1 or anti-BSA were conjugated at a concentration of 1–2 mg/ml activated agarose in 0.1 M phosphate buffer, pH 6.6 (11). 10 ml of the Fc preparation was filtered through the anti-IgG1 or the anti-BSA column, the eluent collected, and concentrated to 10 ml by vacuum dialysis.

**Chromatography of Fc Fragments.** The Fc fragments were chromatographed on a Sephadex G-100 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.) column (2 × 36.5 cm) with a 152-ml bed volume. The flow rate of the column was 30 ml/h and 2-ml samples were collected. The void volume was determined with blue dextran (Pharmacia Fine Chemicals, Div. of Pharmacia Inc.).

**Assay for Endotoxin.** The limulus assay for endotoxin (12) was kindly performed by Dr. David C. Morrison, Scripps Clinic and Research Foundation.

² Morgan, E. L., Spiegelberg, and W. O. Weigle. Submitted for publication.
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TABLE I
Fc Fragment-Induced Proliferative Response of Macrophage-Depleted Mouse Spleen Cells

<table>
<thead>
<tr>
<th>Spleen cell treatment*</th>
<th>cpm ± SE‡</th>
<th>Esterase-positive cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>42,500 ± 2,062</td>
<td>11 ± 1.8</td>
</tr>
<tr>
<td>Sephadex G-10 filtered</td>
<td>7,965 ± 1,101</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Plastic nonadherent</td>
<td>40,010 ± 3,100</td>
<td>6.0 ± 1.5</td>
</tr>
</tbody>
</table>

* 5 × 10⁶ spleen cells/0.2 ml were cultured with 250 μg/ml of Fc and the response measured on day 3.
‡ The background cpm never exceeded 3,000 and were subtracted from the experimental cpm.

Fig. 1. The proliferative response of Sephadex G-10 filtered mouse spleen cells. LPS was used at a concentration of 50 μg/ml and Fc at 250 μg/ml.

Results

The Requirement for Adherent Cells in the Proliferative Response to Fc Fragments. Numerous methods have been employed for the removal of adherent cells from spleen cell populations with varying degrees of effectiveness. We compared adherence to plastic and Sephadex G-10 filtration for the ability to reduce the proliferative response to Fc fragments and endotoxin. The results show that plastic nonadherent spleen cells were capable of responding to Fc, whereas Sephadex G-10 filtered cells were not. In addition, when the two methods were compared for their ability to deplete nonspecific esterase-positive cells, Sephadex G-10 filtration was more efficient (Table I). Sephadex G-10 filtration reduced the number to <0.1%, whereas the plastic adherence procedure reduced the esterase-positive cell number to 6%. In addition, the response to endotoxin was not reduced by G-10 filtration (Fig. 1).

To ascertain whether Sephadex G-10 filtration prevented Fc proliferation through removal of adherent cells and not another cell type, we attempted to restore the response of Sephadex G-10 filtered cells by the addition of plastic adherent cells. When 5 × 10⁶ cells were allowed to adhere to the culture wells, the response was restored to approximately the level of the untreated spleen cell population (Fig. 2). The adherent cells alone did not respond significantly above the background controls. Because untreated spleen was used as the source of adherent cells and other cell types in the population have the capacity to adhere to plastic, spleen cells were exposed to 2,000 R of irradiation (Fig. 3) or treated with both anti-T cell serum and irradiation (Table II) before allowing them to adhere to plastic. The results show that irradiated T-cell depleted adherent cells were as efficient in restoring the response of Sephadex...
G-10 filtered cells as their untreated counterparts. The optimum spleen cell concentration needed for restoration of the proliferative response was $\approx 5 \times 10^5$ (Fig. 3).

**Inability of the C3H/HeJ Mouse to Respond to Fc Fragments.** It was found that both A/J and C3H/St mice responded to Fc fragments with the peak response on day 3 (Fig. 4). In contrast to these results, the C3H/HeJ strain did not respond to the Fc preparation. This observation was not a dose-dependent phenomenon because the addition of Fc in concentration of $>250$ $\mu$g/ml had no effect on the C3H/HeJ response (data not shown).

We have previously shown$^2$ that splenic lymphocytes bound $^{125}$I-labeled Fc fragments, but not Fab fragments. We therefore determined whether spleen cells from the C3H/HeJ strain bound Fc fragments as well as C3H/St and A/J strains. The results indicate that there was not a significant difference in the amount of Fc bound by spleen cells from the three strains (Fig. 5).

Because adherent cells were essential for B-cell proliferation, attempts were made to restore the response of Sephadex G-10 filtered C3H/HeJ cells with plastic adherent cells of two histocompatible strains. Fig. 6 illustrates that irradiated, T-cell-depleted adherent cells from the C3H/St strain were able to restore the proliferative response of Sephadex G-10 filtered C3H/HeJ cells (group VII) to approximately the level of the response obtained by the addition of C3H/St adherent cells to Sephadex G-10 filtered C3H/St cells (group VI). However, irradiated, T-cell-depleted C3H/HeJ adherent cells were unable to restore the responses of either Sephadex G-10 filtered
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Table II
 Restoration of the Fc-Induced Proliferative Response by Irradiated Anti-Thymus Serum-Treated Adherent Cells

<table>
<thead>
<tr>
<th></th>
<th>Spleen cells*</th>
<th>Adherent cells</th>
<th>CPM ± SE‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>--</td>
<td>--</td>
<td>30,265 ± 1,088</td>
</tr>
<tr>
<td>Sephadex G-10 filtered</td>
<td>--</td>
<td>1,978 ± 291</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-10 filtered</td>
<td>Irr.§</td>
<td>29,595 ± 717</td>
<td></td>
</tr>
<tr>
<td>Sephadex G-10 filtered</td>
<td>Irr., Anti-T + C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sephadex G-10 filtered</td>
<td>Irr., Anti-T</td>
<td>29,530 ± 338</td>
<td></td>
</tr>
<tr>
<td>--</td>
<td>Irr.</td>
<td>--</td>
<td>500</td>
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</table>

* 5 × 10⁶ spleen cells/0.2 ml were cultured with 250 µg Fc/ml and the response measured on day 3.
‡ The background cpm never exceeded 3,000 and were subtracted from the experimental cpm.
§ 5 × 10⁶ irradiated spleen cells were allowed to adhere to the culture wells for 30 min before the addition of the filtered cells.
∥ The irradiated cells were treated with anti-T cell serum + C before adhering them to the wells.

Fig. 4. Comparison of spleen cells from three strains of mice for their ability to proliferate in response to 50 µg Fc/ml ● or 100 µg Fc/ml □. C3H/HeJ or C3H/St cells (groups V and VIII). In addition, irradiated, T-cell-depleted adherent cells from C3H/HeN mice restored the response of filtered C3H/HeJ spleen cells to the level of untreated C3H/HeN cells (Fig. 7, group IV). In both series of experiments the adherent cells alone did not respond to the Fc fragments (Fig. 6 and 7) or to LPS (data not shown). These results indicate that the C3H/HeJ strain contains a defect in the adherent cell population. The number of esterase-positive cells in spleens from C3H/HeJ and C3H/St were compared to determine if the C3H/HeJ contained an abnormal number. The results show that both strains contain comparable numbers of esterase positive cells in their spleens (Table III).

Because the C3H/HeJ strain has been shown to be nonresponsive to certain preparations of endotoxin, it was important to rule out the possibility that the Fc preparation was contaminated with endotoxin. The proliferative activity of Fc was found to coincide with the Sephadex G-100 chromatographic profile of the Fc fragment (Fig. 8). In another experiment the Fc preparation was filtered through an Anti-IgG1 affinity column before being used to induce proliferation. The results indicate that the anti-IgG1 column eluent contained no proliferative activity but if a
Comparison of spleen cells from three strains of mice for their ability to bind $^{125}$I-labeled Fe fragments.

<table>
<thead>
<tr>
<th>Group</th>
<th>Untreated C3H/HeJ</th>
<th>Untreated C3H/St</th>
<th>G-10 Filtered C3H/HeJ</th>
<th>Untreated C3H/St</th>
<th>G-10 Filtered C3H/St</th>
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<tbody>
<tr>
<td>I</td>
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<td>X</td>
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</table>

Discussion

B lymphocytes from murine spleen cells proliferated in response to Fe fragments only when splenic adherent cells were present. Sephadex G-10 filtration of spleen cells before stimulating with Fe fragments resulted in a marked reduction of the proliferative response. In addition, Sephadex G-10 filtration almost totally depleted the nonspecific acid esterase-positive cell population. The proliferative response was restored by the addition of irradiated, T-cell-depleted adherent cells. Although Sephadex G-10 filtration has been shown to deplete macrophages and antibody-producing cells (8), it does not alter the splenic B- or T-lymphocyte populations (13). Adherence of the spleen cells to plastic reduced the number of esterase-positive cells by $\approx 50\%$ compared to the untreated spleen cell population. The residual adherent cells were capable of supporting the proliferative response.
The requirement for adherent cells in the proliferative response was further substantiated by the failure of adherent cells from the C3H/HeJ mouse to support such activation. The unresponsiveness of the Sephadex G-10 filtered C3H/HeJ spleen cells was corrected upon the addition of adherent cells from either C3H/St or C3H/HeN mice. The C3H strains employed in the mixing experiments previously have been shown not to react with each other in mixed lymphocyte reactions (14-16). The observation that the C3H/HeJ mouse contained an adherent cell defect is in agreement with published observations (17, 18). Hoffman et al. (17) showed that LPS enabled in vitro spleen cell culture from C3HeB/FeJ but not C3H/HeJ, mice to produce antibody to the antigen, 2,4,6-trinitrophenol-conjugated mouse erythrocytes (TNP-MRBC). This defect was found to be linked to the C3H/HeJ macrophage population. G jade et al. (18) observed that macrophages from C3H/HeJ are resistant to the toxic effect of LPS. In addition, defects have been reported in other non-B-cell populations of the C3H/HeJ mouse. Koenig et al. (19) observed that T-cell precursors of C3H/HeJ mice are unresponsive to induction of differentiation by lipopolysaccharide. It has recently been shown that mitogen-induced differentiation and polyclonal activation of B cells in man (20) and mice (21) are enhanced by T cells. Goodman and Weigle (22) observed that T cells from C3H/HeJ mice are deficient in the capacity to enhance the polyclonal B-cell response.

The mechanism by which Fc fragments stimulate B cells to proliferate is unclear. Although adherent cells are required, the cellular events involved are only speculative at this time. Macrophages have been shown to be essential for the activation of T cells by mitogens (22-24), allogenic cells (25) and antigens (23, 26). Macrophages function in these reactions by uptake and presentation of antigen to T cells (27, 28) and/or by
production of soluble factors (27, 29). In addition, macrophages influence the interaction of B and T cells. The induction of helper T cells to thymus-dependent antigens was shown to be dependent upon the macrophage (8, 30, 31).

T cells do not appear to play a role in Fc-induced proliferation, because their removal from spleen cell preparations has no effect on the proliferative response (1). Therefore, the proliferative signal could be generated from the interaction of the Fc fragments with both adherent cells and B cells or with adherent cells which results in the elaboration of a factor(s) responsible for B-cell activation. The interaction of B cells and macrophages through soluble factors has been extensively reported in the literature (17, 32-35). Peritoneal macrophages were found to enhance the mitogenic response of macrophage-depleted B cells to dextran sulfate (32). Enhancement was also seen with supernatant material from cultured macrophages. Kurland et al. (33) observed that the capacity of B cells to undergo focal proliferation and generation of clones in semisolid agar was dependent upon the presence of macrophages. Nordin (34) recently observed that the in vitro immune response of murine spleen cells to T-cell-independent antigens require the presence of macrophages or 24-h culture supernates from peritoneal macrophages. In addition, Calderon et al. (35) reported that macrophage culture fluid contains at least two distinct biological activities, one which causes the differentiation of memory B cells into antibody-producing cells and another which causes the increase or decrease of antibody production depending upon the immune status of the spleen cell population.

The mechanism(s) involved in activation of B lymphocytes by Fc fragments may be the same as those involved in stimulation of lymphocytes by immune complexes. Mouse spleen cells undergo a proliferative response in the presence of antigen-antibody complexes formed after the separate addition of antigen and antibody to the cultures (3). In addition, immune complexes have been shown to be capable of both suppressing

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**TABLE IV**

<table>
<thead>
<tr>
<th>Fc added*</th>
<th>cpm ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>31,982 ± 491</td>
</tr>
<tr>
<td>Anti-HGG†</td>
<td>4,421 ± 490</td>
</tr>
<tr>
<td>Anti-BSA§</td>
<td>25,990 ± 332</td>
</tr>
</tbody>
</table>

* 250 μg Fc/ml.
† Fc fragments were passed over an anti-HGG affinity column before use.
§ Fc fragments were passed over an anti-BSA affinity column before use.

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**Fig. 8.** Comparison of Sephadex G-100 chromatographic profiles of Fc proliferative activity and protein concentration.
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(36) and enhancing (37) immune responses. Fc fragments have a similar capacity (3). With weak responses, the addition of Fc fragments to in vitro cultures results in an enhanced response, whereas the addition of Fc fragments to strongly responding cultures results in a suppressed response. The role of adherent cells in the regulation of B-cell proliferation is currently under investigation.

Summary

The proliferative response of mouse B lymphocytes induced by Fc fragments was found to be dependent upon an adherent cell population. The adherent cell is esterase positive, irradiation resistant, and not susceptible to lysis by anti-thymus serum and complement. The mechanism(s) by which Fc fragments induce B-cell proliferation could be the result of the interaction of Fc with both B cells and adherent cells or with adherent cells which then release factors that trigger the B cells to proliferate.

Spleen cells from the C3H/HeJ mouse were shown to be unable to respond to Fc fragments. The addition of adherent cells from either C3H/St or C3H/HeN mice to adherent cell depleted C3H/HeJ cells enabled them to respond to Fc, indicating the defect was in the adherent cell population.

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References


