REGULATION OF THE IMMUNE RESPONSE

I. The Potentiation of In Vivo and In Vitro Immune Responses by Fc Fragments*

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The passive administration of antibody can result in both positive and negative signals which regulate the immune response (1-3). Regulation of the immune response by antibody is dependent on the time between the administration of passive antibody relative to antigen (2). In view of these observations, Brysryn et al. (4) proposed that production of antibody is controlled via a dynamic equilibrium among circulating antibody, antigen, and antigen-antibody complexes. Thus, one would predict that both enhancement (5) and suppression (6) of the immune response are dependent upon the ratio of antigen to antibody.

It was shown that passively administered antibody can function independently of the Fc region by directly masking antigenic determinants, thus inhibiting their presentation to lymphocytes (7, 8). On the other hand, other investigators have provided evidence that the Fc portion of the antibody molecule is essential for suppression (9-14). This controversy was resolved by Hoffmann and Kappler (14) who reported that when high concentrations of passive antibody were used, the antigenic determinants were blocked independently of the Fc region, whereas with lower concentration of antibody the Fc portion was required.

Although emphasis has been placed on the suppressive arm of passively administered antibody, enhancement has also been reported in the literature. Such enhancement has been shown with both particulate (15, 16) and soluble antigens (17-19) in mice. The direct activation of normal lymphocytes by antigen-antibody complexes have produced conflicting results. Block-Shtacher et al. (20) and Möller (21) reported that antigen-antibody complexes could stimulate proliferation in human peripheral blood lymphocytes. Stimulation of murine lymphocytes by antigen-antibody complexes has been shown to occur by some investigators (3) but not others (12, 22, 23).

Fc fragments derived from mammalian immunoglobulin (Ig) (3, 24-27) and aggregated human gamma globulin (24, 28), in addition to antigen-antibody complexes (3), are known to be potent activators of murine bone marrow-derived (B) cells...
in that they have the capacity to induce B cells to proliferate and undergo polyclonal activation.

The ability of Fc fragments to mediate a variety of biological functions prompted investigation for their ability to modulate an antibody response. The studies in this report demonstrate that Fc fragments can act as potent in vivo or in vitro adjuvants, and the conditions necessary for obtaining the enhanced responses are described.

Materials and Methods

Animals. Male mice of the C57BL/6J and A/J strains were obtained from The Jackson Laboratory (Bar Harbor, Maine). All mice were between 8 and 14 wk of age when used.

Priming for Secondary In Vitro Antibody Response. Mice were injected with 0.1 ml of 10% suspension of sheep erythrocytes (SRBC)1 (Colorado Serum Co., Denver, Colo.) intraperitoneally. 6–8 wk after priming, they were boosted intraperitoneally with the same dose of SRBC and were used 7 d later.

Tolerization. Mice were tolerized to aggregated human gamma globulin (HGG) (AHGG) as described in Doyle et al. (29). Pooled HGG was obtained as Cohn fraction II through the courtesy of the American Red Cross National Fractionation Center (with the partial support of National Institute 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. The HGG was centrifuged at 150,000 g for 150 min at 4°C in a swinging bucket sw 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The upper quarter of the solution was removed, and 2.5 mg in 0.5 ml was immediately injected intraperitoneally. 30 d posttolerization a portion of the mice were tested for tolerance by immunizing with 400 µg heat-aggregated HGG intravenously followed by another 400-µg injection 10 d later. The spleens were removed 4 d after the second injection and assayed for plaque-forming cells (PFC) to HGG. A tolerized, nonimmunized group was used for experiments involving the use of Fc as an adjuvant.

Preparation of Fc Fragments

HUMAN. A human IgG1 myeloma protein (Fi) was a gift from Dr. Hans L. Spiegelberg, Scripps Clinic and Research Foundation, La Jolla, Calif. The IgG1 was purified by ammonium sulfate fractionation followed by DEAE-cellulose chromatography with 0.01 M phosphate buffer, pH 6, used as the eluent. Fc fragments were obtained by digestion of IgG1 with papain (Sigma Chemical Co., St. Louis, Mo.) in the presence of L-cysteine (Sigma Chemical Co.) and EDTA (J. T. Baker Chemical Co., Phillipsburg, N. J.) for 5 h (30). After digestion, the material was chromatographed on Sephadex G-100 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) to remove any undigested IgG. The Fc and Fab fragments were then separated from each other by DEAE-cellulose chromatography (31).

MURINE. Mouse gamma globulin was obtained from N. L. Cappel Laboratories, Inc., Cochranville, Pa.). Fc fragments were prepared as described for HGG except a 2-h digestion was employed (24).

Generation of Immune Responses

In vivo response to SRBC. Mice, in groups of four to five, were given 0.1 ml of a SRBC suspension intraperitoneally, followed immediately by saline, Fc, Fab, or IgG intravenously. The spleens were assessed for PFC to SRBC 5 d postimmunization.

In vitro response to SRBC. Spleens were removed from primed and boosted mice, and a single cell suspension was prepared by teasing the spleens apart with forceps into phosphate-buffered saline (0.001 M sodium phosphate, 0.15 M NaCl, pH 7.4). A modified Mishell-Dutton culture system was employed for the generation of antibody-producing cells (32). Cells were suspended to a concentration of 6 × 105/ml RPMI-1640 (Flow Laboratories, Inc., Rockville, Maryland).

1 Abbreviations used in this paper: AHGG, aggregated human gamma globulin; BRBC, burro erythrocytes; DHGG, deaggregated human gamma globulin; HGG, human gamma globulin; LPS, lipopolysaccharide; PFC, plaque-forming cell(s); SRBC, sheep erythrocyte(s).
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Md.), supplemented with 2 mM L-glutamine, 1% BME vitamins (Grand Island Biological Co., Grand Island, N. Y.), 100 U penicillin, 100 μg streptomycin (Microbiological Associates, Walkersville, Md.), 5 × 10^{-5} M 2-ME, 7.5% fetal calf serum (Grand Island Biological Co.), and 0.5% fresh normal mouse serum. The spleen cells at a concentration of 6 × 10^5 along with various concentrations of SRBC and Fc, Fab, or IgG were cultured in 0.3-ml final volume in flat-bottom microtiter plates (3040 Micro Test II; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) for 4 d at 37°C in 5% CO₂. At the end of this time duplicate cultures were pooled and assessed for PFC to SRBC.

PFC Assay. PFC to HGG and SRBC were assayed by a slide modification of the Jerne plaque assay (33). For detection of PFC to HGG, burro erythrocytes (BRBC) (Burley Farms, San Diego, Calif.) were conjugated with BRBC-adsorbed Cohn fraction II HGG by using water-soluble carbodiimide (Story Chemical Co., Muskegon, Mich.) (34). Guinea pig serum (Pel-Freez Biologicals Inc., Rogers, Ark.) was used to develop the direct plaques and a combination of guinea pig serum and rabbit anti-mouse immunoglobulin to develop the indirect plaques. Only indirect PFC were determined for HGG responses because only a minimal IgM response is obtained with this antigen. The number of IgG PFC were calculated using a correction factor for inhibition of the IgM PFC by the developing antiserum. Data are expressed as PFC/10^6 spleen cells ± SE. Each experiment was performed a minimum of three times, and the experiments shown are representative of all the data.

Results

Enhancement of the Antibody Response by Fc Fragments. To study whether Fc fragments derived from human Ig had the capacity to act as an adjuvant, the antibody response to SRBC was investigated. The results in the Fig. 1 indicate Fc fragments have adjuvant properties when administered with antigen in vivo. The observed adjuvant effect is extremely dependent upon the dose of antigen employed. The greatest enhancement (~14-fold) of the anti-SRBC response occurred when a suboptimal dose of SRBC was used. Moreover, when conditions were employed where the maximum anti-SRBC response was achieved, the addition of Fc fragments produced little or no enhancement (1,225 vs. 1,395 PFC). There was no polyclonal antibody response upon injection of Fc fragments alone.

Because a dramatic increase in the in vivo anti-SRBC response was achieved with Fc fragments, experiments were conducted to determine if a similar enhancement occurred in vitro. The addition of Fc fragments with SRBC to in vitro cultures resulted in a pronounced enhancement of the IgM anti-SRBC response (Fig. 2). An eightfold enhancement of the response occurred when Fc fragments were administered with low numbers of SRBC. As observed with the in vivo response, when the maximum in vitro response was attained, there was virtually no measurable adjuvant effect (700 vs. 730 PFC).

To ensure that the enhancement of the anti-SRBC response was indeed mediated by the Fc fragments and was not a result of the addition of extraneous protein to the system, the effects of Fab fragments and intact IgG on the in vivo and in vitro responses were assessed. The intact IgG and Fab fragments derived from this myeloma protein did not increase the anti-SRBC response above that obtained with SRBC alone (Table I). On the other hand, the Fc fragments produced a significant increase in both the in vivo (approximately fivefold) and in vitro (approximately eightfold) antibody responses. All experimentation described subsequently employed 0.1 ml of 1% suspension of SRBC (~2.5 × 10^7)/mouse for in vivo work and 1 × 10^4 SRBC/culture for in vitro studies.

To determine if the addition of Fc to in vitro spleen cell cultures altered the peak
day of response to SRBC, kinetic experiments were performed. The response to SRBC was measured over a 5-d period, and the results show that the anti-SRBC response peaked on day 4 of culture regardless of the presence or absence of Fc fragments in culture (Fig. 3).

Concentration of Fc Fragments Needed for Enhancement of the Antibody Response. To determine the optimal amount of Fc fragments needed to enhance the in vivo response various doses of Fc were injected with a constant number of SRBC. The results indicate that maximal enhancement of the in vivo anti-SRBC response occurred when 0.5 mg Fc was injected (Fig. 4, group III). The minimum dose of Fc tested (0.1 mg) produced a significant adjuvant effect (12-fold), but was lower than 0.5 mg (17-fold). To ascertain the optimal concentration of Fc fragments needed to enhance the in vitro anti-SRBC response, different amounts of Fc were added with $1 \times 10^4$ SRBC to the cultures. 100 µg of Fc gave the greatest adjuvant effect (fivefold) (Fig. 5, group III) with both lower and higher amounts being approximately equal (twofold). Experimentation described subsequently employed 0.5 mg Fc/mouse and 100 µg Fc/culture.

Enhancement of the IgG Response by Fc Fragments. The above studies describe the enhancement of the secondary direct or IgM response to SRBC; experiments were similarly conducted to ascertain if the IgG response could be boosted. The results in Table II indicate that the secondary in vitro IgG anti-SRBC response is enhanced by

\[ \frac{\text{Direct Anti-SRBC PFC}}{10^6} \]

Fig. 1. Enhancement of the primary in vivo anti-SRBC response with Fc fragments. Increasing numbers of SRBC even injected with 0.5 µg Fc (m) or alone (○). There were four to five mice/group and the experiment assayed on day 5.
Fig. 2. Enhancement of the secondary in vitro anti-SRBC response with Fc fragments. Increasing numbers of SRBC were added with 100 μg (■) or alone (□) to in vitro cultures. The response was measured on day 4.

**FIG. 2.** Enhancement of the secondary in vitro anti-SRBC response with Fc fragments. Increasing numbers of SRBC were added with 100 μg (■) or alone (□) to in vitro cultures. The response was measured on day 4.

**TABLE I**

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>SRBC</th>
<th>Anti-SRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>In vivo</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>In vivo*</td>
</tr>
<tr>
<td></td>
<td>PFC/10⁶ ± SE</td>
<td>&lt;25</td>
</tr>
<tr>
<td></td>
<td>310 ± 18</td>
<td>106 ± 14</td>
</tr>
<tr>
<td></td>
<td>&lt;25</td>
<td>80 ± 2</td>
</tr>
<tr>
<td></td>
<td>1,287 ± 127</td>
<td>838 ± 70</td>
</tr>
<tr>
<td></td>
<td>430 ± 74</td>
<td>141 ± 31</td>
</tr>
<tr>
<td></td>
<td>266 ± 95</td>
<td>193 ± 41</td>
</tr>
</tbody>
</table>

* The response was measured 5 d after injection.
† The response was measured on day 4 of culture.
§ For the in vivo experiment, mice received 0.1 ml of 1% SRBC.
For the in vitro experiments, cultures received 1 × 10⁴ SRBC.
|| In vivo experiment mice received 0.5 mg. In vitro experiment cultures received 100 μg.
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Fig. 3. Kinetics of the in vitro anti-SRBC response. Spleen cells were cultured with $1 \times 10^4$ SRBC (○) $1 \times 10^4$ SRBC plus 100 μg Fc (●), or 100 μg Fc only (▲) and the response measured on day 3-5 of culture.

Fig. 4. Increasing concentrations of Fc fragments were injected with a constant number of SRBC (0.1 ml of 1%) to determine the optimal concentration of Fc needed for enhancement of the in vivo anti-SRBC response. The response was measured on day 5.

The addition of Fc fragments (fourfold). The conditions for obtaining the optimal adjuvant effect of the IgG response were the same as the IgM response (data not shown).

Effect of Fc Fragment Antigenicity on the In Vivo Adjuvant Effect. Because human Fc fragments are antigenic at the concentrations employed it was important to determine whether the observed adjuvant effect was a result of the antigenicity of the Fc molecule. Mice were rendered tolerant (97% unresponsive) (data not shown) to HGG before use in the following experiments. When the tolerant mice were injected with Fc fragments and SRBC, the anti-SRBC response was enhanced (~17-fold) compared
with the level of normal control mice (Table III, groups III and VII). As described previously, Fab fragments had no effect on the anti-SRBC response (groups IV and VIII).

To delineate further the role of the antigenicity of the Fc molecule, Fc fragments were prepared from murine Ig and injected along with SRBC in mice. The murine Fc fragments were able to enhance the in vivo anti-SRBC response (sixfold) (Table IV, group IV). Intact murine IgG or Fab fragments produced no significant adjuvant effect (Table IV, groups VI and VIII). These results indicate that homologous Fc fragments possess adjuvant properties, thus ruling out the possibility that the adjuvanticity of Fc fragments is related to antigenicity of the molecule.

The enhancement of both the in vivo and in vitro anti-SRBC responses ranged from ~4-fold to ~17-fold, and the experiments shown are representative of all the data.
TABLE III
Enhancement of an In Vivo PFC Response to SRBC in Mice Tolerant to Human Gamma Globulin

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>Fc‡</th>
<th>Fab§</th>
<th>SRBC§</th>
<th>Direct anti-SRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>DHGG</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>&lt;25</td>
</tr>
<tr>
<td>II</td>
<td>DHGG</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>&lt;25</td>
</tr>
<tr>
<td>III</td>
<td>DHGG</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>408 ± 6</td>
</tr>
<tr>
<td>IV</td>
<td>DHGG</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>&lt;25</td>
</tr>
<tr>
<td>V</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>&lt;25</td>
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<tr>
<td>VI</td>
<td>None</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>&lt;25</td>
</tr>
<tr>
<td>VII</td>
<td>None</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>371 ± 48</td>
</tr>
<tr>
<td>VIII</td>
<td>None</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>&lt;25</td>
</tr>
</tbody>
</table>

* Mice were given 2.5 mg deaggregated human gamma globulin (DHGG) 30 d previously. Mice were 97% unresponsive to HGG challenge.
‡ Mice were injected with 0.5 mg Fc intravenously.
§ Mice were injected with 0.5 mg Fab intravenously.
¶ Mice were injected with 0.1 ml of 1% SRBC intraperitoneally.

TABLE IV
Enhancement of an In Vivo PFC Response to SRBC by Murine Fc Fragments

<table>
<thead>
<tr>
<th>Group</th>
<th>Fc*</th>
<th>Fab§</th>
<th>IgG§</th>
<th>SRBC§</th>
<th>Direct anti-SRBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;25</td>
</tr>
<tr>
<td>II</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>205 ± 28</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&lt;25</td>
</tr>
<tr>
<td>IV</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>1,310 ± 281</td>
</tr>
<tr>
<td>V</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>&lt;25</td>
</tr>
<tr>
<td>VI</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>326 ± 75</td>
</tr>
<tr>
<td>VII</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>&lt;25</td>
</tr>
<tr>
<td>VIII</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>270 ± 61</td>
</tr>
</tbody>
</table>

* 0.5 mg murine Fc given intravenously.
‡ 0.5 mg murine Fab given intravenously.
§ 0.5 mg murine IgG given intravenously.
¶ 0.1 ml of 1% SRBC suspension given intraperitoneally.

Discussion

A model is described in this report for the enhancement of both in vivo and in vitro antibody responses by Fc fragments. The injection of Fc fragments along with SRBC or the addition of Fc and SRBC to spleen cell cultures results in a dramatic increase in the number of both IgM and IgG anti-SRBC PFC. The Fc fragment adjuvant effect is a result of the interaction of spleen cells with Fc and not a nonspecific stimulation by protein, as Fab fragments and intact IgG were not able to generate the heightened anti-SRBC responses.

The Fc fragment-induced adjuvant effect was found to be dependent upon the dose of antigen injected or added to the in vitro cultures. The most pronounced increase in the anti-SRBC response occurs where suboptimal amounts of antigen are employed. The adjuvant effect decreases as the concentration of antigen increases, and when the optimal amount of antigen is reached, Fc fragments fail to affect the antibody
response. That antigen concentration apparently plays a critical role in the adjuvanticity of various agents has been suggested in the literature (35, 36). Sjöberg et al. (35) observed that the adjuvanticity of bacterial lipopolysaccharide (LPS) for a primary in vitro response to SRBC occurred only when a low number of SRBC were added to culture. Moreover, Specter et al. (36) found that the synthetic product N-acetylmuramyl-l-ananyl-O-isoglutamine (muramyl dipeptide) enhanced the immune responsiveness of normal lymphocytes most dramatically when suboptimal numbers of SRBC were used. However, Amerding and Katz (37) showed that LPS enhances in vitro responses to SRBC over all antigen concentrations tested. The correlation of adjuvanticity with antigen dose is apparently dependent on a number of variables.

That the cellular events involved in the polyclonal activity and their adjuvanticity are different is suggested by the difference in the kinetics of the two responses. This is concluded from the fact that the Fc fragment induced polyclonal response peaks on day 3 of culture (27), whereas the anti-SRBC response peaks on day 4. The addition of Fc fragments to the SRBC cultures does not alter the kinetics of the response. This observation agrees with those previously published (35), which show that when SRBC are added to LPS-treated spleen cell cultures, the anti-SRBC response is higher than in those cultures receiving only SRBC, but the overall kinetics of the response remain unaltered in the presence of LPS.

Fc fragments derived from murine Ig are as potent an adjuvant as Fc prepared from heterologous Ig. This observation is very important when considering the use of such an adjuvant in the human model. An ideal adjuvant would be a substance which could potentiate the immune response but yet be nonantigenic itself. That antigenicity plays no part in the adjuvant effect is further substantiated by the fact that the anti-SRBC response of mice rendered tolerant to HGG is capable of being enhanced by human Fc fragments to the level of untreated control mice.

The nature of the regulatory role(s) of Fc fragments in the immune response is not clear at the present time. Although we have presented evidence indicating that Fc fragments can potentiate the immune response, data exist which show that Fc fragments have the capacity to suppress an antibody response as well (3). These results are reminiscent of those dealing with immune complexes, where antibody responses are enhanced (5, 15–19) as well as suppressed (6, 14). That homologous Fc fragments are capable of enhancing an antibody response lends credence to the idea that Fc fragments regulate the antibody response in a manner similar to immune complexes. Hoffmann and Kappler (14) postulated that antibody regulated the humoral immune response by affecting the cooperation between T cells and B cells by attaching antigen through its Fc fragment to the surface of cells which carry Fc receptors. Because Fc fragments affect B cells (3, 24–27), T cells (27), and macrophages (26, 27), multiple sites of regulation are possible. The nature of the regulatory event(s) and the cell population(s) affected are the foci of another report.²

Summary

Fc fragments derived from human and murine Ig were found to be potent adjuvants when administered with antigen. Both the in vivo and in vitro anti-sheep erythrocytes (SRBC) responses were significantly enhanced by Fc fragments. The adjuvant effect was shown to be extremely dependent upon the dose of antigen used, with the greatest

enhancement occurring when suboptimal doses of antigen are employed. The antigenicity of the Fc molecule was not related to its adjuvanticity because homologous Fc was as potent an adjuvant as heterologous Fc. Moreover, human Fc fragments enhanced anti-SRBC responses in mice which were tolerant to human gamma globulin.

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References