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Activation of T Lymphocytes by the Fc Portion of Immunoglobulin

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T lymphocytes are stimulated to release T-cell-replacing factors in response to Fc fragments of human IgG. Lyt $1^{+}23^{-}$ T cells are directly triggered to factor production by Fc subfragments, derived from intact Fc fragments by macrophage-dependent enzymatic cleavage. These factor(s) replace T cell function in two Fc-mediated immune responses; induction of polyclonal antibody synthesis, and potentiation of anti-SRBC responses.

Key words: **T cell factors, polyclonal antibody formation, Fc fragments, interleukin 2**

Antigen-antibody complexes, aggregated human gamma globulin, and Fc fragments derived from mammalian immunoglobulin mediate a variety of immunological functions. They are potent activators of B lymphocytes, stimulating proliferation and polyclonal antibody formation, and modulate specific antibody responses both in vivo and in vitro [1–7]. Both macrophages and T lymphocytes are necessary for the Fc-induced polyclonal antibody response [3, 4]. Macrophages process the Fc fragments through enzymatic cleavage to an active subfragment of 14,000 daltons. The subfragment stimulates both proliferation and polyclonal antibody responses in macrophage-deficient B cell preparations [4, 5]. Fc fragment activation of T lymphocytes is less well characterized, although T cells do not proliferate in response to Fc [1]. Since both the Fc-induced polyclonal response and the adjuvant effects of Fc fragments require T cells, these two systems have been employed to analyze Fc effects on T cells. As will be shown, certain T lymphocyte subsets are stimulated by Fc to produce factors which exert T cell replacing functions. These studies have expanded the understanding of the mechanisms of Fc fragment regulation of immune responses and may provide insight into possible regulatory effects exerted by antigen-antibody complexes. A model for the possible regulation of in vivo antibody responses by Fc fragments derived from immune complexes is discussed.

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MATERIALS AND METHODS

Animals

Male mice of the inbred C57BL/6J strain were obtained from the Jackson Laboratories (Bar Harbor, Maine). Inbred C57BL/6 nude mice (N_4F_4) were obtained from the Scripps Clinic and Research Foundation breeding colony. All mice were between 8–10 weeks of age.

Preparation of Fc Fragments

A human IgG1 myeloma protein (Fi) was a gift from Dr. Hans L. Spiegelberg, Scripps Clinic and Research Foundation. The IgG1 was purified by ammonium sulfate fractionation followed by DEAE cellulose chromatography with 0.01 M phosphate buffer pH 8 used as the eluent.

Fc fragments were obtained by a 5-hr digestion of IgG1 with papain (Sigma Chemical Co., St. Louis, Missouri) in the presence of L-cysteine (Sigma) and ethylenediaminetetraacetic acid (EDTA) (J.T. Baker Chemical Co., Phillipsburg, New Jersey) for 5 hr [8]. Following digestion the material was chromatographed on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, New Jersey) to remove any undigested IgG. The Fc and Fab fragments were then separated from each other by DEAE chromatography [9]. Mitogenic Fc subfragments were prepared as described in detail elsewhere [5].

Preparation of Interleukin 2 (IL-2)

IL-2 was prepared as described by Watson et al [10]. Briefly, the spleen cells of 50 mice were cultured in serum-free RPMI-1640 (Flow Laboratories, Rockville, Maryland) supplemented with 0.1 M HEPES, 2 mM L-glutamine, 100 units penicillin, 100 μg streptomycin, and 5×10^{-5} M 2-mercaptoethanol (2-ME) for 24 hours. The culture supernatants were collected by centrifugation, concentrated by vacuum dialysis, and subjected to chromatography on Sephadex G-100. Chromatography was performed with sterile 0.9% saline containing 100 units penicillin and 100 μg streptomycin/ml. Each column fraction was tested for activity in a thymocyte mitogenic assay described in [10]. The active fractions were pooled, dialyzed against 1% glycine, and subjected to preparative isoelectric focusing [11] in a pH gradient from 3–10. The isoelectric focusing bed was divided into 20 fractions and each eluted with 5 ml sterile 0.9% saline. Each fraction was dialyzed to remove the ampholytes, then tested for the ability to replace T cells in the polyclonal response.

Production of Fc-Fragment-Induced T Cell Factors

Spleen cells were incubated 24 hr in RPMI-1640 supplemented with 2 mM L-glutamine, 100 units penicillin, 100 μg streptomycin, 5×10^{-5} M 2-ME and 5% fetal calf serum (FCS) (Grand Island Biological, New York), with 50–250 μg/ml Fc fragments. Culture supernatants were collected and tested for T-cell-replacing activity in the Fc-induced polyclonal response.

Macrophage Depletion

Macrophages were depleted by Sephadex G-10 (Pharmacia) filtration according to the method of Ly and Mishell [12]. 50×10^6 cells were applied to 9-ml columns of Sephadex G-10 which had been equilibrated in Hanks balanced salt solution (BSS) and 5% FCS at 37°C. Cells were eluted with approximately 5–6 mls of warm BSS and 5% FCS.

Antisera Treatment

Antisera and C′ treatment was performed essentially as recommended by Shen and Boyse [13]. 50×10^6 cells/ml were incubated with an appropriate amount of antisera at 4°C for 30 minutes. The cells were washed with phosphate-buffered saline containing 5% FCS, and resuspended in RPMI-1640 containing 5% FCS, 1% sodium azide and 25% C′ in which they were incubated 30 min at 37°C. The complement, a mixture of rabbit and guinea pig shown to be non-cytotoxic for murine lymphocytes, was a gift from Dr. Sharyn M. Walker. After complement treatment the cells were washed in PBS with 5% FCS and resuspended in complete media.

αLyt 1.2 and αLyt 2.2 were obtained from Dr. F. W. Shen, of the Memorial Sloan-Kettering Cancer Center, New York. Under the conditions employed, 5–15% of thymocytes and 20–30% of spleen cells treated were recovered following Lyt antisera and C′ treatment. The anti-IA antisera, A. TH anti-A. TL, was a gift from Dr. J. Ray of the National Institutes of Health. Thirty to forty percent of spleen cells and 20% of thymocytes treated were recovered following treatment with this antisera and C′. Rabbit anti-mouse thymocyte sera (Lot 15088, Microbiological Associates, Bethesda, Maryland) was absorbed with a BALB/c myeloma, XS63, and was cytotoxic for approximately 50% of spleen cells.

Polyclonal Antibody Response Assay

For the generation of the polyclonal plaque-forming cell (PFC) response, spleen cells were suspended to a concentration of 2×10^6 /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, 0.5% fresh mouse serum, and 7.5% FCS. Duplicate cultures of 6×10^5 cells/0.3 ml were incubated in microtiter plates (3042 Microtest II, Falcon Plastics, Oxnard, California) 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-sheep red blood cells (TNP-SRBC) by the slide modification of the Jerne and Nordin plaque assay [14]. Heavily conjugated TNP-SRBC were prepared according to the method of Kettman and Dutton [15] and were used as the indicator RBC. Guinea pig serum (Pel-Freez, Rogers, AR) was the source of complement used to develop the IgM plaques. Results of the plaque-forming assay are expressed as mean PFC/10^6 original cells cultured ± standard error. Each experiment was performed several times, and the experiments shown are representative of all the data.

Generation of Immune Responses

Priming for secondary in vitro antibody response. Mice were injected with 0.1 ml of 10% suspension of sheep red blood cells (SRBC) (Colorado Serum Co., Denver, Colorado) intraperitoneally (IP). Six to eight weeks after priming they were boosted IP with the same dose of SRBC and used 7 days later.

In vitro response to SRBC. Spleens were removed from primed and boosted or untreated mice, and a single cell suspension was prepared by teasing the spleens apart with forceps into phosphate-buffered saline (PBS), (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 [16]. Cells were suspended to a concentration of 6×10^6/ml RPMI-1640 supplemented with 2 mM L-glutamine, 1% BME vitamins (Grand Island Biological Co., New York), 100 units penicillin, 100 μg streptomycin (Microbiological Associates, Bethesda, Maryland), 5×10^{-5} M 2-ME, 7.5% FCS, and 0.5% fresh normal

TABLE I. T Cell Requirement for Fc Fragment Stimulation of a Polyclonal Antibody Response

Cell source	Fc fragments[a]	Anti-TNP PFC/10^6 ± SE
Normal spleen	–	< 10
Normal spleen	+	117 ± 13
T-cell depleted spleen[b]	+	< 10
B cells and T cells[c]	+	193 ± 12
T-cell depleted spleen and IL-2[d]	–	16 ± 4
T-cell depleted spleen and IL-2[d]	+	121 ± 6

[a]50 μg/culture well.
[b]T cells removed for treatment with anti-thymocyte sera + C′ as described in Methods.
[c]2.5 × 10^5 B cells (anti-thymocyte + C′ treated spleen cells) were mixed with 2.5 × 10^5 nylon wool purified T cells.
[d]10 μl IL-2.

mouse serum. The spleen cells at a concentration of 6 × 10^5 along with various concentrations of SRBC and Fc were cultured in 0.3 final volume in flat-bottom microtiter plates for 4 days at 37°C in 5% CO_2. At the end of this time duplicate cultures were pooled and assessed for PFC to SRBC, as described above. Indirect plaques were developed with a combination of guinea pig serum and rabbit anti-mouse immunoglobulin.

RESULTS

Fc-Induced Polyclonal Antibody Response

Fc fragments trigger anti-TNP polyclonal antibody responses in murine spleen cell cultures (Table I). The response was found to be T cell dependent as treatment with anti-thymocyte sera and C′ prevents the response. Polyclonal antibody formation can be restored by adding back nylon wool-purified T cells. Furthermore, the T cell requirement can be replaced by a soluble T cell replacing factor, IL-2. IL-2 completely restores the Fc-induced polyclonal antibody response in T-deficient cultures. IL-2 is generated by Concanavalin A stimulation of murine spleen cells and purified by Sephadex G-100 chromatography and preparative isoelectric focusing. IL-2 displays considerable charge heterogeneity in preparative IEF in the pH range 4–5. Therefore, each individual fraction of a preparative isoelectric focusing gel run on IL-2 was tested for the ability to replace T cell function in two systems: the Fc-induced polyclonal antibody response and the primary anti-SRBC response. As shown in Figure 1, these activities do not reside in the same fractions. The majority of the activity which restores anti-SRBC antibody formation in T-depleted cultures is present in two peaks with pI values of 4.1 and 4.8. In the same preparation, the polyclonal-restoring activity is found in one peak at pH 4.4

The ability of soluble T cell factors to function in the polyclonal response suggested the possibility that Fc might trigger the production of such material. Table II illustrates that Fc fragments incubated with spleen cells for 24 hr do stimulate the release of T cell replacing factors into the culture supernatant. For the sake of clarity, this Fc fragment-induced T cell replacing factor has been called (Fc) TRF to distinguish it from IL-2 pro-

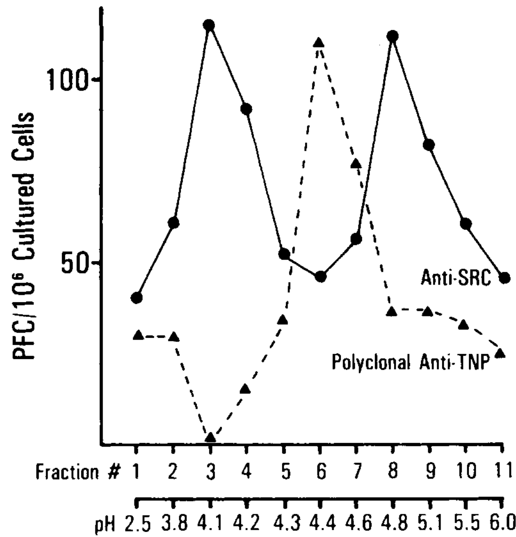

Fig. 1. Preparative isoelectric focusing of interleukin-2. Interleukin-2 prepared by Con A stimulation of murine spleen cells was purified by Sephadex G-100 column chromatography. Fractions containing IL-2 activity were pooled and subjected to preparative isoelectric focusing. The IEF bed was divided into 20 fractions, each fraction eluted with 5 ml sterile saline, dialyzed to remove the ampholytes, and tested for the ability to replace T cell function in primary anti-SRBC responses and Fc-induced polyclonal antibody responses. Only the first ten fractions displayed activity in either of these assays.

TABLE II. Stimulation of TRF Production by Fc Fragments

Stimulating agent[a]	Anti-TNP PFC/10^6 ± SE
–	25 ± 7
50 μg/ml Fc fragments	153 ± 20
100 μg/ml Fc fragments	218 ± 3
150 μg/ml Fc fragments	83 ± 10
2 μg/ml Concanavalin A	160 ± 8

[a]5 × 10^4 spleen cells were cultured for 24 hr with the stimulating agents. The culture supernatants were then tested for TRF activity in the Fc-induced polyclonal response in T-depleted cultures. Ten μl of culture supernatant/culture well was tested in the standard polyclonal assay containing 50 μg/culture Fc fragments.

duced by Concanavalin A stimulation. The cellular requirements for the production of (Fc) TRF are summarized in Table III. Both macrophages and T lymphocytes are essential for (Fc) TRF synthesis. T-deficient nude spleen cells are incapable of factor generation. Use of anti-Lyt antisera and C′ further defines the essential T cell population. Anti-Lyt 1 and C′ completely abolish the competency of spleen cells to produce (Fc) TRF. Anti-Lyt 2 and C′ treatment has no effect on the subsequent ability to respond to Fc fragments in generation if this material. These results suggest that the Lyt $1^+ 23^-$ T cell subset is

TABLE III. Characteristics of Cells Required for (Fc) TRF Production*

Cell source	Direct anti-TNP PFC/10^6 ± SE
Normal spleen	150 ± 4
Nude spleen	< 10
Sephadex G-10 filtered spleen	14 ± 4
αLy 1.2 + C′ treated spleen	< 10
αLy 2.2 + C′ treated spleen	116 ± 2
α Ia + C′ treated spleen	< 10

*Spleen cells were cultured 24 hr with 50 μg/ml Fc fragments. Culture supernatants (10 μl) were tested for the ability to replace T cell function in Fc (50 μg/well)-induced polyclonal antibody responses.

TABLE IV. Generation of TRF by Fc Subfragment in Macrophage-Depleted Cultures

Cells	Fc[a]	Subfragment[b]	PFC/10^6 [c]
Normal	+	–	146 ± 27
Normal	–	+	144 ± 9
αIa + C′ treated	+	–	< 10
αIa + C′ treated	–	+	154 ± 25

[a] 50 μg Fc/ml in generating cultures.
[b] 2.5 μg Fc subfragment/well in generating cultures.
[c] Direct anti–TNP PFC/10^6 cultured cells + standard error. Assay culture contained 6×10^5 cells. 50 μg/well Fc fragments and 10 μl (Fc) TRF containing supernatant.

responsible for factor synthesis. Macrophage depletion by Sephadex G-10 filtration or anti-Ia and C′ prevents factor production. This macrophage requirement can be circumvented by use of macrophage-processed Fc subfragments. Fc subfragments are the biologically active moiety and stimulate B cell proliferation and polyclonal antibody formation in the absence of macrophages [4, 5]. Use of the subfragment allows generation of (Fc) TRF from anti-Ia and C′ treated spleen cells (Table IV). It therefore appears that Fc fragments, following macrophage processing, interact with Lyt $1^{+}23^{-}$ T lymphocytes stimulating the release of (Fc) TRF.

Adjuvant Effect

Fc fragments have adjuvant properties when added with antigen to spleen cell cultures. The observed adjuvant effect is dependent on the dose of antigen employed. An eight-fold enhancement of the secondary IgM anti-SRBC response occurred when Fc fragments were administered with low numbers of SRBC. When the maximum response was obtained, there was no detectable adjuvant effect by Fc (Table V).

The adjuvanticity of Fc appears to be exerted through the T cell population. To determine the role of T cells in the Fc fragment-induced enhancement of the humoral immune response, advantage was taken of the ability of IL-2 to substitute for T cells in an anti-SRBC response [10, 17]. Increasing amounts of IL-2, when added with a constant

TABLE V. Effect of Fc Fragments on Secondary Anti-SRBC Responses* at Several Antigen Concentrations

Culture additions		
Fc[a]	SRBC[b]	PFC/10^6 ± SE
–	10^3	30 ± 2
+	10^3	488 ± 3
–	10^4	57 ± 5
+	10^4	613 ± 45
–	10^5	683 ± 8
+	10^5	790 ± 21

*C57BL/6J were primed and boosted as described in Materials and Methods. Seven days following the boost, spleens were removed and cultured 4 days at 6 × 10^5 cells/well.
[a]100 μg/culture well.
[b]Number of erythrocytes/culture well.

TABLE VI. Inability of Fc Fragments to Enhance Anti-SRBC Responses* When Interleukin-2 Replaces T Cells

Culture additions[a]		IgM Anti-SRBC
IL-2	Fc	PFC/10^6 ± SE
1	–	54 ± 8
1	+	63 ± 3
5	–	128 ± 2
5	+	143 ± 2
10	–	197 ± 23
10	+	155 ± 10
–	–	25 ± 5
–	+	55 ± 10

*Spleen cells from mice primed and boosted as described in Materials and Methods were treated with anti-thymocyte sera and C′. These T-depleted cells were cultured for 4 days with 5 × 10^4 SRBC/well and the other culture additions as described.
[a]Concentrations of IL-2/well in microliters. Fc fragments at 100 μg/well.

low number of SRBC, promote an increasing anti-SRBC PFC response. Adding a constant concentration of Fc fragments does not enhance the response over that obtained with IL-2 alone (Table VI). The enhanced sheep response is not due to the polyclonal response induced by Fc, as the polyclonal response when assayed on day 4 is less than 50 PFC/10^6 cultured cells.

If Fc fragments potentiate humoral immune responses via the release of T cell factors, the Fc-induced TRF might enhance an anti-SRC response in T depleted cultures. As shown in Table VII, such an adjuvant effect is observed when (Fc) TRF is added with

TABLE VII. Enhancement of Anti-SRBC Response* by (Fc) TRF

Culture additions		IgM Anti-SRBC
IL-2[a]	(Fc) TRF[b]	PFC/10^6 ± SE
5	–	74 ± 1
5	+	129 ± 11
10	–	154 ± 2
10	+	244 ± 6
25	–	145 ± 7
25	+	295 ± 8
–	+	7 ± 1

*Mice were primed and boosted as described in Materials and Methods. Seven days following the boost, spleens were removed and treated with rabbit anti-thymocyte sera and C′. T-depleted cells were cultured 4 days at 6 × 10^5 cells/well with 5 × 10^4 SRBC and various culture additives.
[a]Microliters of purified IL-2 added to each culture well.
[b]20 μl culture supernatant containing (Fc) TRF added to each well.

IL-2 to T-deficient cultures. Although the enhancement is not as great as achieved with Fc fragments in intact spleen cultures, the results suggest that the potentiating effect may be mediated by soluble material released by T cells stimulated with Fc.

DISCUSSION

In this report, the role of T cells in the B cell responses induced in murine spleen cell cultures by Fc fragments was examined. Two T-dependent experimental systems were utilized, a polyclonal response triggered by Fc, and the potentiation of an anti-sheep cell response by Fc fragments. In both phenomena, Fc appears to stimulate the generation of helper T cell-replacing factors which exert enhancing effects on proliferating B lymphocytes. Macrophages, which have been shown to be required for the polyclonal antibody response [4], are also necessary for Fc-induced TRF production. Their primary function appears to be to enzymatically cleave the Fc fragments producing biologically active peptides. Purified Fc subfragments bypass the macrophage, triggering both B lymphocyte proliferation [5] and the production of (Fc) TRF by Lyt 1^+23^- T cells. (Fc) TRF provides a differentiative signal to the proliferating B cells, resulting in plasma cell development [18]. The function of this material in mediating the adjuvant effect of Fc fragments is less clear.

Although interleukin 2, prepared by Concanavalin A stimulation of murine spleen cells, contains (Fc) TRF activity, IL-2 and (Fc) TRF may be distinct entities, as both activities are not found in the same fractions of a preparative isoelectric focusing gel. In this case, the interleukin 2 activity was present in two peaks at pH 4.1 and 4.8, while (Fc) TRF was in one peak at pH 4.4. Furthermore, preparations of Fc-induced TRF do not have interleukin 2 activity (data not shown). However, this may be due to differential sensitivities of the assays. The polyclonal response may be sensitive to low concentrations

of factor, and suppressed at the higher concentrations which might be necessary to be detected in the thymocyte mitogenic assay and specific antibody response used to measure interleukin 2 activity. Resolution of this question awaits further characterization of (Fc) TRF. Although these two factors may be chemically distinct, the cellular requirements for the production of both are similar [19–23], as both Lyt $1^{+}23^{-}$ T cells and Ia^{+} macrophages are necessary. The role of the macrophage is defined in the case of (Fc) TRF generation. Macrophages process the Fc fragments to biologically active subfragments. Purified subfragments can bypass the macrophage, directly stimulating T cells to factor production.

Both the requirements for macrophages and T cells in the Fc-induced polyclonal response are somewhat unique to this system. Few, if any, other polyclonal B cell activators have been shown to require macrophages. Lipopolysaccharide [24, 25], dextran sulphate [26], and purified protein derivative [27] are all capable of directly activating B cells. The role of the macrophage in the Fc fragment-induced response is to cleave the Fc fragments, presumably through proteolytic digestion, into mitogenic subfragments that then stimulate B lymphocytes [4, 5].

There have been several reports that T cells modulate the degree of the polyclonal response to bacterial lipopolysaccharide [28, 29] and pokeweed mitogen [30]. The absolute T dependency of the Fc-induced response makes it most similar to the system described by Parker et al [31], in which the polyclonal response triggered by anti-mouse Ig required the addition of culture supernatants from Con A-stimulated murine spleen cells. The capacity to replace both the macrophage and T cell functions with relatively purified soluble factors will allow the analysis of the earliest events in B cell activation, the interaction of the triggers with the target cell membrane, and the biochemical events initiated as a result of such interactions.

The ability of Fc fragments to modulate immune responses is shared by aggregated IgG and immune complexes [1], suggesting complexes might operate by the same mechanism and be involved in regulating ongoing in vivo antibody responses. The following model is proposed for this regulation. Antigen reacts with specific antibody resulting in a conformation change in the antibody which reveals an enzymatic cleavage site within the Fc portion and facilitates binding of the complex to macrophages. Macrophage enzymes cleave the antibody, producing active Fc subfragments. The subfragments interact with B and T lymphocytes, enhancing ongoing antigen-driven responses. In vitro, Fc fragment stimulation results in B cell proliferation and the elaboration of helper T cell-replacing factors from the Lyt $1^{+}23^{-}$ subpopulation of T lymphocytes. These soluble factors potentiate specific immune responses to T-dependent antigens by acting in conjunction with T cell help to enhance the number of specific antibody-secreting cells and by completely replacing T cell action in an Fc fragment-induced polyclonal antibody response. The mechanism of action of this material is not fully understood, or whether the same entity is responsible for both the adjuvant effect, and helper T cell-replacing activity in the polyclonal response.

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