

ANAPHYLATOXIN-MEDIATED REGULATION OF THE IMMUNE RESPONSE

I. C3a-mediated Suppression of Human and Murine Humoral Immune Responses*

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Regulation of the immune response by the third component of complement (C3) has been extensively investigated. C3 and the cleavage products C3b, C3c, and C3d have each received considerable attention in studies of lymphocyte activation and regulation (1–12). Receptors for fragments of C3—such as C3b, C3c, and C3d—have been detected on a number of cells including lymphocytes and macrophages. However, the biological significance of these receptors remains unknown. C3 has been implicated in the activation of macrophages (8) and in modulation of cellular immune functions (1–17). Most of these biological activities have been attributed to C3b, C3c, or C3d with little or no activity associated with the C3a fragment (5, 7).

The C3a fragment has anaphylatoxin properties as evidenced by its potent spasmogenic and tachyphylactic action (17). More recently, Needleman et al. (18) reported that in a serum-free environment, a C3 fragment, presumably C3a, suppresses the antigen- and mitogen-induced proliferative responses of human peripheral blood lymphocytes (PBL). Normal control of C3a action is governed by a serum enzyme (17). Interaction of C3a with endogenous carboxypeptidase N results in the rapid cleavage of the terminal arginine from C3a producing C3a_{des Arg-77}. The des Arg-77 derivative of C3a is incapable of inducing anaphylactic reactions (19).

In this report we have assessed the ability of C3a to modulate both specific and nonspecific immune responses. The anti-sheep erythrocyte (SRBC) responses of both human PBL and murine splenocytes were suppressed by C3a. The nonspecific polyclonal response system mediated by Fc fragments was chosen to determine the cellular level at which C3a suppression occurs. Both macrophages (20–22) and thymus-derived (T) cells (22–24) are an integral part of this response. The results presented in this report indicate that C3a is a potent suppressor of specific and

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¹ *Abbreviations in this paper:* FCS, fetal calf serum; [³H]TdR, tritiated thymidine; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; PBL, peripheral blood lymphocyte; PBS, phosphate-buffered saline; PFC, plaque-forming cell; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SRBC, sheep erythrocytes; TNP, 2,4,6-trinitrophenyl.

nonspecific humoral immune responses and its action is at the level of the helper T cell.

Materials and Methods

Source of PBL. Venous blood was collected in heparinized Vacutainer tubes (Becton, Dickinson & Co., Rutherford, NJ) from healthy adult donors of both sexes.

Animals. Male mice of the C57BL/6 strain were obtained from The Jackson Laboratory (Bar Harbor, ME). BALB/c mice were obtained from Scripps Clinic and Research Foundation breeding colony (La Jolla, CA). All mice were between 8 and 15 wk of age when used.

BCL₁. The in vitro line of BCL₁ was a generous gift from Dr. Samuel Strober Stanford University (Stanford, CA) and its properties have been extensively described (25-27).

Preparation of Fc Fragments. A human IgG myeloma protein (F₁) was a gift from Dr. Hans L. Spiegelberg, Research Institute of Scripps Clinic. 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, NJ) for 5 h (28). The Fc and Fab fragments were separated from each other by DEAE chromatography (29).

Preparation of Plasmin Fc Fragments. Plasmin (Sigma Chemical Co.) digestion of the F₁IgG₁ protein was carried out for 24 h (30). The digested IgG was resolved into two peaks by Sephadex G-150 (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ) chromatography. The small molecular weight peak has been previously shown to polyclonally activate murine B cells in the absence of macrophages (21).

Preparation of Complement Components. Human C3a was isolated from complement-activated serum containing the carboxypeptidase N inhibitor 2-mercaptomethyl-5-guanidinopentanoic acid at 1 mM (19). The isolation procedure has been described in detail (31, 32). The des Arg form of C3a was prepared from complement-activated serum by exactly the same procedure as described for C3a except that no carboxypeptidase inhibitors were employed. These complement products were judged homogenous by single-banding patterns on cellulose acetate electrophoresis at pH 8.6 and by amino acid composition. Human C3a prepared in this manner contains <0.1% contaminating C4a or C5a as determined by radioimmunoassay (33).

Preparation of PBL. Heparinized peripheral blood was diluted with 2 vol of phosphate-buffered saline (PBS):0.001 M phosphate pH 7.2, 0.15 M NaCl. Lymphocytes were separated by Ficoll-Paque (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) gradient centrifugation as described previously (34).

Isolation of Peripheral Blood B and T Cells. PBL suspensions were enriched for B and T cells by the neuraminidase-treated SRBC rosette technique (34). The nonrosetting cells were defined as B cell-and-monocyte-enriched (34) and the rosetting cells as T cell-enriched (34). The T cell populations were subjected to 2,000 rad of irradiation (Gamma Cell 40; Atomic Energy of Canada Ltd., Ottawa) before use in the in vitro antibody response cultures.

Enrichment of T Lymphocytes. The spleen cell population was enriched for T cells by filtration through nylon wool columns (Fenwal Laboratories, Deerfield, IL) (35).

Anti-Lyt Antiserum Treatment. Anti-Lyt 2.2 was obtained from Dr. F. W. Shen, Memorial Sloan-Kettering Cancer Center, New York. Antisera and complement treatment were performed as described previously (23).

Depletion of Macrophages. Spleen cells were filtered through columns of Sephadex G-10 (Pharmacia Fine Chemicals) by the method of Ly and Mishell (36) as described previously (21).

Murine Lymphocyte Response Assays

PROLIFERATION TO FC FRAGMENTS. 2×10^5 BCL₁ cells were cultured in 0.2 ml RPMI 1640 (Flow Laboratories, Inc., Rockville MD), supplemented with 2 mM L-glutamine, 1% BME vitamins (Grand Island Biological Co., Grand Island, NY), 100 U penicillin, and 100 μ g streptomycin (Microbiological Associates, Bethesda, MD). The BCL₁ cells were added to wells of flat-bottomed microtiter plates (3042 Microtest II; Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) to which 2×10^5 irradiated (1,000 rad) BALB/c spleen cells had been allowed to adhere. The cultures were pulsed with 1 μ Ci tritiated thymidine (³H]TdR) 5 Ci/

mM (Amersham-Searle, Arlington Heights, IL)/0.025 ml after 1 d of culture and harvested ~24 h later with an automated cell harvester (M 24V; Brandel, Rockville, MD). The filters were dried, placed in 3 ml scintillation fluid, and counted on a scintillation counter.

PROLIFERATION TO BACTERIAL LIPOPOLYSACCHARIDE (LPS). The response of the BCL₁ B cell tumor line to *Escherichia coli* LPS (055:B5; Difco Laboratories, Detroit, MI) was measured the same as the proliferative response to Fc fragments except no macrophages were mixed with the BCL₁ cells.

POLYCLONAL ANTIBODY RESPONSE TO Fc FRAGMENTS. The Fc fragment-mediated polyclonal antibody response was done as previously described (22). Briefly, 6×10^5 spleen cells in 0.3 ml RPMI 1640 supplemented with 2 mM L-glutamine, 1% BME vitamins, 5×10^{-5} M 2-mercaptoethanol (2-ME), antibiotics, and 7.5% fetal calf serum (Gibco Laboratories). Cultures were harvested on day 3 and the response to 2,4,6-trinitrophenyl (TNP) was measured by slide modification of the Jerne and Nordin plaque assay (37). Results of the plaque assay are expressed as mean PFC/ 10^6 original cells of duplicate pools \pm SE.

IN VITRO ANTI-SRBC RESPONSE. Spleen cells were removed from mice that were injected intraperitoneally with 0.1 ml of 10% suspension of SRBC 4-6 wk previously. A modified Mishell-Dutton culture system was employed for the generation of antibody-producing cells (38, 39). 6×10^5 cells in 0.3 ml RPMI 1640 supplemented as described above with the addition of 5×10^{-5} M 2-ME and 7.5% FCS were cultured for 4 d in flat-bottomed microtiter plates. The direct anti-SRBC response was measured by the slide modification of the Jerne and Nordin plaque assay (37). The results are recorded as direct anti-SRBC PFC/ 10^6 original cells \pm SE.

Human Lymphocyte Response Assays

POLYCLONAL ANTIBODY RESPONSE TO Fc FRAGMENTS. The protocol used has been described in detail previously (34). Briefly, 1×10^5 B cell-enriched (E⁻) cells were cultured with 2×10^5 T cell-enriched (E⁺) cells in 0.3 ml RPMI 1640 supplemented with 2 mM L-glutamine, 1% BME vitamins, antibiotics, and 10% FCS. The number of immunoglobulin-secreting cells was determined 6 d later by the protein A plaque assay (34). Amplifying sera in the protein A assay recognized human IgG + IgM + IgA. The results are recorded as Ig-secreting cells/ 10^6 original B cells \pm SE.

IN VITRO ANTI-SRBC RESPONSE. The primary anti-SRBC response was done as described by Misiti and Waldmann (40). 5×10^6 PBL were cultured in 1 ml RPMI 1640 supplemented as described for the Fc polyclonal assay plus 5×10^{-5} 2-ME, and the SRBC-adsorbed autologous human plasma was substituted for the FCS. The cultures were maintained in gas boxes, which were rocked at 7 or 3.5 cycles/min for 11 d. The cultures were fed a Mishell-Dutton cocktail mixture (38) containing autologous plasma every other day. The direct or IgM response was measured by the Jerne and Nordin plaque assay on day 11 of culture. The results are recorded as direct anti-SRBC PFC/culture \pm SE.

ANTIGEN-INDUCED PROLIFERATIVE RESPONSE. The proliferative response to tetanus toxoid was done as described by Broff et al. (41). Briefly, 1×10^5 PBL were cultured with 5 μ g/ml tetanus toxoid (Department of Public Health, Boston, MA). The response was assayed on day 7 of culture.

MITOGEN-INDUCED PROLIFERATIVE RESPONSE. The mitogen proliferative response was done the same as the antigen proliferative response. Pokeweed mitogen (PWM) (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, California) was used at a concentration of 33 μ g/ml and phytohemagglutinin (PHA-P) (Difco Laboratories, Detroit, MI) was used at 1%. The response was assayed on day 5 of culture.

Results

C3a-mediated Suppression of Both Specific and Polyclonal Antibody Response by Human PBL. Addition of purified human C3a to cultures of PBL caused a suppression in the ability of these cells to produce polyclonal antibody when stimulated by Fc fragments derived from human Ig (Fig. 1). Maximum suppression (84%) was achieved with C3a at 50 μ g/ml. In contrast, C3a_{des Arg-77} (C3a minus the carboxy-terminal arginine) at concentrations up to 200 μ g/ml (Fig. 1) was ineffective in suppressing the

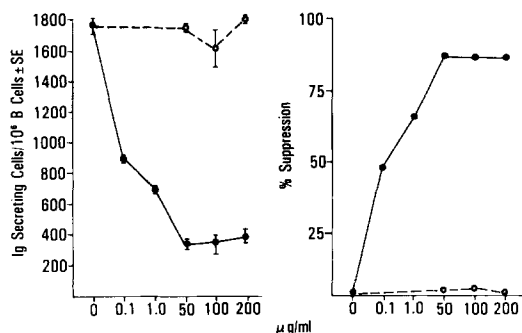


FIG. 1. Human PBL (1×10^5 B and 2×10^5 T cells) were cultured with $5 \mu\text{g/ml}$ of Fc fragments and increasing amounts of C3a (●) or C3a_{des Arg-77} (○). The polyclonal antibody response was measured on day 6 of culture. 1 mM 2-mercaptomethyl-5-guanidinopentanoic acid was present.

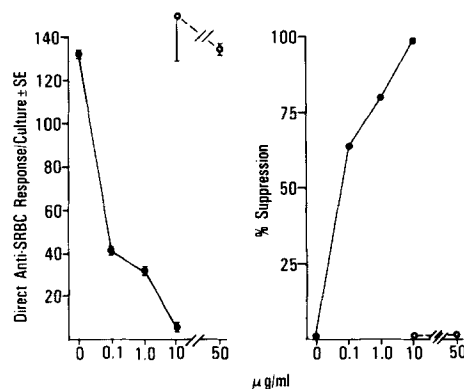


FIG. 2. Human PBL (5×10^6) were cultured with 1×10^6 SRBC and increasing amounts of C3a (●) or C3a_{des Arg-77} (○). The direct anti-SRBC response was measured on day 11 of culture. 1 mM 2-mercaptomethyl-5-guanidinopentanoic acid was present.

polyclonal antibody response. We also examined whether C3a suppressed specific antibody responses. Human C3a was added to cultures containing PBL and SRBC in an attempt to suppress the primary *in vitro* anti-SRBC response. The results depicted in Fig. 2 reveal that C3a also suppresses specific antibody responses. The addition of $10 \mu\text{g/ml}$ C3a to cultures results in $\sim 100\%$ suppression. When C3a_{des Arg-77} was substituted for C3a, no suppression was observed, indicating that the terminal arginyl residue is essential for suppression of both specific and nonspecific antibody responses by human PBL.

Inability of C3a to Suppress T and B Cell-mediated Proliferative Responses. To assess the effect of C3a on specific T cell proliferative responses, human PBL were stimulated with tetanus toxoid. The results in Fig. 3 indicate that addition of C3a to culture does not suppress this response. To further explore the action of C3a on T cell proliferation, stimulation of T cells by nonspecific mitogens was examined in the presence and absence of C3a. Neither C3a or C3a_{des Arg-77} were capable of suppressing the T cell response to PHA or the T and B cell proliferative response to PWM (Table I).

In addition to being a potent polyclonal activator, Fc fragments induce both murine (20) and human (34) B cells to proliferate. To assess the effects of C3a on B cell proliferation, C3a was added along the Fc fragments to murine spleen cell cultures

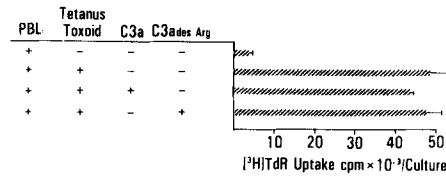


FIG. 3. Human PBL (1×10^5) were cultured with $5 \mu\text{g/ml}$ tetanus toxoid and $50 \mu\text{g/ml}$ C3a. The proliferative response was measured on day 7 of culture. 1 mM 2-mercaptomethyl-5-guanidinopentanoic acid was present.

TABLE I
Inability of C3a to Suppress the PHA- and PWM-induced Proliferative Responses by Human PBL

Stimulator	C3a	C3a _{des Arg-77} *	[³ H]TdR uptake <i>cpm/culture ± SE</i>	Suppression %
—	—	—	$2,432 \pm 486$	—
PHA‡	—	—	$187,750 \pm 5,289$	0
PHA	+	—	$175,502 \pm 1,800$	6
PHA	—	+	$175,154 \pm 5,660$	6
PWM§	—	—	$41,243 \pm 256$	0
PWM	+	—	$39,612 \pm 180$	3
PWM	—	+	$36,407 \pm 3,821$	10

* Both C3a and C3a_{des Arg-77} were present at a final concentration of $100 \mu\text{g/ml}$. 2-mercaptomethyl-5-guanidinopentanoic acid was 1 mM .

‡ PHA was 1% .

§ PWM was $33 \mu\text{g/ml}$.

TABLE II
Inability of C3a to Suppress the Fc Fragment-mediated Proliferative Response of Murine Spleen Cells

Fc*	C3a‡	[³ H]TdR uptake <i>cpm/culture ± SE</i>	Suppression %
—	—	$12,652 \pm 2,194$	—
+	—	$40,312 \pm 749$	0
+	+	$36,135 \pm 2,489$	10

* $250 \mu\text{g/ml}$ of Fc added.

‡ $50 \mu\text{g/ml}$ of C3a added. 2-mercaptomethyl-5-guanidinopentanoic acid was 1 mM .

and the proliferative response measured. The results in Table II show that B cell proliferation is unaffected by C3a.

C3a-mediated Suppression of Both Specific and Polyclonal Antibody Responses by Murine Splenic Lymphocytes. We questioned whether human C3a could suppress the humoral immune response in a more defined model. Therefore, murine lymphocytes were examined for specific anaphylatoxin effects. Human C3a at $50 \mu\text{g/ml}$ suppressed the Fc polyclonal antibody response of murine cells to approximately the 100% level (Fig. 4). In contrast, addition of C3a_{des Arg-77} to the culture produced little, if any, suppression of the response. C3a suppressed the murine anti-SRBC response in a dose-

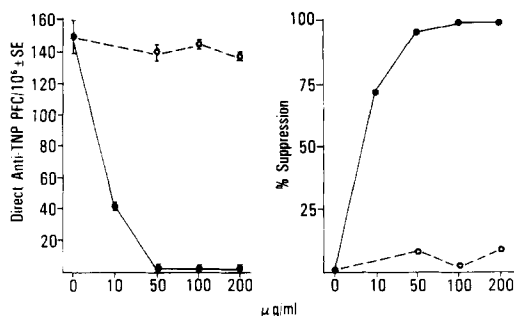


FIG. 4. Murine splenic lymphocytes (6×10^5) were cultured with 250 $\mu\text{g/ml}$ of Fc fragments and increasing amounts of C3a (●) or C3a_{des Arg-77} (○). The polyclonal antibody response was measured on day 3 of culture. 1 mM 2-mercaptomethyl-5-guanidinopentanoic acid was present.

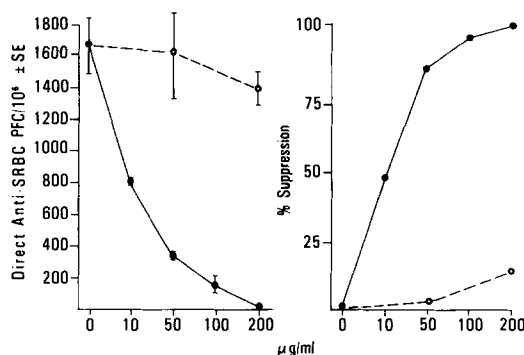


FIG. 5. Murine splenic lymphocytes (6×10^5) were cultured with 1×10^6 SRBC and increasing amounts of C3a (●) or C3a_{des Arg-77} (○). The direct anti-SRBC response was measured on day 4 of culture. 1 mM 2-mercaptomethyl-5-guanidinopentanoic acid was present.

dependent manner, whereas C3a_{des Arg-77} failed to produce a significant suppression even at very high concentrations (Fig. 5). The main difference between the effect of C3a on the human and murine models was the concentration of human C3a needed to produce 50% suppression. Suppression of the murine humoral immune response to the 50% level required ~100-fold greater quantities of human C3a (0.1 vs. 10 $\mu\text{g/ml}$) than were needed to achieve the same level of suppression in the human response. This difference indicates that there is better binding of human C3a to receptor sites on homologous cells than heterologous cells. Such species differences were not discerned for the anaphylatoxin activities of human, rat, and porcine C3a (17).

Kinetics of C3a-induced Suppression. To determine when and how long C3a needs to be present for suppression of the Fc polyclonal response, it was added from day 0 to day 5 of culture and the ensuing polyclonal antibody response measured on day 6. The results indicate that by day 2, the PBL are no longer susceptible to the suppressive effects of C3a (Fig. 6), indicating that C3a acts at an early phase of the response. As a control, C3a_{des Arg-77} was added at the designated times and as shown in Figs. 1 and 2, no suppression was observed.

Requirement for a Carboxypeptidase Inhibitor in Cultures Containing Serum. Up to this point, we used cultures containing either fetal calf serum or human plasma in all the experiments. To measure optimum C3a-mediated suppression the addition of

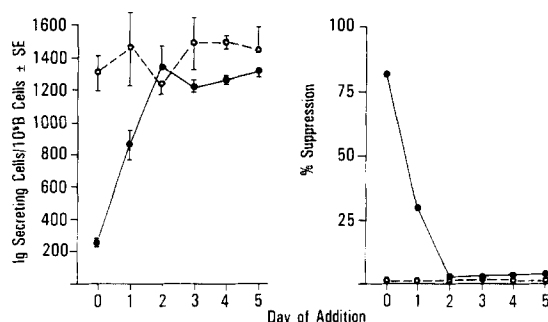


FIG. 6. Human PBL (1×10^6 B and 2×10^5 T cells) were cultured with 5 $\mu\text{g}/\text{ml}$ of Fc fragments. At various time intervals C3a (●) (50 $\mu\text{g}/\text{ml}$) or C3ades Arg-77 (○) (50 $\mu\text{g}/\text{ml}$) was added to culture. The polyclonal antibody response was measured on day 6 of culture. 1 mM 2-mercaptomethyl-5-guanidinopentanoic acid was present.

TABLE III
Suppression of the Fc Fragment-mediated Polyclonal Antibody Response of Human PBL by Human C3a

SCP inhibitor*	Fc‡	C3a§	C3ades Arg-77§	Ig-secreting cells/ 10 ⁶ B cells ± SE	Sup- pression %
+	+	-	-	1,721 ± 131	0
+	+	+	-	341 ± 17	80
+	+	-	+	1,538 ± 174	10
-	+	-	-	1,364 ± 96	0
-	+	+	-	1,415 ± 69	0
-	+	-	+	1,470 ± 76	0

* The serum carboxypeptidase inhibitor 2-mercaptomethyl-5-guanidinopentanoic acid was added to a final concentration of 0.15 $\mu\text{g}/\text{ml}$ (1 mM).

‡ The Fc concentration was 5 $\mu\text{g}/\text{ml}$.

§ The C3a and C3ades Arg-77 concentration was 50 $\mu\text{g}/\text{ml}$.

2-mercaptomethyl-5-guanidinopentanoic acid, a serum carboxy-peptidase inhibitor (19), is required. If the inhibitor is omitted, the endogenous enzyme rapidly cleaves the terminal arginine from C3a producing C3ades Arg-77 (33). To formally prove that the carboxypeptidase inhibitor is required for expression of maximal suppressive activity by C3a, human PBL were cultured in the presence of Fc fragments and C3a in serum-containing medium with and without the inhibitor added. Cultures containing the inhibitor showed a strong suppression (80%), whereas cultures lacking the inhibitor showed no suppression (Table III). Moreover, addition of C3ades Arg-77 plus inhibitor to the cultures had no effect. To determine if a combination of the inhibitor and C3a was responsible for suppression, we took advantage of an Fc polyclonal system where serum was omitted. Fc fragments induce the murine B cell tumor line BCL₁ to proliferate and secrete antibody in the absence of serum (Table III, and E. L. Morgan, M. L. Thoman, and W. O. Weigle, manuscript in preparation). The addition of C3a to BCL₁ cultures resulted in a 69% suppression of the polyclonal antibody response (Table IV). These results indicate that suppression occurs as an effect of the C3a and not the carboxypeptidase inhibitor.

Cellular Level of C3a-mediated Suppression. As mentioned above, Fc fragments induce

TABLE IV
Suppression of the BCL₁ Fc Fragment-mediated Polyclonal Antibody Response by Human C3a

BCL ₁ *	T Cells‡	Fc§	C3a	C3a _{des Arg-77} ¶	Ig-secreting cells/10 ⁶ BCL ₁ ± SE	Suppression %
+	+	-	-	-	3,500 ± 471	—
+	+	+	-	-	14,500 ± 1,967	0
+	+	+	+	-	4,500 ± 1,210	69
+	+	+	-	+	14,750 ± 2,036	0

* 1×10^5 cultured BCL₁ cells + 2×10^5 adherent cells.

‡ 1×10^5 cultured T cells.

§ 250 µg/ml of Fc added.

|| 50 µg/ml of C3a or C3a_{des Arg-77} added.

TABLE V
*Inability of C3a to Suppress the Proliferative Response of the
 BCL₁ Tumor Line**

BCL ₁ *	LPS‡	Fc§	C3a	[³ H]TdR uptake¶ cpm/culture ± SE	Suppression %
+	-	-	-	10,469 ± 610	—
+	+	-	-	97,407 ± 446	0
+	+	-	+	108,105 ± 5,586	0
+	-	-	+	5,778 ± 66	0
+	-	+	-	151,927 ± 6,424	0
+	-	+	+	166,350 ± 5,010	0

* 2×10^5 BCL₁ cells/well were mixed with 2×10^5 irradiated, adherent BALB/c cells in serum-free medium.

‡ 20 µg/ml of LPS added.

§ 100 µg/ml of Fc added.

|| 50 µg/ml of C3a added.

¶ The cultures were harvested on day 2 of culture.

human (34) and murine (20) B cells to proliferate as well as to secrete antibody. The proliferative response requires the presence of macrophages (20, 21), whereas the polyclonal response requires both macrophages and T cells (22-24). The site of C3a-mediated suppression in the Fc-mediated polyclonal response is not at the level of the proliferating B cell. This is shown in Table II, where C3a did not affect the Fc-induced proliferative response of intact spleen cells. To further explore the effects of C3a on B cells, the B cell tumor line BCL₁ was supplemented with macrophages and cultured with Fc fragments and C3a. The results in Table V indicate that C3a did not suppress the Fc proliferative response. Moreover, C3a did not suppress the BCL₁ proliferative response to LPS, another B cell activator (Table V). These results, in conjunction with data obtained with PHA and PWM, indicate that C3a suppression is not at the level of the proliferating T or B cell.

To ascertain whether C3a-mediated suppression occurred at the level of the macrophage, plasmin Fc fragments were used as the stimulating agent. It has been previously reported that the requirement for macrophages could be bypassed in the Fc-induced polyclonal response by use of either Fc subfragments (21, 22) or by plasmin-derived Fc fragments (30). When macrophage-depleted murine spleen cells

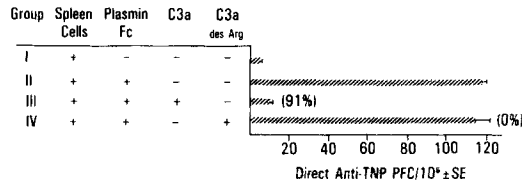


FIG. 7. Macrophage-depleted murine splenic lymphocytes (6×10^5) were cultured with 100 $\mu\text{g/ml}$ plasmin-generated Fc fragments and 50 $\mu\text{g/ml}$ of C3a or C3a_{des Arg-77}. The polyclonal antibody response was measured on day 3 of culture. The number in parentheses indicates percent suppression. 1 mM 2-mercaptomethyl-5-guanidinopentanoic acid was present.

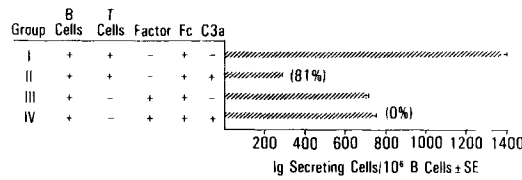


FIG. 8. Human PBL B cells (1×10^5) were cultured with T cells (2×10^5) or Sephadex G-100-purified, PHA-induced factor (25 μl) plus 5 $\mu\text{g/ml}$ Fc fragments. C3a was added to certain cultures at 50 $\mu\text{g/ml}$. The polyclonal antibody response was measured on day 6 of culture. 1 mM 2-mercaptomethyl-5-guanidinopentanoic acid was present.

TABLE VI
Ability of C3a-treated T Cells to Suppress the Human Fc Fragment-induced Polyclonal Antibody Response

Incubation*	Fc‡	Ig-secreting cells/10 ⁶ B cells ± SE	Suppression
<i>h</i>			%
0	-	134 ± 12	
0	+	1,017 ± 50	
C3a, 0.5	+	192 ± 56	82
Medium, 0.5	+	1,283 ± 68	0
C3a, 1.0	+	158 ± 19	85
Medium, 1.0	+	1,142 ± 130	0
C3a, 2.0	+	175 ± 6	83
Medium, 2.0	+	1,234 ± 25	0

* T cells were incubated with 50 $\mu\text{g/ml}$ C3a for the times indicated with inhibitor (2-mercaptomethyl-5-guanidinopentanoic acid), washed, and cultured with B cells in the absence of inhibitor for 6 d.

‡ Fc concentration was 5 $\mu\text{g/ml}$.

were cultured with plasmin Fc fragments, a polyclonal antibody response was generated (Fig. 7). Addition of C3a to plasmin Fc cultures resulted in a dramatic suppression (91%) of polyclonal antibody response (Fig. 7). In contrast, C3a_{des Arg-77} was unable to suppress the plasmin Fc polyclonal response. These results indicate that C3a-mediated suppression is specific and occurs in the absence of macrophages.

The role of the T cell in C3a-mediated suppression of the immune response was examined using the Fc polyclonal model. It has been established that the T cell

TABLE VII
*Inability of Anti-Lyt-2.2 Treatment to Prevent C3a Suppression of the Murine
 Fc Fragment-mediated Polyclonal Antibody Response*

Spleen cell treatment	Fc*	C3a‡	Direct anti-TNP PFC/10 ⁶ ± SE	Suppression %
None	-	-	14 ± 4	—
None	+	-	119 ± 3	0
None	+	+	14 ± 1	100
Anti-Lyt-2.2	-	-	24 ± 1	—
Anti-Lyt-2.2	+	-	168 ± 11	0
Anti-Lyt-2.2	+	+	24 ± 5	100

* 250 µg/ml of Fc added.

‡ 50 µg/ml of C3a added. The concentration of 2-mercaptomethyl-5-guanidinopentanoic acid was 1 mM.

requirement in the murine Fc polyclonal antibody response can be replaced by a factor derived from either concanavalin A- (23) or Fc fragment- (24) stimulated T cells. This finding has been confirmed for a human Fc polyclonal model in that the Sephadex G-100-purified factor derived from PHA-stimulated human PBL is capable of replacing T cells in the human PBL polyclonal response (Fig. 8, group III). Addition of C3a to human cultures failed to suppress the response (Fig. 8, groups III and IV). As a control, cultures with T cells were also supplemented with C3a and the ensuing response was measured (Fig. 8, groups I and II). These results indicate that the C3a-mediated suppression of the immune response occurs through a T cell population. As described above, T cells are required for the Fc fragment-induced murine polyclonal responses as well as the human PBL responses.

To determine the length of time C3a had to be in contact with T cells to induce suppression, purified human T cells were incubated with C3a in the presence of inhibitor, washed, and added to B cell populations in the absence of inhibitor and the polyclonal antibody response measured. The results in Table VI indicate that 0.5 h is sufficient time for C3a to induce an 82% suppression of the human Fc polyclonal antibody response. Moreover, the suppression is not a result of C3a carryover because the residual C3a bound to T cells would be converted to the inactive C3a_{des Arg-77} form by serum carboxypeptidase in the absence of an inhibitor. Similar results were found using C3a-treated murine splenic T cells (data not shown).

The Lyt phenotype of the T cell in the murine Fc polyclonal response is Lyt-1⁺2⁻ (24). Therefore, the presence of a potential Lyt-1⁻2⁺ suppressor T cell was investigated. Intact murine spleen was treated with anti-Lyt 2.2 before stimulation with Fc fragments. Depletion of Lyt-2⁺ cells had no effect on the polyclonal antibody response (Table VII). When C3a was included in these cultures, the response was completely suppressed (Table VI), indicating that the C3a-induced suppression was not dependent on the presence of an existing Lyt-2⁺ suppressor cell.

Discussion

C3a is a complement activation factor from human serum that has the capacity to suppress both specific and nonspecific humoral immune responses. Addition of C3a

to cultures of human PBL or murine splenic lymphocytes results in suppression of both the anti-SRBC response and the Fc fragment-mediated polyclonal antibody response. Moreover, the Fc polyclonal antibody response of the murine B cell tumor BCL₁ was also suppressed. In contrast to these results, mitogen- and antigen-induced T and B cell proliferative responses were not affected by C3a. Concentrations of C3a that are 10-fold greater than those required to fully suppress the anti-SRBC response had no effect on the proliferative response induced by tetanus toxoid. Similar concentrations of C3a failed to suppress the nonspecific proliferative responses to PHA or PWM. In addition, B cell proliferation induced by Fc fragments or LPS was unaffected by C3a. The inability of C3a to suppress either antigen- or mitogen-induced proliferative responses is in contrast to the results of Needleman et al. (18). These authors reported that a small fragment derived from trypsin digests of C3, presumably C3a, was capable of suppressing both antigen- and mitogen-induced PBL proliferative responses. The reason(s) for our inability to suppress mitogenic responses is currently unknown. Several possibilities could account for the discrepancies: (a) the C3a prepared from serum is exposed to extremely low pH conditions during isolation and may be physically different from that obtained by Needleman (18) from purified C3; or (b) multiple C3 factors may be present in their preparation. Studies using synthetic analogue peptides of C3a that are biologically active in other systems indicate that antibody, but not proliferative, responses are suppressed by C3a peptides (E. L. Morgan, W. O. Weigle, and T. E. Hugli, manuscript in preparation).

Immune suppression mediated by C3a occurs at an early phase in the antibody response. This conclusion came from our observation that when C3a was added on day 0, maximal suppression was achieved; however, when added on day 1, only marginal suppression was observed. In addition, interaction of purified T cells with C3a for as little as 30 min results in a pronounced suppression. Moreover, the positive action of the carboxypeptidase inhibitor dictates that C3a must act relatively early in the response because the compound is a competitive inhibitor and protects the factor from conversion for only a few hours.

The carboxy terminal arginine is essential for C3a to exhibit its immunosuppressive properties. When the terminal arginine is removed from C3a the resulting C3a_{des Arg-77} molecule is nonsuppressive. These results correspond to the tactoryphylactic properties of C3a (17). Taken together, our results indicate that the terminal arginine of C3a is at the active center and is required for producing immunosuppressive actions. Therefore, the serum carboxypeptidase serves the same role in controlling the immunosuppressive activities of C3a as it does in restricting spasmogenic action. Work is currently in progress to confirm that synthetic peptide analogues of C3a that exhibit the biological attributes of this factor are also capable of immunosuppression (42).

T lymphocytes are the target of C3a-mediated suppression of the immune response. Substitution of the T cells by a soluble T cell factor in the Fc polyclonal antibody response abrogates the suppressive response of C3a. This is an important finding in that C3a is incapable of suppressing either antigen- or mitogen-induced T cell proliferation. The action of C3a on T cells could be through an interference with the T cell-macrophage interaction as proposed for other C3 cleavage products (11). This pathway is presumably not affected, however, because when macrophages are removed suppression still occurs. In the case of the plasmin Fc-induced polyclonal antibody response, the response is T cell dependent, but unlike Fc activation, it is

independent of macrophages (30). C3a suppresses the plasmin Fc polyclonal response as efficiently as it does the Fc-mediated response. Additional evidence for the T cell being the target of C3a-mediated suppression is drawn from the observation that incubation of purified T cells with C3a renders the T cells incapable of helping in the Fc-induced polyclonal antibody response. Moreover, C3a has been found not to suppress *in vitro* antibody responses to the T cell-independent antigens, TNP-LPS and TNP-Ficoll (E. L. Morgan, unpublished observations). C3a-mediated suppression does not appear to involve the activation of existing suppressor T cells because depletion of Lyt-2⁺ cells has no effect on the level of suppressor cells. This finding does not rule out the possibility that a suppressor cell is being generated in culture, however. Because C3a acts early in polyclonal and specific antibody responses, the possibility exists that C3a could act upon suppressor precursor cells (Lyt-1⁺2⁻), inducing the formation of suppressor cells (44). Preliminary data indicate that C3a does indeed induce the production of suppressor T cells that are capable of suppressing both the Fc polyclonal and anti-SRBC responses (E. L. Morgan, W. O. Weigle, and T. E. Hugli, manuscript in preparation).

C3a was also unable to suppress the LPS- or Fc fragment-mediated proliferative responses. The Fc polyclonal response results from the delivery of two signals to the B cell (22). The first is a proliferative signal that stimulates the B cells, and in the presence of a second T cell-derived signal they may be differentiated to antibody-secreting cells. The B cell proliferative phase is an integral part of the polyclonal response without which little or no antibody is produced. The inability of C3a to suppress the Fc proliferative response in the presence or absence of T cells gives further support to the hypothesis that C3a exerts its action at the level of the helper T cell.

The mechanism by which C3a interferes with helper T cell action is currently unknown. C3a does not interfere with T cell proliferation or the delivery of the (Fc) T cell-replacing factor signal to the B cell because C3a was unable to suppress responses mediated by soluble cell factors when substituted for T cells. If C3a acted by combining with the T cell factor or prevented the factor from interacting with the proliferating B cell, then suppression should occur.

The amount of human C3a needed to suppress human PBL responses was ~100-fold lower than that needed to suppress murine immune responses. It is not currently known whether the species differences are expressions of receptor specificity or of true variations in sensitivity between human and murine helper T cells. It is clear that the level of C3a required to suppress the antibody response falls well within the physiologic range of humans. Significant suppression of human PBL responses was observed at a C3a concentration of 0.1–1 $\mu\text{g}/\text{ml}$, a level well below the potential concentration of 50–60 $\mu\text{g}/\text{ml}$ C3a generated by maximal human C3 conversion.

The physiological significance of C3a-mediated regulation of the humoral immune response is currently a matter of speculation. In contrast to the results obtained with C3a, C5a was found to augment the Fc-mediated polyclonal antibody response of human PBL (E. L. Morgan, W. O. Weigle, and T. E. Hugli, manuscript in preparation). Invasion of the body by a foreign organism results in the synthesis of antibody and the triggering of the complement cascade. The presence of C3a and C5a in the microenvironment where B cells, T cells, and macrophages are interacting could influence the level of antibody produced. Although C3a is relatively short-lived in

circulation, that the suppressor action is mediated by C3a-induced suppressor T cells lends validity to its potential action *in vivo*. In addition, the presence of the serum carboxypeptidase could act as a control mechanism for C3a immunosuppressive action. Thus, in addition to its other biological activities, the anaphylatoxin may be involved in controlling the amount of antibody produced during an infection. The action of C3a in conjunction with other factors could serve as a complex regulatory circuit in controlling an ongoing antibody response.

Summary

The C3a fragment of the third component of complement was found to have immunosuppressive properties. C3a is capable of suppressing both specific and polyclonal antibody responses. In contrast, C3a had no effect on antigen- or mitogen-induced B or T cell proliferative responses. The carboxy-terminal arginine is essential for C3a to exhibit its immunosuppressive properties. The serum carboxypeptidase inhibitor 2-mercaptomethyl-5-guanodinopentanoic acid, which prevents cleavage of the terminal arginine that would produce C3a_{des Arg-77}, allowed us to assay the effects of C3a on *in vitro* immune response systems where serum is required. When the terminal arginine is removed from C3a, the resulting C3a_{des Arg-77} molecule is nonsuppressive. Helper T lymphocytes are the target of C3a-mediated suppression of the immune response. Substitution of T cells by soluble T cell factors was found to abrogate the C3a suppressive activity.

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