

REGULATION OF HUMAN CYTOTOXIC RESPONSES BY COMPLEMENT: C3, C3b
AND C3d PREPARATIONS ENHANCE HUMAN ALLOGENEIC CYTOTOXIC RESPONSES.

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ABSTRACT

Complement components and complement breakdown products have been found to participate in the regulation of the immune response. In the present study we investigated the effect of C3 and its fragments, C3b, C3c and C3d on human allogeneic cell mediated lympholysis (CML). C3 and C3b at a concentration of $275 \text{ M} \times 10^{-9}$ and C3d at a concentration of $330 \text{ M} \times 10^{-9}$ enhanced human allogeneic CML by at least two fold. In contrast C3c did not affect CML responses. Both C3b and C3d had to be present at the initiation of the cultures in order to exert their effect. Similar doses of C3b and C3d did not affect the mixed lymphocyte responses (^3H -thymidine uptake) while higher doses were clearly inhibitory. None of the preparations induced proliferative or cytotoxic responses in the absence of allogeneic stimulating cells. C3b and C3d added to the mixed lymphocyte cultures caused increased production of interleukin 2. We conclude that C3b and C3d facilitate allogeneic cytotoxic responses through increased production of interleukin 2.

INTRODUCTION

Numerous reports have investigated the regulatory effects of complement factors and complement factor breakdown products on human and animal cellular immune responses (1,2,3). In particular, C3 or its

breakdown products have been found to inhibit T cell proliferative responses (4-7); fluid phase C3b induces the production of lymphokines by guinea pig spleen cells (8,9); polymerised C3b enhances the growth of an interleukin 2 (IL 2) dependent T cell line (10); complement carrying immune complexes induce the production of interleukin 1 (IL 1) by human monocytes (11); fluid phase C3b induces the proliferation of murine spleen cells (12) and cross-linked C3 induces the proliferation of lipopolysaccharide activated murine B lymphocytes (13); finally fluid phase C3b inhibits the secretion immunoglobulin by polyclonally activated human B cells (14). Cytotoxic T lymphocytes (CTL) are consistently generated in vitro during mixed lymphocyte culture (MLC) of human peripheral mononuclear cells (MNC) (15). During the interaction of the allogeneic cells lymphokines are produced which promote and sustain the CTL (16).

It was recently reported that C3 inhibits the generation of human allogeneic CTL in vitro (17); earlier observations had shown that in vivo activation of complement had no effect on the generation of CTL, allograft rejection and delayed hypersensitivity reactions (18,19). In this study we confirm that relatively high concentrations of fluid phase human C3 suppress human allogeneic CTL responses in vitro, but interestingly, lower doses have a significant enhancing effect on the same responses. Investigating the effects of the C3 breakdown products, we found that C3b and C3d have a similar enhancing effect. Since production of IL 2 was enhanced during the MLC if low concentrations of C3b and C3d were present we postulate that C3 and its breakdown products C3b and C3d can enhance the human CTL responses through enhanced IL 2 production.

MATERIAL AND METHODS

Mononuclear cell preparation

MNC were obtained by standard Ficoll-Hypaque gradient centrifuging technique from human peripheral blood, drawn aseptically in heparinized syringes.

Cell Mediated Lymphosysis (CML)

CML assays were performed as previously detailed (20). Effector cell were obtained by harvesting MNC produced by a 6 day MLC. Responder cells (1×10^6 /ml) and irradiated (2000 rads) stimulator cell (0.5×10^6 /ml) in medium RPMI-1640 containing 10% heat-inactivated normal human serum were cultured in flat-bottom 24 well plates (Costar, Cambridge, MA). As target cells we used phytohemagglutinin 1 μ g/ml, (Burroughs-Wellcome, Teckenham, England) stimulated human MNC. Prior to use, these cells were labeled with ^{51}Cr by incubation with 200 μCi of ^{51}Cr labeled sodium chromate (New England Nuclear, Boston, MA). Effector and target cells were incubated at 37°C for 4 hours and then the supernatants were collected and counted in a gamma counter. The percentage of specific cytotoxic chromium release was evaluated as follows: % cytotoxicity = $(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm}) \times 100$. Spontaneous release in the present experiments ranged between 18 and 25% in 4 hours. MNC cultured without stimulator cells served as control, unsensitized, effector cells. Specific lysis obtained from unsensitized cells ranged from -1 to 6% and it was subtracted from the percentage of lysis obtained by sensitized cells.

Mixed Lymphocyte Reaction (MLR)

For the estimation of the MLR, MLC (at the same cell concentrations and media) were carried in 96 round-bottom well plates in a total volume of 0.2 ml/well; 18 hours prior to harvesting the cells, 0.5 μCi of methyl tritiated thymidine (New England Nuclear) was added; cell harvesting was done with a multichannel harvester and counted in a scintillation counter.

IL 2 measurements

MNC were put into MLC in the presence of absence of C3b or C3d. The supernatants from these cultures were collected at the end of the fourth day and tested for IL 2. IL 2 concentrations were measured by the use of IL-2 dependent murine T cell line (CTLL-2). One thousand CTLL-2 cells were

cultured with 50 μ l of test supernatants for 24 hours. One half μ Ci of ^3H -thymidine was added during the last 8 hours and the uptake was compared to that achieved by standard IL 2 preparations.

Human C3 breakdown product preparation

Human C3 was partially purified by DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) column chromatography of the plasminogen-depleted 5% polyethelene glycol supernatant of fresh plasma (21,22). C3 was cleaved to C3b with trypsin in the presence of a 50% suspension of activated-Thiol Sepharose (ATS, Pharmacia Fine Chemicals). A portion of the C3b-ATS was eluted with L-cysteine in order to obtain C3b, while the rest was further cleaved by elastase for the production of C3d and C3c fragments. The above techniques have been detailed elsewhere (22,23). The purity of the C3, C3b, C3d and C3c preparations which were used in the present study was checked by SDS-PAGE electrophoresis (Fig. 1).

RESULTS

C3 has a biphasic effect on human allogeneic CTL generation

Human C3 was added at concentrations ranging between 55 and 550 $\text{M} \times 10^{-9}$ into human allogeneic MLC. The effect on the generation of CTL was biphasic. Smaller doses, 138 and 275 $\text{M} \times 10^{-9}$ enhanced the CML responses up to two-fold; in contrast higher doses (550 $\text{M} \times 10^{-9}$) consistently suppressed the response by 80% or more (Fig. 2). This study focuses on the enhancing part of the curve.

C3b enhances human allogeneic CML responses

Fluid phase C3b was added at the initiation of mixed allogeneic lymphocyte cultures, at doses ranging between 28 and 550 $\text{M} \times 10^{-9}$. Addition of C3b, at doses of 138 and 275 $\text{M} \times 10^{-9}$ enhanced significantly the allogeneic CML responses. Lower doses (28-55 $\text{M} \times 10^{-9}$) did not have any effect on the CML responses while higher doses (550 $\text{M} \times 10^{-9}$) were suppressive. Results from five experiments are shown in Fig. 3.

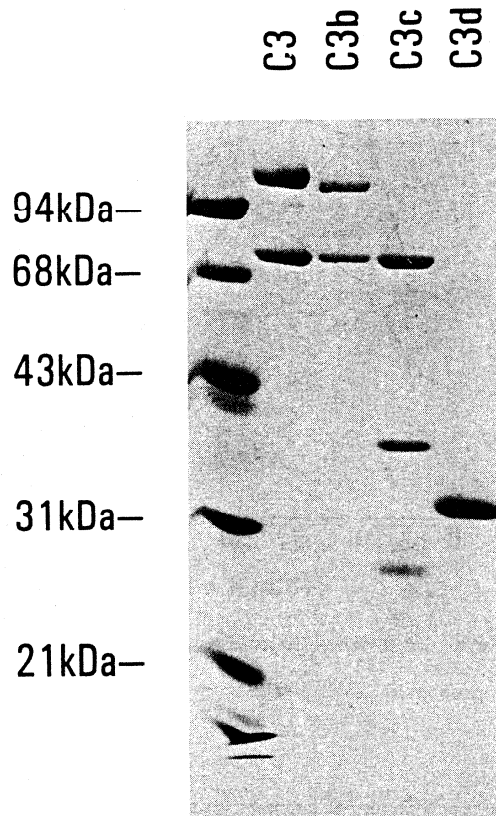


Fig. 1: SDS-PAGE electrophoresis of the complement preparations which were used in the present study.

Unsensitized MNC (MNC cultured alone for 6 days) exhibited minimal ability to lyse allogeneic targets. In this series of experiments these responses ranged between -2 and 6% and they were not affected by the addition of C3b at the same dose range.

In order to exert its enhancing effect, C3b had to be present at the initiation of the cultures. Addition of $275 \text{ M} \times 10^{-9}$ of C3b/ml at the initiation of the cultures enhanced significantly the CML responses.

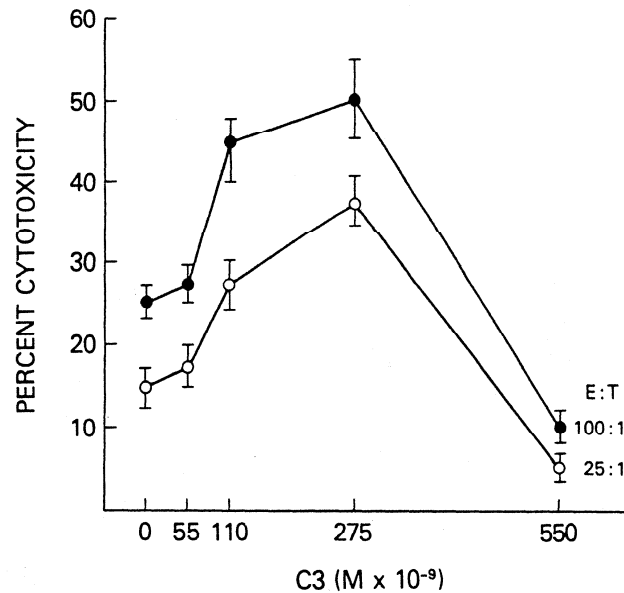


Fig. 2: Effect of C3 on human allogeneic CML. Results from 3 experiments (mean ± SEM) are presented. E:T, Effector:Target ratio.

Addition of C3b 24 hours later had a marginal effect, while if added at any point between day 2 and day 6 of the cultures there was not any effect. Presence of C3b during the 4 hour CML assay had no effect on the rate of chromium release (Fig. 4).

C3d but not C3c enhances human allogeneic CML responses

Breakdown products of C3b, C3c and C3d were tested for their ability to enhance human CML responses. C3c added at the initiation of the cultures at doses ranging between 100 and 1000 M x 10⁻⁹ did not affect the CML responses (data not shown). In contrast C3d added at the initiation of the mixed lymphocyte responses clearly enhanced the CML responses. (Fig. 5).

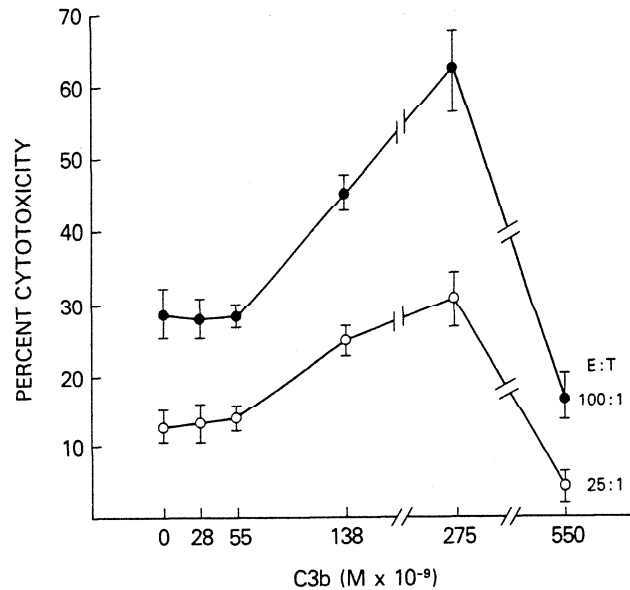


Fig. 3: Effect of C3b on human allogeneic CML. Results from 5 experiments (mean \pm SEM) are presented.

Effect of C3 and C3 breakdown products on human allogeneic MLR

C3, C3b and C3d preparations were added into human allogeneic MLC and the proliferative responses were estimated 6 days later. C3 suppressed MLR if present at concentrations higher than $275 \text{ M} \times 10^{-9}$; C3b and C3d similarly suppressed MLR if present at concentrations higher than 550 and $1650 \text{ M} \times 10^{-9}$ (Fig. 6). The enhancing effect of C3b and C3d on the CML responses occur at doses lesser than those at which the same peptides suppress the mixed lymphocyte reaction.

Enhancement of IL-2 production by C3b and C3d during MLR

In order to investigate the mechanism by which complement preparations cause enhancement of the cytotoxic responses we measured the IL 2 concentrations in the MLC supernatants. IL 2 concentrations in MLC

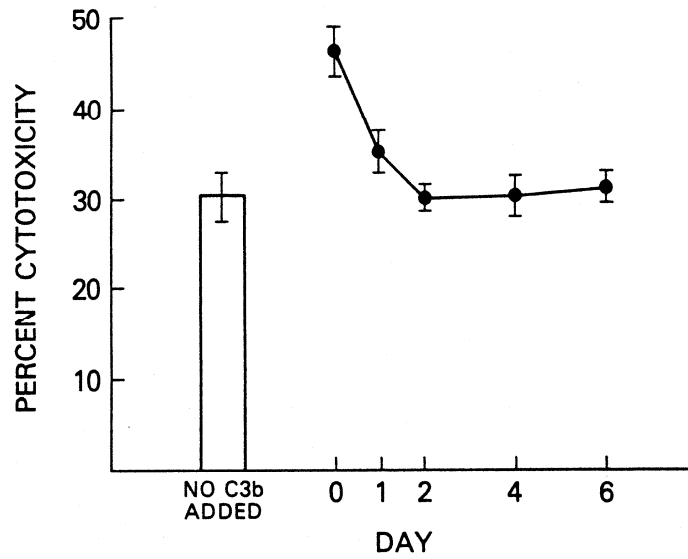


Fig. 4: C3b ($275 \text{ M} \times 10^{-9}$) added to the MLC on different time points. Results (mean \pm SEM) from 2 experiments are presented. Effector:target ratio, 100:1.

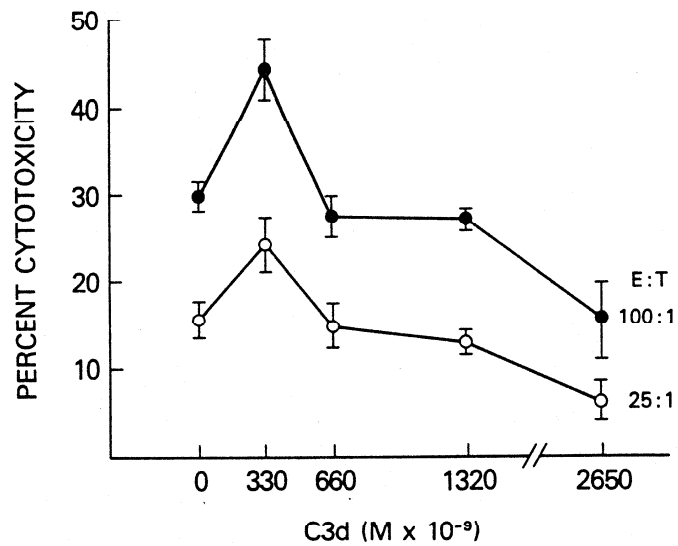


Fig. 5: Effect of C3d on human allogeneic CML. Results (mean \pm SEM) from 4 experiments are shown. E:T, Effector:Target ratio.

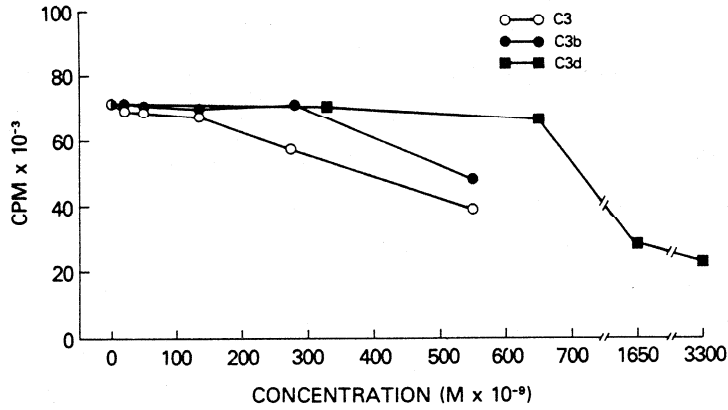


Fig. 6: Effect of C3, C3b and C3d on human MLR. Results (mean \pm SEM) from 3 experiments are shown.

TABLE 1
Effect of C3b and C3d on IL-2 production during MLR

Added	IL 2 (units/ml)
0	3.0 \pm 0.5 [□]
138 ⁺ C3b	3.2 \pm 0.4
275 C3b	7.0 \pm 1.5
550 C3b	1.5 \pm 0.9
330 C3d	6.7 \pm 1.9
660 C3d	6.5 \pm 1.4
3300 C3d	1.6 \pm 0.3

[□] mean \pm SEM (n=3)

⁺ M x 10⁻⁹

are increased up to day three and decline thereafter apparently because it is used up by the expanding cytotoxic cells (unpublished observations). Hence we measured the IL 2 content on the third day of the culture. Results from 3 experiments are shown in TABLE 1. Low concentrations of C3b and C3d caused more than two-fold increase in the IL 2 production while higher concentrations suppressed its production.

DISCUSSION

In the present study we examined the effect of C3 and its breakdown products on the primary in vitro human allogeneic CML. We observed that C3, added at the initiation of the MLC, had a biphasic effect. Low doses increased by at least two fold the specific allogeneic CML response while higher doses had a suppressive effect. The C3 breakdown products (C3b and C3d) had similar biphasic effect on the CML responses. C3c and C3a (data not shown) did not have any modulating effect on these responses.

The suppressive effect (high concentrations) on the human CML is in agreement of a previous report (17) while the enhancing effect exerted by low concentrations is a novel phenomenon. The effect of the above complement preparations on human allogeneic MLR was investigated in parallel. Several C3 breakdown products have been known to suppress the blastic transformation during the MLC (5-7,24). In the present study we confirmed that C3 and the breakdown products C3b and C3d readily suppress the thymidine uptake during the MLC. These suppressive effects parallel the suppression of the CML responses observed at the same dose range by C3, C3b and C3d. MLR responses were not affected by lower doses of the examined complement preparation. Thus, enhanced CML responses are apparently not secondary to enhanced cellular proliferation. Responder lymphocytes cultured alone, without stimulator cells, in the presence of heat inactivated human serum exhibit minimal lytic effect (up to 6% in this series of experiment) of allogeneic targets. Addition of complement

preparations at various concentrations did not enhance this nonspecific cytotoxicity (results not presented); this indicates that an allogeneic stimulus has to be present in order to ignite the response of the stimulator cells; once the cells have been stimulated then they are amenable to either enhancing or suppressive signals provided by complement. Thoman et al (25) reported recently studies on the effect of C3d-K (fragment generated from human iC3b by plasma kallikrein) on proliferative responses which concluded that only "activated" cells are prone to the suppressive effect exerted by this peptide.

C3b and C3d were able to exert enhancing effects only if present at the initiation of the cultures. This observation is in agreement with the fact that IL 2 is produced, and subsequently used, during the early phases of the MLC and the finding of this study that C3b and C3d cause enhanced production of IL 2. Complement preparations were added to the final 4 hour chromium release assay but none of them had any effect. It seems that complement does not participate in the effector-target conjugation and cytolysis phases.

Because IL 2 plays a central role in the proliferation of the cytotoxic T lymphocytes we measured IL 2 content in the supernatants of the MLC. Both C3b and C3d clearly enhanced the production of IL-2 if used at low concentrations and suppressed it if used at high concentrations. It is apparent that the observed changes of the levels of the CML responses caused by C3 and its breakdown products C3b and C3d are secondary to the fluctuations of the IL 2 production caused by these preparations. The mechanism by which IL 2 production is modified was not investigated in the present study. These might be preceded by changes in the IL 1 production or direct effect on the IL 2 producing cells. It was reported recently that complement containing immune complexes induce the release of IL 1 by human monocytes (11).

In conclusion, we have demonstrated that C3 and its trypsin cleavage products C3b and C3d have a biphasic effect on human allogeneic CML responses; small concentrations enhance the production of IL 2 and the generation of CTL during the human allogeneic MLC while larger concentrations suppress both responses.

ACKNOWLEDGEMENT

We wish to thank Mrs. Linda Adams for her assistance in the preparation of this manuscript.

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